Influence of Biotechnological Processes, Speed of Formulation Flow and Cellular Concurrent Stream-Integration on Insulin Production from β -cells as a Result of Co-Encapsulation with a Highly Lipophilic Bile Acid

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Abstract—Introduction—We have shown that incorporation of the hydrophilic bile acid, ursodeoxycholic acid, into β -cell microcapsules exerted positive effects on microcapsules' morphology and size, but these effects were excipient and method dependent. Cell viability remained low which suggests low octane-water solubility and formation of highly hydrophilic dispersion, which resulted in low lipophilicity dispersion and compromised cellular permeation of the incorporated bile acid. Thus, this study aimed at investigating various microencapsulating methodologies using highly lipophilic bile acid (LPBA), in order to optimise viability and functions of microencapsulated β -cells.

Methods—Four different types of microcapsules were produced with (test) and without (control) LPBA, totalling eight different microcapsules. Microencapsulating methodologies were screened for best microcapsule-cell functions and microencapsulating processes were examined in terms of frequency, formulation flow, total bath-gelation time and cellular concurrent stream-integration rate, cell-viability, insulin production and inflammatory profile.

Results—Optimum biotechnological processes include formation frequency (Hz) of 2350, formulation flow (ml/min) of 1.2, total gelation time (min) of 18 and cellular concurrent stream-integration rate (ml/min) of 0.7. In all formulations, LPBA consistently improved cell viability, insulin production, mitochondrial activities and ameliorated inflammation.

Conclusion—The deployed biotechnological processes and LPBA optimised formation and functions of β -cell microcapsules, which suggests potential applications in diabetes mellitus via the creation of more stable β -cell microcapsules capable of delivering adequate levels of insulin to control glycaemia and potentially curing diabetes.

Keywords—NIT-1 β -cells, Chenodeoxycholic acid, Microencapsulation, Diabetes mellitus.

INTRODUCTION

Biotechnological processes used for encapsulation of cells and proteins have radically evolved over recent years, and have adopted multifaceted approach integrating physico and electro chemical methodologies to bioengineering artificial delivery systems capable of housing biologically active cells or proteins.^{9,18} Biotechnological processes have utilized multiple parameters, which are deployed at various stages of the fabrication process of delivery systems. These parameters include wide range of voltages, vibrational frequencies, gelation bath temperatures and pH values as well as electrode tension and flow pressures.⁶³ Delivery systems include microcapsules, liposomes, micelles and scaffolds.^{16,53} Recently, biotechnological processes such as cell-microencapsulation and delivery of therapeutics have gained substantial interests.⁵⁷ One example of cell-encapsulation is pancreatic β -cell microencapsulation for insulin delivery and diabetes treatment.

Diabetes mellitus has two main types, type 1 and type 2, both of which are characterized by high levels of glucose in the blood and chronic inflammation. Insulin was discovered in the 1920s and since then has been the mainstream treatment for diabetes mellitus, and used by all Type 1 diabetics and more than 30% of type 2 diabetics. Due to challenges associated with current insulin delivery systems, such as route of administration, hypoglycemic episodes and stringent storage conditions, β -cell encapsulation is considered novel and innovative method for sustained insulin delivery and glycemic control. However, to date, microcapsules-containing functional β -cells are not

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commercially available, due to lack of appropriate delivery systems that can house and maintain functional β -cells, long term. Our lab is one of the first to introduce new excipients (bile acids) to β -cell microcapsules, which showed potential enhancement of cells' performance and insulin release. However, our new microcapsules remained incapable of permanently maintaining optimum functional β -cells.

Bile acids are amphiphilic molecules produced in the liver as a result of cholesterol catabolism and degradation.^{54,65} They are endogenously produced surfactants and possess wide range of biological activities including facilitating vitamin absorption, lipid uptake and endocrine signalling effects.^{15,25} The applications of bile acids in diabetes have been widely studied in the literature.^{7,23,24} We have studied the interaction between bile acids and potential antidiabetic therapeutics *in vitro*,^{8,20,21,27–32,37,40,41,45,47,49,50,56} *ex vivo*^{3–6} and *in vivo*.^{1,2,11,19,21,22} Our studies have shown potential applications of bile acids as biological agents, permeation enhancers and microcapsule-stabilising excipients. A recent study in our laboratory has illustrated the importance of microcapsules' morphology on functions and long-term viability of β -cells.³⁶ Other studies have demonstrated that bile acid incorporation and effects on pancreatic β -cell is dependent on type of bile acid, biotechnological encapsulation processes, and microcapsules' components.⁴⁵ The incorporation of a highly lipophilic bile acid (LPBA) is anticipated to improve the characteristics of microencapsulating formulation, enhancing cell interaction with the formulation, enhancing physical properties of the microcapsules including swellability and mechanical strength properties, which may result in better cell viability (life span), insulin production, mitochondrial activities and ameliorating the inflammatory profile often associated with β -cell apoptosis.⁵⁹ LPBA may also exert its own biological effects and directly improve the microenvironment within microcapsules, where the cells are encapsulated. This is part of our ongoing work exploring LPBA applications in pancreatic β -cell delivery and effects.³⁰

Thus, this study aimed to optimise biotechnological processes used for β -cell microencapsulation, and study effects of adding LPBA into different microencapsulating formulations, in terms of microcapsules' size distribution, and biological functions of the microencapsulated cells.

MATERIALS AND METHODS

The lipophilic bile acid (LPBA) used (chenodeoxycholic acid, > 98%), sodium alginate, poly-L-ornithine, polystyrenic sulphate, polyallylamine, were all purchased from Sigma Aldrich (Australia).

Cellular Encapsulation and Biotechnological Microencapsulation Processes

NIT-1 cells were cultured on T-25 cm² flasks (Thermo Fisher Scientific®, Australia) using our established methods, using Dulbecco's modified Eagle's medium obtained from Gibco, Life Technologies (USA).¹³ The cells were microencapsulated using Buchi-based microencapsulator according to our previously published work.^{39,44} Microencapsulation of the NIT-1 pancreatic β -cells was done under sterile conditions using vibrational-jet flow technology. Table 1 describes different formulations used, and Table 2 describes the gradually screened processes used. Constituents of the microcapsules contained selected polymers, copolymers and gels based on previous studies, which showed good safety and cell compatibility profiles, and potential applications in microcapsule-fabrication, refined micro-architecture formation and supported microcapsules' surface-stabilising effects.^{32,48} Eight different microencapsulating formulations were prepared, and mixed thoroughly for 6 h prior to encapsulation with the cells. Biotechnological processes and screened parameters were selected based on ongoing research investigating various methodologies, in-house built techniques, and system-managed factors which effect microcapsules' formation and consistent scalable output. Main factors examined were formation frequency, flow, gelation time and integration polymer-cell rate (Table 1).

Formulation Zeta-Potential, Microcapsule Size Distribution, Swellability and Mechanical Strength Analyses

Zeta potential of the formulations was assessed using Zetasizer 3000 (Malvern Instruments, UK) and size distribution of microcapsules was assessed using Mastersizer 2000 instrumentation (Malvern Instruments, UK). Microcapsules swelling and mechanical resistance studies were carried out on each formulation over a period of 2 days. To determine the swelling properties, 200 microcapsules were weighed and placed in 25 mL phosphate buffer. The microcapsules were and swelling index was measured as the final weight over the initial weight.^{42,55} In order to determine the mechanical strength of microcapsules, 200 microcapsules were placed in 25 mL phosphate buffer and shaken back-and-forth using a Boeco Multishaker PSU 20 (Boeco Company, Germany). The number of fractured or damaged microcapsules was counted at regular intervals and the mechanical resistance was measured as the final number of intact microcapsules remaining compared to the initial number as a percentage.^{39,51,52}

TABLE 1. Constituents of microencapsulating formulations used to form microcapsules containing the cells.

Formulation type	Alginate (%)	Poly-L-ornithine (%)	Ultrasensitive gel (%)	Polystyrenic sulphate (%)	Polyallylamine (%)	Lipophilic bile acid (%)
F1	1.8	0.11	0	0.5	0.5	0
F2	1.8	0.11	0	0.5	0.5	4
F3	1.2	0.6	0.9	0.4	0.65	0
F4	1.2	0.6	0.9	0.4	0.65	4
F5	1	0.10	2.1	0.31	0.65	0
F6	1	0.10	2.1	0.31	0.65	4
F7	1.8	1.8	0.4	0.15	1	0
F8	1.8	1.8	0.4	0.15	1	4

TABLE 2. Biotechnological processes deployed for cell-microcapsule fabrication.

Biotechnological process	In-house tailored capacities
Formation vibrational frequency (Hz)	1500–2500
Formulation-flow (ml/min)	1–2
Total bath-gelation time (min)	5–20
Cellular concurrent-integration (ml/min)	0.5–2

TABLE 3. Biotechnological processes of cell-microencapsulation and optimum manufacturing parameters.

Biotechnological process	Optimum working value
Formation frequency (Hz)	2350
Formulation-flow (ml/min)	1.2
Total bath-gelation time (min)	18
Cellular concurrent-integration (ml/min)	0.7

Cell Viability, Confocal Imaging, Insulin Output, Mitochondrial Activities and Inflammatory Profile Analyses

Using in-house developed methods, confocal imaging and MTT assays were carried out to measure cell-microcapsule imaging and cell viability, postmicroencapsulation, followed by insulin and TNF- α and IL-6 measurements using our established methods.³⁰ Samples from supernatant in wells containing the microencapsulated cells were collected at different time points and immediately replaced with media. Samples were analysed for insulin and inflammatory biomarkers' concentrations using ELISA Kits (Mercodia Cooperation, Uppsala, Sweden) and Attune Acoustic Focusing Flow Cytometer (Life Technologies, USA) with BD Biosciences CBA (San Jose, USA), while mitochondrial activities of microencapsulated cells were assessed by using the Seahorse XF analyser.^{26,30,31,33–36,46,49} Microcapsules containing the cells were incubated for evaluation prior to analyses. Multiple time points were examined over 2 week's period. Since cells showed viability and metabolic activities for up to 2 days, postmicroencapsulation, only data over 2 days were represented for accuracy. Data were normalised for viable cell counts and for each assay five microcapsules of equivalent size and encapsulated cell contents were used. Number of viable cells were counted in each microcapsule analysed, and averaged to reflect an accurate biological measure of individual cell's performance per microcapsules, and provides precise information on how each cell performed, in

various experimental conditions, when encapsulated using different formulations.

Statistical Analysis

Statistical data were assessed using one way ANOVA and posthoc testing, or parametric/non-parametric *t* test, as appropriate. GraphPad Prism 7.02 version was used (USA) and values falling below the detectable limit of assay were recorded as zero.

RESULTS

Biotechnological Microencapsulation Processes

Wide ranges of various microencapsulating parameters using Buchi-customized system were examined and refined, and the resulting best working values were selected based on best produced microcapsules in terms of microcapsules' size distribution and constant cell viability (Table 3).

Formulation Zeta-Potential, Microcapsule Size Distribution, Swellability and Mechanical Strength Measurements

Electrokinetic (Zeta-potential) measurements of formulation showed higher values, as the result of LPBA incorporation (Table 4) suggesting better stability, while microcapsule's size distribution was not significantly affected by LPBA incorporation suggest-

ing that the microencapsulating process is capable of maintaining size, regardless of bile acid incorporation or changes to type of formulations used (Table 4).

LPBA incorporation showed consistent reduction in microcapsules' swelling (Fig. 1a) and enhancement of mechanical strength (Fig. 1b) properties, among all formulations examined.

Confocal Imaging, Cell Viability, Insulin Output and Bioenergetics

The fluorescence staining method used for the confocal imaging of the microcapsules involved carboxyfluorescein succinimidyl ester (CFSE) which stains the nucleus of viable cells and allows for the visualisation of the cell population within the microcapsules using fluorescent microscopic techniques. Confocal imaging showed well-spread cell distribution within the microcapsules (Fig. 2), and LPBA incorporation exerted consistent positive effects in all tested formulations and resulted in higher cell viability, insulin output, ATP production (Fig. 3), and ameliorated inflammation (Fig. 4). Results suggest even-cell

distribution within the microcapsules, and excipient-stabilising and biological enhancing effects of the bile acid, on viability, mitochondrial activities and functions of microencapsulated β -cells, and a reduction in the inflammatory profile of the cells.

DISCUSSION

The main objectives of this study were to develop microencapsulation processes needed for optimum production of microcapsules containing β -cells, and measure effects of LPBA incorporation into various types of microcapsules, in terms of microcapsules' physical stability, and biological functions of the microencapsulated cells. Findings showed that using Buchi-based customized system formation-frequency needs to be > 2000 Hz with < 20 min bath-gelation time, in order to produce consistent size microcapsules with similar cell-loading capacity and consistent viability measurements. This is not in line with previous work using basic-Buchi system, where formation-frequency < 2000 Hz and longer bath-gelation time were deemed appropriate.³⁴ However, it is in line with

TABLE 4. Average microcapsule Zeta potential and diameter size for F1 to F8.

Electrokinetic measurements (Zeta-potential; mV)	Microcapsule size in diameter (μm)
F1 = -26 ± 3 & F2 = $-39 \pm 5^*$	F1 = 760 ± 18 & F2 = 790 ± 30
F3 = -23 ± 4 & F4 = $-36 \pm 5^*$	F3 = 750 ± 30 & F4 = 770 ± 50
F5 = -25 ± 3 & F6 = $-32 \pm 4^*$	F5 = 740 ± 30 & F6 = 760 ± 20
F7 = -22 ± 2 & F8 = $-40 \pm 3^{**}$	F7 = 750 ± 10 & F8 = 780 ± 30

Results are expressed as mean $\pm$ SEM, $n = 3$. * $p < 0.05$, and ** $p < 0.01$ compared to corresponding control, $n = 3$.

FIGURE 1. Swelling index (a) and mechanical strength testing (b) of F1-F8 microcapsules. Data are mean $\pm$ SEM, $n = 3$.

FIGURE 2. Confocal cell-imaging of microcapsules containing β -cell in the 10 formulations: F1-F10 (a-j).

FIGURE 3. Microencapsulated cellular viability (a), insulin output (b) and ATP production (c) for the formulations F1 to F8. Data are mean $\pm$ SEM, $n = 3$ (ANOVA followed by Tukey post hoc).

studies carried out by other groups when working with highly viscous formulations.⁶³ Accordingly, the general trends and interplay of these processing conditions

tend to be highly dependent on system configuration and assigned parameter-range, based on particular manufacturer's design and capacity. Our developed

FIGURE 4. Microencapsulated cellular levels of TNF- α (a) and IL-6 (b). Data are mean $\pm$ SEM and $n = 3$.

microencapsulating system uses concurrent, semi-parallel, parallel and gradient-tailored integration of multiple microencapsulating parameters. These parameters provide constant output and minimum fluctuation or pressure variation. Method refinement involved wide-range screening of the parameters, one parameter at a time, while maintaining all other parameters constant, which facilitated informative results and accurate findings, based on manufacturer's design and instrument set-up and the deployed for-

mulation-cell flow model. Optimum values were reported when microcapsules were produced at a constant flow rate, and with consistent shape and size, and contained similar cell numbers. These optimised values produced similar viable cells, per microcapsules, independent of formulation used or the presence of LPBA.

LPBA increased Zeta-potential of all formulations, while having no significant effect on size of microcapsules. The increase in Zeta-potential brought about by

LPBA incorporation may be the result of the negatively charged carboxylic group of LPBA, which brought about an overall increase in the number of charges on surface of particles and suggests optimisation of dispersion stability. This is supported by studies carried out by Gallardo *et al.*¹²; that showed lowering of formulation pH by adding an acid, can reduce sedimentation rate and enhance stability, while studies carried out by Stachurski and MichaLek⁶¹ have demonstrated close correlation between change in Zeta-potential and stability of formulation. The lack of LPBA effects on microcapsules' size suggests that the developed microencapsulating method is robust and provided reproducible and consistent output, regardless of LPBA incorporation or type of formulation used.

LPBA increased physical strength of microcapsules and reduced swellability in all formulations. This is likely the result of microcapsule surface-stabilising effects of LPBA due to an interaction with alginate resulting in more viscous and less permeable surface, which resists swelling and prevents moisture loss. This supports formulation-stabilising effects of LPBA (Table 3) and is in line with previous studies where bile acids have shown surface-stabilising effects when incorporated into alginate-based microcapsules.^{38,43} This is also in line with studies carried out by Takka and Cali which showed that bile salt incorporation into chitosan-based microcapsules resulted in reinforced-surface and stronger microcapsules.⁶² The stabilising effects of LPBA on the microcapsules may be responsible for enhanced cell viability and functions (Fig. 2) and reduced inflammation (Fig. 3). The mechanism by which LPBA optimises cell mitochondrial activities could be by directly enhancing protein expression and nuclear receptor activation within the cells, or could be by optimising the architecture of the microenvironment the cells were encapsulated in, which brings about better cell-to-cell adhesion and improved physical support, resulting in optimised cell mass and survival.

LPBA incorporation brought about consistent increase in cell viability among all formulations, and likely to be the result of not only improved physical properties of microcapsules by LPBA, but also as a result of direct interaction between cells and the bile acid. This is supported by other studies, where bile acids distribution within alginate-microcapsules were found to be throughout all layers of the microcapsules, and thus, it is likely that LPBA has direct contact with the encapsulated cells.^{31,32} Enhanced cell viability was accompanied by increased insulin output and ATP production levels, which illustrates that LPBA incorporation improved physical microenvironments, which

supports cell mass, and also likely to have exerted stimulatory effects on cell biological activities, energy output, ATP production and insulin release. Results also suggests that the strengthening of the microcapsules' surface did not compromise diffusion of insulin and inflammatory cytokines from within the microcapsules to outside of the microcapsules, where samples were taken and analysed for insulin, TNF- α and IL-6 concentrations. F8 seems to possess best mechanical strength (Fig. 11), highest insulin (Fig. 3h) and energy (Fig. 3l) output and lowest TNF- α levels (Fig. 4d). This suggests an association between the physical characteristics and stability of microcapsules, cell mass and survival, mitochondrial activities, insulin output and levels of the inflammatory cytokines, TNF- α produced by the cells. The extent of LPBA impact on the microencapsulated cells' functions seems to be formulation-dependent, since insulin and energy output as well as levels of inflammatory cytokines were not the same among all formulations.

Our findings of LPBA enhancement of cell mitochondrial activities and insulin output are in line with previous studies by Moorianian *et al.* which showed that the bile acid ursodeoxycholic acid enhanced mitochondrial energy production of β -cells.³⁹ Other studies have also demonstrated an association between β -cell functions, diabetes, and disturbances in the bile acid pool. Siow *et al.*⁶⁰ have demonstrated strong association between diabetes induction and β -cell damage, and concentrations of bile acids in the bile acid pool. Possible mechanisms of bile acids effects on cell viability and functions are associated with their effects on nuclear factors and receptors such as FXRs and TGR-5, as described by Houten *et al.*¹⁴; Such bile acids effects on cell biological functions seem to be closely related with their effects on inflammation, especially in diabetes, as demonstrated by Schaumberg *et al.*⁵⁸

The antiinflammatory effects of LPBA is possibly due to its microcapsule-stabilising effects or its direct biological stimulatory effects. These effects are in line with published studies, which showed that bile acids possess antiinflammatory effects in human islets via suppression of TNF- α generation, as illustrated by Yang *et al.*⁶⁴ LPBA antiinflammatory effects may also be due to its effects on cell-polymer interaction which resulted in improved microenvironment within the microcapsules, sustaining cell mass and functionality due to uniform membrane permeability to nutrients or possibly due to LPBA's direct biological effects on the cells which lead to optimisation of cellular respiration and energy production.¹⁰ This is consistent with the literature. Kullmann *et al.*, have shown that the tertiary bile acid ursodeoxycholic acid reduced local

FIGURE 5. Summary and main findings of the study, which illustrates the optimised formulation excipients, best biotechnological processes and best excipient ratios forming the microcapsules.

intestinal inflammation in rats receiving indomethacin.¹⁷ Accordingly, our findings demonstrate that the deployed microencapsulating processes were robust and produced constant size microcapsules and LPBA incorporation enhanced cell functions and biological effects, and also ameliorated inflammation in a formulation-dependent manner (Fig. 5).

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CONFLICT OF INTEREST

Armin Mooranian, Ryu Takechi, Emma Jamieson, Grant Morahan and Hani Al-Salami declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for this article.

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