**Ursodeoxycholic acid effect on polymer-hydrogel system of transplantable NIT-1 cells**

Armin Mooranian[1], Rebecca Negrulj[1], Hani Al-Salami[1] *

[1] Biotechnology and Drug Development Research Laboratory, School of Pharmacy, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia

**Corresponding author:**

Dr Hani Al-Salami
Senior Lecturer of Pharmaceutics, School of Pharmacy
Curtin University
Tel | + 61 8 9266 9816
Fax | + 61 8 9266 2769
Email | hani.al-salami@curtin.edu.au
Profile | https://healthsciences.curtin.edu.au/research/biotechnology/index.cfm

**Abstract**

**Introduction:** The encapsulation of pancreatic β-cells in biocompatible matrix has generated great interest in diabetes treatment. Our work has shown improved microcapsules when incorporating the bile acid ursodeoxycholic acid (UDCA), in terms of morphology and cell viability although cell survival remained low (<28%). Thus, the study aimed at incorporating the polyelectrolytes polyallylamine (PAA) and poly-l-ornithine (PLO), with the polymer sodium alginate (SA) and the hydrogel ultrasonic gel (USG) with UDCA and examined cell viability and functionality post microencapsulation.

**Methods:** Microcapsules without (control) and with UDCA (test) were produced using PLO, PAA, SA and USG. Pancreatic β-cells were microencapsulated and the microcapsules' morphology, surface components, cellular and bile acid distribution, osmotic and mechanical stability as well as biocompatibilities, insulin production, bioenergetics and the inflammatory response were tested.

**Results:** Incorporation of UDCA increased cell survival ($p<0.01$), insulin production ($p<0.01$), reduced the inflammatory profile (TNF-α, IFN-ϒ, IL-6 and IL-1β; $p<0.01$) and improved the microcapsule physical and mechanical strength ($p<0.01$).

**Conclusion:** β-cell microencapsulation using PLO, PAA, SA, USG and the bile acid UDCA has good potential in cell transplantation and diabetes treatment.

**Keywords: pancreatic β-cells, bioenergetics, ursodeoxycholic acid, artificial cell microencapsulation**

**Abbreviations**

DM: Diabetes Mellitus

T1D: Type 1 Diabetes Mellitus

T2D: Type 2 Diabetes Mellitus

ACMT: Artificial Cell Microencapsulation Technology

USG: Ultrasonic Gel

PLO: Poly-l-Ornithine

SA: Sodium Alginate

PAA: Polyallylamine

UDCA: Ursodeoxycholic Acid

DMEM: Dulbecco's Modified Eagle's Medium

OM: Optical Microscopy

SEM: Scanning Electron Microscopy

EDXR: Energy Dispersive X-Ray Spectroscopy

TRITC: Tetramethylrhodamine Isothiocyanate

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

OCR: Oxygen Consumption Rate

ECAR: Extracellular Acidification Rate

PPR: Proton Production Rate

BR: Basal Respiration

MR: Maximal Respiration

PL: Proton Leak

ATPP: Adenosine Triphosphate Production

CE: Coupling Efficiency

SRC: Spare Respiratory Capacity

G: Glycolysis

NGD-ECAR: Non-Glucose-Derived Extracellular Acidification Rate

ELISA: Enzyme-Linked Immunosorbent Assay

CBA: Cytokine Bead Array

TNF-α: Tumour Necrosis Factor-α

IFN-γ: Interferon-γ

IL-1β: Interleukin-1β

IL-6: Interleukin-6

## Introduction

Diabetes mellitus (DM) is a chronic disease which is characterised by uncontrolled blood glucose and inflammation (1, 2). DM is classified as type 1 diabetes (T1D) which is an autoimmune disease and type 2 diabetes (T2D) which is a caused by metabolic and genetic factors (1, 2). T1D and T2D have two main common symptoms, glucose disturbances and inflammation (3). Insulin has shown to possess anti-inflammatory effects as well as being the most effective drug to control glycaemia in diabetic patients (4, 5). Accordingly, to ensure continuous and optimal supply of insulin to diabetic patients, various approaches to supply insulin have been examined (4, 5). Insulin pumps and transplanted devices filled with reservoirs of insulin have been implanted and tested in diabetes patients (6). The aim has been to be able to design a system that can secrete insulin as a response to an increase in glucose levels and the system has sufficient insulin to control glycaemia, permanently (7). One approach is transplanting the cells that can manufacture insulin (known as β-cells of the pancreas). In order to transplant these cells, special devices are needed that are capable to physically carry enough cells, be able to function permanently, have permeable membrane that allow nutrients and oxygen to diffuse but not macrophage and immune cells and being able to stay intact and not trigger any immune response post transplantation (8-11). This can be achieved by using artificial cell microencapsulation technology (ACMT) (12).

ACMT has been used for decades to deliver cells and enzymes (12). Suitable and immunostatic microcapsule need to be large enough to carry cells, strong enough to protect these cells and permeable enough to allow nutrients and oxygen to diffuse through the capsules' membrane and support biological activity of the cells. One of the most commonly used polymer in cell microencapsulation is sodium alginate (SA) due to its good biocompatibility and biodegradability characteristics (13). However, encapsulating cells using only SA for cell transplantation produces microcapsules with many limitations. The limitations of the microcapsules are mainly poor mechanical strength, weak membrane, and uneven membrane porosity and density, which result in membrane deformation, cell leakage and sudden rupture of microcapsules (14). In order to overcome the limitations of SA-microcapsules, additional excipients have been incorporated into the microencapsulating formulation. Various excipients have been shown to enhance the physical structure and mechanical strength of the microcapsules, however, cell viability remains low, especially in the long-term (15). Excipients such as the cationic polyelectrolyte polyallylamine (PAA) and the hydrogel polyethylene glycol (the major component of ultrasonic gel; USG) are anticipated to enhance microcapsule formulation and stability (15-17). PAA and USG interaction with the alginate polymer may form solid gels with enhanced physical structure (14, 15). However, there are many limitations to incorporating USG and PAA, into SA-microcapsules. PAA has been shown to attract inflammatory cells due to its cationic nature (13-15). In addition, PAA in desirable concentrations, may significantly reduce the size of pores of microcapsule's membrane resulting in hypoxic stress, reduced exchange of nutrients and wastes and consequent cell death (15).

The major component of USG is polyethylene glycol (18) and this has been shown to be less immunogenic and more hydrophilic than PAA and thus lowers the risk of immune system activation and

irritation post-transplantation (19). However, USG lacks adequate structural integrity and long term degradation in aqueous environments is a possibility (19).

Clearly, in addition to SA, USG and PAA, there is need for an excipient that can optimise microcapsule stability without compromising cell viability or functions. Ideally, an excipient is required that has been shown to support microcapsules' physical structure and mechanical strength and also support cell viability, functionality and metabolism as well as possess anti-inflammatory effects. An example of such an excipient is the tertiary bile acid, ursodeoxycholic acid (UDCA). In a recent study in our laboratory, UDCA incorporation into SA microcapsules has shown promise in supporting β-cell growth (17).

Accordingly, this study aimed to examine the incorporation of a mixture of ultrasonic gel (USG), poly-L-ornithine (PLO), sodium alginate (SA), polyallylamine (PAA) and the bile acid ursodeoxycholic acid (UDCA), in carrying β-cells and the ability of the microcapsule to maintain cellular functionality in vitro. The study examined the formulation rheology, stability and Zeta potential, the microcapsule structure, morphology, physical strength, the microcapsule size, shape, cell contents, cell viability, functionality, inflammatory profile and insulin secretion post microencapsulation. This study is part of ongoing work in our laboratory investigating microcapsule excipient-cell interactions in terms of using various excipients, at different ratios, in order to optimise cell survival, functionality and inflammatory profile post-microencapsulation (16, 17, 20).

## Materials and Methods

### *β-cell microcapsule preparation*

Cells (generously donated by Professor Grant Morahan at the University of Western Australia) were cultured on T-75 $cm^2$ tissue culture flasks (Thermo Fisher Scientific®, Australia) and fed with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, USA) supplemented with 6 mmol glucose (Sigma Chemical Co, USA), 10% foetal bovine serum (Thermo Fisher Scientific, Australia) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Australia) (21). Sodium alginate (SA, ≥ 99% purity, endotoxin tested, low viscosity with 44% D-mannuronic acid (M), 16% L-guluronic acid (G) and 40% MG-GM block), poly-l-ornithine hydrochloride (PLO; molecular weight 30-70 kDa), ursodeoxycholic acid (UDCA, 99%), calcium chloride dihydrate ($CaCl_2$. $2H_20$, 98%), and poly-allylamine hydrochloride (PAA, molecular weight 58 kDa) were all purchased from Sigma Chemical Co, USA. Ultrasonic gel (USG) was purchased from Australian Medical Association (Australia). All solutions were mixed well using sterilized equipment in ratios: 1.8% SA, 4.5% USG, 1% PLO, 2.5% PAA and 4% UDCA up to 100% in water.

Cells were microencapsulated using an established method (16, 17). Briefly, The β -cells, NIT-1, were incubated in an environment of 5% CO 2 in humidified air at 37°C and the medium was changed every 48 hours. The cells were subcultured weekly by incubating the monolayer with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Australia) for 4 –5 minutes under the same environmental conditions used to culture the cells. The trypsinized cells were added to the

equivalent volume of freshly prepared media and centrifuged at 2500 rpm for 5 min at 25°C using a Beckman Coulter Allegra X-12 centrifuge (Beckman Coulter, USA). The supernatant was then discarded, and cells were resuspended in fresh media ready for microencapsulation under sterile conditions.

Microcapsules were prepared under sterile conditions and Büchi concentric nozzle system was used to form the microcapsules using vibrational frequency of 800 Hz over a period of 10 minutes followed by a lag-period (10 minutes) (16, 17). The concentric nozzle system ensured uniform distribution of β-cells in the mixture. Microcapsules were collected in a hardening bath containing 2% $CaCl_2$ before being transferred into fresh media and incubated for further 48 hours prior to commencing the experiments (16, 17).

***Optical Microscopy (OM)***

Size and shape of the microcapsules were determined with OM utilising Nikon YS2-H optical (light) microscope (Nikon Company, Japan) (16, 17).

***Scanning electron microscopy (SEM) and Energy Dispersive X-Ray (EDXR) spectroscopy***

The surface morphology of the microcapsules was examined using the MIRA3 FE SEM (Tescan, Czech Republic), while the chemical characterisation of the microcapsule surface was examined using the EDXR (Oxford Instruments, INCA X-Act, USA) (22-24).

***Spinning disk confocal scanning microscopy of stained β-cells and chemically conjugated bile acid microcapsules***

Encapsulated NIT-1 cells were stained with the CellTrace carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Kit (Life Technologies, USA) post-trypsinization. Confocal scanning microscopy was carried out using an UltraVIEW Vox spinning disk confocal microscope (Perkin Elmer, USA) equipped with Yokogawa CSU-X1 confocal scanning unit (Perkin Elmer, USA). To ensure optimal encapsulated islet cell viability and utmost sterility during imaging, microcapsules were placed within a fully incubated environmental control unit (fully enclosed microscope and stage unit) enriched with $CO_2$ (10%) and temperature controlled (37 $^0$C). Image analysis was undertaken using the Velocity Multi-Platform 3D Cellular Imaging and Analysis Software (Perkin Elmer, USA) (16).

To determine the partitioning of the bile acid UDCA within all the layers of the microcapsule, a conjugation reaction using a fluorescent compound (tetramethylrhodamine isothiocyanate; TRITC) was done based on the work of Sherman and Fisher (25). Microcapsules were then analysed for their morphology and bile acid distribution/partitioning within the microcapsule matrix via the UltraVIEW Vox spinning disk confocal microscopy (Perkin Elmer, USA) (16, 20). As no NIT-1 cells were stained here and no other substance displayed any fluorescent activity, fluorescent visualization of the microcapsules at 532nm was specific to the TRITC-UDCA conjugate (16, 20).

***Zeta-potential and size analysis***

To determine the electrokinetic stability and size uniformity of the microcapsules, Zeta potential and size distribution for the microencapsulated formulation of F1 and F2 were measured by photon correlation spectroscopy using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK) and by Mie and Fraunhofer scattering technique using Mastersizer 2000 (Malvern Instruments, Malvern, UK) (26).

***Swelling studies***

To determine the swelling properties of the microcapsules, 200 mg dry microcapsules were weighed and placed in 20 ml of phosphate buffer pH 7.4 at a temperature of 37$^{o}$C for 7 days (7, 27, 28). The swollen microcapsules were then removed at periodically predetermined intervals and the swelling index was determined (18, 27).

***Mechanical resistance***

In order to test the mechanical stability of the microcapsules, a mechanical resistance testing was done over a period of 7 days using a Boeco Multishaker PSU 20 (Boeco Company, Germany) (29).

***MTT and bioenergetics assay***

A validated MTT method was used for determining encapsulated cellular activity and viability, as described elsewhere (16, 17). Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared as a 5mg/ml stock solution (Sigma Chemical CO, USA) in phosphate buffer pH 7.4 (Thermo Fisher Scientific, Australia). A well validated method was deployed in order to determine encapsulated cell viability using MTT (16, 17, 30). This method is widely used in the assessment of cell viability within microcapsules without the need to rupture the microcapsules (16, 17, 31, 32). For the MTT assay protocol, 20 µ l of MTT from the stock solution were added into each well of 96-well plates (Thermo Fisher Scientific, Australia) containing freshly made microcapsules that had been placed in 200 µ l of media (pH 7.4) for 48 hours and incubated at 37 ° C with 5% $CO_2$ in humidified air. The MTT conversion to formazan was stopped after 4 hours by washing the microcapsules with MilliQ water for 5 min in order to remove spectroscopic interference. Formazan was dissolved in 100 µL of dimethyl sulfoxide (DMSO) (Sigma Chemical CO, USA) via reverse pipetting and the resultant purple solution was analysed photometrically at 550nm. As this method of determining cell viability is based on calorimetric assessments of mitochondrial activity and does not provide information on cell numbers within microcapsules, it is important to normalise the data for encapsulated cell count using non-calorimetric techniques such as the Trypan Blue assay (16, 17). All data were normalised for live cell count using Trypan Blue selectivity assay via the Countess Automated Cell Counter (Invitrogen, Korea) as previously reported (16, 17). Normalisation of microencapsulated live cell count data using each formulation system allows the effect of excipients on cell viability to be determined based solely on metabolic activity. MTT assessments were also done for free (unmicroencapsulated) cells prior to encapsulation in addition to free cells treated with F1 or F2 formulations as well as free cells treated only with the bile acid UDCA (without the polymer matrix).

Further evaluation of mitochondrial and glycolytic activities of cells, within the microcapsules, including oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and proton production rate (PPR) were carried out, in Real-Time, using an in-house developed method via the Seahorse Flux Analyser XF 96 (Seahorse Bioscience, USA) (16). Various parameters were also measured: Basal Respiration (BR), ATP production (ATPP), Proton Leak (PL), Maximum Respiration (MR), Spare Respiratory Capacity (SRC), Coupling Efficiency (CE), Glycolysis (G) and Non-Glucose-Derived ECAR (NGD-ECAR). Microencapsulated NIT-1 cells were cultured for 48 hours in DMEM as previously described (16). All data were normalised for live cell count using Trypan Blue selectivity assay.

***Insulin and cytokine measurement***

A subset of freshly made microencapsulated NIT-1 pancreatic β-cells from Formulae 1 (control) and 2 (test) were incubated for 48 hours in DMEM medium supplemented with 10% foetal bovine serum, 5% penicillin-streptomycin and 6 mM glucose (33). Aliquots of the media were removed at the end of 48 hours incubation time and secreted insulin content was analytically determined using ultrasensitive mouse insulin ELISA (Mercodia Cooperation, Uppsala, Sweden) (33). Cytokines derived and secreted by encapsulated pancreatic β-cells were measured using BD Biosciences CBA technology (San Jose, California, USA). Freshly made Formulae 1 and 2 microcapsules were cultured for 48 hours and aliquots of the media were removed at the end of 48 hours and analysed. Levels of TNF-α, IFN-γ, IL-1β and IL-6 were measured using Mouse BD Flex Sets (BD Biosciences, San Jose, California, USA) according to the manufacturer's protocols and recommendations. Samples were assayed using an Attune Acoustic Focusing Flow Cytometer (Life Technologies, Carlsbad, California, USA). All data were normalised for live cell count using Trypan Blue selectivity assay.

**Statistical analysis**

The results are expressed as mean ± standard error of the mean from triplicate biological samples of the same batch unless otherwise stated. For statistical analysis, ANOVA with Tukey post-hoc analysis was done, setting the level of significance at $p < 0.05$. All the statistical analysis was done by GraphPad Prism version 6.0 (GraphPad Software, Inc., USA). The results for the *p* value were only reported where significance was noted.

## Results and Discussion

### *Microscopy morphology, size and Zeta potential analysis*

Image analysis of control (Figure 1: a-g) and test (Figure 2: a-l) microcapsules showed uniform and consistent spherical shapes with similar surface composition (mainly Ca and O from SA and $CaCl_2$) and cell distribution within microcapsules. It also showed even UDCA distribution within the microcapsules which is in line with our previously published work (16). The even distribution is anticipated to facilitate UDCA interaction with the microencapsulated cells allowing direct effect on cell viability and also on microcapsule formulation characteristics such as swelling and mechanical strength. It is also apparent that the incorporation of UDCA did not influence the number, distribution and proliferation of viable cells within the microcapsules as microencapsulated cell numbers were similar between F1 and F2 microcapsules (Figure 1: average number of green dots that represent viable cells within each microcapsule formulation are similar). However, the incorporation of UDCA may enhance cellular metabolism, bioenergetics and activity with reduced stress on the cells leading to reductions in cytokine production and enhanced overall biological activity (Table 2). Thus, despite similar live cell counts within the F1 and F2 microcapsules, the incorporation of UDCA to the formulation system could impact on the degree and extent of cellular metabolic and biological activity (Table 2). This is also in line with recent studies that showed dose-dependent positive effects of a UDCA derivative on pancreatic β-cells (34).

Microcapsule physico-chemical stability is also important to consider in the design and evaluation of β-cell microcapsules. Indeed, de Vos et al (35) has demonstrated a strong association between Zeta potential and microcapsule stability by showing that the greater the magnitude of the Zeta potential charge *in vitro* the greater the biocompatibility *in vivo* due to reductions in the inflammatory response post-transplantation. Accordingly, in order to transplant the microcapsules, their surface charge (Zeta potential) and size uniformity are important factors which this study also investigated (Table 1).

Table 1 shows that UDCA incorporation did not alter the microcapsule size and variation in size remained small. Table 1 also shows that UDCA increased the magnitude of the Zeta potential ($p < 0.01$) to -31±2 mV which enhances particle size charges and supports stability of the formulation (35-37). The increase in the negative charge brought about by UDCA may be a direct result from its anionic nature (deprotonation of the carboxylic acid group) in the physiological testing conditions (27, 28).

| | UDCA | | Statistical Analysis (p-value) |
|---|---|---|---|
| | + | - | |
| **Microcapsule Size (µm)** | 996 ± 10 | -17 ± 2 | Not significant |
| **Zeta Potential (mV)** | 995 ± 12 | -31 ± 2 | $p < 0.01$ |

**Table 1: Average microcapsule size and Zeta potential of –ve UDCA (F1) and +ve UDCA (F2) formulations. Data are mean ± standard error of the mean, n=3.**

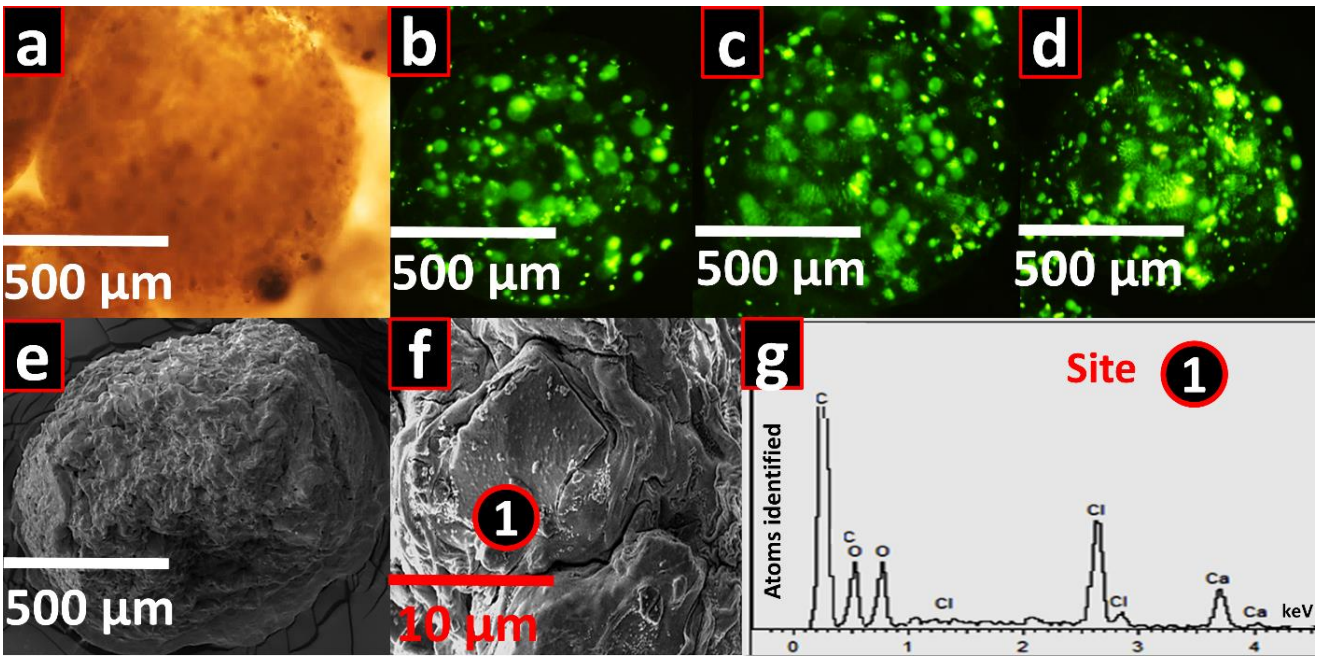


**Figure 1: Micrographs of F1 (control) microcapsules. a: optical image of F1, b-d: confocal images of CFSE stained cells within F1 microcapsules, e: SEM micrograph of F1, f: surface site used for EDXR analysis, g: EDXR spectral analysis.**

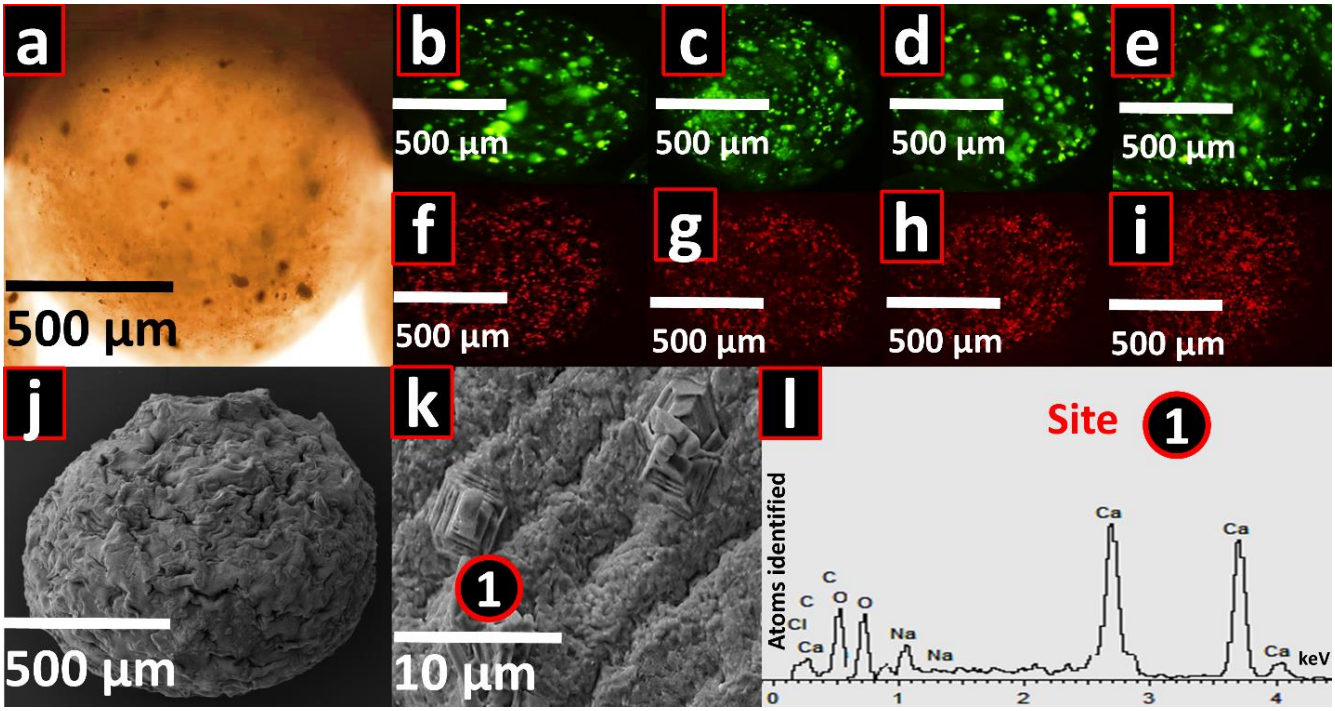


**Figure 2: Micrographs of F2 (control) microcapsules. a: optical image of F2, b-e: confocal images of CFSE stained cells within F2 microcapsules, f-i: confocal images of fluorescent labelled UDCA within F2 microcapsules, j: SEM micrograph of F2, k: surface site used for EDXR analysis, l: EDXR spectral analysis.**

***Osmotic and mechanical stability***

Figure 3 shows that UDCA reduced the swelling ($p < 0.01$) and enhanced the mechanical strength of the microcapsules ($p < 0.01$) after 7 days, supporting the physical properties. The stabilising effects of UDCA on the microcapsule structure may be one of the reasons for its positive effect on cell biological activity, bioenergetics and the inflammatory profile (Table 1). It is also noticeable that, in this formulation, UDCA incorporation did not reduce the membrane porosity of the microcapsules (Figure 1) allowing nutrient and oxygen permeation and waste exchange, thus, not compromising cell activity (Table 1).This is also in line with the bile acid effects reported by Lee et al (34) which showed beneficial effects of a UDCA derivative on β-cell viability and activity brought about by reductions in endoplasmic reticulum stress. Given the beneficial effects of UDCA addition in terms of physico-chemical stabilisation, improvements in β-cell metabolism, respiration, bioenergetics and biological activity could occur and, accordingly, these parameters were evaluated further (Table 2).

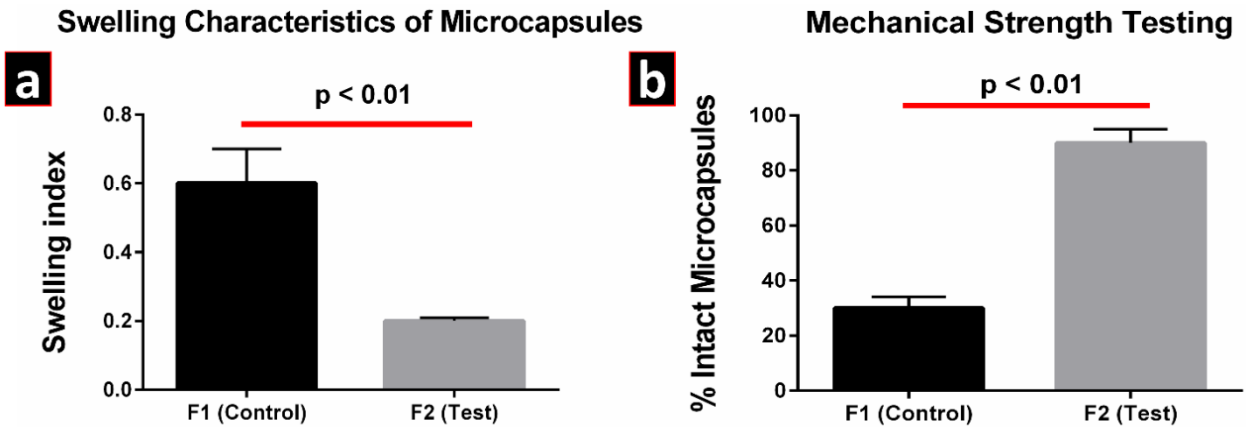


**Figure 3: Swelling characteristics of F1 (control) and F2 (test) microcapsules (a) and mechanical strength testing of F1 (control) and F2 (test) microcapsules (b) at the end of 7 days. Data are mean ± standard error of the mean, n=3.**

***Mitochondrial activity, bioenergetics, cell viability, insulin and cytokine production***

Free (unmicroencapsulated) cells displayed MTT viability at 99 ± 1% and this was not significantly different compared with free cells treated with F1 (99 ± 0.8%) and F2 (97 ± 1.2%) formulations. In addition, free cells treated with only UDCA displayed MTT viability readings of 99 ± 1% which was comparable to free cells pre-microencapsulation as well as free cells treated with either F1 or F2 formulation mixtures. These calorimetric data reveal that neither the formulation matrix system nor the bile acid UDCA have any effect on cellular metabolic activity and any effects noticed would be due to the microencapsulation system enclosing and encapsulating the cells.

Consistent with results from Figure 1 showing even distribution of UDCA within the microcapsules, Table 2 shows improved mitochondrial and cell viability ($p < 0.01$), improved insulin production ($p < 0.01$) and increased cellular bioenergetics, respiration, energy and ATP production ($p < 0.01$) while coupling efficiency remained constant suggesting better cell biological activity without significant cell stress (38, 39). Compared to free cells pre-microencapsulation, there was a drop in viability post-microencapsulation for both formulations and this may be attributed to the microencapsulation process or direct cell-polymer interactions occurring in the suspension before microcapsule formation.

Cell stress can result in higher levels of cytokines involved in inflammatory response (38-40). Table 2 shows that UDCA reduced secretions of cytokines involved in inflammatory response as well as other cellular roles such as proliferation and apoptosis (38-40). Specifically, there were significant reductions in TNF-α ($p < 0.01$), IFN-γ ($p < 0.01$), IL-6 ($p < 0.01$) and IL-1β ($p < 0.01$) secretions. The reduction of these cytokines by UDCA may have been brought about by its direct effects on the cells or as a result of the amplified proliferative cell signalling and reduced apoptosis signalling. UDCA effects on the cytokines may have also been the result of stabilising effects on the microcapsules in terms of providing more

mechanical support, which enhanced cell activity and reduced cell stress and eventual apoptosis. In the literature, UDCA has been shown to have direct and significant anti-inflammatory and anti-apoptotic effects (41-44).

The effects of UDCA on microencapsulated cell viability and activity seem to be strongly influenced by the nature and proportion of excipients such as the polymer, hydrogel or polyelectrolyte system used. Specifically, in a recently published work, UDCA enhanced the metabolic activity of microencapsulated β-cells when added at a concentration of 3% to a formulation system consisting of 10% SA with 1% PLO (17). Interestingly, the addition of 1% UDCA to a more complex formulation system consisting of 1% SA, 4.5% USG, 1% PLO, 0.7% polystyrene sulphonate (PSS) and 2.3% PAA resulted in a drastic reduction in microencapsulated β-cell viability, metabolism and bioenergetics with a negative impact on biological activity (16). It thus appears that the incorporation of UDCA into formulations used to microencapsulate pancreatic β-cells exerts biological effects that are strongly dependant and heavily influenced by the nature and proportions of the polymers, hydrogels and polyelectrolytes used.

Intriguingly, despite the contrasting biological effects of UDCA incorporation into β-cell microcapsules made by varying formulation systems, there are consistent findings in the literature with regards to the microcapsule membrane stabilising effects brought about by bile salts irrespective of the nature of the other excipients used (45). In line with the literature findings, our data supports these effects (Figure 3).

| **Calorimetric Assessment of Microencapsulated β-Cell Viability (Mitochondria-Dependant MTT Assays)** | **Average levels** | | ***Statistical Analysis (p-value)*** |
|---|---|---|---|
| | **F1 (- UDCA)** | **F2 (+ UDCA)** | |
| Microencapsulated Cellular Calorimetric-Based Viability (%) | 31 ± 1.5 | 55 ± 2.5 | $p < 0.01$ |
| **Insulin Secretion** | **Average levels** | | ***Statistical Analysis (p-value)*** |
| | **F1 (- UDCA)** | **F2 (+ UDCA)** | |
| Insulin Levels (μg/L) | 0.03 ± 0.01 | 0.09 ± 0.02 | $p < 0.01$ |
| **Bioenergetics, Respiration and Metabolism (Mitochondrial and Glycolysis Stress Testing)** | **Average levels** | | ***Statistical Analysis (p-value)*** |
| | **F1 (- UDCA)** | **F2 (+ UDCA)** | |
| Oxygen Consumption Rate (OCR; pmol $O_2$/min) | 32 ± 0.5 | 50 ± 1.6 | $p < 0.01$ |
| Extracellular Acidification Rate (ECAR; mpH/min) | 18 ± 1.3 | 28 ± 1.5 | $p < 0.01$ |
| Proton Production Rate (PPR; pmol/min) | 31 ± 0.9 | 63 ± 1.1 | $p < 0.01$ |
| Basal Respiration (BR; pmol $O_2$/min) | 17 ± 0.5 | 32± 0.5 | $p < 0.01$ |
| Maximal Respiration (MR; pmol $O_2$/min ) | 25 ± 0.6 | 48 ± 0.6 | $p < 0.01$ |
| Spare Respiratory Capacity (SRC; pmol $O_2$/min) | 8 ± 0.8 | 16 ± 0.6 | $p < 0.01$ |
| Proton Leak (PL; pmol $O_2$/min) | 3 ± 0.5 | 6 ± 0.5 | $p < 0.01$ |
| Coupling Efficiency (CE; %) | 82 ± 0.8 | 81 ± 0.5 | Not Significant |
| ATP Production (ATPP; pmol $O_2$/min) | 14 ± 1.7 | 26 ± 1.7 | $p < 0.01$ |
| Glycolysis (G; mpH/min) | 7 ± 1.8 | 14 ± 1.1 | $p < 0.01$ |
| Non-Glucose-Derived ECAR (NGD-ECAR; mpH/min) | 7 ± 0.5 | 10 ± 0.3 | $p < 0.01$ |
| **Inflammatory Cytokine Production** | **Average levels** | | ***Statistical Analysis (p-value)*** |
| | **F1 (- UDCA)** | **F2 (+ UDCA)** | |
| Tumour Necrosis Factor-α (TNF-α; pg/ml) | 17 ± 1.5 | 5 ± 0.4 | $p < 0.01$ |
| Interferon-γ (IFN-γ; pg/ml) | 13 ± 1.4 | 4.9 ± 0.9 | $p < 0.01$ |
| Interleukin 1-β (IL-1β; pg/ml) | 10 ± 0.9 | 3 ± 0.8 | $p < 0.01$ |
| Interleukin-6 (IL-6; pg/ml) | 11 ± 1.1 | 3 ± 0.8 | $p < 0.01$ |

**Table 2: Microencapsulated cell viability, biological activity, bioenergetics, respiration, metabolism and cytokine production. Data are mean ± standard error of the mean, n=3.**

When comparing our microcapsules to other cell microencapsulating systems, there are similarities as well as differences depending on the cell type and nature of formulation excipients used. Lim and Sun (46) first fabricated β-cell microcapsules consisting of a simple system of alginate, poly-l-lysine and polyethyleneimine. The microencapsulated β-cells showed short-term reversal of diabetes in rats. Other researchers have used polymeric microspheres, microcapsules and subcutaneous macrodevices with some success (47). Our study used the commonly used polymer sodium alginate and also examined cell response to the microencapsulating polymer as well as viability and cell-polymer biochemical interactions. However, our study was different to others in terms of standardisation and normalisation of cell numbers and measuring in real-time cellular mitochondrial activities, respiration and bioenergetics post-microencapsulation and pre-transplantation. Moreover, our study examined the incorporation of an unconjugated tertiary bile acid which has recently shown significant anti-inflammatory effects *in vivo* (43). Our new and distinguished formulation showed good viability and metabolic activity as well as a reduction in the production of cytokines involved in inflammation, suggesting unique properties and potential use in future long-term transplantation studies.

Overall, it appeared that the addition of UDCA into the formulation system exerted beneficial effects not only in terms of cell activity but also via enhancing microcapsule physico-chemical stability without compromising microcapsule size, shape or encapsulated cell content. This is summarised in Figure 4.

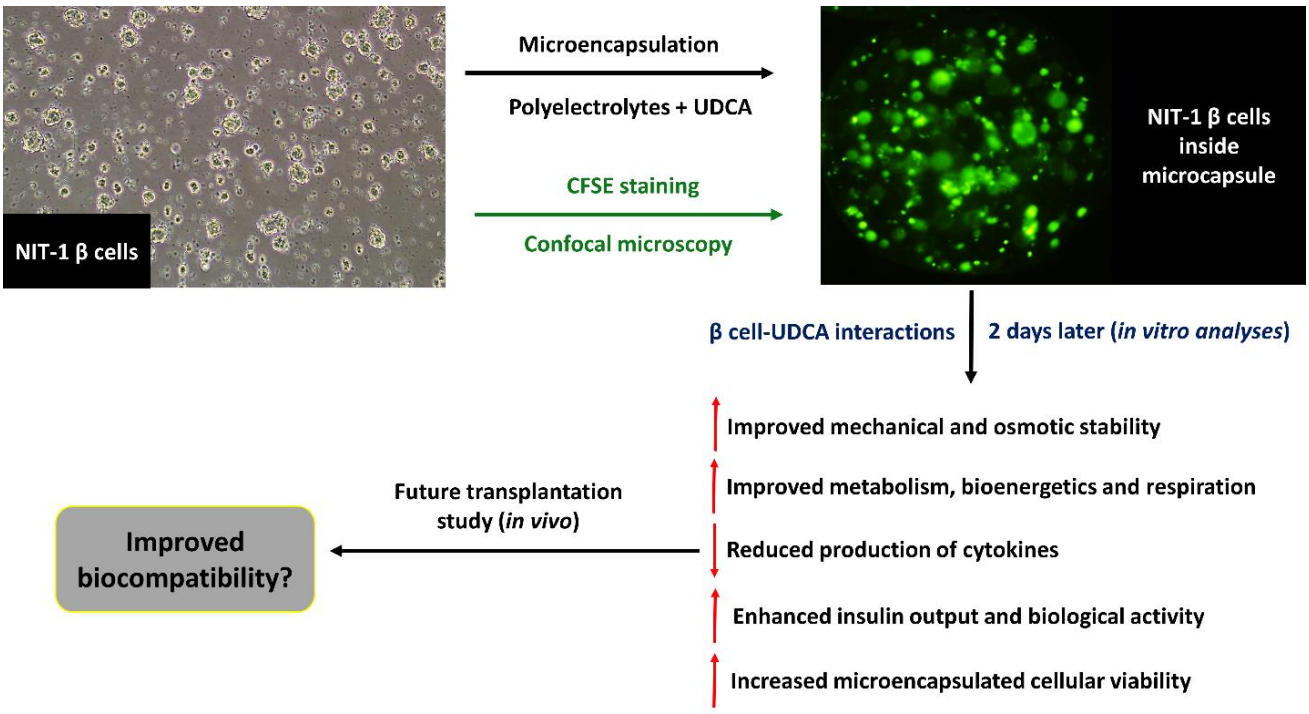


**Figure 4: Summary of the key effects brought about by incorporating UDCA into β-cell microcapsules**

## Conclusion

In summary, this study examined the effects of 1.8% SA, 4.5% USG, 1% PLO and 2.5% PAA formulation on microcapsule formation, characteristics and the biological functions of the microencapsulated β-cells with and without the bile acid UDCA (4%). Our findings show positive and desirable effects of UDCA on encapsulated cellular viability and biological activity, *in vitro*. However, the study was carried out over a short period of time (48 hours) and thus future studies will aim to prolong the study period and test the performance of the microcapsules *in vivo*. This study is part of ongoing work in our laboratory focusing on the effect of various combinations of polymers, hydrogels and polyelectrolytes with bile acids on β-cell microcapsules for subsequent transplantation in an animal model of diabetes mellitus (16, 17, 20, 48, 49).

## Acknowledgments

The authors acknowledge Australian Postgraduate Award (APA) + Curtin Research Scholarship (CRS) for their support. The authors acknowledge the Curtin Health Innovation Research Institute for provision of laboratory space and technology platforms utilised in this study and also acknowledge the use of laboratory equipment, scientific and technical assistance of the Curtin University, Microscopy and Microanalysis Facility, which has been partially funded by the University, State and Commonwealth Governments. The authors also acknowledge the ARC Centre of Excellence in Plant Energy Biology (University of Western Australia) for training, support and access to equipment. The NIT-1 pancreatic mouse β-cells were a generous donation from Professor Grant Morahan at the University of Western Australia.

## Declaration of interest

The authors declare no conflict of interest.

**References**


1. Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, et al. Mechanisms of β-cell death in type 2 diabetes. Diabetes. 2005;54(suppl 2):S108-S13.

2. Vehik K, Dabelea D. The changing epidemiology of type 1 diabetes: why is it going through the roof? Diabetes Metab Res Rev. 2011;27(1):3-13..

3. Negrulj R, Mooranian A, Al-Salami H. Potentials and Limitations of Bile Acids in Type 2 Diabetes Mellitus: Applications of Microencapsulation as a Novel Oral Delivery System. J. Endocrinol. Diabetes Mellit. 2013;1(2):49-59.

4. Beck J, Angus R, Madsen B, Britt D, Vernon B, Nguyen KT. Islet encapsulation: strategies to enhance islet cell functions. Tissue Eng. 2007;13(3):589-99.

5. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet. 2005;365(9467):1333-46.

6. Sutherland DE. Current status of beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus. Transplant Proc. 2003;35(5):1625-7.

7. Mooranian A, Negrulj R, Mathavan S, Martinez J, Sciarretta J, Chen-Tan N, et al. Stability and Release Kinetics of an Advanced Gliclazide-Cholic Acid Formulation: The Use of Artificial-Cell Microencapsulation in Slow Release Targeted Oral Delivery of Antidiabetics. J Pharm Innov. 2014;9:150-157.

8. Hafiz MM, Faradji RN, Froud T, Pileggi A, Baidal DA, Cure P, et al. Immunosuppression and procedure-related complications in 26 patients with type 1 diabetes mellitus receiving allogeneic islet cell transplantation. Transplantation. 2005;80(12):1718-28.

9. Steele JA, Halle JP, Poncelet D, Neufeld RJ. Therapeutic cell encapsulation techniques and applications in diabetes. Adv Drug Deliv Rev. 2014;67-68:74-83.

10. Lacík I. Polymer chemistry in diabetes treatment by encapsulated islets of Langerhans: review to 2006. Aust J Chem. 2006;59(8):508-24.

11. de Vos P, Lazarjani HA, Poncelet D, Faas MM. Polymers in cell encapsulation from an enveloped cell perspective. Adv Drug Deliv Rev. 2014;67-68:15-34.

12. Vaithilingam V, Tuch BE. Islet transplantation and encapsulation: an update on recent developments. Rev Diabet Stud. 2011;8(1):51-67.

13. Orive G, Maria Hernández R, Rodríguez Gascón A, Calafiore R, Swi Chang TM, Vos Pd, et al. History, challenges and perspectives of cell microencapsulation. Trends Biotechnol. 2004;22(2):87-92.

14. Schmidt JJ, Rowley J, Kong HJ. Hydrogels used for cell-based drug delivery. J Biomed Mater Res A. 2008;87(4):1113-22.

15. Bhatia SR, Khattak SF, Roberts SC. Polyelectrolytes for cell encapsulation. Current opinion in colloid & interface science. 2005;10(1):45-51.

16. Mooranian A, Negrulj R, Chen-Tan N, Fakhoury M, Arfuso F, Jones F, et al. Advanced bile acid-based multi-compartmental microencapsulated pancreatic beta-cells integrating a polyelectrolyte-bile acid formulation, for diabetes treatment. Artif Cells Nanomed Biotechnol. 2014:1-8.

17. Mooranian A, Negrulj R, Arfuso F, Al-Salami H. Characterization of a novel bile acid-based delivery platform for microencapsulated pancreatic β-cells. Artif Cells Nanomed Biotechnol. 2014(0):1-7.

18. Mooranian A, Negrulj R, Arfuso F, Al-Salami H. The effect of a tertiary bile acid, taurocholic acid, on the morphology and physical characteristics of microencapsulated probucol: potential applications in diabetes: a characterization study. Drug Deliv and Transl Res. 2015:1-12.

19. Prakash S, Soe-Lin H. Strategy for cell therapy: polymers for live cell encapsulation and delivery. Trends Biomater Artif Organs. 2004;18(1):24-35.

20. Mooranian A, Negrulj R, Al-Salami H. The incorporation of water-soluble gel matrix into bile acid-based microcapsules for the delivery of viable β-cells of the pancreas, in diabetes treatment: biocompatibility and functionality studies. Drug Deliv and Transl Res. 2015 (in press).

21. Hamaguchi K, Gaskins HR, Leiter EH. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. Diabetes. 1991;40(7):842-9.

22. Mooranian A, Negrulj R, Mikov M, Golocorbin-Kon S, Arfuso F, Al-Salami H. Novel chenodeoxycholic acid-sodium alginate matrix in the microencapsulation of the potential antidiabetic drug, probucol. An in vitro study. J Microencapsul. 2015;32(6):589-97.

23. Mooranian A, Negrulj R, Chen-Tan N, Al-Sallami HS, Fang Z, Mukkur T, et al. Novel artificial cell microencapsulation of a complex gliclazide-deoxycholic bile acid formulation: a characterization study. Drug Des Devel Ther. 2014;8:1003.

24. Mooranian A, Negrulj R, Mathavan S, Martinez J, Sciarretta J, Chen-Tan N, et al. An advanced microencapsulated system: a platform for optimized oral delivery of antidiabetic drug-bile acid formulations. Pharm Dev Technol. 2015;20(6):702-9.

25. Sherman I, Fisher M. Hepatic transport of fluorescent molecules: in vivo studies using intravital TV microscopy. Hepatology. 1986;6(3):444-9.

26. Mooranian A, Negrulj R, Mathavan S, Martinez J, Sciarretta J, Chen-Tan N, et al. An advanced microencapsulated system: a platform for optimized oral delivery of antidiabetic drug-bile acid formulations. Pharm Dev Technol. 2015;20(6):702-9.

27. Negrulj R, Mooranian A, Chen-Tan N, Al-Sallami HS, Mikov M, Golocorbin-Kon S, et al. Swelling, mechanical strength, and release properties of probucol microcapsules with and without a bile acid, and their potential oral delivery in diabetes. Artif Cells Nanomed Biotechnol. 2015:1-8.

28. Mooranian A, Negrulj R, Al-Sallami HS, Fang Z, Mikov M, Golocorbin-Kon S, et al. Release and swelling studies of an innovative antidiabetic-bile acid microencapsulated formulation, as a novel targeted therapy for diabetes treatment. J Microencapsul. 2015;32(2):151-6.

29. Mooranian A, Negrulj R, Al-Sallami HS, Fang Z, Mikov M, Golocorbin-Kon S, et al. Release and swelling studies of an innovative antidiabetic-bile acid microencapsulated formulation, as a novel targeted therapy for diabetes treatment. J Microencapsul. 2015;32(2):151-6.

30. Uludag H, Sefton MV. Colorimetric assay for cellular activity in microcapsules. Biomaterials. 1990;11(9):708-12.

31. De Castro M, Orive G, Hernandez RM, Gascon AR, Pedraz JL. Comparative study of microcapsules elaborated with three polycations (PLL, PDL, PLO) for cell immobilization. J Microencapsul. 2005;22(3):303-15.

32. Bertolotti A, Borgogna M, Facoetti A, Marsich E, Nano R. The effects of alginate encapsulation on NIT-1 insulinoma cells: viability, growth and insulin secretion. In vivo. 2009;23(6):929-35.

33. Vaithilingam V, Kollarikova G, Qi M, Lacik I, Oberholzer J, Guillemin GJ, et al. Effect of prolonged gelling time on the intrinsic properties of barium alginate microcapsules and its biocompatibility. J Microencapsul. 2011;28(6):499-507.

34. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, Park SG, et al. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. Biochem Biophys Res Commun. 2010;397(4):735-9.

35. de Vos P, de Haan BJ, Kamps JA, Faas MM, Kitano T. Zeta-potentials of alginate-PLL capsules: a predictive measure for biocompatibility? J Biomed Mater Res A. 2007;80(4):813-9.

36. Labhasetwar VD, Dorle AK. A study on the zeta potential of microcapsules during ageing. J Microencapsul. 1991;8(1):83-5.

37. Xie HG, Li XX, Lv GJ, Xie WY, Zhu J, Luxbacher T, et al. Effect of surface wettability and charge on protein adsorption onto implantable alginate-chitosan-alginate microcapsule surfaces. J Biomed Mater Res A. 2010;92(4):1357-65

38. Naldini A, Carraro F, Silvestri S, Bocci V. Hypoxia affects cytokine production and proliferative responses by human peripheral mononuclear cells. J Cell Physiol. 1997;173(3):335-42.

39. Zheng X, Zheng X, Wang X, Ma Z, Gupta Sunkari V, Botusan I, et al. Acute hypoxia induces apoptosis of pancreatic [beta]-cell by activation of the unfolded protein response and upregulation of CHOP. Cell death & disease. 2012;3:e322.

40. Donath MY, Böni-Schnetzler M, Ellingsgaard H, Ehses JA. Islet Inflammation Impairs the Pancreatic β-Cell in Type 2 Diabetes. Physiology (Bethesda, Md). 2009; 24:325-31.

41. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol. 2009;15(14):1677-89.

42. Kullmann F, Arndt H, Gross V, Ruschoff J, Scholmerich J. Beneficial effect of ursodeoxycholic acid on mucosal damage in trinitrobenzene sulphonic acid-induced colitis. Eur J Gastroenterol Hepatol. 1997;9(12):1205-11.

43. Martinez-Moya P, Romero-Calvo I, Requena P, Hernandez-Chirlaque C, Aranda CJ, Gonzalez R, et al. Dose-dependent antiinflammatory effect of ursodeoxycholic acid in experimental colitis. Int Immunopharmacol. 2013;15(2):372-80.

44. Amaral JD, Viana RJ, Ramalho RM, Steer CJ, Rodrigues CM. Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 2009;50(9):1721-34.

45. Takka S, Cali AG. Bile salt-reinforced alginate-chitosan beads. Pharm Dev Technol. 2012;17(1):23-9.

46. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science. 1980;210(4472):908-10.

47. Dufrane D, Goebbels RM, Gianello P. Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. Transplantation. 2010;90(10):1054-62.

48. Mooranian A, Negrulj R, Al-Salami H. The effects of Ionic Gelation- Vibrational Jet Flow technique in fabrication of microcapsules incorporating beta-cell: applications in Type-1 Diabetes. Curr Diabetes Rev. 2015.

49. Mooranian A, Negrulj R, Al-Salami H. Viability and topographical analysis of microencapsulated β-cells exposed to a biotransformed tertiary bile acid: an ex vivo study. Int. J. Nano and Biomaterials. 2015 (in press).