Journal of Controlled Release 335 (2021) 619–636

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review article

Cell microencapsulation technologies for sustained drug delivery: Latest advances in efficacy and biosafety

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ARTICLE INFO

Keywords:
Cell encapsulation
Efficacy
Biosafety
Biocompatibility
Biomaterial
Alginate

ABSTRACT

The development of cell microencapsulation systems began several decades ago. However, today few systems have been tested in clinical trials. For this reason, in the last years, researchers have directed efforts towards trying to solve some of the key aspects that still limit efficacy and biosafety, the two major criteria that must be satisfied to reach the clinical practice. Regarding the efficacy, which is closely related to biocompatibility, substantial improvements have been made, such as the purification or chemical modification of the alginates that normally form the microspheres. Each of the components that make up the microcapsules has been carefully selected to avoid toxicities that can damage the encapsulated cells or generate an immune response leading to pericapsular fibrosis. As for the biosafety, researchers have developed biological circuits capable of actively responding to the needs of the patients to precisely and accurately release the demanded drug dose. Furthermore, the structure of the devices has been subject of study to adequately protect the encapsulated cells and prevent their spread in the body. The objective of this review is to describe the latest advances made by scientist to improve the efficacy and biosafety of cell microencapsulation systems for sustained drug delivery, also highlighting those points that still need to be optimized.

1. Introduction

For more than four decades, different materials, both of natural and synthetic origin, have been used to manufacture sustained drug delivery systems. Among them, we find those that allow the sustained release of encapsulated growth factors, proteins or drugs; but also others, of greater complexity, that are capable of immobilizing and protecting living cells, selectively isolating them from their environment while they secrete the therapeutic molecules of interest. Cell encapsulation systems have shown wide applicability in pathologies with very diverse characteristics, such as diabetes mellitus (DM), anemia, hemophilia B or pathologies of the central nervous system (CNS), among others [1]. They are especially convenient for pathologies in which maintaining a strict control over the release of the therapeutic molecule is essential.

Cell encapsulation can be classified based on the size of the system. On the one hand, we find cell macroencapsulation systems, in which the cells are immobilized in relatively large diffusion chambers, with semipermeable properties. They can have different shapes, such as discs, flat sheets or hollow fibers. The application of cell macroencapsulation devices have shown very good results *in vivo* demonstrating their undeniable therapeutic potential. However, macrocapsules are characterized by a relatively small surface/volume ratio, which is probably their worst disadvantage, since this implies the need for large amounts of nutrients and oxygen to achieve an adequate diffusion into the chamber and limits the amount of cells that can be encapsulated without creating necrotic nuclei in the innermost and inaccessible areas [2].

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https://doi.org/10.1016/j.jconrel.2021.06.006
Received 14 November 2020; Received in revised form 4 June 2021; Accepted 6 June 2021
Available online 9 June 2021
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Cell microencapsulation represents a very interesting alternative, greatly improving the surface/volume ratio and increasing the diffusion of nutrients and oxygen inside the capsules. Cell microencapsulation strategy is based on the immobilization of cells that produce therapeutically relevant molecules in spherical particles between 100 and 1500 μm in diameter, approximately. The particles are elaborated with biocompatible materials and usually surrounded by a semi-permeable polymeric membrane that prevents the passage of high molecular weight molecules — antibodies and other components of the immune system —, protecting these cells from the host's immune response and from the mechanical stress that may occur when the implant is placed in the selected tissue [3]. In addition, the microcapsule must exert a tight control over the bidirectional diffusion of molecules — entrance of nutrients and oxygen; and release of *de novo* synthesized therapeutic factors and metabolic subproducts —, and provide cells with a suitable environment to enhance and modulate their function. This technology also suppresses, or at least reduces, the chronic administration of immunosuppressive agents, thus avoiding some of the adverse events associated with organ and tissue transplantation. On the other hand, the constant improvements in imaging techniques and robotic surgery procedures allow the access to difficult to reach areas for implantation [4].

Today, the results obtained in the various clinical trials carried out to date, make clear the advantages and potential applications of this promising technology. However, there are still aspects that need to be improved so that cell microencapsulation systems can be applied routinely in clinical practice. For this reason, in the last years, researchers have directed efforts towards trying to solve some of the key aspects that still limit efficacy and biosafety, the two major criteria that must be satisfied to reach the clinical practice. Those two concepts are closely related to each other and must be carefully defined and regulated due to their implications regarding patient well-being. The objective of this review is, therefore, to group and describe the extensive work carried out with the aim to improve these criteria, emphasizing the points that still need to be optimized.

2. Efficacy - biocompatibility

When talking about efficacy of cell microencapsulation, biocompatibility is one of the most important aspects to be considered. It will determine implant's viability, functionality and durability, becoming in many cases a limiting factor to succeed. The biocompatibility of the implant must be given in 2 directions *(Fig. 1)*. From outside to inside, the materials used must protect the immobilized cells, avoiding direct toxicity or the blockage of nutrients and oxygen diffusion. From inside to outside, none of the system components — cells, biomaterials, cross-linking agents, etc. — or procedures used must be toxic for the patient or elicit an immune response in the host. When this occurs, the foreign body reaction *(Box 1)* can eventually isolate the implant within a fibrotic capsule, thereby preventing the access of essential molecules and leading to graft failure. In addition, the biocompatibility must last over time, since live cell therapies are normally used for long-term treatments. In this sense, several experts in the field of cell encapsulation have decided to define the term "biotolerability", considering it more appropriate than "biocompatibility" [5].

Despite the undeniable improvement occurred in recent decades, the biomaterials and cells that are used today continue to produce, to a greater or lesser extent, an inflammatory response by the host, so searching for suitable components remains a priority. The final performance of the device will depend not only on the biomaterials and cells used, but also on the site of implant, the local application of immunosuppressive drugs, or even the size and shape of the implant.

2.1. Biomaterials, crosslinkers and coatings

2.1.1. Biomaterials and cross-linkers

On the one hand, the elaborated devices must present a suitable structure, resistant to unwanted degradation that avoids contact between the encapsulated cells and the host immune system. Furthermore, the biomaterials must guide the processes of proliferation and differentiation of encapsulated cells, enhancing their viability and functionality. On the other hand, the choice of all the materials must be made taking into account possible toxicities. The latter includes, in addition to the main materials, cross-linkers, physicochemical modifications and possible degradation byproducts.

Today, the materials used include ceramics, plastics and various polymers, among others. The latter can be classified as natural (polysaccharides, polypeptides and polynucleotides) or synthetic. Among natural polymers, polysaccharides are the most used because they allow relatively smooth encapsulation processes that are compatible with cell viability. Examples of natural polymers are alginate, agarose, collagen, or cellulose. On the other hand, polyethylene glycol (PEG) continues to be the most widely used option among synthetic polymers, along with poly(lactic-co-glycolic acid) (PLGA) and polyvinyl alcohol (PVA) [11,12].

Among all the available polymers, alginate is by far the most widely

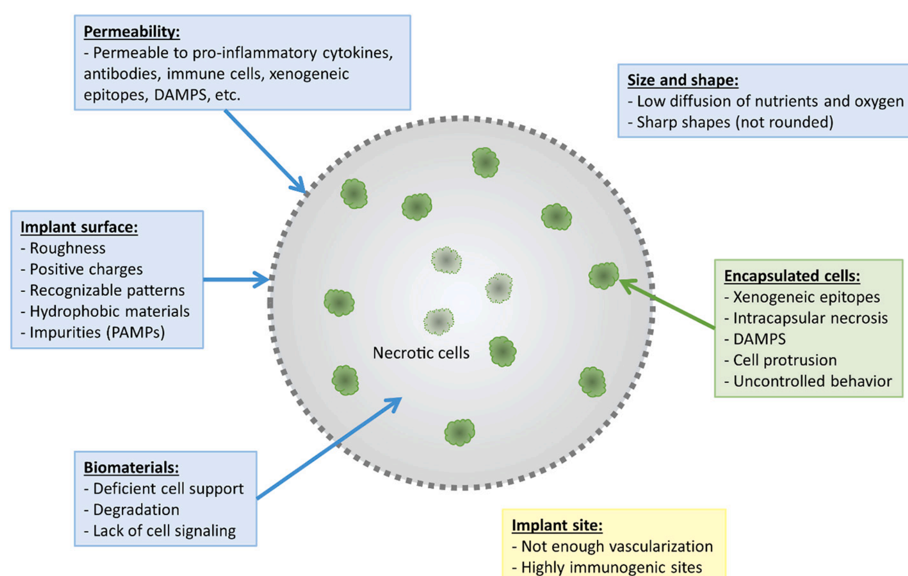

Fig. 1. Factors that may compromise implant biocompatibility/biotolerability. Regarding the biomaterials and coatings used, factors such as the permeability of the microcapsule to pro-inflammatory molecules, the characteristics of the implant surface, the size and shape of the sphere or structural deficiencies can trigger an immune response against the capsules. The encapsulated cells can also release pro-inflammatory molecules or protrude outside the implant. Futhermore, the characteristics of the implant site should also be considered in detail when trying to improve the biocompatibility/biotolerability of the system.

Box 1
Foreign body reaction against biomaterials.

Although the materials and therapeutic applications differ, the process by which the body produces rejection against implants has many points in common. This is known as "foreign body reaction" and consists of the following phases:

Immediately after the implantation and depending on the characteristics of its surface —material, shape, roughness, electrostatic charge, etc. — and the injury caused during the surgical process, various host proteins — such as albumin, fibronectin or complement molecules — will start to adhere to the surface of the implant. This creates a chemoattractive gradient for the innate immune response [6]. Neutrophils are the first cell type present at the implant site and their function is to engulf the microorganism remains and dead cells. Neutrophils also secrete proteases, lysozymes, reactive species and other enzymes to eliminate any type of biodegradable material. At the same time, they secrete cytokines and other factors that cause the activation of macrophages (differentiated from the recruited monocytes), which will be the predominant cell type in the following phases of the foreign body response. This acute phase of the inflammatory reaction would end with a return to homeostasis if the material recognized as foreign disappears completely.

On the contrary, if the host cannot destroy the implant, its continued presence can lead to a second phase of chronic inflammation. The "frustrated" macrophages start to fuse into multinucleated cells around the implant, giving rise to foreign-body giant cells [7]. At the molecular level, pro-inflammatory cytokines, such as IL-6, TNF-α, interleukin 4 (IL-4), and interleukin 13 (IL-13), have been reported to be overexpressed [8]. In this phase, there is a continuous presence of monocytes and lymphocytes and a constant activation of macrophages and neutrophils, which secrete enzymes and reactive species. At the same time, neovascularization phenomena are observed, with the appearance of functional capillaries.

In the final phase, fibroblasts, activated by macrophages, deposit collagen fibers to form a dense and fibrous acellular capsule that isolates the implant from the surrounding tissue [9]. This prevents the passage of nutrients and oxygen, and eventually leads to compromising the viability of the encapsulated cells.

I*n vivo* the chronology varies depending on the organism, ranging from the appearance of fibrosis in just 7 days, in the case of mini-pigs, to 14 days in rats [10].

used biomaterial in cell microencapsulation systems, due to its excellent biocompatibility and easy handling [13]. Alginate is a natural anionic polysaccharide that creates three-dimensional structures, going from sol to gel, when it reacts with divalent ions. It is made up of different proportions of residues of β-D-manuronic acid (M) and α-L-guluronic acid (G) that create different structures according to the ratio of G and M. Determining and standardizing this proportion is essential since it has a great influence on some of alginate hydrogels properties, such as their biocompatibility, stability, mechanical resistance and permeability, among others [13]. In general, alginates with a higher proportion of G blocks are stiffer, compared to those with a higher proportion of M blocks that have better elastic properties, due to the greater affinity of guluronic acid for divalent ions, and these physical-mechanical differences affect the way the immune system reacts against the implant [14].

On the other hand, the purity degree of the alginate is directly related to its biocompatibility. Low purity alginates contain endotoxins, proteins and polyphenols that reduce the biocompatibility of the implants and can damage the encapsulated cells [15]. Several commercial alginates have been described to contain pathogen-associated molecular patterns (PAMPs). These are potent initiators of inflammatory responses [16] and produce the release of small proinflammatory cytokines — such as interleukin 1β (IL-1β), the tumor necrosis factor α (TNF-α) or interleukin 6 (IL-6) —, which can come into contact with the encapsulated cells and cause damage. The most common endotoxin that can be found in alginate are lipopolysaccharides (LPS), which can bind to toll-like receptors 4 (TLR-4) [17], producing an inflammatory response mediated by a variety of cells of the immune system [18]. Therefore, in recent years, different purification methods have been developed in order to obtain ultra-pure alginates with less immunogenicity *in vivo* [19–22]. However, there is great variability between the procedures used in the different research groups and it is still necessary to improve the tools for the screening and elimination of these and other impurities, such as peptidoglycans and lipoteicoic acid [23–27].

In this sense, there are divided opinions on whether or not it will be possible to achieve an adequate and sufficient level of purification of the alginate — so that it becomes nearly inert to the immune system — or whether it will also be necessary to chemically modify its structure. Indeed, pericapsular fibrosis has been one of the major drawbacks in clinical studies carried out to date with alginate as the main material. However, the composition of the alginate and the variability between administration protocols, cell types or the concomitant use of different coating materials, among others, make the comparison complicated.

In recent years, alginate purification protocols have been refined [27], while some groups have begun to include chemical modifications in the alginates [28,29]. A few years ago, Paredes-Juarez *et al.* created a platform that allows the identification of pattern recognition receptor (PRR) activating polymers, in order to identify contaminants in the biomaterials [16]. On the other hand, in a study carried out by Vegas *et al.*, a combinatorial approach was used to generate a wide range of alginate variants with the aim of finding those that were able to decrease the foreign body response [28]. After a first selection, the most promising hydrogel spheres were evaluated *in vivo*, in rodents and non-human primates (cynomolgus macaques). Three triazole-containing analogues (Z2-Y12, Z1-Y15 and Z1-Y19) were identified, which significantly reduced the foreign body response, when compared to conventional SLG20 alginate spheres inhibiting macrophage recognition and fibrosis formation *(Fig. 2)*. When implanted intraperitoneally into non-human primates (n = 3 each alginate variant), Z2-Y12, Z1-Y15 and Z1-Y19 spheres with 1.5-mm diameters displayed substantially reduced fibrotic responses after 4 weeks compared to 1.5-mm SLG20 spheres. SLG20 spheres had more extensive immune macrophage and fibrosis-associated activated myofibroblast coverage, consistent with the visible fibrotic overgrowth seen in the phase contrast imaging. Z1-Y19 spheres displayed more coverage by macrophage and myofibroblasts than either Z2-Y12 or Z1-Y15, but less compared to SLG20. Z2-Y12 and Z1-Y15 spheres had markedly lower levels of smooth muscle actin (SMA) protein, the major morphological characteristic of myofibroblasts, as compared to SLG20 spheres, but Z1-Y19 spheres did not, despite the lower average SMA measured. Hydroxyproline quantification revealed lower collagen levels for all three lead formulations compared to SLG20.

In an interesting and more recent study, Liu *et al.* proposed a group of zwitterionic sulfobetaine (SB) and carboxybetaine (CB) modifications of alginate (SB-SLG20 and CB-SLG20) to reduce cell accumulation and

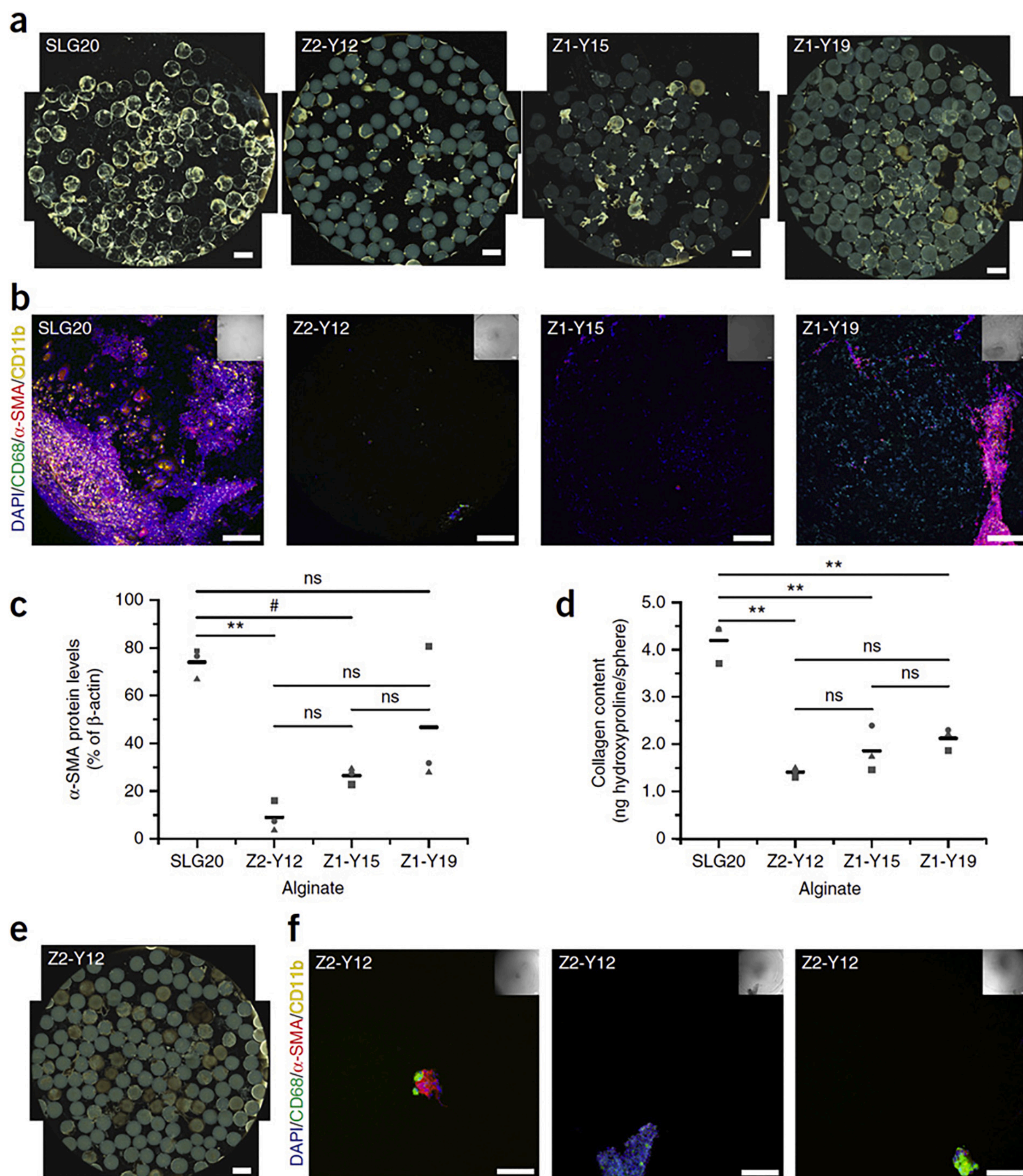

Fig. 2. Modified hydrogels mitigate foreign body response in non-human primates. Z2-Y12, Z1-Y15 and Z1-Y19 alginate spheres significantly reduce fibrosis in cynomolgus macaques, while conventional SLG20 alginate spheres become fibrotic. **a** Phase contrast imaging of spheres retrieved after 4 weeks in the intraperitoneal space show less fibrosis on Z2-Y12, Z1-Y15 and Z1-Y19 spheres than on SLG20. Scale bars, 2,000 μm; n = 3. **b** Confocal imaging of retrieved spheres from a after 4 weeks in the intraperitoneal space show significantly less macrophage (CD68, CD11b), myofibroblast (SMA) and general cellular deposition (DAPI) on Z2-Y12 spheres. Scale bars, 200 μm; n = 3. Brightfield images of the stained spheres are inset; scale bars, 100 μm. **c** Western-blot analysis of protein extracted from the top three alginate analog spheres and control spheres in a; n = 3. Blots were stained for SMA and loading was normalized to β-actin. Dots represent measurements from individual biological replicates, and lines show the average of the three replicates. One-way ANOVA with Bonferroni correction was used to allow for statistical comparison of multiple means. #P < 0.05; **P < 0.001; ns, not significant. **d** Collagen content using a hydroxyproline quantification assay of protein extracted from the top three alginate analog spheres and control spheres in a. n = 3. Dots represent measurements from individual biological replicates and lines show the average of the three replicates. One-way ANOVA with Bonferroni correction was used to allow for statistical comparison of multiple means. #P < 0.05; **P < 0.001; ns, not significant. Reprinted from ref. [28], with permission from Springer Nature.

fibrotic processes around the capsules [30]. Studies in mice, dogs, and pigs showed a significant reduction in these processes. Finally, rat pancreatic islets immobilized in SB-SLG20 microbeads (control group with SLG20), were transplanted, for 200 days, into the peritoneal cavity of streptozotocin (STZ)-induced C57BL/6J diabetic mice and four out of six mice maintained normoglycemia by the end of the study (the shortest duration of glycemic control was ~135 days) *(Fig. 3)*. An intraperitoneal glucose tolerance test (IPGTT) 200 days after transplantation, showed that the mice (cured ones, $n = 3$) in the SB-SLG20 group cleared blood glucose (BG) and restored normoglycemia at a rate comparable to that of non-diabetic mice. An *ex vivo* glucose-stimulated insulin secretion (GSIS) of islets retrieved from cured mice ($n = 3$) indicated again the normal function of islets. Dark-field microscopic images and hematoxylin-eosin (H&E) histological analysis of retrieved SB-SLG20 microcapsules from normoglycemic mice after 200 days revealed no or minimal cellular deposition on the microcapsules and the presence of numerous functional islets inside.

Microencapsulation systems based on the gelation of alginate mainly use barium and calcium as crosslinking agents and, in some cases, strontium [31]. Today there is no clear preference between calcium or barium and its use depends mainly on the protocol adopted in the different research groups. The arguments in favor of using calcium as a crosslinking ion revolve around the lower toxicity [32]. However, the resistance of the spheres cross-linked with calcium is lower than that achieved with barium, and that is why other groups opt for this option when developing their systems [28,29]. Some authors argue that, barium-cross-linked beads could be significantly less immunogenic as they do not need subsequent coatings to increase the immunoisolation, which are usually necessary when calcium is selected as the crosslinker [33]. This last statement is still in doubt, since some studies seem to indicate that the level of immunoisolation would not be sufficient in the barium beads if they do not have posterior coatings. In addition, the *in vivo* implantation of alginate and barium beads have originated a fibrotic response to the implant in different administration routes [26]. On the other hand, the release of cross-linking ions must also be taken into account, especially when barium is selected, due to its toxicity [34].

However, despite the advantages of alginate, there are still aspects that need to be optimized. Among them its mechanical properties, since the systems made with alginate and different ions tend to undergo changes in size due to the osmotic processes that occur in the physiological environment, increasing the permeability of the capsule, weakening its structure and finally causing rupture of the system [31].

Ion concentration, the selected crosslinking agent or the alginate composition are determining factors in obtaining adequate and homogeneous gelation. Simply varying the gelling conditions, the spatial distribution of the alginate chains in the microsphere can vary from homogeneous to very heterogeneous, with up to 10 times more concentration on the surface than in the nucleus [31]. Some studies have suggested that a truly homogeneous distribution of alginate chains can only be achieved by internal and external gelation applied simultaneously [35].

Release of components from the microcapsules can also stimulate an inflammatory response. This includes degradation products that may arise from reactions occurring under physiological conditions, detachments of parts of the system or ion exchange, among others. Alginate is subjected to hydrolytic and enzymatic degradation. It has been described to have a very low rate of hydrolysis at physiological pH and the low molecular weight chains released are excreted via the urinary tract. These degradation processes have been extensively studied, *in vitro* and *in vivo* [36,37], in the subcutaneous space, the peritoneum and in

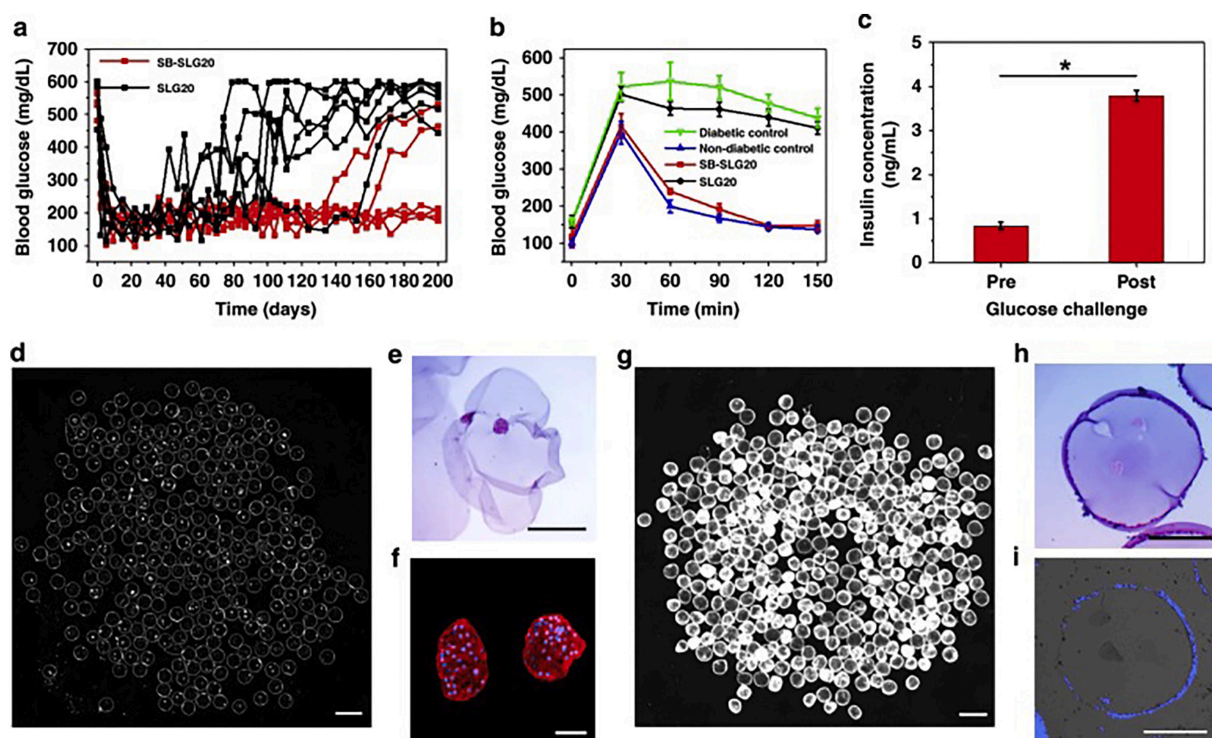

Fig. 3. Sulfobetaine-alginate (SB-SLG20) microcapsules improve diabetes correction in mice in a 200-day study. a Blood glucose concentrations of mice ($n = 6$ mice per treatment group). **b** Intraperitoneal glucose tolerance test (IPGTT) before retrieval ($n = 3$). **c** *Ex vivo* glucose-stimulated insulin secretion test (GSIS) of the retrieved rat islets from SB-SLG20 microcapsules, $n = 3$, Mean ± SEM, $^*P < 0.05$. **d** A dark-field phase contrast image of retrieved islet-containing SB-SLG20 microcapsules. ($n = 6$; scale bar, 2 mm). **e** An hematoxylin-eosin (H&E) stained cross-sectional image of retrieved islet-containing SB-SLG20 microcapsules. Scale bar, 500 μm. **f** Immunohistochemical staining of rat islets in retrieved SB-SLG20 microcapsules. Insulin is stained red and nuclei are stained blue (Scale bar: 50 μm). **g** A dark-field phase contrast image of retrieved islet-containing SLG20 microcapsules. ($n = 6$; scale bar, 2 mm). **h** An H&E stained cross-sectional image of retrieved islet-containing SLG20 microcapsules. Scale bar, 500 μm. **i** Immunohistochemical staining of rat islets in retrieved SLG20 microcapsules. Insulin staining is negative and nuclei are stained blue. Scale bar, 500 μm. Reprinted from ref. [30] http://creativecommons.org/licenses/by/4.0/.

some areas of the brain.

Some groups have devised strategies to improve the mechanical stability of alginate hydrogels, covalently crosslinking it with different polymers, via photocrosslinking solutions or enzymatic reactions, for example [38–40]. In a recent study, sodium alginate was functionalized, with cross-reactive PEG derivatives presenting a terminal thiol and carbon electrophile functionalities, and the spheres formed by a combination of Ca-alginate interactions and sulfur-carbon covalent bonds. The resulting spheres showed greater mechanical resistance and better preserved shape, compared to the simple alginate and calcium beads. When these spheres were implanted in the intraperitoneal space of immunocompetent mice, tissue adherence was not observed and integrity was not compromised in the 30 days of the study [41]. In another interesting study, it was shown that modifying the alginate with 2-aminomethyl methacrylate hydrochloride can decrease immune reactions against the implant. The authors performed a first ionic crosslinking, followed by the application of UV light to form the covalent bonds. This showed greater mechanical stability when it was evaluated *in vivo* for 3 weeks [42].

Material selection is especially demanding in this type of system since the resulting particles must not only have a high durability after implantation, they must also be capable of responding to the biological needs of the immobilized cells for long periods of time. However, many materials, such as alginate, do not have cellular signaling motifs and must be biofunctionalized to improve their interaction with encapsulated cells. In this regard, in recent years, different proteins — such as collagen, laminin or fibronectin — or small short peptides — such as RGD (arginine-glycine-aspartic acid) — have been incorporated into microcapsules, trying to imitate the physical and biomechanical characteristics of the native environment of the encapsulated cells to improve and control cellular behavior [43–46]. In this regard, there are divided opinions on whether it is more appropriate to use complete extracellular matrix proteins, such as fibronectin or collagen, or small synthetic peptides, such as RGD [47,48]. Interestingly, the best strategy in every case seem to be strongly cell-dependent.

For example, Garate *et al.* evaluated the influence of RGD functionalization of alginate encapsulating C_2C_{12} myoblasts, baby hamster kidney (BHK) fibroblast or stromal mesenchymal cells (MSCs) and the results showed different optimal concentrations of RGD in every case [47,49,50]. In this sense, Gonzalez-Pujana *et al.* designed a sensitive analytical tool that permits the evaluation of different cell adhesion kinetics, but also the integrin profiling and their contribution to cell attachment and adhesion strengthening via clustering, which allows the design of specific biofunctionalization strategies depending on the cell type [51].

Other components of the extracellular matrix (ECM), such as hyaluronic acid (HA) have also been added to the alginate matrix of microcapsules. Recently, pancreatic pseudo-islets derived from MSCs were immobilized in alginate-HA microcapsules and the results showed better cell viability, with lower levels of initial apoptosis [52]. Moreover, the inclusion of HA in the alginate matrix, enhanced the differentiation of the MSCs towards pancreatic progenitors and increased the insulin release [53,54].

2.1.2. Coating materials

In some cases, and depending on the application, the microbeads composed of different biomaterials and cells, are the final product to be administered. However, obtained pore size in most cases is too large and does not present a real barrier against the threats that the implant will face once implanted. Therefore, many groups coat these beads with different polymers to elaborate microcapsules that control the molecules and cells that can come into contact with the immobilized cells. Currently, this filtering is carried out by defining a minimum molecular weight — molecular weight cut-off (MWCO) — of solute that is totally excluded by the semipermeable membrane [55]. This definition can be misleading since molecules of similar molecular weight can have very different sizes, as is the case with proteins and polysaccharides.

Today, there is still no consensus regarding what should be the most optimal criterion for the exclusion of molecules that can access the interior of the microcapsule, despite Chick *et al.* already named the concept of the immuno-barrier in 1977 [56]. Adequately defining this concept is essential to develop biocompatible and biotolerable systems and it should be a priority issue.

To create a biocompatible and biotolerable environment, the semi-permeable membrane must first avoid contact of the encapsulated cells with the cellular components of the immune system and the antibodies. A MWCO of around 70 kDa seem to be adequate for many drug delivery applications, but it has been found that this is not enough if bi-directional flow of antigenic, chemotactic and cytotoxic molecules — such as reactive oxygen species or pro-inflammatory cytokines — is allowed. With the classic approach of size-exclusion, low molecular weight molecules such as IL-1β (17.5 kDa) or TNF-α (51 kDa) will be able to easily access the interior of the capsule and cause damage to the encapsulated cells, as they are even smaller than some of the therapeutic molecules that are usually released from these systems. On the other hand, encapsulated cells secrete antigens — e.g. chemokines as low as 8-13 kDa in molecular weight — to the exterior of the microcapsules that are responsible for recruiting cells from the host immune system.

For the elaboration of the semi-permeable membrane, different polymers have been used, such as chitosan, oligo-chitosan or poly (methylene-co-guanidine) (PMCG), but both in preclinical studies and in human trials, the most used molecules are poly-L-lysine (PLL) and poly-L-ornithine (PLO) [57–59]. However, both molecules are known to be immunogenic, so many groups choose to add a last layer of alginate on the particles to mask the positive charges that would otherwise be exposed to the components of the immune system. Resulting microcapsules are known as APA (alginate-poly-L-lysine-alginate or alginate-poly-L-ornithine-alginate) [60,61]. This strategy has been intensely debated since there are studies showing that this second layer of alginate may not be sufficient to inactivate all the exposed positive charges [62–64]. In fact, the studies carried out to analyze the surface of the microcapsules coated with these polycations, showed that these molecules are exposed — and in great quantity — in the outermost 1-2 monolayers of the membrane, thus the outer alginate layer appears to overlap with the PLL layer, rather than form an additional outer membrane [65,66]. In addition, both polycations show limited physicochemical properties but most works chose PLO for apparently having greater mechanical stability, biocompatibility and permeability [63].

The increased immunogenicity of microcapsules coated with this type of polycations is mainly due to the physicochemical changes that affect protein adhesion on the surface of the microcapsules, such as zeta potential, hydrophobicity or roughness. On the one hand, the zeta potential of this type of implant must be negative and similar to that of the membranes of adjacent cells. In a study carried out by De Vos *et al.* [67], it was found that the zeta potential of APA-PLL microcapsules showed a more negative value before they were implanted. Although all the values were less negative than those described in other studies for the uncoated alginate microbeads [68]. On the other hand, in a study carried out by Lekka *et al.* [69], a lower surface roughness, of less than 1 nm deep, was associated with the uncoated alginate microspheres and with the PLL-coated microcapsules, compared to much higher values, of up to 14.4 nm, of PMCG-sulfate coated microcapsules. Finally, the addition of coatings to the alginate microbeads increases the hydrophobicity of the system [63], although the type of alginate used in each case also influences the final result.

In a very interesting study by Rokstad *et al.* [62] a lepirudine-based human whole blood model was used as a tool for measuring the biocompatibility of different microcapsules. The results showed that alginate polycation (AP) or APA capsules trigger the complement activation, whereas Ca/Ba alginate do not. *Fig. 4* shows that the deposition of complement component 3 (C3) on the bead surface is higher in AP or APA beads than Ca/Ba beads.

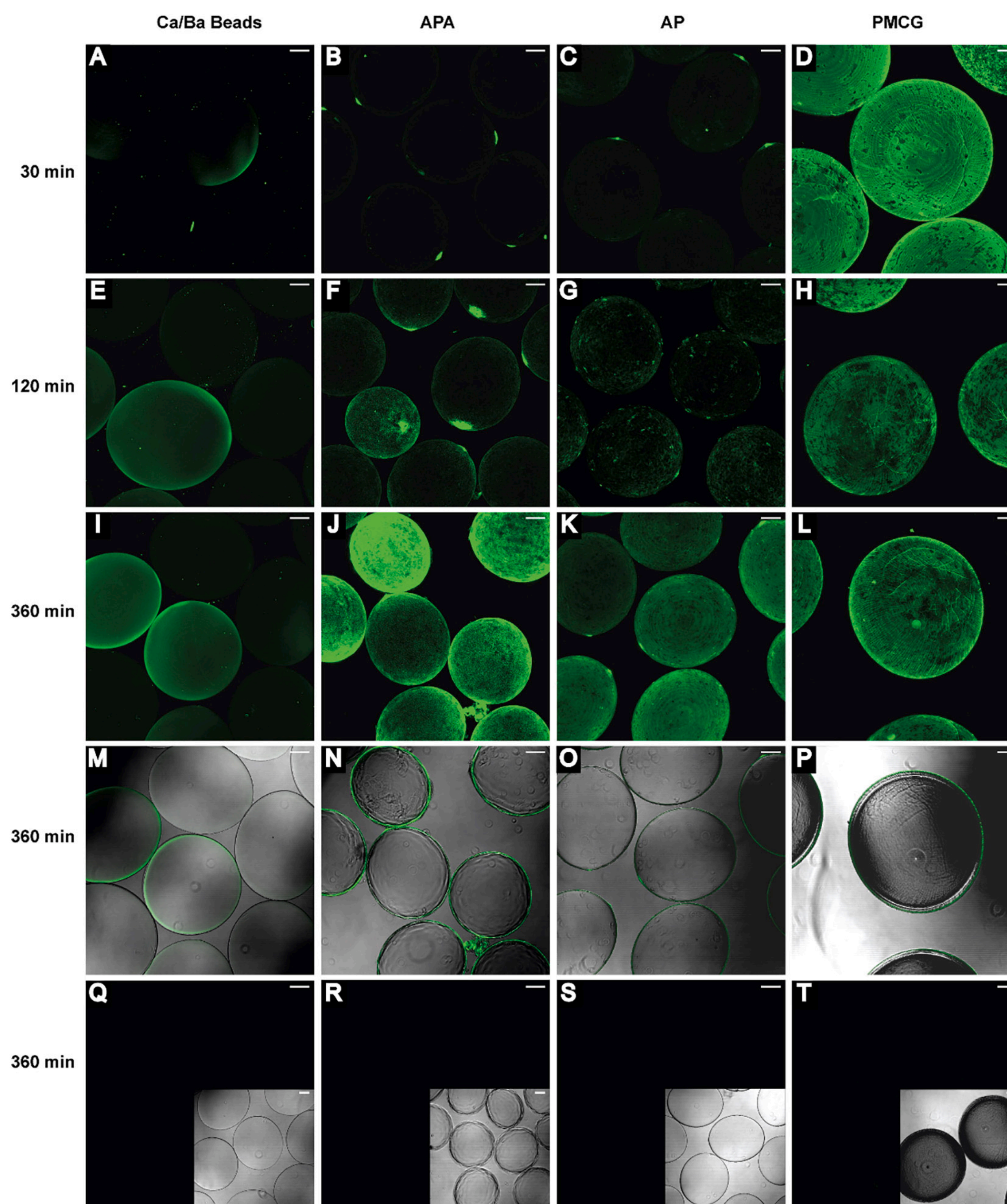

Fig. 4. Deposition of C3 on the microsphere surface after incubation in human lepirudin anti-coagulated whole blood. **A–L** 3D projections made by sectioning entire microspheres after incubation for 30, 120 and 360 min. **M–P** Projections through the equator overlaid with transmitted light images after 360 min. **Q–T** Controls are given in the lower panels as projections (black pictures). The inserts show transmitted light equatorial sections for visualization. Bars are 100 μm. Reprinted from ref. [62] with permission from Elsevier.

Taking into account the obvious need to improve the coatings of these particles, in recent years several groups have analyzed other molecules that may be appropriate, both to substitute the PLL/PLO molecules or in combination with them [70–72]. In a recent study, genipin was used in association with PLL [73]. Using force spectroscopy-based simultaneous topographical and mechanical characterization to study polymer to polymer interaction, the study concluded that genipin crosslinking avoided membrane detachment in alginate microspheres with double polycation coatings.

Attempts have also been made to improve the biocompatibility of the microcapsules by coating them with polymers capable of reducing protein adsorption and the fibrotic response to the implant. By coating the alginate microcapsules with hydrophilic polymers such as PEG [74–76], the biocompatibility of the implant can be improved, although the level of protein adsorption will depend on the density, length and conformation of its chains. In one study, alginate-PEG microcapsules containing allogenic islets were evaluated and their biocompatibility was improved when transplanted into the intraperitoneal space, but not into the epididymal fat pad [77]. The strategy of coating the alginate microcapsules with PEG and rapamycin, evaluated by another group, was also able to reduce macrophage proliferation and fibrotic response [78].

Modifying the surface of the microcapsules with a patented macromolecular heparin conjugate has also been shown to improve biocompatibility and significantly reduce the fibrotic response against the implant, in syngeneic and allogeneic transplant models [79]. With a similar strategy, but coating the alginate microcapsules with the C-X-C motif chemokine 12 (CXCL12) (also known as "stroma-derived factor 1", SDF-1), the biocompatibility was also improved but, in this case, a long-term improvement in xenogeneic pancreatic islet survival and

functionality was also achieved, due to the recruitment of immunosuppressive regulatory T cells to the implant site [80]. Recently, in another study, coating the alginate spheres with chitosan also significantly reduced the fibrotic response against the implant, improving its biocompatibility, while maintaining glucose levels for one year, in a canine allogeneic transplant model and in a xenotransplant in rodents [81].

Another strategy that may be very interesting is to incorporate motifs that have anti-inflammatory properties into the design of the microcapsule. Sulfated alginates [82] or the interleukin-1 receptor (IL-1R) [83] are good examples of this, as they decrease the production of some cytokines and improve the viability of encapsulated cells.

In some systems, such as those made by the alginate-PLL combination, it is not possible to independently adjust the mechanical stability and the permeability of the microcapsules, which is a notable limitation [84]. However, there are studies in which this permeability-stiffness relationship has been divided using various polymers [85]. The possibility of independently modifying critical parameters for cell encapsulation, such as capsule size, thickness, mechanical resistance and membrane permeability, offers great advantages in the design of this type of system.

Poor mechanical resistance can lead to protrusion of encapsulated cells, a phenomenon that needs to be fixed when designing these systems [86]. In this sense, Johnson *et al.* carried out an analysis quantifying cell protrusion in alginate microcapsules, coated with PLL and 50% hydrolyzed poly(methylvinylether-alt-maleic anhydride) (PMM). According to the results obtained, around 30% of the encapsulated INS-1E β cells were located in the last 20 μm of the alginate-PLL-PMM50 layer, with 7% of the cells protruding [87]. Reinforcing the capsules with cross-linked shells may help preventing cell exposure and scape.

Lastly, in some cases, for example if the secreted molecule is especially large, it will be necessary to optimize the system so that it allows the passage of the therapeutic molecule out of the capsule, without compromising the protection of the encapsulated cells. In this sense, recently Montanucci *et al.* modified the permeability of alginate microcapsules to allow the continuous secretion of immunoglobulin M (IgM), with no signs of inflammation [88].

2.2. Cell source and target pathology

Both allogenic and xenogeneic cells have been incorporated into the microencapsulation systems. In case of human origin cells, their acquisition can be complicated and expensive. Besides, they can be subject to biological, ethical and legal limitations. Therefore, the use of xenogeneic cells has spread in the field of cell microencapsulation, thanks to the immunoisolation produced by the semipermeable membrane [89]. However, the systems used to date to encapsulate both cell types have been practically identical, without taking into account that the different immunological responses caused by allogenic or xenogenic cells require capsular configurations capable of protecting the cellular content against variable immunological environments.

In the case of allogeneic transplants, it is probably sufficient to avoid contact between the encapsulated cells and the cells of the host's immune system [90]. Therefore, the simplest microcapsules of cations and alginate, without great limitations in the diffusion of molecules, may be suitable. When a xenotransplantation is performed, the scenario is more complex and the simplest systems may not be effective in avoiding immune rejection (*Fig. 5*). These cells produce xenogenic epitopes, such as galactosyl (Gal) residues, that are secreted outside the capsule and are recognized by the immune system of higher mammals, including humans. In addition, in recent years the role of N-glycolyl neuraminic acid (Neu5Gc), another pig xenoantigen, is being studied as a possible obstacle in xenotransplantation [91].

The complexes formed by Gal residues and the antibodies linked to them, are powerful activators of the classical complement pathway. As these complexes begin to accumulate on the capsular surface, chemotaxis of different cell types, such as neutrophils [92] occurs, which initiate powerful inflammatory reactions. During this first phase, many

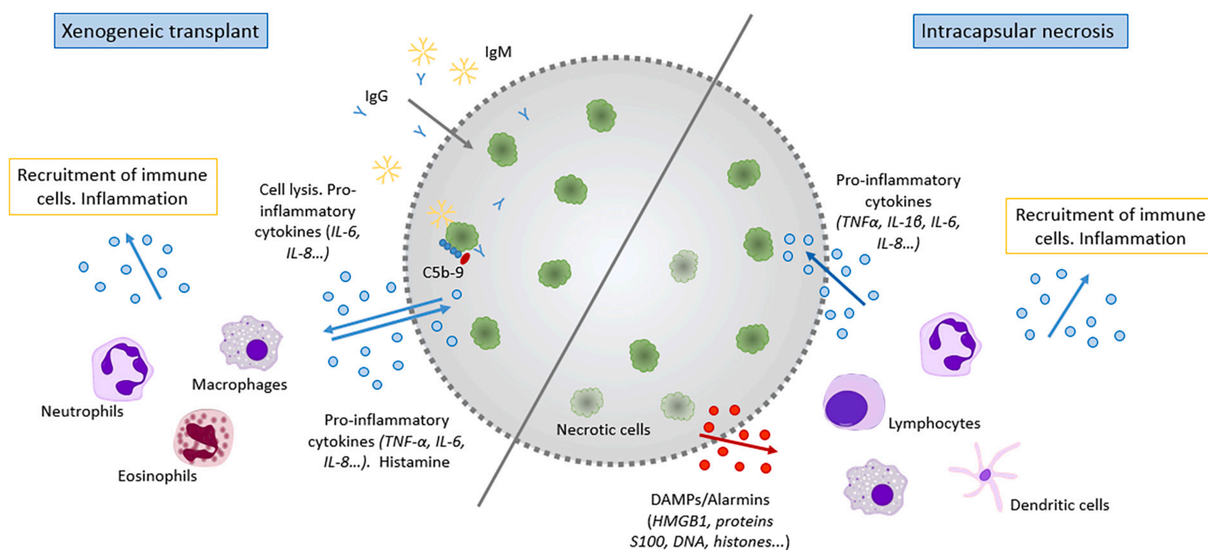

Fig. 5. Pro-inflamatory molecule secretion from the microcapsules activates the immune response in different scenarios. Xenogeneic transplant. Some antibodies are able to enter the capsule or contact the encapsulated cells located in the most superficial layers of the implant. These antibodies recognize sequences that are not present in the host species, such as the Gal carbohydrate in the case of primates, and this leads to the activation of complement pathways. This activation produces direct cell lysis and the release of molecules that promote inflammation and the recruitment of immune cells, such as neutrophils, to the implant site. The recruited cells release small cytokines that can cross the microcapsule membrane and damage the encapsulated cells. In more advanced phases, fibrotic processes appear, which could end up isolating the implant and compromising the supply of nutrients and oxygen to the interior of the capsule. **Intracapsular necrosis.** Necrotic cells release the so-called DAMPs or alarmins, such as high-mobility group box-1 (HMGB1), heat shock proteins (HSPs), S100 proteins, DNA/RNA fragments etc., to the extracellular fluid. These small molecules can diffuse outside the microcapsules and activate cells of the immune system, such as macrophages, dendritic cells, neutrophils or lymphocytes, binding PRRs, such as TLRs. These cells, in response, will secrete pro-inflammatory cytokines, such as IL-1β, IL-6, interleukin-8 (IL-8), or TNF-α, which will produce inflammation and recruit more immune cells to the area. These molecules can enter the microcapsules and damage the encapsulated cells. If the situation persists over time, the adaptive immune response may be activated.

small cytokines are able to cross the semipermeable membrane of the microcapsules, causing damage to the encapsulated cells.

Following the first innate response, a second IgM-mediated [92] delayed hypersensitivity response to xenogeneic epitopes begins, promoting the recruitment of new cells of the immune system to the implant site and the secretion of more chemokines and cytokines. After these events, the microcapsules are usually surrounded by inflammatory cells and fibroblasts that hinder the passage of nutrients and oxygen, compromising the survival of the encapsulated cells. Finally, the appearance of fibrosis can lead to total isolation of the implant.

Due to these differences between allo and xenografts, the latter require systems that protect encapsulated cells against more potent threats. The membranes must be less permeable and, ideally, prevent the passage of molecules produced by the immune system, while preventing the exit of hyperinflammatory xenogeneic epitopes, such as Gal residues.

Another way of activating the immune response may occur when cell necrosis appears inside the microcapsules [93] *(Fig. 5)*. Unfortunately, this is still quite common, when there are problems in the diffusion of nutrients and oxygen, due to insufficient permeability of the biomaterials, fibrotic processes associated with foreign body reaction or an excess of encapsulated cell mass [94]. Necrotic phenomena are directly related to damage-associated molecular patterns (DAMPs). These molecules are normally found inside the cells, but are released outside when cell damage occurs [95]. Some examples are heat shock proteins or DNA/RNA fragments. The mammalian immune system has specific receptors for this type of signals, the PRRs, such as Toll-like receptors (TLRs). The DAMPs released from the microcapsules are powerful activators of the immune system, activating inflammatory and angiogenesis processes, which mediate the release of large amounts of cytokines that jeopardize the survival of the encapsulated cells [96].

In this sense, there are different studies that tried to improve the viability of microencapsulated cells, incorporating chemical compounds capable of generating oxygen [97,98] or through strategies that promote the vascularization of the implant. For the latter, several strategies have been tested. On the one hand, the ability of different angiogenesis-inducing growth factors, such as fibroblast growth factor (FGF) [99,100] or vascular endothelial growth factor (VEGF) [101,102] has been exploited to promote the neovascularization of the implant, thus improving the results of the therapy. On the other hand, the implantation of the microcapsules in pre-vascularized spaces is also considered as a beneficial option, either generated in the host's organism or in macrodevices [103,104].

Even if the risks associated with xenotransplantation are being reduced, the advances in the field of stem cell use have unlocked an unthinkable potential. The ability to differentiate human stem cells, from different sources, to obtain the desired cell type or the possibility of reprogramming adult cells to induced pluripotent stem cells (iPSCs) [105,106] have defined the path to a sufficient source of human cells. Furthermore, in the particular case of iPSCs, there are not ethical restrictions [107].

In this vein, the studies carried out to date have shown that it is possible to obtain fully functional beta cells or pancreatic progenitors, starting from human pluripotent stem cells (hPSCs) — either human embryonic stem cells (hESCs) [108–113] or iPSC [114]. In recent years, these cells, known as human stem cell derived β cells (SC-β), have been seen as an excellent source of unlimited pancreatic cells [30,111,115]. For example, Vegas *et al.* implanted human SC-β, immobilized on alginate beads, in the intraperitoneal space of immunocompetent C57BL/6J mice previously treated with streptozocin. C-peptide levels and blood glucose concentration showed therapeutically relevant results up to 174 days, without the need for immunosuppressive treatment [116]. In another recent study, the maturation of SC-β was stimulated by forming aggregates, similar in size to pancreatic islets, which make them respond to glucose stimulation in just 3 days after transplantation [117].

Likewise, stem cells from other origins, such as amniotic fluid or adipose tissue, can also be transformed into insulin-producing cells, which can be encapsulated and transplanted in diabetic animal models to normalize blood glucose values [118–120]. For example, Montanucci *et al.* managed to remit hyperglycemia in diabetic mice, implanting human umbilical cord Wharton jelly-derived mesenchymal stem cells (hUCMS), immobilized in alginate and PLO microcapsules [121]. Moreover, in a more recent study by the same authors [122], hUCMS cells were co-encapsulated with human pancreatic islet-derived progenitor cells (hIDC) and implanted into non-obese diabetic mice. The objective of this synergy was to maintain tracer insulin output by hIDC, while exploiting the immunoregulatory properties of hUCMS. A decline of blood glucose levels was observed *in vivo*.

MSCs have demonstrated to be very suitable for their use in cell encapsulation systems, due to their hypoimmunogenic and immunomodulatory characteristics [123–125]. These cells inhibit immune responses by secreting cytokines and soluble growth factors that produce a local immunosuppressive effect in the surrounding cells [126]. In recent studies, efforts have focused on analyzing the behavior of immortalized MSCs, genetically modified to secrete erythropoietin (EPO), for the treatment of anemia [50,127–129]. In addition, their benefits have also been evaluated in hepatic pathologies [130,131], as an alternative to porcine hepatocytes [132,133]. Moreover, MSCs not only are a very interesting option as a secretory cell [50,128], but also as a co-encapsulated auxiliary cell [134–137]. In a recent study, pancreatic islets and MSCs were co-encapsulated in alginate and PEG microcapsules and implanted into the intraperitoneal space of a diabetic mouse model [138]. The results showed that MSCs interact with N-cadherin and increase insulin secretion, in addition to providing structural support to the islets, improving their viability and functionality.

It is also important to highlight that the customizable environment generated in these 3D structures can notably improve cell viability and cellular function. In this regard, cell microencapsulation technologies are used far beyond sustained release purposes, for example, for enhanced cell culture [139] or for recapitulating tumor microenvironment or *in vitro* disease models [140], among others.

2.3. Microcapsule size and shape

The optimal size for cell microencapsulation systems remains a matter of debate. On the one hand, it is evident that a larger capsule size could be an obstacle for the diffusion of nutrients and oxygen to the nucleus of the system. This would lead the encapsulated cells to situations of hypoxia and cell death, as well as to slower responses to the stimuli from their environment [141]. In fact, in a recent study, it was suggested that, in the case of pancreatic islets, the maximum distance between these and the extracapsular fluid should not be more than 100 μm, to allow adequate exchange of nutrients and oxygen [142].

Therefore, many have been the studies aimed at obtaining smaller capsules. Coaxial air flow and flow focusing technologies were presented as attractive alternatives to the usual methods of making microcapsules by means of electrostatic dripping, making it possible to manufacture capsules of 100-200 μm in diameter, that allow for more complicated routes of administration, such as intracranial administration (in the case of CNS pathologies) or even intravitreal [143,144]. Furthermore, trying to reduce the size of the capsules as much as possible, in recent years nanoencapsulation strategies have also been evaluated — such as conformal coating or layer-by-layer coating —, especially for the immunoprotection of the islets of Langerhans [124].

Conformal coating is a form of non-spherical encapsulation that reduces the diffusion distance and the volume of the implant [145]. However, the process often involves multiple steps that can cause damage to the encapsulated cells, and a conclusion has not yet been reached regarding whether these type of coatings are thick enough for their use in clinical practice [146,147]. Some studies [148] have suggested that the conformal coating has a lower immunoprotective capacity, compared to hydrogel microcapsules, but in recent years several new strategies have demonstrated the potential of this technology

[149–151].

In case of layer-by-layer coatings, layers of polymers of opposite charges alternate on the surface of a group of cells, decreasing the biomaterial/cell ratio and thus improving the diffusion of the therapeutic molecule. In theory, this strategy should improve some of the most characteristic limitations of conformal coatings, such as inadequate mechanical stability or limited immunoprotection. Layer-by-layer coatings have been evaluated in several studies in rodents, with promising results [152–154] and in a recent study in non-human primates, layer-by-layer encapsulated pancreatic islets with 3 layers of PEG had 100% survival during 150 days after xenotransplantation — in the presence of immunosuppressive treatment —[155,156].

However, the polymers used to nanoencapsulate therapeutic cells are usually less biocompatible than other hydrogels normally used in the field of cell microencapsulation. Furthermore, the shape and roughness of the implant surface produce notable differences in the immune response that it causes. Therefore, in nanoencapsulation, it might be interesting to add a second type of coating that attenuates the shapes and complements the system [157].

On the other hand, and in opposition to the idea of reducing size as much as possible, Veiseh *et al.* published a complete study analyzing the influence of the size of the alginate microspheres (containing or not Langerhans islets) in rodents and non-human primates. Their conclusions, validated also with other materials such as ceramics, metals and plastics, indicated that the larger microspheres (1.5 mm) had a smaller number of immune system cells and fibrotic processes in all cases [158] *(Fig. 6)*. When implanted for 14 days into the intraperitoneal space of C57BL/6 mice, the Ba^{+2}-crosslinked SLG20 alginate hydrogel spheres, in eight different sizes (0.3-1.9 mm), showed a marked reduction in cellular deposition and fibrosis onto spheres as their size increased. Cellular deposition on spheres was examined using Z-stacked confocal imaging using DAPI (nucleus marker), F-actin (cellular cytoskeleton marker) and α-SMA (myofibroblast marker). Additional immunostaining for the host immune cell markers CD68 (macrophage), Ly6G/Ly6C-GR1 (neutrophil) and TGF-β (inflammation marker) also showed reduced immune cell deposition on larger spheres, and this result was confirmed by qPCR expression analysis of additional fibrosis markers - namely collagen 1a1 (Col1a1), collagen 1a2 (Col1a2) and α-SMA - and by western blot analysis of α-SMA expression within the cellular overgrowth on spheres.

This study has led to an opinion division among the experts in the field [27]. In fact, some of them have criticized that the study did not take into account the degree of purity of the alginate used, one of the factors considered key to predict the expected inflammatory response [159].

Finally, it is important to highlight that there are many factors influencing bead formation and that many of them are not properly described yet. In a recent study, the impact of several parameters (such as, critical electric potential, needle size or distance between the needle and the gelation bath) on electrostatic-droplet formation was investigated and their influence was described through equations to make procedures more reproducible and allow optimal control of capsule size and properties [160].

2.4. Implant site and administration procedure

The choice of a suitable implant site can greatly determine its biocompatibility and viability. Ideally, this space should be large enough to accommodate the required number of microcapsules and easily accessible for implant removal. Likewise, the neovacularization of the implant must be favored, while the immune response must be limited to avoid an excessive fibrotic response that may condition the supply of nutrients and oxygen to the microencapsulated cells. In this sense, when a systemic effect is pursued, the intraperitoneal and subcutaneous cavities appear as simple and minimally invasive routes [37,161]. In fact, most of the studies with pancreatic islets have focused on the intraperitoneal route, despite the high activity of macrophages and the need for larger numbers of cells compared to other areas [162]. However, the neovascularization capacity in these two pathways is not sufficient in some cases, resulting in inadequate therapeutic molecule release pharmacokinetics and a shortage of nutrients and oxygen for encapsulated cells, especially when pancreatic islets are used [26,157]. In this case, the hepatic and renal subcapsular cavities are considered to have some advantages over the intraperitoneal or the subcutaneous spaces [162,163].

In recent years, preserving the benefits of the intraperitoneal route but improving the disadvantages, in some studies the surgery to create an omental pouch has been proposed. This surgery can ensure a space large enough to accommodate the necessary number of microcapsules, with greater vascularization and improving one of the main problems of biped hosts: the aggregation of the spheres in the lower part of the peritoneum, which increases the shortage of nutrients and oxygen to the implant. This strategy has demonstrated its long-term benefits, keeping diabetic rodents in normoglycemia [164]. More recently, Bochenek *et al.* described the implantation of allogeneic pancreatic cells encapsulated in Z1-Y15 alginate beads in a similar omental pouch in non-human primates [29] *(Fig. 7)*. To perform the bursa omentalis transplantation technique, a small incision was introduced laparoscopically into an avascular section of the gastrocolic ligament. The spheres were then infused into the bursa omentalis. Over time, the spheres distributed uniformly as a monolayer between the two vascularized omental layers. This administration site was compared to other anatomical sites. The bursa omentalis showed a trend of increased average pO2 compared to the general intraperitoneal space, but this was not found to be significantly different (35.1±3.2 mmHg compared with 30.7±1.6 mmHg, respectively). The kidney capsule was found to yield the highest pO2 measurements compared to other sites (48.7±1.3 mmHg).

Regarding the renal subcapsular space, although the vascularization in this area is greater than in other areas, the space is more limited and it is difficult to administer the necessary large implant volumes. As a mere example, in a study carried out in 7 *macaca fascicularis* xenotransplanted in the renal subcapsular space with microencapsulated pancreatic islets, porcine C-peptide was detectable in 2 of the animals for 60 days after administration [165].

Finally, regarding the subcutaneous route, some authors argue that it may still be attractive due to some remarkable advantages such as being less invasive, allowing the easy monitoring and retrieval of the implant and showing a lower immunogenic activity [26].

On the other hand, when a local release of the therapeutic molecule is sought, the administration must be carried out near the target tissue. Clear examples of this are the eye [166] or CNS [167], in which the natural barriers prevent the systemic administration. These two routes are considered to be immune-privileged and thus lower immunological responses are expected after implantation. Another situation that requires local administration is when the objective of the microencapsulated cells is the conversion of a prodrug into an active molecule, exclusively near a tumor, to avoid the adverse events derived from a systemic exposure [168].

In any case, the viability of the implant will largely depend on the lack of any fibrotic capsule that could isolate the encapsulated cells. In order to reduce the fibrotic response, some groups have described the benefits of concomitant administration of immunosuppressive molecules locally or temporarily, just a few days after implantation. The incorporation of drugs, such as ketoprofen or dexamethasone within the microcapsules has been tested in several studies, in which the fibrotic response has been reduced [169–171]. More recently, the CXCL12 molecule was co-encapsulated together with SC-β derived from hPSCs, with the aim of reducing the fibrotic pericapsular response, in the absence of systemic or local immunosuppressive treatments. CXCL12 produced an increase in insulin secretion by the encapsulated cells and the implant remained viable for more than 150 days in immunocompetent mice [172]. The same strategy was also used in non-human

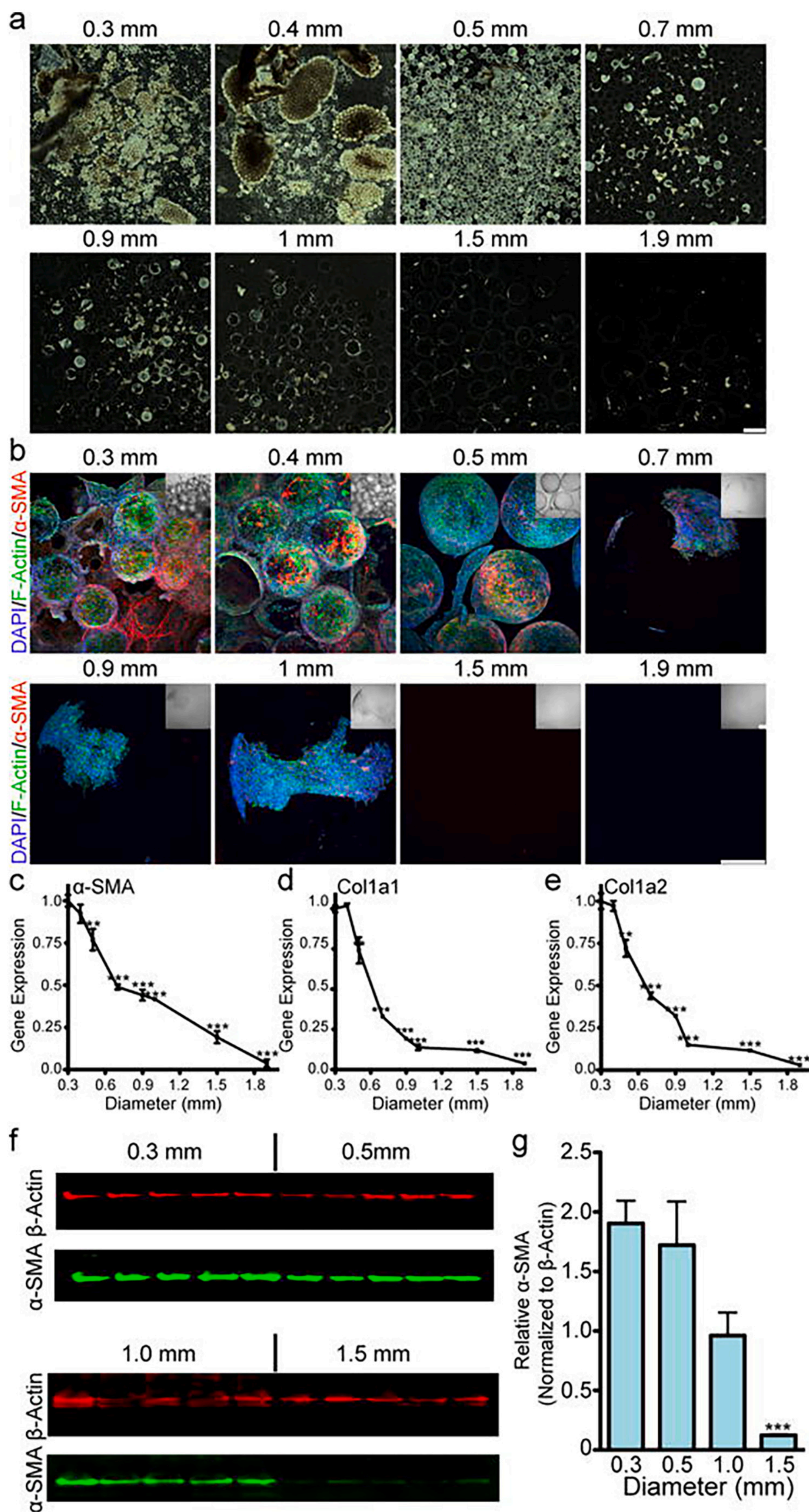

Fig. 6. Increasing alginate sphere size results in reduced cellular deposition and fibrosis formation on the spheres. SLG20 alginate spheres (0.5ml in volume) of different sizes (0.3, 0.4, 0.5, 0.6, 0.7, 1, 1.5 and 1.9 mm) were implanted into the intraperitoneal space of C57BL/6 mice, where they were retained for 14 days and analyzed for degree of fibrosis upon retrieval. **a** Dark phase contrast images obtained from retrieved spheres reveal a significant decrease in level of cellular overgrowth with increase in sphere size; scale bar = 2mm. **b** Z-stacked confocal images of retrieved spheres immunofluorescence stained with DAPI (highlighting cellular nuclei), phalloidin (highlighting F-actin) and α-Smooth Muscle Actin (α-SMA, myofibroblast cells); Scale bar = 300 μm. **c** q-PCR based expression analysis of fibrotic markers α-SMA, **d** collagen 1a1 and **e** collagen 1a2 directly on the 8 various sized (0.3, 0.4, 0.5, 0.7, 0.9, 1, 1.5, 1.9 mm) spheres plotted normalized to relative expression levels on 300 μm sized spheres. **f** Semi-quantitative western-blot analysis of α-SMA expression in cell overgrowth from on microspheres (1-5 labeling of bands corresponds to individual mice). **g** Plot of analyzed band intensities from western blot images shown in f. Error bars, mean ± SEM. N = 5 mice per treatment. All experiments were performed at least three times. qPCR and western blot statistical analysis; one-way ANOVA with Bonferroni multiple comparison correction *:$p < 0.05$, **: $p < 0.001$, and ***: $p < 0.0001$. Reprinted from ref. [158] with permission from Springer Nature.

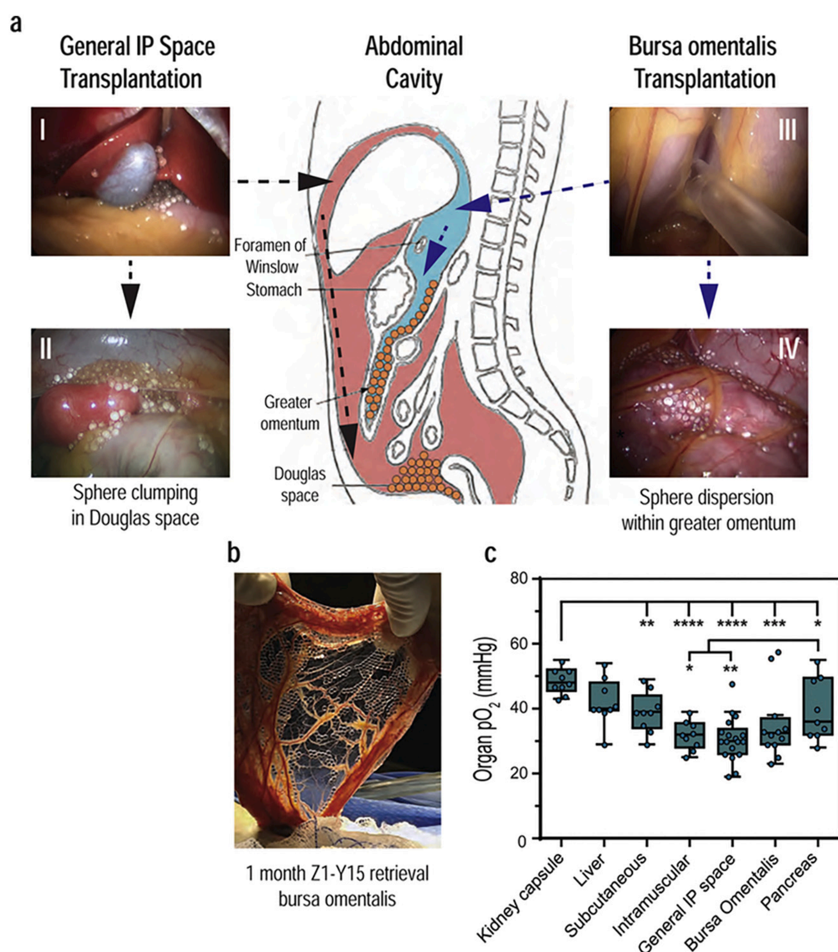

Fig. 7. Transplantation method (general IP space VS. bursa omentalis) causes differential spatial distribution of the Z1-Y15 alginate spheres post-implantation. a General intraperitoneal space transplantation: Z1-Y15 spheres were laparoscopically distributed around the left and right medial lobes of the liver within the intraperitoneal space (pink) (I). At 1-month post-implantation, the non-fibrosed spheres had settled and clumped within the Douglas space (II). Bursa omentalis transplantation: the stomach was lifted with a laparoscopic grasper and a small incision was made into an avascular section of the gastrocoli ligament. The Z1-Y15 spheres were then infused into the bursa omentalis (blue) (III). At 1-month post-implantation, the Z1-Y15 spheres remained spatially dispersed within the bilayer of the greater omentum (IV). A schematic of the two transplantation methods provides the location of the anatomical sites and a summary of the spatial distribution of the Z1-Y15 spheres during the 1-month retrievals (center). The general IP space transplantation was repeated independently for n = 10 NHP and the bursa omentalis transplantation for n = 7 with resultant similar spatial distributions. **b** The greater omentum was extracted through the supra umbilical midline incision at 1 month and shows translucent, unattached Z1-Y15 spheres with encapsulated allogeneic islets within the omental tissue bilayer. **c** Partial oxygen pressures (pO_2) of various transplantation sites that have been previously investigated for encapsulated islet transplantation. The kidney capsule has the highest pO_2 measurements compared to the other anatomical sites. The intramuscular space (rectus abdominis) and general IP space have lower pO_2 compared to the pancreas, liver, subcutaneous and kidney capsule. The bursa omentalis site was not found to be statistically significantly different than the pancreas or the general IP space. (* $p < 0.05$; one-way analysis of variance (ANOVA) with Fisher's LSD for multiple comparisons; 3 steady-state measurements were taken for each anatomical site from the same primate; n = 3 NHP; box and whisker with median, upper and lower quartile ranges, outliers, 1.5 x IQR, individual data points overlaid). Reprinted from ref. [29] with permission from Springer Nature.

primates (n = 4) but the results are still preliminary [173]. Toll-like receptor 2- modulating pectin – polymers, with varying degrees of methyl-esterification (DM) have also been used in alginate-based microcapsules, in order to attenuate immune responses and support islet-xenograft survival [174]. DM18-pectin/alginate microcapsules showed a significant decrease of DAMP-induced Toll-Like Receptor-2 mediated immune activation *in vitro* and, when implanted *in vivo*, pericapsular fibrosis was reduced. In another study [175], a crystalline Colony Stimulating Factor 1 Receptor (GW2580) inhibitor was encapsulated with human islets in alginate microbeads, and transplanted into the IP space or injected subcutaneously in STZ-induced diabetic C57BL/6 mice. Drug-loaded beads in both IP and SC sites were equally capable of restoring glycemic control in diabetic mice for extended periods of time up to 72 days. In contrast, SC capsules without drug did not provide control of blood sugar.

3. Biosafety: dose control, monitorization and extraction

Once the implant has been placed, real-time monitoring of its location and correct operation can give us valuable information. The exact location of the microcapsules can be determined by cellular labeling with fluorescent dyes or radiolabels [176,177], traditional imaging systems, such as ultrasounds [178] or, more commonly, by high resolution and contrast magnetic resonance imaging (MRI) [179–183]. There are different types of contrast materials that can be used with these techniques. One of the most studied examples are the super-paramagnetic iron oxide nanoparticles (SPIO) [184].

It is also possible to detect microcapsules by X-ray, if we encapsulate contrast agents such as barium sulfate or bismuth sulfide, which make them opaque [185]. In one study, a method based on gold nanoparticles was developed as a contrast agent to monitor alginate microcapsules by X-ray and micro-CT [186] techniques. The same group demonstrated that it is possible to detect alginate microcapsules both *in vitro* and *in vivo*, through low exposure to X-rays, if they are coated with gold nanoparticles [187]. On the other hand, different groups have found other innovative ways to monitor implanted microcapsules. For example, researchers have recently developed a type of microcapsule with intrinsic capacity for *in vivo* imaging by incorporating genipin into its own design [188]. Different volumes (50-200 μL) of capsules were implanted into the subcutaneous space of NSG mice. Taking advantage of the natural fluorescence of this compound, they demonstrated the linear correlation between the implanted microcapsule volume and the signal emitted by the microcapsules for 35 days. Thus, through this strategy, it is possible to assess the actual injected dose — volume of microcapsules — after administration and monitor the position of the implant over time, which improves in a remarkable manner the biosafety and efficacy of the therapy *(Fig. 8)*.

Molecular imaging techniques not only allow us to monitor the exact location of the implant, but also to simultaneously confirm the viability and functionality of the encapsulated cells by including reporter genes that emit fluorescent and/or bioluminescent signals [189–191]. These techniques provide us with quantitative and real-time information, in a non-invasive way. Recently, Spanoudaki *et al.* combined fluorine MRI and unsupervised machine learning to monitor over time the spatial arrangement and the oxygen content of implants encapsulating pancreatic islets *in vivo* [192].

Regarding biosafety, another critical point may occur at the end of the therapy, or in an event where significant adverse effects are

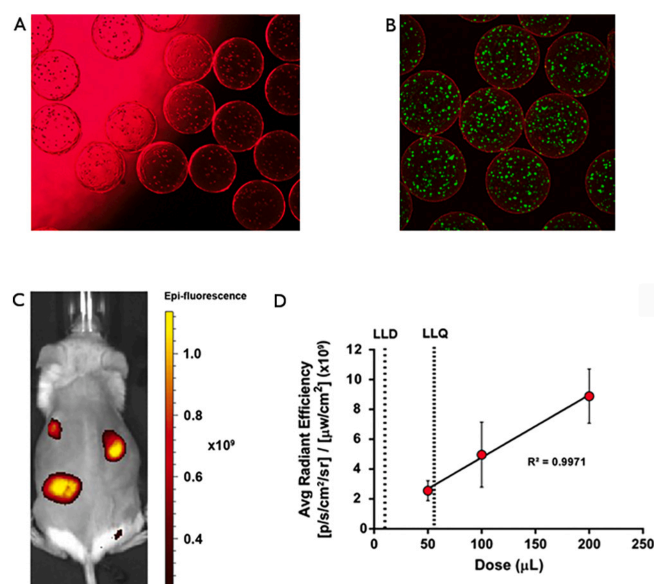

Fig. 8. Monitoring implantable immunoisolation devises with intrinsic fluorescence of genipin. **A** Representative epifluorescence micrograph. **B** Representative confocal fluorescence image of cells encapsulated in genipin-cross-linked double poly-L-Lysine membrane (GDP) microcapsules and probed with LIVE/DEAD viability kit (Green, living cells; Red, dead cells) 14 days after encapsulation. **C** Representative image of a mouse 21 days after injection of GDP microcapsules. The fluorescence from the microcapsules was imaged with 570 nm excitation and 620 nm emission. Scale bar denotes range of photons displayed on a pseudocolor scale with yellow and dark red denoting highest and lowest values, respectively. **D** Graph displays dose-dependent response of average radiant efficiency for GDP microcapsules. Reprinted from ref. [188], Copyright 2018, with permission from John Wiley and Sons.

detected. In these situations, a system that allows us to ensure a total inactivation and/or removal of the implant can be necessary. For such aim, one of the main strategies is the inclusion of suicide genes into the genome of the encapsulated cells [193–195]. The enzyme-activated prodrug mechanism, thymidine kinase/ganciclovir system that targets actively dividing cells is the most frequently studied gene therapy strategy [195]. However, these strategy could present some disadvantages when the encapsulated cells are in a slowly dividing state. In order to improve this problem, Wong *et al.* equipped glial cell-derived neurotrophic factor (GDNF)-secreting cells with a proliferation independent Tet-on regulated pro-caspase 8 apoptotic gene switch for a safer ocular drug delivery [196,197].

Recently, Delcassian *et al.* developed functionalized iron oxide nanoparticle-loaded alginate microcapsules that enabled graft retrieval under an applied magnetic field [198]. In addition, this system facilitates graft localization via MRI. These capsules containing islets were evaluated both *in vitro* and *in vivo*, in immunocompetent diabetic mice, and they were able to restore normoglycemia for at least 6 weeks. The application of a magnetic field for 90 s, 24h after implantation, allowed the retrieval of up to 94% of the transplant volume.

On the other hand, there is the possibility of introducing the microcapsules in a physical support that prevents their dispersion. These retention elements can be, for example, cements based on calcium phosphate or hydrogels of different composition. Acarregui *et al.* managed to improve the administration, retention and extraction of the APA microcapsules, administering them in injectable or preformed alginate hydrogels. Likewise, this system significantly reduced the post-implant inflammation that normally occurs in the first days after administration [171].

Finally, in some cases, in order to avoid the need to remove the implant after treatment, biodegradable systems that can be eliminated when necessary could be the best option [199].

The field of synthetic biology has opened new perspectives for cell microencapsulation technologies. Using genetic engineering techniques, it is possible to reprogram the metabolic activities of eukaryotic cells so that they produce the therapeutic molecule at the right time. The secretion can be activated by a specific inducer or even in direct response to the needs of the patient [200–202] — when working with more advanced systems —, capable of interpreting and reacting to various pathophysiological stimuli [203,204]. Taking control over the therapeutic molecule expression levels allows us to administer the necessary dose, increasing the efficacy and minimizing adverse events.

Finally, when these functionalities are incorporated into encapsulated cells, an adequate control over the system is mandatory, avoiding possible genetic construct-transfers to the host's cells or checking that these systems do not interfere with the metabolic processes of the host.

4. Concluding remarks and future prospects

The clinical application of cell microencapsulation technologies has remained elusive for decades, even if the first clinical trials took place more than 20 years ago [205]. One of the most limiting factors slowing the development of these systems is the great variability between the protocols used in different research groups. This, together with the lack of detail in the descriptions of the materials and processes used, makes the comparison between systems and the extraction of solid conclusions very complicated. Despite promising preclinical results and decades of technology development, phase I/II clinical trials performed to date resulted in poor outcomes, with fibrotic reaction being the major factor responsible for graft failure [32,205–209]. Thus, combating the foreign body reaction is crucial for improving implant outcomes, along with ensuring an appropriate supply of nutrients and oxygen to the encapsulated cells.

In recent years, the improvements made in the alginate — either by optimized purification protocols [19–22] or the recent proposals to modify its chemical structure [28] — and the emergence of stem cell therapies, have greatly improved key biocompatibility issues, taking the technology an step forward in terms of efficacy.

Finally, as previously discussed, the shape and size of the implant [158], which is still a controversial topic [27], or the administration route, are also other factors influencing the outcome of graft viability. In this regard, the optimal site should be accessible via minimally invasive methods, ensure a good vascularization of the implant, and not elicit a strong immune reaction.

In the upcoming years, cell microencapsulation technologies are expected to finally reach the market, but their success will mainly depend on their ability to meet the strict regulations applied to cellular therapies and the possibility to set up large-scale practices that allow the safe and efficient production of the microcapsule batches. Even if there are still some aspects that need to be optimized, the extensive work carried out to date, in terms of improving key aspects such as efficacy and biosafety, have taken cell microencapsulation technologies closer than ever to the clinical practice.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgements

Authors gratefully acknowledge the ICTS "NANBIOSIS", specifically by the Drug Formulation Unit (U10) of the CIBER-BBN at the University of the Basque Country (UPV/EHU) in Vitoria-Gasteiz. Tania B. Lopez-Mendez thanks the Basque Government (Department of Education, Universities and Research) for the Ph.D. fellowship.

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