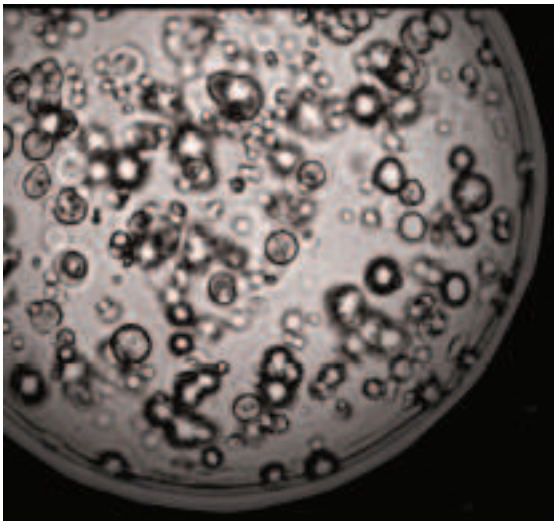

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Cell Microencapsulation for therapeutic purposes:
towards greater control over biocompatibility

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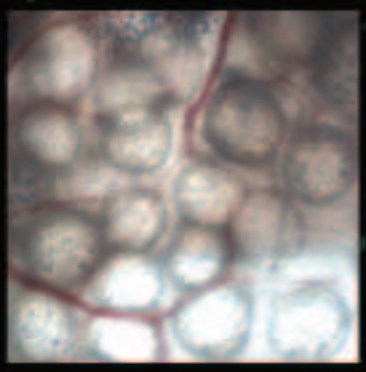
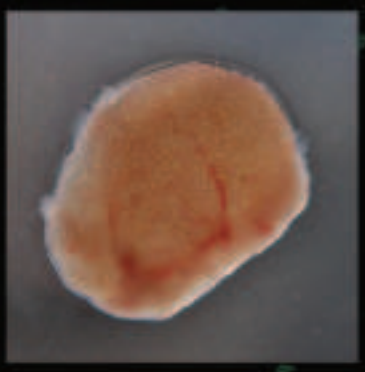

Cell microencapsulation for therapeutic purposes: towards greater control over biocompatibility

Microencapsulación de células con fines terapeúticos: avances en la biocompatibilidad

AINHOA MURUA UGARTE

Laboratorio de Farmacia y Tecnología Farmacéutica
Universidad del País Vasco / Euskal Herriko Unibertsitatea

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A mis padres Luis Mª y Mª Asun,
a mi hermano Mikel y a Sergio

The dimensions are minuscule,
the potential enormous

Ruth Duncan

GLOSSARY

6-OHDA: 6-hydroxydopamine
Ab: antibody
ACD: anemia of chronic disease
AD: Alzheimer's disease
ALS: amyotrophic lateral sclerosis
APA: alginate-poly-L-lisine-alginate
APH: alginate-protamine-heparine
ATSC: adipose-tissue stromal cell
AV: adenovirus
BBB: blood-brain barrier
BDNF: brain-derived neurotrophic factor
BHK: baby hamster kidney
BMP: bone morphogenetic protein
CCK-8: cell counting kit-8
CEpo: carbamylated Epo
CERA: continuous erythropoietin receptor activator
CHO: Chinese hamster ovary
CKD: chronic kidney disease
CNS: central nervous system
CP: choroid plexus
CS: cellulose sulfate
CsA: cyclosporine A
CSF: cerebrospinal fluid
cβR: common beta receptor
DMEM: Dulbecco's modified Eagle medium
DMSO: dymethylsulfoxide

DNA: deoxyribonucleic acid
DPO: darbepoietin alfa
DXM: dexamethasone
ECM: extracellular matrix
EFP: Epo fusion protein
ELISA: enzyme-linked immunoabsorbent assay
EMEA: European medicine agency
Epo: erythropoietin
Epo-R: erythropoietin receptor
ESA: erythropoiesis stimulating agent
ET-1: endothelin-1
FBR: foreign body reaction
FBS: fetal bovine serum
FBS: fetal bovine serum
FDA: Food and Drug Administration
FK-506: tacrolimus
FOB: follow-on biologics
G: α-L-guluronic acid
GLP-1: glucagon-like peptide 1
GM-CSF: granulocyte macrophage colony-stimulating factor
GMP: good manufacturing practice
H&E: hematoxilin & eosin
HA: hyaluronic acid
HAMC: hyaluronan and methylcellulose
HBSS: Hank's balanced salt solution
HD: Huntington's disease
HEK293: human epithelial kidney 293 (cells)
HEMA-MMA: hydroxyethyl methacrylate-metacrylic acid
hEpo: human erythropoietin

HIF: hipoxia-inducible transcription factor
HIV: human immunodeficiency virus
IKLLI: isoleucine-lysine-leucine-leucine-isoleucine
IKVAV:isoleucine-lysine-valine-alanine-valine
IL: interleukin
IM: intramuscular
JAK-2: janus kinase 2
kDa: kilo dalton
LRE: leucine-arginine-glutamine
LV: lentiviral vector
LVG: low viscosity and high guluronic (alginate)
M: β-D-mannuronic acid
MAPK: mitogen-activated protein kinase
mEpo: murine erythropoietin
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Mr: molecular mass
mRNA: messenger ribonucleic acid
MSC: mesenchymal stem cell
MTT: (method of transcriptional and translational) assay
MVG: medium viscosity and high guluronic (alginate)
MW: molecular weight
MWCO: molecular weight cut-off
O/W: oil in water
PAM: pharmacologically active microcarriers
PBS: phosphate-buffered saline
PCL: poly (3-caprolactone)
PD: Parkinson's disease
pDADMAC: poly-diallyl-dimethylammonium chloride
pDNA: plasmid DNA

PDSGR: proline-aspartic acid-serine-glycine-arginine

PEG: polyethylene-glycol

PERV: porcine endogenous retrovirus

PEX: hemopexin like protein

PGA: poly glycolic acid

PHI: prolyl hydroxylase inhibitors

PI3K: phosphoinositide 3-kinase

PLA: polylactic acid

PLGA: poly (lactic-co-glycolic acid)

PLL: poly-L-lysine

PMCG: polymethylene-coguanidine

PMN: polymorphonuclear

PSS: poly(styrene sulfonate)

PVA: poly (vinyl alcohol)

QA: quinolinic acid

RBC: red blood cells

RGD: arginine-glicine-aspartic acid

rHuEpo: recombinant human erythropoietin

RPE: retinal pigment eplithelial

SA: sodium alginate

SC: subcutaneous

SCI: Spinal cord injury

SGA: second generation antipsychotic

STAT: signal transducers and activators of transcription

TGF-β: tissue growth factor-β

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

W/V: weight/volume

WHO: World Health Organization

WIGSR: tyrosine-isoleucine-glycine-serine-arginine

WST: water-soluble tetrazolium

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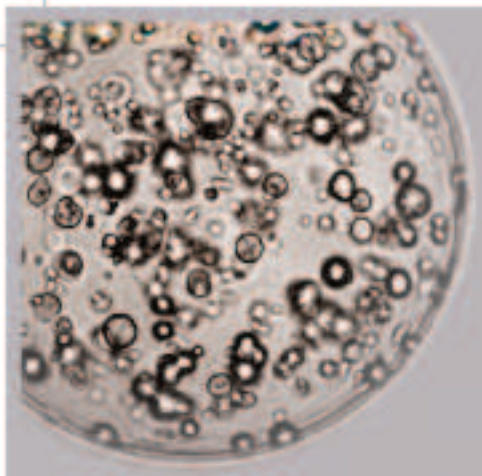

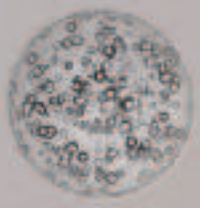

INTRODUCTION

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Microcapsules and microcarriers for *in situ* cell delivery*

Rosa Mª Hernández, Gorka Orive, Ainhoa Murua, José Luis Pedraz *

Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, 01006, Vitoria-Gasteiz, Spain

Networking Biomedical Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, SLFPB-EHU, 01006, Vitoria-Gasteiz, Spain

ABSTRACT

In recent years, the use of transplanted living cells pumping out active factors directly at the site has proven to be an emergent technology. However a recurring impediment to rapid development in the field is the immune rejection of transplanted allo- or xenogeneic cells. Immunosuppression is used clinically to prevent rejection of organ and cell transplants in humans, but prolonged usage can make the recipient vulnerable to infections, and increase the likelihood of tumorigenesis of the transplanted cells. Cell microencapsulation is a promising tool to overcome these drawbacks. It consists of surrounding cells with a semipermeable polymeric membrane. The latter permits the entry of nutrients and the exit of therapeutic protein products, obtaining in this way a sustained delivery of the desirable molecule. The membrane isolates the enclosed cells from the host immune system, preventing the recognition of the immobilized cells as foreign. This review paper intends to overview the current situation in the cell encapsulation field and discusses the main events that have occurred along the way. The technical advances together with the ever increasing knowledge and experience in the field will undoubtedly lead to the realization of the full potential of cell encapsulation in the future.

* Corresponding author: J.L. Pedraz

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Keywords Cell encapsulation; Alginate–PLL–alginate; Cell therapy; Drug delivery; Engineered cells; Stem cells.

Contents

1. Introduction

Over the last decades various cell types including primary cells [1], stem cells [2] or bioengineered cells [3] have been considered potentially therapeutic for the treatment of many diseases including those with deficient hormone production, such as insulin in diabetes [4] erythropoietin in anemia [5] and factors VIII and IX in hemophilia [6]. Moreover, delivering therapeutic products from nonautologous engineered cell lines has also been assayed in cancer therapy [7] and bone repair [8].

In general, 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 synergy 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 immunosuppressant drugs can be eliminated or at least reduced [9,10].

At present, the burgeoning number of cutting edge discoveries is leading to the design of biomimetic and biodegradable microcarriers that can easily combine with stem cells. These devices 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 review, we discussed the state of the art in the field of cell encapsulation technology. The key elements in the design and development of cell-loaded microcarriers are summarized. 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.

2. Microcapsules and microcarriers as a tool for regenerative medicine

Cell therapy is one of the most exciting fields in translational medicine. It stands at the intersection of a variety of rapidly evolving scientific disciplines: biomaterials, immunology, molecular biology, stem cell biology, tissue engineering, transplantation biology, regenerative medicine, and clinical research. The aim of cell therapy is to replace, repair, or enhance the function of damaged tissues or organs [11]. However, the success of any medical treatment depends not only upon the

pharmacokinetic / pharmacodynamic activity of the therapeutic agent, but to a large extent, on its bioavailability at the site of action in the human body [12–15].

Since the pioneering study by TMS Chang in the early 1950s [16], when it was originally introduced as a basic research tool, the entrapment of cells has since been developed based on the promise of its therapeutic usefulness in tissue transplantation and nowadays represents an evolving branch of biotechnology and regenerative medicine with numerous applications [17].

Cell encapsulation is a strategy that aims to physically isolate a cell mass from an outside environment within the confines of a semipermeable membrane barrier without the use of long-term therapies of modulating and/or immunosuppressive agents, which have potentially severe side effects [18–21]. Microcapsules are almost exclusively produced from hydrogels since they hold a number of appealing features. They provide a highly hydrated microenvironment for embedded cells that can present biochemical, cellular, and physical stimuli that guide cellular processes such as differentiation, proliferation, and migration [22]. Additionally, the frictional or mechanical irritation to the surrounding tissue is reduced by the soft and pliable features of the hydrogel. Moreover, some authors mention that due to the hydrophilic properties of the material, there is virtually no interfacial tension with surrounding tissues and fluids which minimizes cell adhesion and protein adsorption. Combination of these two factors results in high biocompatibility [10]. Moreover, hydrogels provide a high degree of permeability for low-molecular-mass (Mr) nutrients and metabolites.

In addition to using natural biomaterials, synthetic polymers as well as inorganic compounds have also been used [23]. Although synthetic materials provide researchers with large flexibility in material design, they do not have an intrinsic mechanism for interacting with cells, and cell adhesion is typically mediated by non-specific cell adhesion [24,25]. This limits their use in applications that require defined control over cell–matrix interactions, but this can be achieved by functionalizing these matrices with bioactive molecules, as it will be discussed later.

Microcapsule surrounding membranes are expected to be amenable to nutrient diffusion and molecules such as oxygen and growth factors essential for cell survival [10]. Furthermore, the elimination of cell secretions and catabolic products must be possible while keeping out all high molecular weight immune system components such as immunoglobulins and immune cells. The permselective capsule environment has been shown to support cellular metabolism, proliferation, differentiation and cellular morphogenesis [10,26,27].

The primary impetus behind the development of cell encapsulation technologies has been the aim to transplant cells across an immunological barrier without the administration of immunosuppressant drugs, an important issue to be considered in organ

transplantation due to their important adverse effects. Non-specific suppression of the immune system may lead to a variety of undesired complications in patients (e.g., opportunistic infections, failure of tumor surveillance) [28–30]. By surrounding a transplant with a membrane barrier, the access of the host's immune system to the transplant can be physically prevented, acting as an "artificial immunopriviledged site" shielding the graft from destruction, which has initiated a flurry of research into bioartificial organs and tissue engineering [31,32].

The encapsulation of cells has therefore two major potential benefits: 1) transplantation without the need for immunosuppressive drugs, and 2) use of cells from a variety of sources such as primary or stem cells, or genetically engineered cells which can be modified to express any desired protein *in vivo* without the modification of the host's genome [33–37].

Immunoprotection of transplanted cells and tissues by size-based semipermeable membranes allows the *in situ* delivery of secreted proteins to treat different pathological conditions such as CNS diseases, diabetes mellitus, hepatic diseases, amyotrophic lateral sclerosis, hemophilia, hypothyroidism and cardiovascular diseases among others [38–42]. Such cell-based devices are thought to hold great promise in applications requiring site-specific and sustainable drug delivery of cell-synthesized molecules.

Cell immobilization shows an important advantage compared with encapsulation of proteins, allowing a sustained delivery of '*de novo*' produced therapeutic products giving rise to more physiological concentrations.

Furthermore, if the encapsulation device is broken, the toxicity caused by a quick delivery of high concentrations of the drug could be avoided. However if cells manage to exit the encapsulation device the host's immune system might attack them compromising their survival. Moreover, the use of an inducible genetic system to avoid excess expression of the therapeutic protein (which in many cases might become hazardous) is an important challenge in the development of these delivery systems.

Numerous immune isolation procedures have been developed over the years. These techniques are generally classified as macroencapsulation (large usually flat-sheet and hollow-core fibers) and microencapsulation (involving small spherical vehicles and conformally coated tissues). Regarding microcapsules, their spherical shape is considered advantageous from a mass transport perspective, offering optimal surface-to-volume ratio for protein and nutrient diffusion, and thus cell viability compared to other immobilization scaffolds, which improves oxygen and nutrients' permeability [43]. The small size of the capsules (from 100 μm to 500 μm) allows their implantation in close contact to the blood stream, which could be beneficial in specific applications later discussed for the long-term functionality of the enclosed cells due to an enhanced oxygen transfer into the capsules. Moreover, microcapsules are typically more durable than macrocap-

sules and difficult to mechanically disrupt [10].

Microcapsules can be classified in 3 categories: matrix-core/shell microcapsules manufactured by gelling alginate droplets in a solution containing a bivalent ion followed by a surface treatment with a polycation (multi-step technique) [44–49], liquid-core/shell microcapsules produced by dropping a cell suspension containing bivalent ions into an alginate solution (one-step technique), and cells-core/shell microcapsules (or conformal coating). Matrix-core/shell microcapsules in which cells are hydrogel-embedded, exemplified by alginates capsule, are by far the most studied method. Many refinements of the technique have been attempted over the years such as correct biomaterial characterization and purification, improvements in microbead production procedures, and new microbead coating techniques.

All techniques typically start with a scheme to generate a controlled-size droplet, followed by an interfacial process to stabilize the droplet and to obtain a solid microcapsule membrane around the droplet. However, aside those more traditional techniques (either matrix-core or liquid-core shells), new techniques are emerging in response to shortcomings of existing methods. More recently, conformal coating, where the surface of a cell mass is surrounded with a membrane, has also been attempted to minimize membrane thickness, internal mass transfer resistance and implant size [50,51].

Microcapsules and hollow spheres can be developed efficiently using many techniques well described for drug delivery and other non-pharmacological applications [52,53]. However, in cell encapsulation applications, complex and conflicting requirements have to be met. Reproducible methods using very precise parameters (permeability, size, and surface) are of outstanding importance, but these procedures should also support cell viability and integrity during the encapsulation process and after implantation. Last but not least, the preparation method must ensure adequate flux across the capsule membrane for cell survival and functions.

The polyelectrolyte complexation of alginate with polycationic poly(L-lysine) (PLL), initially developed by Lim and Sun [45], has been the most widely employed system for a variety of applications (*in vivo* and *in vitro* three-dimensional (3D) cell cultures, clonal selection of desired cell phenotypes, bioengineering, large-scale production of cell-derived molecules in the biotechnology industry, reproductive biotechnology, gene or cell therapy, etc.) [10,54,55]. This is a gentle, cell compatible method which has seen adaptation of the initial technique by independent laboratories in the last decades, naturally leading to process improvements and development of superior encapsulation materials. Many attempts have been made to optimize the performance of the capsules, and numerous encapsulation techniques have been developed over the years. Table 1 summarizes main production methods of microbeads.

Antosiak-Iwańska *et al.* recently proposed the use of alginate–protamine–

heparine (APH) capsules as a more resistant alternative to the conventional alginate–poly-L-lysine–alginate (APA) microcapsules. However, long-term experiments indicate that immune isolation with APA microcapsules is more effective than with APH microcapsules [32].

Few cell immobilization technologies have allowed to obtain very small micrometric biocompatible microcapsules (30–60 μm) with high mechanical stability, of controlled size and uniformity. On the basis of this new technology of producing very small microcapsules with a high mechanical stability, Herrero *et al.* succeeded in employing a spraying technique (using atomization nozzles) to encapsulate mesenchymal stem cells and monocytes [11]. This method is advantageous in terms of ease to set-up and scale up for the proposed industrial, automatic dropwise of the polymer solution and obtained spherical and uniform particles. This spraying technique and alginate microparticle formulations can further be optimized for oral delivery of several pharmaceutical peptides and proteins [56].

Haeberle *et al.* [57] presented a novel technique which can process highly viscous biopolymer solutions (up to 50,000 times the viscosity of water) while being sufficiently gentle to maintain the viability of the cells. In this scheme, a commercially available polymer micronozzle [58] was spun on a centrifuge to dispense alginate droplets through an air gap into a standard Eppendorf tube ('Eppi') mounted on the flying bucket rotor. The tube contained an aqueous $CaCl_2$ solution to perform diffusion-based hardening to Ca-alginate beads.

A novel encapsulation system (five-component/three-membrane hybrid capsule) of sodium alginate (SA), $CaCl_2$, polymethylene-coguanidine (PMCG), cellulose sulfate (CS), and poly-L-lysine (PLL) has recently been proven efficient in pancreatectomized canine allotransplantation experiments [33]. To improve the performance, a thin interwoven PMCG–CS/PLL–SA membrane was fused onto the PMCG– CS/$CaCl_2$–SA capsule, forming permanent bonds. This union improves the immunoprotection function without jeopardizing the influx of nutrients and oxygen and efflux of therapeutic products and waste.

Table 1
Main production methods of microbeads.

Methods	Core polymer	References
Solid-core		
Extrusion methods	Alginates/Ca^{2+} or Ba^{2+}	[44,45,163]
Emulsion (thermal gelation)	Agarose	[161]
Microfluidics	Alginates/Ca^{2+}	[47]
Microlithography	Alginates/Ca^{2+}	[48]
Hollow-core		
Interfacial precipitation	HEMA-MMA, PLGA	[159]
Complex coacervation	Cellulose sulfate/pDADMAC	[160]
Conformal coating		
Polyelectrolyte cell coating	Alginates, PDADMAC/PSS	[33]
Interfacial precipitation coating	Methacrylates, alginates/Ba^{2+}	[50]
Interfacial polymerization	PEG-dyacrilates	[51,52]

HEMA–MMA, hydroxyethyl methacrylate–methacrylic acid; PLGA, poly(lactic glycolic) acid; pDADMAC, poly-diallyl-dimethyl-ammonium chloride; PSS, poly(styrene sulfonate); PEG, poly(ethylene glycol).

To shield the PMCG and PLL on the surface of the capsule, a third (outer) membrane of $CaCl_2$–SA [59] was added to encase the system.

Conformal coating may be thought of as a special case of microencapsulation where the term is used to describe a method of forming a barrier directly on a small cell mass or a small piece of

tissue. The method eliminates unutilized space in a microcapsule core by surrounding the cell mass with the encapsulation membrane. This theoretically provides an improved mass transport between the capsule exterior and the cell mass, and increases the effectiveness of cell packing (hence, minimizes implant size). Despite its potential, *in vivo* performance of conformally coated islets remains to be reported in the literature [50,51].

In addition to incorporating the living material, some approaches employing microcapsules as "microcarriers" have also been described in the literature, where cells are attached to the surface of the biomaterial employed (Fig. 1). Tatard *et al.* developed pharmacologically active biodegradable microcarriers (PAM) made with poly (D,L-lactic-coglycolic) acid (PLGA) and coated with adhesion molecules to serve as a support for cell culture [60]. Stover *et al.* have proposed the use of Spheramine, an active component of cultured human retinal pigment epithelial (hRPE) cells, attached to an excipient part of cross-linked porcine gelatin microcarriers which was in Phase II clinical trial [61] but had to be stopped recently due to adverse effects encountered [62]. Gelatin microcarriers are also under study to be used in three-dimensional cartilage- and bone like tissue engineering [63].

Integrated biodegradable devices have also been proposed recently based on the integration of two techniques: microcapsules and surface-coated poly (3-caprolactone) PCL capsules (diffusion chambers) [38]. Microcapsules provide a 3D microenvironment for spatial cell growth with good viability and proliferation. Coating biocompatible and hydrophilic PEG gelatin on the PCL surface could mediate the inflammatory response, prevent fibrosis formation, and maintain controllable performance. Most importantly, the dual nanoporous construct provides a unique way to allow superior cell growth, immunoprotection, fibrosis prevention and controllable release of secreted products in a biodegradable device.

Cell immobilization has long been suggested as an efficient delivery method for cell transplantation, but it was recently reported that cell immobilization can lead to modification of cell wall and cell membrane compositions [64]. An increased understanding of the chemical signals that direct cell differentiation, migration and proliferation, advances in scaffold design and peptide engineering that allow this signaling to be recapitulated and the development of new materials, such as DNA-based and stimuli-sensitive polymers, have recently given engineers enhanced control over the chemical properties of a material and cell fate. Additionally, the immune system, which is often overlooked, has been shown to play a beneficial role in tissue repair, and future endeavors in material design will potentially expand to include immunomodulation.

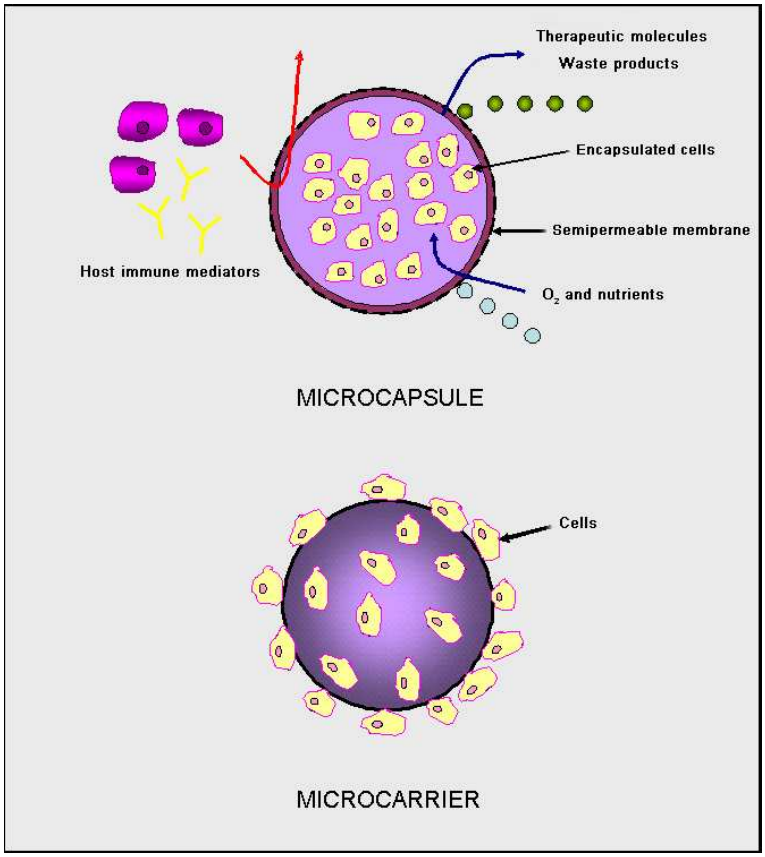

Fig. 1. Comparison between microcapsules and microcarriers.

It is apparent that cell fate in growing tissues relies heavily on the adhesion ligands presented by the matrix, and the development of methods to functionalize materials with these molecules is central in recapitulating these matrix effects and supporting the growth of functional tissue.

3. Biomaterials in cell microencapsulation

Biomaterials are increasingly important in the development of drug delivery systems and tissue engineering approaches and play key roles in overcoming the inherent insufficiency of tailored therapies. Polymers of many types are used to create drug vehicles providing sustained delivery of potentially therapeutic agents, including proteins, genes, cells and oligonucleotides. Biomaterials also make excellent scaffolds suitable for delivering cells to the host or immobilizing them for long-term delivery of molecules to the surrounding tissue. Scaffolds can be loaded with proteins and/or have a surface

morphology or extracellular matrix (ECM) capable of controlling cell attachment, growth, and differentiation. In the last few decades, the field of cell microencapsulation has also raised much interest in part due to the advancement and optimization of the biomaterials used to elaborate the capsules [65]. These living cell-containing particles can be modified with surface characteristics that allow them to control the proliferation and differentiation of the enclosed cells [66–68].

It was recently acknowledged that the success of this therapeutic approach requires a detailed analysis, at the atomic and molecular levels, of the types of biomaterials employed and especially of the mechanisms driving cell–material interactions. One of the first issues in this endeavor is the immunogenicity of the biomaterials used to fabricate the microcapsules and the biocompatibility of the microcapsule system in its final form. One critical limitation has been the persistent lack of reproducibility of the different biomaterials and the requirements to achieve a better understanding of the chemistry and biofunctionality of the biomaterials and microcapsule system. More detailed and in-depth knowledge will lead to the production of standardized transplantation-grade biomaterials and biocompatible microcapsules.

3.1. Alginates for cell encapsulation

Alginates are certainly the most frequently employed biomaterials for cell immobilization due to their abundance, easy gelling properties and apparent biocompatibility. Although the suitability of other natural and synthetic polymers is under investigation [69,70], none has reached the same level of performance as alginates. As natural polymers, alginates exist in brown seaweeds and bacterium [71] and their compositions vary depending upon the source from which they are isolated [72]. The production of alginates with specific structures can also be made by enzymatic modification using mannuronan C-5 epimerases [73]. Alginates are a family of unbranched binary copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), of widely varying compositions and sequential structures. Determining and standardizing these differences is of paramount importance since they have a significant impact on some of the alginate gel properties including biocompatibility, stability, mechanical resistance, permeability, biodegradability and swelling behavior.

One particular critical issue is the biocompatibility of the alginates and alginate microcapsules. A very high level of biocompatibility is essential assuming that the final aim of the encapsulation device is to protect the enclosed cellular tissue from the host's immune response. It is necessary to improve our knowledge about the biomaterial and device properties, and to optimize and characterize each of the steps related to the cell encapsulation technology, from the alginate extraction and purification to the elaboration and administration of the microcapsule.

As a natural polymer, alginate's performance as a biomaterial is limited by its tendency to be largely contaminated. In addition, the industrial processes used for extracting alginates from seaweed could introduce further contaminants into the raw alginates. Some of these impurities include endotoxins, certain proteins and polyphenols. The latter can be dangerous for humans as reported by the World Health Organization (WHO) [74] and can possibly accumulate in the body [75]. Moreover, endotoxins and proteins have been associated with reduced biocompatibility of the alginate. Therefore, a key element in the validation of the alginate for implantation purposes is an efficient purification process to monitor and remove all its contaminants. In the last few years, several research groups have developed their own in-house protocols for alginate purification [76–81]. The first published method described by Zimmermann *et al.* used a free-flow electrophoresis technique [76] but, since it was difficult and expensive, it was abandoned in favor of chemical extraction procedures. Even, the first comparative evaluation of some of these in-house alginate purification protocols was published [82]. Results from this study showed that in general all of the studied purification methods reduced the amounts of endotoxins and polyphenols but were less effective in eliminating proteins. A commercially purified alginate was also analyzed in order to provide a comparison between the in-house and commercial purification processes. Interestingly, the commercially purified alginate also presented residual proteins in amounts that may be enough to compromise microcapsule biocompatibility [82]. Overall, the results of this study reflected that currently employed methods to purify alginates may not be efficient enough to completely remove contaminating and potentially immunogenic species. It has been demonstrated that purifying the alginate induces a number of changes in the polymer's characteristics. Alginate hydrophilicity was shown to increase by 10 to 40% following purification by different methods, in correlation with a decrease in protein and polyphenol content. This increased hydrophilicity correlated with lower immunogenicity of the alginate gel. In this study, reducing the contamination level of the alginate also correlated with an increased solution viscosity, a property that will influence the morphology of the final microcapsule.

The composition of the alginate is another critical issue to be considered. In fact, alginate composition regulates some main properties of the alginate gels including stability, biocompatibility and permeability. In the last decade, biocompatibility of the alginates in relation to their composition has been a matter of much debate and controversy. Some groups have reported that alginates with a high content in M evoke an inflammatory response by stimulating monocytes to produce cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF). This mechanism may be driven via binding to CD14 [83,84]. Furthermore, antibodies to

alginates were found when high-M alginates were transplanted but not in the case of high-G alginates [85]. Soon-Shiong *et al.* also observed a cellular overgrowth of 90% of the capsules when high-M alginate was used [86]. In contrast, Clayton *et al.* found guluronic acids to be associated with more severe cell overgrowth [87]. De Vos and co-workers have also reported that after transplantation in rats, the majority of high-G alginate capsules are overgrowth by inflammatory cells and are adherent to the abdominal organs whereas intermediate-G capsules (with higher M content) are free of any adhesion and are floating freely in the peritoneal cavity [88,89].With the aim of shedding light on this discussion, we evaluated the *in vitro* and *in vivo* biocompatibility properties of microcapsules elaborated with alginates of different composition and purity. Our results suggested that the purity of individual alginate preparation, rather than their chemical composition, was probably of greater importance in determining microcapsule biocompatibility [90]. All this controversy might be caused in part by the lack of a standard definition for high-G alginates and high-M alginates as well as for the different purity levels of both monomeric units and the different geometry of the capsules employed in the experimental studies [27]. However, further efforts are needed to develop standardized assays that facilitate the evaluation of the biocompatibility of alginates and other hydrogels. Recently, a highly sensitive cell assay based on the induction of apoptosis in Jurkat cells, capable of detecting low levels of immunogenic impurities present in alginate samples has been reported [91]. This *in vitro* test, as well as other similar assays, is certainly a useful tool to evaluate, select and improve alginate preparations. Nevertheless, it should always be kept in mind that only the results of *in vivo* implantations can provide definitive information on the immunogenicity of alginates. In general, further research is still needed to precisely identify the alginate properties that can reliably predict its *in vivo* performance. This information is necessary to establish strictly outlined criteria for alginate selection and purification and obtain results that are reproducible between research groups.

3.1.1. Functionalizing and modifying alginate gels

In general, biomaterials have been considered as simple inert scaffolds in which cells were merely entrapped. One current exciting approach consists on modifying the biomaterials with different peptides and proteins that provide control over cell fate. By tailoring the polymers with sequences that mimic the extracellular matrix (ECM) it is feasible to control cell proliferation and even cell differentiation. Some examples of molecules that have been used to decorate the biomaterials include RGD, IKLLI, IKVAV, LRE, PDSGR and YIGSR [92–94]. These moieties trigger a cascade of intracellular signaling events through the focal contacts providing tight control over cell–matrix interactions [24].

The most widely employed peptide sequence is arginine–glycine–aspartic

acid (RGD) derived from fibronectin, a natural protein present in ECM [95,96]. The coupling of RGD sequences to alginate hydrogels has been extensively studied by Mooney *et al.* This group showed how it was possible to direct cell fate by controlling RGD density on alginate gels [97,98]. In addition, the influence of different nanopatterned islands of RGD on cell behavior has been extensively evaluated [99]. Recently, they reported the development of novel tools that allow for quantifying the interactions between cells and presenting ligands [100,101]. Such advances make a step forward in the understanding of cell–ECM interactions and confirm how integrin expression varies depending on the stage of cell differentiation.

The elaboration of biomimetic scaffolds has also been applied to cell encapsulation technology by our research group [67]. By designing biomimetic cell–hydrogel capsules we were able to promote the *in vivo* long-term functionality of the encapsulated myoblast cells and improve the mechanical stability of the capsules. Biomimetic capsules were fabricated by coupling the adhesion peptide arginine–glycine-aspartic acid (RGD) to alginate polymer chains and by using an alginate mixture providing a bimodal molecular weight distribution. The biomimetic capsules provided cell adhesion for the enclosed cells, potentially also leading to mechanical stabilization of the cell–polymer system. Strikingly, the novel cell–hydrogel system significantly prolonged the *in vivo* long-term functionality and drug release, providing a sustained erythropoietin delivery during 300 days without immunosuppressive protocols. Additionally, regulating the cell-dose within the biomimetic capsules enabled a controlled *in vitro* and *in vivo* drug delivery [67].

Another modification under evaluation is that focused on the control over the biodegradation rate of the alginates. The easily biodegradable alginates result in interesting tissue engineering approaches, especially when the repair, remodelling or regeneration of tissues is intended. In such an approach, the alginate is designed to degrade once the biomaterial has met its biological function. The degradation rate should be adjusted to the time required by grafted and host cells to replace the scaffold and provide new tissue. One interesting example is the oxidation of alginate chains by generating functional groups that are more susceptible to hydrolysis [102,103].

3.2. Collagen

Collagen is the major component of mammalian connective tissue and has been used in cell immobilization due to its biocompatibility, biodegradability, abundance in nature, and natural ability to bind cells. It is found in high concentrations in tendon, skin, bone, cartilage and, ligament, and these tissues are convenient and abundant sources for isolation of this natural polymer. Collagen can be readily processed into porous sponges, films and injectable cell immobilization carriers. Collagen may be gelled utilizing changes in pH, allow-

ing cell encapsulation in a minimally traumatic manner [104,105]. It may also be processed into fibers and macroporous scaffolds [106,107]. Its natural ability to bind cells makes it a promising material for controlling cellular distribution within immunoisolated devices, and its enzymatic degradation can provide appropriate degradation kinetics for tissue regeneration in micro and macroporous scaffolds. Challenges to using collagen as a material for cell immobilization include its high cost to purify, the natural variability of isolated collagen, and the variation in enzymatic degradation depending on the location and state of the implant site [108]. Collagen has been used to engineer a variety of tissues, including skin [109,110], bone [111,112], heart valves [113], and ligaments [114].

3.3. Chitosan

Chitosan is a deacetylated derivative of chitin, which is widely found in crustacean shells, fungi, insects, and molluscs. Chitosan forms hydrogels by ionic or chemical cross-linking with glutaraldehyde, and degrades via enzymatic hydrolysis. Chitosan and some of its complexes have been employed in a number of biological applications including wound dressings [115], drug delivery systems [116] and space-filling implants [117]. Due to its weak mechanical properties and lack of bioactivity, chitosan is often combined with other materials to achieve more desirable mechanical properties. Specifically, chitosan has been combined with calcium phosphate to increase its mechanical strength for micro and macroporous scaffold applications [117], and has been combined with collagen to provide a more biomimetic microenvironment in nanoporous cell encapsulation applications [118].

3.4. Agarose

Agarose, similar to alginate, is a seaweed derived polysaccharide, but one that has the ability to form thermally reversible gels. Mainly used for nanoencapsulation of cells, agarose/cell suspensions can be transformed into microbeads by utilizing a reduction in temperature [119]. A possible drawback to its use in this application is cellular protrusion through the membrane after gelation. Other uses of agarose in cell immobilization include the fabrication of microporous gels seeded with chondrocytes for the repair of cartilage defects [120].

3.5. Other polymers and types of biomaterials for cell encapsulation

Other biomaterials have been investigated in the field of cell microencapsulation, although none of them is as much characterized and studied as alginates. On the way to obtaining alternative cell-based therapeutic strategies, we could benefit from the advantages that other biomaterials could offer. 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 [121,122].

4. Critical properties for the elaboration of microcarriers

Although advances of outstanding importance have already been achieved in the field of cell microencapsulation, there are some critical aspects that should be carefully taken into consideration if the clinical success of the technology is aimed. A compilation of important capsule properties is provided in recent reviews [123,124].

4.1. Microcapsule permeability and MWCO

The mass transport properties of an encapsulation membrane are critical since the influx rate of molecules (essential for cell survival) and the efflux rate of therapeutic products will ultimately determine the extent of entrapped cell viability. Moreover, membrane pore size must be carefully controlled to avoid the undesired entrance of immune system components from the host that might destroy the inner cells. The metabolic requirements of different cell types are diverse and, hence, in principle optimal membrane permeability depends on the choice of cells [10]. Although the role of permeability for particular elements essential for cell survival has been explored (for example, oxygen) [125], no systematic approach has been taken to determine the permeability requirements of each cell type. As a consequence, an empirical approach has been typically taken to tailor capsule permeability for cell survival. The upper limit of capsule permeability, i.e., molecular weight cut-off (MWCO; size of the largest molecule that is not substantially blocked by the semipermeable membrane), will be application dependent. In the case of transplantation, the MWCO is expected to be different whether xenogeneic or allogeneic tissues are destined for engraftment [10].

4.2. Mechanical integrity / stability / durability

The mechanical role performed by the semipermeable barrier ensures that no direct cell–cell contact occurs between transplanted and host cells, while allowing for paracrine interaction between the biological environment (host) and the transplant graft.

The assessment of capsule mechanical properties is important, not only to determine the durability of capsules during production and handling, but also as an indication of the capsule membrane integrity. The latter is most informative when long-term studies are carried out.

4.3. Microcapsule size and morphology

Another important issue that should be taken into account is the diameter of the capsule as it could influence the immune response against capsules. Sakai *et al.* observed that cellular reaction was much lower when employing

smaller microcapsules in comparison to bigger size microcapsules [126].

Rough surfaces of capsules must also be avoided due to the fact that they may elicit immunological reactions when implanted. In addition to a biomaterial's chemical properties, researchers have realized that structural aspects of the membranes can also have profound influences on cell function, fate and tissue formation [126–129].

A smooth and clean device surface, controlled geometry and dimension, and polyethylene glycol (PEG) or gelatin modification on the capsule surface could mediate the acute inflammatory response and minimize fibrosis formation [38].

Moreover, to guarantee a sufficient diffusive mass transport, in overall, the diameter of the microcapsules should not exceed 300–400 μm [130,131].

4.4. Biocompatibility and low immunogenicity

Biocompatibility is defined as the ability of a biomaterial to perform with an appropriate host response in a 'specific application' [132]. Biocompatibility of microcapsules and their biomaterials' components is a critical issue if the long-term efficacy of this technology is aimed. Usually, a fully biocompatible system is considered to be a system manufactured of membranes which elicit no or not more than a minimal foreign body reaction. The host response is a potentially serious and deleterious problem to the clinical implementation of the technology.

A key element in the validation of alginate for implantation purposes is the efficient purification process to monitor and remove all its contaminants (inflammatory components) which include endotoxins, polyphenols and certain proteins. Not surprisingly, the purity of the alginate has been found to be a pertinent factor in the biocompatibility of alginate–PLL capsules. Although most purification methods have been found to succeed in reducing endotoxins and polyphenols, these methods have not achieved a correct elimination of the protein content [82]. In addition, the purification process might induce a number of changes in the polymers' features which should be carefully controlled [133].

The surgical implantation method is believed to be an additional parameter that influences the host reaction or biocompatibility to such implanted devices.

Several experiments have demonstrated that the surgical implantation method can influence and activate a non-specific response against implanted devices. Moreover, although it has been described as a transient response, it is difficult to avoid as it cannot be solved by chemical modification of the capsule. In order to overcome this obstacle, the use of transient immunosuppressive protocols has been proposed [134,135].

Upon transplantation of encapsulated alien cells, the host response is initiated by an acute inflammatory reaction caused by the disruption of host vasculature (associated with the release of bioactive proteins from the host such as fibrinogen, thrombin,

histamine and fibronectin) (Fig. 2). Activated platelets, polymorphonuclear leukocytes, humoral components of serum, clot constituents, cell debris, and extracellular matrix are initially present at the host–material interface. Tissue macrophages are recruited to the site and mediate the process of clean-up and initial wound healing. Mast cells and macrophages produce bioactive factors such as IL-1β, TNF-α, TGF-β and histamine, which stimulate cells in the capsules. Finally, mesenchymally-derived cells mediate matrix production and contracture coupled with a neovascularization response which rounds out the process. Within two weeks, basophils and granulocytes gradually disappear from the graft site while macrophages and some migratory cells that are primarily fibroblastic remain attached to an average of 2–10% of the capsules. These attached macrophages remain activated and contribute to the deleterious circle of activation. As a consequence, although the loss of 2–10% of capsules might not be crucial for the functionality of the remaining 90–98%, different studies show that it is mandatory to completely delete overgrowth surrounding microcapsules [43,122,136] due to the fact that it may interfere with diffusive transport of molecules and oxygenated blood supply [76,86,137].

In addition to the interaction between the biomaterials and the host tissue, a significant interaction is the one between the biomaterial and the encapsulated donor tissue. The response varies in degree and in the specific cell types involved depending upon the site of implantation. Neovascularization is another critical process which may determine the success of encapsulation therapy. A number of studies have showed that the outer microarchitecture of the encapsulation membrane exerts a profound influence on the neovascularization response, and not necessarily the membrane surface chemistry [138–141]. Membranes with surface pores that allow host cell colonization without inducing significant cell spreading, in general, have resulted in the formation of vascular structures very near the host–material interface [10].

De Vos *et al.* have reported an interesting advance to predict biocompatibility where the measurement of the electrical charge of the surface by means of zeta potential was found to predict the interfacial reactions between the biomaterial and the surrounding tissue [142].

4.5. Cell choice

The choice of cells depends upon the intended application, such as the secretion of a particular naturally occurring bioactive substance like neurotransmitter, cytokine, chemokine, growth factor, growth factor inhibitor, angiogenic factor; or the metabolism of a toxic agent, or the release of an immunizing agent; or based on a sense and release function such as oxygen partial pressure and Epo or glucose and insulin.

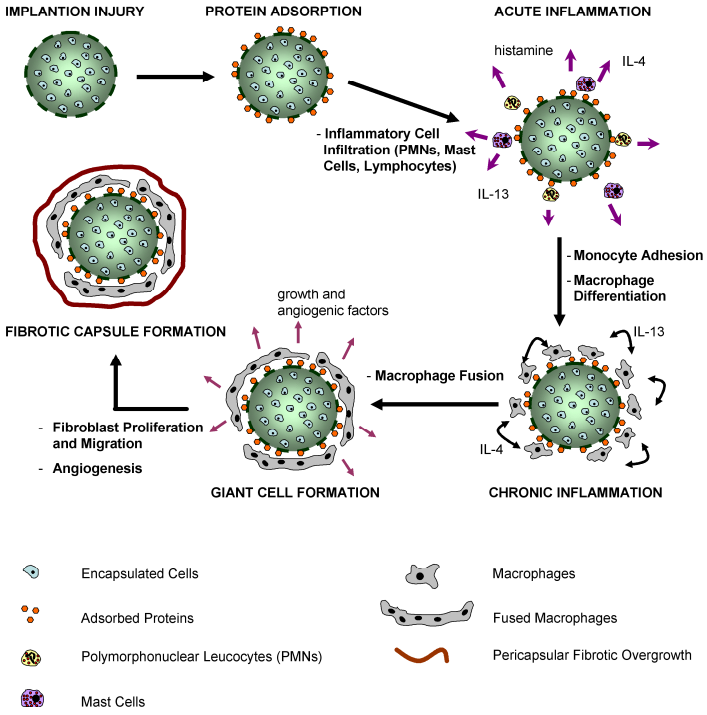

Fig. 2. Diagram of the process of acute and chronic inflammatory responses in the termed foreign body reaction against implanted biomaterials. Reproduced, with permission, from Ref. [122] © 2009 Landes Bioscience.

Cell encapsulation technology has in part failed to reach clinical approval so far mainly due to the high immunogenicity of the encapsulated cells (seed cells for therapeutic function), which eventually evoke an inflammatory reaction in the microenvironment surrounding the microdevices that leads to suffocation and death of the encapsulated cells [9,65,143]. The key issue to overcoming this problem could be to use cells that can downregulate or reduce this immune response [144].

The encapsulated nonautologous cells secrete cytokines and shed antigens, which eventually initiate a host immune response and lead to inflammatory tissue surrounding the microcapsules. This inflammatory reaction leads to cell suffocation and decreased encapsulated cell viability [65,143]. One promising solution to reduce host immune reaction is by administering anti-inflammatory drugs along with the therapeutic system [135,145]. Another approach under study to reduce host immune reaction is

to replace the cell lines commonly used for cell encapsulation with naive cells, such as stem cells. Human mesenchymal stem cells (hMSCs) show promising properties as a cell of choice for cell microencapsulation and cell-based therapy. MSCs improve the biocompatibility of the microcapsules *in vivo*, and can serve as a platform for continuous long-term delivery of therapeutic factors, including potent cancer therapies [144].

4.6. Other issues

As previously mentioned, a gentle encapsulation technique is required if viability of the entrapped cells is aimed. In addition, an important issue that involves the use of spherical-shaped microcapsules mainly, is the formation of local domains of necrotic spots due to inadequate internal oxygen mass transfer. Various alternatives have been proposed to overcome this obstacle. On the one hand, as previously mentioned, Sakai *et al.* developed alginate–agarose subsieve-size capsules of less than 100 μm in diameter to improve oxygen transfer into the capsule where cell viability was observed not to be affected by the small size of the capsules [146]. Alternatively, Khattak *et al.* included synthetic oxygen carriers (perfluorocarbons) in alginate gels to improve oxygen supply. An enhancement in metabolic activity and cell viability was detected due to a reduction in anaerobic glycolysis which resulted in an increase in glucose consumption/lactate production efficiency [147].

Another challenge in the field of cell microencapsulation is the ability to monitor the implanted devices. Once microcapsules are transplanted, the only way until recently was to assess their functional state is through invasive recovery surgery. Fortunately, imaging technologies have made possible an accurate non-invasive follow-up of engrafted tissues [148,149]. Non-invasive imaging techniques using various reporter genes are complementary to *ex vivo* molecular–biological assays and include additional spatial and temporal dimensions.

An alternative interesting approach to overcome this situation has also been recently proposed by Barnett *et al.* using alginate-based radiopaque microcapsules containing either barium sulfate or bismuth sulfate which could be monitored by X-ray [150]. However, although cell viability and capsule permeability were not affected by radiopaque agents it should be mentioned that the metals employed in this work are toxic both for the encapsulated cells and the recipient. In a recent study by Fu *et al.*, the group demonstrated that incorporation of perfluorooctylbromide into alginate–PLL microcapsules may allow easy X-ray tracking, potentially providing scientists in the field with a further tool to understand and improve cellular distribution following implantation [151]. Additionally, magnetic resonance-guided imaging of magnetocapsules (alginate microcapsules elaborated using Feridex®) has also been proposed and could be considered an interesting non-invasive

approach which might ease the *in vivo* detection of implanted devices [152].

Regarding the use of polymers for cell encapsulation, while both natural and synthetic polymers can be used for the preparation, natural polymers are more cell compatible, react under milder conditions and allow for the encapsulation of fragile cells, but the challenge in producing such uniform capsules is to ensure excellent repeatability and reproducibility both within and between batches [31]. A great deal of research work is still needed in order to obtain an increased number of commercially available and clinically successful natural-based systems. Undoubtedly, natural-origin polymers or nature-inspired materials appear as the natural and desired choice for the referred applications [153].

Despite many advances, researchers in the field of cell microencapsulation still face significant challenges regarding the optimization of scaffolds for each specific application. Scaffolds play an essential role as the extracellular matrix but they are often unable to mimic the exact microenvironment to promote the correct and accurate response. The emerging and promising next generation of engineered biomaterials is directed to producing scaffolds with an informational function, e.g., biomaterials containing sequences of growth factors which ease cell attachment, proliferation and differentiation; far better than non-informational polymers. The use of growth factors has been considered as an alternative to modify not only the host healing response at the site of injury to facilitate tissue repair, but also to manipulate and enhance the *in vitro* tissue growth in order to produce more biofunctional tissues. Hence, the strategy is to model the extracellular matrix and provide the necessary information or signaling for cell attachment, proliferation and differentiation to meet the requirement of dynamic reciprocity for tissue engineering and drug delivery.

5. Therapeutic applications

In this part of the article, the effect of microencapsulated-cell therapies on different disorders will be presented in addition to commenting on available scientific data in this area.

5.1. Diabetes

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Current research efforts towards therapy of type 1 diabetes are aimed at developing approaches for restoration of regulated insulin supply. Transplantation of islets of Langerhans has been proposed as a safe and effective method for treating patients with insulin-dependent diabetes mellitus, although it is still, an experimental procedure. In fact, the exciting improvements in outcomes following clinical islet transplantation using the 'Edmonton protocol', have renewed hope for patients with type 1 diabetes [154]. The protocol is based on the use of human islets from cadaveric donors, which are implanted in the liver of carefully selected diabetic recipients via portal vein injection. However, the limited availability of human tissue and

the need for lifelong immunosuppression which results in long-term side effects, makes the widespread application of this therapy difficult.

Using islets of Langerhans from other species is an obvious way of providing the large amounts of functional tissue required for transplantation therapy. In 1980 Lim and Sun implanted microencapsulated xenograft islet cells into rats and the microencapsulated islets corrected the diabetic state for several weeks [45]. Since then, there has been considerable progress toward understanding the biological and technological requirements for successful transplantation of encapsulated cells in experimental animal models, including rodents and non-human primates. Bioartificial pancreatic constructs based on islet microencapsulation could eliminate or reduce the need for immunosuppressive drugs and offer a possible solution to the shortage of donors, as it may allow for the use of animal islets or insulin-producing cells engineered from stem cells [4,155,156].

Different polymers have been used for islet encapsulation and immunoprotection, photopolymerized poly (ethylene glycol) (PEG) [157], water insoluble polyacrylates [158,159], sodium cellulose sulfate [160], agarose [161], chitosan [162] and alginate [163]. Among others, alginate-based microcapsules are widely used vehicles for introducing islets into the body. Several experiments have demonstrated that these polymeric microcapsules could be useful in the treatment of diabetes. Elliot *et al.* [164] have tested some microencapsulated piglet islet formulations into mice and monkeys and noted amelioration of disease. In another study with a placebo-controlled design [165], researchers assessed the safety and clinical activity of alginate-encapsulated porcine islets in a non-human primate model of streptozotocin-induced diabetes. They noted worsening of the disease in control animals: six out of eight control monkeys required increased doses of daily insulin; in contrast, six of the eight islet-transplanted monkeys had reduced insulin requirements. After islet transplantation, individual blood glucose values varied and one monkey was weaned off insulin for 36 weeks. In a recent study which reports the use of intraperitoneally implanted encapsulated allografts, type 1 diabetic patients remained nonimmunosuppressed but were unable to withdraw exogenous insulin [166,167].

In the last few years, the renewed interest in porcine islet xenotransplantation has generated some controversy about the human clinical trials carried out. The study by Living Cell Technologies Ltd. with the Diabecell® device (neonatal porcine islets encapsulated in alginate microcapsules) provided evidence of improvement in glycemic control individuals and showed no evidence of porcine viral nor retroviral infection. Moreover, they reported evidence of residual, viable, encapsulated porcine islets being retrieved from a patient 9.5 years after transplantation [168]. However, this approach has been criticized by the International Xenotransplantation Association as being premature and

potentially risky [169]. Recent progress in this area, like the use of closed, porcine endogenous retroviruses free (PERV-free) herds or advances in immunoisolation may help to improve the formulations. In fact, a new open-label investigation about the safety and effectiveness of Diabecell® in patients with diabetes type 1 is currently recruiting patients (NCT00940173) [170].

Besides alginate, polyethylene glycol (PEG) is widely used for islet encapsulation. The immobilization of PEG chains to the cell or tissue surface creates a molecular barrier preventing molecular recognition between cell surface receptors and soluble ligands. Therefore, surface PEGylation has been used to improve the biocompatibility of islets [171,172]. Islet surfaces have been isolated either with a conformal PEG coating, a technique in which a polyethylene glycol pre-polymer is photopolymerized around an islet [173], or by direct covalent modification of the protein surfaces of islets [174]. In an *in vitro* study performed with PEG-grafted islets cultured with peritoneal macrophages and splenic lymphocytes, it was concluded that the grafted PEG molecules onto the islets could efficiently prevent the activation of immune cells and secretion of cytokines. However, grafted PEG molecules do not completely prevent the infiltration of the cytotoxic molecules into the islets [175]. Subsequently, these authors [176] have evaluated the clinical potential of a new combinatorial therapy based on PEGylation and immunosuppressant therapy with low doses of cyclosporine A (CsA). For 1 year after transplantation, PEGylated islets firmly controlled blood glucose levels, and enabled normal blood glucose responsiveness, hormone synthesis, and the existence of PEG molecules at transplanted islets, suggesting that a PEGylation/CsA combinatorial therapy could semipermanently protect transplanted islets from immune reactions at least in the rodent model. This technology is currently the basis for Phase I/II clinical trials by Novocell for encapsulated human islet allografts implanted into the subcutaneous site. The trials began in 2005 (NCT00260234) [170].

Eventhough alginate and PEGylated microcapsules are being tested in clinical trials, biocompatibility, immunoprotection and hypoxia [143,177] are main issues that need to be improved. A number of different strategies have been proposed as potential solutions to overcome these problems. The use of growth factors may be useful for therapeutic stimulation of neovascularization, which may improve the survival and function of microencapsulated islets at the transplantation site by allowing for adequate oxygen and nutrient exchange, as well as removal of waste products between encapsulated islets and the systemic circulation [178,179]. Besides avoiding islet hypoxia, the improvement of the biocompatibility of islets after transplantation is essential. On this respect, in a recent paper, Teramura & Iwata [180] have proposed a novel method using a layer of HEK293 living cells for islet encapsulation (Fig. 3). In this context the use of bioactive peptides like the glucagon-like peptide 1 (GLP-1) analog

features an innovative strategy to modify PEG hydrogels which can significantly enhance the efficacy of islet encapsulation [181]. Finally, in order to avoid acute inflammation and its harmful effects on transplanted islets, different approaches have been developed.

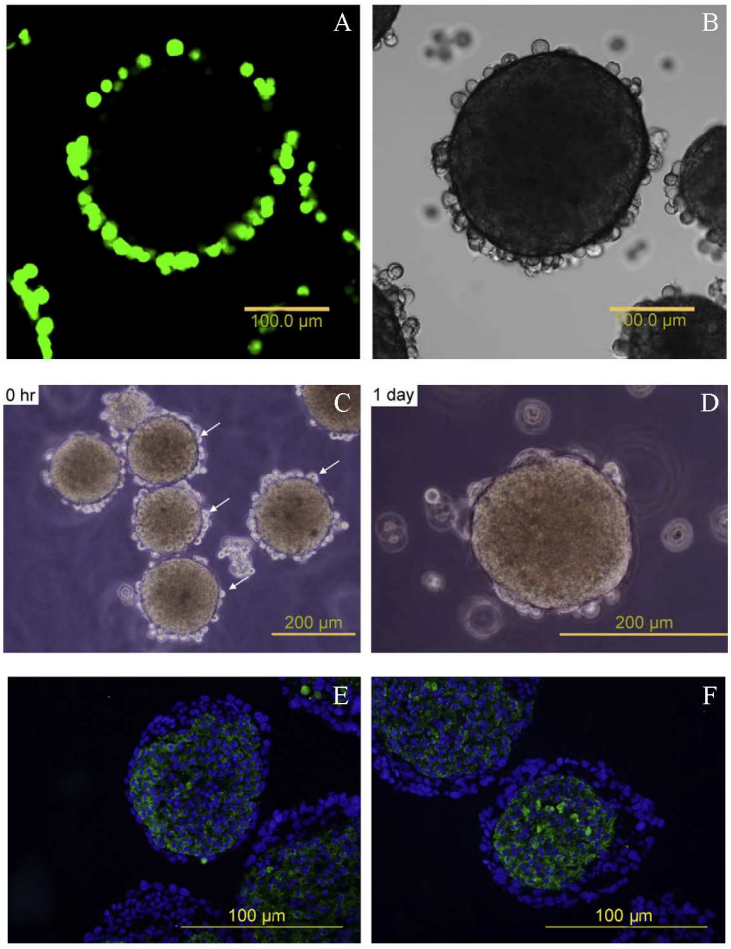

Fig. 3. (A, B) Confocal laser-scanning and differential interference microscope images of surface-modified cells and islets. Hamster islets modified with biotin–PEG–lipid and immobilized with streptavidin-immobilized HEK293 cells. The HEK293 cells were labeled with CellTracker®. (C, D) Phase-contrast microscopy of HEK293 cell-immobilized islets in culture at 0 and 1 days. HEK293 cells were immobilized on the surface of the islets and cultured on a non-treated dish in Medium 199 at 37 °C. Arrows indicate immobilized HEK293 cells. (E, F) Histochemical analysis of HEK293 cell-immobilized islets cultured for 3 and 5 days in medium. Frozen sections of HEK293 cell-immobilized islets were stained with Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. The pictures are merged images from insulin and Hoechst 33342 staining. Reproduced, with permission, from Ref. [180] © 2009 Elsevier Ltd.

For example co-administration with anti-inflammatory drugs [134,182] or modulation of macrophage activation [183,184] among others, are being studied to generate bioactive barriers that locally modulate host response to microencapsulated cells.

Much work is clearly needed before microencapsulated cell therapy for diabetes can be advanced to the clinic. The challenges center on generation of an abundant source of regulated insulin-producing cells and some aspects of the cell-based encapsulation methods should be improved in order to increase the transplant longevity and functional performance of the capsules *in vivo* [4].

5.2. Bone and cartilage defects

Bone defects resulting from trauma and tumor resection are common clinical problems. Bone tissue usually has the ability to regenerate, but when a defect of critical size needs to be bridged, the repair attempt fails in most cases.

The current standard tissue used is autologous tissue, which is usually harvested from the iliac crest of the patient. Although autografting has been a major treatment, it has several limitations including patient pain, cost, and limited supply. As an alternative, allografting has been studied due to its abundant source. However, its drawbacks, including the uncertainty of biocompatibility and disease transmission, have limited its use [185]. On the other hand, articular cartilage has limited capability for healing after trauma and only few long-tem treatments are available today, including mosaicplasty, periosteal transplantation and autologous chondrocyte implantation. To overcome these drawbacks, investigators are considering alternative therapies in which mesenchymal stem cells are involved. MSCs are multipotent progenitor cells that can be isolated from bone marrow, adipose tissue, muscle tissue, umbilical cord blood, peripheral blood, and other tissues [186–188] and have the capability to differentiate into multiple tissue-forming cell lineages, such as osteoblasts and chondrocytes, which contribute to the regeneration of bone and cartilage.

Lately, some works have showed that microcapsules could create a 3D microenvironment that would provide a niche for stem cell growth and differentiation [189]. In this respect, Endres *et al.* [190] have confirmed that, *in vitro*, these hMSC were able to differentiate along the chondrogenic lineage when encapsulated in Ca-alginate microcapsules and stimulated with TGF-β3. The size of these microcapsules is in the injectable range (mean diameter of 600–700 μm) making this administration easier. Furthermore, Ca-alginate might also protect the cells against shear forces during the injection process and overload until they form their own functional extracellular matrix in the defective site. In another study, Abbah *et al.* [191] investigated the effect of confinement within calcium cross-linked alginate microcapsules on the survival of murine adipose-tissue stromal cells (ATSC) with osteogenic potential and their subsequent ability to elicit osteogenic response. It is impor-

tant to emphasize that these microcapsules were superior for murine ATSC proliferation and osteogenic differentiation when compared to the 2D monolayer plastic tissue culture surface. Similar results have been obtained by these authors using rabbit bone marrow cells [192].

Moreover, MSCs can be genetically engineered to over-express the required protein. Thus, using this *ex vivo* gene therapy approach, the microcapsule containing the cells can be both a growth factor delivery carrier and a 3D matrix for cellular activities in repair. Previous studies have demonstrated that bone morphogenetic proteins (BMPs), especially BMP-2, are the most effective in inducing complete bone morphogenesis [193,194]. In the MSCs expressing BMP-2 an important advantage accrues to the MSCs, since the genetically engineered cells feature not only a paracrine effect on the host, but also an autocrine effect on the MSCs themselves [195]. Zilberman *et al.* [8] immobilized adult MSCs expressing rhBMP-2 within APA microcapsules and studied the effect on mice. After subcutaneous administration of capsules a physiological response was elicited and formation of ectopic cartilage and bone in the host was observed. The authors concluded that the angiogenic and osteogenic activities observed outside the capsules are consequences of the paracrine effect of the engineered MSCs. In a parallel experiment, when encapsulated cells were transplanted into a local segmental bone defect, they were also able to form massive bone tissue in the defect. The bone in this case comprised the host cells' response to the paracrine effect of the secreted rhBMP-2. However, either during subcutaneous or bone defect administration, encapsulated MSCs differentiated inside the capsules mainly to cartilage cells. Therefore, microencapsulation of genetically engineered MSCs can be a useful tool to study and distinguish between autocrine and paracrine mechanisms and intercellular interactions.

This approach based on genetically engineered cells to release growth factors has also been assayed for cartilage regeneration. In an interesting study, Paek *et al.* [196] examined the survival and the maintenance of functionality of microencapsulated genetically modified fibroblasts in allogeneic and xenogeneic models. The growth factor released from Ca^{2+}-alginate immobilized cells was human transforming growth factor-β1 (hTGF-β1). This substance is of particular importance in intraoperative procedures for cartilage regeneration because it can induce chondrogenic differentiation or synovial cells. Both allogeneic and xenogeneic transplants could survive and maintain the hTGF-β1 secretory function in mice during 3 weeks. This period of time is long enough since therapy format of intraoperative cartilage repair envisions only 1-week *in situ* delivery of hTGF-β1.

A new design to obtain better cell-based therapies for bone regeneration involves co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres. Together with osteoprogenitor cells, endothelial

cells can regulate their osteogenic potential in bone defects. Recent studies have already shown that osteoblastic differentiation is improved by endothelial cells in 2D culture systems [197,198]. In an elegant study, Grellier *et al.* [199] co-immobilized these two types of cells within RGD-modified alginate microcapsules. Both *in vitro* and *in vivo* studies revealed that osteoprogenitor cells enhanced their mineralization potential when they were co-encapsulated with endothelial cells, thus setting up a promising new injectable strategy for bone tissue engineering.

Other approaches currently under investigation for their potential to repair bone and cartilage include the use of cell-seeded microcarriers [200]. Polymeric microcarriers made up of PLGA have been shown to be suitable as an injectable delivery system for chondrocytes. In a recent report [201] PLGA/gelatin microspheres modified with RGD peptides were used to culture chondrocytes *in vitro*. The results observed were found to be particularly interesting due the fact that RGD sequences significantly improved attachment, proliferation and viability in addition to glycosaminoglycan secretion from chondrocytes. Bioceramics [202], calcium titanium phosphate [203] and hydroxyapatite [204], among others, have been proposed as cell delivery systems. These materials have osteoconductive nature and are extensively used in bone reconstruction.

In a recent paper Wang *et al.* [205] have proposed a novel gellan gel-based microcarrier for anchorage-dependent cell delivery. In this study, the gellan microspheres were covalently coated with gelatin layers to create the cell binding ligands on which human cells, including fibroblasts and osteoblasts, were cultivated for appraisal of cell delivery and developmental efficacy. The *in vivo* results [206] suggest that these microcarriers, combined with a hydrogel, facilitate both survival and differentiation of osteoprogenitor cells, while maintaining their favorable spread morphology in hydrogel matrices. This novel composite system could be beneficial to clinical regenerative medicine in the field of bone engineering. Moreover, specific growth factors or extracellular matrix proteins can be included in the microcarrier to further aid proliferation and differentiation [207]. Hence, microcarriers can play a dual role as both delivery systems of bioactive factors and scaffolds for proliferation and differentiation of cells.

In conclusion, microcarriers made from a wide range of established and novel biomaterials are being evaluated for their ability to facilitate growth and differentiation, in addition to their capability to meet the criteria for resorption post tissue-implantation. This is the driving force for emerging opportunities and the further application of the microcarrier culture in the field of bone and cartilage tissue engineering [200].

5.3. Neurological diseases

Human neurological disorders such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease

(AD), stroke, and spinal cord injury (SCI) are caused by a loss of neurons and glial cells in the brain or spinal cord. In recent years, the transplantation of cells to the brain and/or spinal cord has been pursued as a potential curative treatment for a broad spectrum of human neurological diseases [208,209]. Promising results have already been achieved in clinical trials, but much remains to be done before cell-based therapies can be practiced extensively.

One source of transplantable cells is the choroid plexus (CP). In addition to its well-defined role in cerebrospinal fluid (CSF) production and maintenance of extracellular fluid concentrations throughout the brain, the CP secretes numerous endogenous neurotrophic factors with therapeutic potential [210]. These primary cells are currently under study for the treatment of neurodegenerative disorders. Emerich *et al.* developed an alginate-based encapsulation system where CP were immobilized in an effort to achieve an appropriate delivery of neurotrophic factors to the brain in rodent [211] and primate models of Huntington's disease. In this latter study [212], cynomolgus primates received stereotaxic transplants of either empty capsules or porcine CP-loaded capsules directly into the caudate and putamen. One week later, they received unilateral injections of the excitotoxin quinolinic acid (QA). Researchers reported that QA administration produced a large lesion in both the caudate and putamen in monkeys receiving implants of empty microcapsules. In contrast, the size of the lesion was significantly reduced (5-fold relative to control implanted monkeys) in animals receiving identical QA lesion but implants of encapsulated CP. It seems then that implants of alginate-encapsulated porcine CP prevent the degeneration of striatal neurons typically occurring after QA intrastriatal injections. Similar results have been obtained in a rodent model of stroke [213]. The *in vivo* studies in a well-established middle cerebral artery occlusion model demonstrated that encapsulated CP significantly reduced the extent of cerebral infarction and associated behavioral deficits.

Another important neurodegenerative disorder to take into consideration is Parkinson's disease, characterized by an extensive loss of dopamine neurons in the substantia nigra pars compacta and their terminals in the striatum. An interesting approach, using gelatin microcarriers (Spheramine), has been tested as a new drug delivery system for the treatment of this disease. It consists of an active component of cultured human retinal pigment epithelial cells (hRPE), attached to a cross-linked porcine gelatin microcarrier. In this case immunosuppression was not required because RPE cells are isolated from post mortem human eye tissue. Currently, it is postulated that the ability of hRPE cells to produce levodopa in the biosynthetic pathway for melanogenesis may serve as the rationale for a therapeutic effect, but a role of trophic factors cannot be excluded. The studies carried out in PD animal models of unilateral 6-hydroxydopamine (6-OHDA) striatal-lesioned rats and

MPTP-induced hemiparkinsonian Macaca mulatta monkeys determined the preclinical efficacy of hRPE cells [214,215]. Afterwards, in a Phase I clinical trial in humans the safety and efficacy of Spheramine implanted in the putamen of patients with advanced PD was evaluated [61]. However, the therapeutic potential of this platform has been questioned due to the recently announced failure of the commercially sponsored Phase II clinical trial [216]. While an in-depth analysis of this disappointing result must await publication of the details of this trial, Falk *et al.* postulated that the failure to account for the role of neurotrophic factors could be an important aspect and the verification of high expression of these factors, and not only for levodopa should be considered [62].

Another interesting therapeutic application employing genetically engineered cells, instead of primary cells includes the immobilization of growth factor-producing fibroblast for delivery of therapeutic products to the injured spinal cord (SCI). This pathology results in the disruption of ascending and descending axons that produce a devastating loss of motor and sensory function. Several strategies have been used to provide trophic and antiapoptotic molecules that can alter the environment of the injured central nervous system. In this sense, various studies [3,217] have pointed out that primary fibroblasts genetically modified to produce brain-derived neurotrophic factor (BDNF) survived in the injured spinal cord of adult Sprague-Dawley rats rescuing axotomized neurons, promoting regeneration and contributing to recovery of locomotor function. However, immunosuppression was needed to prevent rejection of grafts. As an alternative, cell microencapsulation could replace immune suppression by protecting the cells after grafting. In 2005 Tobias *et al.* [218] reported that alginate–poly-L-ornithine microcapsules containing BDNF-producing fibroblasts grafted into a nonimmunosuppressed SCI murine model resulted in partial recovery of forelimb usage in a test of vertical exploration and of hindlimb function while crossing an horizontal rope. Moreover, compared with the animals that received unencapsulated BDNF-producing cells without immunosuppression, the recovery was significantly higher. However, results were similar to those of immunosuppressed animals that had received unencapsulated cells. The study also showed no evidence of regeneration of rubrospinal axons in mice implanted with encapsulated cells presumably because the amounts of BDNF available from the encapsulated graft were substantially less than those provided by the much larger numbers of cells grafted in the nonencapsulated formulation in the presence of immunosuppression. Taken together, these results suggest that improvements in cell encapsulation systems to release higher concentrations of neurotrophin are required to stimulate regeneration of axotomized brainstem neurons. Thus, after optimization of the drug delivery system, nonautologous engineered cells immobilized in microcapsules could be a feasible

approach for recovery of lost function in the injured spinal cord.

Future efforts will need to systematically approach each potential clinical indication with emphasis on optimizing the cell source, determining which cells are most beneficial, identifying the optimal post injury timing, transplant location and dosage of cells to be grafted. In addition, reducing the diameter of cell-enclosing capsules designing subsieve-size capsules [219], could be an interesting alternative for drug delivery in the CNS.

5.4. Cancer

One attractive approach to the treatment of disseminated cancer is to enhance the immunogenicity of tumor cells, thus allowing systemic immune-mediated tumor cell death. Several different immune-mediating products, such as cytokines, assayed in the clinic, result in enhanced immunity and decreased tumorigenicity. For example, IL-12 and TNF-α encapsulated in polylactic acid microspheres have been implanted intratumorally into an experimental fibrosarcoma model (MCA205 cell line) generating a systemic anti-tumor immune response capable of eradicating distant disease [220]. However, the major disadvantage of these polymeric biodegradable microspheres lies in the fact that multiple capsules would be required to maintain continuous delivery. To overcome this problem the use of microcapsules containing cells secreting cytokines has been proposed. Cirone *et al.* [7] described a sustained release of IL-2, into tumor-bearing mice, after a single implantation of nonautologous mouse myoblasts (C_2C_{12}), genetically modified to secrete the cytokine, immobilized in microcapsules. The treatment with these encapsulated cells led to a delay in tumor progression and prolonged survival of the animals. However, the long-term efficacy was limited by an inflammatory reaction against the implanted microcapsules probably because of the secreted cytokine and the antigenic response against the xenogeneic fusion protein itself. Hence, although efficacious in suppressing tumor growth initially, this immune therapy protocol only produced a transient effect, with the tumors resuming growth after a delay of 15 days.

Another focus of interest in the treatment of cancer is angiogenesis inhibition. Tumors are dependent for their growth on the development of a blood vessel network and may trigger angiogenesis by release of specific growth factors such as the vascular endothelial growth factor (VEGF). Thus, the growth of tumors can be suppressed by inhibiting angiogenesis. In 2001 two independent groups [20,221] treated glioma models of cancer with encapsulated xenogenically derived cell lines genetically modified to secrete endostatin, one of the most potent antiangiogenic drugs that can directly induce apoptosis in tumor cells. Both groups reported that local delivery of endostatin significantly inhibited tumor growth. Nevertheless, local delivery of drugs might not be feasible in the treatment of many tumors, especially for metastasis. In this respect, in a

recent study by Teng *et al.* [222], Chinese hamster ovary (CHO) cells engineered to secrete human endostatin encapsulated in APA microcapsules were transplanted into peritoneal cavities of mice bearing subcutaneous B16 melanoma. The results proved that the intraperitoneally implanted microencapsulated cells could significantly inhibit the subcutaneous growth of melanoma in mice. Using a similar approach Cirone *et al.* [223] tested angiostatin to treat a murine model of melanoma/breast cancer. When angiostatin was delivered systemically by implanting microencapsulated cells genetically modified to express angiostatin, tumor growth in the recipient animals was suppressed by >90% 3 weeks post-tumor induction, while survival at this date was 100%, compared to 100% mortality in the untreated or mock-treated controls.

Currently, simultaneous delivery of cytokines and antiangiogenic drugs is being explored for cancer therapy. Cirone *et al.* examined a two-pronged strategy by delivering interleukin 2 fusion protein (immunotherapy) and angiostatin (antiangiogenic therapy) concurrently via microencapsulated cells, to evaluate their potential synergism in tumor suppression. Two different methods have been used, capsules fabricated to contain a mixture of both cell types, each type delivering the desired therapeutic product [224] or a mixture of different capsules, each one containing a single specific cell type [225]. The best results were obtained with IL-2-secreting cells and angiostatin-secreting cells encapsulated in separate microcapsules and implanted at different times post-tumor induction. Thus, the use of a combined strategy with microcapsules containing cells engineered to release different molecules, with anti-tumor properties targeting multiple pathways, opens up new possibilities in the treatment of cancer.

The use of encapsulated cells overexpressing enzymes that can activate chemotherapeutic agents or prodrugs, offers a promising mean to treat tumors [226]. The first demonstration to prove the use of this method in treating solid tumors was provided in 1998, in a murine model of pancreatic cancer [227]. In this study, feline kidney epithelial cells genetically modified to over-express a cytochrome P450 enzyme (CYP) were encapsulated in polymers of cellulose sulfate. In this study, encapsulated CYP overexpressing cells were implanted into xenograft tumors and this was then followed by multiple administrations 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 result in tumor reductions and, in some mice, even complete loss of the tumor. The data could be reproduced using encapsulated HEK 293 cells overexpressing the same CYP [228]. Based on these results NovaCaps®, an encapsulated cell therapy product was developed and tested in a Phase I/II clinical trial. 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 [229,230]. Therapeutic benefit includes a 100% improvement in median survival over a control group and 1 year survival rates, which were almost twice as high as those documented after treatment with the current gold standard treatment, gemcitabine. Recently [231], 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).

The findings reviewed demonstrate the advantages of this delivery system, which allow continuous release of biologically active products for the treatment of cancer. However, the encapsulated nonautologous cells secrete cytokines and shed antigens, which evoke a host immune response and lead to inflammatory tissue surrounding the capsules that may cause damage to the graft. In order to overcome this shortcoming, stem cells could replace the cell lines commonly used in cell microencapsulation. Moreover, as previously mentioned, mesenchymal stem cells are hypoimmunogenic and can be genetically modified to express a variety of therapeutic agents. In a recent work, Goren *et al.* [144] encapsulated genetically engineered hMSCs to express hemopexin like protein (PEX), an angiogenesis inhibitor, in alginate–PLL microcapsules and tested the efficacy of the microencapsulation system in a model of human glioblastoma. The results revealed a significant reduction in the tumor volume 22 days post-treatment (89%) when compared with the tumor sizes of the control groups. On the other hand, immunohistological studies demonstrated a decrease in blood vessel formation and tumor cell proliferation and an increase in tumor cell apoptosis. These findings indicate the potential of microencapsulated engineered hMSCs to serve as a platform for therapeutic applications in several pathologies like cancer.

Finally, immunoisolation of cells to deliver vaccines is another concept in cancer therapy. The administration of recombinant purified antibodies is an expensive process. Hence, delivery of antibodies by encapsulated cells could be an alternative and more cost-effective method [232].

In conclusion, the use of encapsulated cells has great potential as the basis for the treatment of various forms of cancer. This section has attempted to summarize preclinical and clinical data from some of the more promising strategies involving encapsulated cells to treat tumors. The authors believe that these results represent a glimmer of hope particularly for those tumors representing an unmet medical need.

5.5. Heart diseases

Heart failure is a leading cause of morbidity and mortality worldwide. The critical cause of heart failure is myocardial ischemia, resulting in dysfunction and death of cardiomyocytes. The main cardiac response to myocardial infarction can be seen as cardiomyocyte hypertrophy, apoptotic myocyte loss, progressive collagen replacement, and

enlargement of the left ventricle [233]. Progenitor or stem cells constitute a new prospect in the confrontation of cardiac insufficiency [234]. The beneficial action of transplanted cells is under investigation and numerous trials examine their potential active contribution to myocardial contractility, indirect improvement of cardiac mechanisms, induced neovascularization, and other likely actions in the biological operation of the heart [235–237]. The mechanism by which stem cell-based therapy causes beneficial effects on cardiac function remains unclear and it may be possible that the beneficial effects observed in cell therapy are due to multiple mechanisms; one system alone may not account for all observed improvements in cardiac function. However heart regeneration is still confronted with great controversy. Moreover, several delivery routes have been investigated for cell administration [238], but cell retention following implantation remains a major challenge in such cases. The heart constantly contracts which contributes to the mechanical loss by squeezing the injected cells out of the myocardium. Of all the injected cells, not more than 5–15% are retained within the myocardium. In a recent study performed using fluorescent microspheres similar in size to mesenchymal stem cells, it has been suggested that mechanical loss may occur early, as cells are "squeezed" by the mechanical forces of the heart into the vasculature. Artificial stem cells (APA microcapsules containing stem cells) with a diameter of 200 μm, significantly increased the amount of retained microspheres in rat hearts because the size of APA microcapsules was larger than the blood vessel diameter and the contractive forces of the heart were unable to wash out the capsules into the bloodstream. The microencapsulated stem cells may exert a beneficial influence on myocardial regeneration by means of a paracrine growth factor effect, but if a mechanism of transdifferentiation or fusion for tissue regeneration is required, the microcapsule can be elaborated to be more biodegradable [24] so as to promote or enhance cell–cell contacts. These results suggest that these microencapsulated stem cells may have much greater potential for heart regeneration in comparison to free stem cells [31]. Fig. 4 shows retention of microencapsulated cells in heart muscle in comparison to free cell delivery.

There is another very interesting and attractive cell-based microencapsulation therapy that has been applied in heart regeneration. A growing body of evidence has indicated that supplementation of angiogenic factors can stimulate new blood vessel growth (neovascularization) and restore perfusion in damaged or ischemic myocardium [239,240]. Thus, the use of angiogenic factors in the infarcted area could be an alternative especially for patients who are not suitable for conventional revascularization treatment [241]. Using this approach, Zang *et al.* [242] enveloped xenogeneic CHO cells engineered to express VEGF into APA microcapsules and delivered them into the infarcted myocardium of rats 3 weeks after left anterior artery ligation. The major finding in this 21-day *in vivo*

study was that VEGF secreted by locally implanted microencapsulated engineered CHO cells could augment angiogenesis and improve global heart function in the post-infarction myocardium. In addition, the microcapsules could suppress or delay the immune response to the implanted xenogeneic graft for at least three weeks. Thus, the results obtained suggest that microencapsulated xenogeneic cell-based gene therapy might be a novel alternative strategy for therapeutic angiogenesis in ischemic heart disease.

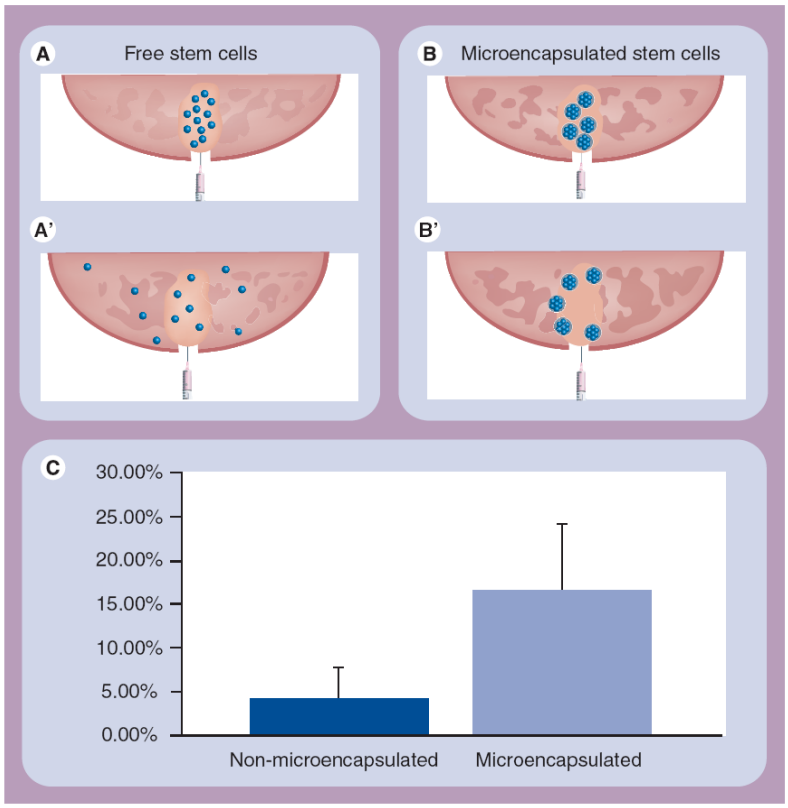

Fig. 4. Mechanism for retention of microencapsulated stem cells for cellular cardiomyoplasty. Stem cell delivery without (A) and with microencapsulation (B) into the beating heart through intramyocardial injection. Pictures denote the myocardial cross-sections with numerous blood vessels where the needle disrupts the vascular bed once pierced into it. A 'complete washout' of the injected free stem cells into the blood vessels is seen in (A′) while (B′) depicts a retention of the microcapsules, encapsulating the stem cells. A comparison of the percentage retention of free microspheres and microspheres encapsulated in microcapsules in the beating heart of rat models is shown in (C). There was a statistically significant difference in the percentage of retention between the non-microencapsulated versus the microencapsulated microspheres ($P<0.05$). Reproduced, with permission, from Ref. [31] © 2009, Future Medicine Ltd.

5.6. Other diseases

As already mentioned above, cell microencapsulation has been considered a promising possibility for the long-term treatment of chronic pathologies, since it could alleviate the shortcomings of conventional formulations which usually require multiple administrations to obtain a therapeutic effect; Jeon *et al.* [243] have recently proposed the implantation of encapsulated bovine-derived chromaffin cells for the treatment of chronic neuropathic pain. Adrenal chromaffin cells have been reported to release catecholamines and metenkephalin which produce analgesic effects in the central nervous system [244]. Using a rat model of neuropathic pain, the intrathecally implanted xenogeneic cells resulted in high levels of catecholamines and metenkephalin demonstrating the effectiveness of the developed system.

In the field of liver failure, microencapsulation has been used as an alternative to directly injecting hepatocytes, creating a living cell-based replacement system. *In vivo* studies in Gunn rats showed that alginate–PLL encapsulated hepatocytes decreased serum bilirubin levels for up to 4 weeks while increasing the survivability of these animals by up to 80% when subjected to a model of fulminant hepatic failure [245,246]. However, no such function has been demonstrated over longer periods of time. Research in recent years has suggested that there are several different methods that have the potential to maintain the specific function and phenotype of the bioencapsulated hepatocytes such as the co-encapsulation of hepatocytes with other types of cells, the so called "feeder cells". In this respect, Liu [247] and Chang [248] have reported that both in syngeneic and xenogeneic *in vivo* transplantation studies, viability of hepatocytes can be maintained longer when encapsulated with bone marrow cells. In addition, transplantation of both cell types co-encapsulated improved the ability of hepatocytes to correct congenital hyperbilirubinemia in Gunn rats. Thus, bone marrow cells play an important role as a new type of feeder cells for bioencapsulated hepatocytes for liver disease cellular therapy. Moreover, this group [249] has demonstrated that cell encapsulation can enhance *in vivo* transdifferentiation of bone marrow cells into hepatocyte-like cells. These findings suggest the potential of stem cell microencapsulation as a new alternative to employing hepatocytes.

Last but not least, our research group succeeded in a long-term *in vivo* assay where genetically engineered erythropoietin (Epo)-producing C_2C_{12} myoblasts immobilized in APA microcapsules were implanted in allogeneic and syngeneic mice [5,44,250]. High and constant hematocrit levels were maintained during the study periods after only one shot of cell-loaded microcapsules and without implementation of immunosuppressive protocols. Subsequently, on a recent *in vivo* study carried out in our laboratory, we have shown that combination of cell encapsulation and transient immunosuppression (FK-506, 4 weeks)

can induce host acceptance of xenogeneic cells [27]. Finally, in an attempt to investigate the possibility of designing biomimetic cell–hydrogel capsules to promote the *in vivo* long-term functionality of the enclosed cells and improve the mechanical stability of the capsules, we fabricated biomimetic alginates by coupling the RGD peptide to alginate polymer chains. These novel biomimetic capsules provided cell adhesion for the enclosed cells and prolonged their long-term functionality and drug release for more than 300 days [67]. An important challenge to overcome in the future is the regulation of the delivered Epo in order to avoid too high hematocrit levels which could lead to undesired side effects such as polycythemia. This alternative technology could avoid the repeated weekly injections currently practiced in anemic patients.

6. Concluding remarks

Since the early pioneering period, the technology of mammalian cell encapsulation has developed significantly. These examples represent only some of the current applications of cell encapsulation but authors believe this technology may see exciting improvement in the next few decades. Future research must focus on the development of a technically advanced capsule technology to satisfy the demands of the GMP guide lines for large-scale transplantation. This ambitious goal requires the interdisciplinary and integrated effort of scientists with different areas of expertise such as genetics, materials science, physicochemistry and chemical engineering, pharmaceutical technology, biology and medicine. Issues on long-term viability, risk of immune development, related safety and retrieval of the unwanted cells, together with the development of high biocompatible polymeric membranes, with sufficient durability and appropriate permeability, should be addressed to further explore their possible clinical applications. Due to the major advantages cell microencapsulation offers as a living drug delivery system, its practical importance will continue increasing in the future.

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Emerging Technologies in the Delivery of Erythropoietin for Therapeutics

Ainhoa Murua, Gorka Orive, Rosa Mª Hernández and José Luis Pedraz

Laboratory of Pharmacy and Pharmaceutical Technology, Networking Biomedical Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, SLFPB-EHU, Faculty of Pharmacy, University of the Basque Country, 01006, Vitoria-Gasteiz, Spain

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Abstract: Deciphering the function of proteins and their roles in signaling pathways is one of the main goals of biomedical research, especially from the perspective of uncovering pathways that may ultimately be exploited for therapeutic benefit. Over the last half century, a greatly expanded understanding of the biology of the glycoprotein hormone erythropoietin (Epo) has emerged from regulator of the circulating erythrocyte mass to a widely used therapeutic agent. Originally viewed as the renal hormone responsible for erythropoiesis, recent in vivo studies in animal models and clinical trials demonstrate that many other tissues locally produce Epo independent of its effects on red blood cell mass. Thus, not only its hematopoietic activity but also the recently discovered non-erythropoietic actions in addition to new drug delivery systems are being thoroughly investigated in order to fulfill the specific Epo release requirements for each therapeutic approach. The present review focuses on updating the information previously provided by similar reviews and recent experimental approaches are presented to describe the advances in Epo drug delivery achieved in the last few years and future perspectives.

Keywords Erythropoietin; Sustained delivery; Cell-based therapy; Cell microencapsulation; Neuroprotection; Angiogenesis.

1. Introduction – Erythropoietin biology and mechanism of action

Erythropoietin (Epo) is a low-molecular (30.4 kDa) pleiotropic glycoprotein consisting of 165 amino acids, which plays an hormonal role in the stimulation and maintenance of erythropoiesis (maturation of erythroid progenitor cells into mature red blood cells (RBC)) and erythrocyte differentiation [1]. The molecule contains up to 14 sialic acid residues [2,3]. Epo exists as a mixture of several isoforms differing mainly in their glycosylation (which results in different plasma half-lives) [4]. Differences have been found in isoform compositions of serum and urinary Epo [5] and among Epos obtained from subjects under different physiological conditions [6].

Epo leads to inhibition of apoptosis of the blast cell lineage (thus increasing their survival) [7–10] by regulating the dynamic balance between erythropoiesis and erythrocyte loss within the circulation to provide adequate tissue oxygenation [11,12], but can also mediate other effects directed toward optimizing oxygen delivery to tissues demonstrating the concept of communication and interaction between organs and systems [13–17].

Epo is biosynthesized during the fetal life from hepatocytes and by renal instersticial fibroblast-like cells in the peritubular capillary bed of the kidney cortex and perivenous hepatocytes in the liver in adults [18,19]. The liver accounts for 10–20% of the Epo production: hepatocytes surrounding central veins are responsible for most of the Epo [20,21]; however, other Epo producing cells are present in the liver and they share many similarities with the fibroblast-like interstitial cells of the kidney [19].

Its gene expression is induced by hypoxia-inducible transcription factors (HIFs) mainly at the mRNA level [22]. HIF-1 is a transcription factor and regulates the transcription of genes whose protein products are involved in energy metabolism, angiogenesis, erythropoiesis and iron metabolism, cell proliferation, apoptosis, and other biological processes [23]. Human genetics can offer important insights into proteins that play key roles in medically relevant pathways. In recent years, there have been advances in understanding the mechanism of oxygen sensing in mammals and its relevance to human disorders of RBC control. This has enhanced our understanding of the molecular mechanisms by which RBC mass is regulated in humans [24].

Epo binds to a specific membrane receptor molecule (Epo-R), a 66 kDa membrane protein with 507 amino acids, that belongs to the cytokine class I receptor superfamily. Some members of this family include the IL-3 receptor, the IL-4 receptor, the IL-6 receptor, the prolactin and growth hormone receptor, and the GM-CSF receptor [25,26]. The binding of Epo to its receptor triggers a conformational change of the preformed Epo dimer receptor, and subsequent activation of several downstream pathways that promote cell survival [27–30]. Autophosphorylation of the Janus kinase (JAK-2) signal

transducer activates STAT-family, phosphatidylinositol 3-kinase (PI3K)/Akt, and the Ras-mitogen-activated protein kinase (MAPK) signal transduction pathways (Fig. 1) [31].

Other compounds such as second-generation antipsychotics (SGAs) have also been found to regulate the Epo/Epo-R expression. Pillai *et al.* suggested that SGAs may exert a neuroprotective effect and trigger neuroplasticity through expression of Epo [32].

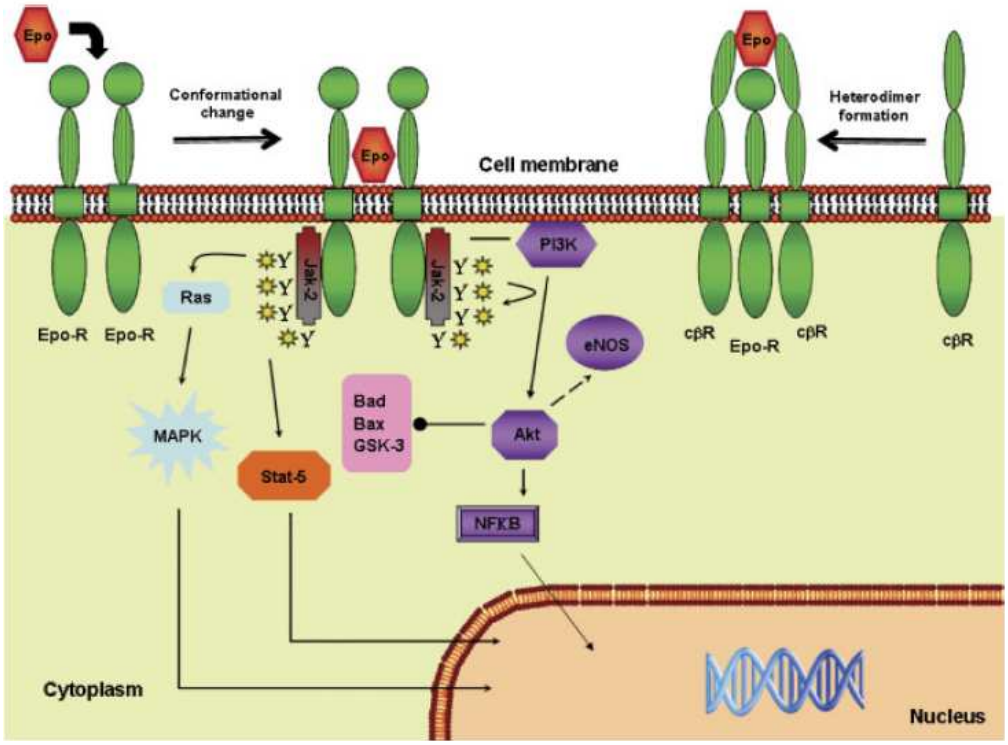

Fig. 1. Simplified scheme showing the effects resulting from Epo binding to its cognate receptor (Epo-R) on the cell surface. After Epo binds to Epo-R the following steps can be identified: Epo-R conformational change, JAK-2 autophosphorylation, Epo-R phosphorylation at several tyrosine residues creating docking sites for different transduction pathways. At least three pathways are subsequently involved. In addition, the common ß receptor (cßR/CD131) is also described (related to tissue protection function related to Epo) and the proposed conformational change to activate signaling cascades that mediate tissue protection.

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, Epo might also be in part removed from circulation by uptake into erythrocytic and other cells possessing the Epo receptor [33].

2. Extra-erythropoietic actions of Epo: opening new frontiers

For many years, Epo has been identified as the renal hormone that stimulates erythroid progenitors within the bone marrow to mature into RBCs. However, there is increasing evidence suggesting a wider biological role of Epo/Epo-R not related to erythropoiesis. Many experimental and clinical studies have stated however that in addition to the two main sites of secretion, low levels of Epo mRNA have also been detected in lungs [34], testes [35], enterocytes, breast gland and human milk [36], spleen [37], bone marrow macrophages [38], placenta [39], astrocytes [40], neurons [41], and the mouse ischemic heart [42,43], suggesting that Epo is a multifunctional trophic factor, with many other physiological roles, a tissue-specific regulation, and several mechanisms of action [44].

Trophic factors are proteins that support and protect specific cellular subpopulations [45]. The ability of one compound to elicit multiple effects is a general characteristic of biological systems. While these actions can be complementary, the physiological roles are often qualitatively different. Typically, multifunctional molecules that trigger widely different biological responses do so by utilizing different receptor isoforms with markedly different binding affinities for the cognate ligand [46].

In fact, the action of Epo does not seem to be limited to controlling proliferation and differentiation of erythroid cells. It overcomes the effect on the hematopoietic system [47–49] and functional receptors for Epo have been found in both nonerythroid blood cell lines and a variety of nonhematopoietic cells, suggesting new roles in nonhematopoietic tissues. These include brain (astrocytes and neurons, both provided with specific Epo-R), cardiovascular system (cardiomyocytes, endothelium, and vascular smooth muscle), spinal cord, reproductive organs, skeletal muscle, retina, gastrointestinal tract (gut and pancreas), and lung [50–62]. Moreover, Epo expression seems to be regulated in a tissue-specific manner (Table I) [44]. Many studies (including a phase II clinical trial in ischemic stroke) demonstrate that rHuEpo protects the brain, spinal cord, heart, kidney, and retina from injury and improves cognition [63–65].

However, effective use of Epo as therapy for tissue protection requires higher doses than for hematopoiesis and thus the long plasma half-life of Epo might potentially trigger serious adverse effects due to the continuous activation of the hematopoietic receptor and thus erythropoiesis. The good news is that ongoing studies are enabling to better understand both the Epo molecule and its receptors (Epo-R) in which locally produced Epo has been found to

signal through a different receptor isoform and to be a major molecular component of the injury response reducing pro-inflammatory cytokine-induced injury [46].

The use of nonhematopoietic, tissue-protective Epo derivatives, for example, carbamylated Epo, could overcome these difficulties (the activation of erythropoiesis when tissue protection is only aimed) [66]. All lysines in Epo are transformed to homocitrulline by carbamylation producing carbamylated-Epo (CEpo) [67]. This molecule does not bind to the classical Epo-R receptor and does not show any hematopoietic activity. Nevertheless, it binds to a hetero-receptor resulting from physical association between a molecule of Epo-R and a molecule of common ß receptor (cßR), also named CD131: this heterodimer shows tissue protective activity [68–75]. When compared to Epo, desialylated-Epo shows similar Epo-R affinity, the same neuroprotective and cardioprotective properties, but a shorter plasma halflife [70]. Because of this short half-life, only a small proportion of erythrocyte precursors are protected from apoptosis. Accordingly, desialylated-Epo does not increase erythrocyte mass but surprisingly it is protective in animal models of stroke, spinal cord injury, and peripheral neuropathy [76].

Moreover, regions within the Epo molecule mediating tissue protection have been identified and this has enabled the development of potent tissue-protective peptides, including some mimicking Epo's tertiary structure but unrelated in primary sequence [46].

Although the specific functions of Epo/Epo-R in all sites are not yet completely clarified, angiogenesis, the process through which new blood vessels arise from pre-existing ones, has also been indicated. The potential role of Epo in the process of angiogenesis should be considered as a subset of its possible function in improving overall tissue oxygenation and anti-apoptotic role [13].

Table I. Extra-Hematopoietic Functions Related to Epo

Brain	Neuroprotection	50,51
CNS	Cognition improvement	65
Spinal Cord	Neuroprotection	56
Cardiovascular system	Cardioprotection	52,53
Kidney	Renoprotection	55
Lung	Cytoprotection	57
Reproductive organs	Effect on male reproductive function	61
Skeletal muscle	Tissue bioenergetics	62
Gastrointestinal tract	Cytoprotection	58–60
Retina	Angiogenesis	54

2.1. Central nervous system

The delivery of peptides and regulatory proteins holds great promise as therapeutic agents for the central nervous system (CNS) [77]. The specialized vascular system of the CNS, formed by endothelial cells, pericytes, and astrocyte, end-feet present with specific properties which are collectively called the blood–brain barrier (BBB). The complexity of the BBB (made of tight junctions between endothelial cells and an ensemble of enzymes, receptors, efflux pumps for many therapeutic agents, and transporter systems) [78,79] not only makes work difficult but also offers diverse opportunities for drug development. Delivery across the vascular BBB by way of delivery systems is promising as is harnessing of endogenous transporters for delivery of their ligands [80].

In the last years, Epo and Epo-R have been widely investigated in the nervous system. Epo and Epo receptors have been found to be upregulated in the spinal cord and brain after injury [81,82] and their protective role has been proven in ischemic animal models [83–88]. Neuroprotective functions (after local administration of Epo) associated with anti-apoptosis, antioxidation, neurotrophic action, and angiogenesis can be applied to several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease (protecting neurons and even restoring dopaminergic function), glaucoma, and SCI [89].

Although the BBB is a major obstacle to the delivery of these potential therapeutics to their site of action, considered impermeable to large molecules, recent studies clearly demonstrate that some high weight molecules can be specifically transported into the brain across the capillary endothelium. However, there is still a controversial discussion whether Epo crosses the BBB or not. It is important to underline that BBB crossing has been observed only when high concentrations are employed (and even in these cases, little passage is observed) [90]. This passage has been found to increase in conditions of neural injury such as hypoxia, meningitis, or intraventricular hemorrhage, and thus it may be an ideal molecule to test in terms of delivery strategy in the context of neuroprotection (slowing or halting disease progression and functional decline) of the central nervous system.

Recently, Campana *et al.* [91] have identified a 17 amino acid sequence of Epo, possessing neurotrophic activity but no erythropoietic activity. At present, the mechanisms and the signal pathways by which Epo acts as a neuroprotective and neurotrophic agent in the CNS is not well understood, and several theories have been proposed. One of the most exciting, but under-explored, mechanisms for peptide and protein delivery is the use of their endogenous transporters [77]. In conclusion, the discovery of Epo and Epo-R production by neural cells in humans happened only 10 years ago, but an important body of evidence demonstrates that this

hormone has a significant impact on the pathophysiology of the brain [92].

2.2. Cardiovascular system

It was not until 2003 that Epo's cardioprotective role was first demonstrated against ischemia or ischemia-reperfusion [93–97]. Moreover, the chronic administration of Epo, when started well after myocardial infarction, has been found to reduce adverse remodeling (infarct size) and preserve long-term left ventricle dilation in ischemia-reperfusion and permanent coronary artery occlusion animal models [98–101]. The direct effects of Epo upon cardiac contractile and secretory functions through a mechanism involving the inhibition of cellular damage and apoptosis and an improvement in contractile recovery (by stimulating neovascularization, in part enhancing endothelial progenitor cell mobilization from the bone marrow) all contributing to this cardioprotective effect, are under study [102–104].

Epo treatment in renal patients has been associated with changes in the levels of known paracrine regulators of cardiac function, such as ET-1 (endothelin-1) [105], catecholamines [106], prostaglandins [107], and agents of the renin–angiotensin system [108]. Taken together, all these findings suggest that Epo may have important direct cardiac effects independent of its effects on RBC mass.

As previously mentioned, the presence of the Epo receptor in the heart has been localized to endothelial cells, vascular smooth muscle cells, cardiac fibroblasts, and cardiomyocytes [109–111]. Thus, the complete Epo system appears to be present in the heart and mediates its cardioprotective actions [112].

Albeit only small groups of patients have been studied so far, several trials are currently being performed to try and translate the cardioprotective effect to patients presenting with an acute myocardial infarction. At the present time, several large-scale studies are underway to determine the effect of Epo or its analogue (CEpo) [113], in patients with myocardial infarction [103].

2.3. Cancer

Anemia that frequently occurs in cancer patients is often derived from a combination of different factors. Recent studies reveal that patients with head, neck, and breast cancers expressing Epo-Rs may promote tumor growth via the induction of cell proliferation and angiogenesis [114,115]. Nevertheless, several preclinical studies have shown a beneficial effect of Epo on delaying tumor growth through reduction of tumor hypoxia and its deleterious effects on tumor growth, metastasis, and treatment resistance [1].

Epo also increases survival in anemic patients undergoing radiation treatment or chemotherapy [116]. In fact, multiagent chemotherapy has a spectrum of side effects, including persistent, severe anemia, and fatigue [117]. The severity of anemia and fatigue vary with chemotherapy, disease type, age, and other factors [118–123]. Usually, physicians treating patients with adjuvant chemotherapy for breast cancer often focus on

potentially life-threatening toxicities such as febrile neutropenia or toxicities that require immediate symptomatic intervention or dose reductions, such as diarrhea, neuropathy, or mucositis. Fatigue and anemia are often more insidious, but their impact on patient compliance is an outstanding concern for cancer patients [124]. Experimental data show that Epo treatment is able to improve quality of life, asthenia, mood, and cognitive functions in cancer patients undergoing chemotherapy [125,126].

These observations have led to speculation that Epo might improve tumor control and patient survival; however, the mortality rate observed in patients administered with Epo was higher than among placebo patients during the Breast Cancer Erythropoietin Trial [115]. Due to the hazards Epo treatment may pose in cancer patients, therapies must proceed with strong caution in patients with malignancies, according to the directive of the US Food and Drug Administration.

For instance, the treatment of other types of cancer as multiple myeloma is a more complex issue, as any concomitant anemia might be multifactorial (e.g. infiltration of the bone-marrow by the myelomatous cells, severe renal changes) [44].

Careful investigation of the Epo-R signaling cascades present in specific tumor cells may reveal further potential targets [127].

3. rHuEpo derivatives - Pharmaceutical products

The cloning of the Epo gene in 1983, beginning of human recombinant Epo (rHuEpo) therapy in 1985, and approval for its clinical use nearly 20 years ago (in 1989) has revolutionized the management of anemia, providing the opportunity for safe long-term correction without the attendant risks related to blood products. The success of this strategy in chronic kidney disease (CKD) has slowly allowed anemia associated with other chronic states (e.g. heart failure, zidovudine-treatment for HIV infection, diabetes, and cancer chemotherapy) to be also tackled [128].

Many factors may contribute to anemia: blood loss, kidney failure, nutritional factors, and inflammation. The management of anemia in CKD patients has contributed to the understanding of treatment of anemia in many human disorders [30]. In particular, the anemia of inflammation (often called anemia of chronic disease; ACD) has received intense study in the last 20 years; the prototype being the anemia related to rheumatoid arthritis [129]. The striking feature of ACD is that despite high total body iron stores [130], there is still restricted iron available for erythropoiesis [129,131]. The resolution of this clinical paradox has revealed a new understanding of iron biochemistry.

RHuEpo is an erythropoiesis stimulating agent (ESA) produced from Chinese hamster ovary (CHO), baby hamster kidney (BHK), or cultivated human cells by recombinant DNA technology [132]. Both endogenous Epo and rHuEpo exhibit several isoforms that differ in glycosylation and, hence, biological activity [133].

Before rHuEpo became available for therapy, about 25% of patients with CKD needed regular transfusions of erythrocytes [134]. However, the complications and side effects of blood transfusions such as allergic reactions, alloimmunization, immunological reactions, and transmission of viruses and parasites should be carefully considered against the cost and benefits of rHuEpo (Table II) [135,136].

A variety of rHuEpo are used in the clinic. Two forms of these rHuEpo have been commercially available from the very beginning: Epoetin α and Epoetin ß (CHO cell-derived rHuEpos). Epoetin α (Epogen®, Procrit®, Eprex®, Erypo®, and Espo®) is widely available, while Epoetin ß (NeoRecormon® and Epogin®) is marketed only outside the USA. The pharmacokinetic and pharmacodynamic properties of both preparations are very similar [137]. In addition, rHuEpo (Epoetin omega; Epomax®) engineered in transfected BHK (Mesocricetus auratus) cell cultures [138] has, at times, been applied in some Eastern European and Asian countries. With respect to the sequence of their [165] amino acids, the Epoetins are identical with human urinary Epo (rHuEpo) [139].

A fourth type of rHuEpo has been approved by the European Union authorities for the treatment of anemia associated with CKD: Epoetin delta (Epoδ; DynEpo). Epoδ is expressed from cultivated human cells and for this reason the molecule is expected to have more similarities in oligosaccharide residues to the endogenous human Epo [19].

The addition of two N-linked glycosylation sites and two acidic oligosaccharide side chains increases plasma half-life three times [3] and reduces Epo-R binding affinity, thus improving the stability and pharmacokinetic properties [140,141] Darbepoetin alfa (DPO; Aranesp®, Nespo®) is produced by glycoengineering. The amino acid sequence of darbepoetin differs from the isoform of human Epo in five positions [133,142], allowing for additional oligosaccharide attachments [141]. Darbepoetin is expressed from genetically engineered CHO.

Table II. Benefits and Cost of Epo Therapy

Pros	Cons
Improved quality of life (due to renal disease, chemotherapy…)	Hypertension
Reduced need of blood transfusions	Seizures
Prevention of iron overload	Fistula thrombosis
Improved nutritional status, physical capacity, cognition, immune function, sexual capacity, pruritus, and glucose metabolism	Pure red cell aplasia (Epo Abs)
Reverses myocardial hypertrophy	Epo receptors in tumors
Reduced risk of sensitization	Polycythemia (using sustained delivery systems)
Reduced coagulation disorders	High dose required for tissue protection
Reduced angina	BBB controversial crossing

In the light of the medical and economic success of the first generation rHuEpo preparations, a generation of biotechnology-derived therapeutic agents is reaching the end of their patent lives (Epoetin-α and ß are no longer protected by patent in the European Union, heralding the market entry of biosimilars). Several biopharmaceutical congeners and synthetic erythropoiesis stimulating compounds have already been launched as anti-anemic drugs or are currently in clinical trials [139].

Since the manufacturing process will be different from that used by the innovator, subtle changes in post-translational modifications including glycosylation, conformation, and impurities might be encountered, resulting in safety, efficacy, and consistency concerns of the clinical effects, which might become a limiting factor in the licensing/marketing of future biosimilar Epos [143]. Currently, it is not possible for another manufacturer to duplicate exactly the product profile of the innovator. Thus, the term "generic" is not used to describe rHuEpo molecules made by different manufacturers. Instead, the descriptors "follow-on biologics" or FOBs, "generic biosimilars," or "generic biopharmaceuticals" are used [144,145].

Last but not least, rHuEpo injections remain an expensive treatment, which requires frequent delivery injection repeats and which can lead to anti-Epo antibodies production by the patient [146].

4. Recent advances in Epo controlled delivery systems

4.1. Novel strategies for stimulation of erythropoiesis

Marketed pharmaceutical products of rHuEpo require frequent injections or high-dose systemic administration, which may cause undesired side effects. Moreover, large protein molecular weight and instability have significantly limited the clinical application of Epo. In addition to the rHuEpo forms, various exciting and innovative genetic engineering strategies to anemia correction have been investigated for enhancing Epo bioavailability and decreasing side effects. Some of them stand on or are close to the threshold of yielding products ready for clinical use, including PEGylation of Epo [147], use of antibodies, or aptamers to enhance crossing of biological barriers or targeted delivery [89], continuous Epo-R activator (recently licensed in Europe) [148], Epo mimetic peptides [149,150], Epo fusion proteins (EFP) [151], synthetic erythropoiesis stimulating protein [152], HIF-PHI (prolyl hydroxylase inhibitors) [153], GATA inhibitors [154], hematopoietic cell phosphatase inhibitors [155], and Epo gene therapy strategies [89,128].

Epo PEGylation (the process of connecting a hydrophilic polymer to Epo) changes the stability, immunogenicity, and pharmacokinetics properties of proteins by reducing the renal clearance (prolonging the duration of Epo in the circulation) [156,157] and also enhancing proteolytic resistance and eschewing recognition of immune cells. As a result, the plasma half-life of PEG–Epo is longer than Epoetin and DPO.

In the clinical setting, PEG–Epo (Mirceras) is approved by the US FDA for the treatment of anemia associated with chronic renal failure to maintain stable haemoglobin levels by monthly administration.

HIF-PHI may offer several safety and efficacy advantages over current ESA therapy including correction of anemia with normal physiological levels of Epo and correction of anemia without increasing blood pressure and treatment of dialysis patients who are hyporesponsive to ESAs. FG-2216 and FG-4592, FibroGen's first two erythropoietic HIF-PHI, have been the subject of clinical studies involving nearly 700 subjects. Proof of principle has been demonstrated in dialysis and nondialysis settings of anemia associated with CKD, and both investigational drugs have been found generally safe and well tolerated in clinical studies conducted to date [153].

Fusion proteins are created by expressing a hybrid gene made by combining two or more genes, which alter characters of target proteins and even have multiple functions. These EFPs have longer half-life or greater biological activities in erythropoiesis by different mechanisms, such as the enhancement of binding affinity to Epo-R, the extension of Epo-R phosphorylated state, and the increase of carbohydrate content [158,159]. Table III gives an overview and further information of novel pharmacological approaches to stimulate erythropoiesis.

4.2. New Epo drug delivery systems

Epo gene therapy appears to be a promising alternative to the current treatments of severe anemia since it requires less frequent administrations and may allow sustained Epo secretion and constant patient coverage. Epo gene transfer has already been tested on normal and pathological (ß-thalassemia and chronic renal failure) animal models.

Table III. Novel Pharmacological Approaches to Stimulate Erythropoiesis.

Epo PEG-ylation (CERA)	146
Antibodies/aptamers (targetting)	89
Continuous Epo-R activator	148
Epo mimetic peptides	149,150
EFP	151
Synthetic erythropoiesis stimulating protein	152
HIF stabilizers (prolyl hydrolases)	153
GATA inhibitors	154
Hematopoietic cell phosphatase inhibitors	155
Epo gene therapy	89,128

To this end, several gene transfer strategies have been employed such as: (1) naked DNA injection (by electrotransfer [160], electroporation [161], naked plasmid DNA (pDNA) [162], and poloxamer/DNA formulations) [163]; (2) viral gene delivery using adenovirus (AV) [164], helper-dependent AV [165], adeno-associated virus [166,167], and lentivirus [168]; (3) nonviral strategies including gastrointestinal patches [169], polytetrafluoroethylene chambers [170] human dermal cores (Biopump) [171], poly(lactic-co-glycolic acid) (PLGA) microparticles [172], hyaluronan and methylcellulose (HAMC) hydrogels [173], hollow fibers [174], and microcapsules (Table IV) [175–177].

Intramuscular (i.m.) injection of pDNA encoding Epo has been shown to be efficacious in eliciting significant amounts of circulating Epo and prolonged hematocrit increase in mice [178]. Unfortunately, the efficiency of transduction upon plasmid injection decreases from mice to larger animals [179,180]. This limitation has discouraged research on this type of gene correction strategy to humans. Several strategies have been devised to increase muscle fiber transduction [181]. Gene electrotransfer can increase the uptake and expression of plasmids DNA up to 100-fold [182]. However, even under these enhanced conditions levels of Epo reached a plateau at high plasmid doses after a single site injection [160]. In conclusion, understanding how to improve protein production and secretion in muscle cells might become an important field of research for effectively using muscle tissue as a bioreactor for the production of therapeutic proteins *in vivo*. Electroporation is already in use in clinical trials to promote the uptake of chemotherapeutics in malignant tumors [183].

Table IV. Epo Gene and Cell-Based Therapy Strategies

Naked DNA injection	Intramuscular electro-transfer of genes	160
	Intramuscular electroporation	161
	Intramuscular plasmid DNA (pDNA) injections	162
	Intramuscular poloxamer/DNA formulations (block copolymers)	163
AV	Intramuscular injections	164
Helper-dependent AV	Intramuscular injections	165
Adenoassociated virus	Intramuscular injections	166,167
Lentivirus	Intramuscular injections	168
Gastro-intestinal patch	Intestinal implantation	169
Polytetrafluoroethylene chambers	Implantation under the stomach	170
PLGA microspheres	Subcutaneous implantation	172
Human dermal cores	Dermal implantation of ex vivo transduced dermal cores	171
Hollow fibers	Intraperitoneal implantation	174
HAMC hydrogels	Intrathecal implantation	173
Microcapsules (alginate)	Intraperitoneal, subcutaneous implantation	175–177

Of particular interest is gene transfer to muscle tissue, as long-term, high levels of expression of the transferred genes can be obtained [184–186]. Interesting preclinical experiments using electroporation include gene transfer of Epo to treat anemia [187–189] or ß-thalassemia [190,191].

Despite the many contributions to generate suitable Epo expression, there have been few reports to produce Epo in a disease model of chronic renal failure to demonstrate long-term correction of anemia as would apply in the clinical setting. In this regard, lentiviral vectors (LV) have many attractive features that make them an ideal candidate for Epo gene transfer. The split-genome design of the LVs as initially published by Naldini *et al.* [192] have the advantage over early developed murine leukemia retroviral vectors by enabling provirus integration into predominantly quiescent, nondividing cells [192–195]. In terms of Epo gene transfer, Seppen *et al.* [196] showed that LV administration to nonuremic rat muscle increased hematocrit levels for up to 1 year.

Oral delivery of drugs is the most attractive route of administration. However, oral administration of protein and peptide drugs is not an easy task. To overcome this drawback, Venkatesan *et al.* designed an intestinal patch delivery system in such a way that the protein drug is protected from the intestinal enzymes and is capable of delivering the drug through the mucosal surface of the small intestine [169]. Once the patch is administered orally, it is stable in the gastric condition, whereas when it reaches the small intestine, the pH-sensitive layer is dissolved and the mucoadhesive layer (when incorporated in the formulation) enables its attachment to the intestinal wall. Once the patch is attached, the drug and absorption enhancer are released simultaneously. On completion of the release and when the mucoadhesive property is lost, the patch detaches itself from the intestinal wall and is subsequently excreted in feces. However, further studies are required owing that this study was carried out by placing the patches in the intestine through surgery, rather than administering them orally.

Another interesting nonviral strategy includes the use of composite PLGA microspheres wherein the protein is protected in polysaccharide fine particles dispersed in the polymer matrix. By the process of stabilized aqueous–aqueous emulsification, Epo can be loaded in polysaccharide glassy particles without contact with water–oil or water–air interfaces. Being preloaded in the polysaccharide particles, Epo native state can be preserved during the successive microencapsulation process, leading to sustained-release microspheres. Geng *et al.* have recently developed a microsphere formulation of Epo prepared with the present method, which showed a prolonged efficacy in mice without compromising the development of anti-Epo antibodies [172].

Over the past few decades, significant advances in molecular and cell biology have enabled scientists to identify a number of chronic and malignant disease mechanisms and develop various therapeutic drugs [197]. The treatment of diseases involving dysregulation of

endogenous and often essential cellular processes is challenging. As previously mentioned, drug therapies are often plagued by a rapid loss of bioactivity and subsequently limited therapeutic efficacies [198,199]. Recently, cells have been increasingly exploited as alternative drug delivery devices. The development of polymer-based encapsulation devices where various types of cells could be embedded to act as drug depots enabling the delivery of therapeutic products in a sustained manner over time could be considered a promising therapeutic alternative to the current administration schemes [200].

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. Moreover, cell encapsulation strategy would improve the pharmacokinetics of easily degradable peptides and proteins (protecting them from proteolytic cleavage), which often have short half-lives *in vivo* [201].

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 [174,202,203]. Aebischer *et al.* have intensively worked in this field, using hollow fibers and important improvements in their encapsulation strategy have been achieved, evidenced indirectly by higher Epo release rates from the immobilized cells. In addition, a high secretion cell line was achieved in order to assure a suitable *in vivo* therapeutic response.

To achieve a fully biocompatible therapeutic strategy in xenogeneic approaches, the need of transient immunosuppressive protocols demonstrated to have a positive effect on macroencapsulation systems as evidenced by improved outcomes in comparison with the nonimmunosuppressed groups [204]. One important advantage of this macroencapsulation approach lies in the easy removal of the implanted devices.

Microencapsulation systems (100–500 μm diameter beads) produced from polymer based hydrogels offer potential advantages in comparison with the macroencapsulation approaches (1 cm long, 550 μm outer diameter hollow fibers). The spherical nature of the microcapsule beads maximizes the surface area and their small size facilitates biomolecular transport. These facts improve permeability of the membrane and thus cell viability [205]. Finally, they can be implanted with minimal-invasive surgery into the peritoneal cavity [206], subcutaneous tissue [207], myocardium [208], or elsewhere.

Our research group has recently 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) [175].

Results showed that implantation of Epo-secreting cell-loaded microcapsules leads to high and constant hematocrit levels for more than 100 days in all implanted mice without implementing immunosuppressive protocols. Due to the angiogenic and immunomodulatory

properties related to Epo, the formation of a vascularized network surrounding the microcapsule graft was observed (and no major host reaction) especially in the subcutaneous space, highlighting the feasibility of cell encapsulation technology for the long-term delivery of Epo.

One important consideration to enhance long-term Epo delivery from the enclosed cells may rely on studying and improving the biocompatibility of materials and capsules [209,210]. Previous analyses carried out by our research group evidenced that a careful selection of purified alginates, selection of cell lines with adequate features, and the development of small and uniform microcapsules are key requirements to ensure an optimal biocompatibility and long-term functionality of the therapeutic molecules [205,211–213]. 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.

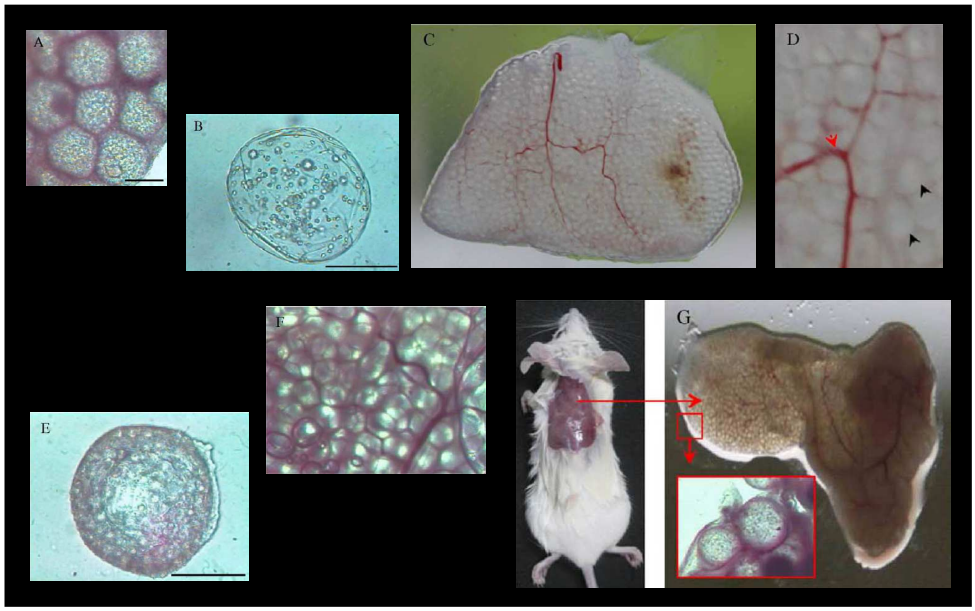

Fig 2. (A–D) Photographs of explanted microcapsules 150 days postimplantation in syngeneic C3H mice. (A, B) Microcapsules retrieved from the peritoneum. (C, D) Microcapsules explanted from the subcutaneous tissue. (E–G) Images of explanted microcapsules 150 days postimplantation in allogeneic Balb/c 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 Ref. [175] © 2005 American Society of Gene Therapy.

Factors limiting the long-term efficacy of microencapsulated cells have been extensively studied [210,214]. In an effort to evaluate the importance of the biocompatibility of the biomaterials employed, a next step toward 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 [215]. 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. The vital importance of the biomedical grade alginates used in cell encapsulation technology is evidenced in undesired effects observed *in vivo* (higher degree of fibrotic overgrowth).

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, a complete morphological and mechanical evaluation of microcapsules containing Epo-secreting C_2C_{12} myoblasts was carried out [176,216], followed by a successful xenogeneic approach where Epo-secreting murine C_2C_{12} myoblasts were subcutaneously implanted for 14 weeks in Fischer rats, using transient Tacrolimus (FK-506) immunosuppression [177].

The parallel control of the scaffold structure, processing, and function may lead to further improvements in the therapeutic efficacy of cells. Various nanoscale and microscale techniques will probably provide significant benefits in modulating individual properties of polymeric biomaterials to continuously control the cellular response. Engineering the physiological environments of microencapsulated-cell implants may also improve the clinical results of cell-based drug release.

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.

5. Concluding remarks

One of the main challenges in human disease treatment is no longer the development of efficient drugs, but the improvement of drug selectivity. For systemically secreted products, such as Epo, it will also be necessary to use an inducible genetic system to avoid excess expression and dangerous polycythemia. A variety of systems are under development, including a rapamycin-regulated expression [217], a tetracycline-regulated system [218], and an antiprogestin-regulated expression [219,220]. In addition, considering that the concentrations used for tissue protection are far too high to avoid erythrocytosis, the use of nonerythropoietic Epos might be considered an efficient alternative strategy.

The current Epo treatment strategies are limited by some serious shortcomings. These include the complexity and high cost of manufacture, strict requirements for correct storage and administration, nonconvenient routes of administration (subcutaneous and intravenous, but not oral), and toxicity/immunogenicity [221]. As a result, much effort continues to be spent to advance other techniques to achieve anemia correction. Optimum Epo developed delivery systems will offer low dose and administration frequency, and fewer side effects for patients requiring long-term Epo treatments.

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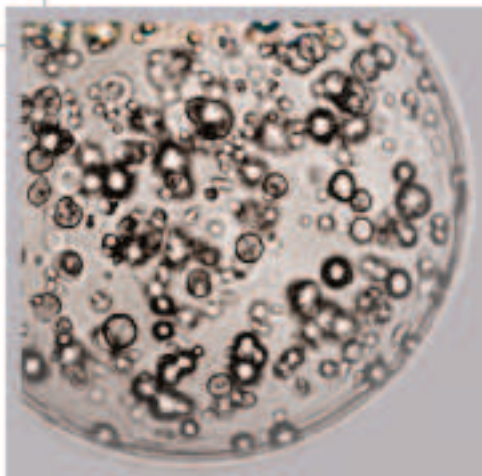
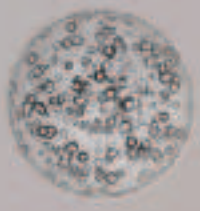
Ainhoa Murua *graduated in Biology and Biochemistry at the University of Navarra. She continued her scientific training at the Laboratory of Pharmaceutical Technology, University of the Basque Country, where her doctoral thesis is under way (therapeutic applications of cell microencapsulation technology) while also working on additional scientific CIBER-BBN (Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine) projects. Her main research interests include biotechnology and gene therapy, research areas which are making important step forwards and may enable a therapeutic alternative to the treatment of diseases that lack efficient treatment at present.*

Gorka Orive *is Ph.D. in Pharmacy and currently Assistant Professor of Pharmacy & Pharmaceutical Technology at the University of the Basque Country (Vitoria, Spain). He is the Director of research publications and Scientific responsible of the field of oral implantology for Biotechnology Institute (BTI, Vitoria, Spain). His interests include polymer-based cell therapy for long-term and controlled protein and growth factor delivery to different tissues including brain. He is also interested in the potential use of autologous platelet's growth factors and fibrin scaffold for regenerative medicine. He has published more than 100 articles in national and international journals and edited several books focused on cell microencapsulation for therapeutic purposes and the use of plasma rich in growth factors in medicine.*

Rosa Mª Hernández *is a Professor of Pharmacy and Pharmaceutical Technology since 2009. She obtained her Ph.D. in Pharmacy in 1992 from the University of Salamanca, Spain, after which she joined the University of the Basque Country in 1993. She has supervised 10 Ph.D. thesis and co-authored more than 170 original articles and book chapters. Her main research and development interests are focused on the design and evaluation of new drug delivery systems by using microparticles, for cell-based therapies and vaccines.*

José Luis Pedraz *is Ph.D. in Pharmacy (University of Salamanca, Spain). He is a Professor of Pharmacy and Pharmaceutical Technology at the Faculty of Pharmacy in the Basque Country University. He is the cofounder and director of the Pharmaceutical Development Unit of the Basque Country. His interest is focused on the development and evaluation of pharmaceutical dosage forms (microcapsules, micro- and nanoparticles) for the administration of genes, proteins, peptides, vaccines, and cells. He has published over 200 scientific articles and edited several books focused on cell microencapsulation.*





OBJECTIVES

As previously described in the introductory part, cell microencapsulation could be considered a promising alternative for the long-term treatment of pathologies requiring chronic drug administration, since it could alleviate the shortcomings of conventional formulations which usually require multiple administrations to obtain a therapeutic effect. However, several challenges still remain unsolved if the clinical application of the technology is aimed. On the one hand, the development of suitable storage protocols seems necessary, considering the increasing inter-laboratory collaborations. On the other hand, a recurring impediment to rapid development in the field is the immune rejection of transplanted allo- or xenogeneic cells which should be overcome in order to achieve full biocompatibility and long-term functionality of the implanted devices.

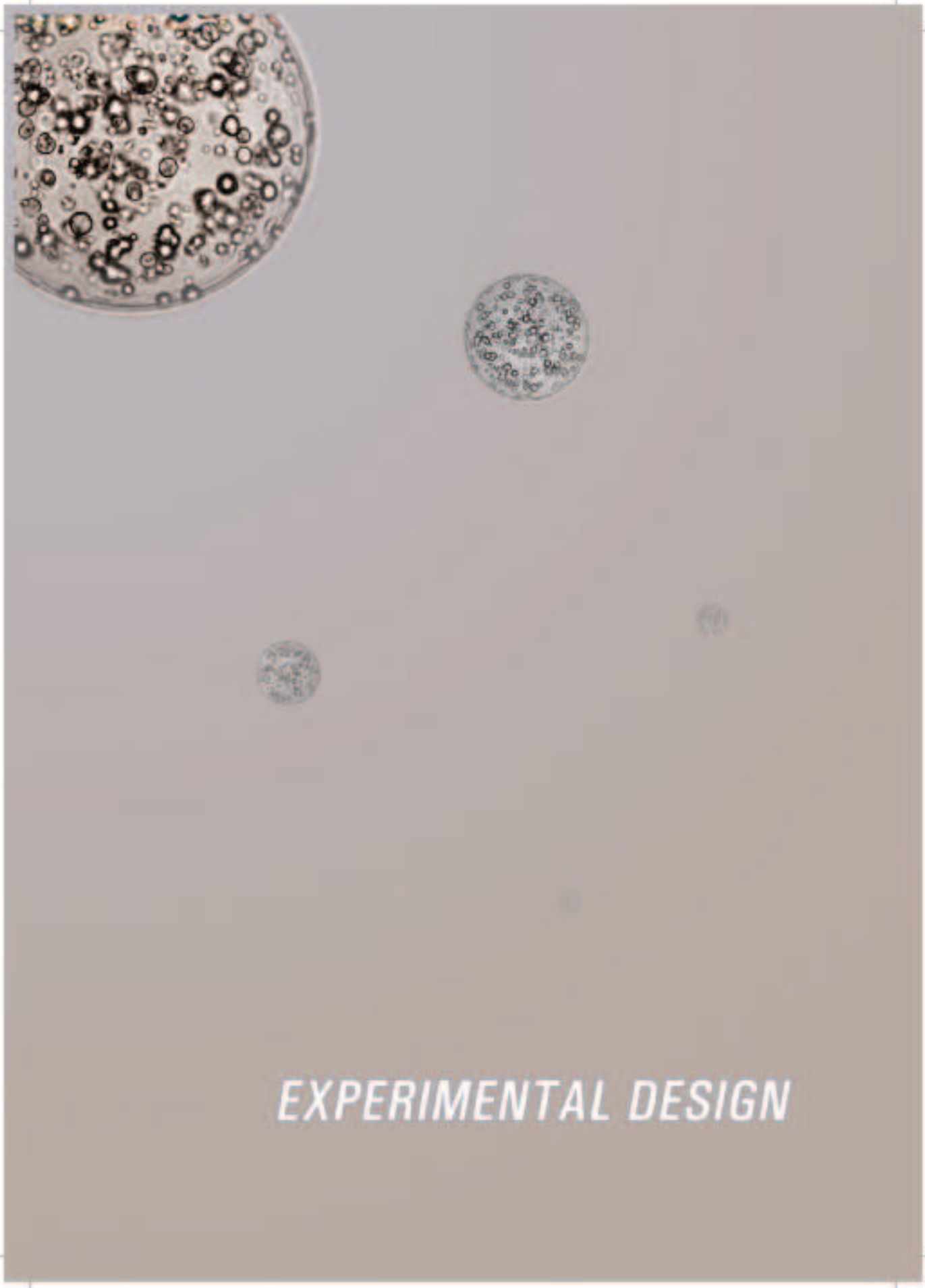
Taken these issues into consideration, the objectives of the present study are the following:

1. To fully characterize (both *in vitro* and *in vivo*) mEpo-secreting C_2C_{12} myoblasts embedded in APA microcapsules. *In vitro*, morphology and mechanical properties of microcapsules and viability of the enclosed cells will be analized. Long-term *in vivo* functionality and biocompatibility of the encapsulated cells implanted in the subcutaneous space of allogeneic mice will be studied.

2. Using DMSO as cryoprotectant, a selective identification of cooling protocols, cryoprotectant concentrations and cryopreservation periods will be carried out, in order to determine the optimal cryopreservation parameters for successful storage of microencapsulated cells.

3. To evaluate the functionality and biocompatibility of cell-based scaffolds in a xenogeneic environment, using tacrolimus-based transient immunosuppression.

4. To develop an independent composite drug delivery system secreting dexamethasone to enhance and prolong the functionality of the cell-loaded graft in a more physiological manner, even using low cell doses.



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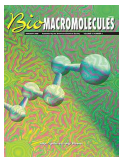

In vitro characterization and *in vivo* functionality of erythropoietin-secreting cells immobilized in alginate-poly-L-lysine-alginate microcapsules

Ainhoa Murua, María de Castro, Gorka Orive, Rosa Mª Hernández, José Luis Pedraz *

Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, 01006 Vitoria-Gasteiz, Spain

ABSTRACT

The *in vitro* and *in vivo* characterization of cell-loaded immobilization devices is an important challenge in cell encapsulation technology for the long-term efficacy of this approach. In the present paper, alginate-poly-L-lysine-alginate (APA) microcapsules containing erythropoietin (Epo)-secreting C_2C_{12} myoblasts have been elaborated, characterized, and tested both *in vitro* and *in vivo*. High mechanical and chemical resistance of the elaborated microcapsules was observed. Moreover, the *in vitro* cultured encapsulated cells released 81.9 ± 8.2 mIU/mL/24 h (by 100 cell-loaded microcapsules) by day 7, reaching the highest peak at day 21 (161.7 ± 0.9 mIU/mL/24 h). High and constant hematocrit levels were maintained over 120 days after a single subcutaneous administration of microcapsules and lacking immunosuppressive protocols. No major host reaction was observed. On the basis of the results obtained in our study, cell encapsulation technology might be considered a suitable therapeutic strategy for the long-term delivery of biologically active products, such as Epo.

* Corresponding author: J.L. Pedraz.

Keywords Alginate; Cell encapsulation; Cryopreservation; Dimethylsulfoxide; Erythropoietin; Myoblast.

1. Introduction

In the few last decades, researchers involved in the development of pharmaceuticals have understood that drug delivery is a fundamental part of drug development. This issue is particularly relevant considering that over 95% of all new potential therapeutics have poor pharmacokinetics and biopharmaceutical properties [1]. In addition to reducing the frequency of drug administration and thus improve patient comfort, novel drug delivery systems would offer protection and improve the pharmacokinetics of easily degradable peptides and proteins, which often have short half-lives *in vivo* [2].

Although standard drug therapy is usually effective in treating the symptoms of a disorder, a patient may be required to take the drugs for an extended time, and there may be serious or unpleasant side effects. However, a patient may be cured with few negative consequences if the treatment can be targeted directly at the specific cause of the disease (the gene defect) or if that cause can be neutralized or reversed. Therefore, gene therapy provides an attractive alternative to drug therapy as it seeks to provide treatment strategies that will be more complete and less toxic to the patient. Furthermore, gene therapy may provide a way of treating diseases that cannot be managed by standard therapies. As an example, the search for alternative therapies to the continuous injections that recombinant human erythropoietin (rHuEpo) treatments require is at its peak.

One of the emerging technologies that has gained the attention of the scientific community is cell encapsulation. Cell encapsulation results in the immobilization of cells in biocompatible as well as chemically and mechanically stable devices that deliver "*de novo*" produced therapeutic products in a sustained and controlled manner. Besides, the protection of the inner cell content from both mechanical stress and the host's immune response is ensured. This could be an advantage, as chronic administration of immunosuppressants could be avoided, improving the quality of life in patients again.

This strategy has provided a wide range of promising therapeutic treatments for central nervous system diseases [3-7], diabetes [8-12], hemophilia [13,14], and anemia [15] among others. This technology offers a safe and manufacturable method for the local and systemic delivery of therapeutic molecules from the enclosed cells. It can be considered as a "living drug delivery system" where the transplanted cells 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. The microcapsule's membrane can serve as an immunoisolation barrier to keep the host's immune system away from the living cells, but at the same time it allows nutrients, oxygen, waste, and cell products to pass through without much difficulty.

Scientists are now taking steps to properly resolve some of the main challenges of this field [16-18] including the selection of clinical-grade biopoly-

mers [19], the development of a standardized, repeatable, and reproducible technology [20,21], the control of permeability, mechanical stability, and durability of the microcapsules [22], and, last but not least, the suitable *in vivo* evaluation of the microcapsules.

Recently, as a proof of principle, we have studied cell encapsulation technology by implanting encapsulated Epo-secreting cells in the peritoneum and subcutaneous tissue of syngeneic and allogeneic mice [23]. Epo was selected as a model drug because of its emerging therapeutic effects and due to the ease of monitoring its expression and bioactivity *in vivo* by following the hematocrit level. In addition, due to its short half-life, we suggest that cell encapsulation technology could avoid the tiresome repeated Epo injections currently practiced. Despite our initial positive results, further characterization and evaluation of the microcapsules and longer *in vivo* evaluation periods were suggested to properly evaluate the safety and long-term functionality of this approach.

In the present paper, a complete morphological and mechanical evaluation of microcapsules containing Epo-secreting C_2C_{12} myoblasts has been carried out. Furthermore, the *in vitro* characterization and the *in vivo* functionality and biocompatibility of the encapsulated cells during 4 months have been studied and discussed.

2. Experimental Procedures

2.1. Cell culture

Murine C_2C_{12} myoblasts derived from the skeletal leg muscle of an adult C3H mouse and genetically engineered to secrete murine Epo (mEpo) were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne (EPFL), Lausanne, Switzerland). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose, and 1% antibiotic/antimycotic solution. Cultures were plated in T-flasks, maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere standard incubator, and were passaged every 2-3 days. All reagents were purchased from Gibco BRL (Invitrogen S.A., Spain).

Before cell encapsulation, Epo secretion from 10^6 cells/mL/24 h was determined using a sandwich enzyme-linked immunoabsorbent assay (ELISA) kit for human Epo (R&D Systems, Minneapolis, MN). Cross-reaction of the kit allowed detection of mEpo in culture supernatants.

2.2. Cell encapsulation

C_2C_{12} myoblasts genetically engineered to release Epo were immobilized into alginate-poly-L-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [24]. Low viscosity and high guluronic (LVG) alginate was purchased from FMC Biopolymer (Norway), and poly-L-lysine (PLL; hydrobromide Mw, 15 000-30 000 Da) was obtained from Sigma (St. Louis, MO). Briefly, cells were suspended in 1.5% (w/v) LVG-alginate sterile solution, obtaining a cell density of $2x10^6$ cells/mL of alginate. This suspension was extruded through a 0.35 mm needle using a 10 mL sterile syringe with a peristaltic pump. The resulting alginate beads were maintained in agitation for 10 min in a $CaCl_2$ solution (55 mM) for complete ionic gelation and were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a coating with 0.1% alginate for other 5 min. Microcapsules were prepared at room temperature, under aseptic conditions, and were cultured in complete medium. The diameters and overall morphology were characterized using inverted optical microscopy (Nikon TSM) and confocal microscopy (Olympus FluoView FV500).

Fluorescence images were obtained applying a viability/cytotoxicity test for mammalian cells purchased from Invitrogen.

2.3. Mechanical stability studies: compression and osmotic resistance tests

The compression resistance of microcapsules was determined as the main force (g) needed to generate 70% uniaxial compression of a sample of microcapsules using a Texture Analyzer (TA-XT21, Stable Microsystems, Surrey, U.K.). The force exerted by the probe on the microcapsule was recorded as a function of the compression distance leading to a force *versus* strain relation. Thirty microcapsules per batch were analyzed to obtain statistically relevant data.

The swelling behavior of the microcapsules was determined after 1% citrate solution (w/v) treatment. In short, 100 μL of microcapsule suspension (50-100 microcapsules) was mixed with 900 μL of phosphate-buffered saline (PBS) and placed in a 24-well cell culture cluster. Four wells were used for each group. The cell cluster was placed in a shaker at 500 rpm and 37 °C for 1 h. Afterward, supernatants were eliminated, and 800 μL of a sodium citrate solution was added. The cluster containing the microcapsules was maintained at static conditions at 37 °C for 24 h. On the following day, the diameters of 20 microcapsules of each group were measured. The washing and shaking step with PBS and the static condition were repeated during the following days until a 6-day period was completed.

2.4. mEpo production and metabolic cell activity

The cellular activity and Epo secretion of the entrapped cells were evaluated *in vitro* for 21 days. The viable cell number per microcapsule was determined by the tetrazolium assay (MTT) (Sigma, St. Louis, MO). Briefly, 25 μL of a 5 mg/mL solution of MTT in PBS was added to a known number of microcapsules (around 40) placed in a 96-well cell culture cluster and incubated at 37 °C for 4 h. Afterward, the MTT solution was removed by vacuum aspiration, and 100 μL dimethylsulfoxide was added. The resulting purple solution was read 5 min later on a microplate reader (Multiskan EX Labsystems) at 560 nm with 690 nm as the reference wavelength. Results are expressed as mean ± standard deviation.

Conditioned media samples (cell supernatants) were assayed using the Quantikine IVD Epo ELISA kit purchased from R&D Systems. Standards and samples were run in duplicate according to the procedure specified in the kit. The detection limit of this assay was 2.5 mIU/mL. The mEpo secretion of around 200 cell-loaded microcapsules was measured in conditioned medium for an 8 h release period to calculate the C_4C_{12}-mEpo-microencapsulated cells daily secretion rate. Results are expressed as mean ± standard deviation.

2.5. Microcapsule implantation

Adult female Balb/c mice (Harlan Interfauna, Spain) were used as allogeneic recipients. Animals were anesthetized by isoflurane inhalation, and a total volume of 0.5 mL of cell-loaded microcapsules ($2x10^6$ cells/mL) suspended in Hank's balanced salt solution (HBSS) was implanted subcutaneously using a 18-gauge catheter (Nipro Europe N.V., Belgium). Control animals received 1 mL of HBSS by the same route. Before implantation, microcapsules were washed several times in HBSS. Upon recovery, animals had access to food and water *ad libitum*. No immunosuppressant protocols were applied to the animals during this study.

2.6. Hematocrit measurements

Blood was collected weekly by retroorbital puncture using heparinized capillary tubes (Deltalab, Spain). Hematocrits were determined after centrifugation at 3000 rpm for 15 min of whole blood using a standard microhematocrit method. Results are expressed as mean ± standard deviation.

2.7. Histological analyses

At day 130 after implantation, some animals were sacrificed, and microcapsules were retrieved and fixed in a 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2. Serial horizontal cryostat sections (14 μm) were processed for hematoxylin & eosin (H&E) staining.

2.8. Statistical analyses

Data are presented as mean ± standard deviation. All statistical computations were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL). Data between control and experimental groups were analyzed for statistical significance using Student's t-test according to the results of the Levene test of homogeneity of variances. A P-value of $P<0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Microcapsule characterization

All cell-loaded microcapsules had a uniform and spherical morphology without irregularities on their surface as shown in Figure 1. Previous studies have reported the relevance of the materials employed in the elaboration of microcapsules to obtain biocompatible microcapsules [25]. However, not only the materials used but also the spherical and smooth shaped morphologies of the microcapsules have been observed to be of great importance to elude the host's immune response [26]. Furthermore, the fluorescence analysis of the microcapsules demonstrated the high viability of the enclosed cells (Figure 1B), leading to the conclusion that enclosed cells were correctly adapted to the surrounding polymer scaffold.

3.2. Integrity and stability of microcapsules

Another important consideration is the study of the integrity and stability of the cell-loaded microcapsules. Alginates are nowadays the most frequently used biomaterials and generally present low immunogenicity, low toxicity, and thus good biocompatibility (which is one of the most important preconditions for biomaterials to be used clinically) [27].

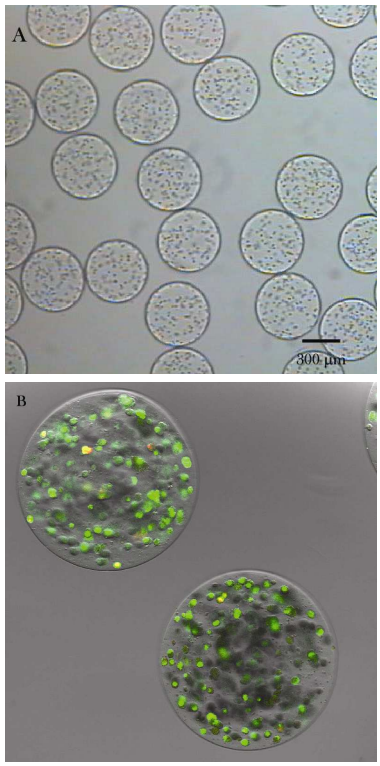

Fig. 1. Morphologies of microencapsulated Epo-secreting myoblasts. (A) Optical microscopy. (B) Fluorescence image of cells stained with calcein-AM (green, live cells) and ethidium homodimer (red, dead cells).

These positive features have made alginate the polymer of choice in the field. Alginates create three dimensional structures when they react with divalent cations such as calcium, barium, and strontium. Moreover, the election of adequate biomaterial compositions has been optimized by the combination of an alginate core surrounded by a polycation layer that at the same time is

covered by an outer alginate membrane [28]. This microencapsulation design (alginate-polycation-alginate) is nowadays the most often described system in the scientific literature [29]. Alginate-PLL-alginate microcapsules have showed suitable mechanical strength and resistance to swelling in previous experiments carried out by our research group [30].

In the present study, we performed a thorough *in vitro* characterization of the cell-loaded microcapsules to determine their suitability for the following *in vivo* assays. The mechanical resistance of the cell-loaded microcapsules against compression was 34.5 ± 7.5 g/microcapsule, which corroborates the membrane's resistance to bursting forces. On the other hand, the swelling assay showed that microcapsules swelled and increased their diameter by approximately 10% after the first citrate treatment, but afterward their size remained stable (Figure 2). These results confirm the high mechanical and chemical resistance of the microcapsules elaborated in this study.

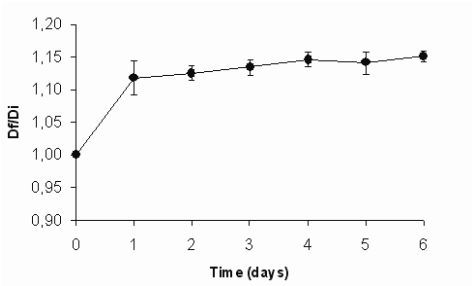

Fig. 2. Osmotic pressure resistance of microcapsules after a 6-day treatment with citrate. The error bars on each point correspond to the standard deviation of the mean.

3.3. Microencapsulation of C_2C_{12} myoblasts: in vitro viability and Epo production

The metabolic activity and Epo production of the encapsulated cells was analyzed over the course of 21 days. The level of viability detected is an indicator of the mitochondrial cell activity and therefore the physiological state of the enclosed cells. Thiazolyl Blue Tetrazolium Blue is a yellowish solution and is converted to water-soluble MTT-formazan of dark blue color by mitochondrial dehydrogenases of living cells. As seen in Figure 3A, C_2C_{12} myoblasts showed similar viabilities over the 21 days. A slight decrease was observed during the first week, but after this period entrapped cells maintained their viability, which supports the idea that the diffusion of nutrients and oxygen was not affected by an inappropriate membrane behavior.

C_2C_{12} myoblasts have been selected as a model cell line for immobilization by many research groups [31-33] in part due to the fact that they can be easily cultured and can afterward be terminally differentiated into myotubes [34] after immobilization both *in vitro* and *in vivo* [35]. Besides, myoblasts present a relative lack of major histocompatibility expression on the surface, which may lead to a decrease in the stimulation of humoral immune response [36].

Regarding the cell line used in this study, a full characterization of the Epo production before and after encapsulation was carried out with the aim of adjusting the therapeutic dose of the microencapsulation product. The clone

selected for immobilization released 46.3 ± 1.5 IU/mL Epo/10^6 cells/24 h.

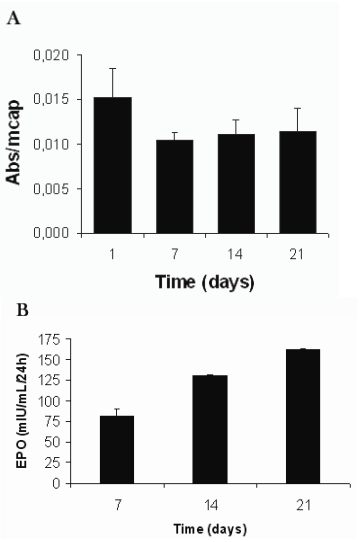

Fig. 3. (A) *In vitro* viability of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. (B) Epo secretion by entrapped cells (mIU/mL/24 h/100 microcapsules).

The reduction in the Epo release rate following encapsulation has been previously reported by our research group [23] observing a 66% reduction. Having a look at the Epo release by the immobilized cells (Figure 3B), the amount of Epo produced at day 7 by 100 cell-loaded microcapsules was 81.9 ± 8.2 mIU/ mL/24 h. After this period, Epo release showed an important increase, reaching the highest production rate at day 21, 161.7 ± 0.9 mIU/mL/24 h. This could be explained by the fact that encapsulated myoblasts maintained *in vitro* were not induced to terminally differentiate into myotubes, leading to a slight increase in the overall cell density. In contrast, this induction into myotubes is promoted after the *in vivo* administration of the encapsulated cells, which enables a better control of the dosage.

3.4. Long-term hematocrit levels of Balb/c mice with subcutaneously implanted Epo-secreting microencapsulated cells

On the basis of the *in vitro* Epo production, we estimated that 0.5 mL of cell-loaded microcapsules ($2x10^6$ cells/mL alginate) might result in a therapeutic dose to provide significant increase in mice hematocrit levels over time. To address this issue, adult female Balb/c mice were used as recipients, and cell-loaded microcapsules were implanted in the subcutaneous space. As it is observed in Figure 4, a significantly higher hematocrit level was observed in all the animals implanted with alginate microcapsules when compared with the HBSS (control) group ($P<0.05$).

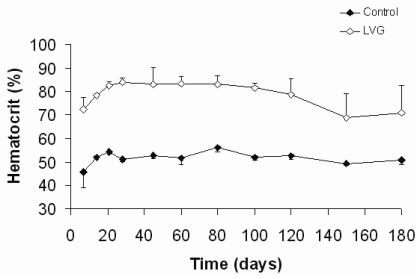

Figure 4. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. Values represent mean ± standard deviation. $^*P<0.05$ versus the control.

Regarding the implanted group, the hematocrit levels of the animals increased to 84 ± 1.9% during the first 3 weeks of study, and afterward they

remained asymptotic until day 120 post-implantation. Although the hematocrit level decreased slightly after this period, levels at day 120 post-implantation remained statistically high in comparison with the control group (78.7 ± 6.8% *versus* 52.5 ± 1.5%, respectively) ($P<0.05$). However, a slight increase was also observed in the control group during the first 3 weeks followed by plateau until the end of the study.

Some interesting conclusions can be highlighted from these experiments. First, a 4-month release of Epo was observed after a single shot of cell-loaded microcapsules and following subcutaneous administration in allogeneic recipients. In previous studies, this route has been reported to result in poor implant viability and inconsistency in the hematocrit response to Epo secretion [37]. This long-term efficacy might be due to the optimized volume-surface relation of the microcapsules, which improves the cell product kinetics and oxygenation of the cells. Second, no remarkable side effects were observed during the treatment period although the high hematocrit levels obtained may be responsible for the appearance of polycythemia in the animals (expanded red cell mass) [38].

3.5. Microcapsule retrieval and histological analysis

The implanted cell-loaded microcapsules from the treatment group were explanted at day 130 post-implantation. The macro- and microscopic appearances are shown in Figures 5A and 5B. Microcapsules retrieved from the subcutaneous tissue were mostly aggregated, forming an irregular structure in which immobilized cells remained viable. The microcapsule network was easily harvested as one piece after a small skin incision, as illustrated in Figure 5A. This could be an advantage, as one important challenge in the field of cell microencapsulation is the sometimes difficult removal of the implanted graft.

The histological analyses of the explanted microcapsules revealed the formation of some blood capillaries within the microcapsule aggregates. We hypothesized the latter could be due to the angiogenic effects reported for Epo. In fact, the Epo molecule has been reported to act as an angiogenic factor by different pathways [39-41]. This situation might be helpful as the access of oxygen and nutrients to the entrapped cells might be improved. Interestingly, although highly purified alginates were used for microcapsule elaboration and this process was done under aseptic conditions, a weak fibroblast overgrowth was detected surrounding the microcapsules (Figures 5C and 5D).

The data presented in this study demonstrate a proof-of-principle for cell encapsulation technology for the long-term delivery of Epo. The correct characterization of the immobilization systems and the genetically modified cell lines used are of paramount importance to optimize the final cell encapsulation product.

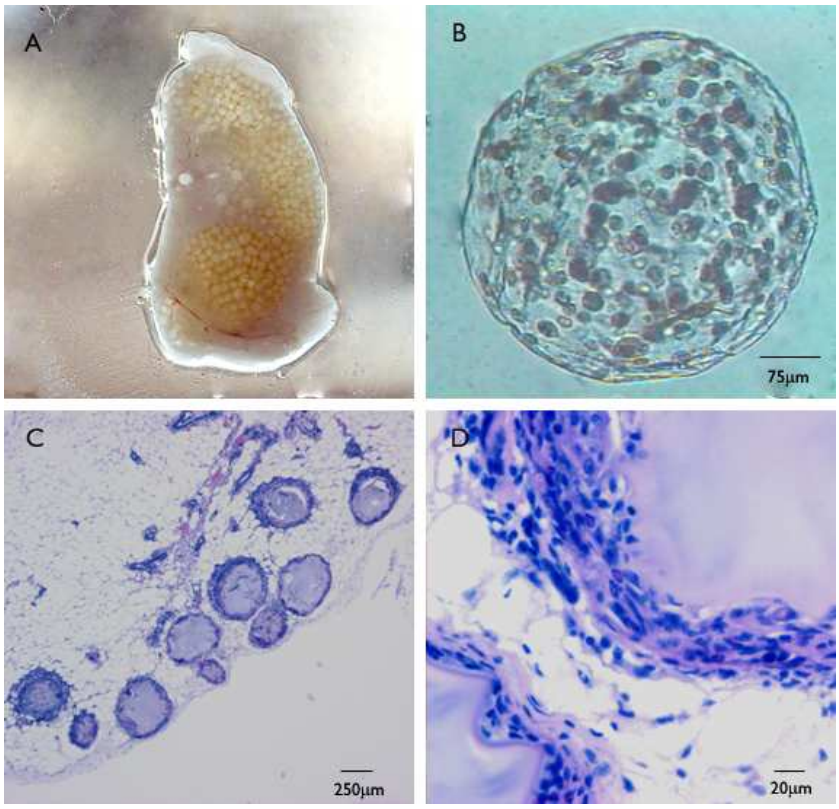

Fig. 5. (A and B) Photographs of microcapsules explanted from the subcutaneous tissue 130 days post-implantation. (C and D) Histological analysis of explanted cell-containing microcapsules (H&E).

Animals implanted with microencapsulated cells showed elevated hematocrit levels during 4 months of study, with no remarkable side effects. The presence at explantation of a cell-loaded microcapsule aggregate surrounded by several blood capillaries might be a consequence of the angiogenic effects of the Epo molecule. The latter may suggest the interesting role that this or any other type of angiogenic molecule could have in the long-term functionality of this type of cell-loaded microcapsules.

On the basis of the aforementioned advantages of this technology, this "living drug delivery system" can be considered as an alternative method for the systemic delivery of Epo from genetically engineered cells. Viable and functional cells will be able to release the desired products in a more physiological manner. Moreover, to overcome the current organ donor shortage, the

immunoprotective properties of this device make this strategy suitable for allotransplantation therapy, turning this technology into an alternative therapy to whole organ transplantation.

4. Conclusion

In the present study, subcutaneous implantation of alginate-poly-L-lysine-alginate microcapsules containing Epo-secreting C_2C_{12} myoblasts in allogeneic mice recipients resulted in an important increase of hematocrit levels. High and constant levels were maintained over 120 days after a single administration of microcapsules and lacking immunosuppressive protocols. At explantation, a thin fibrotic layer was observed surrounding the microcapsules. The pharmacodynamic characteristics of Epo added to its poor pharmacokinetics make this molecule a candidate for its delivery using cell microencapsulation technology. Our results demonstrate that cell encapsulation technology might be a suitable therapeutic strategy for the long-term release of this therapeutic product.

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Supporting information available

Scheme of the cell encapsulation procedure using an electrostatic droplet generator. This material is available free of charge via the Internet at http://pubs.acs.org. doi 10.1021/bm070194b.

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Cryopreservation based on freezing protocols for the long-term storage of microencapsulated myoblasts

Ainhoa Murua [a,b], Gorka Orive [a,b], Rosa Mª Hernández [a,b], José Luis Pedraz [a,b,*]

[a] *Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, 01006 Vitoria-Gasteiz, Spain*

[b] *Networking Biomedical Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, SLFPB-EHU, 01006 Vitoria-Gasteiz, Spain*

ABSTRACT

One important challenge in biomedicine is the ability to cryogenically preserve not only cells, but also tissue-engineered constructs. In the present paper, alginate-poly-L-lysine-alginate (APA) microcapsules containing erythropoietin (Epo)-secreting C_2C_{12} myoblasts were elaborated, characterized and tested both *in vitro* and *in vivo*. Dimethylsulfoxide (DMSO) was selected as cryoprotectant to evaluate the maintenance of physiological activity of cryopreserved microencapsulated myoblasts employing procedures based on freezing protocols up to a 45-day cryopreservation period. High chemical resistance of the cryopreserved microcapsules was observed using 10% DMSO as cryoprotectant following a standard slow-cooling procedure. Although a 42% reduction in Epo release from the microencapsulated cells was observed in comparison with the non-cryopreserved group, the *in vivo* biocompatibility and functionality of the encapsulated cells subcutaneously implanted in Balb/c mice was corroborated by high and sustained hematocrit levels over 194 days and lacking immunosuppressive protocols. No major host reaction was observed. Based on the results obtained in our study, a slow-cooling protocol using 10% DMSO as cryoprotectant (confirmed for cryopreservation periods up to 45 days) might be considered a suitable therapeutic strategy if the long-term storage of microencapsulated cells, such as C_2C_{12} myoblasts is pretended.

* Corresponding author: J.L. Pedraz.

Keywords Alginate; Cell encapsulation; Cryopreservation; Dimethylsulfoxide; Erythropoietin; Myoblast.

1. Introduction

Since encapsulation and transplantation of cells are labor-intensive tasks, cryopreservation has emerged as an attractive system for the long-term storage of microencapsulated cells. The increasing inter-laboratory collaborations make transport facilities a basic need in terms of biosafety and correct transport conditions for their research material interchange. Assuming this, the cryopreservation of not only cells or tissues, but also cell encapsulation devices, and even laboratory-produced whole organs in the future, may be essential.

Several strategies for cryopreservation, depending on the application field, have been reported: ultrarapid freezing and thawing [1], controlled-rate freezing [2], freezing with non-penetrating polymers [3], vitrification [4,5] and equilibrium freezing [6]. An important issue that needs to be considered in any method employed is the rate of cooling, due to the dramatic effect it may have on these phenomena. Moreover, there are several factors important for successful cryopreservation including: composition of the cryopreservation medium and nature of the cryoprotectants [7,8], the freezing procedure [9,10], the thawing procedure [11] and the intrinsic susceptibility of the cells to freeze damage [12].

In 1964, T.M.S. Chang described the first approach of microencapsulating biological materials within a semipermeable membrane [13]. Since then, a variety of biological materials have been successfully encapsulated within semipermeable membranes developed with a wide range of polymers [14–19]. Cell encapsulation technology offers a safe and manufacturable method for the chemically stable immobilization of cells resulting in a controlled and sustained release of '*de novo*' produced therapeutic products. The inner cell content is immunoprotected from both mechanical stress and the host's cellular immune response (reducing need for immunosuppressants) by a suitable membrane which at the same time allows the entrance of nutrient and oxygen supply for the encapsulated cells and the exit of therapeutic products and waste.

However, the water content of the hydrogels (over 90%) together with the relatively large size (300–400 μm) and the fragile semipermeable membrane make microcapsules particularly prone to cryodamage by ice crystal development [20]. Cryoprotectants minimize damage caused by ice formation and encourage formation of an amorphous state in cells, rather than ice crystals, during the cooling-cryostorage-warming cycle [3,8]. Additionally, maintaining frozen cells at the proper long-term storage temperature as liquid nitrogen, minimizes damage to frozen cells [11].

Based on the potential advantages of microencapsulation aforementioned, and considering that little information exists regarding *in vivo* approaches and immune response analysis, we evaluated all the parameters that might suffer from cryodamage during cryopreservation such as cell functionality, chemical stability of the immobilization devices and *in vivo* functionality and biocompatibility in a rodent model, in order to

develop an adequate freeze–thaw protocol.

Using DMSO as cryoprotectant, the present study was undertaken to selectively identify the cooling protocols, cryoprotectant concentrations and cryopreservation periods to facilitate banking of Epo-secreting myoblasts.

2. Materials and methods

2.1. Cell culture

Murine C_2C_{12} myoblasts derived from the skeletal leg muscle of an adult C3H mouse and genetically engineered to secrete murine Epo (mEpo) were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne, EPFL, Switzerland). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic/antimycotic solution. Cultures were plated in T-flasks, maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere standard incubator and were passaged every 2–3 days. All reagents were purchased from Gibco (Spain).

2.2. Cell encapsulation

C_2C_{12} myoblasts genetically engineered to release Epo were immobilized into alginate-poly-L-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [21]. Low viscosity and high guluronic (LVG) alginate was purchased from FMC Biopolymer (Norway) and poly-L-lysine (PLL; hydrobromide Mw: 15 000–30 000) was obtained from Sigma (USA). Briefly, cells were suspended in 1.5% (w/v) LVG alginate sterile solution, obtaining a cell density of $2x10^6$ (for the first approach) and $5x10^6$ cells/mL alginate (for the rest of the *in vitro* and *in vivo* experimental procedures). This suspension was extruded through a 0.35mm needle using a 10 mL sterile syringe from a peristaltic pump. The resulting alginate beads were maintained in agitation for 10 min in the $CaCl_2$ solution (55 mM) for complete ionic gelation and were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a coating with 0.1% alginate for other 5 min. Microcapsules were prepared at room temperature, under aseptic conditions and were cultured in complete medium. The diameters and overall morphology were characterized using inverted optical microscopy (Nikon TSM).

2.3. Cryopreservation protocols

For all the solutions prepared in the experimental assays, the cryoprotective agent DMSO (ATCC, USA) was diluted to the desired concentration in fresh growth medium prior to adding it to the cell suspension. This minimizes the potentially deleterious effects of chemical reactions such as generation of heat, and assures a more uniform exposure to the cryoprotective agent when it is added to the microencapsulated cell suspension, reducing potential toxic effects. Solutions were sterilized by filtration using a 0.2 μm nylon syringe filter (Iwaki, Japan) to minimize the risk of contamination and moisture introduction due to repeated use from one container.

After considering previous protocols employed by the scientific community, the first approach of a series of experiments to find the optimal cryopreservation conditions for microencapsulated C_2C_{12} myoblasts, comprised a non-cryopreserved group, and four different freezing protocols using 20% DMSO as cryoprotectant. Table 1 summarizes the different freezing protocols applied. Based on the results obtained in this first approach, once the optimal freezing protocol was selected (a protocol derived from slow-cooling freezing: slow-cooling [SC], Table 1) a range of DMSO concentrations were evaluated (1%, 5%, 10%, 20% and 30%) in order to corroborate the analyzed literature regarding optimal cryoprotectant concentrations.

Regarding cryovial volumes, 0.2 mL of microcapsules were transferred under aseptic conditions to cryovials (Nalgene, Spain) containing 1.3 mL cryoprotectant solution.

Table 1
Cryopreservation protocols

	SC	SCSh	SpC	MC
DMSO (%)	20	20	20	20
Freezing protocol	1 h: −20 °C	1 h: −20 °C		
	23 h: −80 °C	23 h: −80 °C	2 h: −80 °C	
	Liquid N_2	Liquid N_2	Liquid N_2	Liquid N_2
Shaking[a]	No	Yes	No	No

SC, Slow-cooling; SCSh, Slow-cooling + shaking; SpC, Super-cooling; MC, Maxicooling.

[a]Exposure of microcapsules to cryoprotectant medium on a horizontal shaker for 20 min at room temperature.

2.4. Thawing of microencapsulated cells

For cell reconstitution, frozen vials were placed in a 37 °C water bath until ice disappeared. The external surface of the cryovials was disinfected with Desinmur spray (Fagesa, Spain) to minimize the risk of contamination prior to opening. Immediately after thawing, several washes using fresh culture medium were carried out for complete cryoadditive removal in 1.5 mL eppendorf tubes. Microcapsules were finally transferred into 25 cm^2 T-flasks in fresh culture medium and cultured overnight at 37 °C (5% CO_2, 95% air) in humidified air.

2.5. mEpo production

The Epo secretion of the entrapped cells was evaluated *in vitro* before and after cryopreservation. Conditioned media samples (cell supernatants) were assayed using the Quantikine IVD Epo ELISA kit purchased from R&D Systems (USA). Standards and samples were run in duplicate according to the procedure specified in the kit. The detection limit of this assay was 2.5 mIU/mL. The mEpo secretion of around 200 cell-loaded microcapsules (in triplicate per study group) was measured in conditioned medium for an 8 h release period in order to calculate the C_2C_{12}–mEpo-microencapsulated cells daily secretion rate. Results are expressed as mean ± S.D.

2.6. Mechanical stability study: osmotic resistance test

After a 24 h *in vitro* culture period for microcapsules to recover from the stress derived from the encapsulation process, swelling behavior of microcapsules was determined after 1% citrate solution (w/v) treatment. In short, 100 μL of microcapsule suspension (50–100 microcapsules) were mixed with 900 μL of PBS and placed in a 24-well cell culture cluster. Four wells were used for each group. The 24-well cell culture cluster was put in a shaker at 500 rpm and 37 °C (heater) for one hour. Afterwards, PBS supernatants were eliminated by decanting microcapsules in the wells and 800 μL of sodium citrate solution was added. The cluster containing the microcapsules in citrate medium was maintained at static conditions at 37 °C for 24 h. The following day, the diameters of 20 microcapsules of each group were measured. The washing and shaking step with PBS for 1 h were repeated on days 3 and 7 of the assay during which the cell cluster was maintained at static conditions at 37 °C in the heater.

2.7. Microcapsule implantation

Adult female Balb/c mice (Harlan Interfauna, Spain) were used as allogeneic recipients. Animals were anesthetized by isoflurane inhalation and a total volume of 0.2 mL of cell-loaded microcapsules ($5x10^6$ cells/mL) suspended in Hank's Balanced Salt Solution (HBSS) to a final volume of 1 mL was implanted subcutaneously using an 18-gauge catheter (Nipro Europe N.V., Belgium). Microcapsules cryopreserved for 72 h following slow freezing and using either 10% or 20% DMSO were evaluated along with a longer cryopreservation period group consisting of microcapsules implanted after 15 days at cryopreservation temperature (SC freezing and 10% DMSO). One final group evaluated for a longer *in vivo* period aimed at corroborating the results obtained in the first approach (cryopreserved for 45 days using SC freezing and 10% DMSO). Control animals received 1 mL HBSS by the same route. Before implantation, microcapsules were washed several times in HBSS. Upon recovery, animals had access to food and water *ad libitum*. No immunosuppression protocols were applied to the animals during this study. All experimental procedures were performed in compliance with protocols approved by the institutional animal care and use committee.

2.8. Hematocrit measurements

Blood was collected weekly (every fortnight after one month post-implantation) by retro-orbital puncture using heparinized capillary tubes (Deltalab, Spain). Hematocrits were determined after centrifugation at 3000 rpm for 15 min of whole blood using a standard microhematocrit method. Results are expressed as mean ± S.D.

2.9. Histological analysis

At day 180 after implantation some animals were sacrificed and microcapsules were retrieved and fixed in a 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2. Serial horizontal cryostat sections (14 μm) were processed for hematoxylin–eosin (H&E) staining.

2.10. Statistical analysis

Data are presented as mean ± S.D. Student's t-test was used to detect significant differences when two groups were compared. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni, Scheffé or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. All statistical computations were performed using SPSS 16.0 (SPSS, USA).

3. Results

3.1. Cell functionality and microcapsule morphology evaluation

The evaluation of different freezing methods (summarized in Table 1), which was carried out using the same DMSO concentration in all cases (20%) revealed that the most suitable protocol was the slow-cooling protocol (1 h: -20 ºC; 23 h: -80 ºC; Liquid N_2: -196 ºC). The 20% slow-cooling group showed a 64% reduction in Epo release whereas the rest of the approaches showed higher reduction rates. As described in the experimental procedure section, some microcapsules were kept in the cryoprotectant solution on a horizontal shaker for 20 min at room temperature, with the aim of evaluating whether the semipermeable membrane of the microcapsules somehow limited the diffusion of DMSO into the microcapsules. A 91% reduction in Epo release was observed in comparison with the non-cryopreserved group, confirming the correct permeability of the microcapsule membrane, as stated by considerable effects provoked by the 20 min exposure (Fig. 1).

Based on the results obtained in this first approach, the slow-cooling protocol was selected to continue with the assays.

The evaluation of the mEpo release reduction of the different cryoprotectant concentrations showed that the concentrations of choice where 10% and 20% as the lowest Epo reduction rates were found in these groups (42% and 45% respectively).

It should be mentioned that the cell load employed in this second assay ($5x10^6$ cells/mL alginate) was higher than the cell load employed in the first assay ($2x10^6$ cells/mL alginate). The experiment outcomes showed that the higher cell load resulted in a lower Epo reduction (64% *vs.* 45% respectively).

Considering this positive fact, and the benefits derived from it (the need of a lower implant dose to produce a therapeutic effect *in vivo*), the high cell load was selected for the following experiments of the study.

On the other hand, reduction rates for the rest of the groups were as follows: 95% (1% DMSO), 73% (5% DMSO), 90% (30% DMSO) (Fig. 2A). These results made it possible to refine the cryopreservation technique for the

in vitro stability assay and the *in vivo* approach.

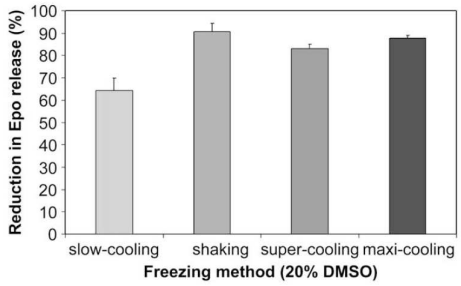

Fig. 1. Reduction in Epo release using different freezing protocols and sharing a common DMSO concentration, 20%, as summarized in Table 1, in comparison with the non-cryopreserved control group. Epo release unit: mIU/mL/24 h/100 microcapsules.

Moreover, in order to evaluate the possible cryodamage caused during the freeze–thaw process, post-thawed morphology of microcapsules cryoprotected with different DMSO concentrations was examined using inverted optical microscopy. Samples were allowed to recover for 24 h prior to survival assessment. As shown in Fig. 2B, important morphological differences were observed on the surface and overall integrity of microcapsules among the range of cryopreservation concentrations evaluated [22,23]. Whereas the groups treated with 1% and 5% DMSO showed important irregularities on their surface (Fig. 2B-a, b) as well as shrinking behavior (Fig. 2B-a), higher cryoprotectant concentrations (10%, 20% and 30% DMSO) turned out to have a more favorable effect on microcapsule morphology (Fig. 2B-c–e). As previously reported, the highest cryoprotectant concentration cells can tolerate should be used [24,25]. However, a major drawback of this high cryoprotectant concentration is cell toxicity.

3.2. Stability of microcapsules

The correct integrity and stability of cell-loaded microcapsules are key requirements in the development of biocompatible devices. In the present study, one of our main goals was to determine the extent of damage cryopreservation could provoke on microcapsule integrity. Once the overall morphology was assessed, we aimed at evaluating swelling properties of the beads and hence, their suitability for the following *in vivo* assays. The swelling assay showed that both non-cryopreserved and cryopreserved microcapsules, regardless of the DMSO concentration and the cryopreservation period (72 h or 15 days) employed in the cryopreservation procedure, swelled and increased their diameter at approximately 10% after the first citrate treatment [26]. However, their size remained stable afterwards during the one-week period the assay lasted (Fig. 3). These results confirm the high chemical resistance of the microcapsules elaborated in this study.

3.3. Hematocrit levels of Balb/c mice following subcutaneous implantation of cryopreserved Epo-secreting microencapsulated cells

Based on the *in vitro* Epo production, we estimated that 0.2 mL of cell-loaded microcapsules ($5x10^6$ cells/mL alginate) might provide a detectable increase in mice hematocrit levels over

time. Cell-loaded microcapsules were implanted in the subcutaneous space of adult female Balb/c mice. In the first approach, the assay comprised four groups: a control group with mice administered with HBSS and three other groups with implanted microcapsules cryopreserved for 72 h using the freezing protocols previously chosen as probably the most suitable ones (SC freezing using 10% or 20% DMSO) and the evaluation of a longer period of cryopreservation: 15 days (using 10% DMSO and SC freezing). As it is observed in Fig. 4, a significantly higher hematocrit level was observed in all the animals implanted with microcapsules when compared with the HBSS (control) group ($P<0.05$).

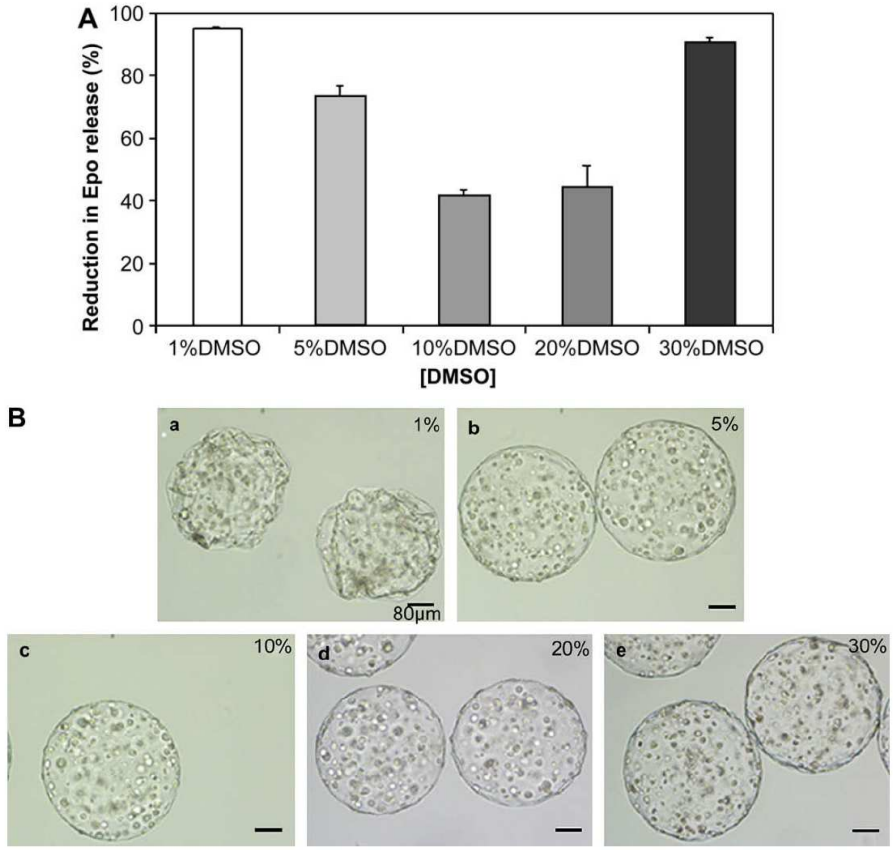

Fig. 2. A. Reduction in Epo release using different cryoprotectant concentrations, in comparison with the non-cryopreserved control group. Epo release unit: mIU/mL/24 h/100 microcapsules. B. Post-thawed morphology of microencapsulated Epo-secreting myoblasts using different DMSO concentrations. Optical microscopy. (a) 1% DMSO; (b) 5% DMSO; (c) 10% DMSO; (d) 20% DMSO; (e) 30% DMSO.

Regarding the implanted groups, no significant differences were found between the non-cryopreserved and the 10% DMSO group (81 ± 5% vs. 85 ± 2% respectively) (P<0.05) by day 45. Additionally, no significant differences were found between the non-cryopreserved and the 20% DMSO group (81 ± 5% vs. 74 ± 8%) (P<0.05).

However, significant differences were found between the 10% DMSO and the 20% DMSO group (85 ± 2% vs. 74 ± 8% respectively) (P< 0.05), making the 10% DMSO group the most suitable choice. Moreover an additional group consisting of microcapsules cryopreserved using the slow-cooling protocol and 10% DMSO but maintained at -196 °C for 15 days (instead of 72 h) showed no significant difference in comparison with the 10% DMSO group (86 ± 4% vs. 85 ± 2% respectively) (P<0.05).

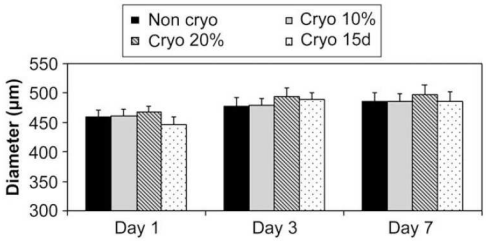

Fig. 3. Osmotic pressure resistance of microcapsules after a one-week treatment with citrate. The error bars on each point correspond to the standard deviation of the mean.

3.4. Hematocrit levels of Balb/c mice using long-term cryopreserved microcapsules: success in increasing the in vivo assay period

One last *in vivo* approach employing the slow-cooling freezing protocol and 10% DMSO chosen as optimal in the first and second assays and also tested *in vivo* in the previously developed animal study, evaluated the effect of a prolonged cryopreservation period (45 days) before implantation. The benefits of being able to preserve the microcapsules cryopreserved for a longer period of time (considering some means of transport usually cause trouble to sending of material) were evaluated. Results showed no significant differences by day 194 between the non-cryopreserved and the cryopreserved group (92 ± 3% vs. 87 ± 9% respectively) (P<0.05), confirming a positive outcome for a longer *in vivo* study period in comparison with the previous *in vivo* experiment (Fig. 5).

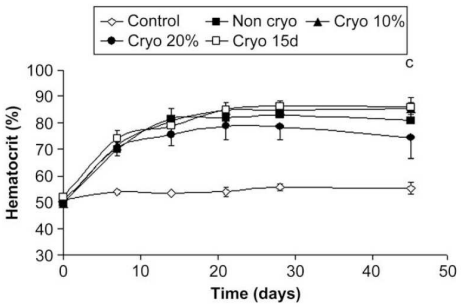

Fig. 4. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. In addition to a negative control group (HBSS, no microcapsules), non-cryopreserved microcapsules were tested *vs.* cryopreserved microcapsules (using SC freezing and either 10% or 20% DMSO) and an additional group for the evaluation of a longer period of cryopreservation: 15 days (using 10% DMSO and SC freezing). Values represent mean ± S.D. Significance (day 45) P<0.05; a: Non Cryo *vs.* Cryo 10%; b: Non Cryo vs. Cryo 20%; c: Cryo 10% *vs.* Cryo 20%; d: Cryo 10% *vs.* Cryo 15d (letter not shown when P>0.05).

3.5. Microcapsule retrieval: histological analysis

By the end of the second *in vivo* experiment (day 180 post-implantation), some of the implanted cell-loaded microcapsules from the non-cryopreserved and the cryopreserved group (microcapsules cryopreserved for 45 days using 10% DMSO and SC freezing) were explanted. The microscopic appearance is shown in Fig. 6.

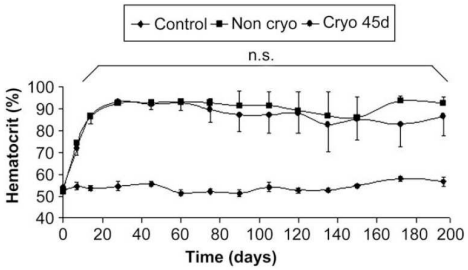

Fig. 5. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. Evaluation of non-cryopreserved microcapsule implantation *vs.* microcapsules cryopreserved for 45 days (using 10% DMSO and SC freezing). Control group: HBSS, no microcapsules. Values represent mean ± S.D. Significance: $P<0.05$*; $P>0.05$ n.s.: Non Cryo *vs.* Cryo 45d.

Microcapsules retrieved from the subcutaneous tissue were mostly aggregated forming an irregular structure in which immobilized cells remained viable as stated indirectly by the elevated hematocrit levels during the study period. The microcapsule network was easily harvested as one piece after a small skin incision. This could definitely be an advantage, as one important challenge in the field of cell microencapsulation is to achieve complete removal of the clump at explantation.

The histological analyses of the explanted microcapsules revealed the formation of some blood capillaries within the microcapsule aggregates. Epo has been reported to act as an angiogenic factor by different pathways [27–29].

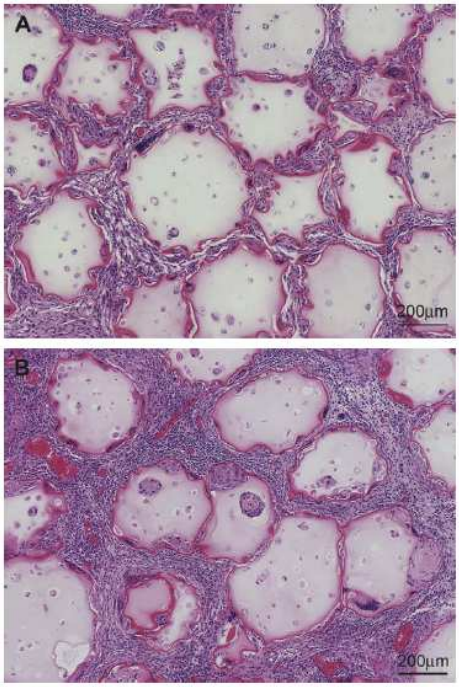

Fig. 6. Microcapsules explanted from the subcutaneous tissue of individuals belonging to the non-cryopreserved and the group cryopreserved for a period of 45 days (using 10% DMSO and SC freezing). 180 days post-implantation. Histological analyses (H&E). A. Non-cryopreserved group. B. Cryo 45d group.

The vascular outgrowth leads to a more suitable microenvironment, where the access of oxygen and nutrients to the entrapped cells might be improved.

Interestingly, although highly purified alginates were used for microcapsule elaboration and this process was done under aseptic conditions, a weak foreign body reaction was observed surrounding the microcapsules although it should be highlighted that no significant adverse effects were encountered as a result of the slight fibrotic layer (Fig. 6).

4. Discussion

The entire process of cryopreservation involves three major phases: a prefreezing phase in which the cells are exposed to a cold shock; a critical freezing phase in which cell membranes are exposed to osmotic and thermal stresses; and finally, a thawing phase wherein the reverse process occurs [30]. During all of these phase transitions, cell membranes are highly vulnerable to variations in thermal and osmotic conditions.

As cryoprotectives, glycerol and DMSO are commonly agents of choice and they are used in various concentrations [31–34]. In general, the use of the highest concentration the cell can tolerate without toxicity is recommended. The main task these solutions fulfill is the decrease of the osmotic imbalance, which occurs across the cell membrane during the freezing process. The cooling rate is also an important factor of this phenomenon. During slow-cooling, ice forms mainly external to the cell before intracellular ice begins to form [35]. This results in extensive cellular dehydration ("solution effect"). On the other hand, rapid cooling leads to more intracellular ice ("mechanical cell damage"). Both effects can be detrimental to cell survival and can inactivate the cells [36]. The formation of intracellular ice as well as the solution effects, which occur during freezing are largely responsible for diminished cell recovery. However, the slow-cooling rate allows the cells to dehydrate by maintaining equilibrium with the partially frozen extra-cellular solution [37]. Although with only a few exceptions, a cooling rate of 1 °C/min is recommended [38]. However, a regimen found effective for one cell type may not be effective for others. Like controlled-rate freezing, a protocol for one cell type may be unsuitable for another that may differ in the diffusion rate of the cryoprotectants and in its osmotic tolerance [11]. Not only the cryoprotectant employed, or the cooling rate but also the storage conditions have also an influence on cell recovery and viability. The storage temperature affects the length of time after which cells can be recovered. In general, the lower the storage temperature, the longer the viable storage period for the cells [10].

Cryopreservation plays a critical role in cell and tissue banking. With respect to the actual donor shortage, the development of immunoprotective devices to enclose cells that deliver therapeutic agents seems to be a promising approach making this strategy suitable for allotransplantation. Thus, considering encapsulation and transplantation procedures are labor-intensive, cryopreservation could be considered as an attractive system for the long-term maintenance and transport of these cell-constructs.

In attempting to develop cryopreservation protocols for microencapsulated cells, a logical starting point would be to examine the cryopreservation protocols previously developed for the enclosed mammalian cells. The relatively large sizes of microcapsules (diameter ~400 μm) makes them particularly susceptible to cryodamage incurred by ice crystallization. Moreover, it must be noted that the high water content of the hydrogel (over 90%) together with the fragile semipermeable membrane and because microcapsules have a substantially large volume to surface area, they are more likely to come into contact with developing ice crystals during cryopreservation, and hence are much more susceptible to cryodamage [20].

Several research groups have already succeeded in freezing and recovering of microencapsulated cells although their effectiveness is variable in terms of viable cell recovery, based on differences in the cryopreservation method [8,39–42]. These differences include cell density, cryopreservation media, cooling rate and storage temperature. However most of these experiments are usually based on *in vitro* approaches and therefore in general lack long-term *in vivo* animal studies which at a final stage evidences results obtained *in vitro* [43].

The data presented in this study demonstrate a first *in vivo* approach to the cryopreservation of microencapsulated C_2C_{12} myoblasts. Animals implanted with the freezed/thawed microencapsulated cells using the 10% DMSO cryoprotectant solution showed higher levels of hematocrit levels in comparison with the 20% DMSO group (Fig. 4). The angiogenic effects of Epo might be responsible for the presence at explantation of several blood capillaries surrounding the cell-loaded microcapsule clump. This neovascularization may suggest the interesting role that this or any other type of angiogenic molecule could have in the long-term functionality of this type of cell-loaded systems.

Previous studies also suggest that unlike the freezing process, rapid thawing of frozen cells is necessary to maintain high viability in order to reduce the exposure of cells to the potentially cytotoxic, cryoprotective agent DMSO [44]. Additionally, several washes placing the entire content of the vials into fresh culture medium, seem to effectively remove the residual cryoadditive.

Overall, the data provided in this study might be of interest to the scientific community working on *in vivo* approaches using cell microencapsulation technology. The multidisciplinarity of this field (from design of polymeric matrices to *in vivo* explantation studies of the devices) promotes interlaboratory collaborations, which can result in more accurate, precise and complete experimental outcomes if optimal storage and transport of cell-based products is achieved.

To our knowledge, microencapsulated cells cryopreserved for as long as 45 days have not been previously proven to be efficient and valid as confirmed in the present study stated by high hematocrit levels maintained in mice implanted with these long-term

cryopreserved microcapsules showing no adverse side effects (Fig. 5). The benefits of preserving the microcapsules for a longer period of time (considering some means of transport usually cause trouble when sending material to other laboratories or companies) were evaluated and no significant differences were found by day 194 between the non-cryopreserved and the cryopreserved group, thus confirming the safety of employing microcapsules cryopreserved for as long as 45 days even having the objective of developing *in vivo* animal studies.

A thorough *in vitro* and *in vivo* evaluation of the cryoprotectant concentration and cryopreservation period was carried out in addition to supporting the use of a slow-cooling protocol and results confirm most of the revised literature on similar approaches previously developed. Although plenty data are derived from all the experimental procedures carried out during the development of this study, the most valuable data are obtained by the success in animal studies evidenced by long-term sustained high hematocrit levels in implanted groups and lack of serious side effects surrounding the implants.

In spite of the encouraging results obtained in this study, the reduction in Epo release after cryopreservation of microcapsules (around 50%) should be minimized by future improvements in the development of suitable cryopreservation protocols.

Some interesting conclusions can be highlighted from these experiments. As a result of the thorough investigation carried out studying most cryopreservation variables involved during freezing and thawing, a long-term sustained release of Epo was achieved after a single subcutaneous administration of post-thawed microcapsules (cryopreserved using slow-cooling freezing and 10% DMSO as cryoprotectant) in allogeneic recipients. No remarkable side effects were observed during the treatment period although the high hematocrit levels obtained may be responsible for the appearance of polycythemia (expanded red cell mass) in the animals [45].

Future challenges may be based on the use of polysaccharides (such as trehalose or sucrose) or serum-based cryoprotectant solutions to improve recovery of microencapsulated cells from the cryopreserved state, a matter of increasing interest. Its low cost along with the easy experiments involved in the procedure make this trial a handy possibility to work on. As a result, vitrification procedures are making increasing improvements in the field [5,40,46,47]. The development of simple and reliable procedures that eliminate the need for slow freezing and enable cryopreservation by direct transfer to liquid nitrogen (employing highly concentrated cryoprotective solutions) will allow much more widespread use of cryopreserved cell-based systems.

5. Conclusions

Freezed/thawed microencapsulated cells using the 10% DMSO cryoprotectant solution and following a slow-

cooling protocol showed the most suitable features in terms of Epo release from the microencapsulated myoblasts, scaffold integrity and *in vivo* hematocrit levels, with no remarkable side effects in terms of reaction of foreign body. In addition, long storage cryopreservation periods were assayed (up to 45 days) which could be beneficial if a successful inter-laboratory exchange of microcapsules or even cell banking is aimed. In conclusion, adequate cryopreservation of encapsulated C_2C_{12} myoblasts merely changes the physiological characteristics of the cells *in vitro* and *in vivo* fulfilling the aim of this study which was to establish a cheap and convenient cryopreservation technique with minimized cell injury during the freeze–thaw process.

Acknowledgements

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Appendix

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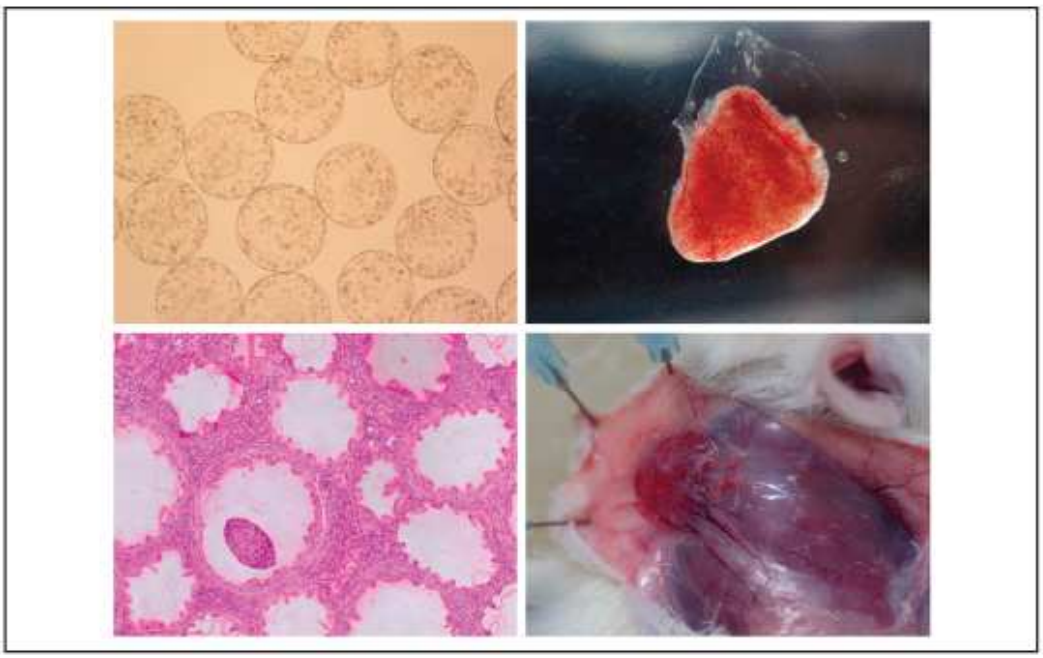

COVER STORY
Xenogeneic delivery of therapeutic products using transient immunosuppression

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Xenogeneic transplantation of erythropoietin-secreting cells immobilized in microcapsules using transient immunosuppression

Ainhoa Murua [a,b], Gorka Orive [a,b], Rosa Mª Hernández [a,b], José Luis Pedraz [a,b,*]

[a]Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, 01006 Vitoria-Gasteiz, Spain

[b]Networking Biomedical Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, SLFPB-EHU, 01006 Vitoria-Gasteiz, Spain

ABSTRACT

Cell encapsulation technology holds promise for the sustained and controlled delivery of therapeutic proteins such as erythropoietin (Epo). Transplantation of microencapsulated C_2C_{12} myoblasts in syngeneic and allogeneic recipients has been proven to display long-term survival when implanted subcutaneously. However, xenotransplantation approaches may be affected by the rejection of the host and thus may require transient immunosuppression. C_2C_{12} myoblasts genetically engineered to secrete murine Epo (mEpo) were encapsulated in alginate-poly-L-lysine-alginate (APA) microcapsules and implanted subcutaneously in Fischer rats using a transient immunosuppressive FK-506 therapy (2 or 4 weeks) to ameliorate immunoprotection of microcapsules. Rats receiving short-term immunosupression with FK-506 maintained high hematocrit levels for a longer period of time (14 weeks) in comparison with the non-immunosuppressed group. In addition, a significant difference in hematocrit levels was detected by day 65 among rats immunosuppressed for 2 or 4 weeks, corroborating the need of a minimum period of immunosuppression (4 weeks) for this purpose. These results highlight the importance of applying a minimum period (4 weeks) of transient immunosuppression if the host acceptance of xenogeneic implants based on microencapsulated Epo-secreting cells is aimed.

* Corresponding author: J.L. Pedraz.

Keywords Alginate; Cell encapsulation; Cryopreservation; Dimethylsulfoxide; Erythropoietin; Myoblast.

1. Introduction

Grafting of primary or genetically engineered cells of allo- or xenogeneic origin has emerged as a promising approach for the sustained and regulated delivery of '*de novo*' produced therapeutic agents with potential capability to treat many diseases or disorders involving hormone or protein deficiencies [1–8]. However, the clinical application of this approach has not yet been fully achieved mainly due to the deleterious side effects of current immunosuppression protocols for preventing host rejection of transplanted cells.

It has long been hypothesized that the use of systemic immunosuppression could be avoided by physically immunoprotecting transplanted cells and tissues in semipermeable membranes [9] preventing direct contact between the transplanted cells and recipient T cells, but not against indirect recognition [10,11]. Since the pioneering study carried out by TMS Chang [12] and Lim and Sun's introduction of alginate-encapsulated islets [13], decades of extensive research has focused on the design and application of immunoisolation devices capable of protecting transplanted allo- and xenogeneic cells from the host, while maintaining unimpeded the exchange of oxygen, nutrients and therapeutic factors (released by the encapsulated cells). This requires the development of efficient, validated and well-documented technology, which could represent a major problem-solving approach thereby circumventing the restrictions in human cell tissue procurement [14,15].

Several studies have suggested that when xenogeneic cells are implanted in sites other than the CNS (survival displayed for 6 months) [16], microencapsulation is beneficial but insufficient for the avoidance of the host indirect immune response [17–19]. This reaction is commonly known as the foreign body reaction (FBR). Several complications are associated with the deposition of FBR-tissue around medical devices [20]. The major factor complicating the implantation of foreign materials is that adherent inflammatory cells or fibrous capsule contraction may cause damage to the implant [21].

In spite of the simplicity of the concept of cell microencapsulation, xenogeneic approaches may usually require the administration of a short-term treatment of immunosuppressive drugs due to specific transplantation antigens, which are well known to be associated with serious side effects or even graft failure [22]. The extent of induced graft acceptance depends both on the immunomodulator and dosing regimen used.

Recently our research group demonstrated that subcutaneous implantation of alginate-poly-L-lysine-alginate (APA) microcapsules containing Epo-secreting C_2C_{12} myoblasts in syngeneic and allogeneic murine recipients resulted in an important increase of hematocrit levels with only one shot of cell-bearing microcapsules and using no immunosuppressive protocols [23–25].

In an attempt to make a step forward in this technology, the long-term func-

tionality and biocompatibility of this cell-based product in a xenogeneic environment was evaluated. As observed in similar procedures carried out by other research groups, xenotransplants often require the use of transient immunosuppression in order to be accepted by the host's immune system. Based on the successful outcomes observed in similar approaches, we opted to choose FK-506 (tacrolimus) as the immunosuppressant [26,27].

Thus, microencapsulated Epo-secreting murine C_2C_{12} myoblasts were subcutaneously implanted in Fischer rats and the efficacy of two immunosuppressive protocols involving FK-506 for either 2 or 4 weeks was evaluated and compared against Epo-treated animals receiving no immunosuppression.

2. Materials and methods

2.1. Cell culture

C3H-mouse derived C_2C_{12} myoblasts genetically engineered to secrete murine Epo (mEpo) were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne, EPFL, Lausanne, Switzerland). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic/antimycotic solution. Cell cultures were plated in T-flasks, maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere standard incubator and were passaged every 2–3 days. All reagents were purchased from Gibco BRL (Invitrogen S.A., Spain).

2.2. Cell encapsulation

C_2C_{12} myoblasts genetically engineered to release Epo were immobilized into alginate-poly-L-lysine-alginate (APA) microcapsules using an electrostatic droplet generator (800 V) with brief modifications of the procedure designed by Lim and Sun [13]. Low viscosity and high guluronic (LVG) alginate was purchased from FMC Biopolymer (Norway) and poly-L-lysine (PLL; hydrobromide Mw: 15,000–30,000) was obtained from Sigma (St. Louis, USA). Briefly, cells were suspended in 1.5% (w/v) LVG-alginate sterile solution, obtaining a cell density of 5×10^6 cells/mL alginate. This suspension was extruded through a 0.35 mm needle using a 10 mL sterile syringe from a peristaltic pump (flow rate: 5.9 mL/h). The resulting alginate beads were maintained in agitation for 10 min in the $CaCl_2$ solution (55 mM) (Sigma, St. Louis, USA) for complete ionic gelation and were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a coating with 0.1% alginate for other 5 min. Microcapsules were prepared at room temperature, under aseptic conditions and were cultured in complete medium. The diameters and overall morphology were characterized using inverted optical microscopy (Nikon TSM).

2.3. Measurement of mEpo secretion

The Epo secretion of the entrapped cells was evaluated *in vitro* before and after implantation (at explantation) to confirm correct functionality of encapsulated cells. Conditioned media samples (cell supernatants) were assayed using the Quantikine IVD Epo Elisa kit purchased from R&D Systems (Minneapolis, MN). Standards and samples were run in duplicate according to the procedure specified in the kit. The detection limit of this assay was 2.5 mIU/mL. The mEpo secretion of around 200 cell-loaded microcapsules (in triplicate per study group) was measured in conditioned medium for an 8 h release period in order to calculate the mEpo daily secretion rate. Results are expressed as mean ± S.D.

2.4. Microcapsule implantation

Adult male Fischer rats (Charles River, Spain) were used as xenogeneic recipients. Animals were housed in specific pathogen free facility under controlled temperature and humidity with a standardized 12 h light/dark cycle and had access to food and water *ad libitum*. Recipients were anesthetized by isoflu-

rane inhalation and a total volume of 0.4 mL of cell-loaded microcapsules (5×10^6 cells/mL) suspended in Hank's Balanced Salt Solution (HBSS) (to a final volume of 2 mL) was implanted subcutaneously using an 18-gauge catheter (Nipro Europe N.V., Belgium). Control animals received 2 mL HBSS by the same route. Before implantation, microcapsules were washed several times in HBSS. All experimental procedures were performed in compliance with protocols approved by the institutional animal care and use committee.

2.5. Immunosuppression

Prior to implantation, a 3-day FK-506 pretreatment was applied to the immunosuppressed groups (1 mg/kg body weight, i.m.) (kindly provided by Fujisawa GmbH, Munich, Germany). After implantation, these groups were treated daily (5 days per week) for either 2 or 4 weeks (1 mg/kg body weight, i.m.). FK-506 doses were injected into the quadriceps muscle, alternating daily between the left and right legs. The tacrolimus dosing scheme employed in this study was selected from similar approaches previously developed [27].

2.6. Hematocrit measurement

Blood was collected weekly (during the first month and every fortnight onwards) by retro-orbital puncture using heparinized capillary tubes (Deltalab, Spain). Hematocrits were determined after centrifugation at 3000 rpm for 15 min of whole blood using a standard microhematocrit method. Results are expressed as mean ± S.D.

2.7. Histological and macroscopical analysis

At day 65 after implantation, 3 animals from each group were sacrificed and capsules were explanted and fixed in 4% paraformaldehyde solution for histological analyses (H&E). Photographic images were taken using a Canon EOS-1D Mark III.

2.8. Tissue disgregation to evaluate explanted microencapsulated cells

Briefly, a mix of collagenase H (2 mg/mL) (Roche Diagnostics, Germany) and hyaluronidase (1 mg/mL) (Sigma, St. Louis, USA) was prepared using DMEM. This enzyme solution was filter-sterilized prior to use. Using 50 mL tubes, 5–6 mL of disgregation solution was added to around 3–4 mL of a microcapsule aggregate. Once tubes were carefully sealed, they were incubated in a shaker bath at 37 °C at 100 rpm for 4 h. Once the surrounding tissue had been disgregated, the solution in the tubes was filtered using 40 μm pore size filters to recover tissue-free capsules.

2.9. Statistical analysis

Data are presented as mean ± S.D. The Student's t-test was used to detect significant differences when two groups were compared. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. All statistical computations were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL).

3. Results and discussion

3.1. Cell functionality and microcapsule morphology evaluation

All microcapsules had a uniform and spherical morphology (diameter: 450–480 μm) without irregularities on the surface (Fig. 1). As reported by previous studies, not only the materials used [28] but also the spherical and smooth morphology of microcapsules have been observed to be of great importance in eluding the host's immune response [29]. Regarding cellular functionality, indirect measurements of Epo production confirmed the correct functionality of enclosed cells. The *in vitro* Epo production of pre-implanted microencapsulated cells was 296 mIU/mL/100 microcapsules/24 h. In addition, 0.4 mL of cell-loaded micro-

capsules (implanted dose) released 22.8 IU/mL/24 h.

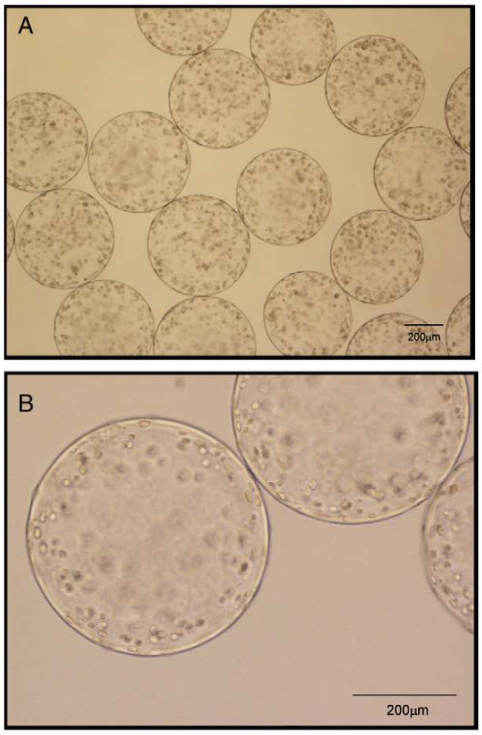


Fig. 1. Morphology of microencapsulated Epo-secreting myoblasts. Optical microscopy.

3.2. Long-term functionality of subcutaneously implanted mEpo-secreting microencapsulated cells in Fischer rats with or without immunosuppression

On the basis of the *in vitro* Epo production, we estimated that 0.4 mL of cell-loaded microcapsules (5×10^6 cells/mL alginate) might result in a therapeutic dose to provide significant increase in rat hematocrit levels over time. As it is observed in Fig. 2, a significantly higher hematocrit level was observed in all the animals implanted with encapsulated cells when compared with the HBSS (control) group (day 65).

As expected, rats receiving FK-506 maintained high hematocrit levels for a longer period of time in comparison to the non-immunosuppressed group (Fig. 2). Moreover, comparing the results obtained in the group immunosuppressed for 2 weeks with the 4-week treated group, a significant difference in hematocrit levels was detected by day 65 (66 ± 5% *vs.* 79 ± 5% respectively) ($P<0.05$), corroborating the need of a minimum period of transient immunosuppression (4 weeks) for this purpose. Moreover, these results are in agreement with similar xenogeneic approaches previously developed [27], thus offering an alternative cell immunoisolation device for this specific application which might benefit from advantages such as an optimal volume–surface ratio (for an adequate nutrition of the totality of the enclosed cells) and small size which could be favorable if the transplantation of encapsulated cells in reduced spaces is aimed.

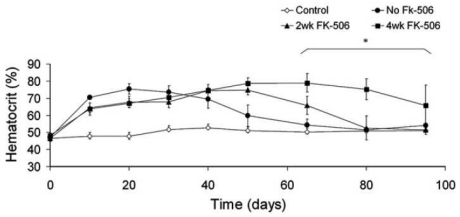


Fig. 2. Hematocrit levels of Fischer rats. 2 wk FK-506 vs. 4 wk FK-506. Significance $^*P<0.05$. Control (n=4). Rest of the groups (n=7).

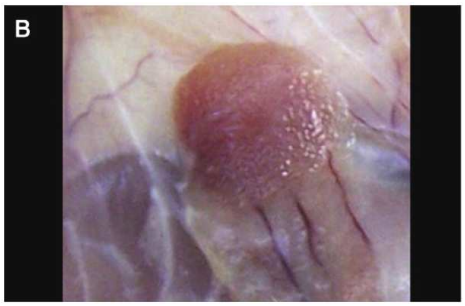
Cell encapsulation within a semipermeable polymer membrane prevents cell contact-mediated death of enclosed cells after *in vivo* implantation. How-

ever, cytokine release from the host (not requiring cell contact) might manage to destroy encapsulated cells. The site of transplantation plays a significant role in determining the fate of xenogeneic cells [16,30–33]. It has been hypothesized that xenoantigens secreted by the encapsulated cells can lead to activation of the host immune system [17]. Once the immune response is activated, increasing populations of lymphocytes, macrophages, granulocytes, and multinucleate giant cells surround the microcapsule aggregate, leading to encapsulated cell death [17]. However, it has been hypothesized that prolonged, low-level release of antigens can lead to tolerance [34].

3.3. Microcapsule retrieval: cell functionality at explantation and histological analysis

The implanted cell-loaded microcapsules of several rats from the treated and non-treated groups were removed at day 65 postimplantation. The macroscopic appearance is shown in Fig. 3. Capsules retrieved from the subcutaneous tissue were mostly aggregated forming an irregular structure in which immobilized cells remained viable as stated indirectly by the elevated hematocrit levels during the study period. The microcapsule network was easily harvested as one piece after a small skin incision. This could be considered as an advantage in comparison with free-floating microcapsules carrying Epo-secreting myoblasts usually observed when the intraperitoneal cavity is used as implantation site [23–25].

Both the macroscopic (Fig. 3) and the histological analyses of the explanted microcapsules revealed some blood capillaries surrounding the microcapsule aggregates (Fig. 4, asterisks), mainly observed in the immunosuppressed individuals.



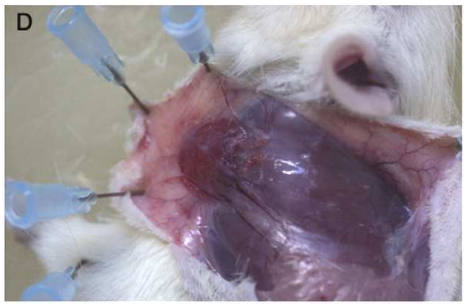
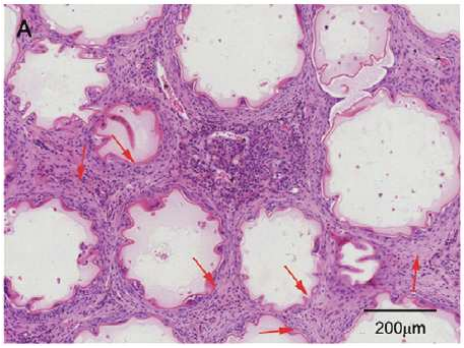


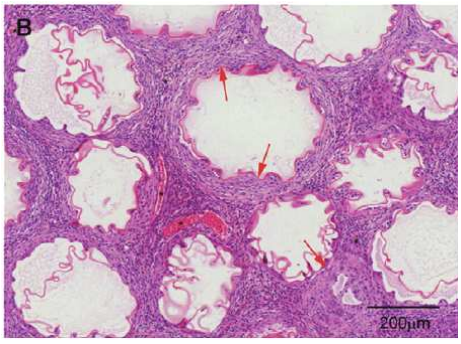
Fig. 3. Photographs of explanted microcapsules and evidence of blood capillaries observed. A. Non-treated rat; B. 2-week FK-506 treated rat; C,D. 4-week FK-506 treated rat.

We hypothesized this could be due to the angiogenic effects reported for Epo [35–37]. The vascular outgrowth leads to a more suitable microenvironment, where the access of oxygen and nutrients to the entrapped cells might be improved.

Histological analyses revealed the formation of a fibrotic layer, and this fact might be responsible (one of many) for Epo delivery limitations. Nevertheless, the angiogenic and immunomodulatory properties attributed to erythropoietin and the increased Epo release from the microencapsulated myoblasts might prevail and thus contribute to enough Epo plasma levels to induce a response, in terms of hematocrit enhancement. Furthermore, no indication of total graft rejection was observed in the hosts.

Albeit the microcapsules were fabricated using highly purified alginates and under aseptic conditions, a slight foreign body reaction was observed surrounding the microcapsules, mainly detected in non-immunosuppressed individuals (Fig. 4, red arrows). This fibrotic layer (developed as a result of implantation of xenogeneic Epo-secreting myoblasts) might be one of the factors responsible for the hematocrit difference found between non-immunosuppressed and immunosuppressed rats. Limited graft survival has generally been associated with pericapsular cell overgrowth (promoted by interleukin-1β and tumour necrosis factor-α) leading to a thick fibrotic layer [38]. This might result in a diminished nutrition to the inner cells and therefore, in diminished cell function and viability with time [22].





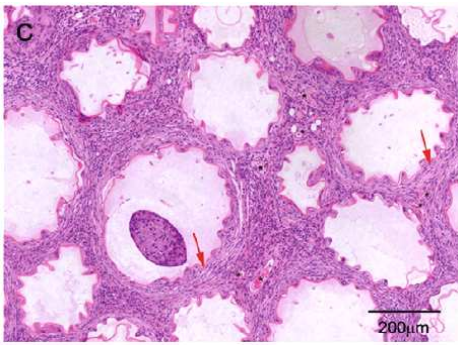


Fig. 4. Histologicalmicrophotographs of explantedmicrocapsules.A. Non-treated. B. 2-week FK-506. C. 4-week FK-506. Asterisks: blood capillaries. Red arrows: foreign body reaction.

Nonetheless, as observed in previous allogeneic approaches carried out by our group, the long-term effectiveness of the developed system was confirmed with high hematocrit levels maintained in the individuals for up to 120 days. Despite detection of a slight fibrotic layer, Epo delivery from the encapsulated cells was not hampered [25].

Finally, Epo delivery from the explanted encapsulated cells (65 days post-implantation) yielded interesting results showing a low Epo production from non-immunosuppressed encapsulated cells (10 mIU/mL/24 h) and the 2-week FK-506 group (10.4 mIU/mL/24 h) in comparison to the 4-week FK-506 group (60 mIU/mL/24 h), which again confirmed the importance of using a minimum period of transient immunosuppression (4 weeks) if the long-term survival of encapsulated xenogeneic cells is aimed. It is important to note that capsules secreting as little as 60 mIU mEpo/day are able to maintain rats at their threshold hematocrit levels, making Epo secretion at explant a more quantitative means of evaluating the survival of encapsulated xenogeneic myoblasts. Moreover, these results confirm that FK-506 therapy had no negative effect on the survival nor the ability to secrete Epo from encapsulated myoblasts.

Novel insight shows that not only the capsules' material but also the effect of cytokines released from the enveloped cells should be held responsible for a significant loss of the immunoisolated cells and, thus, failure of the grafts on the long term [39]. New approaches in which newly discovered inflammatory responses are silenced bring the technology of transplantation of immunoisolated cells close to clinical application [39].

It is likely that future directions in using encapsulated xenogeneic cells will build on incremental improvements and further optimization of diverse immunosuppression protocols, i.e. applying immunosuppression in alternating weeks (immunosuppression during one week, resting period during the following one and so on) to evaluate the potential positive immunomodulator effect of alternative protocol schemes. In addition, the increasing understanding of the biology of the disease, polymer chemistry, and particularly the interaction between cells and polymers will further enhance feasibility of using immunoisolation for therapeutic treatments.

4. Conclusion

The described findings provide a means of transplanting genetically modified xenogeneic myoblasts in a peripheral immunoreactive site (SC) while ensuring their long-term survival. Fischer rats rendered unresponsive during 94 days to encapsulated C_2C_{12} mEpo cells by transient immunosuppression with FK-506 (4 weeks). In particular, the importance of the length of initial immunosuppression on the survival of cells within the implant was confirmed.

Acknowledgements

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Appendix

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Design of a composite drug delivery system to prolong functionality of cell-based scaffolds

Ainhoa Murua [1], Enara Herran [1], Gorka Orive [1], Manoli Igartua [1], Francisco Javier Blanco [2], José Luis Pedraz [1], Rosa Mª Hernández [1,*]

[1] *Laboratory of Pharmacy and Pharmaceutical Technology, Networking Biomedical Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, SLFPB-EHU, Faculty of Pharmacy, University of the Basque Country, 01006, Vitoria-Gasteiz, Spain.*

[2] *CIBER-BBN-Bioscaff Cartílago, INIBIC-Hospital Universitario A Coruña, 15006, A Coruña, Spain.*

ABSTRACT

Cell encapsulation technology raises hopes in medicine and biotechnology. However, despite important advances in the field in the past three decades, several challenges associated with the biocompatibility are still remaining. In the present study, the effect of a temporary release of an anti-inflammatory agent on co-administered encapsulated allogeneic cells was investigated. The aim was to determine the biocompatibility and efficacy of the approach to prevent the inflammatory response. A composite delivery system comprised of alginate-poly-L-lysine-alginate (APA)-microencapsulated Epo-secreting myoblasts and dexamethasone (DXM)-releasing poly(lactic-co-glycolic acid) (PLGA) microspheres was implanted in the subcutaneous space of Balb/c mice for 45 days. The use of independently co-implanted DXM-loaded PLGA microspheres resulted in an improved functionality of the cell-based graft, evidenced by significantly higher hematocrit levels found in the cell-implanted groups by day 45, which was found to be more pronounced when higer cell-doses (100 μL) were employed. Moreover, no major host reaction was observed upon implantation of the systems, showing good biocompatibility and capability to partially avoid the inflammatory response, probably due to the immunosuppressive effects related to DXM. The findings of this study imply that DXM-loaded PLGA microspheres show promise as release systems to enhance biocompatibility and offer advantage in the development of long-lasting and effective implantable microencapsulated cells by generating a potential immunopriviledged local environment and an effective method to limit the structural ensheathing layer caused by inflammation.

* Corresponding author: R.M. Hernández

Keywords Alginate; PLGA; Biocompatibility; Microencapsulation; Dexamethasone; Erythropoietin.

1. Introduction

Microencapsulation of living cells is a promising approach for the continuous delivery of therapeutics. This technology is based on the immobilization of cells within a polymeric matrix surrounded by a semipermeable membrane. The inner cells release the therapeutic agent continuously, while the semipermeable membrane immunoprotects the cells from the host immune system allowing the exchange of nutrients, oxygen and waste products (Ricci *et al.*, 2005; Lee *et al.*, 2000).

Since the successful approach developed by Lim & Sun in 1980, using the APA system to entrap islets of Langerhans, extensive research has been carried out using microencapsulation technology as an alternative treatment for a wide range of disorders and significant achievements with claims of remarkable success including a few non-human primates and human pilot clinical trials have been obtained (Lim et al., 1980; Elliot *et al.*, 2005; Dufrane *et al.*, 2006; Calafiore *et al.*, 2006; Hernández *et al.*, 2010).

Nevertheless, if scalability of the technology into clinical practice is aimed, an optimal composite system must be designed. Failure to achieve optimal biocompatibility and immune acceptance has often been ascribed to the inflammatory response eventually evoked towards the transplanted microencapsulated cells leading to limited immunobarrier competence, hypoxia and finally encapsulated cell apoptosis due to the great distance between the encapsulated cells and the blood supply (De Groot *et al.*, 2004; Orive *et al.*, 2006; de Vos *et al.*, 2009). As far as biocompatibility is concerned, implantable devices can elicit a foreign body reaction. An acute inflammatory response, characterized by neutrophils as the primary cellular infiltrate, is followed by a chronic inflammation characterized by monocyte and lymphocyte. Monocytes, differentiated into macrophages, lead into the granulation tissue development (Babensee *et al.*, 1998; Anderson *et al.*, 1999).

In spite of interesting and significant advances in the field already achieved, some challenges still remain unsolved. Optimal biocompatibility of the cell-based system upon *in vivo* implantation seems to be a pending issue, both in allogeneic and specially in xenogeneic approaches. Hence, the development of a temporally immunoprotected transplantation microenvironment might be a newsworthy approach. Considering the toxicity and side effects related to the implementation of general immunosuppression, the use of a temporal protocol locally administered in order to generate an immunopriviledged environment, represents an interesting alternative approach (Calafiore *et al.*, 1999). Moreover, the labor-intensive constant administration of anti-inflammatory drugs needed to reduce host response against transplanted microcapsules could be avoided and hence a lower dose of drug permitted compared to its systemic administration, with favorable impact on typical treatment side effects due to chronic exposure (Safley *et al.*, 2008).

Several different strategies have been considered to overcome the harmful effects related to systemic immunosuppression, including the use of high purity (pyrogen and endotoxin-free) polymers (already optimized by most researchers in the field) and combined cell microencapsulation systems where a secondary anti-inflammatory drug release system is incorporated along with the cells (Omer *et al.*, 2003; Bunger *et al.*, 2005; Baruch *et al.*, 2009). However, considering the possible decrease in pH as a result of the biodegradation of PLGA, the incorporation of PLGA microparticles within the cell-loaded capsules was discarded (Baruch *et al.*, 2009).

In the present study, an independent composite system was developed comprising APA microcapsules embedding Epo-secreting C_2C_{12} myoblasts and PLGA microspheres loaded with DXM as a model anti-inflammatory drug, hence creating an immunopriviledged environment.

Over the past years, much interest has been focused on the development of PLA, PGA and PLGA copolymer microspheres as delivery carriers of interesting pharmacological agents (Benny *et al.*, 2008; Bae *et al.*, 2009; Anderson *et al.*, 1997) due to their potential usefulness in increasing efficacy (Panyam *et al.*, 2004), reducing enzymatic degradation (Rosler *et al.*, 2001) and controlling release rates (Jain *et al.*, 2000).

Dexamethasone is a clinically widely used glucocorticoid anti-inflammatory and immunosuppressive agent. It is considered a safe drug, being associated with a relatively low risk of adverse gastrointestinal effects and renal effects at anti-inflammatory doses (Brunton *et al.*, 2006). Glucocorticoids are used to prevent or suppress the inflammatory response given by many irritating phenomena such as radiant, mechanical, chemical, infectious and immune stimulus.

Thus, the objectives of this study were on the one hand to develop an independent composite drug delivery system secreting DXM to enhance and prolong the functionality of the cell-loaded graft. On the other hand, the composite system was evaluated for different microencapsulated Epo-secreting cell-doses with the aim of achieving more physiological hematocrit levels to test its therapeutic efficacy.

2. Materials and methods

2.1. Preparation of microspheres

Poly (DL-lactide-co-glycolide) (PLGA) (Resomer® RG 752H) with a copolymer ratio of 75:25 (lactic/glycolic (%)) was provided by Boehringer Ingelheim (Germany). Dexamethasone was purchased from Fagron Iberica (Barcelona, Spain). Poly (vinyl alcohol) (PVA; average MW=30,000-70,000) was obtained from Sigma (St. Louis, USA).

Microspheres were prepared by modification of a previously described technique (Garcia *et al.*, 2009). PLGA microspheres loaded with dexamethasone were prepared by oil-in water (O/W) emulsion/solvent evaporation technique. The organic phase consisted of 200 mg PLGA (75:25) and 40 mg dexamethasone dissolved in 1 ml of methylene chloride. This organic phase was sonicated for 1 min (Branson Ultrasonic Sonifier® 250, CT, USA). The resultant dispersion was added to 2.5 mL 1% PVA aqueous solution and homogenized at 8,000 rpm for 2 min (Ultra Turrax T25, IKA-Labortechnik, Staufen, Germany). Then, 5 mL of 0.1% PVA

aqueous solution was added to the obtained emulsion and sonicated again for 1 min. The final O/W emulsion was added to 50 mL of 0.1% PVA aqueous solution and stirred on a magnetic stir plate at room temperature for 3h to complete evaporation of the solvent. The resulting microspheres were collected by centrifugation at 10,000 xg (Sigma 3-30K), washed three times with distilled water to remove any remaining solvent or PVA and finally, freeze-dried for 24h (LyoBeta 15, Telstar, Tarrasa, Spain). Microspheres without dexamethasone, were prepared using the same method and parameters described above.

2.2. Characterization of microspheres: particle size analysis and morphological evaluation

The mean particle diameter and size distribution were determined by laser diffractometry with a Coulter Counter LS 130 (Amherst, MA, USA). Microsphere morphology and surface characteristics were examined by scanning electron microscopy (SEM; Jeol® JSM-7000F).

2.3. Dexamethasone loading efficiency

5 mg of microspheres were dissolved in 10 mL of acetonitrile, filtered and analyzed by high performance liquid chromatography (Alliance 2795 Waters) coupled to an UV detector. The analytical column was Nucleosil 120 C18 (15 cm x 4 mm, 5 µm,Technocroma) and mobile phase consisted of acetonitrile: water: fosforic (30:70:0.5 (v/v/v)) at pH 6. The injection volume was 20 µL, the flow rate was 1mL/min and UV/Visible absorbance detector was set at 238 nm. The retention time of DXM was 7 min at room temperature (Zolnik *et al.*, 2008; Splanger *et al.*, 2001). The assay was linear over DXM concentrations ranging from 5 µg/mL to 60 µg/mL.

2.4. In vitro release studies

The release profile of DXM from PLGA microspheres was determined by incubating 5 mg of microspheres in a test tube containing 1 mL of PBS 20 mM (pH 7.4) and shaking with a rotator shaker at 25 rpm at 37 ± 0.5 ºC. At defined time intervals, all the release media was removed by centrifugation and replaced with 1 mL of fresh medium. The amount of DXM released in the supernatant was determined by HPLC, using the same method described above. The release test was performed in triplicate and protected from direct light exposure. DXM release profiles were generated for this microsphere formulation in terms of cumulative DXM release versus time.

2.5. Cell culture

C3H skeletal muscle derived C_2C_{12} myoblasts genetically modified to deliver murine Epo (mEpo) were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne, EPFL, Lausanne, Switzerland). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic / antimycotic solution. Cell cultures were plated in T-flasks, maintained at 37 °C in a humidified 5% CO_2 / 95% air atmosphere standard incubator and were passaged every 2–3 days. All reagents were purchased from Gibco BRL (Invitrogen S.A., Spain).

2.6. Cell encapsulation

C_2C_{12} myoblasts genetically engineered to secrete murine Epo were entrapped into APA microcapsules using an electrostatic droplet generator (800 V) with brief modifications of the procedure designed by Lim and Sun (Lim *et al.*, 1980). Low viscosity and high guluronic (LVG) alginate purchased from FMC Biopolymer (Norway) and poly-L-lysine (PLL; hydrobromide Mw: 15,000–30,000) obtained from Sigma (St. Louis, USA) were employed. Cells were suspended in 1.5% (w/v) LVG-alginate sterile solution, obtaining a cell density of 5×10^6 cells/mL alginate. This suspension was extruded through a 0.35 mm needle using a 10 mL sterile syringe from a peristaltic pump (flow rate: 5.9 mL/h). The resulting alginate beads were maintained in agitation for 10 min in the $CaCl_2$ solution (55 mM) (Sigma, St. Louis, USA) for complete ionic gelation and were ionically crosslinked with 0.05% (w/v) PLL for 5 min, followed by a coating with 0.1% alginate for additional 5 min. Microcapsules were prepared at room temperature, under aseptic conditions

and cultured in complete medium. The diameters and overall morphology were characterized using inverted optical microscopy (Nikon TSM).

2.7. Metabolic cell activity

The cellular activity of the entrapped myoblasts was evaluated *in vitro* during 4 weeks post-encapsulation. The viable cell number per microcapsule was determined by the Cell Counting Kit-8 (CCK-8 assay) (Fluka, Buchs, Switzerland). CCK-8 allows very convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4- disulfophenyl)-2H-tetrazolium, monosodium salt] (patent No WO97/38987) produces a yellow coloured and water-soluble product (formazan), upon reduction by dehydrogenases in cells, which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Briefly, 10 µL of the CCK-8 solution was added to a known number of microcapsules (around 40) placed in a 96-well cell culture cluster and incubated at 37 °C for 4 h in humidified conditions. After 4 hours, the resulting solution was read on a microplate reader (Multiskan EX, Labsystems) at 450 nm with 690 nm as the reference wavelength. Results are expressed as mean ± standard deviation.

2.8. Mechanical stability: osmotic resistance test

The swelling behavior of the microcapsules was determined after 1% citrate solution (w/v) treatment. In short, 100 µL of microcapsule suspension (50-100 microcapsules) was mixed with 900 µL of phosphate-buffered saline (PBS) and placed in a 24-well cell culture cluster. Each group was run in quadruplet. The cell cluster was placed in a shaker at 500 rpm and 37 °C for 1 h. Following this, supernatants were eliminated, and 800 µL of a sodium citrate solution was added. The cluster containing the microcapsules was maintained at static conditions at 37 °C for 24 h. On the following day, the diameters of 20 microcapsules of each group were measured. The washing and shaking step with PBS and the static condition were repeated during the following days until a one week period was completed.

2.9. Surgical procedure: subcutaneous implantation of APA microcapsules and PLGA microspheres

Adult female Balb/c mice (Harlan Interfauna, Spain) were used as allogeneic recipients. Animals were housed in specific pathogen free facility under controlled temperature and humidity with a standardized 12 h light/dark cycle and had access to food and water *ad libitum* upon recovery. Recipients were anesthetized by isoflurane inhalation and a total volume of 100 µL or 50 µL of cell-loaded microcapsules (5×10^6 cells/mL) suspended in Hank's Balanced Salt Solution (HBSS) (to a final volume of 500 µL) was implanted subcutaneously using an 18-gauge catheter (Nipro Europe N.V., Belgium). Treatment groups also received 6.75 mg of DXM-loaded PLGA miscropsheres (1mg DXM/mouse), based on previous reports (Hickey *et al.*, 2002a; Zolnik *et al.*, 2008), co-administered with microencapsulated cells, suspended in HBSS. Two control groups were assayed. One of them consisted of 100 µL of empty APA microcapsules and the other control group received empty APA microcapsules along with empty PLGA microspheres (microspheres without DXM), all suspended in HBSS, by the same route. Before implantation, microcapsules were washed several times in HBSS. All experimental procedures were performed in compliance with protocols approved by the institutional animal care and use committee.

2.10. Hematocrit measurement

Blood was collected during 45 days (weekly during the first month) from the submandibular vein using safety lancets and collection vials (Sarstedt, Spain). Hematocrits were determined after centrifugation at 3,000 rpm for 15 min of whole blood using a standard microhematocrit method. Results are expressed as mean ± standard deviation.

2.11. Histological and macroscopical analysis: evaluation of the immune reaction

At day 60 after implantation, 3 animals from each group were sacrificed and capsules were explanted and fixed in 4% paraformaldehyde solution for histological analyses. The overall evaluation of the immune reaction towards transplanted microcapsules was performed blindly by a pathologist. H&E stained slides of each sacrification time point and from each treatment group were evaluated. Masson's trichrome and alcian blue staining were also performed for further evaluation. Photographic images were taken using a Nikon D-60.

2.12. Statistical analysis

Data are presented as mean ± standard deviation. Data between control and experimental groups were analyzed for statistical significance. The Student's t-test was used to detect significant differences when two groups were compared. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. All statistical computations were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL).

3. Results

Figure 1 shows an schematic illustration of the immunopriviledged microenvironment generated in the subcutaneous space of Balb/c mice after implantation of Epo-secreting encapsulated cells and dexamethasone-releasing microspheres.

3.1. APA microcapsule morphology evaluation

All cell-loaded microcapsules had a uniform and spherical morphology without irregularities on their surface and a narrow size distribution as shown in Figure 2A. Previous studies have reported the relevance of the materials employed in the elaboration of microcapsules to obtain biocompatible microcapsules (Ríhová *et al.*, 2000); Santos *et al.*, 2010). However, not only the materials used but also the spherical and smooth shaped morphologies of the microcapsules have been observed to be of great importance to elude the host's immune response (Santos *et al.*, 2010; Ponce *et al.*, 2006), leading to the conclusion that in this study, enclosed cells were correctly adapted to the surrounding polymer scaffold.

3.2. Prepararation and characterization of PLGA microspheres

The mean particle size for the obtained microspheres was 11 ± 0.3 μm for DXM loaded microspheres and 13 ± 0.2 μm for empty microspheres. When observed under scanning electron microscopy (SEM) the spheres appeared spherical with a smooth and uniform surface. (Fig. 2B). Considering that the theoretical loading was 20 %, the loading efficiency for the developed formulation was 74%. Total DXM loading was 15.6 %.

3.3. Cell functionality and stability of microcapsules

The metabolic activity of the encapsulated cells was analyzed *in vitro* over the course of 30 days. CCK-8, being nonradioactive, allows a sensitive colorimetric assay for the determination of viable cells in cell proliferation. As seen in Figure 3A, C_2C_{12} myoblasts showed similar viabilities over the 30-day assay period, supporting the idea that the diffusion of oxygen and nutrients was not influenced by an inappropriate

membrane behavior. A slight increase in viability was observed after the third week, probably due to a slight proliferation of the enclosed myoblasts.

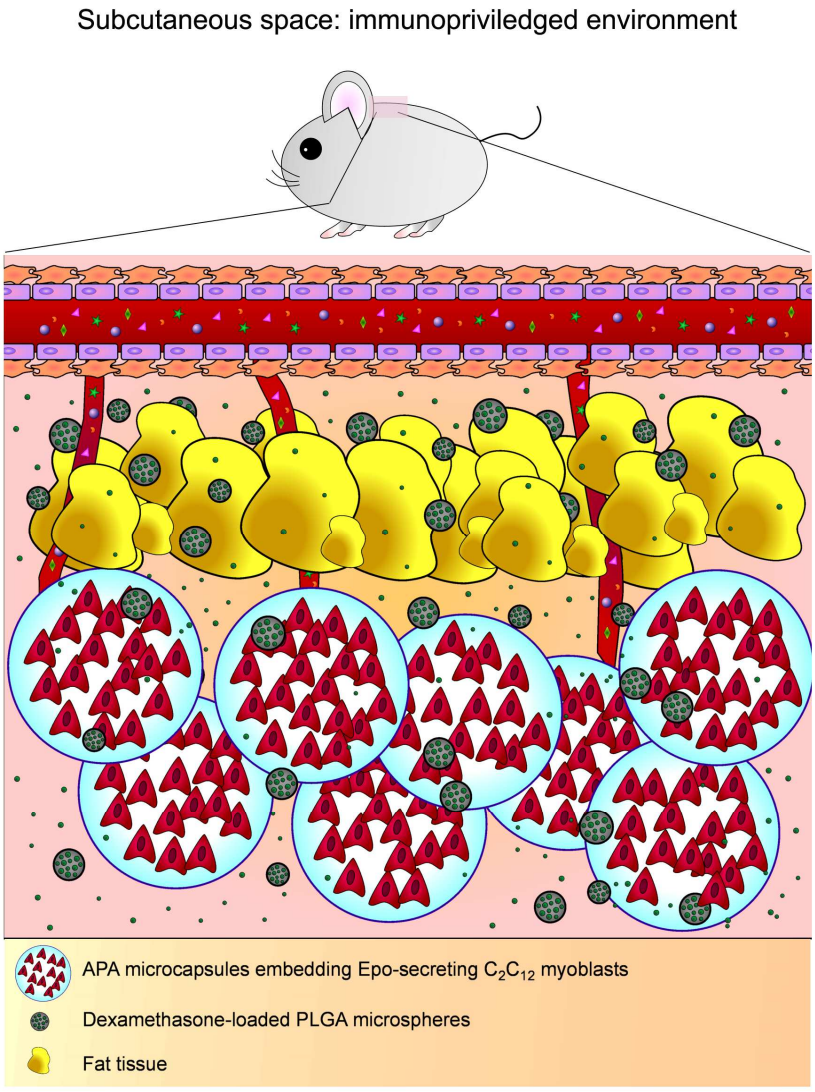

Fig. 1. Schematic illustration of the immunomodulatory environment created in the subcutaneous space of implanted mice.

Once the overall morphology of APA microcapsules was studied, the swelling behavior of the beads was evaluated and hence, their suitability and adaptability for the following *in vivo* assay was assessed. The swelling assay showed that microcapsules swelled and increased their size at approximately 10% after the first citrate treatment (Ponce *et al.*, 2005). Nonetheless, after this initial accommodation of matrices, stability of their size was maintained during the one-week assay period. (Fig. 3B). These results confirm the high chemical resistance of the developed microcapsules.

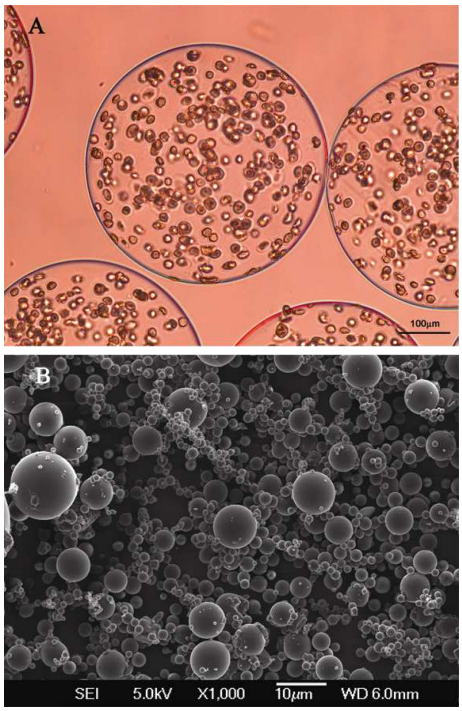

Fig. 2. Morphological evaluation of A. alginate microcapsules and B. PLGA microparticles.

3.4. In vitro dexamethasone release kinetic studies

Figure 4 shows the release profile of DXM from PLGA microspheres. The release profile was triphasic, with an initial burst of 40.1% of the total loaded protein. Between days 2 and 3, release was followed with a mean constant of 11.3 µg DXM/day/mg microspheres. From day 4 to the end of the release assay, a release rate of 1.54 µg DXM/day/mg of microspheres was observed.

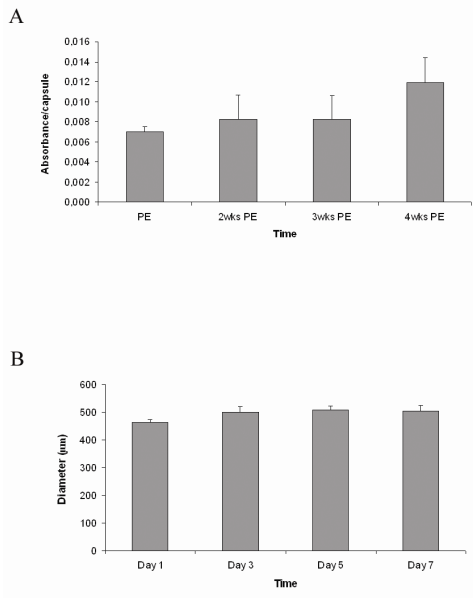

Fig. 3. A. Viability evaluation of encapsulated cells (CCK-8); PE: post-encapsulation. B. Osmotic pressure resistance of microcapsules after a one-week treatment with sodium citrate. The error bars on each point correspond to the standard deviation of the mean.

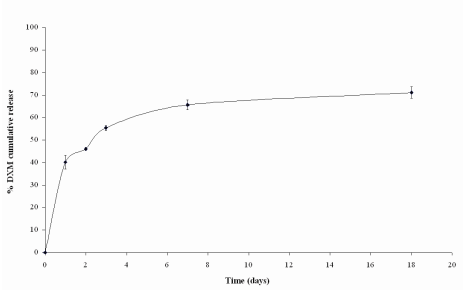

Fig. 4. *In vitro* dexamethasone release profile from PLGA microspheres at 37 ºC in PBS buffer (pH 7.4). Values are represented as mean ± SD (n=3).

3.5. Long-term functionality of subcutaneously implanted mEpo-secreting microencapsulated cells in Balb/c mice. Epo dosing evaluation and anti-inflammatory effect of dexamethasone on implants

The anti-inflammatory effect of dexamethasone may ease to avoid the formation of pericapsular fibrosis, which is mainly responsible for the failure of the implanted devices. To address this issue, adult female Balb/c mice were used as recipients, and cell-loaded microcapsules were implanted in the subcutaneous space. As it is observed in Figure 5, a significantly higher hematocrit level was observed in all the animals implanted with alginate microcapsules when compared with the control group ($P< 0.05$).

The use of an independent composite system resulted in an improved functionality of the cell-based graft, which was found to be more pronounced in the 100 µL-dose group, from day 20 to the end of the study ($P<0.05$).

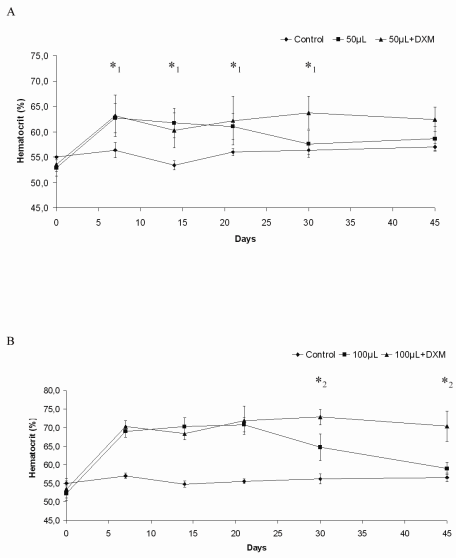

Fig. 5. Hematocrit levels of Balb/c mice over time (45 days). A. 50 µL cell-microcapsule dose. Control: empty alginate microcapsules. B. 100 µL cell-microcapsule dose. Control: empty alginate microcapsules + empty PLGA microparticles. Some groups received dexamethasone-loaded PLGA microparticles while others didn't. Significance: $P<0.05$; *1: control vs. cells. *2: No DXM vs. DXM group.

3.6. Microcapsule retrieval: cell functionality at explantation and histological analysis

The implanted delivery systems of several mice from both control and treatment groups were removed at day 60 postimplantation. A macroscopic image of the implantation site can be observed in Figure 6. Retrieval of microcapsules from the subcutaneous tissue revealed the formation of an irregular structure where capsules were mostly aggregated. Moreover, viability of the entrapped cells could be indirectly stated by the elevated hematocrit levels during the study period. The

microcapsule network was easily harvested in one or two pieces after a small skin incision.

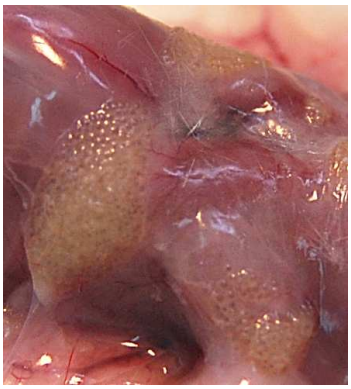

Fig. 6. Subcutaneous location of alginate microcapsules and PLGA-dexamethasone microparticles previous to their explantation on day 60.

Both the macroscopic (Fig. 6) and the histological analyses of the explanted microcapsules revealed some blood capillaries surrounding the microcapsule aggregates (Fig. 7, black arrow), mainly observed in the DXM-treated 100 μL-dose group. This might be due to the angiogenic effects reported for Epo (Murua *et al.*, 2009b; Ribatti *et al.*, 2007; Müller-Ehmsen *et al.*, 2006; Benelli *et al.*, 2006). Histological analyses revealed the formation of a mild fibrotic layer, specially in cell-implanted individuals not treated with dexamethasone. In order to assess the clinical-grade purity and transplantation suitability of the implanted devices alone, two control groups were included in the present study. As observed in Figure 7, control groups showed no evidence of pericapsular overgrowth (they were practically free of inflammation), thus confirming the immuno acceptance of the implanted drug delivery systems. Regarding the cell-implanted groups, eventhough no significant difference was observed (blindly analyzed by an independent pathologist) between the DXM-implanted and non-treated groups in terms of fibrotic reaction, there is a tendency towards a milder fibrotic overgrowth in DXM-treated groups as confirmed by the therapeutic outcome which resulted in enhanced functionality of the cell-based grafts.

4. Discussion

Extensive work has been carried out in recent years aiming at reducing or eliminating the immune reaction towards encapsulated cells implants. Many studies focused on improving the purity of the biomaterials employed in the elaboration of the microdevices and their adequate shape and morphology. In spite of the huge progress made in reducing the immune reaction, particularly in the case of xenotransplantation approaches, much work lies ahead. Short-term systemic immunosuppression has also been proposed as a possible alternative therapy towards eliminating the immune reaction from the host, by actively suppressing the inflammatory response generated against the transplanted encapsulated cells. However, the side effects derived from the systemic delivery of immunosuppressants cannot be avoided up to date so alternative locally-secreted solutions need to be investigated (Weiss *et al.*, 2006).

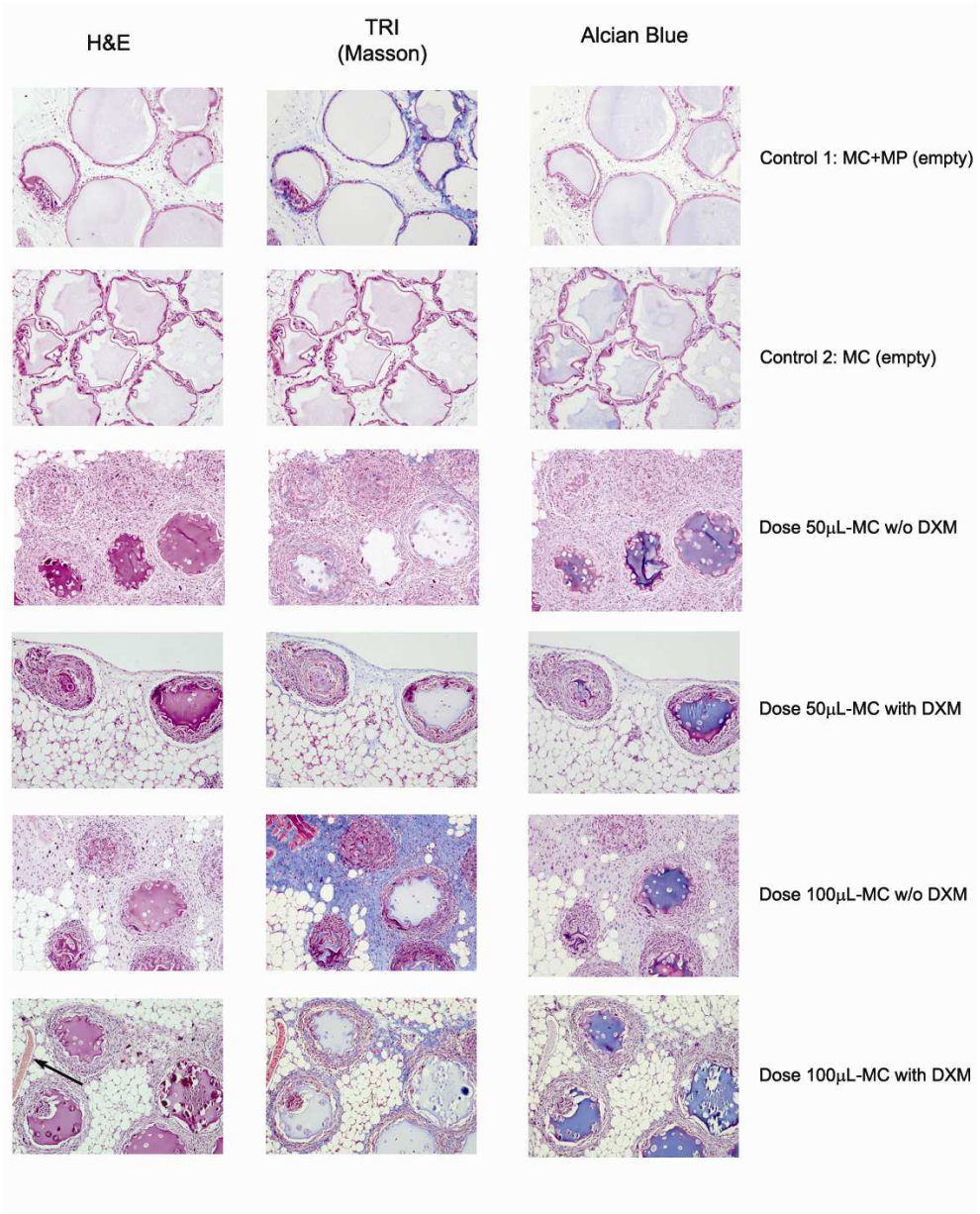

Fig. 7. Histological evaluation of subcutaneously implanted alginate microcapsules [MC] embedding Epo-secreting C_2C_{12} myoblasts (with or without dexamethasone-loaded PLGA microparticles [MP]). Black arrow: blood capillary.

In the present study, we aimed to address this important issue by designing a composite drug delivery system, by the co-administration of PLGA-loaded dexamethasone microspheres. We hypothesized that the anti-inflammatory drug delivery system would provide a local and continuous release of the immunosuppresive agent to the transplantation site, thus decreasing the inflammatory reaction directed towards the microencapsulated cells and improve the system's long-term efficacy (Bhardwaj *et al.*, 2007).

Dexamethasone was selected as a model anti-inflammatory drug due to its safety and wide clinical use (Bunger *et al.*, 2005; Ratner *et al.*, 2005). A temporal but continuous delivery was proposed to diminish the inflammatory response to subcutaneously implanted cell grafts. To suppress inflammation, glucocorticoids inhibit the production of different factors that are important to the emergence of the inflammatory response. DXM acts by decreasing the release of vasoactive and quimioatractive factors, the secretion of lipolytic and proteolytic enzymes, the extravasation of leukocytes into injury areas and finally fibrosis. It also decreases the expression of proinflammatory cytokines like COX-2 and NOS 2 (Ratner *et al.*, 2005). Glucocorticoids are used in combination with other immunosuppressive drugs to treat transplant rejection. Overall, glucocorticoids, have anti-inflammatory effects in the cellular immune response. In addition, glucocorticoids, limit the allergic reactions that occur with other immunosuppressants.

To preserve scaffold functionality over weeks or months, it might result of paramount importance to minimize the immune response activity over the tissue environment surrounding the implanted device. The surgery, even if minor, the implantation of foreign materials (de Vos *et al.*, 2002) and cytokine release from the encapsulated cells (Murua *et al.*, 2009a) are involved in the immediate post-transplant inflammatory response and cannot always be avoided or controlled.

A major problem upon implantation of medical devices and other scaffolds is the tissue injury which triggers a cascade of inflammatory responses that may compromise their functionality in a short period of time (Hickey *et al.*, 2002a).

The inflammation, wound healing, and foreign body reaction are generally considered as parts of the tissue or cellular host responses to injury. This immune response can be defined as the reaction of vascularized living tissue to local injuries that contain, dilute, neutralize, or wall off the injurious agent or process (Medzhitov *et al.*, 2008; Barton *et al.*, 2008; Hickey *et al.*, 2002b; Dungel *et al.*, 2008; Anderson, 2001; Jayant *et al.*, 2009; Koschwanez *et al.*, 2008).

Therefore, *in vivo* functionality of scaffolds can be significantly improved using immunosuppressive anti-inflammatory drugs, as dexamethasone (Patil *et al.*, 2007). By using the local delivery of DXM, it is possible to avoid the peripheral side effects of chronic use (Kim *et al.*, 2007).

In this work we investigated the potential of a composite drug delivery system to modulate the local microenvironment and provide an improved long-term response of a cell-loaded graft. The local release of DXM can prevent peripheral side-effects that occur when immunosuppressive drugs are used by systemic administration. The efforts are targeted to achieve a local temporary release instead of a permanent release (Bunger *et al.* 2005; de Vos *et al.*, 2002).

Interestingly, most of the DXM was released during the first day (~41%). Probably, the most important time frame for anti-inflammatory therapy may be day one after transplantation. Macrophages, the main cells involved in the early pericapsular overgrowth, are recruited during the first day and no changes occur until day 7, when a decrease is observed (de Vos *et al.*, 2002).

Our research group has previously shown the efficacy of PLGA microspheres as drug release systems for the continuous delivery of therapeutics *in vitro* and *in vivo* (Gutierro *et al.*, 2002; Mata *et al.*, 2007). These synthetic polymers are the most extensively studied and used because of the number of advantages they provide. They have already been approved in medical implants and generated tremendous interest due to their excellent biocompatibility, biodegradability and their long term safety in humans (Ratner *et al.*, 2004; Rajeev, 2000).

PLGA microspheres have multiple benefits as local controlled drug delivery systems. A continuous and controlled drug concentration may be achieved in addition to reducing frequency of administration, dose dumping possibility and systemic effects (Hickey *et al.*, 2002a).

Therefore, we decided to combine DXM-loaded PLGA microspheres as a sustained delivery system with APA microcapsules entrapping cells.

The use of an independent composite system resulted in an improved functionality of the cell-based graft, which was found to be more pronounced when higher cell-doses were implanted. On the basis of previously reported studies (Murua *et al.*, 2009a; Murua *et al.*, 2007; Orive *et al.*, 2005), we estimated that 100 μL of cell-loaded microcapsules ($5x10^6$ cells/mL alginate) might result in a therapeutic dose to provide significant increase in mice hematocrit levels over time. However, given the angiogenic and immunomodulatory effects related to Epo, a tendency was also oberved in a lower cell-dose (50 μL). Additionally, the systems showed good biocompatibility and capability to partially avoid the inflammatory response and the pericapsular cell overgrowth, probably due to the immunosuppressive effects related to DXM (Sorianello *et al.*, 2002). This system may open doors to future new alternative composite systems.

5. Conclusions

Taking the aforementioned results altogether, it may be concluded that the co-administration of dexamethasone-loaded PLGA microspheres along with the encapsulation of Epo-secreting

myoblasts may enhance performance of the encapsulation system and may hence be considered very promising and interesting to prevent inflammation leading to pericapsular fibrosis, which may reduce the probability of a successful graft. Further improvement of the composite system is required in order to provide a long-term efficacy of the system, with a suitable therapeutic effect employing lower cell-dose grafts. The release of dexamethasone from PLGA microspheres might provide a useful pharmacological way to prevent the acute inflammatory response due to both biomaterials and surgical manoeuvres employed during the implantation procedure. In a very fast-trak developing area, such as bioartificial devices, this preliminary study might give venue to properly address strategies for cell-based therapies and tissue engineering.

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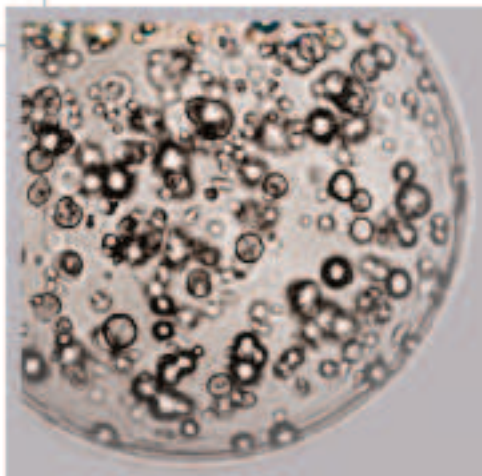

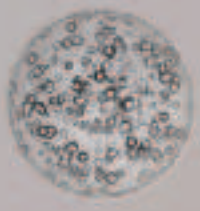

DISCUSSION

Mean life expectancy of the population in the developed world has encountered a consistent increase in the last decades. Healthy life expectancy, however, has not increased concurrently. Hence, a larger proportion of our lives may be spent in poor health so there is a growing demand for the replacement of diseased and damaged tissues. While traditionally tissue grafts have been found adequate for this purpose, the demand for tissue now exceeds the supply. As a result, research in regenerative medicine is growing fast to cope with this new demand. There is now a trend towards supplying cells enveloped into a biomaterial in order to expedite the healing process. Hydrogel encapsulation provides cells with a three dimensional environment similar to that experienced *in vivo* and therefore may allow the maintenance of normal cellular metabolism in order to mimic the conditions found in the body.

In the present work, we have addressed several unsolved issues with the goal of shedding some more light on some of the pending challenges in the field of cell microencapsulation, and the aim of coming closer to a realistic proposal for clinical application.

IN VITRO & *IN VIVO* CHARACTERIZATION OF APA-MICROENCAPSULATED EPO-SECRETING C_2C_{12} MYOBLASTS

A thorough morphological and mechanical evaluation of microcapsules in addition to a complete *in vitro* characterization and *in vivo* functionality and biocompatibility analyses of the encapsulated allogeneic cells during 4 months were performed.

Biomaterials for regenerative medicine require specific and controllable properties. If the long-term *in vivo* stability and functionality of the implanted device is aimed, the mechanical integrity of the cell-loaded microcapsules must be carefully controlled. In addition, it may at times be desirable to promote specific interactions of cells with alginate gels [1].

Alginates are polysaccharides isolated from brown algae such as *Laminaria hyperborea* and *lessonia* found in coastal waters around the globe. The structure of alginate and the relationship of the chemical structure to its gel-forming abilities have been widely studied [2,3]. Alginates are the most widely employed biomaterials in the field and generally show low toxicity, low immunogenicity, and hence good biocompatibility. Alginates create three dimensional structures when they react with divalent cations such as calcium and barium. Moreover, optimization of the polymeric scaffold has been achieved by the combination of an alginate core surrounded by a double membrane comprised of a polycation layer covered by an outer alginate membrane [4]. This microencapsulation design (alginate-polycation-

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1 Orive G, De Castro M, Kong HJ, Hernández RM, Ponce S, Mooney DJ, Pedraz JL. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J. Control. Release 135 (2009) 203-210.

2 Tønnesen HH, Karlsen J. Alginate in drug delivery systems. Drug Dev. Ind. Pharm. 28 (2002) 621-630.

3 Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol. Biosci. 6 (2006) 623–633.

4 Orive G, Tam SK, Pedraz JL, Hallé JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27 (2006) 3691-3700.

alginate) is the most often described system in the scientific literature at present (Figure 1) [5].

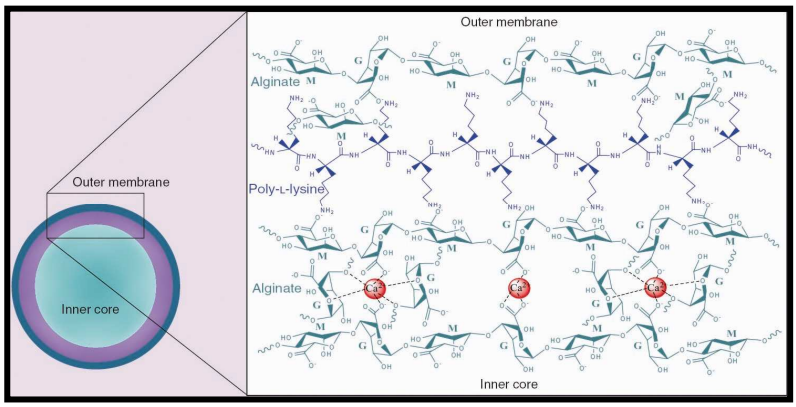

Figure 1. Microcapsule membrane design: alginate–poly-L-lysine–alginate (APA) membrane concept and layer-by-layer molecular structure [5].

In vitro characterization of microcapsules: morphology, integrity, functionality

All microcapsules elaborated during the experiments carried out in this set of works showed a uniform and spherical morphology without irregularities on the surface as shown in Figure 2. The relevance of the materials employed in the elaboration of microcapsules to obtain biocompatible microcapsules has been previously reported [6]. However, not only the biomaterials used but also the smooth and uniform spherical morphology of the microcapsules is of paramount importance to circumvent the immunological system of the recipient host upon transplantation [7]. High viability of the enclosed cells was confirmed by means of

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5 Goren A, Dahan N, Goren E, Baruch L, Machluf M. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J. 24 (2010) 22-31.

6 Říhová B. Immunocompatibility and biocompatibility of cell delivery systems. Adv. Drug Deliv. Rev. 42 (2000) 65-80.

7 Ponce S, Orive G, Hernández RM, Gascón AR, Pedraz JL, De Haan BJ, Faas MM, Mathieu HJ, De Vos P. Chemistry and the biological response against immunoisolating alginate–polycation capsules of different composition. Biomaterials 27 (2006) 4831-4839.

fluorescence imaging (Figure 2B), metabolic activity and protein delivery assays, thus concluding that embedded cells were suitably adapted to the polymer scaffold.

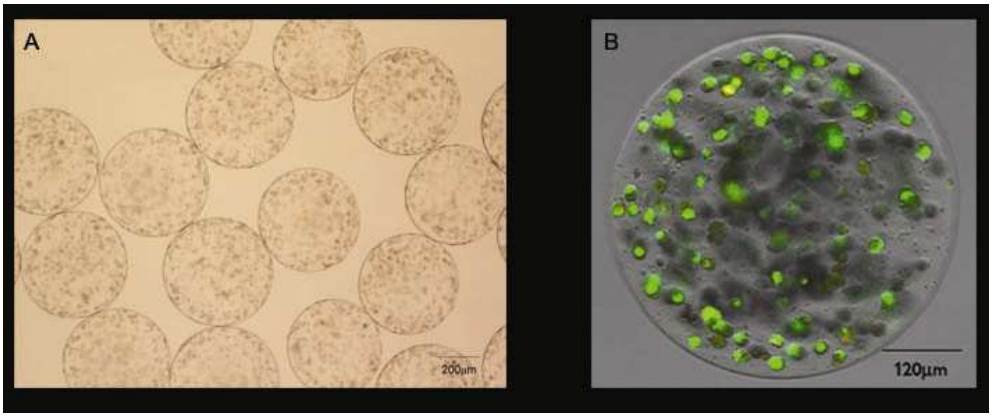

Figure 2. Morphology of microencapsulated Epo-secreting myoblasts. (A) Optical microscopy. (B) Fluorescence image of cells stained with calcein-AM (green, live cells) and ethidium homodimer (red, dead cells).

Alginate-PLL-alginate microcapsules have showed suitable mechanical properties and long-term functionality in previous experiments carried out by our research group [8,9]. The present *in vitro* characterization studies confirmed our previous data; the membrane's mechanical resistance to bursting forces and chemical resistance to swelling conditions was corroborated. These results support the high mechanical and chemical resistance of the developed microcapsules, required if the *in vivo* success of the cell-grafts is aimed [10].

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8 Grandoso L, Ponce S, Manuel I, Arrúe A, Ruiz-Ortega JA, Ulibarri I, Orive G, Hernández RM, Rodríguez A, Rodríguez-Puertas R, Zumárraga M, Linazasoro G, Pedraz JL, Ugedo L. Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats. Int. J. Pharm. 343 (2007) 69-78.

9 Orive G, De Castro M, Ponce S, Hernández RM, Gascón AR, Bosch M, Alberch J, Pedraz JL. Long-term expression of erythropoietin from myoblasts immobilized in biocompatible and neovascularized microcapsules. Mol. Ther. 12 (2005) 283–289.

10 de Vos P, Bucko M, Gemeiner P, Navrátil M, Svitel J, Faas M, Strand BL, Skjak-Braek G, Morch YA, Vikartovská A, Lacík I, Kolláriková G, Orive G, Poncelet D, Pedraz JL, Ansorge-Schumacher

C_2C_{12} myoblasts have been selected as a model cell line for immobilization by many research groups in part due to their favourable transfection possibilities and to their inherent myogenic differentiation potential [11]. Besides, myoblasts present a relative lack of major histocompatibility expression on the surface, which may lead to a decrease in the stimulation of humoral immune response [12].

In vivo characterization. Long-term hematocrit levels in allogeneic mice.

Based on the *in vitro* Epo release assays, we estimated that 0.5 mL of cell-loaded microcapsules (2 x 10^6 cells/mL alginate) might result in a therapeutic dose to provide significant increase in mice hematocrit levels over time. This cell-dose was later optimized in the following studies, in order to minimize the implanted dose, thinking of its future clinical application, where a high-secreting cell line would also be estimated to reduce implantation volume, and thus increase patient confort.

To address this issue, adult female Balb/c mice served as recipients for subcutaneously implanted cell-grafts. Hematocrit levels significantly increased (up to 84%) during the first 3 weeks of study, and remained asymptotic until day 120 post-implantation. Thus a sustained 4-month release of Epo was achieved in allogeneic mice after a single subcutaneous administration of cell-loaded microcapsules and lacking immunosuppressive protocols. This long-term efficacy might be due to the optimized volume-surface relation of the microcapsules, which improves the cell product kinetics and oxygenation of the cells. No remarkable side effects were observed during the treatment period although the high hematocrit levels obtained may be responsible for the appearance of polycythemia in the animals (expanded

MB. Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials 30 (2009) 2559-2570.

11 Haider HKh, Lei Y, Ashraf M. MyoCell, a cell-based, autologous skeletal myoblast therapy for the treatment of cardiovascular diseases. Curr. Opin. Mol. Ther. 10 (2008) 611-621.

12 Garlepp MJ, Chen W, Tabarias H, Baines M, Brooks A, McCluskey J. Antigen processing and presentation by a murine myoblast cell line. Clin. Exp. Immunol. 102 (1995) 614-619.

red cell mass). A pharmacologically controllable cell-based Epo delivery system is under study by our research group at present to overcome this problem (data not published).

At explantation, microcapsules were found to form irregular aggregates with viable cells embedded. The microcapsule network was easily harvested as one piece after a small skin incision as illustrated in Figure 3. This could be an advantage, as one important challenge in the field of cell microencapsulation is the sometimes difficult removal of the implanted graft. Additional device systems are nowadays being investigated to envelop the implantable cell-graft dose, which would assure the complete removal of the drug delivery system upon explantation (Figure 4) [13]. Histological analyses revealed the formation of some blood capillaries within the microcapsule aggregates probably due to the angiogenic effects reported for Epo [14,15]. This might result in a suitable microenvironment where the access of oxygen and nutrients to the entrapped cells might be enhanced. The weak fibroblast overgrowth reported did not question functionality of the cell-grafts.

Figure 3. Microcapsule clump explanted from the subcutaneous tissue (day 130 post-implantation). Scale bar: 500 µm.

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13 Paek HJ, Campaner AB, Kim JL, Golden L, Aaron RK, Ciombor DM, Morgan JR. Lysaght MJ. Microencapsulated cells genetically modified to overexpress human transforming growth factor-β1: viability and functionality in allogeneic and xenogeneic implant models. Tissue Eng. 12 (2006) 1733-1739.

14 Müller-Ehmsen J, Schmidt A, Krausgrill B, Schwinger RHG, Bloch W. Role of erythropoietin for angiogenesis and vasculogenesis: from embryonic development through adulthood. Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H331-H340.

15 De Vos P, de Haan BJ, Kamps JA, Faas MM, Kitano T. Zeta-potentials of alginate-PLL capsules: a predictive measure for biocompatibility? J. Biomed. Mater. Res. Part A 80 (2007) 813-819.

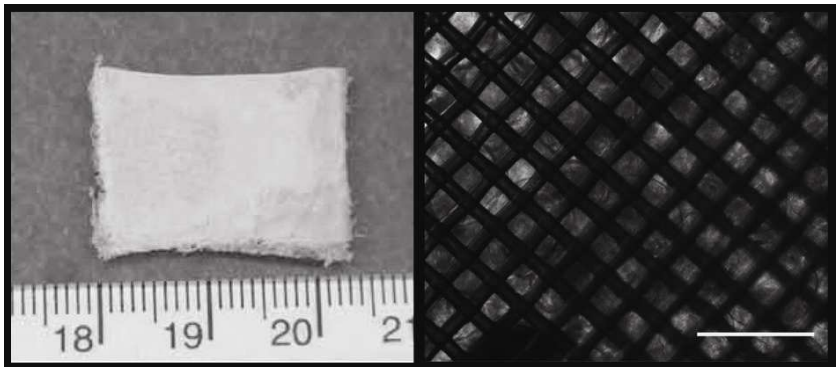

Figure 4. Microcapsules in a nylon mesh implant device at low and high magnification. Scale bar: 500 μm [13].

The data presented in this first study demonstrated a proof-of-principle for cell encapsulation technology if the long-term delivery of Epo is aimed. The correct characterization of the immobilization systems and an optimal cell source election are of paramount importance to optimize the final cell encapsulation product [16]. Moreover the immunoprotective properties of this device make this strategy suitable for allotransplantation therapy, turning this technology into an alternative therapy to whole organ transplantation.

16 Mancuso F, Basta G, Calvitti M, Luca G, Guido L, Racanicchi L, Montanucci P, Becchetti E, Calafiore R. Long-term cultured neonatal porcine islet cell monolayers: a potential tissue source for transplant in diabetes. Xenotransplantation 13 (2006) 289-298.

Considering the increasing inter-laboratory collaborations established to achieve successful, competitive and high quality multidisciplinary research, our next objective became the development of a suitable cryopreservation protocol to correctly preserve and storage microencapsulated cells in the long-term.

LONG-TERM STORAGE OF MICROENCAPSULATED C_2C_{12} MYOBLASTS. CRYOPRESERVATION PROTOCOLS

Cryopreservation plays an important role in cell and tissue banking and will presume yet larger value when increasing cell-based products will routinely enter the clinical arena. The promises of the field, however, depend on the ability to physically distribute the products to patients in need. For this reason, the ability to cryogenically preserve not only cells, but also cell-based systems, and one day even whole laboratory-produced organs, may be desirable. Cryopreservation can be achieved by conventional freezing and alternative procedures recently introduced in the field such as vitrification (ice-free cryopreservation which demands high concentrations of cryoprotective agents) [17]. The present study is based on a freezing protocol procedure.

The cryopreservation process comprises three major phases: a pre-freezing phase in which the cells are exposed to a cold shock; a critical freezing phase in which cell membranes are exposed to osmotic and thermal stresses; and finally, a thawing phase wherein the reverse process occurs [18]. During all of these phase transitions, cell membranes are highly vulnerable to variations in thermal and osmotic conditions.

As cryoprotectives, glycerol and DMSO are agents of choice used in various concentrations [19,20]. In general, the highest concentration the cell can tolerate is recommended as cryoprotective agents decrease the osmotic imbalance across the cell membrane during the freezing process. The cooling rate is also an important

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17 Agudelo CA, Iwata H. The development of alternative vitrification solutions for microencapsulated islets. Biomaterials 29 (2008) 1167–1176.

18 Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology 30 (1993) 423-431.

19 Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 183 (1959) 1394–1395.

20 Meryman HT. Cryoprotective agents. Cryobiology 8 (1971) 173–183.

factor of this phenomenon. During slow-cooling, ice forms mainly external to the cell before intracellular ice begins to form [21]. This results in extensive cellular dehydration ("solution effect"). On the other hand, rapid cooling leads to more intracellular ice ("mechanical cell damage"). Both effects can be detrimental to cell survival and are largely responsible for diminished cell recovery [22]. Additionally, a regimen for one cell type may be unsuitable for another that may differ in the diffusion rate of the cryoprotectants and in its osmotic tolerance [23]. Last but not least, the storage conditions may also influence cell recovery and viability. The storage temperature determines the lapse of time for cell recovery. In general, the lower the storage temperature, the longer the viable preservation period for the cells [24].

The relatively large size of microcapsules (diameter 300-400 μm) makes them particularly prone to cryodamage incurred by ice crystallization. The high water content of the hydrogel (over 90%) together with the fragile semipermeable membrane and their large volume to surface area makes them susceptible to come into contact with developing ice crystals during cryopreservation, and hence are much more amenable to cryodamage [25]. Several research groups have already succeeded in developing diverse freezing protocols for microencapsulated cells although their effectiveness varies in terms of cell recovery and most lack *in vivo* assays [26,27,28].

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21 Farrant J. General observations on cell preservation. In: Ashwood-Smooth MJ, Farrant J, editors. Low temperature preservation in medicine and biology. Kent, UK: Pitman Medical Limited; 1980. p. 1–18.

22 Mazur P, Leibo SP, Chu EH. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. Exp. Cell Res. 71 (1972) 345–355.

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24 Mazur P. Freezing of living cells: mechanisms and implications. Am. J. Physiol. 247 (1984) C125–142.

25 Chin Heng B, Yu H, Chye NgS. Strategies for the cryopreservation of microencapsulated cells. Biotechnol. Bioeng. 85 (2004) 202–213.

26 Wu Y, Yu H, Chang S, Magalhaes R, Kuleshova LL. Vitreous cryopreservation of cell–biomaterial constructs involving encapsulated hepatocytes. Tissue Eng. 13 (2007) 649–658.

This study represented the first *in vivo* functionality demonstration of cryopreserved microencapsulated mEpo-secreting C_2C_{12} myoblasts. Animals implanted with freezed/thawed microencapsulated cells using the slow-cooling protocol and 10% DMSO as cryoprotectant, showed higher levels of hematocrit levels in comparison with the 20% DMSO group for up to 45 days (Figures 5 and 6). In accordance with the previous study, several blood capillaries were observed at explantation surrounding the cell-graft clump, probably due to the angiogenic effects reported for Epo.

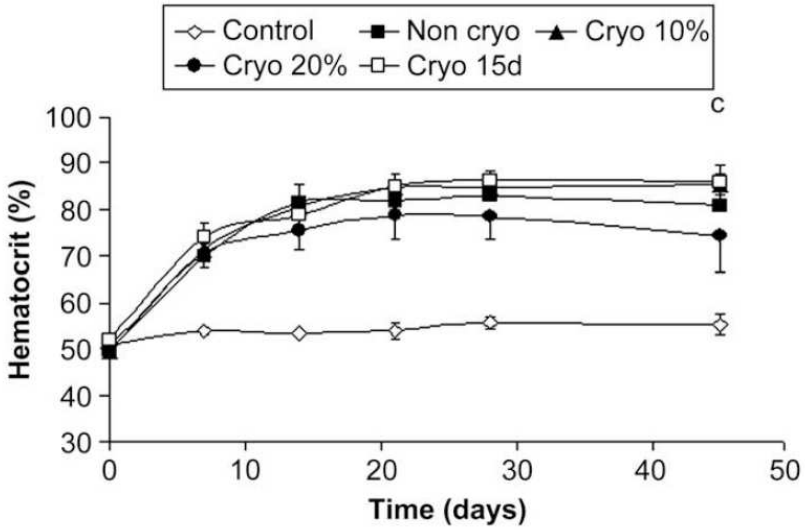

Figure 5. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. In addition to a negative control group (HBSS, no microcapsules), non-cryopreserved microcapsules were tested vs. cryopreserved microcapsules (using slow cooling freezing and either 10% or 20% DMSO for 72h) and an additional group for the evaluation of a longer period of cryopreservation: 15 days (using 10% DMSO and SC freezing). Values represent mean ± S.D. Significance (day 45) $P<0.05$; a: Non Cryo vs. Cryo 10%; b: Non Cryo vs. Cryo 20%; c: Cryo 10% vs. Cryo 20%; d: Cryo 10% vs. Cryo 15d (letter not shown when $P>0.05$).

27 Lee KW, Park JB, Yoon JJ, Lee JH, Kim SY, Jung HJ, Lee SK, Kim SJ, Lee HH, Lee DS, Joh JW. The viability and function of cryopreserved hepatocyte spheroids with different cryopreservation solutions. Transplant. Proc. 36 (2004) 2462–2463.

28 Hardikar AA, Risbud MV, Bhonde RR. Improved post-cryopreservation recovery following encapsulation of islets in chitosan-alginate microcapsules. Transplant. Proc. 32 (2000) 824–825.

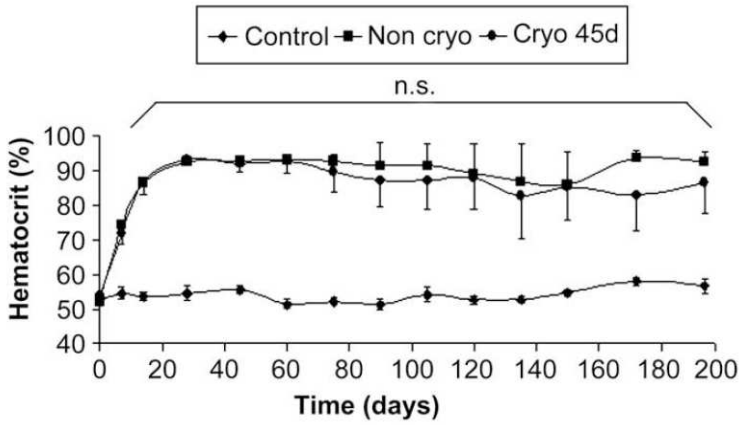

Figure 6. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. Evaluation of non-cryopreserved microcapsule implantation vs. microcapsules cryopreserved for 45 days (using 10% DMSO and SC freezing). Control group: HBSS, no microcapsules. Values represent mean ± S.D. Significance: $P<0.05$*; $P>0.05$ n.s.: Non Cryo vs. Cryo 45d.

Unlike the freezing process, rapid thawing of frozen cells (to avoid excess contact with the cytotoxic cryoprotective agent) is necessary to maintain high cell viability [29]. Additionally, several washes using fresh culture medium, succeed in effectively removing the residual cryoadditive.

Overall, the data provided in this study might be of interest to the scientific community working on *in vivo* approaches using cell microencapsulation technology. The multidisciplinarity of the field (from matrix design to immunohistochemistry analyses at explantation of the devices) promotes inter-laboratory collaborations, which may turn into more accurate, precise and successful experimental outcomes.

29 Li AP, Gorycki PD, Hengstler JG, Kedderis GL, Koebe HG, Rahmani R, de Sousas G, Silva JM, Skett P. Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel. Chem. Biol. Interact. 121 (1999) 117–123.

To our knowledge, microencapsulated cells cryopreserved for as long as 45 days have not been previously proven to be efficient and valid as confirmed in the present study stated by high hematocrit levels maintained in mice implanted with these long-term cryopreserved microcapsules showing no adverse side effects. Cryoprotectant concentration and cryopreservation period were optimized *in vitro* and *in vivo* and the use of a slow-cooling protocol was supported. Longer cryopreservation periods were evaluated and no significant differences were found by day 194 between the non-cryopreserved and the cryopreserved group, thus confirming the safety of employing microcapsules cryopreserved for as long as 45 days. In spite of the encouraging results obtained in this study, the reduction in Epo release after cryopreservation of microcapsules (around 50%) should be minimized by future improvements in the development of suitable cryopreservation protocols.

With the aim of shedding light on the organ shortage issue and in an attempt to make a step forward in the applicability of cell encapsulation technology, we proceeded with a xenotransplantation approach, where Epo-secreting murine myoblasts were implanted in the subcutaneous tissue of Fischer rats, along with two different intramuscularly administered tacrolimus (FK-506) protocols, in order to evaluate its effectiveness to avoid host rejection.

XENOTRANSPLANTATION. FK-506 TREATMENT

Fischer rats received 0.4 mL of cell-loaded microcapsules (5×10^6 cells/mL alginate) which significantly increased rat hematocrit levels over time (day 65). As expected, rats receiving FK-506 maintained high hematocrit levels for a longer period of time in comparison to the non-immunosuppressed group. Moreover, results confirmed the need of a minimum 4-week immunosuppression period for this purpose, as stated by significantly higher hematocrit levels detected by day 65 in the 4-week FK-506 treated group (79%) in comparison with the 2-week treated group (66%) (Figure 7). These results are in agreement with similar xenogeneic approaches previously developed based on the use of macroencapsulation devices, such as hollow fibers [30]. Cell microencapsulation technology may be an alternative for this specific application which might benefit from advantages such as an optimal volume–surface ratio and small size.

Cell encapsulation within a semipermeable polymer membrane prevents cell contact-mediated rejection of enclosed cells upon transplantation. However, cytokine release from the host might manage to destroy encapsulated cells. The site of transplantation plays a significant role in determining the fate of xenogeneic cells [31,32]. As hypothesized, xenoantigens released by the encapsulated cells can switch the host immune system on. Once the immune response is activated, increasing populations of immune cells envelop the microcapsule clump, leading to

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30 Peduto G, Rinsch C, Schneider BL, Rolland E, Aebischer P. Long-term host unresponsiveness to encapsulated xenogeneic myoblasts after transient immunosuppression. Transplantation 70 (2000) 78–85.

31 Elliott RB, Escobar L, Tan PL, Garkavenko O, Calafiore R, Basta P, Vasconcellos AV, Emerich DF, Thanos C, Bamba C. Intraperitoneal alginate-encapsulated neonatal porcine islets in a placebo-controlled study with 16 diabetic cynomolgus primates. Transplant. Proc. 37 (2005) 3505–3508.

32 Dufrane D, Goebbels RM, Saliez A, Guiot Y, Gianello P. Six-month survival of microencapsulated pig islets and alginate biocompatibility in primates: proof of concept. Transplantation 81 (2006) 1345–1353.

encapsulated cell death [13]. Nonetheless, it has also been questioned that prolonged, low-level release of antigens can lead to tolerance [33].

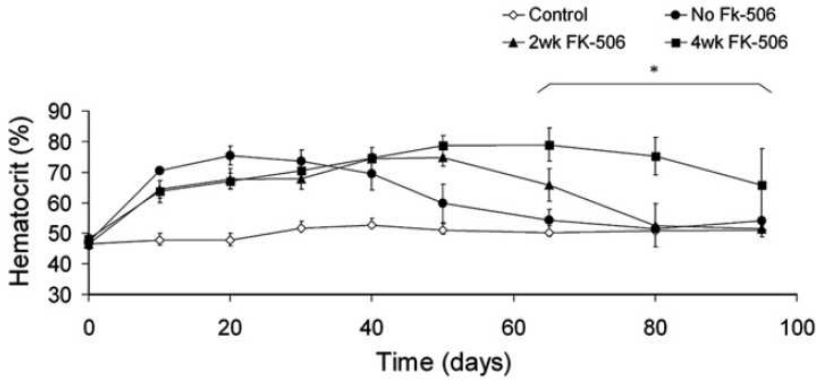

Figure 7. Hematocrit levels of Fischer rats. 2-week FK-506 vs. 4-week FK-506. Significance $^{*}P<0.05$. Control-HBSS (n=4). Rest of the groups (n=7).

In accordance with the previous study, capsules retrieved from the subcutaneous tissue were mostly aggregated forming an irregular structure and surrounded by blood capillaries, mainly detected in the immunosuppressed groups. This could be an advantage in comparison with free-floating microcapsules carrying Epo-secreting myoblasts usually observed when the intraperitoneal cavity is used as implantation site [9,34]. Moreover, a slight fibrotic layer was observed, more prominent in the non-treated group which might be one of the factors responsible for the hematocrit difference found between non-immunosuppressed and immunosuppressed rats.

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33 Zinkernagel RM, Hengartner H. Regulation of the immune response by antigen. Science 293 (2001) 251–253.

34 Ponce S, Orive G, Hernández RM, Gascón AR, Canals JM, Muñoz MT, Pedraz JL. In vivo evaluation of EPO-secreting cells immobilized in different alginate-PLL microcapsules. J. Control. Release 116 (2006) 28–34.

Restrained cell-graft survival has generally been related to pericapsular cell overgrowth (promoted by interleukin-1β and tumour necrosis factor-α) leading to a thick fibrotic layer [35]. Consequently, diminished nutrition to the inner cells might result in unsuitable cell function and viability with time [36]. New approaches based on silencing of inflammatory responses may bring the technology of cell-based transplantation closer to clinical application [37]. It is likely that future directions in using encapsulated xenogeneic cells will build on incremental improvements and further optimization of diverse temporal release protocols of immunosuppressive agents, encapsulated in biodegradable matrices and administered in combination with the cell-graft. Thus, the increasing understanding of the biology of the disease, polymer chemistry, and particularly the cell-biomaterial interactions will further enhance feasibility of using immunoisolation for therapeutic treatments.

35 de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J. Surg. Res. 121 (2004) 141–150.

36 Figliuzzi M, Plati T, Cornolti R, Adobati F, Fagiani A, Rossi L, Remuzzi G, Remuzzi A. Biocompatibility and function of microencapsulated pancreatic islets. Acta Biomater. 2 (2006) 221–227.

37 de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 27 (2006) 5603–5617.

In an attempt to enhance the biocompatibility of the implanted scaffolds, avoiding the daily and continuous administration of immunosuppressive agents, we decided to develop a composite system based on the temporal delivery of dexamethasone from PLGA microspheres, implanted in combination with the cell-graft.

LOCALIZED INFLAMMATION CONTROL: GENERATION OF AN *IMMUNOPRIVILEDGED MICROENVIRONMENT BY CO-ADMINISTRATION OF ENCAPSULATED STEROIDS*

In the last years, extensive work has been carried out aiming at eliminating the immune reaction towards cell-based encapsulated implants. In spite of the huge progress made in reducing the immune reaction, particularly in the case of xenotransplantation approaches, much work lies ahead. Short-term systemic immunosuppression has also been proposed as a possible alternative therapy towards eliminating the immune reaction from the host, by actively suppressing the inflammatory response generated against the transplanted encapsulated cells [38]. In addition, the use of hypoimmunogenic cells [5] or genetically modified cell lines delivering specific factors (i.e. interleukin-10) which downregulate the xenogeneic immune response [39] are under study too.

The main objective of this last experimental study was to develop a composite drug delivery system comprised of PLGA-loaded dexamethasone microspheres and Epo-secreting C_2C_{12} myoblasts enclosed in APA microcapsules. The anti-inflammatory drug release system would provide a local and sustained delivery of the immunosuppressive agent to the transplantation site, thus decreasing inflammation generated by the microencapsulated cells and improve the system's long-term efficacy [40].

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38 Weiss MJ, Ng CY, Madsen JC. Tolerance, xenotransplantation: future therapies. Surg. Clin. North Am. 86 (2006) 1277-1296.

39 Surzyn M, Symes J, Medin JA, Sefton MV. IL-10 secretion increases signal persistence of HEMA-MMA-microencapsulated luciferase-modified CHO fibroblasts in mice. Tissue Eng. 15 (2009) 127-136.

40 Bhardwaj U, Sura R, Papadimitrakopoulos F, Burgess DJ. Controlling acute inflammation with fast releasing dexamethasone-PLGA microsphere/PVA hydrogel composites for implantable devices. J. Diab. Sci. Technol. 1 (2007) 8-17.

Dexamethasone was selected as a model anti-inflammatory drug due to its safety and wide clinical use [41,42]. To suppress inflammation, glucocorticoids inhibit the production of key factors in the emergence of the inflammatory response such as vasoactive and chemoattractive factors provoking the secretion of lipolytic and proteolytic enzymes which leads to extravasation of leukocytes into the injury area and finally fibrosis. It also decreases the expression of proinflammatory cytokines like COX-2 and NOS 2 [42].

In this work we investigated the potential of a composite drug delivery system to modulate the local microenvironment and to provide an improved long-term response of a cell-loaded graft (Figure 8).

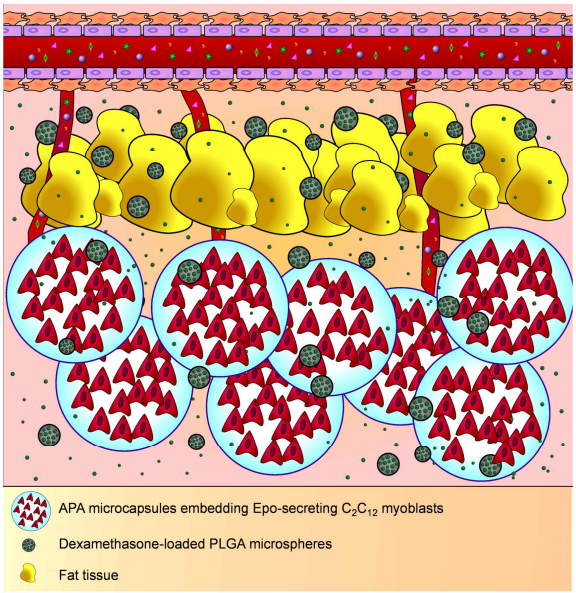

Figure 8. Schematic illustration of the immunomodulatory environment created in the subcutaneous space of implanted mice.

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41 Bunger CM, Tiefenbach B, Jahnke A, Gerlach C, Freier T, Schmitz KP, Hopt UT, Schareck W, Klar E, de Vos P. Deletion of the tissue response against alginate-pll capsules by temporary release of coencapsulated steroids. Biomaterials 26 (2005) 2353-2360.

42 Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. Biomaterials Science: An introduction to materials in medicine, 2nd ed., Elsevier, Amsterdam, 2004.

The local release of DXM can prevent peripheral side effects that occur when immunosuppressive drugs are used by systemical administration. The efforts are targeted to achieve a local temporary release instead of a permanent release.

PLGA microspheres have multiple benefits as local controlled drug delivery systems. A continuous and controlled drug concentration may be achieved in addition to reducing frequency of administration, dose dumping possibility and systemic effects [41].

The use of an independent composite system resulted in improved functionality of the cell-based graft, which was found to be more pronounced when higher cell-doses were implanted (Figure 9).

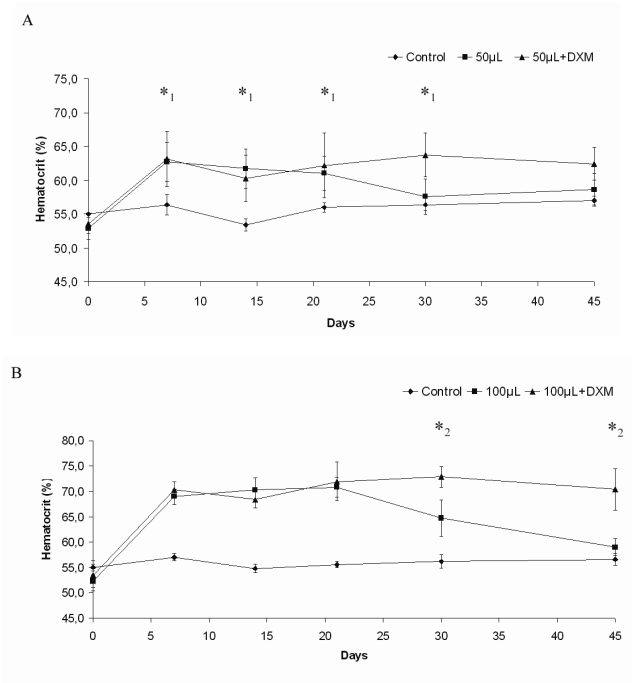
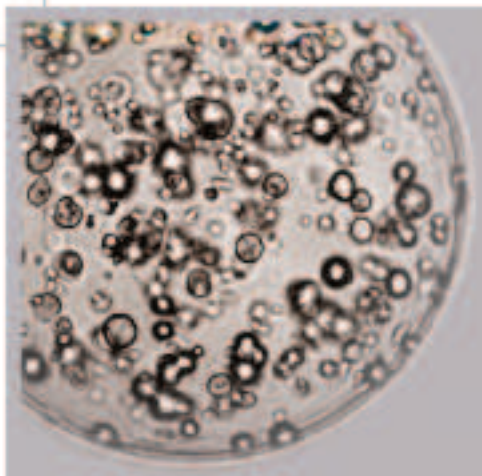
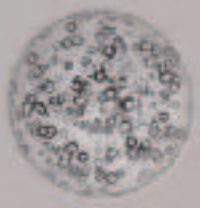


Figure 9. Hematocrit levels of Balb/c mice over time (45 days). A. 50 µL cell-microcapsule dose. Control: empty alginate microcapsules. B. 100 µL cell-microcapsule dose. Control: empty alginate microcapsules + empty PLGA microparticles. Some groups received dexamethasone-loaded PLGA microparticles while others didn't. Significance: $P<0.05$; *1: control vs. cells. *2: No DXM vs. DXM group.

On the basis of previously reported studies, we estimated that 100 µL of cell-loaded microcapsules (5 x 10^6 cells/mL alginate) might result in a therapeutic dose to provide significant increase in mice hematocrit levels over time. However, given the angiogenic and immunomodulatory effects related to Epo, a tendency was also observed in a lower cell-dose (50 µL). Moreover, the systems showed good biocompatibility and capability to partially avoid the inflammatory response and the pericapsular cell overgrowth, probably due to the immunosuppressive effects related to DXM [43]. This system may open doors to future new alternative composite systems.

43 Sorianello E, Schillaci R, Chamson-Reig A, Lux-Lantos V, Libertun C. Actions of immunosuppressor drugs on the development of an experimental ovarian tumor. Exp. Biol. Med. 227 (2002) 658-664.





CONCLUSIONS

On the basis of the results obtained in the experimental studies of this dissertation, the following conclusions were derived:

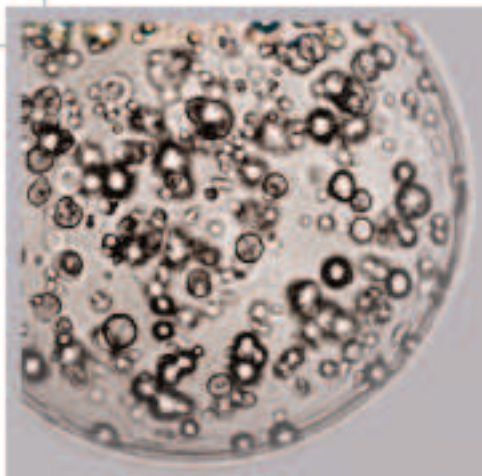
1. A complete *in vitro* and *in vivo* characterization of Epo-secreting C_2C_{12} myoblasts, encapsulated in APA microcapsules was carried out. *In vitro* viability and Epo delivery studies confirmed suitability of the cells to the polymeric scaffold while swelling and compression studies corroborated adequate integrity of the microcapsules. Subcutaneous implantation of microcapsules in allogeneic mice resulted in an important increase of hematocrit levels for 120 days, lacking immunosuppressive protocols.

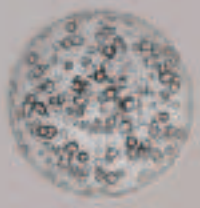
2. As a result of the thorough investigation carried out studying most cryopreservation variables involved during freezing and thawing, a long-term sustained release of Epo was achieved after a single subcutaneous administration of post-thawed microcapsules (cryopreserved using slow-cooling freezing and 10% DMSO as cryoprotectant) in allogeneic recipients. No remarkable side effects were observed during the treatment period.

3. Long storage cryopreservation periods were evaluated (up to 45 days) which could be beneficial if a successful inter-laboratory exchange of microcapsules or even cell banking is aimed. It may be conluded that adequate cryopreservation of encapsulated C_2C_{12} myoblasts merely changes the physiological characteristics of the cells *in vitro* and *in vivo* fulfilling the aim of this study which was to establish an affordable and convenient cryopreservation technique with minimized cell injury during the freeze–thaw process.

4. Long-term survival of genetically modified xenogeneic myoblasts in a peripheral immunoreactive site (SC) was achieved. Fischer rats rendered unresponsive during 94 days to encapsulated C_2C_{12} mEpo cells by transient immunosuppression with FK-506 (4 weeks). In particular, the importance of the length of initial immunosuppression on the survival of cells within the implant was confirmed.

5. The co-administration of dexamethasone-loaded PLGA microspheres along with the encapsulation of Epo-secreting myoblasts may enhance performance of the cell-baed system and could thus be considered very promising and interesting to prevent inflammation and pericapsular overgrowth. The release of dexamethasone from PLGA microspheres might provide a useful pharmacological way to prevent the acute inflammatory response due to both biomaterials and surgical manoeuvres employed during the implantation procedure.





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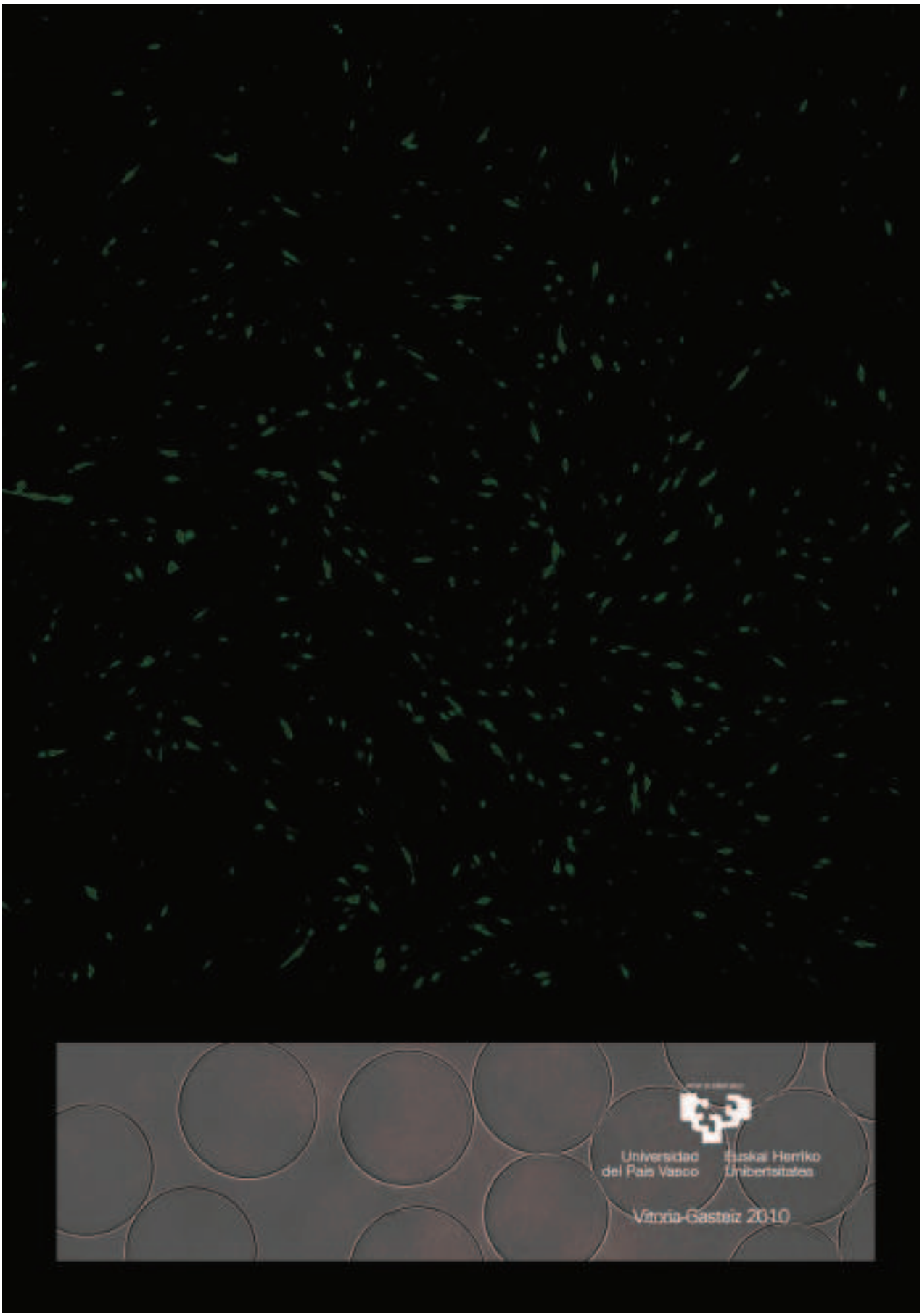

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