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REVIEW

MICROENCAPSULATION FOR THE CELL AND MOLECULAR THERAPY OF TYPE 1 DIABETES MELLITUS: ACTUAL STATE AND FUTURE PERSPECTIVES BETWEEN PROMISE AND PROGRESS

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Abstract

The history of microencapsulation of live cells starts from an old idea of Thomas M. S. 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 not immunosuppressed recipients, at pre-clinical (rodents), and subsequently clinical level. Among the different chemistries potentially suitable for microencapsulation of live cells, alginic acid (AG)-based polymers, originally proposed by Sun, proved to be superior to all others, in the following decades. In fact, only AG-based microcapsules, containing allogeneic islets, ultimately entered pilot human clinical trials in patients with type 1 diabetes mellitus, since immuno-selectiveness and biocompatibility of AG-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 use of novel polymeric formulations, under actual scrutiny. Use of embryonic and adult stem cells, within microcapsules, should address the restricted availability of cadaveric human donor-derived islets, while a new generation of newly-engineered microcapsules could better fulfil issues with graft site and long-term retention of biopolymer properties.

Keywords: Alginate, Microcapsules, T1D

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease due to selective killing of pancreatic islet β-cells, resulting in abolishment of the endogenous insulin secretion. In particular, the inflammatory process and immune response are key factors in the development of the disease (1): 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 T1D may only be treated by "life-saving" exogenous insulin supplementation (4). While actually indispensable for granting T1D patients' survival, exogenous insulin does not represent a cure for this metabolic disorder, and it may 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.

1.1 Mapping the field: role of alginate-based microencapsulation in cell therapy for T1D

1.1.1 Cell therapy by pancreatic islet transplantation for T1D

In an attempt to find a cure for T1D, intrahepatic grafts of healthy pancreatic islets, retrieved from cadaveric donor organs, into totally immunosuppressed T1D patients, initially seemed to represent the most elegant and effective solution to the problem of substituting destroyed, with healthy and functional insulin-producing cells. Unfortunately, this procedure has been associated with limited clinical success in few Centers (5), mainly due to either 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 80s'. In effects, 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 case of human islets shortage (6). Several studies have clearly reported that AG is particularly "islet-friendly". Moreover, microcapsules are three-dimensional extracellular matrix (ECM)-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 (hMSC), mouse and human embryonic stem cells (mESCs, hESCs), neural stem cells and hepatocytes (7,8).

On a physical perspective, microcapsules provide an optimal volume-to-surface area ratio, which promotes effective diffusion of nutrients and oxygen to the encapsulated cells (9). Nevertheless, some technical issues regarding material biocompatibility and the encapsulated islets system bio-performance, are still pending (10,11).

1.1.2 Molecular therapy for T1D

A possible alternative to cell therapy by graft of islets or insulin-producing cells, could be microencapsulation of cell types, like hMSC, releasing immunomodulatory molecules that may interrupt the early T1D autoimmune disease process, in order to arrest progression of the β-cells

destruction. Hence, the approach could advantageously apply to early-onset T1D, 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 may recover under conditions that restrain their destruction (12,13,14). In case the disease process is more advanced, a type of hMSC derived from umbilical cord (hUMSC) 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 may serve for delivery systems of bioactive molecules (ie, monoclonal antibodies), deriving for instance from hybridoma cell lines, targeted to prevent early autoimmune destruction mechanisms of the islet β-cells, and ultimately, the disease onset (17).

1.2 Ag-based Microcapsules

1.2.1 General Principles

AG derivatives still represent the most popular materials associated with good biocompatibility and favourable porosity/permeability properties, for microencapsulation of live cells. *Ad hoc* meticulous purification technologies of the raw AG product, originally extracted from brown seaweeds, have enabled fulfilment 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 targeted to islet cell graft immuno-protection, throughout three decades, we had selected microcapsules basically made of AG complexed with aminoacidic polycations (18).

1.2.2 Basic properties

a) Chemistries

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 from either plain or complexed polysaccharides (ie, alginic acid, chitosan, agarose etc.) or polyethylene glycol or acrylates derivatives, just to cite some of those that entered pilot in vitro and in vivo pre-clinical trials (19).

Unfortunately, the majority of these polymers, except for few, did not associate, partially or in full, with 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, immune-barrier 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 like Barium, or upon complexing the AG-gelled beads (mainly with Calcium) with aminoacidic poly-cations, like poly-L-lysine (PLL) or poly-L-ornithine (PLO), the latter originally and uniquely developed by our laboratory (18).

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+}, 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, use of different gelling cations would possibly affect the basic capsular architecture with no major implications on wall's strength and bio-elasticity of the final product. Hence, in term of human application, use of ultra-purified alginate is mandatory since there are no capsules that are in absolute better than others (20), 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, that 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, able to lodge isolated either allogeneic or xenogeneic islets, that performed very satisfactorily in pre-clinical graft trials in diabetic animal models and paved the way for initiating pilot clinical trials.

b) Microcapsules size and immunobarrier competence

As touched elsewhere, the spherical shape harmonizes volume with surface of the microcapsules, thereby favoring diffusion of humoral factors and molecules from the inside of the capsules to the outer environment. While easier to fabricate, using semi-automated techniques, larger-size capsules may encounter graft site problems, due to the high islet mass necessary to reverse hyperglycemia in the diabetic recipients. In fact, the final encapsulated islet graft volume, for an

average individual capsule's equatorial diameter of 500-600μm (standard size microcapsules), virtually may 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 membrane and the embodied cells, would occupy a very limited graft volume, and could be eligible for alternative graft sites. In principles, pro's and con's affect both types of microcapsules. On one hand, standard-size microcapsules offer a 3D ECM-like matrix that enhance 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 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 that are devoid of a 3D biomatrix, and still today suffer for restricted long-term chemical endurance: in addition, they likely offer lower immune-protection 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 pre-clinical and clinical trials of microencapsulated islet grafts, in terms of functional outcome, among different Centers, continues to constitute a limit of the approach. This likely depends upon variable purity grade of the employed basic AG biopolymers. Poor biocompatibility means foreign body tissue reaction that results in limited nutrients/oxygen diffusion and ultimately in intracapsular live cells death. Performing composition of the microcapsules' membrane texture, means beneficial effects on chemical endurance and retained membrane's molecular weight cut-off selectivity. Hence, 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 better local micro-environment, and superior immuno-barrier competence.

1.3 Clinical Experience

1.3.1 Early pilot clinical trials of AG-microencapsulated islet allografts

a) with patient's immunosuppression

The first human study of encapsulated islets graft was carried out in 1994 by Soon Shiong *et al*. on a T1D 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 IEQ/kg) microencapsulated within AG-poly-L-lysine microcapsules followed by a second graft (5000 IEQ/kg) at 6 months of the first injection. Fair glycemic control apparently was 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 role of the microcapsules as an effective immune-protective barrier.

In 2013, Jacobs-Tulleneers-Thevissen *et al*. reported on a human graft clinical trial with Ca^{2+}/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 T1D patient under maintenance immunosuppression for an intraportal islet transplantation procedure performed 5 years earlier. Plasma C-peptide levels increased above the pre-transplant levels in the first 12 weeks, (threshold reached within the first week). However, there was no reduction in the exogenous insulin requirements and 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 observed (23).

b) with no patient's immunosuppression

Almost 12 years earlier, we carried out a pilot clinical graft trial of human islets, enveloped in AG-PLO microcapsules, with no recipient's immunosuppression, at the University of Perugia Hospitals and Clinics. In this study, four patients, with long-standing T1D (average 25 years) and on intensive exogenous insulin treatment, were grafted intraperitoneally with encapsulated islets ranging on 5000-15,000 IEQ/kg (under local anaesthesia and ultrasound guidance, using an indwelling catheter). All the treated patients tested positive for serum C-peptide, previously undetectable, a marker of islet graft function, throughout 3 years of follow-up. The study also reported significant reduction in the exogenous insulin requirement (50-75%) in all the patients, for several months post-transplant, with transient insulin independence been achieved only in one patient. There was no induction of anti-HLA class I or II antibodies and all the patients tested negative for anti-GAD65 antibodies, proving immune-protection capacity. However, at months post-transplant, full insulin dependence ultimately resumed in all patients. Microcapsules retrieved

5-year post-transplant from a patient showing a small opacity under CT scan, the microcapsules were found 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 immune-barrier competence of the microcapsules for very long periods of time, in absence of recipient's immunosuppression.

In 2009, Tuch et al (26) transplanted four T1D 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, while decline in both blood glucose and insulin requirements was reported. Unfortunately, at 1–4 weeks post-transplantation, C-peptide was undetectable. Only 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 employed AG and/or insufficient immune-barrier competence of the microcapsules. Anti-GAD (but not ICA512) antibodies were detected in three recipients. The antibody titter became elevated 4 weeks after the first infusion in two recipients, and it 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 indicating that the employed microcapsules had not rendered the embodied islet grafts "bio-invisible".

1.3.2 Other pilot clinical trials of microencapsulated human islet allografts

Between 2005 and 2006, two companies, Amycte Inc. and Novocell Inc. (now ViaCyte, Inc.), planned clinical trials with encapsulated islets in type 1 diabetic patients. Amycte, undertook microencapsulation of human islets in AG-poly-L-lysine, thereby embedded into a macro-device before implantation into twelve T1D patients. Novocell Inc. started a phase1/2 clinical trial, employing PEG encapsulated islets that were grafted in twelve patients subcutaneously. However, the study was terminated by the Company since only minor efficacy was observed in the first two cases. Currently, there is not much information on the outcome of these clinical trials (27).

1.3.3 Pilot clinical trials of microencapsulated porcine islet xenografts

Transplantation of microencapsulated porcine islets in T1D patients was initiated in 2007 by Living Cell Technologies (LCT) of Auckland NZ. This Company performed a larger clinical study using special "Specific Pathogen-Free" (SPF) pig islets within AG-PLO microcapsules (product

name: “Diabecell”), where eight patients received varying islet doses (5000–10,000 IEQ/kg body weight). Six patients showed reduced exogenous insulin requirements throughout eight months post-transplant, thereby demonstrating the potential use of this technology as a safe, effective and possibly alternative approach for cell therapy of T1D (28). In particular, Matsumoto et al. reported in 2016 (29) on 8 patients, 4 receiving 2 doses of 5000 porcine IEQ/kg. and 4 receiving 2 doses of 10,000 IEQ/kg. No immunosuppression was administered, while reduction in either daily exogenous insulin dosage, or HbA1c levels, or hypoglycemic episodes were communicated. Nevertheless, reliability and impact of this study look quite limited, due to insufficiency of the exhibited data.

1.4 New strategies for cell and molecular therapy of T1D

1.4.1 Cells

Islet transplantation, in spite of all restrictions that, in fact, prevented diffusion of this approach, at least proved a principle, namely the possibility to reverse hyperglycaemia in T1D by cell therapy. However, the limited availability of donor human islets, only slightly mitigated by the still far possibility to employ pig islets, is pushing research to validate new strategies to generate insulin-producing cells, starting from stem and progenitor cells. Several study protocols have been, and are on development to systematically promote the differentiation of human embryonic stem cells (hESC), and more recently, induced pluripotent stem cells (iPSC), 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 towards 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).

1.4.2 New AG formulations and blends for microencapsulation

In an attempt to reduce induction of peri-capsular fibrosis, upon graft, new AG formulations are being developed (ie, Z1-Y15, Z1-Y19, Z2-Y12- amine) keeping on Ba as a gelling divalent cation of basic AG (sodium salt) (Table 1). In a preclinical study, glucose-responsive hESC-derived mature β-cells were encapsulated in modified AG hydrogels (32,33). Size of the resulting microcapsules was quite large (near 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. While 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 progress of this approach.

To avoid peri-capsular fibrotic overgrowth of the grafted microcapsules, often causing intra-capsular islet-cell death and graft failure, an immunomodulatory chemokine, CXCL12, was incorporated into purified sodium alginate, with the aim to microencapsulate SC-β cells (Table 1) (34,35,36). 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 enveloped in AG-CXCL12 microcapsules evaded the pericapsular fibrotic response, resulting in long-term functional competence and persistent glycemic correction (>150 days) in absence of systemic immunosuppression, in immunocompetent C57BL/6 mice. Application of these results into larger-size mammalians is awaiting.

PEG plus Alginate microcapsules: SC-β cells upon coating with PEG and derivates plus AG showed unaltered viability and insulin secretory capacity. Fabrication of hybrid PEG-ALG interpenetrating polymer networks was employed to prepare microcapsules with study of physical-chemical properties, 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 epidydimal fat pad of immuno-incompetent NOD/scid mice with no available further developments/communication (37).

Recently, coating surface of AG-based microcapsules with zwitterionic block co-polymers 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 model apparently emerged. These zwitterion-modified AGs may contribute to the development of cell encapsulation therapies for type 1 diabetes and other hormone-deficient diseases. This approach may 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 may be (38).

1.4.3 New types of coating biopolymers for microencapsulation

Chemically cross-linked hydrogels capsules and cell coatings based on human elastin-like recombinamers (ELRs) are being under development. ELRs consist on the repeating sequence of Elastin-Like polypeptide (VPGVG) found in the mammalian elastin, in an attempt to mimic biology and physical chemistry of the normal ECM (39,40). These sequence patterns have a proven biocompatible profile and comply with thermo-responsiveness and elasticity expected for this kind of polymers. Presence of high concentration of lysine is intended to provide the 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 (N3) and cyclooctyne (cyclo). The hydrogel microcapsules generated by this technology have been assessed for their properties and function towards a possible application for cell therapy, especially as far as perm-selectivity, 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 viability of the encapsulated sub-cellular 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. 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 vs 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 vs. control uncoated spheroids. Peritoneal cellular response to graft was incomparably lower for coated vs. uncoated hiPSC spheroids (41).

1.4.4 Other encapsulated stem cell types

Post-partum Wharton Jelly-derived human adult mesenchymal stem cells (hUCMS) may 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 pre-treatment of hUCMS with the pro-inflammatory cytokine IFN-γ induced expression of IDO1, 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. Moreover, 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 foetal-maternal interface. In particular, it has been recently described that HLA-G, released from human mesenchymal stem cells, may promote the expansion of Treg populations. We proved that microencapsulated hUCMS transplanted in NOD mice with recent-onset diabetes restored normoglycemia that persisted for long term (15). 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 may successfully manage diabetes in as a strong animal model of spontaneous T1D 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 extraordinary long periods of time (216 days post-TX). In light of these pre-clinical results, it may be possible to speculate on translation of the obtained data into a phase-1 pilot clinical trial in patients with recent-onset T1D.

1.4.5 Microcapsules for drug delivery system for prevention of T1D

Induction of an acquired state of immune tolerance in patients with T1D could prevent the autoimmune destruction of pancreatic islet β-cells. We studied in our laboratory whether the G3C hybridoma cell line-derived monoclonal antibodies (mAb), triggering the glucocorticoid-induced TNFR-related (Gitr) costimulatory receptor, would promote the expansion of regulatory T cells (Tregs) upon graft in SV129 (wild-type) and diabetic-prone NOD mice. The delivery of the G3c mAb 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 IgM that usually are prevented from entering/exiting the microcapsular barrier (42,43) (Figure 1). 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 nonconventional $Cd4^+Cd25^-$

$^{/low}$Foxp3^{low}Gitr$^{int/high}$ (Gitr single-positive) Tregs. Both Cd4^{+}Cd25^{+}Gitr^{high}Foxp3^{+}and Gitr-sp Tregs (including also 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 may have promising clinical potential for the treatment of inflammatory and autoimmune diseases (17).

2. Summary and Future Trends

Despite the fact that three full decades of work with microencapsulation have elapsed, no substantial application of this technology to islet/insulin-producing cells grafts in humans has yet taken off. Nevertheless, great deal of technical advances has been accomplished towards 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. Noteworthy, advanced purification process of the raw AG, coupled with new technologies for the basic AG polymers engineering may offer avenues to implement the basic microencapsulation product. Moreover, new research pathways are underway for validating non-AG molecules (ie, ELR etc.) for cell coating, within conformal microcapsules. While the controversies on about whether larger or smaller microcapsules could better fulfill chances for efficient cell grafts immunoprotection are unsolved, the real critical issues should focus on: 1) efficiency of the immunobarrier competence; 2) 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. On the other hand, conformal microcapsules fit quite 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 provision of direct oxygenation or by promoting neo-vessels formation is quite difficult to apply. The less invasive site, in this regard, could be creation of an omental pouch which couples short distance from the liver (the first site of insulin action) with appropriate vascularization. However, also this option may expose to the risk of eliciting an intense inflammatory response, regardless of basic polymers purity and advanced formulations. If

limitations to diffusion of the microencapsulation technology regard the polymeric composition and configuration of these microcarriers, the other arm, not certainly less relevant pertains to the embodied cells. Whether may be these islets or insulin-producing cells (ie, 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, thanks to their powerful immunoregulatory potential, due to humoral factors outflowing the capsular membranes, may interrupt the early disease process thereby helping rescue of the remainder still healthy β-cells. This simple effect might spare early initiation of exogenous insulin supplementation. However, in case of a more advanced stage of islet β-cells destruction, insulin, even at trace concentrations, as those afforded by progenitor endocrine cells, may 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 T1D.

Disclosure

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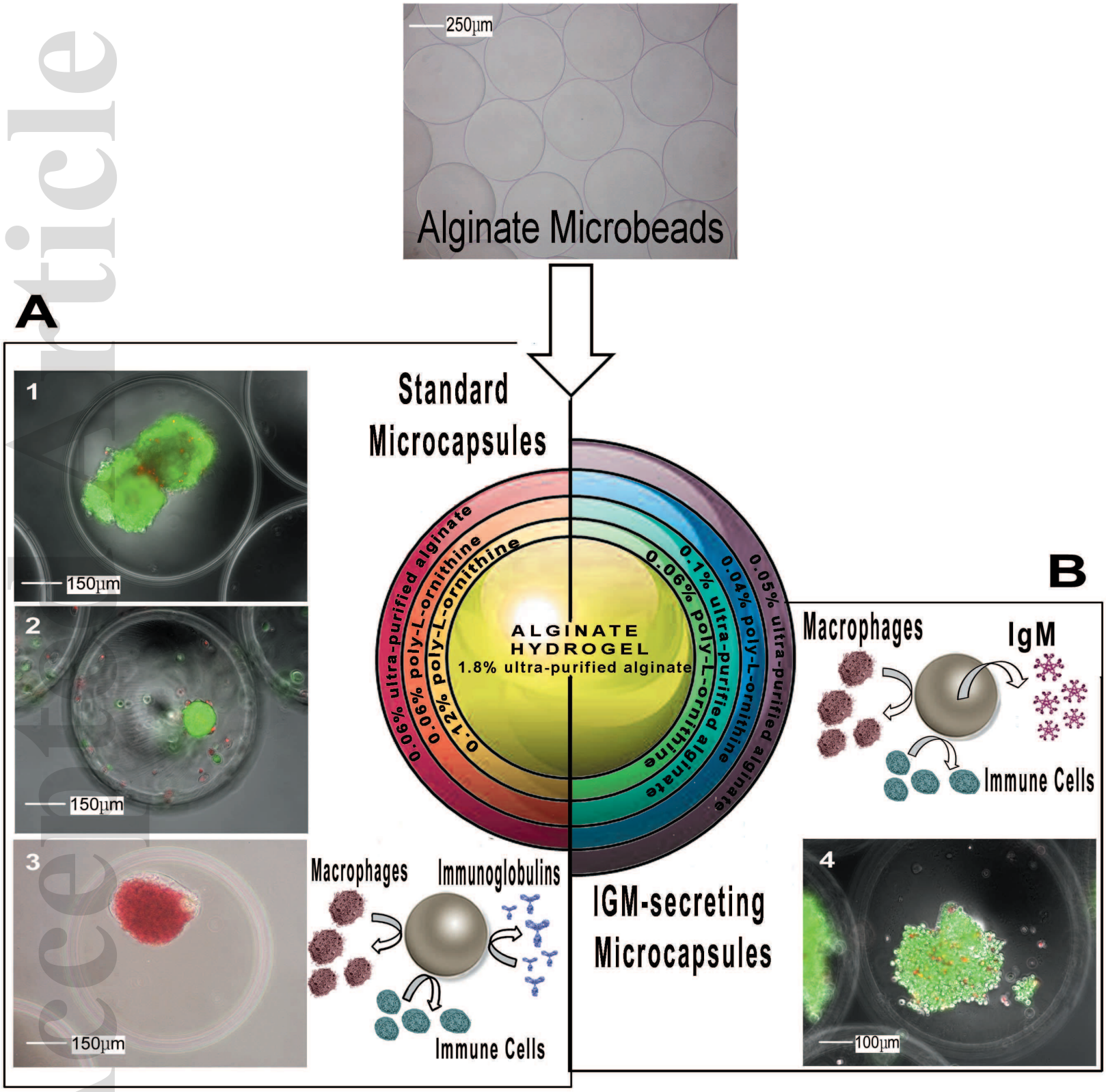
Figure and Table legends

Figure 1. Versatility of AG-based standard size microcapsules: A) standard-size microcapsules: typical immuno-barrier 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 hiPSC; 2) hUCMS aggregates; 3) human islets; B) Differently permeable (IgM-secreting) microcapsules: the modified membrane's properties allow for Ig outflow (in this instance IgM released from G3C hybridoma cell line) but prevent access to immune cells; 4) G3C hybridoma cells, very viable upon staining with ethidium bromide and fluorescein diacetate.

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

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

Authors	Type pf encapsulation	Type of Alginate	Cell source	Implantation site	Immunosuppression
Vegas AJ et al. (2016)[33]	microencapsulation	triazole-thiomorpholine dioxide (TMTD) alginate	SC-β	intraperitoneal space	no
Bochenek MA et al. (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 DA et al. (2019)[35]	microencapsulation	Sodium alginate with CXCL12	SC-β cells	peritoneal cavity	no
Sremac M et al. (2019)[36]	microencapsulation	high-mannuronic acid (LVM) alginate with or without recombinant human CXCL12	allogeneic and xenogeneic islets	intraperitoneal space	no
Liu Q et al. (2019)[38]	microencapsulation	Zwitterionically modified alginates	xenogeneic islets	peritoneal cavity	no



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