RESEARCH OUTPUTS / RÉSULTATS DE RECHERCHE

Alginate@TiO₂ Hybrid Microcapsules as a Reservoir of beta INS-1E Cells with Controlled Insulin Delivery

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ABSTRACT: Designing biocompatible materials to encapsulate xenogeneic insulin-releasing β-cells for transplantation has been considered as a promising alternative to avoid the immunosuppression and drawbacks of the treatment of Type 1 *diabetes mellitus* (T1D) by direct islet transplantation. The current work for the first time studied a hybrid alginate@TiO_2 microcapsule as a reservoir for rat insulinoma-derived INS-1E cells, as a β-cell surrogate, towards the treatment of T1D. The hybrid microcapsule is composed of an alginate core as a biocompatible matrix for cell encapsulation and a crack-free TiO_2 shell as a semi-permeable membrane to prevent cell leakage, protect encapsulated cells from immune attacks, as well as allow the diffusion of nutrients and the secretion of insulin. Compared to most-commonly used pure alginate microcapsules, the insulin-secreting INS-1E cells encapsulated in our alginate@TiO_2 microcapsules revealed higher metabolic activity and maintained the insulin secretion over more than 6 weeks. This study highlights that our designed alginate@TiO_2 hybrid microcapsules can serve as an ideal reservoir for cell encapsulation towards the treatment of T1D, thus further promoting the development of artificial organs for cell therapy.

KEYWORDS: hybrid hydrogels, cell encapsulation, biocompatibility, insulin secretion, type 1 *diabetes*

INTRODUCTION

Type 1 *diabetes mellitus* (T1D), as a kind of human disorders involving hormone or proteins deficiency, has increasingly become a big human health threat nowadays[1]. It consists in the autoimmune destruction of insulin secreting β-cells and hampers thus the glucose homeostasis. Currently the most common treatment is the discontinuous insulin injection which remains however a major burden in everyday life and cannot eliminate all complications of the disease[2]. Transplantation of islets has evolved into a prospective treatment as it allows the recovery of an endogenous insulin secretion and the control of glycaemia in insulin-dependent patients[3-4]. However, this treatment still presents various restrictions such as very limited donor supply, in general one donor for 300 patients. Furthermore, a patient requires generally 2-3 donors[5]. About 90 % of insulin-dependence recurrence 5-years post-transplantation has been observed[6]. A strong and life-long immunosuppressive medication is also required for patients, which leads to various side-effects[7-8].

An alternative to avoid immunosuppression and its drawbacks is to separate the islets (or β-cells) from the host immune system by introducing a semi-permeable membrane that would prevent the entry of immune defenders while allowing the diffusion of essential nutrients, oxygen, metabolites and insulin[9-13]. This kind of cell therapy methodologies has been largely used since first introduced by Bisceglie 80 years ago[14-17]. Alginate-based microcapsules are the most-used hydrogels for this medical treatment, because of their high biocompatibility, low cytotoxicity, low cost, and mild gelation conditions[18-21]. For instance, alginate microcapsules have been used for the encapsulation of insulin-secreting islet cells in order to restore long-term euglycemic maintenance without immunosuppression[22-23]. However, they usually present poor mechanical resistance and thus low stability *in vitro* and *in vivo*.

Hybridization of hydrogels with inorganic compounds has been developed as a promising method to improve the properties of the hydrogels or equip the hydrogels with new functions

to widen their application range or improve their efficiency in applications[24-25]. For example, the incorporation of carbon materials within hydrogels can enhance their electronical conductivity[26], thus realizing the applications in cell therapy towards cardiac or neural diseases or bone injury[27-30]; hybridizing microcapsules with gold nanoparticles enables to reinforce the mechanical strength of hydrogels and facilitate cell adhesion, spreading, and proliferation, which makes possible the precise cell differentiation control[31]; coordinating minerals with hydrogels can introduce the hydrogels with controllable stiffness to control the behaviours of encapsulated cells precisely[32-33]. Recently, new structured hybrid microcapsules which are composed of inorganic shells and an organic core have been reported and attracted a lot of attention[34-35]. The inorganic shell enhances the stability of the microcapsule and provides controlled permeability, while the organic core offers a highly biocompatible environment for the encapsulated cells, which makes this structured microcapsule a promising cell reservoir with long-term viability and activity for cell therapy[36-38]. For example, alginate@TiO_2 hybrid microcapsules have been demonstrated to be a suitable reservoir of HepG2 cells. They showed biocompatible and possessed controlled porosity to confirm the long-term cell viability, the cell activity, and the release of the cell metabolic[39]. Importantly, these microcapsules also presented high *in vivo* biocompatibility and *in vivo* stability when implanted into rats, thus contributing to their potential in cell therapy. However, the previous studies were implemented based on the model HepG2 cells which do not present any therapeutic use, so that further investigation is still highly needed towards practical application of hybrid microcapsules in cell therapy.

Herein, we first time investigate a hybrid alginate@TiO_2 microcapsule as a reservoir for insulin-secreting β-cells to maintain glucose homeostasis towards the cell therapy of T1D. Compared with the most-commonly used pure alginate microcapsules, hybrid alginate@TiO_2 microcapsules present a hard porous inorganic shell which shows enhanced mechanical

stability without compromising the nutrient diffusion and insulin secretion, thus preventing the leakage of encapsulated cells and providing them with a long-term stable environment for growth, proliferation and metabolism. Biocompatible hybrid alginate@TiO_2 microcapsules are prepared through a facile one-step process under mild conditions. Alginate serves as a matrix for cell encapsulation and a template for the formation of TiO_2 shells by the hydrolysis and condensation of the precursor. Rat insulinoma-derived INS-1E cells, which are able to secrete insulin in response to glucose stimulation in the physiological range[40-41], are encapsulated in our hybrid alginate@TiO_2 microcapsules. The morphology and structure of the hybrid microcapsules are investigated in detail. The viability of the encapsulated INS-1E cells are monitored, as well as their glucose-stimulated insulin secretion, in order to advance the development of cell encapsulation techniques for the treatment of T1D.

MATERIALS AND METHOD

Chemicals. Titanium(IV) bis(ammonium lactato)dihydroxide solution (TiBALDH, 50 *wt.%* in H_2O), poly(diallyldimethylammonium) chloride solution (PDDAC, average Mw 100,000-200,000, 20 wt. % in H_2O), alginic acid sodium salt, from brown algae (for immobilization of microorganisms), calcium chloride (dihydrate, 99 %), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, tetrasodium salt, 97 %), hydrochloric acid (Molecular Biology Grade, 36.5-38.0 %), sodium hydroxide (reagent grade, 98 %, anhydrous), chloroform (anhydrous, 99 %), glutaraldehyde (50 wt. % in H_2O), fluorescein diacetate (FDA, used as cell viability stain), glucose solution (BioUltra, for molecular biology, ~20 % in H_2O), sodium chloride (ACS reagent, 99,0 %), potassium chloride (for molecular biology, 99.0 %), potassium phosphate monobasic (plant cell culture tested, 99 %), magnesium sulfate heptahydrate (Plant Cell Culture Tested), sodium bicarbonate (Plant Cell Culture Tested), albumin, from bovine serum (BSA, 98 % (agarose gel electrophoresis, lyophilized powder, essentially fatty acid free,

essentially globulin free)) and dimethyl sulfoxide (BioReagent, for molecular biology, suitable for plant cell culture, 99.9 %) were purchased from Sigma-Aldrich. Ethanol (absolute) was purchased from Fisher. PBS (Phosphate Buffered Saline, 0.0067 M (PO_4), For Cell Culture) was purchased from Lonza. Forskolin (*Coleus forskohlii* in DMSO) was purchased from Merck Millipore. Roswell Park Memorial Institute medium (RPMI, 1640), foetal bovine serum, HEPES buffer solution (1 M), sodium pyruvate (100 mM, 100X), L-Glutamine (200 mM, 100X) and 2-mercaptoethanol (50 mM) were purchased from Invitrogen (Casbald, USA).

Sodium alginate powder was purified according to the method proposed by de Vos *et al*[42]. Briefly, the commercial powder was dissolved in an aqueous solution of EGTA 1 mM to remove Mg^{2+} and Ca^{2+} cations. Subsequently, the solution was filtered and washed by HCl, chloroform and butanol to eliminate remaining ions and proteins. Finally, the purified alginate powder was obtained by adjusting pH to 7.0, adding absolute ethanol for precipitation and drying using a vane pump.

Cell culture. Rat insulin-secreting cell line INS-1E sensitive to glucose stimulation was provided by Prof. Pierre Maechler (University of Geneva, Department of Cell Physiology and Metabolism) and Prof. Patrick Gilon (Université Catholique de Louvain, EDIN, Endocrinology, Diabetes and Nutrition division). The Roswell Park Memorial Institute medium (RPMI, 1640) used for the culture of INS-1E cells was supplemented with HEPES (10 mM), sodium pyruvate (1 mM), glutamine (2 mM), 2-mercaptoethanol (0.05 mM) and 10 % of foetal bovine serum. Cells were incubated in 75-cm^2 flasks (Costar, Lowell, USA) at 37°C in a humid atmosphere of 5 % CO_2, and the medium was replaced by fresh one every 2 days. Once the cells were 100 % confluent (4 or 5 days), they were washed with PBS (Phosphate Buffer Saline) before treatment with a trypsin/EDTA mixture. Trypsinized cells were suspended in fresh medium for encapsulation. For further culture, cells were diluted and placed in new 75-cm^2 flasks containing 15 mL of fresh culture medium with serum.

Cell encapsulation. The pH of the solutions used for cell encapsulation were previously adjusted to 7.4.

Cell encapsulation in pure alginate microcapsules. An INS-1E cell suspension was mixed with 3 *wt.%* sodium alginate. The final cell density was $1.1*10^6$ cells mL^{-1}. This mixture was dropped in a $CaCl_2$ solution (100 mM) using sterilized 3.0 mL plastic Pasteur pipettes (VWR). The droplets were incubated for 5 min to obtain microcapsules with solid calcium alginate external crusts and inner cell-containing sodium alginate core. The microcapsules were finally recovered, placed in test tubes filled with fresh culture medium with serum and maintained in cell culture conditions (37 °C, 5 % CO_2).

Cell encapsulation in alginate@TiO_2 microcapsules. The encapsulation procedure of INS-1E cells in alginate@TiO_2 microcapsules is showed in Figure 1 A. In detail, an INS-1E cell suspension was first mixed with 3 *wt.%* sodium alginate supplemented with titanium precursor (TiBALDH, 250 mM). The final cell density was $1.1*10^6$ cells mL^{-1}. This mixture was extruded into a buffered aqueous solution containing $CaCl_2$ (100 mM) and PDDAC (100 mM). The droplets were incubated for 5 min to obtain microcapsules with a solid white crust. The microcapsules were finally recovered, placed in test tubes filled with fresh culture medium supplemented with serum and maintained in cell culture conditions (37 °C, 5 % CO_2).

Material characterization. Microcapsules were dried through supercritical drying with liquid carbon dioxide, after being incubated in 3 % glutaraldehyde and dehydrated with ethanol. Morphological properties were analyzed using field emission scanning electron microscopy (FE-SEM, JEOL JSM-7500F), after being sputter-coated with gold. The elemental composition of the internal core and external shell of alginate@TiO_2 microcapsules was determined using an energy-dispersive X-rays (EDX) analysis system with an acceleration voltage of 15.00 kV and a working distance of 8 mm.

Cell viability. The viability of encapsulated INS-1E cells was monitored by their oxygen consumption using a Clark's cell vessel (HansaTech). Briefly, 3 microcapsules were put in the device, containing 1.0 mL of fresh culture medium. The relative respiration of INS-1E cells was determined by oxygen consumption. The oxygen consumption of the INS-1E cells just after the encapsulation was set as the reference value (100 %). Cell survival was confirmed using fluorescein diacetate (FDA), a vital dye stain. INS-1E cells were incubated in FDA solution (5 μM) for 5 minutes at room temperature. Pictures were then taken using a fluorescence microscope (Multizoom AZ100, Nikon) either in brightfield mode or in fluorescent mode at 536/40 nm with a colour camera (DSRi1, Nikon).

Glucose-stimulated insulin secretion. An evaluation of insulin secretion of the microcapsules with entrapped INS-1E cells was performed as a function of time. Briefly, culture medium was removed from test tubes containing hybrid microcapsules. Microcapsules were washed twice with Hank's balanced sodium salt solution (HBSS) (114 mmol L^{-1} NaCl, 4.7 mmol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 1.16 mmol L^{-1} $MgSO_4$, 20 mmol L^{-1} HEPES, 2.5 mmol L^{-1} $CaCl_2$, 25.5 mmol L^{-1} $NaHCO_3$ and 0.2 % BSA, pH 7.2). The first wash was just a quick rinse, while for the second wash, the microcapsules were incubated in HBSS for 2 hours at 37°C (5% CO_2). Then, the secretagogues diluted in HBSS were added another 2-hour incubation. A glucose solution of 3 mM was used for the determination of basal insulin secretion level, while 15 mM glucose and 15 mM glucose supplemented with 1 μM forskolin were used for stimulated secretion, using independent samples for each assay. The supernatants were finally recovered to measure insulin concentration using a Rat/Mouse Insulin ELISA Kit provided by Merck Millipore.

Statistical analysis. The concentration of insulin secreted by encapsulated INS-1E cells when stimulated by 15 mM glucose (or 15 mM glucose supplemented with 1 μM forskolin) was compared to basal secretion (stimulation by 3 mM glucose). Results were analyzed by

unpaired Student's *t*-test, using XLSTAT 2013 software (Addinsoft) and with $p < 0.05$ being considered as significant.

RESULTS AND DISCUSSION

Microcapsule formation and cell encapsulation. The cell-laden alginate@TiO_2 microcapsules were formed by dropping the mixture of alginate, TiBALDH and INS-1E cells into $CaCl_2$ / PDDAC solution, as depicted in Figure 1A. When the drops were immersed into the solution, a series reactions took place in the outmost layer of the drops, resulting in the formation of microcapsules. The reactions mainly include three ones as listed in Figure 1C. The sodium alginate in the interface of the drops and the solution reacted with calcium ions by cross-linking to form calcium alginate shells around the drops. Simultaneously, PDDAC adsorbed on the formed calcium alginate shells by electrostatic force. The adsorbed PDDAC further induced the hydrolysis and condensation of the TiBALDH in the outmost layer of the drops to form a TiO_2 layer. With the prolongation of incubation, three above-mentioned reactions continuously occurred to thicken the calcium alginate and TiO_2 shells, and finally contributed to the formation of alginate@TiO_2 microcapsules (Figure 1B). The INS-1E cells were mainly encapsulated in the core of alginate@TiO_2 microcapsules which was most composed of biocompatible alginate. The calcium alginate and TiO_2 shells could protect the encapsulated cells from immune defenders and maintain the nutrient diffusion and insulin secretion.
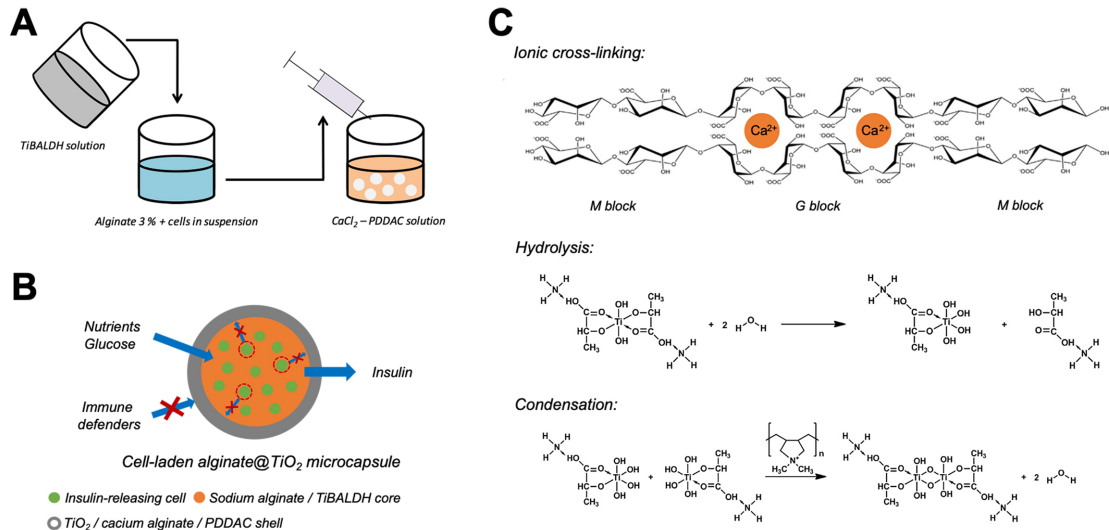
Figure 1. Formation of cell-laden alginate@TiO₂ microcapsules. (A) Encapsulation procedure of INS-1E cells in alginate@TiO$_2$ microcapsules. (B) Schematic diagram of an INE-1E cells-laden alginate@TiO$_2$ microcapsules. (C) Ionic cross-linking of alginate and hydrolysis and condensation processes of TiBALDH in the formation of alginate@TiO$_2$ microcapsules.

Material characterization. The morphology, structure and elemental composition of alginate@TiO$_2$ microcapsules with encapsulated INS-1E cells were characterized. Pure alginate microcapsules with encapsulated INS-1E cells were set as a control. Both cell-laden microcapsules were supercritically dried with CO$_2$, in order to preserve their original structure. The dried microcapsules were characterized using scanning electron microscopy (SEM) equipped with an energy-dispersive X-rays (EDX) analysis system.
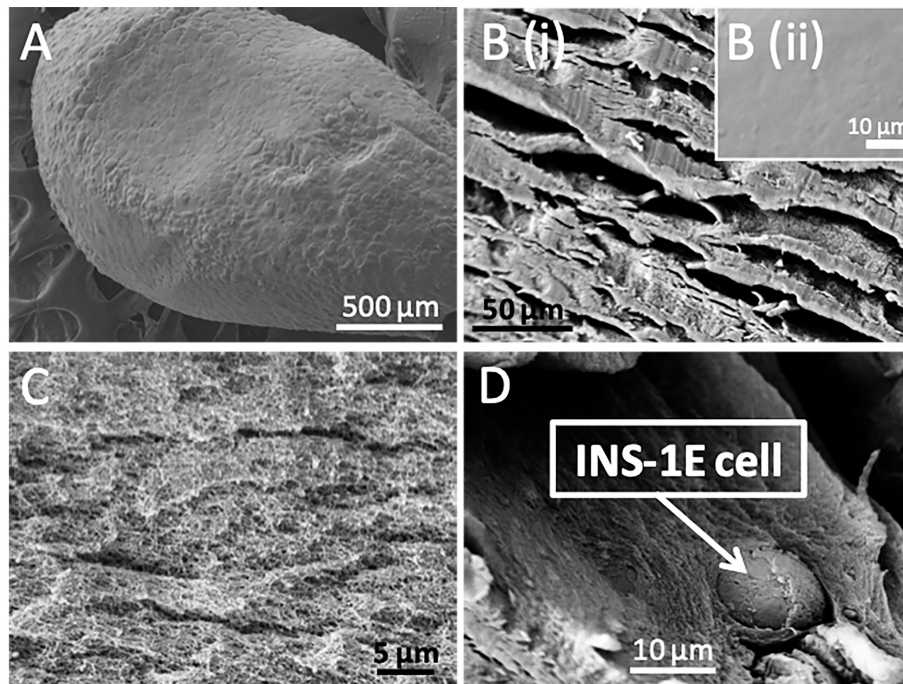
Figure 2. Morphology and structure of a cell-laden alginate microcapsule. SEM micrographs of a cell-laden alginate microcapsule (A), its surface (B) and core (C), as well as encapsulated INS-1E cells (D). The microcapsule was incubated in culture medium for 6 weeks, except for the microcapsule shown in B(ii) which was observed directly after preparation and set as a control. The microcapsules were supercritically dried with CO_2 prior to analyses.

Figure 2A depicts the SEM micrograph of a pure alginate microcapsule with encapsulated INS-IE cells after 6-week incubation in culture medium. The microcapsule shows spherical morphology with a diameter of ~2000 μm. Figure 2B (i) gives the enlarged SEM micrograph of the external surface of the microcapsules in which some cracks can be observed. On the contrary, a very smooth surface can be seen in pure alginate microcapsules without any further incubation (Figure 2B (ii)). The difference between the two surfaces indicates that the surface evolution of pure alginate microcapsules from smooth to cracked by long-term incubation, demonstrating the lack of long-term stability of pure alginate microcapsules. The cracks of the surface could resulted from the dissolution of calcium alginate layers or the degradation of
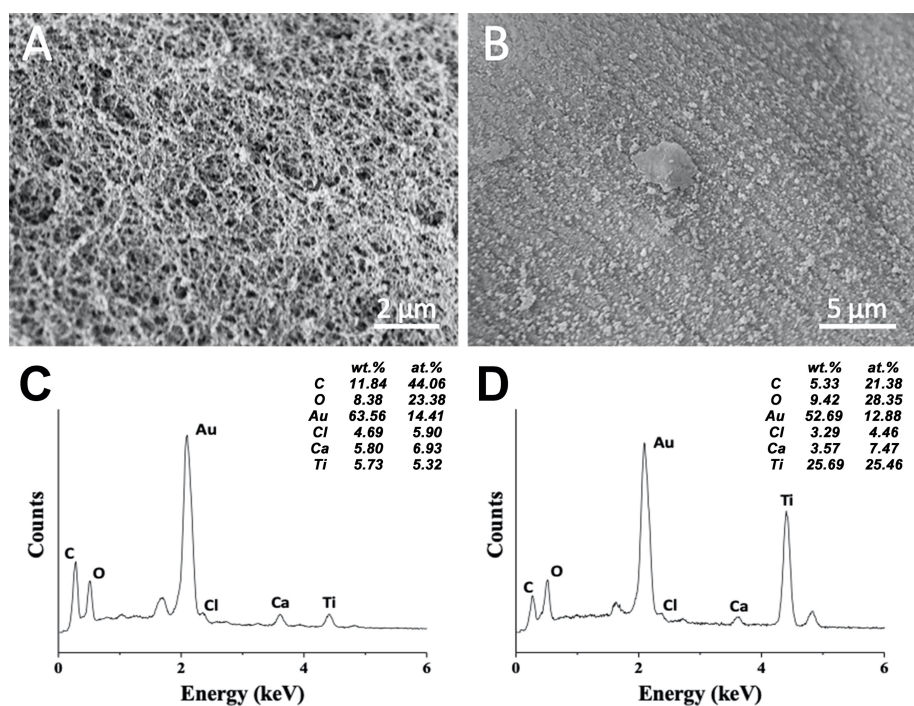
alginate cores. The core of the microcapsule presents a sponge-like structure with highly porosity (Figure 2C), which was formed by the loss of a large content of aqueous solution during the supercritically drying. This sponge-like structured core could not only provide enough space to be filled with nutrient for cell growth, proliferation and metabolism, but also maintain the mass transport and the cell-to-cell communication inside microcapsules. Figure 2D shows encapsulated INS-1E cells in the microcapsule, confirming the presence of the cells in this cell-laden microcapsule.
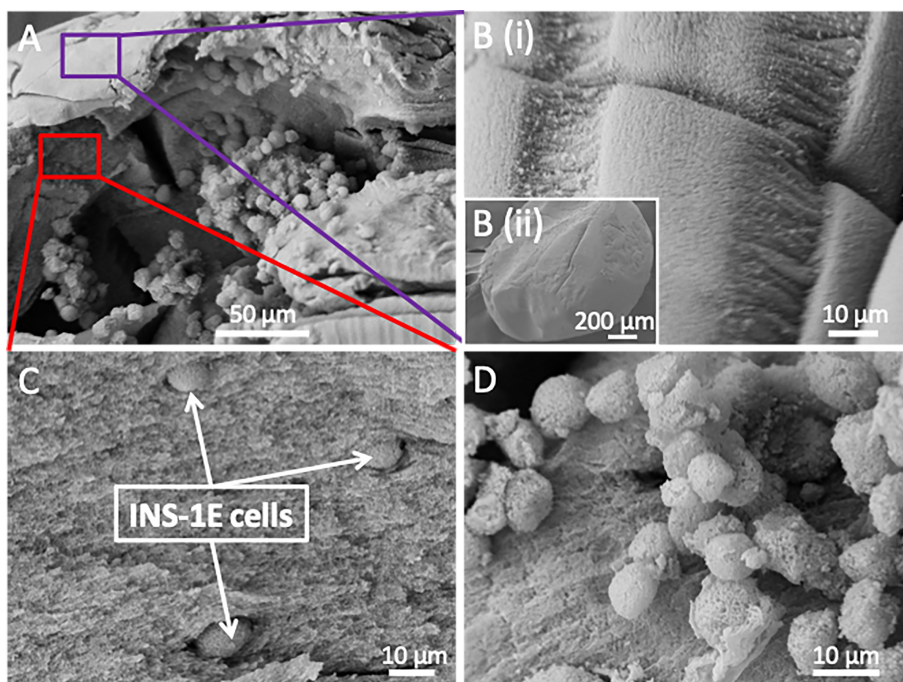
Figure 3. Morphology and structure of a cell-laden alginate@TiO_2 microcapsule. SEM micrographs of a cell-laden alginate@TiO_2 microcapsule (A), its surface (B) and core (C), as well as encapsulated INS-1E cells (D). The microcapsule was kept in culture medium for 6 weeks and supercritically dried with CO_2 prior to analyses.

Figure 3A presents a cross-section of an alginate@TiO_2 microcapsule, highlighting the presence of well dispersed cells in the core and a condensed shell. Figure 3B shows that the surface of the alginate@TiO_2 microcapsules is still very dense without cracks even after 6-week incubation. The dense layer could be attributed to the adsorption of PDDAC and the formation

of TiO$_2$. PDDAC is a positive charged linear polymer. When it was adsorbed on the surface of the microcapsules, it would contribute to the condensation of alginate on the outmost layer owing to its interaction with the negative charged alginate, thus consolidating the calcium alginate shell. Meanwhile, PDDAC induced the hydrolysis and condensation of TiBALDH to form a uniform and robust TiO$_2$ layer which could further enhance the mechanical stability of shells. As a comparison, on the surface of pure alginate microcapsules some cracks of about 10 μm are observed after 6-week incubation which is shown in Figure 2B(i). Since the molecular size of immunoglobulin G (IgG)[43], the smallest defender of our immune system, is around 17-20 nm on the basis of the equation of Stokes[44-46], these immunoglobulin molecules can easily penetrate through the cracks of 10 μm and kill encapsulated INS-1E cells encapsulated. In addition, these large cracks would lead to the leakage of encapsulated cells, thus decreasing functional activity of cell-laden microcapsules in cell therapy. Therefore, pure alginate microcapsules present serious defects in terms of stability to be applied in cell encapsulation towards cell therapy. Whilst alginate@TiO$_2$ hybrid microcapsules remain intact after long-term incubation in culture medium, evidencing their enhanced mechanical stability and suggesting their more effective protection against immune defenders. The core of the alginate@TiO$_2$ microcapsule (Figure 3C) presents the same structural properties as those of pure alginate microcapsules (Figure 2C), which indicates that the formation of TiO$_2$ mainly occurred at the external surface of alginate microcapsules and would not influence the inner structure of alginate, thus confirming their biocompatible structure towards the encapsulated cells. It is worth to note that the encapsulated INS-1E cells were grouped into small clusters (Figure 3D), which is in the same manner as free cells during culture, in addition to the presence of extracellular matrix at their surface, thus suggesting a good health state of the encapsulated cells and proving biocompatibility of the alginate@TiO$_2$ matrix.

Figure 4. Elemental composition of an alginate@TiO$_2$ microcapsule. SEM micrographs of the core (A) and the shell (B) of an alginate@TiO$_2$ microcapsule. EDX spectra and elemental percentage of the core (C) and the shell (D) of the alginate@TiO$_2$ microcapsule. The microcapsule was supercritically dried with CO$_2$ immediately after synthesis prior to analyses.

EDX analyses were performed on alginate@TiO$_2$ microcapsules to identify the elemental composition of the inner and the outer parts of the microcapsules. Figure 4 presents the morphology and EDX spectra of the inner core (A, C) and the external shell (B, D) of alginate@TiO$_2$ microcapsule. The presence of the CKα, OKα and CaKα peaks in either the sponge-structured core (Figure 4A) or the dense shell (Figure 4B) indicates that the microcapsules are mainly made of alginate and calcium alginate. Ti element can be detected at the external surface of the alginate@TiO$_2$ microcapsule (25.46 *at.%*) (Figure 4D), indicating that a TiO$_2$ layer was formed around the microcapsule as a shell which could enhance the stability of microcapsules. A small portion of Ti element (5.32 *at.%*) can be detected in the core of the microcapsule (Figure 4C), which suggests that the formation of TiO$_2$ also took part

in the inner core but with a slow reaction rate due to the absence of polycations. The different percentage of Ti element between the core (5.32 *at.%*) and the shell (25.46 *at.%*) of the microcapsules confirms their core@shell structure which not only maintains the biocompatibility of the alginate core towards the encapsulated cells but also enhances the stability and mechanical strength of the microcapsules through the introduction of an inorganic shell for long-term applications.

Cell viability. The viability of INS-1E cells encapsulated in either pure alginate or alginate@TiO_2 microcapsules was monitored over time by the evaluation of oxygen consumption and FDA staining, in order to test the biocompatibility of the microcapsules towards encapsulated cells for their potential application in cell therapy.

The oxygen consumption of encapsulated cells was measured using a Clark's cell device. This parameter is directly proportional to the number of living cells in the microcapsules. Figure 5 presents the relative respiration as a function of time with 100 % corresponding to the oxygen consumption of INS-1E cells encapsulated in one microcapsule just after encapsulation. The viability of INS-1E cells was maintained for 6 weeks as the relative respiration slightly increased over the whole period. It is worth to note that the respiration activity of the cells encapsulated in alginate@TiO_2 microcapsules increased up to ~250 %, whereas that of the cells encapsulated in pure alginate microcapsules remained ~100 %. The rapid increase in respiration activity of INS-1E cells encapsulated in alginate@TiO_2 microcapsules can be attributed to cell proliferation, suggesting very good biocompatibility of our designed hybrid microcaspules towards INS-1E cells. Please note that the relative respiration of alginate@TiO_2 varied more largely at 14 days and 42 days, which could result from the shell crack of few alginate@TiO_2 microcapsules after long-term incubation. Owing to the cell proliferation and the degradation of alginate core, the shell of few microcapsules appeared cracked after a long-time incubation, while the most others were still crack-free. Pure

alginate microcapsules are highly compatible towards encapsulated cells and support their growth and proliferation, however, the oxygen comsuption which can be linked to the number of living cells has not increased. One possible explanation of this phenomenon is the cell leakage from the pure alginate microcapsules due to the surface crack appearance of the most of the microcapsules upon incubation.
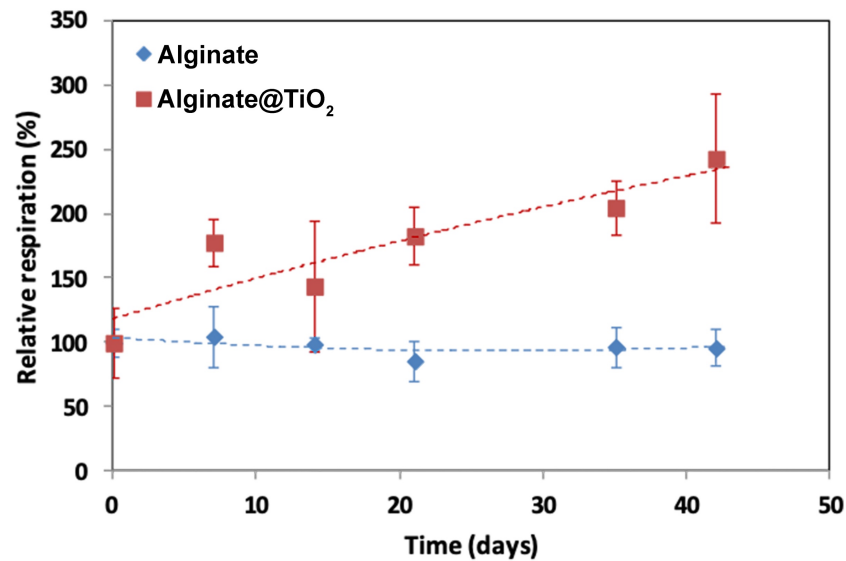
Figure 5. Oxygen consumption of encapsulated INS-1E cells. Oxygen consumption of the INS-1E cells encapsulated in alginate and alginate@TiO_2 microcapsules. 100 % corresponds to the oxygen consumption of INS-1E cells encapsulated within one microcapsule ($1.3*10^2$ $\mu mol\ h^{-1}$ microcapsule^{-1}). Results are presented as means $\pm$ 1 S.D. (n = 3).

The viability of the cells encapsulated in alginate@TiO_2 microcapsules was further confirmed by the characterization of FDA-stained cells using fluorescent microscope. Living cells with intact plasma membrane can produce green fluorescein after stained by FDA due to the presence of active esterase in cytoplasm.
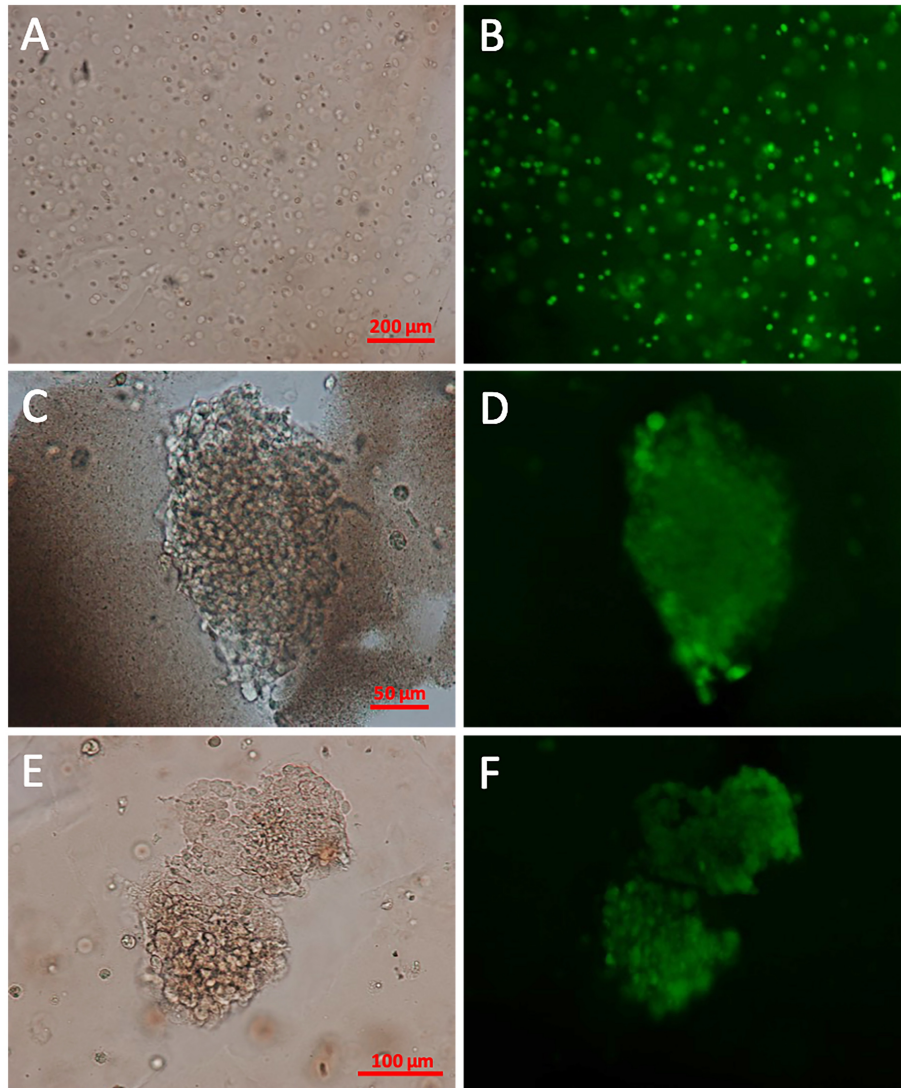
Figure 6. Viability of encapsulated INS-1E cells. Optical micrograph of the FDA-stained INS-1E cells encapsulated in alginate@TiO$_2$ microcapsules 1-h (A), 35-day (C) and 42-day (E) post-encapsulation. Fluorescent micrograph of the FDA-stained INS-1E cells encapsulated within alginate@TiO$_2$ microcapsules 1-h (B), 35-day (D) and 42-day (F) post-encapsulation.

Optical and fluorescent micrographs of FDA-stained INS-1E cells encapsulated in alginate@TiO$_2$ microcapsules are depicted in Figure 6. 1-h post-encapsulation, cells were homogeneously dispersed in microcapsules (Figure 6A) and more than 90 % of them were alive (Figure 6B). After 35 days, the encapsulated INS-1E cells were present as clusters in the microcapsules (Figure 6C and 6E), which was in accordance with the observations made by

SEM (Figure 3A and 3D) and the growth state of free cell in culture plate. In addition, the cell clusters exhibited green fluorescence (Figure 6D and 6F), demonstrating the high viability of the encapsulated cells. Combined with the results of oxygen consumption, it can be concluded that our designed alginate@TiO_2 hybrid microcapsules, in comparison with pure alginate microcapsules, could be a better candidate as a cell resevoir to encapsulate INS-1E cells with long-term viability for the treatment of T1D.

Glucose-stimulated insulin secretion. In order to evaluate the potential of our designed cell-laden alginate@TiO_2 microcapsules used for the treatment of T1D, the insulin secretion of encapsulated INS-1E cells stimulated by glucose were studied by the measurement of insulin concentration in the supernatant of the secretagogue media 2- and 6-week post-encapsulation. Cell-laden pure alginate microcapsules were set as a control. The results were indicated as a stimulation index which is defined by the increase in insulin secretion when stimulated by glucose or glucose supplemented with forskolin minus the basal secretion. Forskolin is an activator of adenylate cyclase that stimulates the release of insulin.
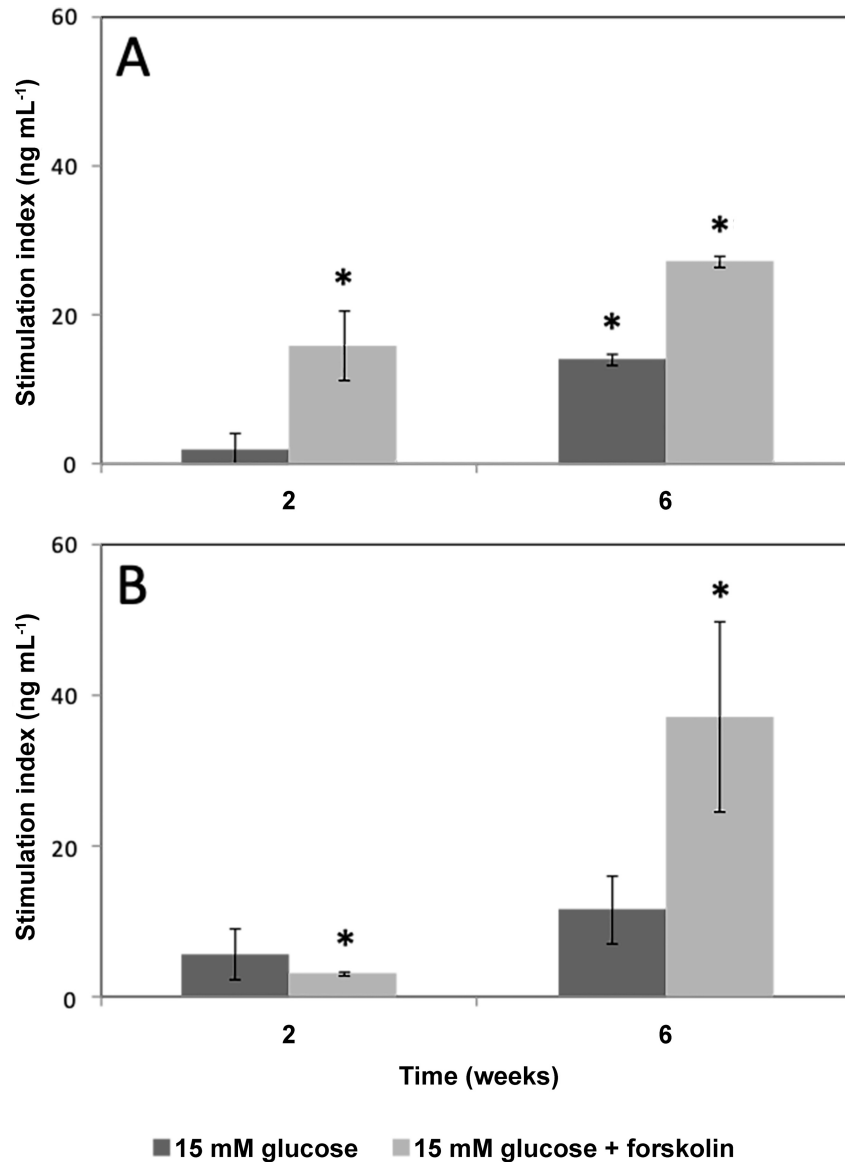
Figure 7. Insulin secretion of encapsulated INS-1E cells. Insulin secretion of the INS-1E cells encapsulated in alginate (A) and alginate@TiO$_2$ (B) microcapsules under the stimulation of glucose (15 mM) or glucose (15 mM) supplemented with forskolin (1 μM). Stimulation index is defined by the increase in insulin secretion (ng mL^{-1}) when stimulated by glucose or glucose supplemented with forskolin minus the basal secretion (3 mM). Values are means ± 1 S.D. (n = 3). * p < 0.05 compared to the basal insulin secretion.

The stimulated insulin secretion of INS-1E cells encapsulated in alginate and alginate@TiO$_2$ microcapsules are presented in Figure 7A and 7B, respectively. Under the stimulation of only

glucose, the insulin secretion of the cells encapsulated in pure alginate microcapsules or in alginate@TiO$_2$ microcapsules and incubated for 2 weeks shows no difference to that of basal levels, however, an increased insulin secretion could be detected for the cells encapsulated in the pure alginate microcapsules or in alginate@TiO$_2$ microcapsules and incubated for 6 weeks because of cell proliferation. In order to confirm the responses of encapsulated cells to glucose more precisely, forskolin was used as an additive stimulator to amplify insulin secretion. After 2-week or 6-week incubation for the cells encapsulated in pure alginate microcapsule and in alginate@TiO$_2$ hybrid microcapsules, the insulin secretion stimulated with glucose supplemented with forskolin shows a highly significant difference ($p < 0.05$) in comparison with basal levels. Both experiments indicate that encapsulated INS-1E cells can significantly respond to glucose stimulation and maintain their metabolic activity. It is worth to note that the cells encapsulated in alginate@TiO$_2$ microcapsules 6-week post-encapsulation show significantly increased insulin secretion levels compared to that 2-week post-encapsulation. The increase of insulin secretion over time was attributed to the enhanced metabolic activity of encapsulated cells and cell proliferation, supporting the results presented in Figures 4 and 5. Please note the cells encapsulated in pure microcapsules showed a less increase of insulin secretion levels between 2-week and 6-week post-encapsulation. This could result rather from the metabolic activity enhancement of encapsulated cells than cell proliferation, as the amount of encapsulated cells was not increased based on the result presented in Figure 5. In addition, this result evidences that alginate@TiO$_2$ microcapsules displayed well adapted porosity for the entry of nutrients and the secretion of insulin. Although pure alginate microcapsules also allow the diffusion of nutrients and insulin as shown by the results presented in Figures 5 and 7, these microcapsules suffer from crack formation during incubation. These cracks could result in the cell leakage and the loss of their immune-isolation.

On the basis of the previous study[39], alginate@TiO$_2$ hybrid microcapsules, in comparison with the most-used pure alginate microcapsules, not only present long-term mechanical stability and high biocompatibility towards HepG2 cells, but also possess well-controlled porosity to maintain the nutrient diffusion and metabolic releasing of the encapsulated HepG2 cells. However, HepG2 cells are only a kind of model cells for the fundamental investigation and show no function in cell therapy. The practical application of alginate@TiO$_2$ hybrid microcapsules in cell therapy still remains a lot of problems. In this work, the functional insulin-secreting INS-1E cells were first time encapsulated in alginate@TiO$_2$ hybrid microcapsules, thus being further developped from the last research and much closer to the practical treatment of T1D. In the case of INS-1E cells, the core of the alginate@TiO$_2$ hybrid microcapsules provided the encapsulated cells with a biocompatible aqueous environment for their growth, proliferation and metabolism. In addition, their robust TiO$_2$ layer not only offered a long-term stability to prevent cell leakage, but also presented well controlled porosity for the diffusion of glucose as well as insulin secreted by the encapsulated cells (Figure 1B). This makes them an ideal reservoir of INS-1E cells for the controlled delivery of insulin in the treatment of T1D.

CONCLUSIONS

The highlights of this work can be summarized into the points listed below:

- Alginate@TiO$_2$ hybrid microcapsules were synthesized by the ionic cross-linking of alginate and the hydrolysis and condensation of TiBALDH, thereafter first time being applied to encapsulate rat insulinoma-derived INS-1E cells, aiming at the treatment of T1D.

- Alginate@TiO$_2$ hybrid microcapsules, compared to the most-used pure alginate microcapsules, demonstrated improved long-term stability and less cell leakage, making them a better reservoir for INS-1E cells to protect them from immune attacks for a long time.

- The rat insulinoma-derived INS-1E cells encapsulated in alginate@TiO$_2$ maintained insulin secretion as a response to glucose stimulation 6-week post-encapsulation, evidencing the potential application of the cell-laden alginate@TiO$_2$ microcapsules in the treatment of T1D.

Consequently, alginate@TiO$_2$ hybrid microcapsules could be an ideal reservoir for the encapsulation of INS-1E cells towards the treatment of the increasingly widespread T1D, as well as offer a draft to design artificial organs for cell therapy in future.

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Notes

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