Multifunctional microcapsules for pancreatic islet cell entrapment: design, preparation and in vitro characterization

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Abstract

Great advances in cell transplantation have been made, including the recent, remarkable success in pancreatic islet transplantation for the treatment of type 1 diabetes mellitus. Unfortunately, the transplanted cells are very susceptible to oxidative stress that cause severe damage to either allo- or xenogeneic islets upon graft in diabetic patients. Consequently, the transplanted islet functional life span is significantly shortened. The aim of this study was to examine the possible effects of antioxidants on in vitro cultured adult rat islets, and to evaluate the effects of a prolonged-release formulation, in form of cellulose acetate (CA) microspheres, on Vitamin D3 activity. Isolated rat islets, both free and entrapped in microspheres were treated with Vitamin D3. The effects of the vitamin were studied at 3, 6 and 9 days of in vitro cell culture. According to insulin secretory patterns, treatment with Vitamin D3 of both free and CA entrapped microspheres, increased the insulin output as compared to untreated controls. Such positive effects were confirmed under islet static incubation with glucose at day 6. These results suggest that pancreatic islets can be advantageously treated with anti-oxidising vitamins before implantation, and speculatively, with the help of special delivery systems, throughout the islet cell life span, in the post-transplant time period.

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1. Introduction

Pancreatic islet cell transplantation might represent the final solution to the therapy of type 1 diabetes mellitus (T1DM) as demonstrated by the very promising results obtained by ongoing allograft clinical trials [1]. These have so far shown restoration of euglycemia in 70% of the grafted patients at 2 years of post-transplant follow up. However the islet supply problem is still open, depending upon scarce availability of human organ donors, while immunosuppressive therapy is associated with adverse long-term side effects.

To possibly solve the above indicated drawbacks, both alternate tissues as a resource for donor islets, and innovative strategies for islets transplant immunoprotection are currently being investigated.

It is well known that only very few islet xenograft protocols in animals have been proven successful [2]. Immunological reactions, as well as not immune events, such as inflammation or apoptosis, are the main causes for graft failure [3–7]. Recent clinical data from the above mentioned trials, have demonstrated that success of human islet transplantation is largely due to characteristics of the implanted cells (with special regard to number and viability [1]).

Although mastered in a few laboratories, microencapsulation technology, consisting of physical envelopment
of the islets within highly biocompatible and selective permeable membranes, was shown to fully immuno protect islet allo- and xenografts, mainly in diabetic rodents, throughout very long periods of time [8–11]. However, the potential advantages associated with use of the microcapsules, are shadowed by as many apparent pitfalls that might attenuate the impact of this approach on islet transplantation in diabetic higher mammals. In fact, with the exception of one relevant, yet isolated report [12], no substantial evidence of function of microencapsulated allo/xenogeneic islet graft has been achieved by trials conducted in diabetic dogs, or preliminarily, in T1DM-patients. These data demonstrated only partial and transient remission of hyperglycemia. Historically, in our own experience, diabetic patients undergoing complex vascular surgery, were grafted with microencapsulated human islets embodied in a co-axial vascular chamber, directly anastomosed to blood vessels: insufficient grafted islet cell mass was held responsible for inconsistency of the obtained results [13], while no certain evidence of immune rejection was observed. Finally, so far of two patients with T1DM, receiving intraperitoneal graft of microencapsulated neonatal porcine islets, neither one showed evidence of clinical correction of hyperglycemia, while only negligible raise in porcine C-peptide urinary levels was detected (unpublished data).

It is known that pancreatic islets may be vulnerable to adhesion of activated leukocytes, cytokines, and free oxygen radicals released by macrophages that may induce cell damage. In an attempt to solve or alleviate these unwanted effects, some laboratories have recently proposed use of vitamins as antioxidants to prolong β-cell function in both patients or islet graft recipients with T1DM [14–16]. For instance, it has been reported that dietary supplement of Vitamin E can help prevention of rejection of pancreatic xenografts [17]. The xenografted animals were fed with Vitamin E at low (150 mg/kg) or high (8000 mg/kg) doses. These experiments demonstrated that orally administered Vitamin E, was able to attenuate leukocyte-endothelial cell interactions, thereby preserving the islet microvasculature. Also recently, the effects of Vitamins E and C and N-acetyl-L-cysteine on β-cell function in diabetic C57BL/KsJ-db/db mice, were studied [18]. The findings suggested that antioxidants could be beneficial to diabetes, evidencing, on the contrary, the negative impact of oxidation on β-cell function. In addition, we have recently demonstrated that Vitamin D3 (cholecalciferol) and E (t-α-tocopherol) indeed improve the in vitro viability and islet performance of free neonatal porcine cell clusters (NPCCs) [19], possibly confirming beneficial effects of antioxidants on cell apoptosis.

Recently, Vitamin D3 has also been shown to act on novel target tissues not related to calcium homeostasis [20]. There have been reports characterizing Vitamin D3 receptors and activities in diverse tissues such as brain, pancreas, pituitary, skin, muscle, placenta, immune cells and parathyroid [20]. The receptor hormone complex becomes localized in the nucleus, and undergoes phosphorylation by reacting with a kinase. This form of the receptor then interacts with the Vitamin D responsive element of target gene and modifies the transcription of those genes to develop the action. The modulation of gene transcription results in either the induction or repression of specific messengers. Genomic action of Vitamin D also explains the biosynthesis of oncogenes, polyamines, lymphokines and calcium binding proteins. However, there is a possibility that some of the actions of 1,25(OH)2D3 may be mediated by non-genomic mechanisms and may not require the binding to Vitamin D receptor (VDR). Vitamin D offers a protection from genotoxic effects of Vitamin D deficiency by increasing the insulin receptor gene expres-sion and BSP (bone sialoprotein), bone-remodeling by decreasing the osteopontin (OPN) m-RNAs, maintaining the normal epidermal structure and enamel matrix. Further role of Vitamin D is envisaged in identifying cyclin C as an important target for Vitamin D in cell-cycle regulation [20].

More importantly, for the aim of the present paper, Vitamin D at physiological concentration has been found to protect cell proteins and membranes against oxidative stress by inhibiting the peroxidative attack on membrane lipids [21–24].

Free radicals generated from intermediates of metabolism are highly reactive since they contain non-paired electrons. These reactive species are capable of initiating lipid peroxidation by reaction with polyunsaturated fatty acids, inactivating proteins and enzymes by reacting with amino acid and damaging RNA and DNA by reacting with guanine. If the cell is insufficiently protected by enzymatic and non-enzymatic antioxidants, free radicals can react with biomolecules and thus damage cellular structure. Antioxidants may prevent genetic changes by pre-venting DNA damage directly induced by free radical attack. Immuno-enhancing mechanism of Vitamin D could lower immunosuppressive lipid peroxides, stabilize lysosomal membrane and protect nuclear structure. It is believed that presence of extensive systems of conjugate double bonds in a drug molecule is responsible for impairing antioxidant properties of the compound [21]. The ability of Vitamin D to inhibit iron-dependent lipid peroxidation in liposomes has been discussed by Wiseman [22]. Wilson has studied the critical role of Vitamin D in free radical induced biological damage [23]. Finally, Sardar et al. have documented Vitamin D as an effective in vivo antioxidant effect on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague–Dawley rats [24].
The overall research aim of the present paper is to introduce a new approach for the integrated immunoprotection of the transplanted islets, irrespective of the islet origin and maturation level. In this respect, we describe here newly designed microcapsules, basically formulated with the well-validated polymer alginate coated with polyornithine, but containing multiple compartments that can interact with the encapsulated cell. In this approach, the “islet compartment” will benefit from a “chemical/pharmacologic agent compartment” constituted of a sustained release formulation able to deliver the included biological response modifiers for long periods of time. In fact, different biological modifiers (with special regard to those associated with antioxidizing or immunomodulating properties) have individually shown to improve the islet viability and/or functions.

Multifunctional microcapsules, while maintaining immunobarrier properties, should also favor retention of the islet cell viability and functional competence. Although apparently complex, multifunctional microcapsules can be fabricated, as demonstrated here on a reproducible basis and they are expected to constitute a new and more effective type of bioartificial pancreas.

Finally, we would like to underline that throughout the paper text we use the term “multifunctional” since the entire alginate capsule is able to give different function to the graft. “multifunctional” related to the final task which the microcapsules should be entrusted with, namely: (a) insulin release: from the entrapped islets; (b) immunobarrier: with the external polyornithine layer, the multifunctional microcapsule offers the immunoprotective function allowing the graft implantation and survival without treatment of the recipients with immunosuppressive protocols; (c) mechanical support: the internal alginate matrix give raise to a mechanical support to the encapsulated cell, this feature it has been demonstrated to prolong both the functionality and viability of pancreatic B cells; finally (d) sustained release: the co-encapsulated cellulose acetate (CA) microspheres allow the sustained release of the Vitamin D$_3$ that gives to the encapsulated islets a prolonged viability and superior ability to produce insulin.

The adjective “multifunctional” reflects thus the final aim associated with use of the microcapsules: anti-rejection immunobarrier for transplant and anti-oxidizing agent for the encapsulated islets.

2. Experimental section

2.1. Materials and donor animals

CA was a gift from Eastman Chemical Company (Kingsport, TN, USA). Male Sprague/Dawley (S/D) rats, weighing approximately 150 g, were supplied from Charles River (Charles River Laboratories, Wilmington, MA, USA). Vitamin D$_3$ was purchased from Fluka (Buchs, Switzerland). When not specified, all other chemicals and reagent were of the highest purity grade commercially available.

2.2. Production of Vitamin D$_3$ containing CA microspheres

Microspheres were prepared, as follows, by an oil-in-water emulsion (o/w) method. CA was dissolved in CH$_2$Cl$_2$, and after solubilization of Vitamin D$_3$, the organic solution was emulsified with an aqueous phase containing hydrolysed poly(vinyl alcohol) (PVA) (Airvol 205, Air Products Corp., PA, USA) as stabilizer. The obtained emulsion was maintained under continuous stirring with a four-blade turbine impeller, typically at 750 rpm. At different time intervals, samples were observed microscopically throughout complete evaporation of CH$_2$Cl$_2$, usually occurring in 3–5 h.

2.3. Microparticle morphology

Microparticle morphology, size and size distribution were evaluated under light as well as electron microscopy examination, considering at least 300 particles/batch. In order to study the internal morphology, dried microparticles were sectioned under a binocular microscope. Sectioned particles were gold coated (Edwards Sputter coating S150). Internal and external morphology was analyzed at 15–20 kV by a scanning electron microscope (Cambridge S 360).

2.4. Microparticle recovery

Microparticle recovery efficiencies were calculated as percentage of weight of the obtained microparticles taking as reference the total amount of polymer used for the preparation.

2.5. Drug content of microparticles

In order to determine the amount of Vitamin D$_3$ per unit weight of microparticle 10 mg of CA microparticles were dissolved in 1 ml of CH$_2$Cl$_2$, vitamin concentration was determined by UV spectrophotometric analysis at 242 nm taking as reference a vitamin calibration curve previously made.

2.6. In vitro drug release studies

In vitro release of Vitamin D$_3$ was performed by dialysis method as previously reported. One hundred milligrams of microspheres were introduced into a dialysis tube (molecular weight cutoff 10 000–12 000
plets were allowed to fall into an isotonic CaCl₂ solution supplemented of 5% (w/v) tween 80, as solubilizing agent. One millilitre samples were removed at various time and analysed spectrophotometrically for Vitamin D₃ content with an UV-VIS Spectrophotometer Specord M 42 (Karl Zeiss Jena, Germany) using a previously constructed calibration curve. The same volume of fresh receiving medium was added to replace the volume of the withdrawn samples.

2.7. Adult rat pancreatic islet isolation and in vitro culture maintenance

Rat pancreatic islets (RPI) were isolated according to original methods [25] slightly modified in our laboratory, from male Sprague/Dawley (S/D) rats, weighing approximately 120–130 g each. Briefly, the rats after general anesthesia, and upon laparotomy were injected with a collagenase solution in the common bile duct previously cannulated by an indwelling polyethylene catether, after the duct had been ligated at the merging point with the duodenum, so as to permit retrograde distention of the pancreas. The pancreas, carefully detached from the small and large intestine, stomach, spleen and lymphonodes, was removed. Pancreas digestion went on at 37°C, under gentle shaking, until the pancreatic tissue came finely apart. After several washes, the digested tissue was then centrifuged through Eurocollins/Ficoll density gradients (the islets were collected at the 1.096–1.060 interface). The resulting pancreatic islets were intact, viable and fairly pure.

2.8. Production of alginate multifunctional microencapsules for pancreatic islet encapsulation

RPI were suspended into 1 ml of a 1.6% solution of sodium alginate. The cell suspension was then extruded through a droplet generator, based on a air-driven jethead, which resulted in the continuous formation of spherical droplets entrapping the islets. In the particular case of the multicompartimental microcapsules, CA microspheres (containing Vitamin D₃) were added to the islet/alginate suspension. Optimal results were obtained with the following experimental parameters: air flow: 31/min; pumping rate of the islet suspension: 15 ml/min; alginate temperature: 10°C. The generated microdroplets were allowed to fall into an isotonic CaCl₂ solution placed at 3–4 cm from the jethead.

The resulting simple or multifunctional calcium alginate microspheres were washed twice with isotonic solution and placed in contact for 10 min with a 0.12% solution of poly l-ornithine (PLO) (MW 17 kDa, Sigma) in saline. The interaction between the negatively charged carboxyl groups exposed on the surface of the alginate capsules, and the cationic functions on the PLO side chains resulted in the formation of a polyionic hydrogel membrane surrounding the capsules. The microcapsules were then overlayered with PLO at lower concentration (0.04%) and finally, with a diluted solution of sodium alginate (0.04%) which formed in the outer coat of the alginate–polycornithine microcapsules (Ag/PLO Caps).

2.9. Vitamin treatment

Batches of 20 RPI were in vitro cultured for 9 days in 2 ml of HAM F12 medium (Euroclone Ltd.), supplemented with 10 mM Nicotinamide (Sigma Aldrich), 3-isobutyl-1-methylxhantine, IBMX (Sigma Aldrich), 10% foetal bovine serum (Euroclone Ltd.), and antibiotics Pen-Strep (Sigma Aldrich) in a 24 wells culture plate at 37°C in air/CO₂ 95%. Medium was changed and collected for insulin determination at days 3, 6 and 9. The amount of free Vitamin D₃ was added daily.

During the experiment RPI were cultured in the following conditions: (a) control untreated free RPI; (b) free RPI in the presence of 2 μM free Vitamin D₃ (added daily), (c) free RPI in the presence of encapsulated Vitamin D₃ (20 μM) in CA microspheres (added only at the beginning of the experiment), (d) control RPI encapsulated in Ag/PLO micropcapsules, (e) RPI encapsulated in Ag/PLO micropcapsules, in the presence of encapsulated Vitamin D₃ (20 μM) in CA microspheres and finally (f) RPI co-encapsulated in Ag/PLO multifunctional microspheres containing Vitamin D₃ (20 μM) in CA microspheres.

3. Results

3.1. Preparation and characterization of CA microspheres (co-embodied microspheres)

The aim of the first part of the present study was to investigate the experimental parameters controlling the procedure of vitamin encapsulation in CA microparticles. Namely, microparticle morphology, size and encapsulation efficiency were investigated. These parameters are particularly important since the small volume of the location site (within the external alginate capsule) can only accept a limited amount of microparticles. On the other hand, microparticles should contain sufficiently high proportion of vitamin to assure long-term local drug delivery and extracellular concentration within the therapeutic protective range.

For this purpose, for microparticle preparation, CA was chosen as a highly lipophilic polymer providing (in reason of its very low swellability and solubility in water), for long-term release.

CA microspheres were prepared by an in-liquid-drying process (solvent evaporation) as described in
the Section 2. The choice and the adjustment of the manufacturing parameters for the preparation of microspheres of defined size were performed according to the following general equation [21]:

\[
d \propto K \frac{D_v R}{N \eta_a \eta_o C_s}\]

where \(d\) is the average particle size, \(K\) is a variable depending on the apparatus geometry (e.g. type and dimension of stirrer), \(D_v\) and \(D_s\) are the diameter of the vessel and of the stirrer, respectively, \(R\) is the volume ratio between aqueous and oil phases, \(\eta_a\) and \(\eta_o\) their respective viscosities, \(N\) is the stirring speed, \(\gamma\) is the surface tension between the two immiscible phases and \(C_s\) is the stabilizer concentration.

Microparticles of size compatible with the encapsulation within the external alginate layer (diameter 30–70 \(\mu\)m), were obtained by using the following experimental parameters: 50 mm diameter vessel, 35 mm four-blade turbine rotor, and a volume ratio of polymeric solution to aqueous phase of 0.21. In addition, other variables such as type of oil and water phases, stirring speed, polymer concentration and surfactant type and concentration were analysed. The effects of these variables on morphological characteristics and encapsulation efficiency of microspheres are described in the following sections.

Light and scanning electron microscopy were employed to analyse the external and internal structure of the microparticles since it is well known that the microcapsule’s morphology affects the release of the contained drug and their in vivo general performances. Fig. 1 depicts electron micrographs of empty CA microparticles (Panel A) and those containing Vitamin D \(_3\) (Panels B and C). As clearly evident, the preparation procedure did not lead to aggregation/agglomeration of the microparticles that on the contrary showed regular spherical geometry. Moreover, the internal morphology of the microspheres demonstrated that the particles were compact and homogeneous with the drug appearing molecularly dispersed into the polymer matrix (see higher magnification in Panel B).

### 3.2. Type of organic solvent

The choice of the organic solvent to dissolve the polymer was found to play a key role either to obtain microparticles or to regulate their general morphological characteristics, recovery and aggregate formation. As preliminary experiment, solubility of CA, in different organic solvents or solvent mixtures (at different ratios) was investigated (including: chloroform, chloroform/acetone, chloroform/acetonitrile, chloroform/propanol, ethyl acetate, diethyl ether, dichloromethane, dichloromethane/chloroform. Special attention was paid to finding solvent mixtures resulting in low viscosity solutions, since it was observed that highly viscous polymeric solutions, invariably failed to yield satisfactory microparticles, always resulting in large clusters or fused material in form of blobs or filaments (see Fig. 2, Panels B and C). A complete series of experiments was performed using pure solvents (i.e. chloroform, methylene chloride, acetone, ethyl acetate and isopropanol) as well as their binary mixture in different proportions. In only few cases (see list in Table 1), we succeeded in obtaining particles with acceptable morphological characteristics, while with the majority of solvent mixtures it was impossible to isolate particles of acceptable quality. For instance, a mixture 50:50 (%, v/v) \(\text{CH}_2\text{Cl}_2/\text{CHCl}_3\)
was found to yield the best results in terms of microsphere quality (see comments in Table 1 and Fig. 2, Panel A), as compared to other tested pure solvents or mixtures in different proportions of chlorinated solvents, ethyl acetate, acetone and isopropanol.

### 3.3. Stirring speed

Since our aim was to obtain microspheres to be further encapsulated in alginate beads, we modulated the stirring speed, and succeeded in obtaining microspheres within suitable dimensional range by using 1.0 g of polymer and a stirring speed of 750 rpm in the presence of 2% PVA solution as an emulsion stabilizer. In fact, these conditions resulted in the formation of microspheres with an average diameter of 53 ± 8 μm (see Table 2 and Fig. 3). Nevertheless, we found that by varying the stirring speed (500 or 1000 rpm), particles with diameter ranging from 17 ± 5 to 104 ± 6 μm, and still suitable for preparation of multicompartmental capsules or potentially, for administration throughout other routes such as oral could be obtained.

### 3.4. Polymer concentration

By changing the amount of CA used for microsphere preparation, a progressive modification in surface characteristics and size distribution (see Fig. 3B) was observed. Relevant changes of the microspheres’ surface were clearly appreciable by scanning electron microscopic analysis, for instance, the surface of microspheres prepared with 2.0 or 3.0 g (see Fig. 3 Panel C) of polymer showed a more pronounced roughness.

### 3.5. Stabilizer concentration

Finally, we tested the effects of different PVA concentrations, namely 0.5, 1 and 2% (w/v), on the size as well as size distribution of the microspheres prepared with 1.0 g of CA and a stirring speed of 750 rpm. As clearly appreciable from the obtained results (see size distributions in Fig. 3D), the addition of progressively larger amount of the polymeric emulsifier, led to stabilization of the emulsion and to reduction in size of the polymer solution droplets, during the emulsification step. Both these effects allowed for significant improvement in the final microspheres quality and monodispersity, together with a size decrease.

### 3.6. Drug encapsulation efficiency

With respect to the encapsulation efficiency exploited by CA microspheres, the crucial role of the chemical–physical characteristics of the employed drug should be stressed with care been taken to assess the hydrophobic–hydrophilic balance of the drug’s molecule. Indeed, hydrophobic drugs can be quantitatively incorporated in microspheres, whilst molecules with hydrophilic portions display a much reduced trapping efficiency (data not shown). For instance, Vitamin D₃ that is highly lipophilic and almost water insoluble, displayed a high encapsulation efficiency (> 95%) in all CA microsphere formulation tested. In particular, the encapsulation efficiency displayed by the optimized formulation for CA microspheres production (Mcc 2 in Table 2) was of 96.4% ± 6 (n = 3 ± SD).
3.7. In vitro release characteristics

The experimental set up and the data analyses of release profile determinations need a short premise for the correct interpretation of the results.

In fact, low-solubility drugs (as Vitamin D₃) are lipophilic and thus the drug solubilization in the receiving buffer usually represents the rate limiting process other than the release of the compound from the microparticle.

In vivo, the dissolution process depends on many and more complex physicochemical parameters that characterize the microenvironment where the release process occurs, such as the presence of cells with their membranes (where the lipophilic compound could possibly intercalate), lipoproteins, fat tissues, binding-carrier proteins, and target and metabolic enzymes, which deeply and clearly affect the solubility of lipophilic compounds and thus the drug release from the microparticles.

In vitro, the situation is much simpler and the release of drug from microparticles depends (a) on the drug product and (b) the dissolution test conditions such as buffer composition, volume of dissolution medium, pH, type of apparatus and agitation.

Form what stated above it is thus clear that the data obtained form in vitro experiments can only give partial and not predictive results of the real in vivo performance offered by the controlled release formulation. Therefore it is much more informative to perform, at least, ex vivo experiments as reported in the further paragraphs of the paper.

In developing a dissolution test for sparingly soluble or water-insoluble drug products, a variety of mechanisms have been used to increase drug solubility, including adding alcohol, other organic solvents and/or surfactants to the aqueous media, increasing the volume of the dissolution medium, and increasing the rate of agitation.

For determining the release profile of Vitamin D₃ from CA microsphere, we developed a specific release

| Effect of the solvent(s) used for polymer dissolution on the characteristic of cellulose acetate microspheres |
|---|---|---|---|---|
| T (°C) | Solvents (%), v/v | Stabilizer (%), v/v | Recovery (%) | Microsphere general morphology |
| RT | CH₂Cl₂/IP (95:5) | PVA (2) | 65 ± 1 | Microspheres were spherical and smooth but highly polydisperse; some filaments were present |
| RT | CH₂Cl₂/acetone (95:5) | PVA (2) | 59 ± 7 | Microspheres were irregular and highly polydisperse; aggregated particles and filaments were present |
| RT | EA/ethanol (90:10) | PVA (2) | 72 ± 5 | Microspheres were mainly elliptical or elongated; filaments were present |
| RT | EA/acetone (85:15) | PVA (2) | 72 ± 6 | Microspheres were not perfectly spherical and with an irregular surface, polydisperse |
| RT | CHCl₃/IP (95:5) | PVA (2) | 81 ± 3 | Microspheres were spherical and smooth; some aggregates were present |
| RT | CHCl₃/acetone (95:5) | PVA (2) | 79 ± 3 | Microspheres were spherical with a reflective surface; polydisperse |
| RT | CHCl₃/acetone (90:10) | PVA (2) | 78 ± 5 | Microspheres were spherical with a reflective surface; polydisperse |
| 50 (after 1 h) | CH₂Cl₂/IP (95:5) | PVA (2) | 51 ± 6 | Microspheres were spherical and smooth some of them were elongated; some aggregates were also present |
| 50 (after 1 h) | CH₂Cl₂/IP (90:10) | PVA (2) | 76 ± 1 | Microspheres were smooth but elongated and not homogeneous; some aggregates were present |
| 50 (after 1 h) | CH₂Cl₂/IP (90:10) | PVA (2) | 89 ± 8 | Microspheres were small, smooth but rather polydisperse, some aggregates were present |
| RT | CH₂Cl₂/CHCl₃ (50:50) | PVA (2) | 81 ± 2 | Microspheres were spherical, smooth, monodisperse and small; no aggregates were present |

Data represent the mean of three independent batches ± SD.

IP: Isopropanol; EA: Ethyl acetate; microsphere were produced at the indicated temperatures with a stirring speed of 750 rpm. PVA: hydrolysed poly(vinyl alcohol).

*Percentage (w/w) of isolated microparticles with respect to the starting amount of polymer utilized for microparticle preparation.*
buffer, defined after having preliminarily analysed the effect of (a) pH (using standard aqueous dissolution media as listed in the USP, including 0.1 N HCl, pH 4.5 sodium acetate buffer and pH 6.8 phosphate buffer); (b) type and concentration of surfactant (selecting from non-ionic surfactants, at 1–5%, w/v).

After performing the above step, sufficient information were available on the effects of pH, surfactant type and concentration.
and concentration to determine an acceptable release buffer with the following final composition: isotonic Palitsch buffer (borate buffer, pH 7.4)/ethanol 85:15 (v/v) containing 1.5% tween 85.

Data from Fig. 4 demonstrate that highly lipophilic compact CA microspheres are characterised by a rather slow release profile. After 30h, only 32% of Vitamin D3 was released from microparticles.

3.8. Production of alginate multicompartimental microcapsules

Alginate microcapsules were prepared by an atomizing method (see details in Section 2) resulting in the production of “medium size” microcapsules that were extremely monodisperse with an average diameter of 496.7 ± 12.5 μm (n = 3 ± SD). The production procedure adopted was relatively simple and consisted of a limited number of steps. In order to achieve complete biocompatibility, indispensable for mammalian cells, the encapsulation procedure was conducted at room temperature under physiologic pH and tonicity using a pyrogen-free alginate solution. The resulting calcium alginate multifunctional microcapsules were coated with poly l-ornithine (PLO). The interaction between the negatively charged carboxyl group on the surface of the alginate capsules and the cationic functions on the PLO side chains gave rise to a polyionic hydrogel membrane surrounding the capsules. The microcapsules were then covered by a double PLO layer and then treated with a sodium alginate diluted solution to form an outer coat (see scheme in Fig. 5A). The final microcapsules resulted elastic and transparent thus facilitating the microscopic observation of the islet viability and morphology during the in vitro studies. Microcapsules were assessed by means of optical microscopy, reported in Figs. 5B and C, for analysing the morphological and dimensional characteristics of the capsules as well as the integrity of the coating.

3.9. Effects of vitamin D3 on insulin secretion

In order to assess RPI functional competence, upon 9 days of culture with or without vitamins, insulin levels were measured. Data reported by Fig. 6 showed that the amount of insulin secreted by islets co-cultured with free Vitamin D3 and, particularly, with Vitamin D3
entrapped in CA microspheres, was significantly higher as compared to the islets alone (Panel A). Moreover in vitro insulin release from AG/PLO microencapsulated islets plus Vitamin D3 entrapped in CA microspheres and, particularly, from islets co-microencapsulated with Vitamin D3 in CA microspheres, was significantly
Fig. 7. In vitro insulin production from RPI before and after cell lysis at day 9 of culture. Panel A: insulin released by free RPI. Control untreated RPI (■); RPI cultivated in the presence of 2 μM free Vitamin D₃ (□); RPI cultivated in the presence of encapsulated Vitamin D₃ (20 μM) (▲). Panel B: insulin release by in vitro cultured microencapsulated RPI in Ag/PLO microcapsules. Control untreated RPI in Ag/PLO microcapsules (■); RPI in Ag/PLO microcapsules cultivated in the presence of encapsulated Vitamin D₃ (20 μM) (□); RPI co-microencapsulated in multifunctional Ag/PLO microcapsules containing Vitamin D₃ in CA microspheres (▲). RPI were in vitro cultured for 9 days in 2 ml of HAM F12 medium (Euroclone Ltd.), supplemented with 10 mM Nicotinamide (Sigma Aldrich), 3-isobutyl-1-methylxanthine, IBMX (Sigma Aldrich), 10% foetal bovine serum (Euroclone Ltd.), and antibiotics Pen-Strep (Sigma Aldrich) in a 24 wells culture plate at 37°C in air/CO₂ 95%. Medium was changed at days 3, 6 and 9. The amount of free Vitamin D₃ was added daily. Data represent the mean of five independent cells batches, each insulin determination was performed in triplicate ± SD.

t-test for Insulin release under static glucose incubation on day 6 (300 mg/dl)

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<th>Condition</th>
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<th>Islets + Vit D₃ microspheres</th>
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Fig. 8. In vitro insulin release from RPI, under static glucose incubation at the indicated concentrations, at day 6 of cell culture. Panel A: insulin release by free RPI. Control untreated RPI (■); RPI cultivated in the presence of 2 μM free Vitamin D₃ (□); RPI cultivated in the presence of encapsulated Vitamin D₃ (20 μM) (▲). Panel B: insulin release by in vitro cultured microencapsulated RPI in Ag/PLO microcapsules. Control untreated RPI in Ag/PLO microcapsules (■); RPI in Ag/PLO microcapsules cultivated in the presence of encapsulated Vitamin D₃ (20 μM) (□); RPI co-microencapsulated in multifunctional Ag/PLO microcapsules containing Vitamin D₃ in CA microsphere (●). RPI were in vitro cultured for 6 days in 2 ml of HAM F12 medium (Euroclone Ltd.), supplemented with 10 mM Nicotinamide (Sigma Aldrich), 3-isobutyl-1-methylxanthine, IBMX (Sigma Aldrich), 10% foetal bovine serum (Euroclone Ltd.), and antibiotics Pen-Strep (Sigma Aldrich) in a 24 wells culture plate at 37°C in air/CO₂ 95%. At day 6 RPI were treated with the indicated amount of glucose for 20 h, thereafter medium was collected for insulin determination. The amount of free Vitamin D₃ was added daily. Data represent the mean of five independent cells batches, each insulin determination was performed in triplicate ± SD.
higher in comparison with microencapsulated islets alone (Panel B).

Fig. 7 (Panel A) shows in vitro insulin release before and after islets lysis at day 9 of culture of islets alone, islets plus free vitamin D₃ and islets plus vitamin D₃ entrapped in CA microspheres; Panel B shows in vitro insulin release, before and after islets lysis at day 9 of culture, of NAG/PLO microencapsulated islets, NAG/PLO microencapsulated islets plus vitamin D₃ entrapped in CA microspheres, and co-microencapsulated islets plus vitamin D₃ entrapped in CA microspheres.

RPI, after 6 days of culture with or without vitamin, were compared for the amount of released insulin at low (50 mg/dl/h) or high (300 mg/dl/h) glucose concentrations. The results of this experiment are reported by Fig. 8.

As expected, incubation with high glucose, increased the RPI’s insulin secretory rate; this effect was further magnified by the addition of islets plus free vitamin D₃ and, particularly, islets plus vitamin D₃ entrapped in CA microspheres (Panel A); Panel B shows that in vitro insulin release of NAG/PLO microencapsulated islets plus vitamin D₃ entrapped in CA microspheres and, particularly, when the islets were co-microencapsulated islets with vitamin D₃ entrapped in CA microspheres, was significantly higher as compared to microencapsulated islets alone.

This final data suggests that vitamin D₃ could be eventually used in order to obtain a beneficial, possible synergistic effect on insulin output of the adult RPI.

4. Discussion

Culture maintenance of isolated islets in general and porcine islets in particular, poses a numbers of problems (19). Although neonatal might offer significant advantages over adult pig islets, in terms of retention of in vitro morphologic integrity, near to 50% of the islets will anyway lose their viability within 24–48 h of the isolation. For this specific reason, some groups have recently chosen to transplant human islets with no culture after isolation (1). Nonetheless, this option could be riskful, since appropriate, post-isolation microbiologic monitoring of the islets would better comply with safety requirements. Therefore “ad hoc” methods to implement in vitro culture maintenance of the freshly isolated islet cells look most timely and useful at this time. Success of the in vitro procedure could, in turn, favourably affect the in vivo islet graft survival.

On a different matter, that is not directly touched by this report, islet allograft survival is clearly dependent on how efficiently the anti-immune rejection measures, based upon general immunosuppression of the recipients will perform. Obviously this is even more stringent for islet xenografts, where the host immune reaction is much stronger. In this instance, it is likely that only physical separation of the grafted islets from the host’s immune system could be effective. Islet microencapsulation have indeed shown to prevent rejection of islet xenografts in a number of diabetic animal models.

However, evidence can now be marshalled upon the fact that other events that are not immune-related, such as cell apoptosis, might adversely affect the islet graft outcome. Combination of these with immune events will dramatically curtail the islet graft functional life span.

In this work we have addressed to examine the effects of anti-oxidizing agents such as vitamins D₃ on RPIs in vitro function. Our preliminary results seem to demonstrate that the vitamins may improve the in vitro islet cell performance, possibly confirming the beneficial effects of anti-oxidants on cell apoptosis. While is still premature to speculate on the impact of these findings on islet transplantation which will, per se, warrant “ad hoc” study, introduction of vitamins D₃ in the in vitro standard islet culture maintenance protocols appears to be meaningful. In vivo graft trials with vitamin pre-incubated, free or microencapsulated islets will unfold whether these anti-oxidizing agents may significantly prolong the islet transplant longevity.

5. Conclusion

We have preliminarily observed that treatment with vitamin D₃ both free and, even better, entrapped in CA microspheres, significantly increased glucose stimulated insulin output as compared to untreated controls, thus confirming beneficial effects of anti-oxidizing agents on islet cell viability and function.

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References


