

Microencapsulation of cells and molecular therapy of type 1 diabetes mellitus: The actual state and future perspectives between promise and progress

Giuseppe Basta, Pia Montanucci , Riccardo Calafiore*

Division of Internal Medicine and Endocrine and Metabolic Sciences (MISEM), Department of Medicine, University of Perugia, Perugia, Italy

Keywords

Alginate, Microcapsules, Type 1 diabetes

*Correspondence

Riccardo Calafiore
Tel.: +39-75-585-8134
Fax: +39-75-578-4492
E-mail address:
riccardo.calafiore@unipg.it

J Diabetes Investig 2021; 12: 301–309

doi:10.1111/jdi.13372

ABSTRACT

The history of microencapsulation of live cells started with an idea of Thomas MS Chang in 1964, thereafter applied to isolated pancreatic islets by Anthony M Sun in 1980. The original aim was to provide isolated cells with an immune-protective shield, to prevent physical contact between the transplanted cells and the host's immune system, with retention of the microcapsules' biocompatibility and physical–chemical properties over time. In particular, this revolutionary approach essentially applied to islet grafts, in diabetic recipients who are not immunosuppressed, at a preclinical (rodents) and, subsequently, clinical level. Among the different chemistries potentially suitable for microencapsulation of live cells, alginic acid-based polymers, originally proposed by Sun, proved to be superior to all others in the following decades. In fact, only alginic acid-based microcapsules, containing allogeneic islets, ultimately entered pilot human clinical trials in patients with type 1 diabetes mellitus, as immuno-selectiveness and biocompatibility of alginic acid-hydrogels were never matched by other biopolymers. With problems related to human islet procurement coming into a sharper focus, in conjunction with technical limits of the encapsulated islet grafting procedures, new challenges are actually being pursued, with special regard to developing both new cellular systems – able to release immunomodulatory molecules and insulin itself – and new microencapsulation methods, with the use of novel polymeric formulations, under actual scrutiny. The use of embryonic and adult stem cells, within microcapsules, should address the restricted availability of cadaveric human donor-derived islets, whereas a new generation of newly-engineered microcapsules could better fulfill issues with graft site and long-term retention of biopolymer properties.

INTRODUCTION

Type 1 diabetes is an autoimmune disease due to selective killing of pancreatic islet β -cells, resulting in endogenous insulin secretion being abolished. In particular, the inflammatory process and immune response are key factors in the development of the disease¹; autoreactive T lymphocyte cells and islet cell-reactive B lymphocyte cells play an important role in the islet β -cell-directed destruction process^{2,3}. As a consequence, patients with type 1 diabetes might only be treated with “life-saving” exogenous insulin supplementation⁴. Although it is actually indispensable for granting type 1 diabetes patients' survival,

exogenous insulin does not represent a cure for this metabolic disorder, and it might attenuate/delay, but never eliminate, the risk for developing secondary complications of the disease, such as cardiovascular and renal disease, neuropathy, and retinopathy with often severe, disabling sequelae.

Mapping the field: Role of alginate-based microencapsulation in cell therapy for type 1 diabetes

Cell therapy by pancreatic islet transplantation for type 1 diabetes

In an attempt to find a cure for type 1 diabetes, intrahepatic grafts of healthy pancreatic islets, retrieved from cadaveric donor organs, into totally immunosuppressed type 1 diabetes patients initially seemed to represent the most elegant and effective

Received 25 June 2020; revised 17 July 2020; accepted 20 July 2020

solution to the problem of substituting destroyed cells with healthy and functional insulin-producing cells. Unfortunately, this procedure has been associated with limited clinical success in few centers⁵, mainly due to either the need for life-long immunosuppression of the recipients, with its imminent, related complications, or the restricted availability of cadaveric human donor pancreases. To overcome these hurdles, microcapsules made of highly biocompatible alginic acid (AG)-derived polymers appeared back in the early 1980s. In effect, they would individually envelope the islets destined to graft within an immune-protective shield to circumvent the recipients' general immunosuppression by virtue of preventing any physical contact between donor islets and the host's immune system. This basic principle would obviate both immune rejection and autoimmune recurrence of the disease, while additionally offering the opportunity to use non-human tissue as a resource for donor islets, in the case of human islets shortage⁶. Several studies have clearly reported that AG is particularly "islet-friendly". Furthermore, microcapsules are 3-D extracellular matrix-like microdevices, based on highly purified and almost endotoxin- and protein-free AG, that offer an excellent microenvironment for the embodied islet retention of viability and function. Microcapsules have also been associated with better growth, differentiation and maturation of different cell types, including adult human mesenchymal stem cells, mouse and human embryonic stem cells, neural stem cells, and hepatocytes^{7,8}.

From a physical perspective, microcapsules provide an optimal volume-to-surface area ratio, which promotes effective diffusion of nutrients and oxygen to the encapsulated cells⁹. Nevertheless, some technical issues regarding material biocompatibility and the encapsulated islets' system bio-performance are still pending^{10,11}.

Molecular therapy for type 1 diabetes

A possible alternative to cell therapy by graft of islets or insulin-producing cells could be microencapsulation of cell types, such as human mesenchymal stem cells, releasing immunomodulatory molecules that might interrupt the early type 1 diabetes autoimmune disease process, in order to arrest progression of the β -cells' destruction. Hence, the approach could advantageously apply to early-onset type 1 diabetes, when approximately 30% of the β -cells are still alive/functional, yet damaged, and could be rescued by an immunomodulatory intervention. In fact, insulin-secreting cells might recover under conditions that restrain their destruction^{12,13,14}. In case the disease process is more advanced, a type of human mesenchymal stem cell derived from the umbilical cord could be advantageously co-microencapsulated with islet-derived insulin-secreting progenitor cells, so as to create a biohybrid system incorporating both immunomodulatory and tracer insulin replacement action^{15,16}. Finally, new research frontiers in the field seem to support the idea that AG-based microcapsules might serve as delivery systems of bioactive molecules (i.e., monoclonal antibodies), derived, for instance, from hybridoma cell lines, targeted to

prevent early autoimmune destruction mechanisms of the islet β -cells and, ultimately, the disease onset¹⁷.

AG-based microcapsules

General principles

AG derivatives still represent the most popular materials associated with good biocompatibility and favorable porosity/permeability properties for microencapsulation of live cells. Ad hoc meticulous purification technologies of the raw AG product, originally extracted from brown seaweed, have enabled fulfillment of regulatory criteria for human application. In fact, raw AG is contaminated by high endotoxin levels, pyrogens, proteins and heavy metals that are to be carefully removed from the final preparation. Microcapsules made by ultra-purified or "clinical-grade" alginates, as produced, for instance, by our laboratory, usually do not provoke any inflammatory cell reaction, as extensively proven by our comprehensive *in vivo* graft studies. Since the beginning of our research activities targeting islet cell graft immunoprotection, throughout three decades, we had selected microcapsules basically made of AG complexed with aminoacidic polycations¹⁸.

Basic properties

Chemistry

Since the beginning of the microcapsules' history, many have been the biopolymers potentially eligible and actually proposed for fabrication of microcapsules for cell transplant purposes. The majority of them derived are from either plain or complexed polysaccharides (i.e., alginic acid, chitosan, agarose etc.), polyethylene glycol or acrylates derivatives, just to cite some of those that entered pilot *in vitro* and *in vivo* preclinical trials¹⁹.

Unfortunately, the majority of these polymers, except for a few, did not associate, partially or in full, with the fundamental and indispensable physical-chemical properties necessary for high-performing microcapsules, with special regard to porosity/permeability as well as other features, such as biocompatibility, immunobarrier competence and adequate size, in an attempt to obtain a good transplant product. On the contrary, AGs have represented the mainstay for microcapsules fabrication, either per se, using gelling cations, such as barium, or on complexing the AG-gelled beads (mainly with calcium) with aminoacidic polycations, such as poly-L-lysine or poly-L-ornithine (PLO), the latter originally and uniquely developed by our laboratory¹⁸.

In our system, upon selection of mannuronic (M)-enriched AG, we studied different gelling cations and their combinations, to determine their eventual influence on physical-chemical properties of the microcapsules both *in vitro* and *in vivo*. In particular, we aimed to determine the *in vitro* long-term stability and *in vivo* biocompatibility of microcapsules made of the ultrapure high-M AG thereafter gelled with different divalent cations, namely Ca^{2+} , Ba^{2+} , Sr^{2+} and Mg^{2+20} . The study showed that, regardless of the selected gelling cations, microcapsules' biocompatibility strictly depended on the AG ultra-purification process. In fact, the use of different gelling cations would

possibly affect the basic capsular architecture with no major implications on the wall's strength and bio-elasticity of the final product. Hence, in terms of human application, the use of ultra-purified alginate is mandatory, as there are no capsules that are absolutely better than others²⁰, as long as they have been made of selected AGs.

Nevertheless, many laboratories using AG polymers for microcapsules preparation encountered unsurmountable obstacles in terms of material biocompatibility or immunobarrier competence, which were both lacking, and ultimately resulted in failure of the microencapsulated islet grafts. Using ultra-purified, "clinical grade" AG covalently bound to PLO, we developed a final product that was able to lodge isolated allogeneic or xenogeneic islets, which performed very satisfactorily in pre-clinical graft trials in diabetic animal models and paved the way for initiating pilot clinical trials.

Microcapsules size and immunobarrier competence

As touched on elsewhere, the spherical shape harmonizes the volume with the surface of the microcapsules, thereby favoring diffusion of humoral factors and molecules from the inside of the capsules to the outer environment. Although easier to fabricate, using semi-automated techniques, larger-sized capsules might encounter graft site problems, due to the high islet mass necessary to reverse hyperglycemia in diabetic recipients. In fact, the final encapsulated islet graft volume, for an average individual capsule's equatorial diameter of 500–600 μm (standard size microcapsules), might only fit the large peritoneal cavity. On the contrary, "conformal" microcapsules, made of thin polymer (usually other than AG) films, tightly adhering to individual islets or cell clusters, thereby virtually eliminating any dead space between the membrane and the embodied cells, would occupy a very limited graft volume, and could be eligible for alternative graft sites. In principle, pros and cons affect both types of microcapsules. On the one hand, standard-sized microcapsules offer a 3-D extracellular matrix-like matrix that enhances the survival and function of the embedded islets or other cell clusters, while retaining excellent immuno-isolation properties. The drawback is that the peritoneal graft site, only suitable for these microcapsules, is associated with quite low oxygen tension and limited nutrient supply by passive diffusion, through the capsular membrane, with the risk for loss of viability of the entrapped islets and consequential fibrotic overgrowth of the microcapsules. On the other hand, conformal microcapsules, due to their extremely thin occupied space, certainly are very flexible in terms of graft site, possibly allowing for better lodging of the islet/cell grafts. However, these capsules are devoid of a 3-D biomatrix and still today suffer for restricted long-term chemical endurance; in addition, they likely offer lower immunoprotection with consequential higher grafted cells exposure to the host's immune attack. In summary, while improvements of microcapsules morphology/size are in progress, only standard-size microcapsules have, so far, entered early pilot human clinical trials.

The lack of reproducibility of both preclinical and clinical trials of microencapsulated islet grafts, in terms of functional outcome, among different centers continues to constitute a limitation of the approach. This likely depends on the variable purity grade of the basic AG biopolymers used. Poor biocompatibility means foreign body tissue reaction that results in limited nutrients/oxygen diffusion and ultimately in intracapsular live cell death. Performing composition of the microcapsules' membrane texture means beneficial effects on chemical endurance and the retained membrane's molecular weight cut-off selectivity. Hence, the likelihood of longer-term survival and function of encapsulated islets/cell clusters grafts is much higher when the microcapsules are formulated with clinical grade, ultra-purified AG, due to both a better local micro-environment and superior immunobarrier competence.

Clinical experience

Early pilot clinical trials of AG-microencapsulated islet allografts

Patient with immunosuppression

The first human study of encapsulated islets graft was carried out in 1994 by Soon Shiong *et al.* on a type 1 diabetes patient who already was under general immunosuppression, as he carried a functioning kidney allograft. The patient received an initial intraperitoneal infusion of islets (10,000 islet equivalent [IEQ]/kg) microencapsulated within AG-poly-L-lysine microcapsules followed by a second graft (5,000 IEQ/kg) at 6 months of the first injection. Fair glycemic control was apparently achieved, and the patient remained insulin independent for 9 months post-transplantation, when exogenous insulin supplementation resumed^{21,22}. Although relevant, the fact that the patient already was on general immunosuppression clouded the role of the microcapsules as an effective immune-protective barrier.

In 2013, Jacobs-Tulleneers-Thevissen *et al.*²³ reported on a human graft clinical trial with $\text{Ca}^{2+}/\text{Ba}^{2+}$ AG microbeads containing allogeneic islets. Encapsulated human islets (300,000 IEQ) were injected into the peritoneal cavity of a 61-year-old female type 1 diabetes patient under maintenance immunosuppression for an intraportal islet transplantation procedure carried out 5 years earlier. Plasma C-peptide levels increased above the pretransplant levels in the first 12 weeks (threshold reached within the first week). However, there was no reduction in the exogenous insulin requirements, and the diabetes auto-antibody status remained unchanged, with no induction of cytotoxic antibodies. At laparoscopy, carried out at 3 months post-transplantation, either single or clustered microcapsules, spread throughout the peritoneal cavity, mostly surrounded by fibrous tissue and immune cells, were observed²³.

Patient with no immunosuppression

Almost 12 years earlier, we carried out a pilot clinical graft trial of human islets, enveloped in AG-PLO microcapsules, with recipients with no immunosuppression, at the University of Perugia hospitals and clinics. In that study, four patients, with long-standing type 1 diabetes (mean 25 years) and receiving

intensive exogenous insulin treatment, were grafted intraperitoneally with encapsulated islets ranging 5,000–15,000 IEQ/kg (under local anesthesia and ultrasound guidance, using an indwelling catheter). All the treated patients tested positive for serum C-peptide, which was previously undetectable, a marker of islet graft function, throughout the 3 years of follow up. The study also reported a significant reduction in the exogenous insulin requirement (50–75%) in all the patients for several months post-transplant, with transient insulin independence achieved in only one patient. There was no induction of anti-human leukocyte antigen (HLA) class I or II antibodies, and all the patients tested negative for anti-glutamic acid decarboxylase 65 antibodies, proving immunoprotection capacity. However, for at least 4 through 12 months post-transplant, full insulin dependence ultimately resumed in all patients. Microcapsules retrieved 5 years post-transplant from a patient showing a small opacity under computed tomography scan were found to be intact within a cyst-like formation, although mostly containing no more viable islets, with no adverse effects at any time being observed^{24,25}. So far, this remains the only human pilot clinical trial where the microencapsulated islets grafted into non-immunosuppressed patients retained islet viability/function and immunobarrier competence of the microcapsules for very long periods of time in the absence of the recipient's immunosuppression.

In 2009, Tuch *et al.*²⁶ transplanted four type 1 diabetes patients intraperitoneally with human islets enveloped in barium-AG microcapsules, with no general immunosuppression. Serum C-peptide was detected in the recipients on day 1 post-transplantation, whereas a decline in both blood glucose and insulin requirements was reported. Unfortunately, at 1–4 weeks post-transplantation, C-peptide was undetectable. In one recipient, who received multiple microencapsulated islet infusions, serum C-peptide was detected 6 weeks after the third implant throughout 2.5 years. Laparoscopy examination was associated with microcapsules that were heavily infiltrated with inflammatory cell tissue. This finding possibly reflected either bio-incompatibility of the AG used and/or insufficient immunobarrier competence of the microcapsules. Anti-glutamic acid decarboxylase (but not islet cell antigen 512) antibodies were detected in three recipients. The antibody titer became elevated 4 weeks after the first infusion in two recipients, and it became raised 14 weeks after the fourth infusion in the third recipient. In all these recipients, antibodies continued to remain detectable 1.1–2.5 years after the initial infusion, possibly showing that the microcapsules used had not rendered the embodied islet grafts “bio-invisible”.

Other pilot clinical trials of microencapsulated human islet allografts

Between 2005 and 2006, two companies, Amycte Inc. (Santa Monica, CA, USA) and Novocell Inc. (San Diego, CA, USA) (now ViaCyte Inc., San Diego, CA, USA), planned clinical trials with encapsulated islets in type 1 diabetes patients. Amycte

undertook microencapsulation of human islets in AG-poly-L-lysine, thereby embedded into a macro-device before implantation into 12 type 1 diabetes patients. Novocell Inc. started a phase I/II clinical trial, using polyethylene glycol (PEG) encapsulated islets that were grafted in 12 patients subcutaneously. However, the study was terminated by the company, as only minor efficacy was observed in the first two patients. Currently, there is not much information on the outcome of these clinical trials²⁷.

Pilot clinical trials of microencapsulated porcine islet xenografts

Transplantation of microencapsulated porcine islets in type 1 diabetes patients was initiated in 2007 by Living Cell Technologies of Auckland, New Zealand. This company carried out a larger clinical study using special “specific pathogen-free” pig islets within AG-PLO microcapsules (product name Diabecell), where eight patients received varying islet doses (5,000–10,000 IEQ/kg bodyweight). Six patients showed reduced exogenous insulin requirements throughout 8 months post-transplant, thereby demonstrating the potential use of this technology as a safe, effective and possibly alternative approach for cell therapy of type 1 diabetes²⁸. In particular, Matsumoto *et al.*²⁹ reported on eight patients in 2016; four received two doses of 5,000 IEQ/kg porcine islets, and four received two doses of 10,000 IEQ/kg porcine islets. No immunosuppression was administered, while the reduction in either daily exogenous insulin dosage, glycosylated hemoglobin levels or hypoglycemic episodes was communicated. Nevertheless, the reliability and impact of that study appeared quite limited, due to insufficiency of the data shown.

New strategies for cell and molecular therapy for type 1 diabetes

Cells

Islet transplantation, despite all restrictions that prevented diffusion of this approach, at least proved a principle; namely, the possibility to reverse hyperglycemia in type 1 diabetes patients by cell therapy. However, the limited availability of donor human islets, only slightly mitigated by the still distant possibility of using pig islets, is pushing research to validate new strategies to generate insulin-producing cells, starting with stem and progenitor cells. Several study protocols have been, and still are, in development to systematically promote the differentiation of human embryonic stem cells, and more recently, induced pluripotent stem cells, into pancreatic endoderm. The latter contains cells have been shown to mature *in vivo*, and to function similarly to β -cells for prolonged periods of time upon graft. In other cases, the cells are transplanted upon full differentiation into β -like cells *in vitro*. The results with these methods are encouraging, and recent efforts are directed toward improving the differentiation conditions, the expansion of the cells at specific progenitor stages and the purification of target cell populations to obtain sufficient quantities of functional pancreatic β -like, insulin-producing cells^{30,31}.

New AG formulations and blends for microencapsulation

In an attempt to reduce the induction of peri-capsular fibrosis, upon graft, new AG formulations are being developed (i.e., Z1-Y15, Z1-Y19, Z2-Y12-amine), keeping barium as a gelling divalent cation of basic AG (sodium salt; Table 1). In a preclinical study, glucose-responsive human embryonic stem cells-derived mature β -cells were encapsulated in modified AG hydrogels^{32,33}. The size of the resulting microcapsules was quite large (close to 1 mm), and the only affordable graft site in recipient immunocompetent C57 mice was the peritoneal cavity. Additionally, as an alternative graft site, an omental pouch was created in primates. Although initially encouraging, in terms of reduced fibrotic reaction to control empty microcapsules grafts, encapsulated islet allografts were associated with minor changes of blood glucose of the treated animals, which limited the progress of this approach.

To avoid peri-capsular fibrotic overgrowth of the grafted microcapsules, often causing intracapsular islet cell death and graft failure, an immunomodulatory chemokine, C-X-C motif chemokine 12 (CXCL12), was incorporated into purified sodium alginate, with the aim to microencapsulate stem cell-derived (SC)- β cells (Table 1)^{34,35,36}. The addition of CXCL12 apparently enhanced glucose-stimulated insulin secretory patterns of the microencapsulated SC- β cells, and induced expression of genes associated with β -cell function *in vitro*. SC- β cells in AG-CXCL12 microcapsules were associated with satisfactory insulin secretion in diabetic mice and accelerated normalization of hyperglycemia. Additionally, SC- β cells enveloped in AG-CXCL12 microcapsules evaded the pericapsular fibrotic response, resulting in long-term functional competence and persistent glycemic correction (>150 days) in the absence of systemic immunosuppression in immunocompetent C57BL/6 mice. Application of these results into larger-size mammals is awaited.

SC- β cells upon coating with PEG and derivatives plus AG showed unaltered viability and insulin secretory capacity.

Fabrication of hybrid PEG-ALG interpenetrating polymer networks was used to prepare microcapsules for the study of physical-chemical properties, such as swelling, surface modulus, rheology, compression and permeability. The hybrid networks proved to be resistant to bulk swelling and compressive deformation, with enhanced flexibility and long-term resilience of the membranes. Cell aggregates, upon polymer coating, were grafted in the epididymal fat pad of immuno-incompetent non-obese diabetic/severe combined immunodeficiency (NOD/scid) mice with no available further developments/communication³⁷.

Recently, coating the surface of AG-based microcapsules with zwitterionic block copolymers significantly reduced post-transplant fibrosis, and improved graft survival in a xenotransplantation graft setting. A group of zwitterionic, sulfobetaine and carboxybetaine modifications of AGs reproducibly mitigated fibrotic overgrowth of the implanted alginate microcapsules in mice, dogs and pigs (Table 1). Using these modified AGs, an improved outcome of encapsulated islet grafts in chemically-induced diabetic mouse and dog models apparently emerged. These zwitterion-modified AGs might contribute to the development of cell encapsulation therapies for type 1 diabetes and other hormone-deficient diseases. This approach might be of interest, although it confirms that AG is possibly irreplaceable as a basic matrix for preparing microcapsules, whatever the nature of the outer coating might be³⁸.

New types of coating biopolymers for microencapsulation

Chemically cross-linked hydrogel capsules and cell coatings based on human elastin-like recombinamers (ELRs) are under development. ELRs consist of the repeating sequence of elastin-like polypeptide (VPGVG) found in mammalian elastin, in an attempt to mimic biology and physical chemistry of the normal extracellular matrix^{39,40}. These sequence patterns have a proven biocompatible profile and comply with thermo-responsiveness and elasticity expected for this kind of polymer. The presence of a high concentration of lysine is intended to provide the

Table 1 | Recent experimental trials of microencapsulated islet cell grafts with different alginate formulations

Authors	Type of encapsulation	Type of alginate	Cell source	Implantation site	Immunosuppression
Vegas <i>et al.</i> (2016) ³³	Microencapsulation	Triazole-thiomorpholine dioxide alginate	SC- β	Intraperitoneal space	No
Bochenek <i>et al.</i> (2018) ³⁴	Microencapsulation	Modified alginate derivatives (Z1-Y19-Ba ²⁺ , Z2-Y12-Ba ²⁺ , Z1-Y15-Ba ²⁺) and a plain alginate (SLG20-Ba ²⁺)	Allogeneic pancreatic islet cells	Omental bursa of macaques	No
Alagpulinsa <i>et al.</i> (2019) ³⁵	Microencapsulation	Sodium alginate with CXCL12	SC- β cells	Peritoneal cavity	No
Sremac <i>et al.</i> (2019) ³⁶	Microencapsulation	High-mannuronic acid (LVM) alginate with or without recombinant human CXCL12	Allogeneic and x enogeneic islets	Intraperitoneal space	No
Liu <i>et al.</i> (2019) ³⁸	Microencapsulation	Zwitterionically modified alginates	Xenogeneic islets	Peritoneal cavity	No

CXCL12, C-X-C motif chemokine 12; SC- β , stem cell-derived β -cells.

polymer with reacting radicals that are necessary for the generation of the desired hydrogels by chemical crosslinking by bearing the reacting groups, such as azide and cyclooctyne. The hydrogel microcapsules generated by this technology have been assessed for their properties and function toward a possible application for cell therapy, especially as far as permselectivity, immunogenicity, bioactivity, encapsulation capacity, dynamic and static biomechanics were concerned. As an initial experimental application, ELR coating of human-induced pluripotent stem cell (hiPSC) spheroids, at the transmission electron microscopy examination, was clearly detectable, and it did not alter the viability of the encapsulated subcellular organelles and hormone granules, associated with the hiPSC spheroids. *In vitro* metabolic data showed insulin secretory patterns and content, consistent with the presence of differentiated β -like cells. Coated spheroids survival was preliminarily assessed *in vivo*, upon intraperitoneal graft into immune-incompetent NOD/scid mice and immunocompetent CD-1 mice. The cell/tissue reaction to graft was examined upon peritoneal lavage, and flow cytometry analysis of spleen and lymph nodes cell phenotypes. Two weeks post-transplant, peritoneal lavage in NOD/scid mice was associated with 90% viability retention of the retrieved coated versus control uncoated spheroids. At the same post-transplant time period, and using the same procedure, CD-1 mice showed viability that was higher for the coated versus control uncoated spheroids. The peritoneal cellular response to graft was incomparably lower for coated versus uncoated hiPSC spheroids⁴¹.

Other encapsulated stem cell types

Post-partum Wharton's jelly-derived human adult mesenchymal stem cells (hUCMS) might differentiate into several cell lineage phenotypes, both *in vitro* and *in vivo*. Recently, we have obtained preliminary evidence that microencapsulated hUCMS positively conditioned the immune system by both reducing pathogenic T-cell subsets and potentiating their regulatory counterparts. *In vitro*, overnight pretreatment of hUCMS with the pro-inflammatory cytokine, interferon- γ , induced expression of indoleamine 2,3-dioxygenase 1, a molecule involved in the tryptophan catabolism, and led to an increase in HLA-G5 expression. Both these molecules play an important role in regulating a number of immunoregulatory pathways. Furthermore, hUCMS are immune-privileged, due to both the lack of HLA class II antigens and their intrinsic immunomodulatory properties. These appear predominantly related to the production of humoral factors. hUCMS also express the following three classes of HLA: HLA-E, HLA-F and HLA-G. These molecules are involved in the tolerogenic process occurring at the fetal-maternal interface. In particular, it has been recently described that HLA-G, released from human mesenchymal stem cells, might promote the expansion of Treg populations. We proved that microencapsulated hUCMS transplanted in NOD mice with recent-onset diabetes restored normoglycemia that persisted for the long term¹⁵. This outcome was ascribed to

hUCMS-related immunomodulatory action on regulatory T-cell subsets (Tregs), as well as to hUCMS paracrine effects on islet cells, resulting in preservation of the mouse endocrine pancreatic morphology. We provided evidence that microencapsulated hUCMS grafts might successfully manage diabetes in a strong animal model of spontaneous type 1 diabetes, such as the NOD mouse. These cells, within microcapsules, comply with safety, efficacy and stability requirements. In fact, as far as safety is concerned, they did not transform nor did they require any host's immunosuppression. As for efficacy, hUCMS induced a stable reversal of hyperglycemia in the NODs with recent-onset diabetes. Finally, in terms of durability, microencapsulated hUCMS grafts were associated with normal metabolic control in the treated animals for extraordinarily long periods of time (216 days post-transplant). In light of these pre-clinical results, it might be possible to speculate on the translation of the obtained data into a phase I pilot clinical trial in patients with recent-onset type 1 diabetes.

Microcapsules for drug delivery system for prevention of type 1 diabetes

Induction of an acquired state of immune tolerance in patients with type 1 diabetes could prevent the autoimmune destruction of pancreatic islet β -cells. In our laboratory, the present authors studied whether the G3C hybridoma cell line-derived monoclonal antibodies, triggering the glucocorticoid-induced tumor necrosis factor receptor-related (Gitr) costimulatory receptor, would promote the expansion of Tregs upon graft in SV129 (wild-type) and diabetic-prone NOD mice. The delivery of the G3c monoclonal antibodies required the envelopment of these hybridoma cells in specially formulated alginate-based microcapsules (G3C/cps). The microcapsules were specially engineered to allow the selective outflow of immunoglobulin M that usually is prevented from entering/exiting the microcapsular barrier (Figure 1)^{42,43}. Treatment for 3 weeks induced Foxp3⁺ Treg-cell expansion in the spleen of wild-type mice, but not in Gitr^{-/-} mice. G3C/cps also induced the expansion of non-conventional Cd4⁺Cd25^{-/low}Foxp3^{low}Gitr^{int/high} (Gitr single-positive) Tregs. Both Cd4⁺Cd25⁺Gitr^{high}Foxp3⁺ and Gitr-sp Tregs (also including antigen-specific cells) were expanded in the spleen and pancreas of G3C/cps-treated NOD mice, and the number of intact islets was higher in G3C/cps-treated than in empty cps-treated and untreated animals. Consequently, all but two G3C/cps-treated mice did not develop diabetes and all but one survived until the end of the 24-week study. In summary, we observed that long-term Gitr triggering induced Treg expansion, thereby delaying/preventing diabetes development in NOD mice. This therapeutic approach might have promising clinical potential for the treatment of inflammatory and autoimmune diseases¹⁷.

SUMMARY AND FUTURE TRENDS

Despite the fact that three full decades of work with microencapsulation have elapsed, no substantial application of this

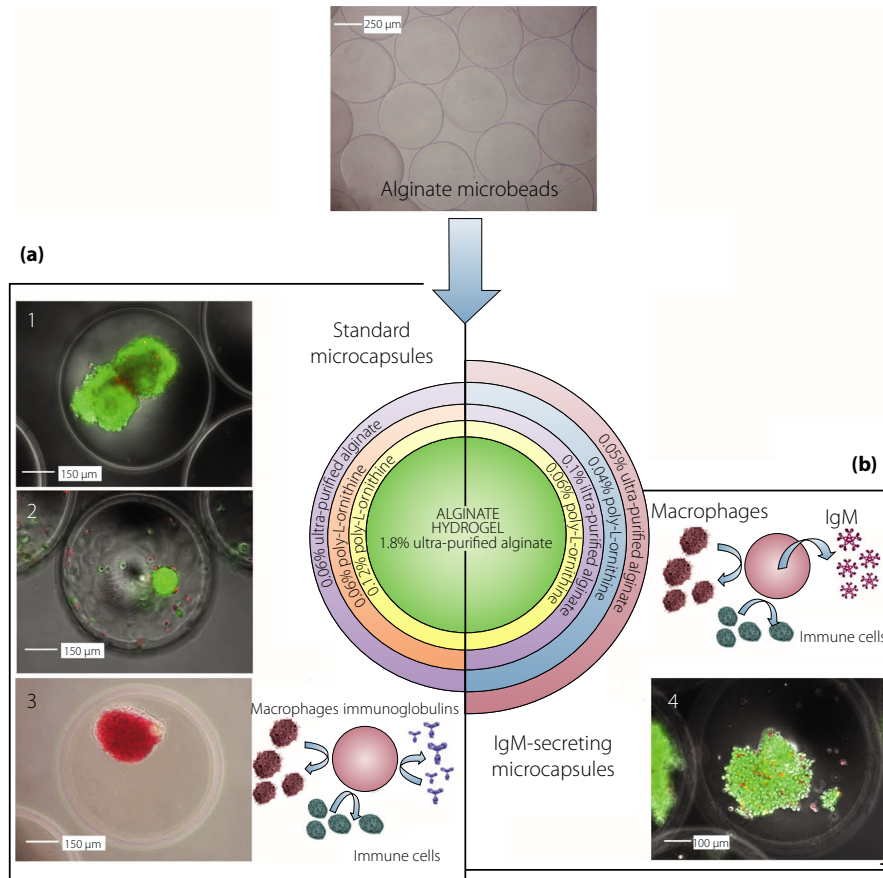


Figure 1 | Versatility of alginic acid-based standard size microcapsules. (a) Standard-sized microcapsules. Typical immunobarrier competence with retention of embodied various cell types cells viability, upon staining with ethidium bromide and fluorescein diacetate (1,2) or diphenylthiocarbazone (3): (1) differentiated human-induced pluripotent stem cells; (2) human adult mesenchymal stem cells aggregates; and (3) human islets. (b) Differently permeable (immunoglobulin M [IgM]-secreting) microcapsules. The modified membrane's properties allow for immunoglobulin outflow (in this instance IgM released from the G3C hybridoma cell line), but prevent access to immune cells; (4) G3C hybridoma cells, very viable upon staining with ethidium bromide and fluorescein diacetate.

technology to islet/insulin-producing cells grafts in humans has been successful yet. Nevertheless, a great deal of technical advances have been accomplished toward the goal of providing islets/cells grafts with stable and performing polymer coating to prevent the recipients' general immunosuppression. In particular, waiting for next-generation polymers, the invariable need for AG use, as a basic constituent polymer for microencapsulation, has clearly emerged. Of note, the advanced purification process of the raw AG, coupled with new technologies for engineering the basic AG polymers might offer avenues to implement the basic microencapsulation product. Furthermore, new research pathways are underway for validating non-AG molecules (i.e., ELR etc.) for cell coating, within conformal microcapsules. Although the controversies about whether larger or smaller microcapsules could better fulfill the chances for efficient cell grafts immunoprotection are unsolved, the real critical issues that should be focused on are: (i) the efficiency of the immunobarrier competence; and (ii) the site of implant. Both depend on the final microcapsules size; the likely advantage for

better immunoprotection afforded by standard size microcapsules is clouded by the limited repertoire of available graft sites. In contrast, conformal microcapsules fit quite a wide array of potential graft sites, in front of inferior immunoprotection capacity, as well as questionable long-term endurance. Certainly, both standard and conformal microcapsules require adequate oxygen/nutrients supply. The idea of bedding the microcapsules within microdevices with the provision of direct oxygenation or by promoting neovessels formation is quite difficult to apply. The less invasive site, in this regard, could be the creation of an omental pouch that couples short distance from the liver (the first site of insulin action) with appropriate vascularization. However, this option might also expose the risk of eliciting an intense inflammatory response, regardless of basic polymers purity and advanced formulations. The limited diffusion of microencapsulated cell clinical trials may be ascribed to either chemical composition and physical configuration of the microcarriers or nature, viability and function of the embodied cells. Whether these might be islets or insulin-

producing cells (i.e., differentiated stem cells and iPSC), the idea of altering the cellular graft composition is very challenging and appealing. For instance, we observed that microencapsulated hUCMS, as a result of their powerful immunoregulatory potential, due to humoral factors outflowing from the capsular membranes, might interrupt the early disease process, thereby assisting in the rescue of the remaining still healthy β -cells. This simple effect might spare early initiation of exogenous insulin supplementation. However, in the case of a more advanced stage of islet β -cells destruction, insulin, even at trace concentrations, as those afforded by progenitor endocrine cells, might synergistically help arrest the disease process, in conjunction with hUCMS-linked immunomodulation. Hence, a biohybrid composite microencapsulated cellular graft, comprised of progenitor or stem-derived β -like cells, assuring an even minimal insulin delivery, coupled with mesenchymal stem cells with their immunoregulatory potential, might represent an advanced prototype for microencapsulated cell-based therapy for type 1 diabetes.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

- Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet* 2014; 383: 69–82.
- Noble JA. Immunogenetics of type 1 diabetes: a comprehensive review. *J Autoimmun* 2015; 64: 101–112.
- Babon JAB, DeNicola ME, Blodgett DM, *et al.* Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. *Nat Med* 2016; 22: 1482–1487.
- Ziegler AG, Rewers M, Simell O, *et al.* Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA* 2013; 309: 2473–2479.
- Shapiro AM, Ricordi C, Hering BJ, *et al.* International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 2006; 355: 1318–1330.
- Sun Y, Ma X, Zhou D, *et al.* Normalization of diabetes in spontaneously diabetic cynomolgus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J Clin Invest* 1996; 98: 1417–1422.
- Qi M, Strand BL, Morch Y, *et al.* Encapsulation of human islets in novel inhomogeneous alginate $\text{Ca}^{2+}/\text{Ba}^{2+}$ microbeads: in vitro and in vivo function. *Artif Cells Blood Substit Immobil Biotechnol* 2008; 36: 403–420.
- Montanucci P, Basta G, Calafiore R. In vitro-cultured human islet cell monolayers: stemness markers and insulin recovery upon streptozotocin exposure. *Tissue Eng Part A* 2009; 15: 3931–3942.
- Montanucci P, Pennoni I, Pescara T, *et al.* The functional performance of microencapsulated human pancreatic islet-derived precursor cells. *Biomaterials* 2011; 32: 9254–9262.
- Papas KK, Long RC, Sambanis A, *et al.* Development of a bioartificial pancreas: II. Effects of oxygen on long-term entrapped beta TC3 cell cultures. *Biotechnol Bioeng* 1999; 66: 231–237.
- Safley SA, Kenyon NS, Berman DM, *et al.* Microencapsulated adult porcine islets transplanted intraperitoneally in streptozotocin diabetic non-human primates. *Xenotransplantation* 2018; 25: e12450.
- Sherry NA, Chen W, Kushner JA, *et al.* Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of β -cells. *Endocrinology* 2007; 148: 5136–5144.
- Chatenoud L, Thervet E, Primo J, *et al.* Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci USA* 1994; 91: 123–127.
- Kroger CJ, Clark M, Ke Q, *et al.* Therapies to suppress β cell autoimmunity in type 1 diabetes. *Front Immunol* 2018; 9: 1891.
- Montanucci P, Pescara T, Alunno A, *et al.* Remission of hyperglycemia in spontaneously diabetic NOD mice upon transplant of microencapsulated human umbilical cord Wharton jelly-derived mesenchymal stem cells (hUCMS). *Xenotransplantation* 2019; 26: e1247.
- Vaithilingam V, Evans MDM, Lewy DM, *et al.* Co-encapsulation and co-transplantation of mesenchymal stem cells reduces pericapsular fibrosis and improves encapsulated islet survival and function when allografted. *Sci Rep* 2017; 7: 10059.
- Cari L, Montanucci P, Basta G, *et al.* Microencapsulated G3C hybridoma cell graft delays the onset of spontaneous diabetes in NOD mice by an expansion of Gitr+ Treg cells. *Diabetes* 2020; 69: 965–980.
- Calafiore R, Koh N, Civantos F, *et al.* Xenotransplantation of microencapsulated canine islets in diabetic mice. *Trans Assoc Am Physicians* 1986; 99: 28–33.
- Calafiore R, Basta G. Clinical application of microencapsulated islets: actual prospective on progress and challenges. *Adv Drug Deliv Rev* 2014; 67–68: 84–92.
- Montanucci P, Terenzi S, Santi C, *et al.* Insights in behavior of variably formulated alginate-based microcapsules for cell transplantation. *Biomed Res Int* 2015; 2015: 965804.
- A process for the ultrapurification of alginates, WIPO (PCT) WO2009093184A1.
- Soon-Shiong P, Heintz RE, Merideth N, *et al.* Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. *Lancet* 1994; 343: 950–951.
- Jacobs-Tulleneers-Thevissen D, Chintinne M, Ling Z, *et al.* Sustained function of alginate encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient. *Diabetologia* 2013; 56: 1605–1614.

24. Calafiore R, Basta G, Luca G, *et al.* Microencapsulated pancreatic islet allografts into non immunosuppressed patients with type 1 diabetes. *Diabetes Care* 2006; 29: 137–138.
25. Basta G, Montanucci P, Luca G, *et al.* Long-term metabolic and immunological follow-up of non-immunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: four cases. *Diabetes Care* 2011; 34: 2406–2409.
26. Tuch BE, Keogh GW, Williams LJ, *et al.* Safety and viability of microencapsulated human islets transplanted into diabetic humans. *Diabetes Care* 2009; 32: 1887–1889.
27. Vaithilingam V, Bal S, Tuch BE. Encapsulated islet transplantation: where do we stand. *Rev Diabet Stud* 2017; 14: 51–78.
28. Elliott RB, Escobar L, Tan PL, *et al.* Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. *Xenotransplantation* 2007; 14: 157–161.
29. Matsumoto S, Abalovich A, Wechsler C, *et al.* Clinical benefit of islet xenotransplantation for the treatment of type 1 diabetes. *EBioMedicine* 2016; 12: 255–262.
30. Agulnick AD, Ambruzs DM, Moorman MA, *et al.* Insulin-producing endocrine cells differentiated in vitro from human embryonic stem cells function in macroencapsulation devices in vivo. *Stem Cells Transl Med* 2015; 4: 1214–1222.
31. Farina M, Alexander JF, Thekkedath U, *et al.* Cell encapsulation: overcoming barriers in cell transplantation in diabetes and beyond. *Adv Drug Deliv Rev* 2019; 139: 92–115.
32. Veisoh O, Doloff JC, Ma M, *et al.* Size- and shape-dependent foreign body immune response to materials implanted in rodents and non-human primates. *Nat Mater* 2015; 14: 643–651.
33. Vegas AJ, Veisoh O, Gürtler M, *et al.* Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immunocompetent mice. *Nat Med* 2016; 22: 306–311.
34. Bochenek MA, Veisoh O, Vegas AJ, *et al.* Alginate encapsulation as long-term immune protection of allogeneic pancreatic islet cells transplanted into the omental bursa of macaques. *Nat Biomed Eng* 2018; 2: 810–821.
35. Alagpulinsa DA, Cao JJJ, Driscoll RK, *et al.* Alginate-microencapsulation of human stem cell derived β cells with CXCL12 prolongs their survival and function in immunocompetent mice without systemic immunosuppression. *Am J Transplant* 2019; 19: 1930–1940.
36. Sremac M, Lei J, Penson MFE, *et al.* Preliminary studies of the impact of CXCL12 on the foreign body reaction to pancreatic islets microencapsulated in alginate in nonhuman primates. *Transplant Direct* 2019; 5: e447.
37. Stock AA, Manzoli V, De Toni T, *et al.* Conformal coating of stem cell-derived islets for β cell replacement in type 1 diabetes. *Stem Cell Rep* 2020; 14: 91–104.
38. Liu Q, Chiu A, Wang LH, *et al.* Zwitterionically modified alginates mitigate cellular overgrowth for cell encapsulation. *Nat Commun* 2019; 10: 5262.
39. Castellanos MI, Zenses A-S, Grau A, *et al.* Biofunctionalization of REDV elastin-like recombinamers improves endothelialization on CoCr alloy surfaces for cardiovascular applications. *Colloids Surf B Biointerfaces* 2015; 127: 22–32.
40. Rodríguez-Cabello JC, González de Torre I, Ibañez-Fonseca A, *et al.* Bioactive scaffolds based on elastin-like materials for wound healing. *Adv Drug Deliv Rev* 2018; 129: 118–133.
41. Basta G, Montanucci P, Pescara T, *et al.* Microencapsulated human induced pluripotent stem cells (hiPSC) for the cell and molecular therapy of type 1 diabetes mellitus (T1D): preliminary in vitro and in vivo data, TERMIS EU 2019 Rhodes, Greece 27–31 May 2019, 1277.
42. Montanucci P, Cari L, Basta G, *et al.* Engineered alginate microcapsules for molecular therapy through biologic secreting cells. *Tissue Eng Part C Methods* 2019; 25: 296–304.
43. US APPLICATION 2018 n. 62/768.249 “Alginate microcapsules for cell and molecular therapy that secrete bioactive immune molecules”.