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Bile acid-based micro/nano capsules for the targeted oral delivery of probucol

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This thesis submitted for the degree of

Master of Philosophy

Of

Curtin University

July 2020

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: Susbin Raj Wagle

Date: 25 July, 2020.

Acknowledgments

This thesis would not be possible without the help, support, and encouragement from my supervisors, laboratory members, friends, and family.

I would like to give a sincere thanks to my supervisor Dr. Hani Al-Salami for his help and guidance during my MPhil study. His expertise in drug delivery and pharmaceutical sciences directed me to produce this quality thesis. I am very grateful for his supervision.

I would also like to acknowledge my secondary supervisor Dr. Armin Mooranian for being my mentor, teaching me experimental skills, and inspiring me all the time whenever I am down.

I would also like to acknowledge the Curtin Health Innovation Research Institute, School of Pharmacy, Curtin University, for providing me the platform to conduct my laboratory work and complete my studies. I am also grateful for all the laboratory members, colleagues, and for the technical staff for their kind assistance and support during my research.

Finally, I am grateful and thankful to my parents and wife for their love, support, and encouragement.

Susbin Raj Wagle

25 July, 2020.

Abstract

One of the most common metabolic diseases, diabetes mellitus, is characterised by hyperglycemia and pathological inflammation. It is categorised into three main types: gestational diabetes, type 1 diabetes, type 2 diabetes mellitus (T2D). The latter accounts for 90% of diabetic incidences worldwide. T2D is primarily caused by environmental and genetic factors where tissues lose sensitivity to insulin secretion/utilization, which leads to dysregulation in glucose levels and eventually to the development T2D. High blood glucose levels, pancreatic β-cell damage, and systemic inflammation are common features observed in T2D patients. The standard treatment for T2D is oral administration of anti-diabetic drugs, with most of them mainly focused on controlling blood sugar levels rather than focusing on diabetes-related inflammation and complications, primarily developed by pancreatic β-cell damage, free radicals, and lipid dysregulation.

Probucol (PB), a commercial available lipophilic drug with anti-oxidative and anti-inflammatory properties and shown its potential to protect pancreatic β-cells; however, PB's use for T2D treatment is limited because of drug's low water solubility, bioavailability, and inter-individual patient variations. The implementation of encapsulation technology, utilising polymer-PB mixtures, results in the production of efficient microcapsules that can be used to increase PB availability and promote steady drug release. Moreover, PB stability can be improved by incorporating absorption enhancers such as bile acids into the polymer-drug mixture. Bile acids are naturally amphipathic steroid compounds, produced in the human body and are shown to be effective microcapsules' excipients, leading to improved drug absorption and delivery properties. Recently, lithocholic acid (LCA) has shown its prominent function as anti-inflammatory and drug formulation-stabilizing effects in atherosclerosis. Therefore, a good approach for treating T2D and improving pancreatic β-cell function can be microencapsulation of the PB together with bile acids like LCA. Previous studies demonstrated that bile acids facilitated the absorption of PB. The encapsulation of PB with polymer resulted in anti-diabetic effects and supported drug release, but targeted, controlled and sustained drug's release *in vitro* remains elusive. Furthermore, PB absorption profile remained to certain extent limited and variable in *in vivo* studies. Therefore, the major challenge is to design new delivery systems for PB using LCA-based matrices to optimize the oral delivery of PB.

Sodium alginate (SA) was used as a polymer base for designed microcapsules. PB-LCA and PB, microcapsules were produced on a Büchi microencapsulation system, with specific jet-flow technology that allows the formation of uniformly shaped polymer capsules. The produced microcapsules were evaluated based on morphology, physicochemical compatibilities, rheological properties, swelling, mechanical strength, buoyancy, stability, and release profiles at different pH values and temperatures

(chapter 2 and 3). Similarly, microcapsules' effects on viability, inflammatory cytokines, and bioenergetics parameters were analysed using pancreatic β-cell lines (chapters 2 and 3).

In brief, chapter 1 describes the hindrance faced by orally administrated lipophilic drugs and defines the possible applications and challenges of encapsulation technologies in the oral delivery of lipophilic drugs by optimizing drug's efficacy and safety profiles. Chapter 2 describes the encapsulation technology and development of robust PB delivery matrices using LCA and the SA. The study characterizes microcapsules based on surface characteristics, morphology, rheological characteristics, physicochemical stability, physical stability, drug release, and also the biological effects of prepared microcapsules' on NIT-1 pancreatic cell line. Similarly, chapter 3 focuses more on microcapsules membrane strength, drug content, release kinetics, stability, and biological effects on a pancreatic β-cell line - NIT-1. Both chapters 2 and 3 describe the impact of LCA on PB microcapsules.

The resulting PB-LCA-SA microcapsules were morphologically consistent and presented good compatibility characteristics (chapter 2). By introducing LCA to the PB-SA mixture, microcapsules retained their physiochemical properties (shape, size, rheological properties, drug content, stability, zeta-potential, surface tension), improvement in mechanical strength, buoyancy, release profile, conductivity, and decreased in the swelling index (chapter 2 and 3). PB-LCA-SA microcapsules improved cell viability, reduced the inflammatory profile, increased anti-inflammatory cytokine, and improved the bioenergetics parameters (chapter 2 and 3) in the hyperglycaemic state.

Hence, the scope of this thesis concentrates on developing, validating, and establishing a robust microencapsulation method, and characterizing prepared microcapsules to study the effects of LCA in PB microcapsules in terms of *in vitro* (formulation study) and *ex vivo* (cell study), and examine the microcapsules' potential effects in treating T2D. In conclusion, from *in vitro* and *ex vivo* models, results suggest that microcapsules produced from PB in combination with LCA showed potential properties for T2D therapy use.

List of publications

List of publication central to the research hypothesis

This thesis contains three publications that are central to the research hypothesis and one publication not central to the research hypothesis but included within this thesis.

Published/in press:

1. **Susbin Raj Wagle**, Bozica Kovacevic**,** Daniel Walker**,** Corina Mihaela Ionescu, Umar Shah, Goran Stojanovic, Sanja Kojic, Armin Mooranian and Hani Al- Salami. Alginate-based drug oral targeting using bio-micro/nano encapsulation technologies.
   Journal: **Expert Opinion on Drug Delivery** (in press).

2. **Wagle, S.R**., Walker, D., Kovacevic, B. *et al.* Micro-Nano formulation of bile-gut delivery: rheological, stability and cell survival, basal, and maximum respiration studies. *Sci Rep* **10,** 7715 (2020) Journal: **Scientific Reports.**

3. **Susbin Raj Wagle**, Bozica Kovacevic[1]**,** Daniel Walker**,** Corina Mihaela Ionescu, Goran Stojanovic, Sanja Kojic, Armin Mooranian and Hani Al- Salami. Pharmacological and advanced cell respiration effects, enhanced by toxic human-bile nano-pharmaceuticals of probucol cell-targeting formulations: **Pharmaceutics** (in press).

List of additional publications

This thesis contains the following publication published in one of the Nature's journals (Scientific Reports).

1. Mooranian A, **Wagle SR**, Kovacevic B, Takechi R, Mamo J, Lam V, Watts GF, Mikov M, Golocorbin-Kon S, Stojanovic G, Al-Sallami H. Bile acid bio-nanoencapsulation improved drug targeted-delivery and pharmacological effects via cellular flux: 6-months diabetes preclinical study**. Scientific Reports**. 2020 Jan 9;10(1):1-5.

Introduction and structure of the thesis

DM (Diabetes Mellitus) is a metabolic disorder triggered by hyperglycaemia and inflammation (1). With high economic pressure on health systems around the world, diabetes is a major global epidemic that affects millions of people every year (2, 3). With the number of diabetic patients is increasing, Australia alone, has a significant yearly cost of more than $15 billion, revealing that one person is diagnosed with DM every five minutes (4). Due to poorly controlled diabetic symptoms, it is projected that by the year 2040, DM will be the seventh leading cause of death and around 700 million individuals will suffer from DM, unless new potential and potent drugs are introduced to the market (5).

DM is mainly divided into three types; type one DM (T1D), type 2 DM (T2D), and gestational diabetes (GD). GD is a type of DM that occurs during the pregnancy and normally goes away after the pregnancy; however, it leads to high risk for T2D development later in life. T1D occurs due to auto-immune destruction of pancreatic β cells in which β cells produce insufficient amounts or unable to produce insulin at all. T2D is one of the common types of DM occurring when insulin-producing pancreatic β-cell are not able to produce enough insulin (due to tissue desensitization to insulin), resulting in high blood glucose levels. Environment and genetic factors are the primary causes of T2D, with T2D accounting for 90% of the total diabetic population (only 10% accounts for T1D and GD).

Insulin is the major hormone which regulates blood glucose level in the human body. In a healthy body, when the blood sugar level is higher than 5mmol/L, insulin is stimulated and released from the pancreatic β-cells to reduce blood sugar level through the receptors, most commonly the Glucose transporter type 4 (GLUT-4). Insulin stimulates GLUT-4 and promotes glucose intake and storage into the muscle, liver, and adipose tissues. Normally, glucose intake and uptake (gluconeogenesis, glycogenolysis, and lipogenesis) are controlled by the feedback mechanism to ensure proper use of energy whenever needed in the body. However, in T2D patients, initially, because of the damaged pancreatic β-cells, there is impaired insulin secretion that causes dysregulation of glucose haemostasis. Initially, chronic tissue exposure to glucose causes increased insulin production. However, β cells later cannot bear the production pressure, which causes a decrease in insulin production and leads to tissue desensitization, and, eventually, T2D development (6). Lately, T2D is generally allied with hypertension, hyperlipidaemia, and high levels of free radicals and inflammation (7). Obesity is the primary risk factor for T2D, which can induce inflammation and oxidative stress at the early stage of T2D and worsen glycaemic control (8). Recently, a strong link was established between diabetes symptoms and chronic inflammation. Therefore, pro- and anti-inflammatory

biomarkers such as interferon-gamma (IFN-γ), interleukin (IL-6), IL-10 have been used to monitor the success of anti-diabetic drugs, potential complications, and inflammatory profile (9).

Pre-diabetes is the condition where pancreatic β-cells have difficulty producing insulin, and if it is successfully diagnosed, diabetes incidence and its complications can be minimized (10). It is clear that, together with anti-diabetic drugs, a healthy diet and an active lifestyle play an essential role in reducing the number of pre-diabetes patients and those prone to developing T2D (11, 12). Previously, T2D was mainly noted as dysregulation of blood sugar level. However, It is now accepted that T2D is caused by inflammation and oxidative stress progression present at the pancreatic site (13, 14). Studies have proved that β-cell inflammation is linked to diabetes development by worsening diabetic symptoms, long-term prognosis, lipid dysfunction, and insulin-resistance, particularly because normal pancreatic β-cells have limited natural defence mechanisms from inflammation and free radicals (15-17). This is the driving mechanism behind T2D`s substantial complications: cardiovascular disorder, gastroparesis, metabolic syndrome, renal diseases, neuropathy, retinopathy, hearing problems, slow healing, and skin problems.

To date, marketed anti-diabetic medications such as sulphonylureas, metformin, and thiazolidinedione have been effective mainly in controlling blood glucose levels either by improving tissue sensitivity or by decreasing glucose uptake (18). It is worth mentioning that less researches are focused on pancreatic β-cell damage, caused by free radicals and toxins along with inflammation, which is critical in diabetes treatment and its complications (19, 20). Therefore, there is a genuine need for novel and effective medication that is superior to current treatment in diabetes prevention and development, as such medication will be not only effective in controlling hyperglycaemia, but also be able to reduce local inflammation and have strong anti-oxidant potential, resolving underlying inflammation.

PB is a lipophilic drug with potential anti-inflammatory and anti-oxidant properties, shown to have a protective effect from inflammation and free radicals on pancreatic β-cells (21, 22). The drug was initially developed and prescribed for the treatment of hyperlipidaemia. However, it was withdrawn from some countries after 18 years of use, mainly from the USA and western countries, due to severe adverse effects (lowering high-density lipoprotein and cardiac electrophysiology, high inter-individual variations, extreme accumulation in adipose tissue), and low bioavailability (22-26). *Ma et al.* showed that the protein transporter such as P-glycoprotein has a significant impact on the rate of PB cellular uptake in the gut, affecting clinical efficacy and safety profile of the drug (27). Therefore, one possible approach to overcome these obstacles is to develop and optimise novel and stable formulations, with good rheological factors able to protect the drug from unwanted degradation in the gastrointestinal

(GI) site, increase drug's solubility and intestinal permeation, and ultimately release the drug at the target site in a controlled manner without having toxic effects for tissues. This can be achieved with the help of artificial cell microencapsulation (ACM) technology using different kinds of polymers and permeation enhancing agents such as bile acids.

ACM is a technology established by pioneer Professor Thomas Chang at McGill University, Canada, in the 1960s (28). After this, the technology was extensively used for the targeted delivery of drugs, living cells, and bioactive moieties by encapsulating them with polymers (15, 29, 30). One of the most critical challenges of this technique is to produce the ideal shape and size of microcapsules with appropriate drug content. An ideal microcapsule will contain materials that can provide the drug-stabilizing conditions to support the controlled release of the drug. This can be achieved by developing suitable encapsulation methods and parameters, together with appropriate polymers; thus, this is the primary objective of this study.

One of the most used polymers for ACM is sodium alginate (SA) due to its high biocompatibility, biodegradability properties, polymers support pH-sensitive, controlled, and targeted drug delivery, by protecting the active compounds from the hostile condition in the GI tract. Therefore, polymer selection is the primary factor in drug discovery and delivery (31). Generally, at low pH, the hydrated SA transforms into the insoluble and form a porous alginic acid matrix, whereas at pH >7.0, the matrix is converted into a more soluble viscous layer that breaks microcapsule wall and releases the encapsulated drug from its core (32, 33).

Bile acids are steroid acids secreted inside the body, broadly divided into primary and secondary bile acids. They are amphipathic compounds consisting of a steroid structure, four rings with five to eight carbon side chains ending in a carboxylic acid, and numerous hydroxyl groups, divided mainly into two types. Primary bile acids (cholic acid and chenodeoxycholic) are synthesized by the liver, whereas secondary bile acid (deoxycholic acid and lithocholic) are formed in the colon from the bacterial metabolic action. Bile acids act as a digestive surfactant agent and help in the solubilisation of lipophilic drugs (34-36). Also, some bile acids, like chenodeoxycholic, ursodeoxycholic (UDCA), and lithocholic acid, have shown significant protective properties on pancreatic β-cell by their potent anti-inflammatory and anti-apoptotic characteristics (37-39). Moreover, studies demonstrated that bile acids act as enhancers for drug release and formulation excipients, facilitated the absorption of PB encapsulated together with the polymer, resulting in positive anti-diabetic effects and support in drug release.; however, results were not promising because targeted, controlled, and sustained drug release remains elusive (15, 29, 40-42). Also, the PB absorption profile remained limited and variable

in *in vivo* studies. This study hypothesises that bile acid with high structural rigidity and less water solubility can improve PB release pattern.

Lithocholic acid (LCA) has high structural rigidity, unique amphiphilic properties, and reasonable biocompatibility, which helps in self-assemble in nanostructures (43). It is one of the bile acids that have anti-inflammatory and protective effects in inflammatory diseases (44). Recently, studies showed that LCA has effectiveness in the oral administration of anticancer drugs (45, 46). However, to the date, there is not advanced research reporting usage of novel LCA in drug discovery and development area. This study is ongoing research to study possible applications and effects of different bile acids either unaided or with anti-diabetic drugs, and with polymers by developing microcapsules (42, 47, 48). More information is necessary to develop microcapsules that display good structural integrity, and exhibit controlled targeted properties with self-assembling potential. Therefore, it is hypothesized that this study would produce new and robust PB microcapsules with improved delivery profiles appropriate for oral delivery in T2D. Thus, this thesis is the first study to optimize encapsulation techniques and microcapsules production, examine and characterize developed microcapsules by *in vitro* studies, and check their potential anti-inflammatory effectiveness *ex vivo* using PB-LCA microcapsules.

Briefly, chapter one characterizes challenges faced by orally administrated lipophilic drugs (mainly in the gastrointestinal tract), describes the encapsulation technologies, and outlines the significance of bile acid and alginate in microencapsulation of the lipophilic drug for optimizing drug's efficacy and safety profiles. Chapter two describes new PB delivery matrices using LCA, and presents the impact LCA has on microcapsules' morphology, surface characteristics, chemical and thermal compatibilities, and rheological properties *in vitro* and also the biological effects of PB-loaded microcapsules' on the pancreatic β-cell line NIT-1. Chapter three examines the impact of LCA incorporation in PB microcapsules in terms of drug content, production yield, microencapsulation efficiency, zeta potential, conductivity, surface tension, particle size, stability, swelling, membrane resistance, buoyancy, drug release, and biological effects, *ex vivo*. Finally, chapter four presents the discussion, conclusion, limitation, and future direction of the study.

Objectives

The main objectives of the study are:

Objective 1: To develop, validate, and optimize the microencapsulation technique and formulation.

Objective 2: To develop and characterize PB microcapsules in terms of morphology, topography, compatibility, and release profiles.

Objective 3: To investigate the effects of bile acid on encapsulated PB.

Hypothesis

The incorporation of LCA into PB formulations will optimize the PB microcapsules features in terms of release from oral targeted delivery.

Outline of experimental design and the main finding

Microcapsules were made by using the Büchi-based microencapsulating system by Ionic Gelation Vibrational Jet Flow technology (IGVJF), using optimal parameters required to produce uniform and high-efficiency microcapsules. Two kinds of microcapsules were produced, including PB and PB-LCA microcapsules using sodium alginate as a polymer. The produced microcapsules were tested *in vitro* (formulation studies) and *ex vivo*. Three independent lots were prepared, analysed, and tested for each formulation.

Through *in vitro* study, produced microcapsules were examined for morphology, surface topography, atomic composition, physicochemical characterization, rheology parameters, electrokinetic stability, size analysis, conductivity, surface tension, swelling resistance, mechanical strength, buoyancy, drug release at various pH values and temperature, and physicochemical stability.

The shape, size, topography, and surface atomic analysis were undertaken using OM (optical microscopy) (Nikon SM2800, Japan), SEM (scanning electron microscopy) (Neon 40EsB FIB-SEM; Germany) and EDXR (energy dispersive X-Ray) (INCA X-Act; UK) as described (15). Physical characterization of pre- and post-microencapsulation were characterized by DSC (differential scanning calorimetric) (DSC 8000, PerkinElmer Inc., USA). Chemical analysis of prepared microcapsules was carried by FTIR (Fourier-transform infrared spectroscopy) (PerkinElmer Inc., USA). The Visco-88 viscometer was used to analyze the rheological parameters of formulations. Electrokinetic stability was measured by using zeta sizer (Zetasizer 3000HS, UK), and particle size analysis was noted by

Mastersizer (Mastersizer 2000, UK). The tensiometer was used to record surface tension exerted by the formulations (Sigma 703). The conductivity was analysed by a conductivity meter (CDM230, conductivity meter). High-pressure liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan) and UV-spectrophotometer methods were develop and validated to determine the drug content in microcapsules (Shimadzu UV-Vis spectrophotometer 1240, Japan). The swelling resistance, mechanical strength, buoyancy, drug release, and physicochemical stability were tested as described (41, 42, 49, 50).

The ex vivo study was prepared to examine the biological impact of PB microcapsules. Pancreatic β-cell line, NIT-1 was used to measure the biological activities using viability assay, pro-inflammatory (IFN-γ and IL-1-β) and anti-inflammatory (IL-10) biomarkers and bioenergetics parameters such as extracellular acidification rate (ECAR), oxygen consumption rate (OCR), proton production rate (PPR), basal respiration (BR) and maximal respiration (MR). NIT-1 cells were cultured in DMEM media at two different glucose concentrations, 5.5 mmol, and 25 mmol, and treated with prepared microcapsules to see the effect on cytokines production and bioenergetics parameters.

The results revealed that the PB-LCA formulation exhibited best microcapsules in terms of morphology, physicochemical characterization, rheology parameters, PB release, and physicochemical stability. Morphological examination of PB microcapsules showed clear border with spherical shape and size ranges from (800- 1000µm), and assimilation of LCA in PB formulations did not change the shape, size, surface topography and elemental present of microcapsules. Microcapsules had rough surfaces with white deposit granules. In the rheological study, both formulations exhibited non-Newtonian, thixotropic properties, and integration of LCA did not change the rheological properties of the matrix. Physicochemical characterization was investigated in both pre- and post-microcapsules, to assess excipient compatibilities, and showed that assimilation of LCA did not alter the physical and chemical properties of PB (chapter 2).

Encapsulation efficiency, drug content, production yield, electrokinetic stability, surface chemistry, and size distribution were consistent between PB and PB-LCA microcapsules. However, significant changes in electrical conductivity, swelling index, mechanical resistance, micro-floating capacity, and drug release were found when LCA was incorporated into PB microcapsules. The drug release was maximum at pH 7.8 and LCA addition in PB microcapsules showed a more controlled release. In physical stability study, a significant impact of temperature ranges from -20 to 40 ^{o}C, and humidity was noted on both PB and PB-LCA microcapsules (chapter 3).

After 48 hours, NIT-1 cells treated with PB-LCA microcapsules had significantly reduced pro-inflammatory and enhanced anti-inflammatory cytokines in hyperglycemia state. Moreover, a

significant improvement in bioenergetics parameters was found after the 48 hrs treatment with LCA-PB microcapsules on the cell's hyperglycaemic conditions (chapter 2 and 3).

Thesis's structure

This thesis contains four chapters that cover the importance and impact of bile acid on PB microcapsules.

Chapter one: Introduction and literature review

The contents of this chapter are covered by the first manuscript from the publication list.

> **Susbin Raj Wagle**, Bozica Kovacevic**,** Daniel Walker**,** Corina Mihaela Ionescu, Umar Shah, Goran Stojanovic, Sanja Kojic, Armin Mooranian, and Hani Al-Salami. Alginate-based drug oral targeting using bio-micro/nanoencapsulation technologies.
> Journal: **Expert Opinion on Drug Delivery** (in press).

It is a review, chapter one takes into account lipophilic drugs and oral administration, effects of main metabolic enzymes, efflux proteins, and GIT environment in the oral bioavailability of lipophilic drugs and illustrates possible novel approaches in enhancing oral bioavailability of lipophilic drugs. The role of bile acids and microencapsulation to improve the oral bioavailability of lipophilic drugs is also described, which is the central concept of this thesis.

Chapters two: Describes the method validation and optimization, characterization of PB microcapsules, and analyses the impact of bile acid in PB microcapsules.

Chapter two covers the following objectives:

Objective 1: To develop, validate, and optimize the microencapsulation technique and formulation.

Objective 2: To develop and characterize PB microcapsules in terms of morphology, topography, compatibility, and release profiles.

Objective 3: To investigate the effects of bile acid on encapsulated PB.

The contents of this chapter is addressed in the second published manuscript from the publication list.

> Sub-objective (1): To microencapsulate PB with LCA using SA, optimize technique and examine the effect of LCA on the morphology, surface analysis, rheology, thermo-chemical

stability, drug's release, cell viability, inflammatory profiles and bioenergetics parameters of the microcapsules.

Wagle SR, Walker D, Kovacevic B, Gedawy A, Mikov M, Golocorbin-Kon S, Mooranian A, Al-Salami H. Micro-Nano formulation of bile-gut delivery: rheological, stability and cell survival, basal and maximum respiration studies. **Scientific Reports**. 2020 May 7;10(1):1-0.

Chapters three: Explores the new design of delivery systems for probucol using LCA-based matrices, these drug-formulations are tested *in vitro* and *ex vivo.*

Chapter three covers the following objectives:

Objective 1: To develop, validate, and optimize the microencapsulation technique and formulation.

Objective 2: To develop and characterize PB microcapsules in terms of morphology, topography, compatibility, and release profiles.

Objective 3: To investigate the effects of bile acid on encapsulated PB.

The contents of this chapter is addressed in the third under review manuscript from the publication list.

Sub-objective (2): To validate and optimize the technique and microencapsulate PB with LCA using SA, and examine the effect of LCA on drug content, microcapsule efficiency, production yield, drug release, zeta potential, conductivity, surface tension, stability, buoyancy, mechanical resistance, and swelling index. Microcapsules' effects on NIT-1 pancreatic β cells, inflammatory profiles, and bioenergetics parameters were also analyzed.

Susbin Raj Wagle, Bozica Kovacevic**,** Daniel Walker**,** Corina Mihaela Ionescu, Goran Stojanovic, Sanja Kojic, Armin Mooranian and Hani Al-Salami. Pharmacological and advanced cell respiration effects, enhanced by toxic human-bile nano-pharmaceuticals of probucol cell-targeting formulations. Journal: **Pharmaceutics** (in press).

Chapter four: Discussion, conclusion and future perspective.

List of abbreviations:

DM	Diabetes mellitus
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes
GD	Gestational diabetes
PB	Probucol
LCA	Lithocholic acid
ULCA	Unconjugated lithocholic acid (ULCA)
SA	Sodium Alginate
LVSA	Low viscosity sodium alginate
ACM	Artificial cell microencapsulation
GIT	Gastrointestinal tract
CDCA	Chenodeoxycholic acid
UDCA	Ursodeoxycholic acid
PBUDCA	Probucol-Ursodeoxycholic acid
DSC	Differential scanning calorimetry
FTIR	Fourier-transform infrared spectroscopy
OM	Optical microscopy
SEM	Scanning electron microscopy
EDXR	Energy-dispersive X-ray spectroscopy
IF	Interferon
IFN-γ	Interferon-gamma
IL-1 β	Interleukin- 1 beta
IL-10	Interleukin- 10

OCR	Oxygen consumption rate
ECAR	Extracellular acidification rate
PPR	Proton Production rates
BR	Basal respiration
MR	Maximal respiration
NADH	Nicotinamide adenine dinucleotide hydrogen
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
GLUT4	Glucose transporter type 4
Biopharmaceutics	Biopharmaceutics classification system
CYP	Cytochromes P450
ABC	ATP-binding protein
P-gp	P-glycoprotein
MRPs	Multidrug resistance proteins
BCRP	Breast cancer resistance protein
MDR	Multidrug resistance
PXR	Pregnane X receptor
DDI	DDI
SEDDS	Self-emulsifying drug delivery system
IGVJF	Ionic Gelation Vibrational Jet Flow technology
DMSO	Dimethyl sulfoxide

Table of Content

Chapter 1

Chapter 1

Introduction and literature review

Alginate-based drug oral targeting using bio-micro/nano encapsulation technologies.

The contents of this chapter are covered by article first from the publication list:

Chapter one is a review article that provides insights into the lipophilic drug delivery systems. The detailed description of the obstacle associated with the oral bioavailability of lipophilic drugs is discussed. Furthermore, techniques to overcome these obstacles, with much emphasis on optimal safety and efficacy, were addressed. The ionic vibrational jet flow encapsulation technology has an enormous impact on lipophilic drug delivery systems, which is discussed thereafter. The chapter further emphasis the combination of drug-bile acid-alginate compounds by using the microencapsulation method as a promising strategy to improve the oral delivery of lipophilic drugs, which is the central concept of this thesis.

EXPERT OPINION ON DRUG DELIVERY
https://doi.org/10.1080/17425247.2020.1789587

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REVIEW

Alginate-based drug oral targeting using bio-micro/nano encapsulation technologies

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ABSTRACT

Introduction: Oral delivery is the most common administrated drug delivery path. However, oral administration of lipophilic drugs has some limitations: they have poor dose-response due to low and varied dissolution kinetics and oral bioavailability with sub-optimal dissolution within the aqueous gastrointestinal microenvironment. Therefore, there is a need for robust formulating methods that protect the drug until it reaches to its optimum absorption site, allowing its optimum pharmacological effects via increasing its intestinal permeation and bioavailability.

Area covered: Herein, we provide insights on orally administered lipophilic drug delivery systems. The detailed description of the obstacles associated with the oral bioavailability of lipophilic drugs are also discussed. Following this, techniques to overcome these obstacles with much emphasis on optimal safety and efficacy are addressed. Newly designed ionic vibrational jet flow encapsulation technology has enormous growth in lipophilic drug delivery systems, which is discussed thereafter.

Expert opinion: Researchers have shown interest in drug's encapsulation. A combination of drug-bile acid and microencapsulation methods can be one promising strategy to improve the oral delivery of lipophilic drugs. However, the most critical aspect of this approach is the selection of bile acids, polymer, and encapsulation technology.

ARTICLE HISTORY
Received 28 April 2020
Accepted 26 June 2020

KEYWORDS
Bioavailability; encapsulation; gastrointestinal tract; ionic jet flow technology; lipophilic drugs; microcapsules; oral administration

1. Introduction

The solubility of lipophilic drugs within the gastrointestinal tract (GIT) microenvironment is one of the fundamental properties which plays a significant role in oral administration and uptake into the systemic circulation. Lipophilic drugs are insoluble in water but readily dissolve in lipids. Biopharmaceutics classification system (BCS) classified drugs into four classes, based on two key parameters, intestinal permeability and solubility. Overall, class I is defined as drugs with high solubility and high permeability, class II as low solubility and high permeability, class III as high solubility and low permeability, and class IV as low solubility and low permeability. Different approaches such as permeation enhancers, prodrugs, improvement of dissolution rates, physical and chemical modification are employed to increase the solubility and permeability of drugs [1], [2]. Among these four BSC classes: type II and IV are considered most challenging in oral delivery of lipophilic drugs as lower absorbance results in slow bioavailability which in turn result in inter and intrapersonal variability [3]. Different BCS drug groups which exhibit large inter and intrapersonal variability and low bioavailability presented in Table 1.

About 40–60% of the marketed oral medications are either BCS group II or group IV and considered nearly insoluble (<100 µg/mL, lipophilic in nature), resulting in weak-aqueous solubility and poor dissolution in the GI fluids with subsequent decrease in bioavailability and higher inter-individual variability of oral uptake [3,4]. The current challenge is to overcome these problems faced by orally administrated medication that also has a low bioavailability. Different approaches are ongoing to improve the oral bioavailability of lipophilic drugs such as modifying the chemical structure with the aim of enhancing solubility using prodrugs, encapsulation, surface modification and coating with various functional groups [5,6]. Among them, the encapsulation method has gained more attention [7]. Ionic gelation vibrational jet flow is an encapsulation method that shields volatile drugs from premature degradation, oxidation, and hydrolysis and caters for controlled and targeted delivery by allowing for site-specific targeting, which in turn positively impact on pharmacokinetics/pharmacodynamics profiles [8]. For targeted drug delivery, different types of delivery vehicles such as liposomes, polymeric micelles, biodegradable particles, dendrimers and nano-particle drug carriers are prepared by the encapsulation method [9–12]. The ideal drug delivery vehicle must be biocompatible, biodegradable, nontoxic and non-immunogenic to the host [5]. Nano or microparticles formation by using biodegradable polymers such as sodium alginate, poly (lactic-co-glycolic acid) (PLGA), Polylactic acid (PLA) have been

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Article highlights

- Lipophilic drugs are poorly water-soluble but dissolve readily in lipids and rapidly growing in the drug market despite its low bioavailability and poor water-solubility.
- Oral delivery is the most common method of drug delivery, mainly due to comfort and patient compliance. However, orally administered drugs have to pass via many biochemical and physiological obstructions and eventually decrease the bioavailability of drugs.
- The human intestine expresses an abundant amount of cytochrome P450 iso-enzymes like cytochrome P-450 3A (CYP 3A4) act as a primary barrier for the absorption of lipophilic drugs. Also, efflux proteins are mainly present in the intestinal membrane, where they can expel a range of structurally different drugs, drug conjugates, and metabolites.
- There are numerous techniques and strategies to improve oral bioavailability, and one of them is the drug's encapsulation technique.
- Ionic Gelation Vibrational Jet Flow technology (IGVJF) in drug delivery is one of the newly pioneered physio-chemical microencapsulation methods for medical and biotechnological applications.

Recent roles of bile acids are expanded towards the importance of microcapsules- stabilization and as permeation-enhancing agent for the optimal bioavailability of lipophilic drugs.

This box summarizes key points contained in the article.

studied extensively over the past few years for the controlled and site-specific drug delivery system by tuning the polymer characteristics and surface chemistry and especially useful for drugs with an intracellular target [11]. In this approach, the drug is loaded with polymer and form nano-particles and targeted to a specific site of the body for the absorption. Hence, the main aim of this review is to provide the knowledge of lipophilic drugs and oral administration, the effect of main metabolic enzymes and efflux protein in the oral bioavailability of lipophilic drugs and to illustrate novel approaches in enhancing oral bioavailability of lipophilic drugs using bile acids and microencapsulation technique to produce Nano and Microparticles.

1.1. Lipophilic drugs and oral administration, uptake and possible barriers

Drugs are administered either orally or through intravenous, rectal, ocular, nasal, inhalation, parenteral, cutaneous and transdermal. In general, oral delivery is the preferred route due to its comfort and patient compliance. However, orally administered drugs have to go through many biochemical and physiological obstructions before release into systemic circulation [13].

One of the most difficult hurdles for oral drug delivery is the rapid change in GIT pH values before absorption that can cause deleterious changes to drug chemical composition and stability. Changes in GIT pH can also play a vital role in the mechanism of drug delivery. Weak bases have better solubility in acidic conditions however their solubility in the basic environment is decreased as a result. This in turn also decreases the drugs bioavailability as the target sight for absorption is in an alkaline section of the small intestine [14]. So, the pH differences from the highly acidic environment in the stomach (1–2) to the natural to natural alkaline in the ileum and colon (7.0–7.5) can have a drastic impact on the absorption and bioavailability of a drug[15]. Exposure in these environments can cause oxidation, degradation, or hydrolysis of oral drugs before reaching the target site for the absorption (Figure 1) [16].

Lower down along the GIT; drugs eventually settle in the intestinal tract beginning with the small intestine. The human gut is considered as an essential site for the first-phase drug's metabolism and extraction. It expressed an abundant amount of cytochrome P450 enzymes and effluxes proteins, acts as a primary barrier for the absorption of lipophilic drugs, which act as subtract molecules to endure oxidative metabolism [17,18].

Also, the luminal efflux transporter protein present in enterocyte is another barrier for drug absorption (Figure 1). These efflux transporters present on the top of the enterocyte transfer drugs back to the intestine from the cell, where drugs have already entered and inhibited the drug absorption into the blood [19].

Usually, a drug molecule that enters the enterocyte undergoes three primary fates; diffuse into the hepatic portal flow, undergo metabolism within cells or transported back into GI lumen by efflux transporter, where it can be then reabsorbed [20]. Drugs escape from enterocyte metabolites, and the efflux mechanism reaches the liver through the hepatic portal system [21], which further contains different CYP metabolic enzymes like CYP3A4, which has been another considerable obstruction to the retention of lipophilic competitors [22].

By overcoming all the above obstacles, drugs are absorbed at the enterocyte by using either passive diffusion or carrier-mediated processes (Figure 1). Lipophilic drugs, mainly undergo passive transcellular absorption [23]. The part of the drug that reaches circulation is transferred to the liver via the

Table 1. Lipophilic drugs with corresponding solubility and low bioavailability.

Drug Name	Category/Action of Drug	Treatment	BCS class	Absolute Bioavailability	Ref.
Acebutolol	Beta-blocker	Hypertension	III	40%	[170]
Felodipine	Ca-channel blocker	Hypertension	II	15%	[171]
Candesartan	Angiotensin receptor blocker	Hypertension	II	15%	[172]
Simvastatin	HMG coenzyme- A reductase	High blood cholesterol and triglycerides	II	5%	[173]
Probucol	Anti-hyperlipidemia	Familial hypercholesterolemia, adjunct therapy	II	< 10%	[174]
Aciclovir	Anti-viral medicine	Cytomegalovirus	III	10-20%	[172]
Amphotericin B	Anti-fungal medicine	Fungal infections	IV	< 10%	[175]
Lovastatin	HMG-CoA Reductase Inhibitor	High blood cholesterol	II	<5%	[176]
Atorvastatin	HMG-CoA Reductase Inhibitor	Cardiovascular disease	II	12%	[172]
Verapamil	Ca-channel blocker	Hypertension, angina and supraventricular tachycardia	II	35%	[177]

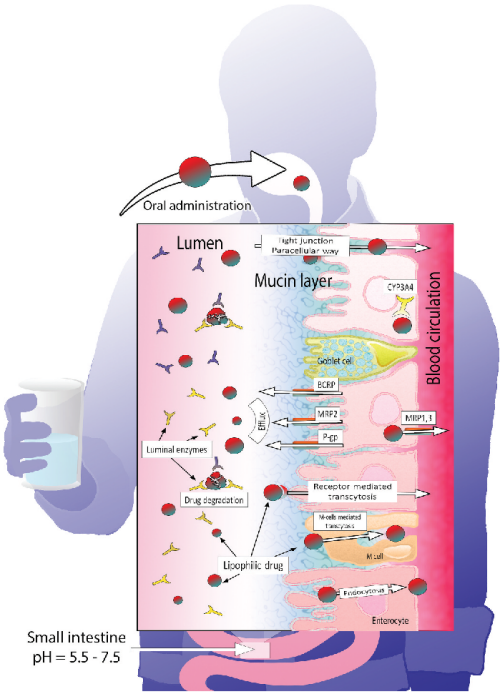

Figure 1. Possible interaction of lipophilic drugs with intestinal barriers and the process of drug transport into circulation.

portal system and undergoes further metabolism and biliary excretion. Therefore, before entering the target site and systemic circulation, lipophilic drugs are typically degraded at different locations along the GI tract, and this can lead to a loss of active compounds and thus decreases the overall bioavailability (Figure 1).

1.2. Effect of Cytochrome P450 on orally administrated drugs

The cytochrome P450 (CYP450) isoenzyme is hemeprotein superfamily that is present on the membrane of the endoplasmic reticulum, the protein is involved in the breakdown of many exogenous compounds (drugs, alcohol) and endogenous substances (steroids, fatty acid and prostaglandins) by oxidation, hydrolysis, or reduction of compounds [24]. The name 'P450' derives from a spectrophotometer absorption peak at 450 nm when it is reduced and bound with carbon monoxide [24]. There are many other metabolic enzymes, but CYP450 is intensely studied because it plays a vital role in most clinically significant metabolic drug interactions, and a considerable hindrance for orally administrated drugs [22,25]. 'CYP' is the root symbol of cytochrome P450 gene for humans, followed by a by an Arabic numeral (e.g., CYP3) indicating the gene family, CYP subfamily is presented by capital letter behind the number (e.g., CYP3A) and individual gene/iso-enzyme is further represented by Arabic numeral in an Italic (e.g. CYP3A*4*) [26]. To date, 14 P450 genes and 20 subfamilies are already reported in the human genome [27]. These genes are primarily connected with hepatic metabolism. CYP1, 2, and 3 are common CYP450 families, which represent 70% of all the hepatic content. Among these three families, CYP3A is the most significant and represents around 30% of absolute hepatic P450. Six isoenzymes CYP1A*2*, 3A*4*, 2C*9*, 2C*19*, 2D*6* and 2E*1* from CYP families are major enzymes that participate in the hepatic metabolism of a wide range of drugs (Table 2). CYP3A*4* is mainly found at a higher concentrations in the mucosa of the small intestine [28]. Usually, CYP3A*4* are structurally diverse and catalyze the breakdown of a wide range of clinically significant drugs [29]. Drugs administrated orally are more likely to have CYP3A4 interaction during absorption, in the small intestine CYP3A*4* blocks the drug absorption and contributes to the general degradation of the drug [30]. Numerous inhibitors, substrates, and inducers of CYP3A*4* exist which have shown to improve or decrease the bioavailability of drugs accordingly (Table 2).

CYP gene polymorphism causes poor, intermediate drug metabolism [40]. In one study examining inter-individual altered drug metabolisms, polymorphisms in CYP3A*4* metabolized more than 50% of the orally administrated drug. Many variants have been identified (more than 26), and many of these variants cause altered enzyme activities ranging from modest to highly decreased drug efficiencies [41,42].

Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) are the nuclear receptors that activate CYP33A4 present in the enterocyte. They are usually activated by different endogenous (e.g., bile acid) or exogenous compound (drugs) and upsurges the metabolic activity of CYP enzymes [43]. For instance, long-term treatment of rifampin decreased atorvastatin bioavailability by rifampin-activated PXR due to induced expression of CYP3A and effluxed transporters [44]. Drug bioavailability can be enhanced by targeting specific membrane transporters using novel drug delivery carrier system.

1.3. Effect of efflux drug transporter on drug intracellular uptake, transport and penetration through the intestinal epithelial membrane

Active efflux proteins are also another factor in poor absorption and low bioavailability of lipophilic drugs along with CYPs enzymes present in an enterocytes [45–51]. The small intestine expresses varieties of transporter proteins implicated in efflux and influx [52]. ATP (adenosine triphosphate)-dependent efflux transporters are the most important transporters impacting drug absorption. ATP-binding cassette or ABC (ATP-binding protein) transporter are active transporters, and part of a large superfamily of proteins that typically transport different compounds including lipophilic drugs across the cell membrane [52]. These transporter proteins are extensive, and functionally extremely diverse in mammals. The human genome contains 48 ABC proteins, the most common and clinically significant of which are the p-glycoprotein (P-gp), Multi-Drug Resistance Proteins (MRPs) and Breast Cancer

Table 2. An overview study investigation of CYP450 isozymes on several drugs, potent inhibitors, inducers and metabolites.

Isoenzymes	Potent inhibitors	Potent inducers	Drugs	Metabolites	Ref.
CYP1A*1*	Propofol	Polycyclic hydrocarbons	Amiodarone	Desethylamiodarone	[22,31,32]
CYP1A*2*	Amiodarone cimetidine ciprofloxacin fluvoxamine	Carbamazepine phenobarbital, rifampin,tobacco	Amiodarone	Desethylamiodarone	[22,33]
CYP2C*8*	Cimetidine	Rifampicin	Gemfibrozil	Gemfibrozil glucuronide	[34,35]
CYP2C19	Fluvoxamine, isoniazid, ritonavir demethoxygeldanamycin	Carbamazepine, phenytoin, rifampi [22,36]	Tanespimycin	7-amino-17-	
CYP2C*9*	Amiodarone, fluoxetine, metronidazole, ritonavir trimethoprim/sulfamethoxazole	Carbamazepine, phenobarbital, rifampin	Warfarin	Hydroxy warfarins	[22,37]
CYP2D*6*	Amiodarone, diphenhydramine fluoxetine, paroxetine ritonavir,	No significant inducer	Sarprogrelate	M-1	[22,38,39]
CYP3A*4*	Grape fruit juice, itraconazole	Carbamazepine, St. John's wort, phenytoin, rifampin	Diltiazem	N,N-didesmethyldiltiazem, N-desmethyl-diltiazem	[39]
CYP3A*5*	Ketoconazole, nefazodone, ritonavir, telithromycin, verapamil, troleandomycin demethoxygeldanamycin	Carbamazepine, phenobarbital, phenytoin, rifampin dexamethasone [22,36]	Tanespimycin	17-amino-17-	

Resistance Protein (BCRP). Among them, P-gp, also named as multi-drug resistance (MDR) code gene ABCB1 has a clear function in the transport of clinically essential drugs [48,53–55]. The essential ABC transport proteins that participated in drug resistance and deposition are mentioned in Table 3.

Efflux proteins are mainly present in the intestinal epithelial membrane, where they can eject a range of structurally different drugs, drug conjugates, and metabolites from the cell. Most of their recognized substrates are hydrophobic compounds such as cyclosporine (BCS class II), verapamil (BCS class II), loperamide (BCS class II), digoxin (BCS class IV), ritonavir (BCS class IV) and hence, have major impact on the absorption and distribution of the lipophilic drug. For instance, P-gp identifies a range of structurally and pharmacologically unrelated neutral and positively charged hydrophobic compound [23]. This transporter uses ATP to translocate substances across the cell membrane and have the capacity to affect bioavailability by forcing drugs out of the enterocyte (Figure 1). In transcellular absorption, the drug passes from lumen to blood (apical-basal membrane surface), needs an uptake through the apical membrane, transport across the cytosol, into the blood through the basolateral layer [57]. P-gp and MRPs are present in higher numbers on the apical membrane of the small intestine. They are considered the rate-limiting obstacle for the absorption of orally administered drugs. Whereas, Mrp3 are situated on the basolateral membrane and eliminate their substrates from enterocytes into the circulation [58]. Studies by *Al-Salami H et al.* presented that gliclazide (BCS class II second-generation sulphonylureas drug) acts as a substrate for Mrp2 and Mrp3, which alter the drug's absorption through the rat's ileum [53,59–61]. Moreover, the study further illustrated gliclazide bioavailability was increased when combining with the bile acid salt (act as a substrate of Mrp3), and produce a hypoglycemic effect in diabetic rats by changing its affinity for or by inducing upregulation of the transport proteins Mrp2 and Mrp3 [53,62]. In the presence of rifampicin and glibenclamide (Mrp2 and Mrp3 inhibitors), increased gliclazide's paracellular permeability and movement [48]. Also, the same group demonstrated that the probiotics alter the gliclazide fluxes in healthy and diabetic rats. The Mrp2 upregulation, which might decrease the absorption of gliclazide by releasing more gliclazide back into the mucosal solution [63].

The study conducted by utilizing MDR1 knock out mice also showed that P-gp has a clear role in drug absorption. This representative study showed that MDR knock-out mice (MDR1A-/-) are shown to have six times higher concentration of paclitaxel in systemic circulation compared to wild type (MDR1+/+) mice. Also, a higher concentration of paclitaxel was noticed in MDR1+/+ when PSC833 (efflux inhibitor) blocked MDR1 function. The study revealed that intestinal absorption of chemotherapy drugs was enhanced when drug administered with P-gp inhibitor [64,65]. Overall, the clinical efficacy of lipophilic drugs mostly relies on their capability to

Table 3. Key transport protein, tissue disputation and their respective function in the human body.

Family	Member/Alias	Tissue distributions	Main Function	Ref.
ABCA	ABCA1	Ubiquitous	Facilitates the efflux of cholesterol/phospholipids	[46,56]
ABCB	ABCB1 (MDR1/PGP)	Blood-brain barrier, intestine, kidney.	Multi-drug resistance, drugs transportation	[46,56]
	ABCB11 (SPGP, BSEP)	Liver.	Drug/peptide transport into the ER Bile salt/bile acid transport	
ABCC	ABCC1 (MRP1)	Ubiquitous.	Drug resistance	[46,56]
	ABCC2 (MRP2)	Liver, intestine, blood-brain barrier, kidney.	Biological anion transport	
	ABCC3 (MRP3)	Intestine, kidney, liver, pancreases, adrenal gland, prostate.	Drug resistance	
	ABCC4 (MRP4)	Lung, Ovary, testis, kidney, prostate.	Nucleoside transport	
	ABCC5 (MRP5)	Liver, brain, testis, skeletal and cardiac muscle.	Nucleoside transport	
	ABCC7 (CFTR)	Exocrine tissues	Chloride ion transport	
	ABCC8 (SUR)	Pancreas	Sulfonylurea receptor	
ABCD	ABCD1(ALD)	Peroxisomes	Fatty acid transport and regulation	[46,56]
ABCE/F	ABCE1 (OABP)	Ovary, testes, spleen	Oligoadenylate-binding protein	[46,56]
ABCG	ABCG2	Placenta, intestine, blood-brain barrier, breast, stem cells	Drug resistance	[46,56]

cross intestinal barriers like efflux proteins to reach their target.

1.4. The interrelation between P-gp and CYP3A

CYP3A4 and Pgp are functionally interactive and widespread overlap in substrates, inhibitors, and inducers and reduce the intracellular concentration of drug substrates, firstly by metabolism and the secondly by transmembrane efflux [19]. PXR is an orphan nuclear receptor that controls both CYP3A4 and P-gp [19]. Hyperforin is a highly potent PXR activator causing stimulation of CYP3A enzymes by P-glycoprotein in the intestine and declines the bioavailability of drugs[66]. Different studies showed that inhibition or inactivation of P-glycoprotein in the small intestine reduces CYP3A-dependent metabolism [20,21] and increases hepatic metabolism [67]. Digoxin clinical efficacy decreased when co-administrated with rifampicin, a strong CYP3A4 and Pgp inducer [68]. As dabigatran is a substrate of P-glycoprotein, inhibitors of P-glycoprotein and CYP3A4 such as amiodarone, ketoconazole, verapamil and clarithromycin may upsurge the peak plasma concentrations of dabigatran [69]. With this, while forming and developing drugs, the interaction between drug-metabolizing enzymes and active transporters should be a high priority.

1.5. Drug-drug interaction and metabolites

Co-administration of multiple drugs is a common practice for many patients. As a result, there is a high possibility of drug-drug interaction (DDI) [30]. In modern pharmaceutical research, the impact of drug metabolites on CYP inhibition and resulting DDI has become a significant concern for new drug formulation. Different metabolites which contributed to clinically relevant DDIs are continuously increasing, and some of them are mentioned in Table 2 [70]. Therefore, in the novel drug formulation, it is vital to assess the effect of drug metabolites on CYPs enzymes before being orally administered. Several other factors directly or indirectly cause DDI, and the most important one is CYP activity and efflux proteins either by induction or inhibition [71]. For instance, the interaction between rifampin (an inducer for CYP3A and ABCB1) and cyclosporine (substrate for CYP3A and ABCB1) resulted in augmented clearance and reduced bioavailability of cyclosporine A [72]. Similarly, prolonged exposer of Saint John's wort (SJW) decreases bioavailability for varieties of drugs as it induces both P-gp and CYP3 expression activity [73]. For performing the pre-clinical assessment and quantitative DDI, the US Food and Drug Administration (2012) published guidelines stated that drug interaction is related to clinically relevant induction when there is a > 25% reduction in plasma drug concentration. Therefore, DDI can alter drug absorption, metabolism, and distribution and can lead to toxicity for cells and also have significant capacity to modify the bioavailability of drugs[70].

1.6. Encapsulation technology

The improvement of oral bioavailability of medications is the most reasonable methodology to expand the bioavailability of drugs as it is the most favored and convenient route of administration [74–77].

There are various methods and strategies paclitaxel, to improve the oral bioavailability of lipophilic drugs such as physical and chemical modification, partition coefficient modifications, and novel drug delivery methods, as mentioned in Figure 2.

In a physical modification, the drug's size is altered and enhance surface-area-to-volume ratio, usually by different techniques such as spray drying, and/or micronization, to increase interaction with the solvent. Similarly, the change of drug's structure by pH adjustment and salt formulation are

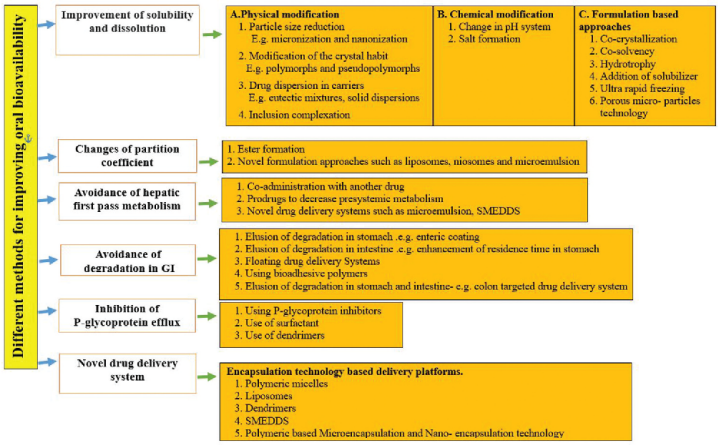

Figure 2. Different methods for improving bioavailability: SMEDDS; self-micro emulsifying drug delivery system.

standard chemical techniques that upsurge drug's solubility and dissolution rate [78–83]. Utilizing partition coefficients, the drug is distributed between two immiscible solvents at equilibrium. The compounds with high partition-coefficient value can effortlessly permeate via a biological membrane, which can improve by increasing drug's solubility using different novel formulation approaches such as liposomes, niosomes and microemulsion [84,85].

Novel drug delivery systems may include self-emulsifying drug delivery systems (SEDDS), liposomes, micelles, niosomes, and dendrimers have shown a significant advantage in drug delivery and formulation [3,86–88].

SEDDS are isotropic stable colloidal mixtures of solvents, surfactants, lipids, and co-solvents/surfactants. In this delivery system, the surfactant and co-surfactant help to stabilize the dispersion system and enhance lipophilic drug penetration via enterocytes. It is one of the promising lipophilic drug delivery methods because of easy formulation and thermodynamic stability and can produce different size particles ranges from micro to macro-emulsion droplets [89].

In drug delivery, liposomes are used as a vehicle having at least one lipid bilayer composed of phospholipids. Contrary to the micelles, liposomes have a bi-layer membrane. One of the advantages of the liposomal formulation is it can encapsulate both hydrophobic and hydrophilic drugs [84]. They have hydrophilic head facing outside and hydrophobic tail surround the fat-soluble nutrient inside (Figure 5 A). The size of liposome is a vital in drug delivery [90].

Liposomes have numerous drawbacks such as short shelf life, toxicity, and chemical stability issues at differing pH values [91]. To overcome these shortcomings, researchers shifted toward niosomes. These are the compound of nontoxic, nonionic surfactant and cholesterol incorporation as an excipient, which provides rigidity to the structure and keeps formulation stable. Because of niosomes unique structure (bi-layered structure with hollow space at the center), niosomes can be used for loading and delivery of lipophilic drugs by encapsulation processes [91].

Dendrimers have an interior dendritic structure, an exterior surface with functional surface groups and a central core. It is a highly branched, novel polymer (e.g., poly-propyleneimine), monodisperse macromolecules [75,92–96]. Usually, lipophilic drugs are encapsulated inside the hydrophobic dendrimer cavity to increase water solubility [97]. Recently, it has been shown; dendrimers are capable of bypassing efflux transporters [10,98].

One common approach to increase the solubility of the drug is by using cyclodextrin [99]. Cyclodextrin is a cyclic oligosaccharide with a macrocyclic ring of glucose subunits linked by α-1,4 glycosidic bonds. It has a hydrophobic interior and hydrophilic exterior and form complexes with lipophilic drug. These processes have been applied for delivery for different types of drugs such as meclizine, *itraconazole* which increase the solubility and stability of the drugs and facilitates penetration via enterocytes [100,101].

There are different methods employed to improve oral delivery as described in Figure 2, but encapsulation technology is the most promising technology and gained more attention for the optimization of oral delivery.

Encapsulation is based on the entrapment of active compound(s) within a carrier material(s) using various techniques. The coated materials are also called a core or internal phase, whereas the coating materials (carrier materials) are named shells, matrix, or external phase [102]. This technology is extensively applied in medical biotechnology and pharmaceutical industries as well as in food industries to encapsulate active compounds by forming protective barriers against human GI physiological conditions or other extreme conditions. Now, microencapsulation technology is being successfully applied to pharmaceuticals and biotechnologies disciplines and is gaining a lot of attention from academic, economic, and scientific bodies [103].

The encapsulation can be done at the micro and nano-level (size dependence), wherein nano and micro range from 1–1000 nm and 1–1000 μm respectively. The size greater than (>1000 μm) is considered to be in macro range [7,8,104]. The terms 'nano' and 'micro' state to particles where the sizes are measured in nanometers (nm) and micrometers (μm), respectively [105,106]. These size-dependent levels depend on various factors, the selection of shell, particle size, zeta potential and viscosities of formulation, drug loading efficiencies, encapsulation efficiency, and release kinetics and drug bioactivity [106]. Nano- capsules have more advantages in drug delivery over the microcapsules because of their large surface area to volume ratio, which makes them to easily pass from the upper GI and enhances absorption [107].

1.7. Application of nano and micro-encapsulation technology in lipophilic drug delivery

Encapsulation technologies have many benefits over other traditional drug delivery systems. Encapsulation encloses the bioactive materials and enhances the bioavailability by protecting the drug from harsh GI environment (rapid changes in pH values ranges from 1.5 to 7.5, enzymatic barriers, efflux proteins barrier, and epithelium barrier). Encapsulation also provides controlled, sustained and targeted drug release. Additionally, encapsulation reduces evaporation or volatility loss, offers protection from reactivity barrier for bioactive compounds, decreases drug efflux by inhibiting efflux mechanism if encapsulated with efflux inhibitor (such as cyclosporine A, rifampicin, glibenclamide, itraconazole) [48,108,109], increases water solubility and dissolution rate, improves of physical stability, shelf life and biological activity of bioactive compounds [110–112]. Based on this, encapsulation technologies have gained significant attention in the targeted drug delivery systems.

1.8. Nano and microencapsulation technology in target drug delivery

Nano and microencapsulation technology have the potential to transport the therapeutically active molecules to the site of action [113]. To date, this encapsulation technology has been used to encapsulate the wide varieties of lipophilic drugs, effective for the oral delivery to treat diabetes, cancer, neurological disorders and inflammatory diseases [105][114]

Usually, encapsulated drugs are protected by the polymer from degradation before reaching target sites. In GI fluid,

polymer like sodium alginate at low pH it form a porous, insoluble acid matrix and protects the drug from insults from the GI tract, when it reaches a more neutral pH, the alginate acid matrix is transformed into a soluble viscous layer and causes the breakdown of polymer and release of the encapsulated compound (Figure 3) [93,94,96,115–117]. As seen in Figure 1, there is low drug bioavailability due to the possible interaction of drugs with different pH values, enzymes, and proteins, causing degradation in the GI tract before reaching the target site. However, the encapsulation process prevents the drugs from unwanted degradation and permits the drug to reach the target site that ultimately increases the bioavailability of drugs (Figure 3)

The uses of nanotechnology have shown strong potential, with numerous types of nanomedicine entering clinical studies to overcome the efflux mechanism. Nanotechnology refines the concept of co-administering drugs with efflux inhibitors by incorporating them into a single drug carrier [118]. Research has established that nucleic acid miRNA founded nano-particles play a vital role in the modulation of drug resistance in tumors by efficiently reducing MDR1 expression *in vivo* [119]. Nano-encapsulation has been revolutionary in selective cancer cell treatment by overcoming the limitations of conventional chemotherapies. Nano-particles that encapsulated chemotherapeutic agents are formed in such a way as directly interact with the affected cells and by engaging ligand-receptor interaction or antibody-antigen recognition [120,121]. Also, nano-encapsulation is considered as one of the most promising methods for the treatment of inflammation at the preferred location with low side effects [122]. Flurosemide that were nano-encapsulated with gelatin and polyions showed prolonged drug release through self-assembly of polymeric shells [123]. It has also been shown to be a promising approach for controlled and targeted release of vitamins [124].

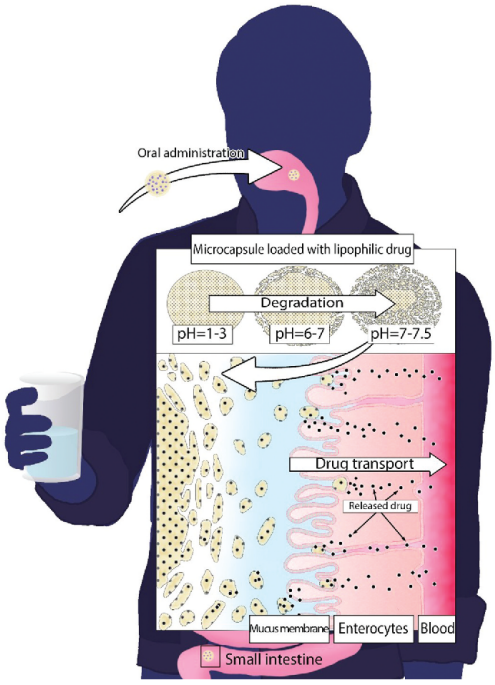

Figure 3. Orally administered encapsulated drug, protection, release and absorption in the GI tract.

1.9. Different carrier agents used in nano and micro technology

Many materials can be used for drug encapsulation but should be safe and biodegradable [125]. Encapsulation agents should have several vital characteristics including being as an excellent emulsifier, having low viscosity even at high concentration, proper dissolution and network-forming features, precision targeting by protecting from the acidic and enzymatic condition as well as from efflux proteins of GI tract, be nontoxic to the human body, hydrophilic nature and so on [126]. Likewise, from the industrial outlook, it should be economical and affordable. Furthermore, while encapsulating bioactive compounds, it is also essential to understand the physicochemical and rheological characters of encapsulated compounds and interaction with a carrier agent [127].

Different natural and synthetic polymers are available for the production of microcapsules. Natural polymers can be divided into polysaccharide-based carrier agents (starches, cellulose, pectin, chitosan, alginate, gums), lipid-based carrier agents (lipids) and protein-based carrier agents (caseins, gelatine, whey proteins, soy proteins, cereal proteins) [125]. Among all the polymers, alginate has gained the most attention, with the majority of studies employing this polymer [128–132]. The reasons behind its high acceptance are favorable properties like its stability and solubility, nontoxicity to human and animal cells, versatility, excellent biocompatibility, and biodegradability [128,133,134]. Some of the disadvantages of alginate microcapsules may include its low mechanical strength, uneven porosity and membrane density, which results in membrane deformation and subsequent capsule rupture and drug leakage [135,136]. Therefore, alginate is usually co-formulated in different polyelectrolyte, such as Poly-L-Ornithine (PLO). Such formulation resulting in ionic complexation and enhanced stability of the microcapsules and decline rapid rupture after administration [137].

1.9.1. Alginate as a polymer

Alginate is a linear copolymer compound which has (1, 4)-linked-β-D-mannuronic (M) and α-L-guluronic (G) acid units (consecutive G residues, consecutive M residues, and alternative M and G residues). It is identified by IUPAC as sodium 3, 4, 5, 6-tetrahydroxyoxane-2-carboxylate, and has molecular weight 216.12 g/mol. It is made mainly from two different sources; algae and bacteria. Most of the commercially existing alginates are produced from brown algae (*Macrocystis pyrifera* is the common specie). Bacterial-derived alginate from *Azotobacter* and *Pseudomonas* are generally not economically and commercially feasible.

The viscosity of the alginate solution is determined by the concentration of alginate in the solution and the number or length of monomer units in the alginate segment. Alginates with high contents of guluronic acid have a high number of open-pore structure which display high diffusion rates for the encapsulated material. On the other hand, the higher number of mannuronic acid alginate units are less porous, softer, and easily broken down in the GI tract before reaching the target site for delivery [138,139]. Alginate rich in guluronic acid is more aqueous soluble than mannuronic acid-rich alginate [140]. Alginate is dependent upon the pH to which it is exposed. A slow and stable decrease of the pH results in a gelatinous precipitate of alginate molecules, while a rapid reduction of pH causes the precipitation of alginate molecules in the form of aggregates. The increase in pH above 10 shows a slow depolymerization process [141]. Overall, alginate is the perfect matrix for entrapment of bioactive compounds and drugs as well as for cell immobilization. The drug encapsulated by alginate can be delivered at the desired proportion in a sustained and controlled release system by diffusional processes through pores. Typically, the release of hydrophilic drugs is controlled mainly by diffusion, whereas the lipophilic drug is mostly dependent on gel erosion [142].

1.10. Sodium alginate in controlled and targeted drug delivery

Sodium alginate (SA) has gained more attention because of its biodegradable, biocompatible, hydrophilic and protective characteristics and has shown excellent compatibility and stability [143]. The properties of the alginate are influenced by the viscosity of the matrix and have well studied pH release kinetics [144]. The low-viscosity SA support controlled and faster drug release than high viscosity SA [145]. In GI fluid, low viscosity SA is changed into the porous, insoluble acid matrix that protects against the release of drug from microcapsule, when it reaches into higher pH (targeted site of the small intestine), then the alginate acid matrix is transformed into a soluble viscous layer and causes the breakdown of polymer and releases of drug [146].

It has also been shown that gliclazide loaded only with sodium alginate has an encapsulation efficiency of more than 90% and showed targeted and control drug release [70,147]. An in-vitro study by *Mooranian et al.* (2015) further demonstrated that sodium alginate incorporation with antidiabetic drugs shows potential in the targeted oral delivery [148]. The study confirmed that sodium alginate does not interfere with the drug's morphology, size, structure, stability, chemical, thermal profile, or drug release profile [148,149].

At present, there are numerous approaches for cross-linking sodium alginate, and ionic cross-linking is the most common technique to make alginate hydrogels [145]. Calcium ions cross-linked with alginate have been extensively used due to its high biocompatibility, economy, reduced immunogenicity, and capacity to form hydrogels under mild conditions and also due to mild reaction with alginate. However, the rapid solubility and concentration of the calcium ions significantly impact the structure and the performance of the microcapsules. Carboxyl group of guluronic acid and calcium interacts, resulting in a three-dimensional network [145,150]. The rapid gelation reduces the preparation time, ensures a constant product and can decrease the loss of hydrophilic active principles in the external aqueous medium. Moreover, the Ca-alginate complex is stable in acidic and degradable in neutral or alkaline conditions, which resulted in the sustained and controlled release of core substances in the GI environment [151].

1.11. Microencapsulation technique

Generally, there are three different techniques commonly used to produce micro and nano-capsules: 1) physical/mechanical method which include air suspension, spray drying, solvent evaporation/extraction and pan/fluid coating, 2) physicochemical method such as ionic gelation, and coacervation and 3) chemical methods such as *in situ* polymerization and interfacial [7,152]. The selection of the encapsulation technique should be made wisely to produce high-efficiency micro or nano-capsules. The method chosen should have a high ability to develop capsules that allow optimal production yield and encapsulation efficiency with a proper size that enhances the high bioavailability of the active compound [110]. Considering all these characteristics and the technical and economic feasibility aspects, ionic gelation vibrational jet flow is the most feasible encapsulation option for a wide range of lipophilic drugs [130,131,153–155].

1.11.1. Ionic Gelation Vibrational Jet Flow technology (IGVJF) in drug delivery

IGVJF uses both mechanical (vibrational force) and chemical (ionic gelation) technologies. It is based upon the principle of forming droplets from a mixture of the drug (active ingredients) and a polymer that passes from a precisely drilled nozzle and splits into equal size droplets by exiting vibrational force aided by gravity and influenced by viscosity and surface tension. After production, the droplets immediately form a spherical shape (capsule) by chemical means. The output of these spherical beads occurs due to the surface tension [8]. It usually produces microcapsules size range from (100–2000 µm).

The encapsulator B-390 (BUCHI labortechnik, Flawil, Switzerland) is one of the instruments which produces microcapsules by using this method [136,147,156]. Figure 4 is the schematic representation of encapsulator B-390, which consists of different elements along with its necessary procedure.

In general, drug and encapsulating polymers are mixed and placed into the pressure bottle and forced into bead producing unit with air pressure. A stable and continuous flow of liquid is needed to get the optimal breakup of the extruded liquid jet into droplets and produce microspheres with a small size distribution. The flow of solution through the nozzle is achieved by the air pressure regulation system. It contains an electrode that is part of the electrostatic dispersion unit; droplets pass through an electric field; they resulted in a surface charged. Electrostatic repulsion disperses the beads collected in the polymerization bath, which is electrically grounded. Bead formation can be observed visually using a stroboscope. The electrostatic charge is transferred to the

polymerization bath and accumulates if the vessel is not grounded. Therefore, the grounding hook attached to the ground wire is put into the polymerization bath so that the electrostatic charges will flow to the ground (Figure 4) [8].

The production of bead size is controlled by different elements such as nozzle size, vibration frequency, flow rate, and physical characteristics of encapsulation mixture [157]. Typically, the capsules size is twice the nozzle diameter. However, by changing jet velocity and the vibration frequency, the size of the bead can be adjusted. The general rules for the high production yield is that higher frequency generates smaller capsules, lower liquid flow rates produce smaller bead sizes, and smaller nozzle produces smaller bead size. Moreover, the larger the beads, the higher the electrostatic voltage is required to separate the jet. The liquid passes through the nozzle, must have enough flow rate so that it can be pass via the nozzle to form a constant steady laminar liquid jet [155].

1.12. Application of IGVJF in drug discovery to treat the disease

IGVJF is used to produce multi-compartment microcapsules for targeted oral delivery of lipophilic drugs such as probucol and gliclazide [105,] [158–161].

Microcapsules made from alginate are insoluble at acidic pH of the stomach, creating the protective barrier for the encapsulated drug. Furthermore, at alkaline pH in the intestine, alginate becomes water-soluble, allowing controlled microcapsule degradation. Unlike lipophilic drugs on its own, probucol entrapped in alginate matrix can cross the mucin layer, delivering a higher concentration of drugs to the absorption sites. The results suggest that microcapsules produced with this method have potential applications in the oral delivery of probucol in type two diabetes [105]. Likewise, *S. Mathavan et al. (2018)* also used the same technique and proved that gliclazide microcapsules found a positive effect on cell viability and control glucose levels in type-1 diabetic animals [147]. Moreover, other recent studies also found that microcapsules produced by using IGVJF showed good morphological structure, chemical and physical compatibility, targeted drug release and high encapsulation efficiency, high production yield and research are ongoing for sustained and targeted delivery for the treatment of diabetes [148,162]. It also produces capsules capable of protecting living cells like pancreatic beta cells from the immune system and allows for their potential transplantation in diabetes patients [163].

The IGVJF can produce beads with required characteristics that facilitate the application of novel drugs and can improve drug efficacy and oral bioavailability. It has been useful in providing a wide range of microcapsules that protect drugs from the GI environment and optimize targeted and sustained drug release. However, this technique is not without its limitations. The method is confined to low production yields as it only yields single droplets at any given time. The production flow rate is mostly reliant on the diameter of the nozzle. However, the other possible option to raise the production rates can be accomplished by increasing the number of nozzles' on the machine [8,164].

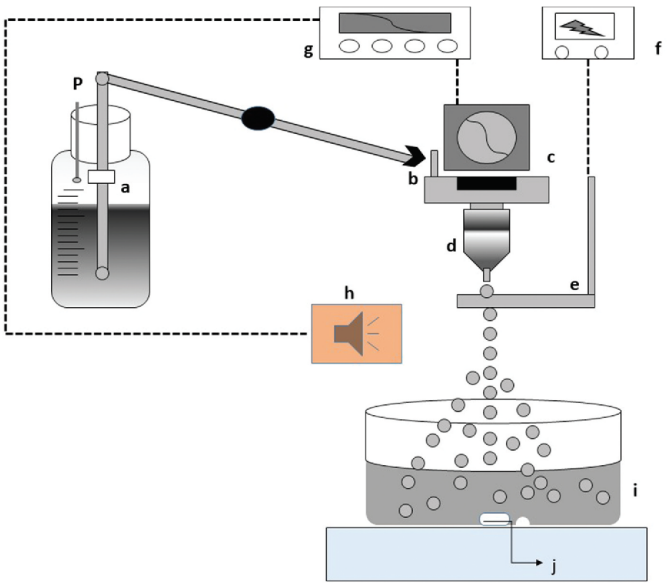

Figure 4. General method for preparation of microcapsules and schematic representation of Ionic Gelation Vibrational Jet Flow technology. Pressure bottle (a), Bead producing unit (b), Vibration unit (c), nozzle (d), electrode (e), dispersion unit (f), vibration control (g), stroboscope (h), polymerization bath (i) and magnetic stirrer (j).

1.13. Bile acid constituents of the microcapsules and role in the lipophilic drug delivery

Bile acids (BAs) are naturally synthesized compounds inside the human body from the catabolism of cholesterol, primarily in the liver. The synthesis of the bile acid is complex, multi-step, and multi-organelle processes. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary bile acids produced in the liver. Before the excretion of the bile acid from the liver cell, primary bile acids conjugated with glycine or taurine, creating bile salts which are hydrophilic, zwitterionic compounds with beneficial amphoteric nature. Once the primary BAs reach the intestinal lumen, they undergo deconjugation, oxidation and dihydroxylation by gut microbiota converting to secondary bile acids, deoxycholic acid (DSC) and lithocholic acid (LCA).

Around 95% of bile acids are reabsorbed from the intestine and conveying them back into the liver, which is called enterohepatic circulation. Remaining 5% (secondary bile acid) are either passively absorbed from the colon, or they are excreted in the faces. BA pool undergoes up to 12 times in a day to maintain bile acid concentration in the body. The reabsorption of bile acid occurs through the acid transporter, mainly apical sodium-dependent bile transporter (ASBT) (conjugated bile acid). This transporter protein is present in the intestinal microvilli border membrane, whereas unconjugated bile acid is transported by sodium independent organic anion transporting protein (OATP).

The primary function of bile acid is cholesterol homeostatic, emulsifying agents (assisting digestion of dietary fats) and helping the absorption of fat-soluble vitamins like D, K and A. The roles of the bile acids are expanded toward microcapsules-stabilizing and permeation-enhancing agent for the optimizing bioavailability of lipophilic drugs. It has been confirmed that the bile acid increased the epithelial transport and penetration of hydrophobic drugs through the transcellular and paracellular routes by binding with calcium ions and opening the tight junction between cells [165].

BAs have a unique chemical structure. They are amphipathic compounds (hydrophobic on the convex, beta side and hydrophilic on the concave alpha side) exhibiting high surface activity and ability to form micelles or polymolecular aggregation with other compounds, increasing the solubility of the compound (Figure 5) [166].

In the last 10 years, the focus has been toward to the role of bile acids as a drug carrier, absorption modulators in order to enhance the bioavailability of lipophilic drugs. Probably because of its unique structural and physical properties, high availability and biocompatibility and ability to act as a drug carrier, it has been demonstrated that bile acids alter the pharmacokinetics and pharmacodynamics properties of the drugs it carries and increases the absorption, solubility and dissolution rate [166]. The study has revealed that bile acid normally presents in the core (internal layers) of microcapsules but not on the surface and interacts with the microencapsulated drug and shows its biological function (Figure 5B) [167]. Rifaximin is a highly lipophilic drug, the combination of the drug-bile acid increases the solubility (60–120 times), bioavailability and anti-microbial effect [168]. Therefore, poor bioavailability of the orally administered lipophilic drugs (Table 1) can be resolved by the bile acid-based microcapsules or bile micelles [169].

A. Mooranian et al., used the IGVJF method to microencapsulate the probucol together with different bile acids (with and without bile acids) and the impact of bile acids based on morphology, physio-chemical stability, drug release, stability, was studied along with drug's bioavailability [161]. The results showed that the probucol-bile acids microcapsules show excellent characteristics proved that bile acid acts as an absorption enhancer and increases the absorption of lipophilic drug probucol [79,161]. The bile acid increased gliclazide permeation and then bioavailability in the rat model of type one diabetes [75]. *Al-Salami et al. (2008)* explained the high level of gliclazide is due to suppression in ABC- mediated transport (Mrp3) by the addition of bile semi-synthetic bile acid [48]. Overall, their studies concluded that the incorporation of the bile acid improved oral drug delivery and enhanced the bioavailability of drugs. Thus, the combination of the bile acid-drug and encapsulation method can be one promising strategy to increase the bioavailability of lipophilic drugs.

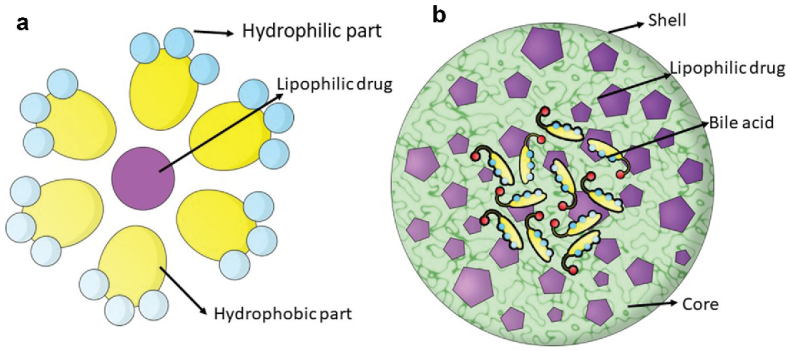

Figure 5. A) Bile acid and solubilization of lipophilic drug B) bile acid interaction with drugs inside microcapsules.

2. Conclusion and future perspective

Oral delivery is the most common administrative drug delivery path. Orally administrated lipophilic drugs have poor aqueous solubility, reduced dissolution rate and inability to withstand various environmental extremes in the upper GI tract, which consequently result in low therapeutic potential with insufficient clinical outcomes. Hence, scientists working at the interface of biotechnology and pharmacy are focused on overcoming these problems by developing newer strategies to administer the lipophilic drug with a controlled delivery. Although a significant number of approaches have shown the potential for oral delivery of lipophilic drugs, encapsulation technologies are by far the most promising. Newly designed IGVJF encapsulation technology has shown promising results for use in encapsulating lipophilic drugs for targeted delivery with sustained-release kinetics. A combination of drug-bile acid and IGVJF method is a promising strategy to increase the bioavailability of lipophilic drugs. This bio-based encapsulation technique needs optimization studies to validate standard process parameters for the encapsulation of a particular drug and delivery in a controlled and targeted fashion.

3. Expert opinion

Oral delivery is the most commonly administrated drug delivery path. The percentage of lipophilic drugs in the pharmaceutical market is escalating each day. However, oral administration of lipophilic drugs have numerous limitations: poor aqueous solubility reduced dissolution rate and inability to withstand upper GI tract environment extremes. Because of these limitations, the oral administration of lipophilic drugs limits clinical efficacy. Different approaches are ongoing to improve the oral bioavailability of lipophilic. One of the novel ways to overcome these obstacles is the micro/nano-encapsulation of lipophilic drugs. Researchers have shown interest in drug's encapsulation, and it seems to be a promising method to design and delivery the drug with the most favorable properties. Detailed insights about various bio-based encapsulating agents with enhanced keeping quality of lipophilic drugs *in vivo*, a factor much required for industrial scale-up, are discussed.

Moreover, encapsulation technology is adopted not only to protect the lipophilic drug from the adverse gastrointestinal environment but also to support controlled and targeted drug release. Controlled drug release provides numerous advantages compared to conventional dosage forms such as improved bioavailability and decreased toxicity. Ionic vibrational jet flow technique is amongst the most preferred encapsulation technology based on its ability to produce best microcapsules supported by morphology, physicochemical stability, membrane strength, controlled drug release, encapsulation efficiency, production yield, biological effects and *in vivo* study [161]. This technology is one of the newly established methods with an enormous growth in the pharmaceutical industry, useful to encapsulate molecules in the range from 1 µm to 2000 µm and support drug efficacy and oral bioavailability. In the last 10 years, attention has been attentive to the role of bile acids as a drug carrier, absorption modulators to enhance the bioavailability of lipophilic drugs. Because of its unique structural and physicochemical properties and ability to act as a drug carrier, it has been demonstrated that bile acids alter the pharmacokinetics and pharmacodynamics properties of the lipophilic drugs and increases the absorption, solubility and dissolution rate. Hence, the combined use of bile acid with other polymers might improve intestinal permeation and oral bioavailability or organ-specific drug delivery; however, choice of polymer and bile acids may have a significant impact on drug's formulation properties and clinical efficiency.

Although the era of lipophilic drug encapsulation is in its early stages, it is progressing fast. Bile acid-based formulations are economically viable for lipophilic drug delivery; hence the combination with sodium alginate or other food-grade hydrocolloids is suggested to improve the clinical functionality of drugs. Currently, bile acid-based formulations and lipophilic drug delivery system mainly focus on physicochemical, thermomechanical, drug loading ability, in-vitro and *in-vivo* studies. In the near future, important issues such as specific integration with human tissues and cells, the effect on human metabolism need to be addressed. Multiple modifications for improved lipophilic delivery with desired functionality needs some more attention. More studies are required in order to suggest formulation-excipient suitability of the bile acid, in the field of drug delivery and controlled release applications. Investigating specific bile acid applications in lipophilic drugs, in terms of cutting edge nano/micro-technologies, gut-cellular targeted deliveries and effects at the cellular levels will be a milestone in lipophilic drug delivery.

Funding

H Al-Salami has been and is currently receiving funding from Beijing Nat-Med Biotechnology Co. Ltd.

Declaration of interest

H Al-Salami's work is partially supported by the European Union's Horizon 2020 SALSETH research and innovation programme under the Marie Sklodowska-Curie Grant agreement No. 872,370. H Al-Salami has been and is currently receiving funding from the Beijing Nat-Med Biotechnology Co. Ltd. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose

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Chapter 2

Chapter 2

Micro-Nano formulation of bile-gut delivery: rheological, stability and cell survival, basal, and maximum respiration studies.

This chapter succeeded in the following objectives and sub-objectives and covered by article two from the publication list:

Objective 1: To develop, validate, and optimize the microencapsulation technique and formulation.

Objective 2: To develop and characterize PB microcapsules

Objective 3: To investigate the effects of bile acid on encapsulated PB.

> Sub-objective (1): To microencapsulate PB with LCA using SA, optimize technique and examine the effect of LCA on the morphology, surface analysis, rheology, thermo-chemical stability, drug's release, cell viability, inflammatory profiles and bioenergetics parameters of the microcapsules.

Study finding: PB-LCA microcapsules showed excellent stability, uniform morphology, and chemical and thermal excipient compatibilities, suggesting overall microcapsules stability. LCA did not alter the shape or size of microcapsules, rheological parameters, suggesting microcapsules stability. LCA support controlled and targeted PB release. PB-LCA microcapsules enhanced the NIT-1 cell viability, bioenergetics and cellular parameters, and reduced inflammatory cytokines and increased anti-inflammatory cytokines ($p < 0.01$) under the hyperglycemic state. Overall, this study suggested that PB-LCA microcapsules have good structural properties and beneficial effects on biological activities and could be suitable for oral delivery of PB in T2D. More studies are required to characterize microcapsules in terms of microencapsulation efficiency, drug content, production yield, mechanical resistance, swelling index, physical stability, and buoyancy index. These parameters are covered in chapter 3.

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Micro-Nano formulation of bile-gut delivery: rheological, stability and cell survival, basal and maximum respiration studies

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Probucol (PB) is a drug that exhibits significant hydrophobicity and substantial intra and inter individual variability in oral absorption, with a miniature bioavailability and complex three compartmental pharmacokinetic modelling due to its high lipid affinity, low stability and high octanol to water partition coefficient. Multiple attempts to formulate PB have not produced satisfactory stable matrices, drug-release profile or rheological flow properties for optimum manufacturing conditions, and with positive and none toxic biological effects. Lithocholic acid (LCA) has recently shown to optimise formulation and cell uptake of drugs. Hence, the aim of this study was to design new PB delivery system, using LCA, and examine its morphology, rheology, stability, and cellular effects. PB was formulated with LCA and sodium alginate (PB-LCA-SA) using various microencapsulation methodologies, and best formulation was investigated *in vitro* and *ex vivo*. Using our Ionic Gelation Vibrational Jet flow technology, PB-LCA-SA microcapsules showed good stability and significantly enhanced cell viability, cellular respiration, and reduced inflammation suggesting potential LCA applications in PB delivery and biological effects.

More than 40% of drugs are lipophilic and exhibit poor water solubility and low bioavailability, despite favourable pharmacological activity[1]. A common and promising approach to improve the drug's low water solubility and bioavailability is using encapsulation technology as well as formulation strategies to design new matrices where drugs can be incorporated, and delivered efficiently in the body, after oral administration[2–5]. Artificial cell microencapsulation (ACM) is widely used to microencapsulate and deliver lipophilic drugs that show poor dissolution and absorption kinetics and low bioavailability[6–8]. Microencapsulation of lipophilic drugs is one of the most promising applications in diabetes therapy and it encompasses the use of polymers/copolymers mixture to encapsulate and engulf a drug or a therapeutic entity conferring improved stability and targeted delivery properties[9–11]. The technology has been widely researched to optimise delivery and stability of therapeutics including drugs, viable cells and proteins[12–15].

Diabetes Mellitus (DM) is a disease classified generally into two forms, type 1 diabetes (T1D) and type 2 diabetes (T2D)[16]. Millions of people are globally affected by DM and it is anticipated to reach more than 438 million by 2030, which is roughly 8% of the adult population[17,18]. Every region in the world has been affected by the disease, and its incidence is high in countries such as Australia, Europe, North America and countries undergoing westernization including India and China[19]. In 2016, it was estimated that total diabetes-related global costs more than 100 billion USD, which is more than 12% of global combined health expenditure[18,20].

Insulin treatment is widely used in diabetes therapy, with all T1D patients, and in T2D more than one-third of T2D patients using insulin. Insulin is produced by pancreatic β-cells located in the islets of Langerhans. One of the common symptoms associated with T2D development and progression is chronic inflammation of β-cells as well as high levels of low-density lipoproteins (LDL), free radicals and oxidants, which have been connected to exacerbation and worsening of diabetes-associated complications[21]. Recent studies have shown that patients

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with lipid-disorders such as hypercholesterolemia are three times more likely to develop T2D and there is a significant association between T2D and cardiovascular diseases[22,23]. Published studies have also shown that significant inflammation and damage of pancreatic β-cell plays a major role in diabetes and cardiovascular disease development and progression, particularly since β-cells have limited defence against free radicals, oxidants and LDL-associated cellular toxicity[24,25]. Accordingly, new or adjunct antidiabetic drugs should exhibit anti-atherosclerotic, antilipidemic, antioxidant, anti-free radical and β-cell protective effects.

Probucol is a drug marketed for hypercholesteremia and remains widely prescribed in some countries such as China, and probably to a lesser extent in other countries such as Japan and India[26]. Despite its potent and powerful effects in lowering low density lipoprotein (LDL), probucol oral delivery has many challenges which resulted in its withdrawal from many countries including Australia and the USA. Probucol has high lipophilicity, poor water solubility, complex 3-compartmental pharmacokinetic modelling with variable and miniature bioavailability resulting in inconsistent oral uptake and severe side effects in some patients[26,27]. To make matters worse, probucol current oral dosage form, a tablet, has been used since its initial formulation development in the 1960s and remains under developed. Accordingly, in order to overcome challenges in probucol oral uptake in its current dosage form, new oral delivery matrices using cutting-edge technologies are needed. Such technologies can include microencapsulation with new excipients, that have demonstrated powerful oral targeted delivery, permeation enhancement properties and substantial consistent release profiles[6,28].

Bile acids (BAs) are endogenously produced in animals and humans and notionally are known to facilitate food digestion, and vitamin absorption from the gastrointestinal tract. Due to their amphiphilic nature, recent studies have suggested potential applications of BAs in the oral delivery of lipophilic drugs, as formulation excipients, and permeation enhancers[29,30]. Common primary bile acids are cholic acid and chenodeoxycholic acid, while secondary and tertiary bile acids are deoxycholic acid, lithocholic acid, taurocholic acid, and ursodeoxycholic acid. Overall, there are more than 100 types of bile acids although their ratios in the gut-contents, blood, and tissues vary widely depending on species and health status[31]. Several studies in our lab, as well as others have attempted to design new and powerful nano and micro based delivery systems for probucol oral uptake, using some bile acids as well as other excipients' combinations[8,10,32–34]. However, in preclinical studies, probucol absorption remains variable, and not ideal[30]. The bile acid lithocholic acid (LCA) has recently shown to optimise formulation and cell delivery[35]. It is one of the most prominent secondary bile acids and has recently displayed the potential application in the oral delivery of anticancer drugs[36]. It can self-assemble to develop nanostructure, and it has unique amphiphilic properties, high structural rigidity and reasonable biocompatibility[37]. LCA has anti-inflammatory properties and has shown positive effects on inflammatory bowel diseases[38].

In previously published work, our lab demonstrated the potential application of bile acids, either alone or in combination with anti-diabetic drugs and polymers by forming microcapsules[8,10,39]. To date, ideal microcapsules for oral delivery of probucol would exhibit significant targeted properties, proper self-assemble profile, and unique amphiphilic properties with structural integrity and excellent biocompatibility, however this remains elusive and inconsistent. Thus, the objective of study is to examine the suitability, stability and biological activity of newly formed probucol microcapsules without and with lithocholic acid.

Results and Discussion

Morphology, size analysis and chemical characterization of microcapsules. Figure 1(A) shows that optical microscopy (OM) examination of both formulations, PB-SA (i) and PB-LCA-SA (ii) maintained their spherical, round shape and similar size. OM results suggest that the incorporation of LCA did not significantly change microcapsules' topography, uniformity, shape, size, or morphology. SEM (scanning electron microscope) results show well defined spherical shape microcapsule, Fig. 1B (i) and Fig. 1C (i). Microcapsules surface were rough, and Fig. 1B (ii), (iii), and (iv) and Fig. 1C (ii), (iii), and (iv) show that PB-SA and PB-LCA-SA microcapsules presented solid structure with small granules on the surface, which suggests that the incorporation of LCA into PB-SA formulation did not affect microcapsules morphology. Energy dispersive X-ray spectrometry (EDS) (Fig. 2) was used to analyse the element components present on the outer layer of the microcapsules. Figure 2A shows the surface analysis of PB-SA microcapsules and Fig. 2B shows the surface analysis of PB-LCA-SA microcapsules at different sites with their corresponding spectra. Three different locations were randomly chosen. These Figs. 2a–f represent the crystal deposition on microcapsules' surface. Spectral analysis displays a high level of the sulphur atom in both formulations which indicate PB deposition[40]. Other elements such as O and C are anticipated to be present on the surface of microcapsules because these elements were part of the polymer and encapsulation processes deployed[41,42]. Figure 2 suggests that incorporation of LCA does not compromise or significantly alter the PB distribution within the microcapsules' layers and potentially, PB release profile.

Rheological, thermal and chemical profiles. For control and test formulations, Table 1 and Figs. 3A–B provide the rheological profiles, while Fig. 3C and Table 2 provide thermal and the chemical profiles.

Torque profile was elevated by higher speed of mixing, and LCA incorporation did not change that, however, at highest speed, Torque remained lower when LCA was present, suggesting an alteration to the turning-power ability and resistance of the PB-SA formulation as a result of LCA incorporation. Similarly, both control and test formulations exhibited Non-Newtonian shear-thinning rheological behaviour with LCA incorporation resulting in sharper decrease in viscosity associated with consistent increase in shear stress. As mixing speed increases, shear rate of both formulation showed consistent increase, supporting a steady rheological profile with or without LCA. In addition, during mixing, both formulations form quick circular motions away from the site of centripetal force at an increasing speed of the stirring rod suggesting both formulations acted in a non-Weissenberg behaviour, which is consistent with previous studies[43,44]. These results provide useful new knowledge that can contribute to design of new formulation systems via demonstrating the effect of selective concentrations of LCA on the formulation rheology and force (and dimentions via Torque) needed for capsule fabrication and stability profiles.

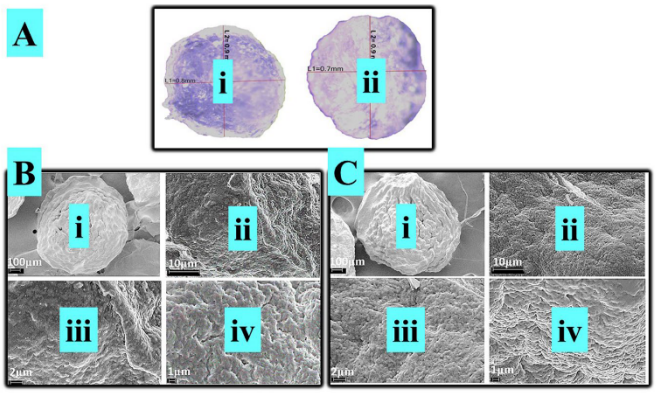

Figure 1. (**A**) Optical microscopy of PB-SA (i) and PB-LCA-SA (ii) microcapsules. (**B**)Scanning electron micrographs of PB-SA revealing microcapsules morphology and surface topography took at different magnifications, (i) 100 μm scale (ii) Surface morphology at 10 μm scale. (iii) 2 μm scale (iv) 1 μm scale. (**C**) Scanning electron micrographs of PB-LCA-SA and surface topography taken at different magnifications, (i) 100 μm scale (ii) Surface morphology at 10 μm scale. (iii) 2 μm scale (iv) 1 μm scale. PB- probucol; SA- sodium alginate; LCA-lithocholic acid.

The differential scanning calorimetry (DSC) thermal peaks provide information on thermal capacity and excipient-excipient and excipient-drug compatibilities. Thermal peak of PB powder is expected around 130 °C region, which correlates to PB melting temperature, while thermal peak of LCA powder is expected around 200 °C region, which correlates to LCA melting temperature. Thermal peak of SA powder is expected around 190 °C region, which correlates to SA melting temperature[45]. With the LCA and SA sharing similar melting temperatures, their peaks were expected to unit, which was observed in Fig. 3C, with potential amorphous phase formation. Results showed that the addition of LCA did not significantly alter the formulation heat capacity and did not result in significant changes of heat emission, or appearance of new peaks but slight endothermic peak shift[46]. Hence, during pre and post microencapsulation processes, the observed endothermic shift in the melting point of PB-SA, and PB-LCA-SA may be influenced by the ionic interaction between the ingredients constituting the microcapsules, alterations in the crystallinity, plasticization, and polymorphism of SA without compromising the overall stability[47]. Thermal compatibility of PB with other bile acids has been published[10], supporting our finding. In our study, DSC analyses were complemented with FTIR (Fourier transform infrared) studies, to describe PB, LCA and SA stability and compatibility profiles.

FTIR studies are commonly used to examine the vibrational frequency levels of different functional groups present within the molecules or between molecules, which reflects chemical bond alteration and stability[48]. FTIR spectra were used to examine chemical stability and compatibility of PB with the polymer SA and the bile acid LCA pre and post-microencapsulation. PB, SA and LCA powders were analysed individually, combined, then pre and post encapsulation. The PB powder spectra analysis displayed three different characteristics peaks at 2955.16, 1421 and 1308 cm^{-1} which confirms the presence of its functional groups, and is in line with other studied (Table 2)[10,48,49]. Likewise, the SA powder analysis exhibited predominant O-H stretching intense peak at 3253 cm^{-1} and three medium intensity peaks at 1569, 1405 and 1025 cm^{-1} which is aligned with other studies[10,49], while the spectra of LCA powder revealed distinct peaks at 3282, 2925, 1701 and 1033 cm^{-1} indicating the presence of LCA (Table 2). The combined mixtures PB-LCA-SA shows the distinct peaks of all compounds without any interference, alternations or dilution which suggests chemical compatibility pre-microencapsulation. PB-SA (control) microcapsules analysis reveals characteristic peaks at 1591, 1416, 1300 and 1026 cm^{-1} which correspond to PB and SA analysis (Table 2). Similar peaks were found after LCA addition (test) and the peak at 1712 cm^{-1} (C = O) confirms the presence of LCA. This result confirms an interaction between LCA and SA but not with PB as found in DSC results. A minor shift towards the right side of the PB peak was seen in both formulation that may have been brought out by SA or LCA. This interaction did not seem to affect PB peaks that remain present suggesting PB stability and excipient-compatibility (Table 2).

LCA incorporation into PB microcapsules did not seem to affect compatibility or stability profiles of the microcapsules, but may influence PB release profiles from the microcapsules (Fig. 4).

Microcapsule disintegration, drug release and dissolution studies. Figure 4 shows PB release from control (PB-SA) and test (PB-LCA-SA) microcapsules in gut-simulating media, at four pH (1.5, 3, 6 and 7.8) over 6 hours at 37 °C. The four (Fig. 4A–D) conditions represent four different sites of PB absorption in the gastrointestinal tract[10,50,51]. PB microcapsules demonstrated significant dependence on the gut media rather than LCA incorporation. At lower pH (stomach & duodenum), there was negligible release from control and test microcapsules, while at higher pH (ileum) there was small drug release, with no significant effects from LCA incorporation. At

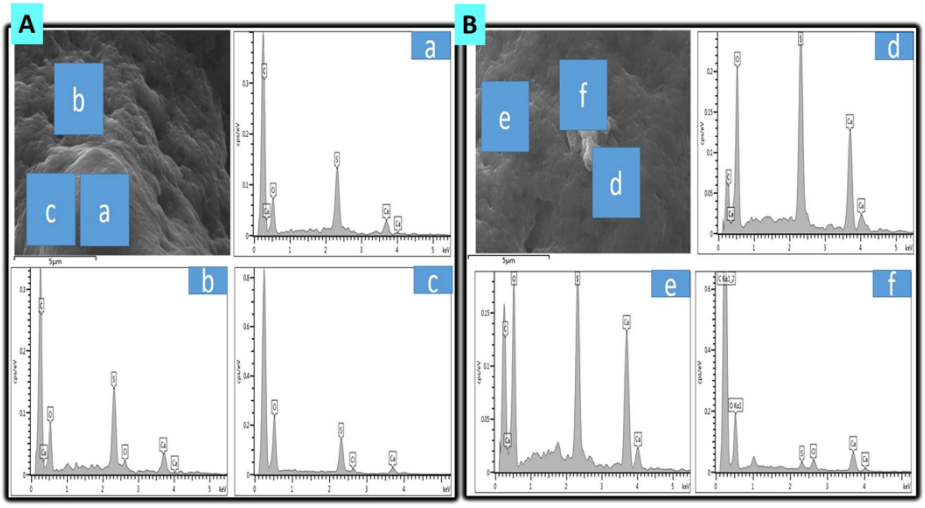

Figure 2. (**A**) EDS of PB-SA microcapsules and the corresponding elemental analysis (a-c). (**B**) EDS of PB-LCA-SA microcapsules and the corresponding elemental analysis (d-f). PB- probucol; SA- sodium alginate; LCA-lithocholic acid.

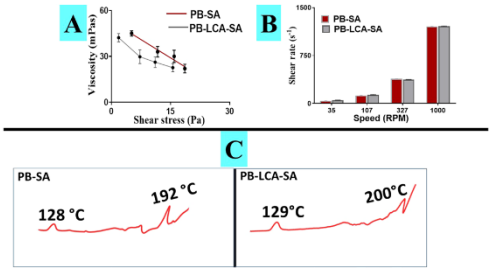

Figure 3. (**A**) Effect of shearing stress on viscosities, (**B**) Effect of speed on shear rate, (**C**) Dominant peaks observed by DSC analysis. PB- probucol; SA- sodium alginate; LCA-lithocholic acid.

Formula code	Set Speed	RPM	Torque(mNm)
PB-SA	2	35	0.1 ± 0.02
	4	107	0.35 ± 0.05
	6	327	0.8 ± 0.1
	8	1000	1.5 ± 0.03
PB-LCA-SA	2	35	BLOD
	4	107	0.14 ± 0.03
	6	327	0.36 ± 0.07
	8	1000	0.68 ± 0.17

Table 1. Rheological parameters for the formulations (n = 3, mean ± SEM, BLOD: Below Limit of Detection. PB- probucol; SA- sodium alginate; LCA-lithocholic acid.

high pH (cecum) there was substantial PB release with more than 50% within the first hour (Fig. 4D). The incorporation of LCA reduced PB release after 3 hours ($p < 0.05$) and the overall release profiles of control and test remain similar at the end of the 6 hours experiment suggesting possibly a slightly more controlled PB release by

Formulation composition	FTIR spectra (λ cm^{-1})	Proposed functional groups
PB powder	2955.16, 1421 and 1308	O-H, C-H, S=O
SA powder	3253, 1569, 1405 and 1025	O-H, C=C, C-H, C-H
LCA powder	3282, 2925, 1701 and 1033	O-H, C-H, C=O, C-H
PB-LCA-SA powder	3270, 2926, 2854, 1697, 1600, 1308 and 1030	O-H, C-H, C-H, C=O, C=C, S=O, C-H
PB-SA microcapsules	3340, 2969, 1591, 1416, 1300 and 1026	O-H, C-H, C=C, C-H, S=O, C-H
PB-LCA-SA microcapsules	3320, 1593, 1712, 1417, 1300 and 1028	O-H, C=C, C=O, C-H, C-H, C-H

Table 2. FTIR spectra of PB, SA and SA powder, PB-LCA-SA mixed powders and microcapsules. PB- probucol; SA- sodium alginate; LCA-lithocholic acid.

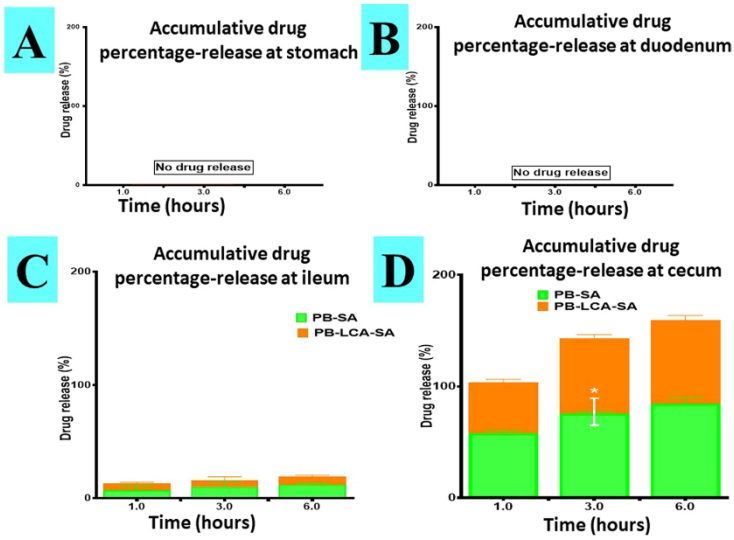

Figure 4. Microencapsules dissolution profiles in simulated gastric media at pH 1.5 (**A**), pH 3 (**B**), pH 6 (**C**) and pH 7.8 (**D**). N = 3, mean ± SD. PB- probucol; SA- sodium alginate; LCA-lithocholic acid. *$p < 0.05$.

LCA incorporation. At low pH (1.5, 3 and 6) alginic acid present on SA matrix results in shrinkage of alginate and thus encapsulated PB remained within core of the microcapsules but in higher pH (> 6) due to rapid dissolution and solubilisation, alginic acid forms a soluble viscous layer[52], resulting in the burst of PB. Similar studies have shown various and inconsistent effects of bile acids on drug release profiles, suggesting drug release from bile acid microcapsules can be formulation dependent[10,51].

Biological activity of PB-loaded microcapsules. Figure 5 shows cell viability at normoglycaemic (5.5 mmol) and hyperglycaemic (25 mmol) states (Fig. 5A), inflammatory (Fig. 5B-D) and bioenergetics (Fig. 5F-I) biological profiles of pancreatic β-cells line exposed for 48 hours to negative control (c; empty microcapsules), PB-SA and PB-LCA-SA microcapsules.

Figure 5A shows that at the hyperglycaemic state, PB-LCA-SA showed significantly higher cell viability compared with PB-SA which was higher than the sham control group, while at the normoglycaemic state, cell viability remained similar regardless of treatments. The higher cell viability, at the hyperglycaemic state as the result of treatments, suggests cellular protective effects brought about by PB, and enhanced by LCA, when encapsulated. In the literature, PB exhibits potent anti-oxidant, anti-inflammatory, and β cells protection properties, and has shown to ameliorate hyperglycaemia-induced oxidative stress and inflammation[53,54]. Results suggest that LCA incorporation did not compromise cell viability, which might be due to modulation of the inflammatory and bioenergetics profiles. Figure 5(B–D) show that PB-SA exerted an anti-inflammatory effects via reducing levels of proinflammatory cytokines (IFN-γ and IL-1 β) and enhancing levels of anti-inflammatory cytokine (IL-10), which is consistent with findings from Fig. 5A, and may relate directly to the controlled-release effects of LCA observed in Fig. 4D. Figure 5(E-I) show mitochondrial activity and cellular respiration of pancreatic β-cells at the normoglycaemic and hyperglycaemic states and results are in line with cell viability and inflammatory profiles and demonstrate significant activation of oxygen consumption rate (OCR), extracellular acidification rate

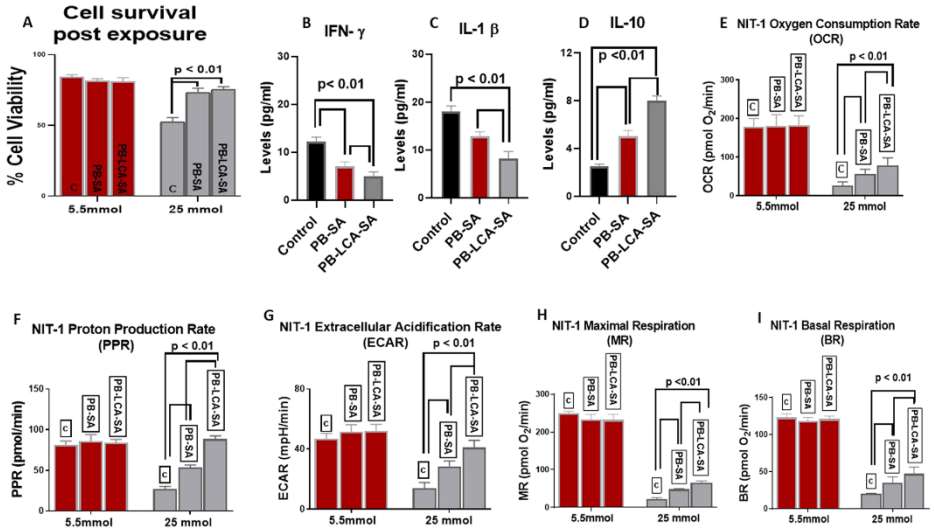

Figure 5. NIT-1 cell viability at 5.5 mmol and 25 mmol glucose (**A**), (**B–D**) NIT-1 level of inflammatory cytokines production, (**E–I**) bioenergetics parameters for NIT-1 beta cells at 5.5 mmol and 25 mmol glucose treated with PB-SA and PB-LCA-SA microcapsules. PB- probucol; SA- sodium alginate; LCA-lithocholic acid. Data values are mean ⊥ SD, N = 3; $p < 0.01$.

(ECAR), proton production rate (PPR), maximal respiration (MR) and basal respiration (BR) values when cells were exposed to PB-SA and PB-LCA-SA microcapsules compare with the hyperglycaemic control. Findings suggest that PB exerted a pancreatic β-cell protective effects possibly via reducing inflammation and enhancing biological bioenergetics, and these effects were optimised by LCA incorporation into PB microcapsules. At the normoglycaemic state, cellular bioenergetics remained similar regardless of treatments, while at the hyperglycaemic state, the treatments showed significant positive effects on biomarkers of cellular metabolism (PPR) and energetics (BR and MR) which were significantly and negatively affected at the hyperglycaemic state. For cellular respiration and acidification rates (OCR and ECAR), both treatments (PB-SA and PB-LCA-SA) showed consistent improvements to cellular bioenergetics and optimised biological functions at the hyperglycaemic states, which supports cellular biological improvements and viability results (Fig. 5). It is worth stating that significant elevation of bioenergetics biomarkers observed when cell treated with both microcapsules at the hyperglycaemic state, suggests that there is increased mitochondrial respiration and higher oxygen molecules serving as electron acceptors and assisting activity within the electron transport chain, with a proportional increase in oxidative phosphorylation, which is consistent with our previous studies[55,56].

Biological measurements (Fig. 5) have shown positive and significant cell-protective, anti-inflammatory and pro-bioenergetics effects of the encapsulated PB and PB-LCA microcapsules on pancreatic β-cells. Compared with the literature, LCA on PB cellular effects are consistent with published data that have shown that bile acids as well as PB-loaded microcapsules enhanced beta-cell viability and functions[8,57]. In one of our recent studies, the bile acid ursodeoxycholic acid was incorporated with drug-containing microcapsules, and showed similar effects on drug release, and cell viability, and these effects were formulation-dependent[31]. Overall, this is the first study to elucidate LCA applications in PB-microcapsules', in the context of cell protection and diabetes treatment.

In conclusion, this study investigated the *in vitro* and *ex vivo* effects of LCA on PB-SA microcapsules in terms of capsules' morphology, rheological, thermal and chemical profiles, and biological effects on pancreatic β-cells. Results showed that the LCA did not compromise the pharmaceutical quality of the PB microcapsules, and it enhanced PB release profile as well as the biological effects at the hyperglycaemic state suggesting potential beneficial effects in diabetes therapy. The overall procedure along with the study results is presented in Fig. 6.

Materials and Method

Materials. Probucol (PB; 99.89%) and sodium alginate (SA; 99%) were obtained from Sigma-Aldrich, (St Louis, MO, USA) respectively. Lithocholic acid (LCA; ≥ 95%) was purchased from Sigma- Aldrich, Co., USA. Calcium chloride dehydrate (98%) was purchased from Scharlab S.L (Australia). All other required chemical and solvents were obtained from Merck and Co, and were of analytical grade and used without any purification.

Cell lines. The mouse pancreatic 3 cells line NIT-1 was gifted from Prof Morahan (The University of Western Australia).

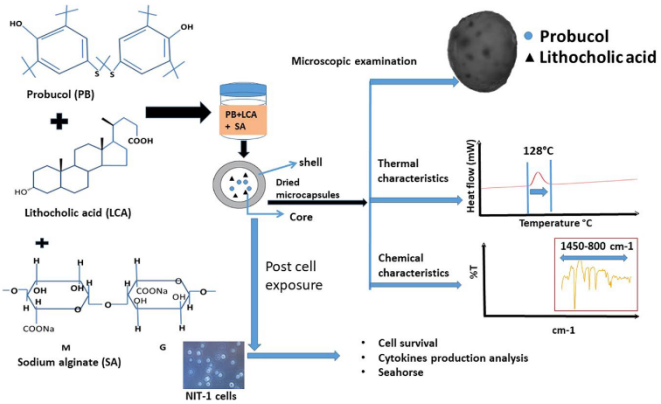

Figure 6. The summary figure of the study.

Drug preparations. Drug stock suspension of PB (2.5 mg/ml) and LCA (1 mg/ml) were made by adding powder with 5% ultra-water-soluble gel. The 2% w/v $CaCl_2$ standard solution was prepared by mixing $CaCl_2$ with deionized water. All chemical and solvents were thoroughly mixed for 10 hours at room temperature, kept in the fridge and processed within 48 hours of preparation.

Microcapsules production. PB-SA and PB-LCA-SA loaded low viscosity SA microcapsule were prepared by using ionic gelation vibrational jet flow technique (BUCHI Labortechnik, Switzerland), in a constant ratio of 1:30 and 1:3:30 respectively. This proportion was based upon the previous publications[9,10]. The parameters were fixed in a frequency range of 1500-2000 Hz with a constant air pressure of 350 mbar with a flow rate of 5 ml/minute. All formulations and microcapsules (PB-SA and PB-LCA-SA) were made, and three independent lots of microcapsules were produced (n = 3).

Characterization of loaded microcapsules. *Morphology, size analysis and chemical characterization of microcapsules.* All microcapsules (both formulations) were prepared and analysed within 48 hours. The morphological analysis and size of microcapsules were undertaken by using OM, SEM and EDS. The microcapsules' diameters were calculated with the help of the software Toup'Tek which is provided within the instrument.

Briefly, 20 freshly prepared microcapsules were dried and randomly taken to access the morphological characteristics and microcapsules' diameter using an optical microscope (Nikon SM2800, Japan mounted with Toup-view Photonics, Co., Ltd Hangzhou, China).

SEM (Neon 40EsB FIB-SEM; Zeiss, Oberkochen, Germany) was used to measure the surface morphology of microcapsules. Multiple pictures from different angles and multiple scales were performed to capture the details of the surface topography. To interpret atoms distribution present in microcapsules, was obtained by using EDS (INCA X-Act; Oxford Instruments, UK). Before analysis, dried microcapsules were mounted on a glass stub and coated under vacuum[10].

Determination of rheological parameters. Rheological parameters including viscosity, shear stress and torque of both formulations were done for freshly made mixtures (prior to gelation), using 2 ml aliquots (n = 3) at room temperature (Visco-88 viscometer, Malvern Instruments, Malvern, UK).

Thermal analysis. Thermal analysis was undertaken by DSC (DSC 8000, PerkinElmer Inc., Waltham, MA, USA). Five mg of PB powder or its microcapsules were loaded into a sealed pan and heated at 30 °C per minute at a flow rate of 20 ml/minutes under nitrogen in the 30- 250 range. For reference (control), an empty aluminium pan was utilised.

Chemical stability studies. FTIR was deployed to determine the chemistry profiles of each formulation and the prepared microcapsules. The spectra of drug, their physical mixtures and formulated microcapsules were measured by FTIR spectrometer-TWO (PerkinElmer Inc., Waltham, MA, USA) in transmission in the frequency range 450-4000 cm^{-1}.

Microcapsule disintegration, drug release and dissolution measurements. Two and a half grams of microcapsules were weighed and suspended in 100 ml of simulated intestinal fluids at four different pH values of 1.5, 3, 6 and 7.8 at 37 °C. The sink condition was maintained throughout the experiment, and the dissolution medium was stirred at 200 rpm for 6hrs[10]. PB concentrations were measured with a UV spectrophotometer (Schimadzu UV-Vis spectrophotometer 1240, Japan) at 242 nm using our published methods[10]. To exclude any

interferences and ensure only PB was being measured at this particular wavelength, microcapsules without drug (SA microcapsules) were also analysed in all four pH values. The study was carried out in triplicate (n = 3).

Pancreatic NIT-1 β cells biological examination. The cells were stored in liquid nitrogen and were cultured on T-75 cm^2 flasks (Thermo Fisher Scientific, Australia) with Dulbecco's Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (Thermo Fisher Scientific, Australia), 5.5 mmol glucose (Sigma-Aldrich, USA) and 1% penicillin streptomycin (Gibco, Life Technologies, USA)[58].

The MTT assays (3- (4, 5 - dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) was used to determine cellular viability of NIT-1 cells after exposure with microcapsules at two glucose concentration (5.5 mmol and 25 mmol) over 48 hours. The MTT stock solution (5 mg/ml) (Sigma Chemical CO, USA) was prepared using phosphate buffer at pH 7.4 (Thermo Fisher Scientific, Australia). A well-established method was used to determine the cellular viability of microcapsules treated NIT-1 cells[8,58,59]. Briefly, after 48 hours of incubation, microcapsules were removed from the 96 wells plates (Thermo Fisher Scientific, Australia) that have been placed in 200 µl of media (pH 7.4) and 20 µl of MTT from the prepared stock solution were added into each 96-wells plates. After 4 hours, MTT conversion to formazan was stopped by adding DMSO (Sigma Chemical CO, USA). The MTT assay was performed by using microplate spectrophotometer system (PerkinElmer Multimode Plate Readers, USA) at 550 nm.

The evaluation of mitochondrial activities of microcapsules treated NIT-1 cells were done in real-time using an in-house developed method with Seahorse Flux Analyser XF 96 (Seahorse Bioscience, USA)[8].

The level of cytokines (pro-inflammatory and anti-inflammatory) production were measured to test the effect of PB loaded microcapsules on treated NIT-1 cells. NIT-1 cells were cultured in DMEM medium with microcapsules at glucose concentration of 5.5 mmol and 25 mmol for 48 hours and microcapsules were removed and aliquots of the media were tested for IL-10, IL-1 beta, and IFN- gamma via cytokine bead array flow cytometric analysis (BD Bioscience cytometric Bead Array Mouse, USA)[8,10] using cell analyzer BD FACSCanto II (BD Bioscience, USA). Data analysis was carried out using the computer software FlowJO (FlowJo, Ashland, Oregon).

Statistical analysis. Graph Pad Prism version X8.2 (Graphpad, Inc., USA) was used to create graph and results are presented as mean ± SD. Statistical measurements were carried out using parametric/non-parametric analysis or using a one way ANOVA and a Tuckey post-hoc, as appropriate set the level of significance.

Received: 2 September 2019; Accepted: 1 April 2020;
Published online: 07 May 2020

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Acknowledgements
The use of laboratory equipment, scientific and technical assistance of Microscopy and Microanalysis Facility at Curtin University which has been partially funded by the University, State and Commonwealth Governments. The work is partially supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 872370. The authors acknowledge the project for Scientific and Technological Development of Vojvodina No. 114-451-2072-/2016-02.

Author contributions
The study was designed by Al-Salami, Mikov, Golocorbin-Kon, Mooranian and analysis and interpretation of research data was done by Wagle, Walker, Kovacevic and Gedawy. Drafting significant parts of the work or critically revising it, which contributes to the interpretation was done by all authors, significant contribution to data interpretation and presentation resulting in significant improvement of quality was done by all authors; providing data or analytical skills, which add significantly to the design, quality and readability of the work was done also by all the authors.

Competing interests
Al-Salami H. has been and is currently receiving funding from Beijing Nat-Med Biotechnology Co. Ltd.

Additional information
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Chapter 3

Chapter 3

Pharmacological and advanced cell respiration effects, enhanced by toxic human-bile nano-pharmaceuticals of probucol cell-targeting formulations.

This chapter succeeded in the following objectives and sub-objective and it is covered by article three from the publication list:

Objective 1: To develop, validate, and optimize the technique.

Objective 2: To develop and characterize PB microcapsules.

Objective 3: To investigate the effects of bile acid on encapsulated PB.

Sub-objective (2): To validate and optimize the technique and microencapsulate PB with LCA using SA, and examine the effect of LCA on drug content, microcapsule efficiency, production yield, drug release, zeta potential, conductivity, surface tension, stability, buoyancy, mechanical resistance, and swelling index. Microcapsules' effects on NIT-1 pancreatic β cells, inflammatory profiles, and bioenergetics parameters were also analyzed.

Study finding: Drug contents, production yield, and encapsulation efficiency were consistent (70-92 %), while both formulations showed consistent electrokinetic properties, size distribution, and surface chemistry. The significant difference was found between two formulations on electrical conductivity, mechano-physical resistance, micro-floating capacity rate, and accumulative drug release at the stomach, duodenum, ileum, and cecum. Both formulations were affected similarly with temperature (range -20 to 40 ^{o}C). NIT-1 pancreatic β-cells were used to study the biological effects of LCA on PB microcapsules. Formulation no.2 (PB-LCA) microcapsules showed a significant reduction in pro-inflammatory biomarkers with significant biological improvement in cellular bioenergetics. Significant anti-inflammatory and favorable bioenergetics effects of ULCA, when combined with probucol under hyperglycaemic condition, suggest the potential application in oral delivery of PB to treat T2D. A primary limitation of this study is the absence of *in vivo* microcapsule treatment studies.

pharmaceutics

MDPI

Article

Pharmacological and advanced cell respiration effects, enhanced by toxic human-bile nano-pharmaceuticals of probucol cell-targeting formulations.

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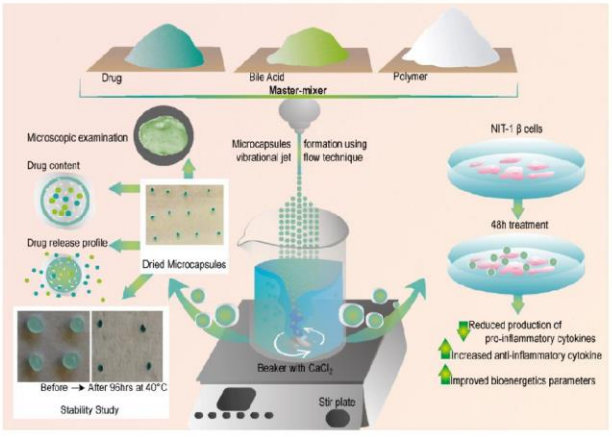
Received: date; Accepted: date; Published: date

Abstract: Bile acids have recently been studied for potential applications as formulation excipients and enhancers for drug release; however, some bile acids are not suitable for this application. Unconjugated lithocholic acid (ULCA) has recently shown drug formulation-stabilizing and anti-inflammatory effects. Lipophilic drugs have poor gut absorption after an oral dose, which necessitates the administration of high doses and subsequent side effects. Probucol (PB) is a highly lipophilic drug with poor oral absorption that resulted in restrictions on its clinical prescribing. Hence, this study aimed to design new delivery systems for PB using ULCA-based matrices and to test drug formulation, release, temperature, and biological effects. ULCA-based matrices were formulated for PB oral delivery by applying the jet-flow microencapsulation technique using sodium alginate as a polymer. ULCA addition to new PB matrices improved the microcapsule's stability, drug release *in vitro* (formulation study), and showed a promising effect in *ex vivo* study ($p < 0.05$), suggesting ULCA can optimize the oral delivery of PB and supports its potential application in diabetes treatment.



Graphical abstract

Keywords: Bile acids; diabetes mellitus; formulation sciences; inflammation; probucol; unconjugated lithocholic acid

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by high blood sugar levels resulting in numerous complications and multiple organ damage. Presently, DM is the most common and significant public health problem worldwide, and its prevalence is increasing [1,2]. It is projected that in the year 2030-2040, around 700 million individuals will suffer from DM, and by then, DM will be the seventh leading causes of death unless new potential and potent drugs are introduced to the market [3,4].

Type one diabetes mellitus (T1DM) is an autoimmune disease resulting in the loss of endogenous insulin production and secretion, while Type 2 DM (T2DM) stems from peripheral insulin resistance and is brought about by genetics and environmental factors such as lifestyle changes, urbanization, aging, obesity, and physical inactivity. T2DM accounts for approximately 90 % of all cases of DM [5-8]. The pancreatic β cells are damaged, massive inflammation, and glucose-derived reactive oxygen species, eventually leading to insulin deficiency; present substantial challenges in diabetes treatment and long terms prognosis [9-11]. To date, marketed anti-diabetic drugs are effective in regulating diabetes-associated high and fluctuating blood glucose levels, via improving tissue sensitivity or increasing available insulin instead of protecting pancreatic β cells from free radicals, oxidants, and subsequent inflammation and cell apoptosis [12,13]. Also, the risk of toxin accumulation at the gut level, and hypoglycaemia are important side effects common to many anti-diabetic drugs, which may compromise the clinical efficiency of the drug [11]. Long term use of an anti-diabetic drug such as sulfonylureas causes β –cells apoptosis due to extreme cell stimulation [14]. Therefore, the use of anti-oxidants (as adjuncts) as potential therapies is based on their ability to protect pancreatic β cells from inflammation, free radicals and oxidation, is currently gaining significant interest as potential therapies in the treatment of T2DM [15-17].

Probucol (PB) is a highly lipophilic compound classified as BCS class-II (Biopharmaceutics Classification System) drug initially developed for the treatment of hyperlipidemia [18]. PB is reported as a cardioprotective agent and is shown that PB–treated rats had markedly reduced myocardial ischemia lesions [19]. It is clarified that one of the causes of DM is due to oxidative stress and inflammation involved in the pathogenesis of islet lesions [20,21]. A study done by Gorogawa and his colleagues has shown that PB can exert anti-diabetic effects by protecting pancreatic β cell function in T2DM animal models [22]. Likewise, another similar study revealed that PB treatment in hamsters partially restored pancreatic β-cell function and decreased serum glucose levels [23]. PB restores insulin secretion by protecting the pancreatic β cells, which supports glucose haemostasis. [15,16,24]. Therefore, PB has great potential in treating diabetes because of its potent anti-inflammatory and antioxidant properties, and pancreatic β-cell shielding effectiveness. However, it failed to demonstrate consistent, desirable effects due to its lipid solubility, three compartmental modelling, high volume of distribution and poor and variable bioavailability; hence, drastically robust orally targeted systems are necessary [19,24-27].

As formulation excipients, bile acids (BAs) can enhance absorption, act as a permeation-enhancing agent and facilitate the drugs' uptake [28-31]. Our lab demonstrated through Chambers diffusion studies that BAs, when incorporated via the Artificial Cell Microencapsulation Technology (ACMT), exert beneficial anti-diabetic effects *in vivo* [32,33] and enhance drug uptake ex vivo [34-36]. BA derivatives have improved anti-diabetic drug absorption via ileum [35]. Findings showed that BA microcapsules prepared using ACMT improved PB oral absorption for T2DM animals [28,29,37]. However, PB's absorption profile remained limited, and its release profile from the microcapsules remained variable. Therefore, we hypothesized that using a more structurally stable, rigid and less soluble BA may improve PB release patterns and promote targeted delivery by increasing water permeation in microcapsules. Unconjugated lithocholic acid (ULCA) is a promising compound, endogenously produced in human, and recently it was shown to possess formulation-stabilizing and anti-inflammatory effects [38,39]. Moreover, ULCA has substantially different chemical structure compared with other bile acids, different hydrophilicity, and contrasting effects *in vivo*. From other bile acids, UDCA (ursodeoxycholic acid) is one of the most studied one. Compered to UDCA, ULCA has vastly deferent ligand potency, particularly in vivo, as ULCA is much more potent ligand for

several bile acid receptors (FXR-Farnesoid X Receptor and TGR5-Takeda G-Protein-Coupled Receptor-5) compared to UDCA, including VDR (vitamin-D receptor), which UDCA is not specific ligand for [40-42].

Recently, researchers have come up with a new concept called "Design of Experiment" (DOF) to understand drug's biophysical properties and manufacturing processes which is used in the formulation development of nanoparticles and microparticles. DOF is one important tool in employing quality by design in formulation development [43]. Latterly, the use of micro/nanoparticles has gained more attention in targeted drug delivery, showing high drug availability and treatment efficacy for many diseases such as cancer, skin disease, a neurodegenerative disorder, and inflammatory diseases [44-47]. Likewise, polymer-based micro/nanocapsules have a particular interest in controlled and targeted drug delivery [48]. The selection of the polymer is a vital step in drug delivery and discovery, as it affects drug release and absorption [49]. Sodium alginate (SA) has shown the most promising result in oral drug administration [31,50]. SA is a natural polysaccharide extracted from brown algae and has characteristic features of being biodegradable and biocompatible, and it has demonstrated excellent stability and compatibility, as well as pH-dependant degradation and release kinetics which are also influenced by its viscosity [51,52]. Specifically, low viscosity SA (LVSA) showed controlled and targeted drug release in comparison with high-viscosity SA (HVSA), primarily when targeting the cecum [53]. In gastrointestinal (GI) fluid, the hydrated LVSA matrix changes into the porous, insoluble acid matrix and, when it reaches higher pH values (the small intestine), the alginate acid matrix is transformed into a soluble viscous layer that breaks down the polymer integrity and releases drugs from the microcapsules [53,54]. Previously, our lab has demonstrated that sodium alginate-based formulation improved pharmacokinetics and pharmacodynamics responses with optimized structural, chemical, and physical compatibility and improved targeted delivery [31,55,56]. This study is a preliminary study to elucidate the application of our Ionic Gelation Vibrational Jet Flow technology in nanocapsules production, before moving a step further toward an *in vivo* animal study of anti-oxidant for T2DM. Therefore, in this study, newly designed PB-LVSA and PB-ULCA-LVSA microcapsule formulation systems are evaluated as a potential drug delivery system in the management of T2DM, which can be extended to other lipophilic anti-diabetic drugs.

2. Materials and Method:

2.1. Materials

PB (99.89%) was purchased from Medisca (USA), sodium alginate low-viscosity (99%) and ULCA (≥95%) were purchased from Sigma-Aldrich CO., (St Louis, MO, USA). Calcium chloride dehydrates ($CaCl_2.2H2O$, 98 %) were purchased from Scharlab S.L (Spain).

2.2. Drug preparations

LVSA (1.2%), PB (4%) and ULCA (2%) stock suspension was prepared by slow addition of powder in 8% water-soluble gel. $CaCl_2$ (5 % w/v) was made by adding $CaCl_2$ powder in ultrapure milliQ water. Prepared formulations were thoroughly mixed for 6 hours at room temperature, stored in the fridge, and used within 24 hours of preparation.

2.3. Microcapsules preparation

PB-LVSA (F1-without ULCA as control) and PB-ULCA-LVSA (F2-with ULCA as test) microcapsules were prepared using our established system (BÜCHI Labortechnik, Switzerland) based on our developed technology: Ionic Gelation Vibrational Jet Flow technique [29,55]. Multiple parameters were used, including a frequency of 1800 Hz with constant air pressure 350 mbar, and liquid flow rate 5mL/minute, and these parameters were constant for both microcapsules. The microcapsules were prepared with or without ULCA to the final concentration of PB:ULCA:LVSA in a ratio of 1:3:30, respectively [12]. This ratio was based on our previous studies [13,57]. Microcapsules were collected in and left for 5-10 minutes in the $CaCl_2$ ionic gelation bath to preserve

the spherical shape of the droplets. To dry microcapsules, the stability chamber (Angelantoni Environmental, Italy) was used and the weight of dried microcapsules was recorded.

Morphology, drug contents, microencapsulation efficiency, production yield, mean particle size, surface tension, conductivity, zeta potential, swelling, mechanical resistance, buoyancy, release patterns, stability, and biological activity of each preparation were analysed in triplicates (n=3) [58,59].

2.4. Characterization of Loaded Microcapsules

2.4.1. Morphological analysis and surface characterization of microcapsules

Morphological characterization was accessed by using Nikon H550S optical microscopy (OM) and scanning electron microscope (SEM) (Neon 40EsB FIB-SEM; Zeiss, Oberkochen, Germany. For SEM, microcapsules were freshly made, dried and mounted on a glass slide stub with double-sided adhesive tape and coated under vacuum in an argon atmosphere with 5nm platinum before examination. Elemental distribution present on the microcapsules' surface was analysed by energy dispersive X-ray spectrometry (EDXR) (INCA X-Act; Oxford Instruments, UK) [28].

2.4.2. Drug Content, Production Yield, and Microencapsulation Efficiency

Dry microcapsules (2g) were ground and dissolved in 200 mL phosphate buffer saline (PBS) pH 7.8. The suspension was stirred for 6 hours, and one mL of the solution was transferred and diluted with phosphate buffer to 20 mL volume. Before analysis, the prepared solution was filtered through a 0.22 μm Millipore filter. The dissolved drug content concentration in the solution was measured with a UV spectrophotometer (Shimandzu UV-Vis spectrophotometer 1240, Japan) at 242 nm against the buffer blank [60]. To confirm the method's accuracy and precision, HPLC (high-pressure liquid chromatography) procedure was done as per our previous research [61]. The analysis was done in triplicate and calculated by our previously established formulae, as mentioned [62].

$$\text{Drug contents (\%)} = \frac{Calculated\ \ amount\ \ of\ \ PB\ \ in\ \ the\ \ microcapsules}{Total\ \ weight\ \ of\ \ microcapsules} \times 100$$

$$\text{Production yield (\%)} = \frac{Total\ \ weight\ \ of\ \ the\ \ microencapules}{Total\ \ weight\ \ of\ \ polymer\ \ and\ \ drug\ \ solution} \times 100$$

$$\text{Microencapsulation efficiency (\%)} = \frac{drug\ \ content}{Theoretical\ \ content} \times 100$$

2.4.3. Electrokinetic stability, size analysis, surface tension and conductivity

Electrokinetic stability and particle size analysis were determined using the zeta sizer and mastersizer, respectively (Zetasizer 3000HS and Mastersizer 2000, Malvern Instruments, UK) [37,62]. Surface tension (ST) was done using a tensiometer (Sigma 703). Conductivity was performed with the help of the conductivity meter (CDM230, conductivity meter) by calibrating with potassium chloride (KCl) standard.

2.4.4. Swelling and mechanical resistance studies

The microcapsules swelling properties were calculated by placing 200 mg of microcapsules (F1 and F2) in 20 mL PBS. The study was conducted at four different pH values (1.5, 3, 6 and 7.8) and at two different temperatures (room temperature and 37°C) over 6 hours. The selection of pH values and temperatures was based upon our previous work [37,62]. Swollen microcapsules net wet weight was measured by weighing on a dynamic balance immediately after blotting them on the filter paper (Whatman #40). The microcapsule swelling index percentage was determined as previously described [63]. For mechanical resistance, briefly, 25 dry microcapsules from each formulation batch (F1 and F2) were placed in 20 mL PBS (pH 7.4) and oscillated at a frequency of 150 rpm for 24 hours

(Boeco Company, Hamburg, Germany). The mechanical resistance index was calculated as per our previously established protocols [62,64].

2.4.5. Buoyancy Test

One hundred dried microcapsules were taken and placed in 200 mL of PBS (pH 7.8). The buffer was stirred for 6 hours at a speed of 50 rpm at a temperature of 37°C using USP dissolution apparatus 24, type II. The temperature was regulated through thermostats. Every hour, the number of floating microcapsules was counted and calculated as previously described [29].

2.4.6. Drug release studies - In Vitro Dissolution Test

2.5 grams of F1 and F2 microcapsules were weighed and suspended in 300 mL of simulated intestinal fluids (SIF), which contained PBS of four different pH values of 1.5, 3, 6 and 7.8 at 37°C. The sink condition was maintained throughout the assay time, and the dissolution medium was stirred at 200 rpm for 6 hours [62]. The solution's absorbance was measured every 30 minutes (2 mL of the solution was taken from the dissolution bath and replaced with the same amount of blank buffer to maintain equilibrium condition throughout the assay). The 2 mL removed from the solution was placed into a 10 mL flask (sink conditions were maintained during this assay). Following this, the transferred solution was diluted with PBS to the volume and further centrifuged to clarify the solution. The amount of released drug in the solution was measured with a UV spectrophotometer at 242 nm against the PBS blank. Additionally, to exclude any interferences and to confirm that only the drug was being measured, microcapsules without PB (LVSA microcapsules) were also analysed at all four pH values.

2.4.7. Physical stability

The stability testing of F1 and F2 was conducted by putting 25 freshly prepared microcapsules on microscope slides and storing them for four days under thermostatically controlled ovens at four different temperatures (-20°C, 5°C, 25°C, and 40°C) with relative humidity set at 35% in the stability chamber [62,64]. After four days, microcapsules were observed under the OM for morphology and appearance change, then drug content and release profile was calculated as mentioned above [62] [62].

2.4.8. NIT-1 pancreatic β cells and biological analysis

The pancreatic β -cell line (NIT-1) were kindly provided by Prof Morahan (The University of Western Australia) and cultured in Dulbecco's Eagle Medium (Sigma-Aldrich, USA) with supplemented 10% bovine serum (Thermo Fisher Scientific, Australia), and 5.5 mmol glucose (Sigma-Aldrich, USA). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The media was changed at 48 hours intervals, and cells were sub-cultured when confluence reached 80% as per the standard method [65].

Cytokine release from NIT-1 cells was performed to examine the biological efficiency of prepared microcapsules. For this, NIT-1 cells were cultured in prepared media, DMEM (pH 7.4) at two different glucose concentrations (5.5 mmol and 25 mmol) and treated with F1 and F2 microcapsules. Microcapsules were put directly in the cells containing wells, and incubated together with the cells for 48 hours. After 48 hours, IFN-γ and IL-10 were examined by removing treated microcapsules and analysing the cell media via cytokine bead array flow cytometry analysis (BD Bioscience cytometry Bead Array Mouse, USA) with the cell analyzer BD FACSCanto II (BD Bioscience, USA). FlowJO software (FlowJo, Ashland, Oregon) was used to interpret data.

For seahorse analysis, cells were treated with microcapsules (F1 and F2) at 5.5 mmol and 25 mmol glucose concentrations for 48 hours. The assay was determined via Seahorse Flux Analyser XF 96 (Seahorse Bioscience, USA) standard [37].

2.4.9. Statistical analysis

Student's t-test was done to analyse drug content, production yield and microencapsulation efficiency and values are presented as mean ± SD where n=3. Correlation, regression and one-way ANOVA/ two-way ANOVA (analysis of variance) were done to analyse the data and Tukey HSD post-hoc comparison of means was conducted when data was statistically significant. GraphPad Prism version X8.2 (Graphpad, Inc., USA) was used for all analyses, and data were considered statistical significance at $p < 0.01$ or $p < 0.05$.

3. Results and Discussion

3.1. Microscopic examination and Surface analysis

The OM images showed (Figure 1-A and 1-G) that both F1 and F2 microcapsules preserved their spherical shape, uniformity, and incorporation of ULCA did not alter the shape and size of microcapsules, which is further ascertained by SEM images (Figure 1-C and 1-I). The average horizontal (L1) and vertical (L2) mean diameter of both microcapsules ranges from 0.7 ± 0.1 mm. Our previous finding showed that microcapsules size ranges from 0.7 to 0.9 mm support targeted gut release, improved cell viability, and hyperglycaemic effect on diabetic animals [31]. The surface topography of F1 (Figure 1-D, and E) and F2 (Figure 1-J and K) presented rough and solid granules on the surface. Furthermore, corresponding spectra analysis on the microcapsules' surface showed the abundance amount of C, Ca, O, and S, which suggest that ULCA did not adversely affect microcapsules morphology and surface topography. The presence of S in analysed spectra indicates that granules on the surface are due to PB deposition because none of the excipients contains S except PB; as PB chemical formulation contains two S atoms (Figure 1-F and 1-L) [18]. The presence of Ca^{2+}, C, and O was expected because they are part of the polymer used, and ionic gelation bath, which was one of the prerequisites of the process during the manufacture of microcapsules [66]. The results support our previous studies that the incorporation of BAs did not change the shape, size, and surface drug content of PB microcapsules, which proved that the microencapsulation method is robust and uniform regardless of any formulation [28,31,67].

Figure 1: Morphological examination of microcapsules. (A-B and G-H) Optical microscopy. (C, I) Scanning electron micrographs. (D-E, J-K) Energy-dispersive X-ray spectra and figures (F and L) with corresponding spectra. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB-probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.

3.2. Drug content, Microencapsulation efficiency, Production yield, Zeta potential, Size analysis, Surface tension, and Conductivity

As shown in Figure 2-A and 2-B, the amount of PB content in both formulations (F1 and F2) remained constant with little variation but not statistically significant (F1= 2.3% ± 0.2 and F2= 2.27% ± 0.32) ($p > 0.05$), which proved that the integration of ULCA in F1 did not alter the drug content of the microcapsules. The total manufacture yield and microencapsulation efficiency of F1 and F2 ranged to 70-92% and was not significantly different between F1 and F2 microcapsules. A good level of PB loading around 85-90% was recorded for both kind of microcapsules. This proves the addition of ULCA has the least effect on the drug content, ability, and yield in both F1 and F2 microcapsules, which lines up with our previous lab studies ($p > 0.05$) [28,31].

The measurement of surface charge provides particle colloidal suspension properties showing which kind of interaction the particles may have, whether they aggregate or disperse in solution. Usually, the higher the negative charge, the better the electrokinetic stability of the particles [68]. Likewise, surface chemistry provides information about the formulation's nature. With higher surface chemistry, the formulation is homogenous and smooth, with impacts on drug release kinetics [69]. Figure 2 (C-F) shows that the surface charge (-60-70 mV) ($p = 0.1167$), size distribution (750-770 μm) ($p = 0.8411$), and surface chemistry (64-67 m/Nm) ($p = 0.2931$) remained constant after addition of ULCA, suggesting that dispersion of microcapsules were stable, and capable to withstanding particle agglomeration and flocculation [70]. However, conductivity was diminished after mixing ULCA in

the PB-LVSA formulation ($p < 0.01$). The conductivity of the formulation provides information on the nature of the main vehicle in terms of current conductivity being predominantly water-based; hence, potential micelle formation and clear hydrophilic-lipophilic balance are significantly influenced by water. The addition of BAs increases the negative charge due to the removal of a proton from the carboxylic acid group resulting in a net decrease of positive surface charge in the mixture, this decreases the conductivity of formulations that support the kinetics of encapsulated drug release [71].

Figure 2: Drug content, production yield, and encapsulation efficiency (A and B), (C) electrokinetic charge, (D) size analysis, (E) surface tension and (F) conductivity. N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.*$p < 0.01$.

3.3. *Swelling index*

Table 1 incorporates the swelling index of F1 and F2 microcapsules. The swelling test was performed at four different pH values (1.5, 3, 6, and 7.8) and two different temperatures (25°C and 37°C). Table 1(A and B) shows that the temperature and pH of the medium affect the swelling properties of the microcapsules. The addition of ULCA in F1 significantly decreased the swelling behaviour of the microcapsules ($p < 0.01$) at high pH and temperature compared to low pH and temperature, suggesting better control of PB release in the target site of the intestine at pH 7.8; which is further complemented by mechanical resistance and buoyancy test (Figure 3 A-B) . Usually, alginate undergoes extensive swelling at higher temperatures and pH because of higher water uptake and an increase in porosity and solubilisation of the polymer [52]. Also, heat often causes the erosion and breakdown of the microcapsule wall (matrix wall), which allows for more significant water infiltration [72]. This result undoubtedly backed our hypothesis that BAs enhance membrane stabilization by cross-linking properties or ionic interaction with the alginate matrix [33-35].

Table 1: Swelling index at pH 1.5, 3, 6 and 7.8 for 25 °C (A) and 37 °C (B) . N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.*$p < 0.01$.

Temperature = 25 °C (A)				
Formula code	**pH 1.5**	**pH 3**	**pH 6**	**pH 7.8**
F1	0.92 ± 0.005	1.873 ± 0.0625*	3.286 ± 0.148*	3.90 ± 0.11*
F2	0.89 ± 0.005	1.383 ± 0.343 *	2.633 ± 0.104*	3.08 ± 0.05*
Temperature= 37°C (B)				
F1	0.99 ± 0.005	2.345 ± 0.005 *	3.83 ± 0.056 *	4.89 ± 0.095*
F2	0.933 ± 0.057	2.12 ± 0.081 *	2.986 ± 0.349 *	3.87 ± 0.161*

3.4. *Mechanical strength, Buoyancy test and Drug release studies*

Mechanical resistance provides ideas about the microcapsules' ability to resist mechanical stress and pressure. Figure 3-A shows the mechanical index of microcapsules in percentage over a 24 hours' time frame. Until 12 hours, there was no statistical alteration in the number of intact microcapsules between the control and test. However, after 12 hrs, control microcapsules started losing their shape and dry contents as well as physical adherence. After 16 hrs, almost 50% of F1 microcapsules became deformed while 80% of F2 microcapsules remained intact ($p < 0.05$); which proved that the addition of ULCA significantly enhanced the microcapsules strength and can prevent premature drug loss due to rapid change in GI pH values (1.5-7.8) when administrated orally [29,31,64].

Figure 3-B shows the *in vitro* buoyancy test of control (F1) and test microcapsules (F2) over 6 hours. In the buoyancy test, the percentage of floating microcapsules is calculated over a time period. At the end of 6 hours, the portion of floating microcapsules for F1 was below 30%, while part of

floating microcapsules was almost 50% for F2 microcapsules ($p < 0.05$). Improved F2 buoyancy proves that microcapsules could maintain this property in the stomach and support and optimize the controlled and targeted release of the carrier drug. Overall, our swelling study findings from Table 1, mechanical resistance (Figure 3-A) and *in vitro* buoyancy test (Figure 3-B) prove that incorporation of ULCA into F1 microcapsules leads to more stable microcapsules by supporting membrane integrity and physical coherence. The release study (Figure 3 C-D) complements the above results and endorses that the addition of ULCA results in more coherent and more potent microcapsules at high pH value of 7.8, which emphases its ability to withstand degradation in lower GI tract, resulting in higher controlled release and promote targeted drug delivery. These results were consistent with our previous studies [29,73].

Figure 3 (C-D) shows PB release at 1.5, 3, 6, and 7.8 pH over 6 hours at a temperature of 37°C for two different formulations (F1 and F2). These pH values were taken based on the sites of anti-diabetic drug absorption in GI and pH gradient system [28,62,73]. The results show that the release of PB is mainly dependent upon pH and formulation type. At lower pH values (1.5, 3 and 6), alginic acid present in the LVSA matrix usually leads to shrinkage, which helps the encapsulated drug to remain within the core of the microcapsules. Still, at higher pH (> 6) values, due to quick dissolution and solubilisation, alginic acid forms a soluble viscous layer [74], resulting in the microcapsule bursting and the subsequent release of encapsulated drug.

As seen in Figure 3-C, at pH 1.5 there was low drug release of only 2-2.5 %, and 3-5% at pH 3 from both F1 and F2 microcapsules. As predicted, the release of PB was higher at pH values of 6 (5-10%) and 7 (60-80%) (Figure 3-D). Notably, the release pattern was significantly higher at pH 7.8 in comparison with pH 6 for both formulations ($p < 0.05$), which coincides with targeted delivery in the distal site of the intestine. After 6 hours, drug release from F1 microcapsules at pH 7.8 reaches up to 80%; whereas drug release from F2 microcapsules peaks at 54-65 % ($p < 0.05$). This feature is important in the development of diabetes therapy, as most of the anti-diabetic drugs are absorbed from the distal site of the intestine (pH 7-7.8) [74]. Other studies presented different and inconsistent effects of drug release such as the biphasic, multiphasic and rapid burst of drug when other bile acids were used to formulate microcapsules [28,62]. In contrast, this study shows the continuous and targeted release of the PB at pH 7.8, probably because of the structural, physical and chemical nature of ULCA. This study further supports that targeted drug release from bile acid microcapsules depends upon the formulation. Compared to other bile acids, ULCA is less soluble but has excellent excipient and stability properties, which assist in resisting dissolution even if pH values are higher. This is achieved by retaining cross-links with LVSA, which results in increased membrane stability and in turn, protects the microcapsule from rapid degradation and allows for a more controlled release of encapsulated PB [75,76] as seen in Figure 3(A and B) and Table 1. Also, EDXR results (Figure 1) showed the potential presence of PB on the surface as well as the inside of the capsules, which is expected and similar to previously published data on drug encapsulation using bile acid-based capsules [29]. This can allows for more controlled and prolonged drug release profile, since initial release can take effect immediately in the intestine, followed by more slowed release. The findings are consistent with previously published data, this release profile suggests successful incorporation of ULCA within the designed capsules, as well as even distribution throughout the layers, including on the surface. Therefore, PB release from the ULCA-based microcapsules showed targeted release and revealed the most favourable characteristics for anti-diabetic drug delivery. The PB release was conducted in SIF in different pH values. In future, the release patterns will teste in an *in vivo* murine model to reflect and compare different fluid dissolution behaviour; fasted state simulated intestinal fluid (FaSSIF), fasted state simulated gastric fluid (FaSSGF), fed state simulated intestinal fluid (FeSSIF) and fed state simulated gastric fluid (FeSSGF).

Figure 3: (A) Microcapsules mechanical strength testing; control (F1) and test (F2). (B) Microcapsules buoyancy index. (C-D) Microcapsules dissolution profiles in simulated gastric media at pH 1.5, pH 3 (C), pH 6, pH 7.8 (D). N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-

ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid. *p < 0.05.

3.5. Stability studies

Figure 4 shows microcapsules (control and test) morphological characteristics before (Figure 4A-D) and after (Figure 4 E-H) accelerated stability testing at various temperatures; -20°C (Figure 4-A and E), 5°C (Figure 4-B and F), 25°C (Figure 4-C and G) and 40°C (Figure 4-D and H) over 96 hours at 35% humidity. Both F1 and F2 microcapsules successfully preserve their original morphological characteristics (shape) during the study period at a lower temperature (-20°C and 5°C). Both microcapsules' size and weight decrease drastically with increasing temperatures (25°C and 40°C) (Figure 4-I and J) due to water content evaporation from the microcapsules [62]. Figure 4-K showed a positive correlation between the size of microcapsules and weight changes. More than 50% reduction in size, and 70% reduction in weight of microcapsules was found, and the most significant effect was seen at temperature 40°C. Microcapsules had become brittle and hard, and lost elasticity on all temperatures expect -20°C. Due to microcapsules moisture content evaporation, many more changes were noticed at 40°C, possibly because of the potential oxidation and dehydration effects on microcapsules. After four days, UV-Vis spectrophotometric analysis was conducted to establish the drug content (post-stability study), concluding that there were no significant changes in drug content. The average percentage of drug content for PB-LVSA was 2.21 ± 0.1, and 2.32 ± 0.2 for PB-ULCA-LVSA, respectively. Similarly, the drug release pattern after post stability study was conducted in two different pH values (6 and 7.8) and the result showed drug release was higher in pH 7.8, and release pattern was continuous and controlled in the presence of ULCA similar to the freshly prepared microcapsules as presented in Figure 3 (C-D)(data are not shown). The post-stability drug content was similar to fresh microcapsules, which proved that there was no significant loss of PB under these testing conditions from both microcapsules. This study is consistent with a previous study where F1 and F2 microcapsules physicochemical compatibility was analysed [39]. Differential scanning calorimetry (DSC) results showed PB did not participate in a cross-linking reaction with LVSA and BA and does not compromise thermal and chemical integrity during the microencapsulation process, which is further supported by Fourier-transform infrared spectroscopy (FTIR) analysis [39]. The addition of ULCA has no impact on the stability of PB microcapsules. Thus, the stability study confirmed uniformity of PB content with no noticeable differences from changing temperatures and conditions.

Figure 4: Effect of temperature on microcapsules' appearance. Pictures were taken before (A-D) and after accelerated stability testing (E-H). (I) % change in weight, (J) % change in diameter and (K) correlation between %changes in diameter vs. % change in weight. N=3, mean ± SEM. F1 (control): PB-LVSA microcapsules and F2 (test): F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA- Unconjugated lithocholic acid.

3.6. Biological activity of PB-loaded microcapsules

3.6.1. Pancreatic β-cell cytokine measurement

Plasma biomarkers like IFN-γ have been linked with inflammation and expressed widely in patients with diabetes [ZZ]. Their expression level is interconnected with the development and progression of diabetes. In Figure 5 (A-B), the level of expression of IFN-γ and IL-10 were measured in NIT-1 pancreatic β-cells exposed to hyperglycemia (25 mmol) and treated with F1 and F2 microcapsules for 48 hours. The cytokines expression in NIT-1 cells after microcapsule treatment was compared to cells treated with empty microcapsules. Figure 5-A shows the level of IFN- γ production was significantly higher in untreated cells (13.50 ± 0.90 pg/mL) ($p < 0.01$), whereas levels decreased in cells treated with microcapsules (F1= 7.90 ± 1.4 pg/mL and F2= 5.80 1.30 ± pg/mL). Basic MTT assay was initially carried out to examine cell biological activity and results were consistent with IFN- γ levels [39]. Likewise, the expression of anti-inflammatory cytokine IL-10 was 3 ± 0.80 pg/mL in control

and increased considerably in treated cells (F1= 5.70 ± 0.85 pg/mL and F2= 8.90 ± 0.72 pg/mL) ($p < 0.01$) (Figure 5-B). The potent anti-inflammatory effect of PB that supports cell proliferation, reduces apoptosis signalling, and increased expression of anti-inflammatory and decreased level of pro-inflammatory cytokine have been previously described [9,78]. Interestingly, the addition of ULCA in PB microcapsules significantly increased the expression of IL-10 cytokine ($p < 0.01$). This suggests that ULCA also may have a decisive role in cell proliferation and support the expression of anti-inflammatory cytokines production. This is due to the stabilizing properties of ULCA, as presented in Table 1 and Figure 3-A, which showed that ULCA act as a stabilizing effect on the microcapsules, thereby reducing swelling property and improving microcapsules mechanical resistance[22,79]. However, no significant difference was noted between F1 and F2 treated cells in IFN-γ expression, possibly because of the different metabolic and secretory pathway compared to other cytokines [80]. The reduction in IFN-γ and enhancement in IL-10, that PB-ULCA microcapsules showed, is expected to decrease cell apoptosis and support cell function, which is supported by bioenergetics analysis [81]. Previously published data have displayed that the anti-oxidant activity of bile acids enhanced β-cell viability [39,81,82] [83].

3.6.2. Seahorse analyses

Different parameters, such as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which assess mitochondrial function, were measured and presented in Figure 5(C-D). NIT-1 cells were treated with empty microcapsules (control), F1, and F2 microcapsules for 48 hours at two glucose concentrations (5.5 mmol and 25 mmol glucose). It shows that there is no cell stress at 5.5 mmol, which causes no significant changes in cellular metabolism biomarkers and bioenergetics parameters between control and test (F1 and F2) cells. At 25 mmol, due to cell stress, significant changes in OCR and ECAR were found between untreated and treated cells ($p < 0.01$). The ULCA incorporation in F1 microcapsules significantly improved β cells OCR (from 57 ± 12 to 79 ± 19 pmol O_2/min) ($p < 0.01$) (Figure 5-C) and ECAR (29 ± 3.9 to 44 ± 5.2 mpH/min) level ($p < 0.01$) (Figure 5-D). This significant improvement suggests a strong positive influence of PB-loaded microcapsules on the β-cells' biological activity such as glycolysis and mitochondrial respiration [84,85]. New oxygen molecules are generated by electron acceptors, stimulating oxidative phosphorylation chain to synthesize ATP (adenosine triphosphate), and ultimately increase insulin secretion from β-cells. However, one major limitation of this study is the lack of insulin secretion data. Different studies also showed that PB-loaded microcapsules have anti-oxidant and anti-inflammatory properties that improve mitochondrial respiration and metabolic activity [37,67,86]. In recent literature, it was shown that PB-ursodeoxycholic acid microcapsules have similar effects on cellular parameters, viability and drug release, proving that these effects were formulation dependent [81]. From these biological results, F2 microcapsules undoubtedly showed a positive and significant protective effect on pancreatic β-cells and improvement in bio-energetic parameters.

Due to the focus of the paper on the effect of ULCA on PB release, biological changes were examined using two different microcapsules, one with ULCA and one without ULCA. Untreated cells were considered as control. Future studies will endeavor to examine biological impact of ULCA alone, using ULCA novel delivery matrices. Overall, to our knowledge, this is the first preliminary study of the application of Ionic Gelation Vibrational Jet Flow technology in producing microcapsules with encapsulation efficiency ≥ 90% as the result of ULCA incorporation, with empowered surface chemistry and electrical conductivity. Furthermore, findings illustrate a clear association between change in weight vs. diameter and clear impact of ULCA integration on the most powerful anti-inflammatory cytokine.

Figure 5: Cytokine production test (A-B) 48 hours' post-treatments and bioenergetics parameters (C-D) using the NIT-1 pancreatic β cell lines at two glucose concentrations 5.5 mmol and 25 mmol. N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.*$p < 0.01$.

4. Conclusion

Our microencapsulation method with set parameters produced excellent and uniform microcapsules. The integration of ULCA on F1 microcapsules did not affect size, shape, uniformity, stability and microcapsules drug content, but enhanced the microcapsules strength. Also, the ULCA addition leads to more stable microcapsules by reduces swelling, and allowing for controlled and pH-targeted drug release. ULCA incorporation enhanced bioenergetics parameters, decreased inflammatory cytokines and increased anti-inflammatory cytokines. Overall, this suggests potential applications of PB in the oral administration in T2D. Future studies will endeavour to evaluate the pharmacology of the microcapsules *in vivo* using diabetic rodent models.

Acknowledgments

The use of laboratory equipment, scientific and technical assistance of Microscopy and Microanalysis Facility at Curtin University which has been partially funded by the University, State and Commonwealth Governments. Al-Salami's work is partially supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872370.

Author contributions

The study was designed by Al-Salami, Mooranian and Stojanovic; significant contribution to data interpretation and presentation resulting in significant improvement of quality was made by Wagle, Kovacevic, Walker, Ionescu, Jones, Kojic, Mooranian and Al-Salami; provide data or analytical skills, which add significantly to the design, quality and readability of the work was done by Wagle and Al- Salami; analysis and interpretation of research data and drafting significant parts of the work or critically revising it, which contributes to the interpretation was made by all the authors and all the authors agree with presented publication draft.

Declaration of conflict of interest

Al-Salami H has been and is currently receiving funding from Beijing Nat-Med Biotechnology Co. Ltd.

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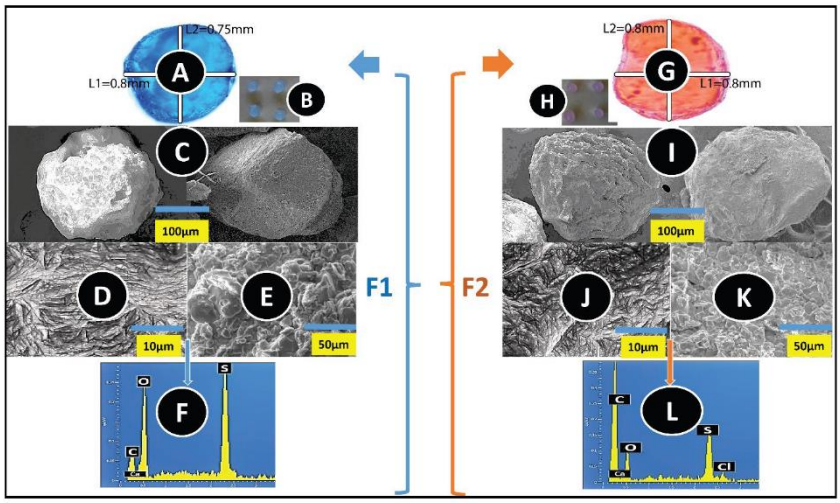


Figure 1. Morphological examination of microcapsules. (A-B and G-H) Optical microscopy. (C, I) Scanning electron micrographs. (D-E, J-K) Energy-dispersive X-ray spectra and figures (F and L) with corresponding spectra. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB-probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.

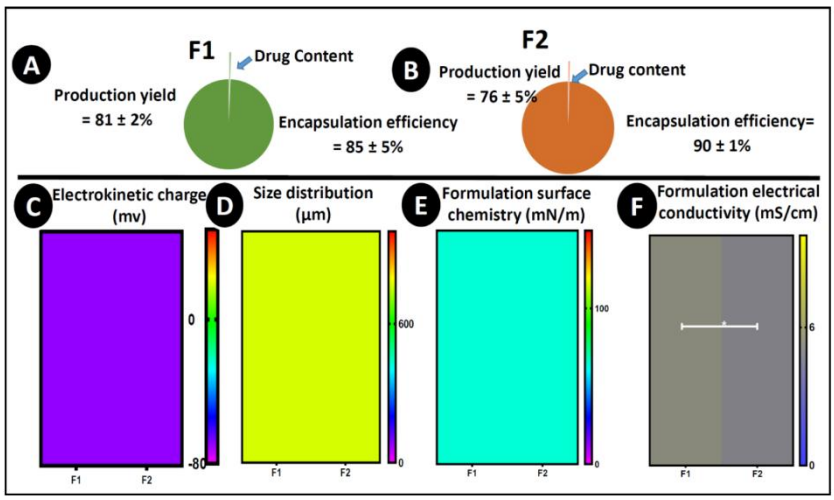
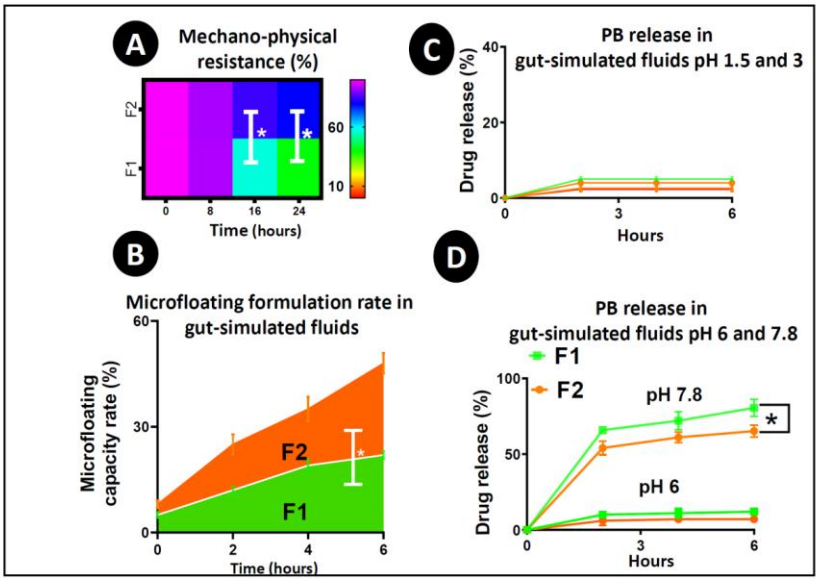


Figure 2. Drug content, production yield, and encapsulation efficiency (A and B), (C) electrokinetic charge, (D) size analysis, (E) surface tension and (F) conductivity. N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.*$p < 0.01$.



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762 **Figure 3.** (A) Microcapsules mechanical strength testing; control (F1) and test (F2). (B) Microcapsules
763 buoyancy index. (C-D) Microcapsules dissolution profiles in simulated gastric media at pH 1.5, pH
764 (C), pH 6, pH 7.8 (D). N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA
765 microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA- Unconjugated lithocholic
766 acid. *$p < 0.05$.

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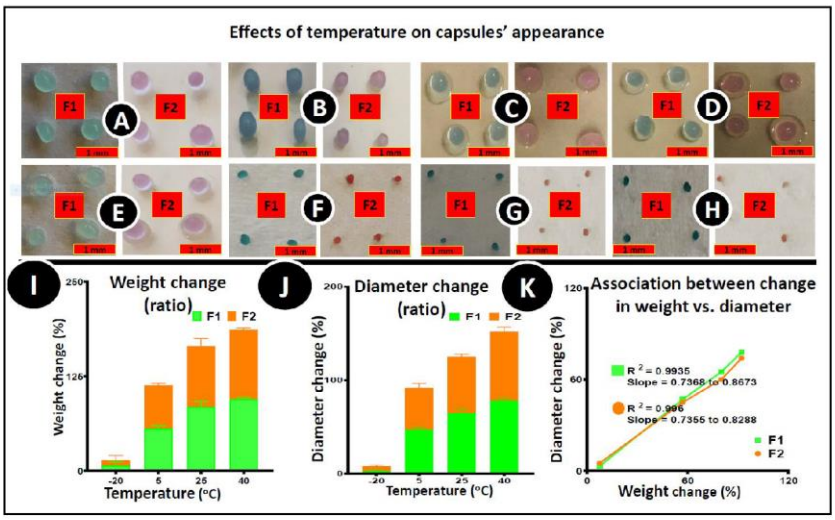

Figure 4. Effect of temperature on microcapsules' appearance. Pictures were taken before (A-D) and after accelerated stability testing (E-H). (I) % change in weight, (J) % change in diameter and (K) correlation between %changes in diameter vs. % change in weight. N=3, mean ± SEM. F1 (control): PB-LVSA microcapsules and F2 (test): PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA- Unconjugated lithocholic acid.

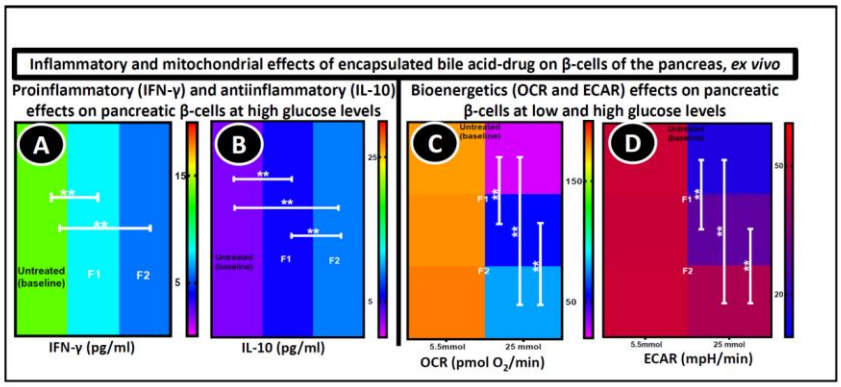

Figure 5. Cytokine production test (A-B) 48 hours' post-treatments and bioenergetics parameters (C-D) using the NIT-1 pancreatic β cell lines at two glucose concentrations 5.5 mmol and 25 mmol. N=3, mean ± SEM. F1: PB-LVSA microcapsules and F2: PB-ULCA-LVSA microcapsules. PB- probucol; LVSA- low viscosity sodium alginate; ULCA-Unconjugated lithocholic acid.*$p < 0.01$.

Chapter 4

Chapter 4

General discussion, conclusion, limitation and future direction of the study

Discussion

This thesis aimed to investigate the influence of LCA on PB microcapsules prepared by using the IGVJF technique*, in vitro* and *ex vivo*. The *in vitro* method was designed to characterize microcapsule combinations of PB-SA and PB-LCA-SA in terms of morphology (shape, size, surface-elemental composition, and internal complexity), excipient thermo-chemical properties, rheological parameters, zeta potential, conductivity, surface tension, physical, thermal and mechanical stability under various temperatures and pH values, buoyancy and release properties. Likewise, the *ex vivo* method was aimed to examine the biological impacts of PB-SA and PB-LCA-SA microcapsules on the pancreatic β cell line. Cell viability, bioenergetics parameters, and cytokines were analysed under the hyperglycaemic and hypoglycaemic state.

In chapter 2, the morphological examination and surface analysis showed that the integration of LCA in PB microcapsules yields no noticeable alteration in size, shape, and surface element present microcapsules. The PB and PB-LCA microcapsules were uniform size, oval or spherical, with the rough outer layer and solid white granules on its surface. To investigate solid granules composition, the elemental analysis was done, showing a high amount of sulfur atoms on the surface of microcapsules (both formulations), which is specific to PB content. The results were parallel with earlier studies, which observed that bile acid incorporation did not change the size nor shape, but it did alter the internal complexity, with bile acid accumulating in the inner layer of microcapsules rather on the surface (15, 41, 51). However, this thesis lacks an analysis of the impact of LCA on the internal complexity of the formulated microcapsules; further studies should address this point. From the above results, we proved that the parameters used to produce microcapsules were optimized and capable of producing uniform microcapsules.

Rheological characters showed that increased mixing speed increased shear rates, torque, shear stress, but lowered the viscosity, which indicates both formations behaved as non-Newtonian fluids with thixotropic pseudoplastic movements. By integrating LCA into the formulation, the rheological properties were not affected (chapter 2) (15, 52, 53). Pharmacodynamics properties (like flow dynamics) are essential for pH targeted drug delivery and for oral-administrated drugs-related time transit (chapter 2) (54, 55).

The thermo-analytical properties of compounds were determined with DSC. The thermal peak of PB physical powder was recorded before and after microencapsulation process (noted around 129 °C),

which resembles with PB melting point. The thermogram of LCA and SA was also analysed which indicative of the LCA and SA's melting point, which was also lined with previously published data (56). There were no significant thermal characteristic changes in PB microcapsule even after the addition of LCA into the mixture (pre and post-microencapsulation), which is supported by the endothermic peak characterize PB melting point. In line with DSC, the chemical composition and crystal lattice structure of the PB, LCA, and SA were analysed using FTIR. Similar to DSC, FTIR spectra were analysed for individual compound powder, mixed powder, and microcapsules. FTIR spectra confirmed the chemical compatibility of all the compounds in powder form, and post microencapsulation process. Both DSC and FTIR results were consistent with previous work as no noticeable changes were detected for PB microcapsules even after the addition of LCA in both pre and post microencapsulation processes (15, 57). However, clear interaction among SA and LCA in both DSC and FTIR analysis was noticed as expected in pre and post microencapsulation processes, as they share similar melting temperature/spectra and minor shift in endothermic peak/spectra could indicate plasticization of the SA (58). Still, this interaction did not impact the drug's thermal and chemical characterization. These properties made pre- and post- PB-SA and PB-LCA-SA microcapsules stable in terms of the drug-polymer matrix`s thermal and chemical reactions without physiochemical changes (chapter 2).

In chapter 3, no significant alteration in PB content, microencapsulation efficiency, production yield, zeta potential, particle size, and surface tension was noticed after incorporation of LCA in PB microcapsules, consistent with previous work (59, 60). The use of IGVJF produces microcapsules with efficiency ≥ 90%, which indicates that the used technique is optimized and robust. Membrane stability studies presented that both microcapsules swelled at a higher pH and temperature (7.8 and a temperature of 37°C). The addition of LCA decreased the swelling properties of PB microcapsules at temperatures of 25 °C and 37 °C at 7.8 pH, protecting the entrapped drug from 'burst release' (61). These results support the hypothesis of the study that bile acids improve SA based microcapsules membrane stability and support the targeted release of PB, which is consistent with the literature (29, 59). The buoyance index was significantly improved when LCA was added to PB-SA microcapsules ($p < 0.05$), which suggests the bile acid-containing microcapsules float in the digestive tract, supporting the gradual release of drug content from the microcapsules (62).

The pH values were chosen based on previous studies, to resemble pH values of gut regions in oral-administrated drug target sites (36, 63, 64). The release of PB from microcapsules largely depends upon pH values and microcapsule`s specific formulation. The *in vitro* release studies showed that at lower pH values (pH 1.5 and 3), drug release from both types of microcapsules was almost undetectable. In contrast, significant drug release was observed in higher pH values (pH 6 and 7.8) from both types of microcapsules, and higher PB release at pH 7.8.

SA is a pH-dependent polymer. At low pH (1.5 and 3), alginate changes to the insoluble structure (alginic acid), reducing its size, effectively preserving the drug within the core of microcapsules (to prevent early burst) (61). On the other hand, at higher pH (6-7.8), due to the fast dissolution and solubilisation of the alginate polymer, the alginic acid develops a soluble viscous layer. It causes a breakdown of the wall and leads to drug release from the core of microcapsule (33). Therefore, there was no release of the drug at low pH (1.5-3), slight release at pH 6, and maximum release at alkaline condition pH 7.8. The drug release from PB-LCA microcapsules was continuous and more controlled compared to PB-SA microcapsules ($p < 0.05$). This release studies suggested that encapsulation protects the drug from unwanted degradation and supports the controlled and targeted release of PB in the presence of LCA, illustrating the importance of LCA in drugs' absorption. Moreover, the release study further shows that LCA presence in PB microcapsules supports the distribution of PB within microcapsules. Furthermore, because LCA has rigid structural integrity and reduced solubility compared to other bile acids, it offers stronger structural support to the alginate matrix by cross-linking with the polymer at different pH values. This increases capsule resistance at different gut environments and prevents the rapid burst of the microcapsule, supporting targeted, controlled, and sustained drug release from the microcapsules. The PB absorption takes place in the distal part of the lower intestine, and adequately formulated microcapsules can maximize PB absorption, optimizing its safety profile, decreasing inter-individual variation, and increasing intestinal permeation and bioavailability (65, 66). Therefore, the PB-LCA microcapsules will probably show better targeted, controlled, and sustained PB release when orally administered to diabetic mice (future study).

Similar studies were done using another bile acid such as CA, TCA (41, 42), but this study shows more convincing drug release over previous research. The drug's release from PB-TCA microcapsules was uniphasic, and PB-CA was monophasic, suggesting a significantly high early release of the drug. Moreover, other studies presented that the release of PB from the alginate microcapsules was not controlled, and continuous even with bile acids incorporated into PB microcapsules (48). This variation in release patterns (slow/quick or quick/slow) causes an initial burst of drug release that reduces the effectiveness and demands a high dose of the drug. This study successfully overcomes the limitations of previous studies, probably because of the robust formulations and impact of LCA physical and chemical properties on PB microcapsules. Controlled and sustained release of probucol is essential for diabetes treatment, as it supports targeted drug delivery, reduces dosage frequency, decreases intrapatient variability, and maintains clinically efficient drug concentration throughout the day leading to the decrease of drug's side effects (67).

A stability study was done to assess the effect of temperatures on the shape, size, texture, and drug content. In line with the previous laboratory experiments, at low temperatures (-20°C and 5°C) both

microcapsules maintained their original size, shape, and texture whereas at 25°C and 40°C, both microcapsules displayed a decrease in size and change in colour (generally from white to yellow). This is probably due to oxidation of the matrix and loss of water content from the microcapsules (57, 68). However, the drug content was not significantly changed compared with the freshly prepared microcapsules for both formulations. Overall, stability results showed that drug content was not affected for both types of microcapsules under the deployed stability conditions, which further signifies that the microencapsulation process is robust and uniform, irrespectively of the addition of LCA (chapter 3).

The *ex vivo* study was conducted to investigate the special effects of PB and PB-LCA microcapsules have on cell proliferation, inflammatory cytokines levels, and bioenergetics parameters at two different states- normoglycemic (healthy condition) and hyperglycaemia (diabetes conditions) using the pancreatic β-cells lines, NIT-1. The viability studied presented that PB microcapsules preserved cell viability even at hyperglycaemia compared with control (untreated cells) ($p < 0.01$), suggesting protective effects on cells. This is probably due to the anti-oxidant and anti-inflammatory effects of PB (69). At normal glucose level (5.5 mmol), no changes in NIT-1 cell viability were noticed between control and test cells, and all the measured values remain similar, and changes in cell viability were only found at hyperglycaemic state (25 mmol) as predicated. No statistically significant difference found on cell viability between PB and PB-LCA microcapsules. However, *Mooranian et al*., showed that PB microcapsules with bile acid enhance cell viability significantly compared to microcapsules without bile acid at hyperglycaemic state (42). However, his next study stated that UDCA produced microcapsules reduced β cell viability (29). Therefore, more studies will be conducted on cell viability using different cell lines and different conditions in the near future to explore the role of LCA on cell viability and survival. Likewise, in line with the earlier studies, this study established that PB microcapsules exhibited strong anti-inflammatory effects, observed in a decrease of IFN-γ and IL-1β levels, and an increase of IL-10. This effect is further pronounced in the presence of LCA (38).

Seahorse XF analyser was used to measure different bioenergetics parameters such as OCR, ECAR, PPR, BR, and MR. Mitochondria and its macromolecules are highly sensitive to stress and inflammation, which could play a significant role in the development of new therapeutic strategies and biomarkers for T2D (70). Results showed these parameters reduced significantly when cells were in the hyperglycaemic state, probably caused by cellular stress developed by high glucose levels. This study showed that the addition of bile acids in PB microcapsules significantly improved cellular parameters at the hyperglycaemic state ($p < 0.01$). These results are in line with other findings (38), which indicate that PB-LCA-SA microcapsules enhance mitochondrial respiration, increase oxidative phosphorylation to produce more ATP, ultimately increasing β-cell activity and insulin secretion (71-

74). The biological effects were more promising when LCA is added to PB microcapsules probably because LCA make microcapsules outer shell stronger that supports controlled drug release, suggesting that PB may have strong anti-inflammatory properties when LCA is added to the formulation. Most of the studies presented that LCA is toxic to cells (75, 76), but this study concludes that proper volume and concentration of LCA encapsulated together with anti-inflammatory and anti-oxidant drugs support cell growth and bioenergetics parameters. Overall, the *ex vivo* study proved that prepared LCA microcapsules improved cell viability and enhanced mitochondrial activity by exerting a noteworthy reduction of pro-inflammatory cytokines and escalation in anti-inflammatory cytokines (chapter 2 and 3).

Summary of the study

In conclusion, this is the first study that investigates probucol encapsulation with LCA in an *in vitro* and *ex vivo* model using sodium alginate and the Ionic Gelation Vibrational Jet Flow technology, developed at Curtin University (the Biotechnology and Drug Development Research Laboratory). Overall, this thesis showed that the developed formulation methodology is successful in the production of microcapsules with uniform morphology, physicochemical stability, and excipient compatibility. The study also examined the PB-LCA microcapsules *ex vivo,* showing enhanced β- cell viability, bioenergetics parameters, and anti-inflammatory cytokines and decreased pro-inflammatory cytokines. This method can be applied to another carrier system for the specific delivery of different drugs in many diseases. Hence, PB-LCA showed better formulation that did not impact PB microcapsules morphology, rheology, stability, while supporting appropriate drug release and biological activities, which can be taken into consideration for further development of potential T2D therapy. The summary figure of this thesis is presented Figure 1.

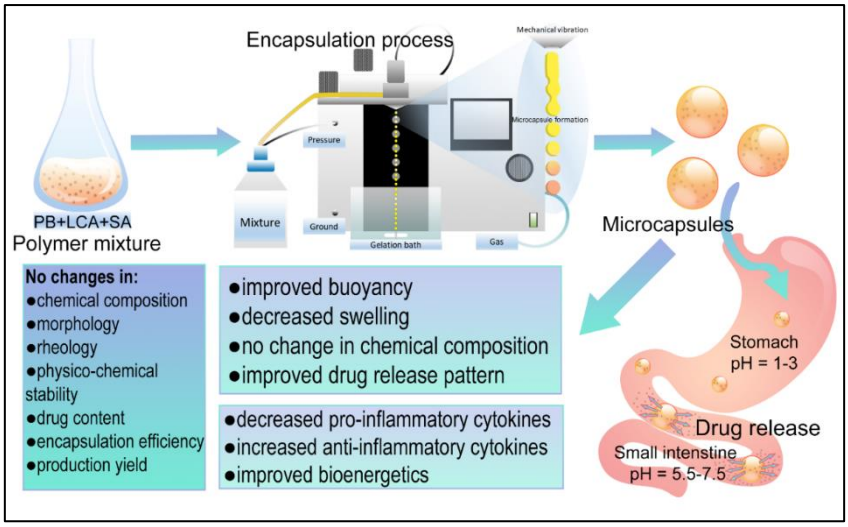

Figure 1: The summary figure of the study.

Limitation of the study

However, this study has some limitations, as well. The study primarily focused on using only one endogenous bile acid and one polymer. Microcapsules were prepared and tested *in vitro* and *ex vivo* only. An *in vivo* study was not conducted due to the time limitation

Future prospective of the study

In the future, developed PB microcapsules will further be tested in a large number in an *in-vivo* mouse model as per our already established T2D *in vivo* group studies:

- empty microcapsules administrated through oral gavage to group 1;
- PB in powder administrated through oral gavage for group 2;
- PB microcapsules administrated through oral gavage for group 3;
- PB-LCA microcapsules administrated through oral gavage for group 4

If the *in-vivo* study is successful, the impact of LCA microcapsules will be tested in humans. Furthermore, the prepared microcapsules will be tested to revolutionise the treatment of hearing loss, aims to revolutionise hearing treatment, with a particular focus on age-related hearing. Likewise, other approaches will be implemented to control blood glucose levels, such as transplantation of encapsulated β-cells in diabetic mice, which can secret insulin in a required manner. Bio-printing, which can be perceived as a form of encapsulation, is the most advanced and recently developed technology in the field of tissue engineering. This technology applies the 3D (there-dimensional) principal printing, where it combines cells, growth factors, and biomaterials to develop tissue-like structures that imitate natural tissues. Moreover, the study will also focus on stem cell biology which is accepted as one of the most favourable strategies for restoring β-cells function. The detailed future study plan is presented in the Figure 2.

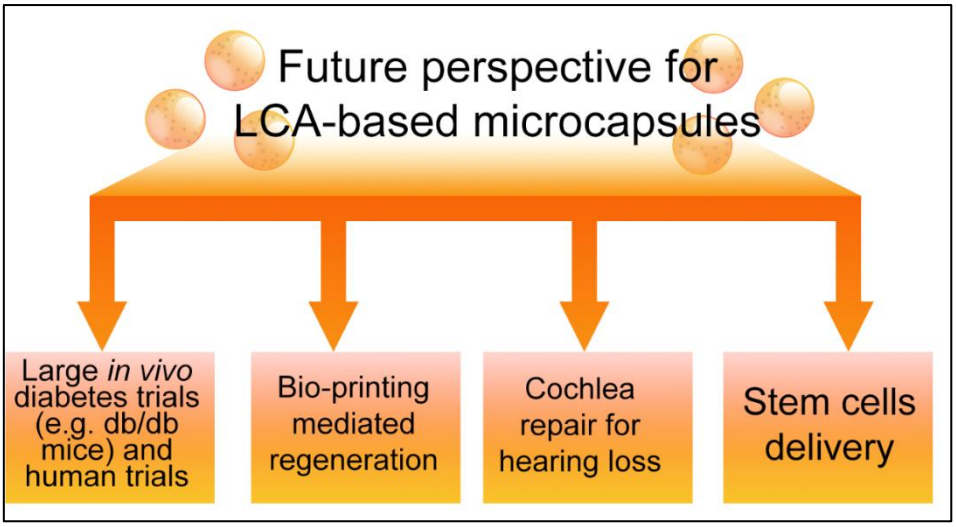

Figure 2: Future prospective study

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Appendix A

Additional publication

This thesis also contains the following publication which is not central to the research hypothesis mentioned but related to the thesis study.

The study examines the effects of UDCA bile acid on PB-microcapsules, *in vitro*, *ex vivo*, and *in vivo*. PB-UDCA microcapsules enhance cell viability, and brought about the hypoglycaemic and anti-inflammatory impact on pre-diabetic mice and shown beneficial therapeutic effects in diabetes treatments.

Mooranian A, **Wagle SR**, Kovacevic B, Takechi R, Mamo J, Lam V, Watts GF, Mikov M, Golocorbin-Kon S, Stojanovic G, Al-Sallami H. Bile acid bio-nanoencapsulation improved drug targeted-delivery and pharmacological effects via cellular flux: 6-months diabetes preclinical study. Scientific Reports. 2020 Jan 9;10(1):1-5.

SCIENTIFIC REPORTS
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Bile acid bio-nanoencapsulation improved drug targeted-delivery and pharmacological effects via cellular flux: 6-months diabetes preclinical study

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The antilipidemic drug, probucol (PB), has demonstrated potential applications in Type 2 diabetes (T2D) through its protective effects on pancreatic β-cells. PB has poor solubility and bioavailability, and despite attempts to improve its oral delivery, none has shown dramatic improvements in absorption or antidiabetic effects. Preliminary data has shown potential benefits from bile acid co-encapsulation with PB. One bile acid has shown best potential improvement of PB oral delivery (ursodeoxycholic acid, UDCA). This study aimed to examine PB and UDCA microcapsules (with UDCA microcapsules serving as control) in terms of the microcapsules' morphology, biological effects *ex vivo*, and their hypoglycemic and antilipidemic and anti-inflammatory effects *in vivo*. PBUDCA and UDCA microcapsules were examined *in vitro* (formulation studies), *ex vivo* and *in vivo*. PBUDCA microcapsules exerted positive effects on β-cells viability at hyperglycemic state, and brought about hypoglycemic and anti-inflammatory effects on the prediabetic mice. In conclusion, PBUDCA co-encapsulation have showed beneficial therapeutic impact of dual antioxidant-bile acid effects in diabetes treatment.

Understanding the link between insulin-resistance, prediabetes and Type 2 diabetes (T2) is anticipated to facilitate better ability to design new interventions in order to control the fast growing epidemic of diabetes. The link encompasses multiple physiological disturbances including obesity. In a review by Qatanani, M. and Lazar, M.A, the authors have examined specific links between insulin resistance and visceral adiposity and excess fat accumulation in blood and tissues[1]. They found that there is a direct correlation between the amounts of lipid represented by biomarkers such as total cholesterol, triglycerides and noneesterified fatty acids (NEFA), and the extent of insulin-resistance and rate of prediabetes development. One of the possible underlying mechanisms to insulin-resistance and prediabetes, has been hypothesized to be oxidative stress and inflammation[2–6]. Oxidative stress and local and systemic inflammation have been shown to be contributing factors in development of insulin-resistance, prediabetes and eventually T2D. Oxidative stress and inflammation have also been linked to worsening of diabetic symptoms and long-term prognosis[7,8]. In addition, diabetes-inflammation has been associated with lipid dysregulation, visceral adipose tissue accumulation and insulin-resistance. Karpe, F. *et al.*; have shown direct association between levels of inflammatory cytokines, with development of visceral fat

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accumulation, insulin-resistance, and prediabetes[9]. Studies published by our laboratory have also demonstrated direct links between concentrations of proinflammatory cytokines: TNF-α, IFN-γ, IL-1β and IL-6 levels with pancreatic β-cell functions and insulin secretions at normoglycemia and hyperglycaemic states[4,10–14]. Overall, better control over prediabetes and diabetes development may be achieved by new and optimised therapeutics which not only target glycemia but also ameliorate or even prevent the underlying inflammation. New and optimised therapeutics can incorporate and encapsulate multiple drugs (e.g. including potent antioxidants such as probucol "PB"), which synergistically target oxidative stress and inflammation in the diabetic hyperglycaemic state.

Of the endogenous bile acids, the tertiary bile acid ursodeoxycholic acid (UDCA) has been shown to be the most potent anti-inflammatory and anti-apoptotic bile acid with significant cell protective properties with its mechanism of action being correlated with its cellular uptake by muscle and β-cells[15–20]. Tsuchida, T., *et al.*; have shown that chronic oral administrations of UDCA in insulin resistant animals have been associated with cardio-metabolic improvement as a result of its cellular uptake[21]. Other studies involving human clinical trials have shown that UDCA is well tolerated and exerts positive effects on glucose and energy homeostasis[22–24]. Many bile acids such as chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) have also shown to be affected by the development and progression of insulin-resistance and diabetes, as well as associated-inflammation[4,5,25,26]. To date and to the best of our knowledge, no studies have examined PBUDCA pharmaceutical properties, their effects on both pancreatic β-cells and muscle cells, their cellular uptake, permeation and interaction with the ABC protein transporters Multi-Resistance Associated Proteins (MRP) 1, 2 and 3, and the preclinical long-term efficacy of these microcapsules on glucose regulation, lipid and inflammatory profiles, as well as their effects on the bile acid profile, in a mouse model of insulin-resistance.

Thus, the aim of this study was to develop microcapsules incorporating PB and UDCA and examine the morphological, physical and chemical compatibility of the microcapsules (*in vitro*), their effects on pancreatic and muscle cells (*ex vivo*), cellular uptake and flux of PB and UDCA, and their potential hypoglycaemic, antilipidemic and antiinflammatory effects in a mouse model of prediabetes, given oral microcapsules for 6 months (*in vivo*). PB oral uptake and concentrations in plasma, tissues (ileum, liver, brain, heart, pancreas, and kidney) and faeces will be measured. The bile acids CDCA, LCA and UDCA concentrations in plasma, tissues and faeces will also be measured. The study is a continuation of ongoing work in our laboratory examining the preclinical efficacy of dual ingredient antioxidant-bile acid microcapsules in diabetes mellitus with published hypoglycaemic effects of controlled groups[3,11,14,27–35].

Results

Microencapsulation fabrication, and stability/shelf life *in vitro* studies. Stability and shelf life *in vitro* studies showed stable microcapsules over a period of 2 weeks, at temperatures $<40\,^{\circ}C$ and a relative humidity of $<35\%$. In addition, Fig. 1 shows the SEM micrographs (UDCA: 1–2, PBUDCA: 3–4), Micro-CT (UDCA: 5, PBUDCA: 6), DSC spectra (UDCA: 7, PBUDCA: 8), FTIR (UDCA: 9, PBUDCA: 10), water saturation index (UDCA: 11, PBUDCA: 12), gut-floating index (13), thermal stability index (14), and PB cumulative drug release at pH 1.5 and 3 (15) and PB cumulative drug release at pH 6.0 and 7.4 (16) of F1 (UDCA microcapsules) and F2 (PBUDCA microcapsules). SEM micrographs showed similar shape and size with some variation between F1 and F2 in terms of F1 having more solid surface with less cracked and pores, which suggests that F2 has porous outer surface, compared with F1. Micro-CT images showed distinct outer surface of the bile acid containing microcapsules, suggesting bile acid accumulation on the surface or outer layers of the microcapsules. DSC and FTIR spectra showed consistent thermal and chemical capacities for both types of microcapsules, suggesting thermo-chemical stability of microcapsules constituents, while water saturation, resistance, gut-floating and thermal indices showed consistency in both F1 and F2 microcapsules with PB release demonstrating pH targeted delivery (Fig. 1).

***Ex vivo* studies.** Figure 2 shows effects of PB and PBUDCA microcapsules on cell viability and oxidative stress (1), their cellular uptake (2), and cellular permeation (3) and efflux protein-transporters effects (4), at normoglycaemic (healthy) and hyperglycaemic (diabetic) states, using two cell types, β-cells and muscle cells.

Cellular viability of pancreatic β-cells and muscle cells were unchanged in normoglycaemic conditions when exposed to F1 and F2, while in hyperglycaemic conditions, β-cell viability was improved by F2 exposure which showed lower fluorescence (Figs. 1–2). Cellular uptake of UDCA was higher when the β-cells were exposed to F1 compared with F2 (normoglycaemic and hyperglycaemic states) while UDCA and PB cellular uptake remained unchanged when cells were exposed to F1 or F2 in normoglycemic and hyperglycaemic states (Fig. 2–2). In normoglycemia and hyperglycaemia, PB (A → B) unidirectional cellular permeation (flux) was higher when combined with G or M, and higher with CA (B → A) with M, and combination and intracellular concentrations of PB showing highest levels when with CA (Figs. 2–3) with hypothesized mechanisms of PB cellular uptake illustrated (Figs. 2–4).

***In vivo* studies.** Figure 3 shows the PB levels, from PB-L, PB-H and PBUDCA groups, in liver, ileum, pancreas, faeces, plasma, heart and kidney.

PB was detected in all tissues and faeces except kidneys. Compared with PB-L, both, PB-H and PBUDCA resulted in increased PB levels in plasma, ileum, liver, brain, heart, pancreas and faeces with higher PB levels in PB-H plasma, tissues and faeces, compared with PB-L. PB-L and PBUDCA (with same dose of PB) resulted in higher PB levels in plasma and tissues with PB-L, implicating MRP3 inhibition. Levels of PB in pancreas were low in PB-H and liver levels showed higher PB concentrations.

Figure 4 shows blood glucose levels (1), the inflammatory biomarkers IFN-γ, IL-1β, IL-6 and TNF-α (2), and the lipid biomarkers total cholesterol, triglyceride and NEFA levels (3) at the end of the experiment and association between IL-1β with blood triglycerides and blood glucose levels among HDF, PBUDCA and UDCA groups.

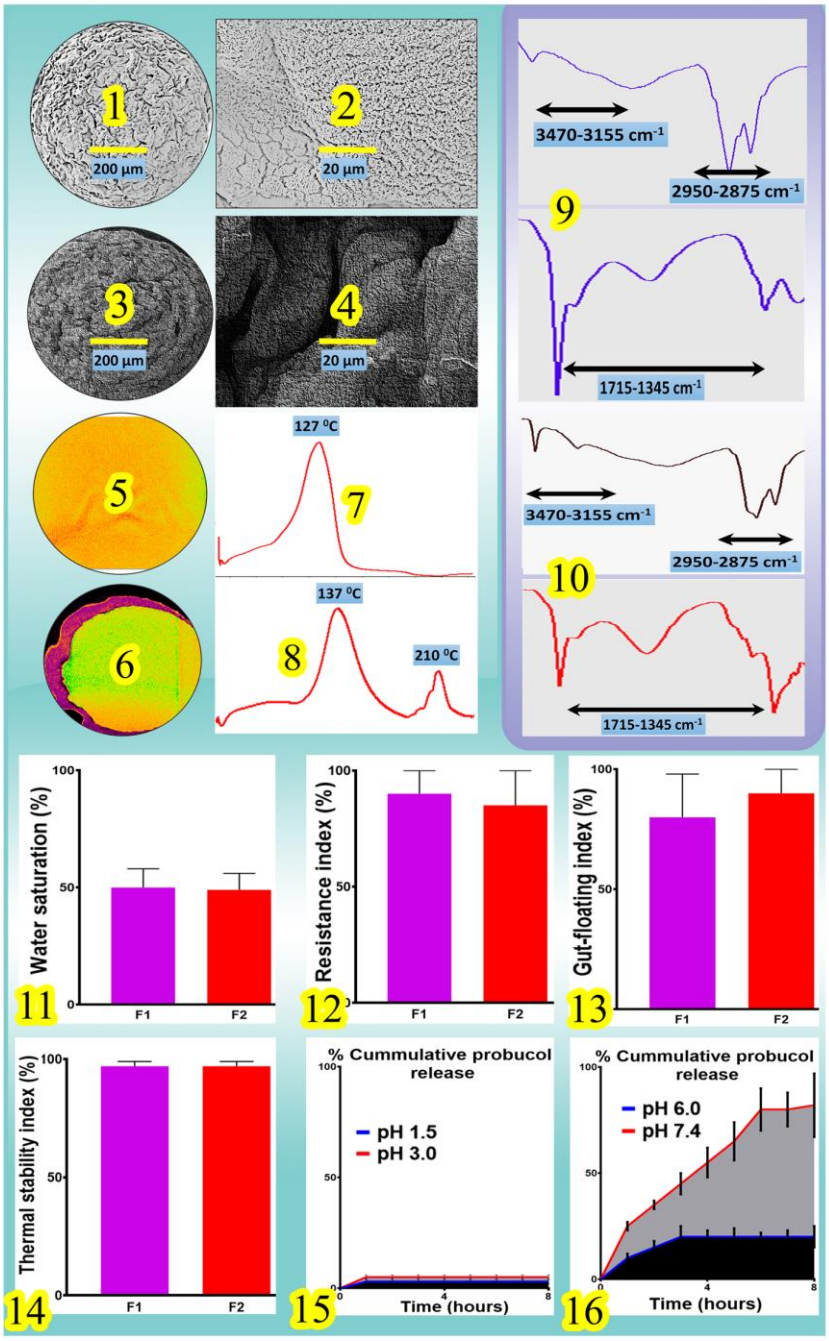

Figure 1. SEM (1–4), Micro-CT (5–6), DSC (7–8), FTIR-IR (9–10), water saturation index (11), microcapsules resistance index (12), PB release at pH 1.5, 3, 6 and 7.4 (13–14), gut-floating index (15) and thermal stability index (16) of UDCA and PBUDCA microcapsules respectively. Data are mean +/− SEM (n = 3).

Compared with control (HFD), all treatments reduced blood glucose (Figs. 1–4), while reduction of inflammatory biomarkers was not consistent among all measured cytokines and by all treatments with PB-L, PBUDCA and UDCA treatments exerting most antiinflammatory effects and PBUDCA showing significant correlation with IL-1β and triglycerides control (Figs. 2–4). Effects of treatment on lipid profile and plasma cholesterol was potent and evident with PB-H treatment, while other treatments had little/no effects on the lipid profile (Figs. 3–4).

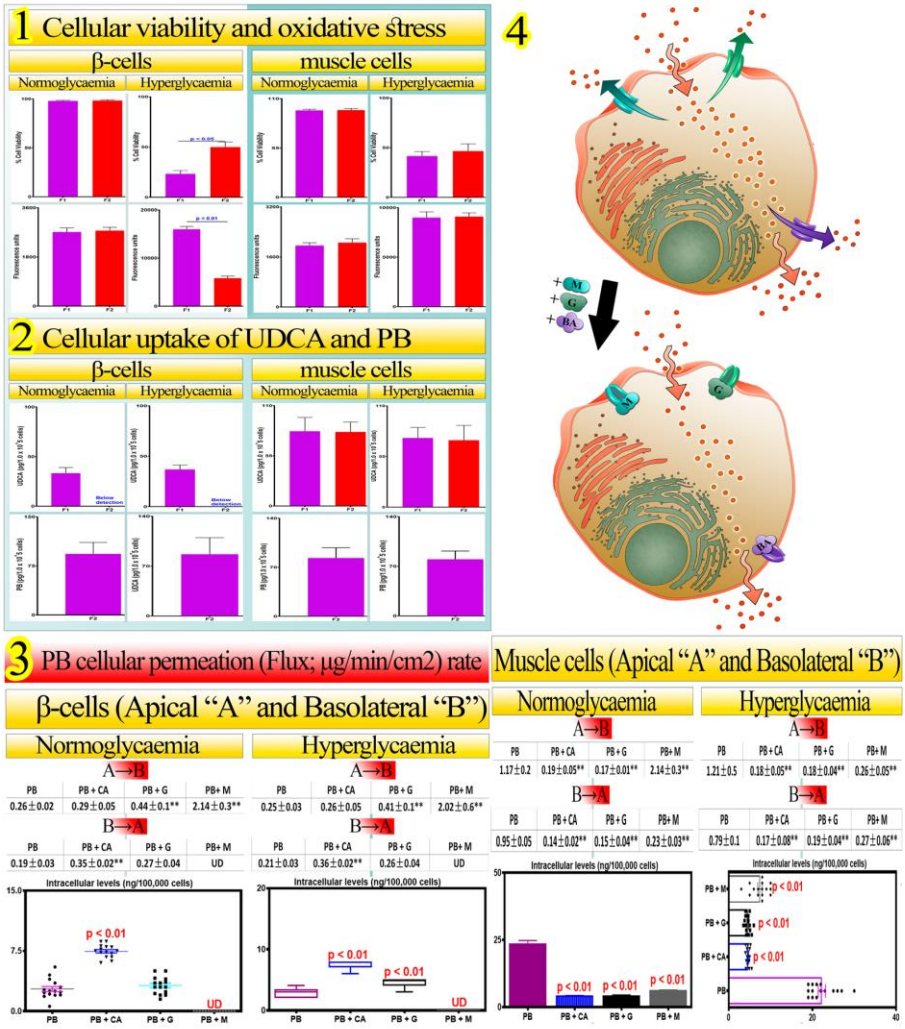

Figure 2. Cellular viability and oxidative stress (1), cellular uptake of UDCA and PB (2), PB unidirectional cellular permeation (flux) and cellular amount retained (3), and cellular efflux protein-transporters competitive inhibition (4), in β-cell and muscle cell, at the normoglycaemic and the hyperglycaemic states. F1: UDCA microcapsules and F2: PBUDCA microcapsules. G-gliclazide; M-metformin; CA-cholic acid. Data are mean +/− SEM (n = 3).

The bile acid profile. Figure 5 shows levels of the primary bile acid CDCA, the secondary bile acid LCA and the tertiary bile acid UDCA in tissues, serum, and faeces.

The bile acid profile is significantly influenced by various treatments with all groups demonstrating significant alteration of bile acid concentrations at the end of the experiment, in ileum, pancreas, serum, heart, liver and faeces. There was significant undetected levels of bile acids in heart suggesting lowered uptake as a result of diabetes development. There was reduction in levels of the primary bile acid, CDCA in serum and due to M, PB-L, PB-H, PBUDCA and UDCA treatments but levels of secondary and tertiary bile acids (LCA and UDCA) were detected in all treated groups suggesting feedback mechanisms compensating for primary bile acid reduction, which was also noticed in heart tissues (Fig. 5).

Discussion

Figure 1 suggests that there was no significant change in morphology, size, multi-surface features in terms of UDCA and PB or UDCA particle distribution within the layers, or surface topographical features between UDCA and PBUDCA microcapsules, despite reduced porosity in F2, which suggests that PB or UDCA presence in microcapsules did not alter topographic properties, multi-layered surface composition, or compromise microcapsules' shape or size. This also suggests that the microencapsulation method was robust and resulted in uniform microcapsules regardless of PB or UDCA presence. DCS and FTIR analyses of UDCA and PBUDCA microcapsules showed small but distinct variation in wavelengths' intensity and range as well as similar melting

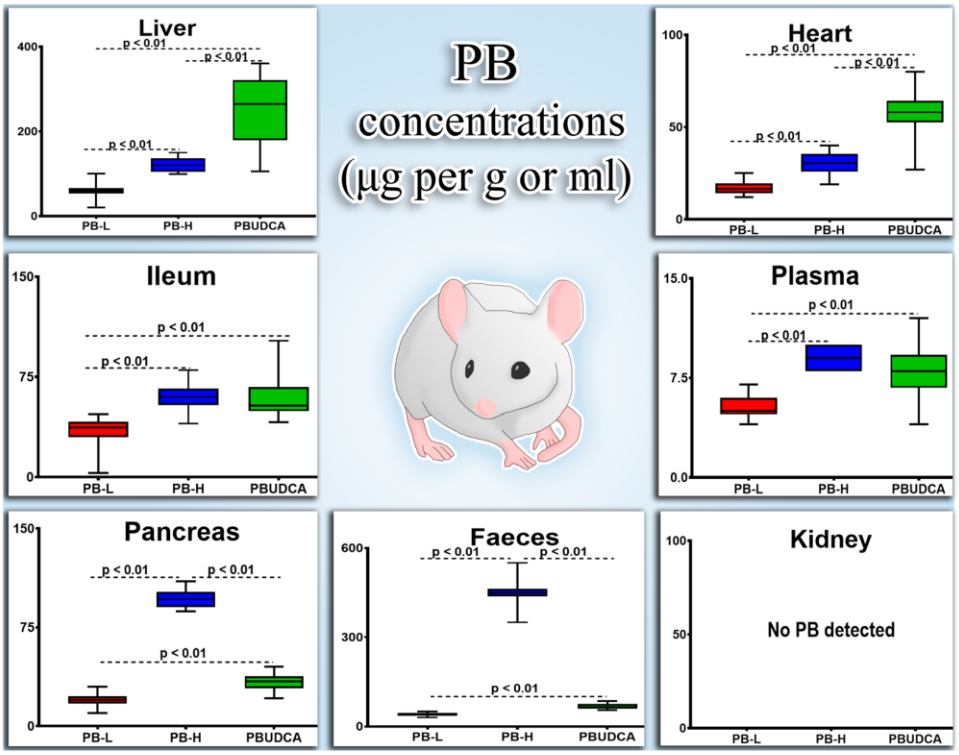

Figure 3. PB concentrations in serum, tissues and faeces. PB-L: low dose probucol, PB-H: high dose probucol and PBUDCA: probucol-ursodeoxycholic acid microcapsules. Data are mean ± SEM. $^{**}p < 0.01$, compared with HFD.

points suggesting stability of ingredients during the encapsulation process. Specifically, in PBUDCA microcapsules compared with UDCA microcapsules, there was a new peak in the 3470–3155 cm^{-1} region as well as alternations in peak-bond activity in the 1715–1345 cm^{-1} region. The new peak and alterations are likely attributed to C-H stretch in the alkane bonds and O-H stretch in the phenol groups within PB's molecular structure as well as C-C stretch within the aromatic rings of PB (occurring around 1420 cm^{-1}) and C-O stretch from the phenol groups of PB (occurring around 1300 cm^{-1})[14,27,29,31]. Such changes in the FTIR and DSC measurements suggests new bond-formation as a result of PB presence, but chemical compatibility was maintained as no bond-activity has completely disappeared nor the new spectra resulting from PB presence was completely different hence a less likely hood of chemical degradation or drug alteration within the microcapsules. This is consistent with our previous studies that showed compatibility between different bile acids and PB presence within the microcapsules[11,14,28,33]. Morphological and compatibility results suggest that PB presence in UDCA microcapsules did not affect the physical characteristics of the microcapsules. Changes in moisture contents (water saturation), physical resistance to stress, floating and thermal stability showed no significant difference between the two microcapsules suggesting that the osmotic stability, mechanical resistance, buoyancy and heat resistance properties remained similar. Results suggest that the presence of PB did not change the physico-chemical properties of the microcapsules and intactness of microcapsules remained consistent, postmicroencapsulation, regardless of PB presence. This supports the notion that our microencapsulation methods was robust and maintained uniformity and preserved the physical properties and structural integrity of the microcapsules. This is also in line with other research groups that showed positive effects of bile components on intestinal drug delivery. Hunt, G.R. and I.C. Jones showed improvement in intestinal drug delivery, to liposomal delivery, by using the bile salts, glycocholate and glycodeoxycholate[36]. However, despite the fact that both microcapsules, PBUDCA and UDCA showed good morphological and physical properties, their impact on cell viability and oxidative stress need to be investigated in order to elucidate beneficial effects at the cellular level. In addition, UDCA potential synergistic effects when combined with PB are likely to be associated with its cellular uptake as UDCA is endogenously produced and metabolised intracellularly.

Cellular protective effects indicative by higher cell viability of PBUDCA microcapsules might be the result of its direct inhibitory effects on oxidative stress of β-cells at hyperglycaemic state, by PB and UDCA, which resulted in normalisation of free radicals and subsequent protection of β-cells from radical damage. Hence, efflux in the presence of PBUDCA resulted in modulation of PB uptake likely by UDCA co-encapsulation affecting specific protein transporters. This is consistent with our studies showing protective antioxidant effects of PB-bile acid microcapsules[3]. This is also consistent with other research groups that have shown strong β-cell protective effects

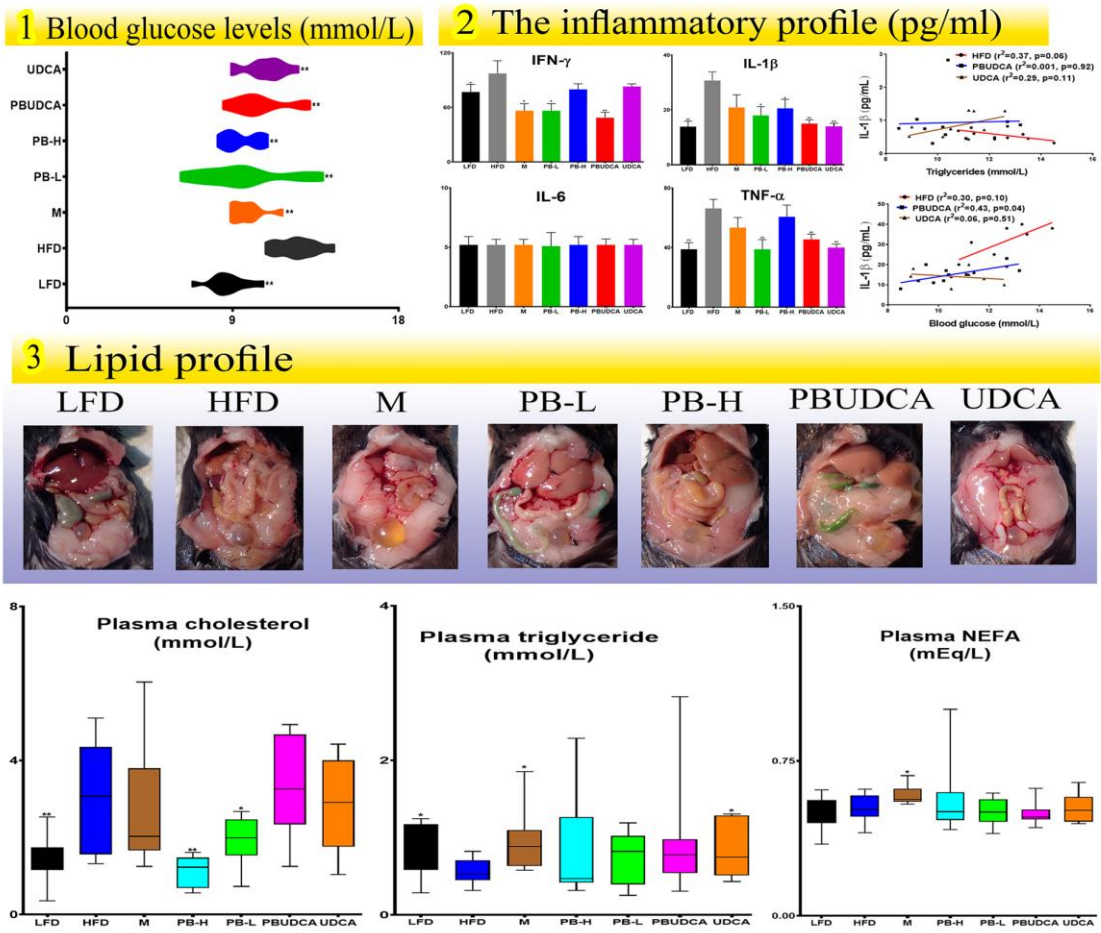

Figure 4. Blood glucose levels (1), plasma inflammatory profile and Linear Regression analyses (2), and plasma lipid profile (3). LFD: low-fat diet, HFD: high-fat diet, M: metformin, PB-L: low dose probucol, PB-H: high dose probucol, PBUDCA: probucol-ursodeoxycholic acid microcapsules, and UDCA: ursodeoxycholic acid microcapsules. Data are mean ± SEM. $*p < 0.05$ and $**p < 0.01$ compared with HFD.

of UDCA or its metabolites. Engin, F. *et al.*; have shown that a conjugated UDCA exerted antiinflammatory effects and reduced loss of β-cell viability and reduced cell apoptosis through its positive effects on unfolded protein response and its mediators[37], while Kim, JY *et al.*; have shown that bile acids are taken up intracellularly into pancreatic cells and influence cell apoptosis by affecting cellular Ca^{2+} signalling pathways[38].

The similar amount of UDCA absorbed by β-cells at normal and hyperglycaemic states suggests that UDCA cellular uptake was independent of glucose concentrations or the glycaemic state. UDCA cellular uptake by muscle cells was significant and independent of glucose levels or the presence of PB in the microcapsules, while UDCA cellular uptake by β-cells was only significant from UDCA microcapsules, and not PBUDCA microcapsules, which suggests that PB selectively inhibited UDCA uptake by β-cells. One possible way by which PB inhibited UDCA uptake by β-cells, was by competitively inhibiting protein-transporters responsible for UDCA uptake into the cells. In one study, Geier, A. *et al.*; have demonstrated that the bile acid UDCA is a substrate of multiple protein transporters in liver such as ABC-transporters and multidrug resistance associated protein 3 (MRP 3) and inhibiting these proteins may affect UDCA cellular uptake[39]. In many other studies, PB has been hypothesized to be capable of competitive inhibition of many protein-transporters responsible for bile acid uptake in liver and pancreas. Rinninger, F *et al.*; have showed strong association between PB cellular transport and the scavenger receptor BI[40]. In another study, Ma, Q, *et al.*; have revealed that the protein transporter which belongs to the ABC transporters, ABCB1, has strong affinity for PB molecules, and ABC transporters are known to also target bile acids[41]. Accordingly, at hyperglycaemic state, PBUDCA microcapsules improved β-cell viability, but impaired UDCA cellular uptake, while both microcapsules exerted similar effects on muscle cells, in terms of viability, oxidative stress and UDCA cellular uptake. The presence of PB exerted favourable cell protective and antioxidant effects on β-cells. Thus, PB concentrations in plasma and tissues should provide an insight on its cellular uptake, as well as its impact on bile acid profile, glucose levels, lipid profile and the inflammatory response.

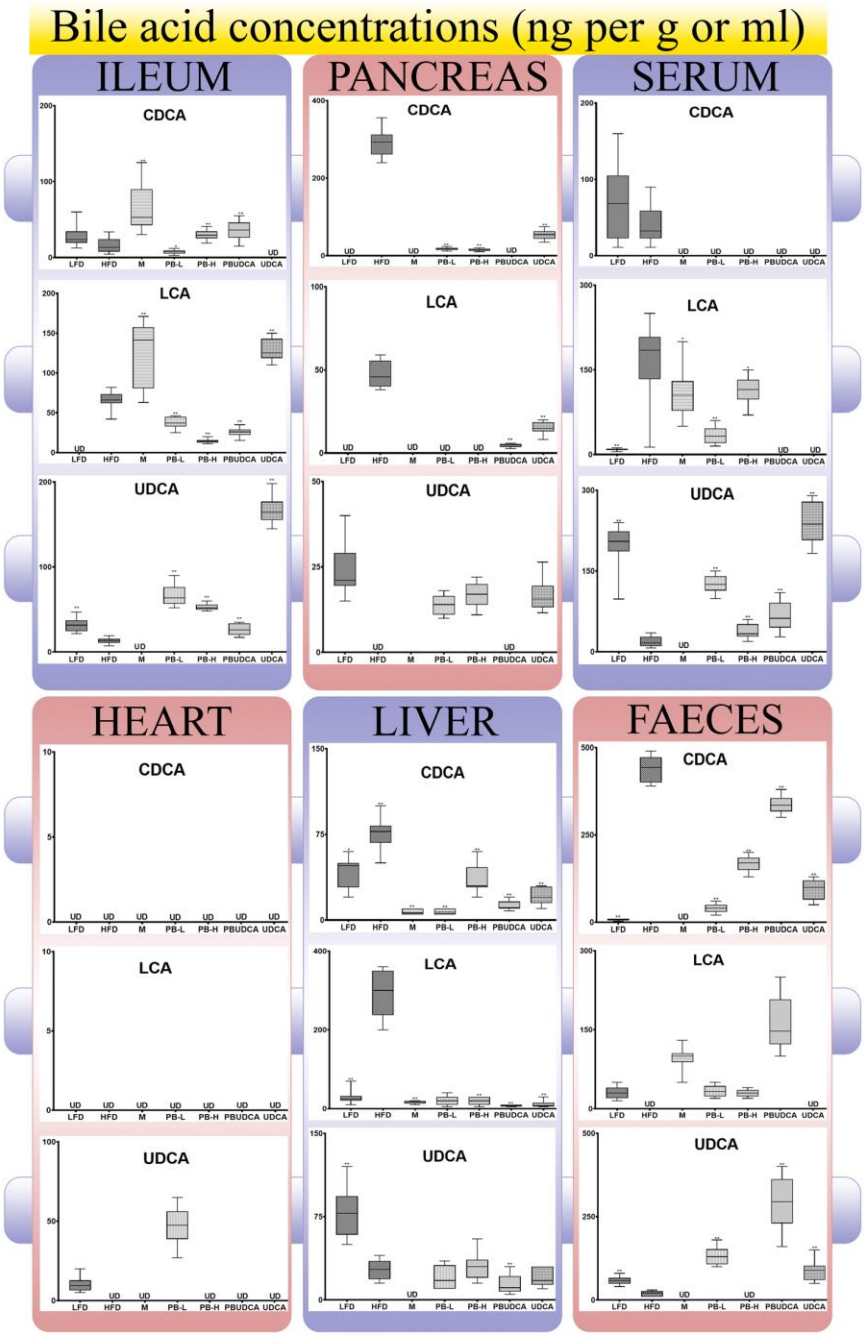

Figure 5. Bile acid concentrations in serum, tissues and faeces. LFD: low-fat diet, HFD: high-fat diet, M: metformin, PB-L: low dose probucol, PB-H: high dose probucol, PBUDCA: probucol-ursodeoxycholic acid microcapsules, and UDCA: ursodeoxycholic acid microcapsules. Data are mean ± SEM. *$p < 0.05$ and **$p < 0.01$ compared with HFD.

Compared with UDCA microcapsules, PBUDCA microcapsules resulted in higher β-cell viability, and lower oxidative stress at hyperglycaemic state, while neither PBUDCA nor UDCA microcapsules had effects on C2C12 cells viability or oxidative stress levels (Figs. 1–2). UDCA uptake by β-cells from UDCA microcapsules was significantly high at both glucose levels, while no UDCA uptake was detected from PBUDCA microcapsules. UDCA uptake by muscle cells was significant and consistent at both glucose levels and irrespective of PB incorporation into microcapsules. PB uptake was significant from PBUDCA microcapsules, at both glucose levels (Fig. 2–2).

	Normoglycaemic state			Hyperglycaemic state		
β-cells						
PB	+BA (−MRP3)	+G (−MRP2)	+M (−MRP1)	+BA (−MRP3)	+G (−MRP2)	+M (−MRP1)
A→B	±	↑	↑	±	↑	↑
B→A	↑	±	↓	↑	±	↓
Cellular retention	↑	±	↓	↑	±	↓
Muscle-cells						
PB	+BA (MRP3)	+G (−MRP2)	+M (−MRP1)	+BA (MRP3)	+G (−MRP2)	+M (−MRP1)
A→B	↓	↓	↑	↓	↓	↓
B→A	↓	↓	↓	↓	↓	↓
Cellular retention	↓	↓	↓	↓	↓	↓

Table 1. Summary of the influence of incorporation of a bile acid (BA), gliclazide (G) and metformin (M) on probucol (PB) cellular permeation (flux) and cellular retention using two cell types (β and muscle cells) at the normoglycaemic and hyperglycaemic states. ±indicates no significant net effect, ↑ indicates increase and ↓ indicates decrease levels, based on Fig. 2–2 measurements.

PB's cellular permeation (flux) and cellular retention were modified (Figs. 2–3) by selective substrates of certain ABC-efflux protein transporters (multi-resistance proteins 'MRP' 1, 2 and 3) which may provide information on mechanisms of PB cellular uptake (Figs. 2–4) relevant to its physiological effects on viability and oxidative stress (Fig. 2: 1 & 2). The addition of gliclazide (G; a selective substrate for MRP2[42]), metformin (M; a selective substrate for "MRP1"[43]) and the bile acid cholic acid (CA; a selective substrate for MRP3[44]) resulted in changes in PB unidirectional apical to basolateral and basolateral to apical flux and subsequent cellular retention in β and muscle cells. The changes of PB flux and cellular retention were possibly via substrate-competitive inhibition by BA, G and M, and these effects were similar at the normoglycaemic versus the hyperglycaemic states, and different when comparing these effects on β-cells versus muscle cells (Table 1). Changes caused by BA, G and M on PB cellular uptake and retention suggest involvement of MRP1, MRP2 and MRP3 in PB cell delivery. The absence of direct and significant effects of hyperglycaemia on PB cell delivery suggests that development of hyperglycaemia and insulin-resistance do not directly affect PB oral uptake or the amount of PB permeating or being retained by cells. In β-cells, MRP3 inhibition resulted in no change of PB unidirectional apical-to-basolateral flux while increasing unidirectional basolateral-to-apical flux and cell retention suggesting that PB has substrate affinity for MRP3 and hence inhibiting MRP3 efflux of PB resulted in constant A→B permeation with significant increase in B→A and cell retention due to increased uptake from the basolateral side of the cells. MRP2 inhibition resulted in increased PB unidirectional apical-to-basolateral flux while having no effects on basolateral-to-apical flux and cellular retention suggesting that PB has substrate affinity for MRP2 on the apical side while its MRP3 efflux is maintained. MRP1 inhibition resulted in increased apical-to-basolateral influx, with reduction in basolateral-to-apical flux and cellular retention suggesting that either inhibiting MRP1 only increased apical-to-basolateral flux with no up-regulation of other efflux transporters transporting cellular PB, or there are other PB efflux transporters, on the basolateral side, that were inhibited by M addition[45,46].

In muscle-cells, MRP3 inhibition resulted in reduction in both, apical-to-basolateral and basolateral-to-apical unidirectional fluxes as well as cellular retention suggesting that MRP3 inhibition brought about reduction in the amount of PB passing through the cells and the overall cellular uptake of PB, likely via either suppression of PB permeation or optimisation of PB overall cellular efflux. Similar to MRP1 inhibition, MRP2 inhibition resulted in reduction in apical-to-basolateral and basolateral-to-apical unidirectional fluxes and less PB cellular retention, and hence a significantly different response in muscle cells compared with pancreatic β-cells suggesting that ABC-efflux protein expression is significantly different between both types of cells, and this is consistent with the literature[47,48]. Similar to MRP1 inhibition in β-cells, muscle-cells MRP1 inhibition resulted in higher apical-to-basolateral flux with reduced basolateral-to-apical flux as well as cellular retention, which suggests PB permeation and retention remain constant between both types of cells and hyperglycaemia did not affect that, although PB apical-to-basolateral flux was reduced indicating direct effects of diabetes on expression and functionality of apical MRP1 in muscle cells. Diabetes-associated alteration of ABC efflux transporters' expression and functionalities are consistent with the literature[49,50] suggesting that diabetes development and progression may have detrimental effects on the functions of selective ABC-efflux transporters and result in variation of drug uptake and absorption in diabetic patients (Table 1).

It is worth stating that there is some ambiguity in the literature regarding selectivity of drugs to specific ABC-efflux transporters and the possibility with multiple simultaneous selectivity and various effects of diseases on expression and functionalities of these transporters. Hence, although we maintain that our MRP1, MRP2, and MRP3 substrates/inhibitors are selective based on our work and others, tissue specific competitive-inhibition remains debatable and a study limitation, particularly when other transporters are also prominent e.g. organic anion transporting proteins and organic cation transporter polypeptide for bile acids[42–44,51,52].

PB was detected in all analysed samples except kidneys, which likely due to its high lipophilic properties and, thus, excretion is mostly by liver rather than kidney, and this is consistent with the literature[53,54]. Compared with PB-L, both, PB-H and PBUDCA resulted in increased PB levels in plasma, ileum, liver, brain, heart, pancreas and faeces. The higher concentrations of PB in PB-H plasma, tissues and faeces, compared with PB-L, was expected and suggests that PB absorption does not reach saturation levels after PB-L, potentially via MRP1 or MRP2 and MRP3 involvement. Despite PB-L and PBUDCA containing the same amount of PB, PBUDCA resulted in higher

Treatments	Glycaemic profile	Inflammatory profile				Lipid profile		
	Blood glucose (mM)	IFN-γ (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	Cholesterol (mM)	Triglycerides (mM)	NEFA (mEq/L)
LFD	↓	↓	↓	±	↓	↓	↑	±
M	↓	↓	±	±	±	±	↑	↑
PB-L	↓	↓	↓	±	↓	↓	±	±
PB-H	↓	±	↓	±	±	↓	±	±
PB-UDCA	↓	↓	↓	±	↓	±	±	±
UDCA	↓	±	↓	±	↓	±	↑	±

Table 2. Summary of the levels of various glycaemic, inflammatory and lipid profiles among treated groups compared with control (HFD). ±indicates no significant net effect, ↑ indicates increase and ↓ indicates decrease levels, based on Fig. 4 measurements.

PB concentrations in plasma and all tissues analysed compared with PB-L, which suggests that incorporation of PB into UDCA-microcapsules enhanced its absorption and tissue accumulation as well as its concentrations in systemic circulation potentially via UDCA inhibition of MRP3 resulting in net enhancement of PB cellular permeation. Pancreas levels of PB remained low with regard to PB-H while liver achieved higher PB levels, possibly due to cellular uptake being limited in the pancreas, but not hepatocytes, hence higher liver accumulation of PB when given encapsulated with UDCA. The improved absorption of PB with microencapsulation is also consistent with findings from other research groups. Fukami, T. *et al.*; have shown that special delivery methods such as nanotechnology can significantly enhance PB's absorption and cellular permeation[55], while Zhang, Z. *et al.*; have demonstrated that using hybrid of surfactants which form negatively charged particles can further enhance the oral delivery of PB[56]. With the significant increase in PB concentrations in plasma and tissues, as a result of PB-H or PBUDCA microcapsule treatments, effects on blood glucose, inflammation and lipid profiles by different treatments, should provide insight on association between PB levels, glycaemic control and pharmacological effects on inflammation and lipid levels, in our insulin-resistance animal model.

With regard to treatments' effects on blood glucose, inflammation and lipid profile (Fig. 4), All treated groups showed significant reduction in blood glucose levels compared with control (with similar magnitudes) which suggests that PBUDCA microcapsules did not produce any additional hypoglycaemic benefits compared with other groups. The similar hypoglycaemic effects between PB-L, PB-H and PBUDCA suggest that the hypoglycaemic effect is independent of PB dosing or its oral delivery formulation, and may relate to treatments' effects on inflammatory or lipid profiles (Fig. 4: 2 & 3). Results from plasma levels of IFN-γ, IL-1β, TNF-α and IL-6 showed that all treatment groups exerted anti-inflammatory effects through reducing at least one of the four pro-inflammatory biomarkers (Table 2). Although none of the treatment significantly changed IL-6 levels, PB-L and PB-UDCA treatments exerted the most antiinflammatory effects via reducing three pro-inflammatory biomarkers IFN-γ, IL-1β, and TNF-α. In addition, PB-L, and PBUDCA also brought about the greatest TNF-α lowering effect similar to that of M. There was weak association between PBUDCA effects on IL-1β and lowering of blood glucose levels which suggests that reducing inflammation assisted with the hypoglycaemic effects, although exact cellular mechanisms remained to be investigated. In addition, the results showed PBUDCA having the greatest anti-inflammatory effects and this may be due to the combined syncretistic biological impact of the potent anti-oxidant PB coupled with the anti-inflammatory bile acid UDCA[26,57,58]. Plasma lipid profiles and visceral fat depositions could impact on the glycaemic and the inflammatory profiles.

Visual examination of the visceral fats showed no visible difference in colure, shape or texture of visceral tissues/fat amongst the groups although such examination was not histopathological or immunophysiological, and hence was considered complementary to the lipid profile analyses. The lipid profile analyses showed that only PB-L and PB-H significantly lowered cholesterol and there was an increase in the triglyceride and NEFA levels with M and triglyceride with UDCA treatments. In addition, none of the treatment groups except M, changed NEFA levels (Table 2). The results of the lipid profile suggest that M has increased triglycerides and NEFA unfavourably affecting the lipid profile, which is consistent with published studies[59]. The lipid-regulatory effects of PB-L and PB-H are expected since PB is a lipid-lowering drug, while UDCA bringing about an increase in triglycerides levels suggests that UDCA administration resulted in negative feedback mechanisms on bile acid synthesis and cholesterol catabolism resulting in higher levels of triglyceride, and modulation of the bile acid profile (Fig. 5).

With regard to the concentrations of CDCA, LCA and UDCA in tissues, serum and faeces, Compared with HFD, all treatments resulted in reduced CDCA plasma concentrations, which suggests direct interference of treatments with either cholesterol catabolism to primary bile acids or accelerated primary bile acid metabolism to secondary bile acids. Ileal CDCA concentrations in LFD were similar to HFD which is consistent with serum levels, and they were higher in M, PB-H and PBUDCA groups while there were not detected in UDCA group suggesting treatments effects on bile acid gut metabolism and ileal cellular uptake. In the pancreas, CDCA levels were the highest in the HDF group and were reduced in all other groups suggesting significant alteration of pancreatic tissue accumulation of the primary bile acid brought about by various treatments that possibly altered cholesterol catabolism and total cholesterol available (Fig. 4) or uptake of metabolised bile acid via gut enterocytes' efflux transport (Fig. 2). Heart tissue showed no presence of CDCA levels, while in the liver, HFD treatment increased CDCA levels and all other various treatments reduced CDCA levels suggesting reduction in CDCA production

Treatments	Primary bile acid profile (CDCA)						Secondary bile acid profile (LCA)						Tertiary bile acid profile (UDCA)					
	serum	ileum	pancreas	heart	liver	faeces	serum	ileum	pancreas	heart	liver	faeces	serum	ileum	pancreas	heart	liver	faeces
LFD	±	±	↑	±	↓	↓	↓	↓	↓	±	↓	↑	↑	↑	↑	↑	↑	↑
M	↓	↑	±	±	↓	↓	↓	↑	↓	±	↓	↑	↓	↓	±	±	↓	↓
PB-L	↓	↓	↑	±	↓	↓	↓	↓	↓	±	↓	↑	↑	↑	↑	↑	±	↑
PB-H	↓	↑	↑	±	↓	↓	↓	↓	↓	±	↓	↑	↑	↑	↑	±	±	↓
PB-UDCA	↓	↑	±	±	↓	↓	↓	↓	↓	±	↓	↑	↑	↑	±	±	↓	↑
UDCA	↓	±	↑	±	↓	↓	↓	↑	↓	±	↓	±	↑	↑	↑	±	±	↑

Table 3. Summary of the levels of bile acids among treated groups compared with control (HFD). ±indicates no significant net effect, ↑ indicates increase and ↓ indicates decrease levels, based on Fig. 5 measurements.

and cholesterol catabolism in hepatocytes and possibly inhibiting the enterohepatic recirculation processes. The reduction of CDCA in the liver by various treatments may be associated with increased hepatocytic concentration (Fig. 3) due to possible cellular efflux inhibition of MRP1, MRP2 or MRP3 by treatments and CDCA accumulation being their substrate (Fig. 2). High CDCA levels in faeces due to HFD is consistent to its increase levels in ileum and their reduced levels by treatments. LCA is considered a potent bile acid and has been associated in the literature with cytotoxicity and inflammation[60–62]. Serum LCA levels were elevated in the HFD group and reduced by treatments. Ileal levels were undetected in the LFD group, significantly high in the HFD group and compared with the HFD group, M and UDCA treatments increased that further while PB treatments reduced those levels suggesting antiinflammatory effects of PB, which is consistent with reduction of the proinflammatory TNF-α observed in Fig. 4. PB antiinflammatory effects were also consistent with previous studies that have shown that PB exerted antiinflammatory and cellular protective effects, *ex vivo*, on pancreatic β-cells[63]. Pancreatic and liver LCA levels changed in a similar way to that in serum, while no LCA was detected in heart tissues. The reduction in LCA levels in pancreas and liver tissues is consistent with that in serum, which suggests that pancreatic and liver cellular uptake is directly proportional to LCA systemic absorption or LCA tissue metabolism is significantly influenced by the treatments. LCA levels in faeces were higher in all treated groups compared with HFD, which is consistent with its lower systemic absorption levels, except in the UDCA group due to possible feedback effects resulting in less metabolic processes and LCA production in the gut, although ileal levels were increased which suggests LCA metabolism occurring in the lower part of the gastrointestinal tract, most likely the colon. Compared with HFD, UDCA serum levels were higher, except in the M group were levels were undetected suggesting significant alteration to bile acid metabolic pathways of primary and secondary bile acids, which is consistent with M effects on CDCA and LCA serum levels. UDCA ileal levels were lowered by HFD, hence higher levels by all treatments except M group were levels were undetected, consistent with serum levels. Pancreatic levels were detected in LFD and all other groups except in HFD, M and PBUDCA groups suggesting significant disturbances of the bile acid metabolism, which is consistent with their effects on primary and secondary bile acids. The effects of M on our bile acid profile is consistent with published studies showing M reducing bile acid gut reabsorption in T2D patients[64], with some studies suggesting M effects on bile acid profile is via ileal protein transporters[65], reducing ileal bile salt reabsorption[66], or influencing crosstalk processes between the nuclear bile acid receptor farnesoid X receptor and the nutrient-sensitive kinase, 5′ adenosine monophosphate-activated protein kinase[67]. UDCA heart levels were detected only in LFD and PB-L groups suggesting that HFD reduced UDCA cardiocyte cellular uptake and only PB in low dose neutralised the HFD effect. Despite the fact that there were no detectable levels for CDCA and LCA in heart tissues, and UDCA being less lipophilic than CDCA, it was present in cardiocytes after prediabetic mice were treated with PB-L possibly as a consequence of high levels of UDCA observed in serum, ileum and pancreas. Similarly, UDCA levels in the liver were reduced in the prediabetic mice with M and PBUDCA treatments lowering the levels further suggesting potential interference of M and PB treatments on bile acid regulation by hepatocytes particularly since PBUDCA microcapsules showed strong pH-targeted release (Fig. 1), PB significant uptake at the hyperglycaemic state (Fig. 2) and PB concentrations in the liver were the highest in the PBUDCA group (Fig. 3). Similar to ileal levels and compared with HFD group, UDCA faecal levels were high in LFD, PB-L, PBUDCA and UDCA groups suggesting minute metabolism within cecum and colon although PB-H treatment showed undetected UDCA levels suggesting strong local effects of high dose PB on gut-UDCA metabolism and systemic absorption, particularly when serum levels of UDCA were higher in the PB-H group compared with untreated HFD control. In general, PB treated groups showed overall decreased primary and secondary bile acids in serum, and overall increased UDCA levels in serum, suggesting reduction in cholesterol levels resulting in less bile acid synthesis compensated by higher levels of tertiary bile acids via feedback mechanisms associated with bile acid enterohepatic recirculation processes[68,69]. Hence, the highest ileal UDCA levels were observed in the UDCA group with corresponding high levels of LCA in the ileum being detected in both the M and UDCA groups. No CDCA was detected in the ileum of UDCA mice and this might be attributed to alterations in the enterohepatic cycling of endogenous bile acids caused by UDCA, increasing the formation of secondary bile acid LCA and the tertiary bile acid UDCA within the gastrointestinal tract (Table 3).

Currently PB is administered orally, as tablets and is widely used for hypercholesterolemia in China and South East Asia. It comes in different strength tablets including 125 mg, with its recommended dosing of 500–1000 mg/day. Similarly, the bile acid UDCA is administered orally as tablets, and is widely used for primary liver cirrhosis, globally. It comes mainly as 250 mg tablets, with its recommended dosing of 500–1000 mg/day. PB and UDCA

tablets are coated to prevent release in stomach, and the tableting formulations have been widely used for several decades.

Conclusion

The microencapsulating method deployed was successful in producing PBUDCA targeted-delivery micro/nano capsules, which are stable, compatible and have desirable and consistent shape and delivery profile in our prediabetic mouse model. PBUDCA enhanced survival of pancreatic β-cells and muscle cells (*ex vivo*) with substrate-selectivity of PB toward the efflux protein transporters MRP1, MRP2 and MRP3, and significant PB oral absorption optimised by PBUDCA microencapsulation, in serum, liver, ileum and heart (*in vivo*); 6-months oral dosing. PBUDCA lowered blood glucose comparable to M, and exerted significant antiinflammatory effects, while anti-lipidemic effects remained insignificant. PBUDCA exerted significant bile acid modulation effects suggesting PB involvement in the enterohepatic recirculation of bile acids, although the exact molecular and cellular pathways and their influence on the bile acid synthesis and feedback mechanisms remained unclear. Future studies should aim to investigate various polymer-bile acid formulation systems to further optimise the delivery and therapeutic impact of dual antioxidant-bile acid microcapsules for the treatment of diabetes mellitus.

Materials and Methods

Materials and drug preparation. Probucol, metformin, low viscosity sodium alginate, ursodeoxycholic acid and sodium alginate were purchased from Sigma Chemical Co, USA, while calcium chloride from Scharlab S.L, Australia. The reagents were purchased from Merck (Australia) and were used without modifications. Stock of PB (20 mg/mL) and UDCA (4 mg/mL) were prepared by vortexing the powders with 10% gel[3,14,27–29]. Preparations were mixed for 7 hours, and used within two day of preparation.

Microencapsulation fabrication, stability/shelf life, and *in vitro* studies. Microcapsules of PBUDCA and UDCA were prepared as established in our laboratory by Ionic Gelation Vibrational Jet Flow Technology, which utilises a Büchi encapsulator (Büchi Labortechnik, Flawil, Switzerland) under a constant liquid flow rate of 1 mL/min. The microcapsules were formed at 2% $CaCl_2$ ionic gelation bath before being washed in water for a few minutes prior to collection and stability/shelf life assessed using Accelerated Stability Chambers using our well-established methods[14,27,28,30–33]. Microcapsule morphology and surface topography were examined using Micro-CT (a SkyScan 1172 A Micro-CT, Kontich, Belgium) and Zeiss-Neon 40EsB FIBSEM (USA) as per our well-established methods[29,70]. The surface characteristics were examined via FIB SEM (Zeiss Neon 40EsB, USA). Osmotic stability of the microcapsules was determined by placing 1 g of microcapsules in phosphate buffered saline for 14 days at 37 °C, and was calculated by weight gain attained compared to initial 'dry' weight[14,27,28]. The mechanical resistance of the microcapsules was determined by placing 200 microcapsules in a shaker and vibrating them over 14 days, and the resistance index was calculated as percentage of damaged microcapsules to intact microcapsules[30,34]. Microcapsules' buoyancy was examined through placing 200 microcapsules in 200 mL of simulated intestinal fluids which consisted of enzyme-based phosphate buffer. The solution was stirred periodically at a set temperature 37.5 °C. The buoyancy index was calculated as the percentage of floating microcapsules[3]. The heat resistance testing was performed by incubating 200 freshly made microcapsules in a climatic chamber (Angelantoni Environmental and Climatic Test Chamber, Italy) set at 37.5 °C for 14 days. The stability index was determined mathematically by calculating the percentage of undamaged microcapsules (no change in colour, texture, appearance or structural integrity) compared to pre-incubated fresh microcapsules[3,11,14].

***Ex vivo* studies.** NIT-1 mouse-cloned pancreatic β-cells and C2C12 mouse-cloned muscle cells were cultured separately in sterile flasks containing growth media optimised with glucose, antibiotics and amino acids using our established methods[3,14]. Viability assays were performed at two glucose concentrations (5.5 mM and 35.5 mM) over a 52 hour period[3,14]. In order to measure oxidative stress, NIT-1 and C2C12 cells were cultured using two different glucose concentrations of 5.5 and 35.5 mM for two days. Stock solutions of Dichloro-dihydro-fluorescein diacetate and azobis-2-methyl-propanimidamide Dihydrochloride were freshly prepared and stored at −20 °C and aliquots used for the antioxidant assay. After two days incubation, microplates containing treated cells were placed in an Enspire Multimode Plate Reader (PerkinElmer, USA) and the fluorescence was read after one hour. Using this method, the intensity of fluorescence directly corresponds to the formation of fluorescent oxidised radical species dichlorofluorescein. The lower the fluorescence reading, the greater the antioxidant activity conferred[71]. The cellular antioxidant assay was done in triplicates and data was normalised for viable cell count. In order to examine UDCA and PB cellular uptake from the microcapsules, at normoglycaemic glucose levels (5.5 mM) and hyperglycaemic glucose levels (35.5 mM), the cells were treated with UDCA or PBUDCA microcapsules for 48 hours, then microcapsules removed and cells washed with PBS, sonicated to rupture, and washed with ice-cold acetonitrile. Similar conditions were used to examine effects of M, G or BA inhibitory effects of PB cellular uptake. The supernatants were removed and analysed using a liquid-chromatography/mass spectroscopy (LC-MS) instrumentation that involved a flow rate of 0.25 mL/min using methanol-water as 65:35 mixture with assay run times of 15 minutes per run based on our established methods[42,49,72,73]. Since UDCA was endogenously produced, PB cellular permeation rate was measured using our well-established methods[74] and selected ABC-transporters substrates were incorporated with PB and UDCA to examine transporters' selectivity to PB and UDCA.

***In vivo* studies.** Six-week old, wild type (C57BL/6J) male mice were attained from the Animal Resources Centre (Australia). Mice were randomly allocated into seven groups, 10 each (n = 70). Group-1 was given low fat diet (LFD; healthy) and empty microcapsules, group-2 was given high fat diet (HFD; insulin-resistance) and empty microcapsules, group-3 was given HFD and metformin (200 mg/kg/day), group-4 was given HFD and low dose PB (80 mg/kg/day), group-5 was given HFD and high dose of PB (800 mg/kg/day), group-6 was given HFD

and PBUDCA microcapsules (PB: 80 mg/kg/day and UDCA 70 mg/kg/day) and group-7 was given HFD and UDCA microcapsules (70 mg/kg/day). HFD consisted of AIN93M rodent chow enriched in 30% (w/w) lard, 0.5% (w/w) cholesterol and 15% (w/w) fructose (Specialty Feeds, Perth, Australia).

All mice were maintained on half-day dark cycle (22 °C) and with water and food *ad libitum*. At the end of 6-months experiment, mice were anaesthetized with isoflurane and euthanised by cardiac puncture followed by cervical dislocation. Blood was collected into EDTA tubes and stored on ice. Plasma was separated by short-speed centrifugation at 4 °C and stored at −80 °C. Tissues of different organs were removed at stored in 4% paraformaldehyde (PFA) at −80 °C. The animal experiments were approved by Curtin University Animal Ethics Committee and all experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes.

Probucol and Bile Acids Analysis

PB HPLC analysis. Standard concentrations and quality control samples of PB in mobile phase acetonitrile: water were prepared for the range of 0.04 to 0.8 mg/ml. volume of injection was 10 µL per injection. Shimadzu HPLC Prominence was used, and consisted of Shimadzu LC-20AT liquid chromatographer, SIL-20A autosampler and SPD-20A-UV/Vis detector (Japan). 160 µL of mobile phase (acetonitrile: water in a 96:4% v/v ratio) was added to 40 µL of purified plasma and vortex-mixed for 5 seconds and centrifuged at 15000 RPM for 15 minutes. Twenty µL of the supernatant was removed and transferred to autosampler vials ready for analysis[20].

Bile acids' LC-MS analysis. Blood, tissue and fecal bile acids analyses were carried out via liquid chromatography mass spectrometry (LCMS). In brief, LCMS (Shimadzu LCMS 2020 system, Shimadzu Corporation, Kyoto, Japan) included a Phenomenex C18 column (Phenomenex Corporation, Torrance, California, USA) 10 cm in length and 2 mm in diameter and with 5 µm particle size. The flow rate set at 0.25 mL/min and the mobile phase was methanol (65%) and water (35%) at pH 2.9, with the standards and quality control samples being within the range of 1–1000 ng/ml. The analysed bile acids CDCA, LCA and UDCA had retention times of 2.6, 5.1 and 1.5 minutes respectively, with a flow rate of 1.5 L/min using our well-established methods[18,42,72,73].

Biological analysis. Blood glucose levels were measured via tail vein venepuncture using Accucheck (Roche Laboratories, Switzerland) and HbA1c measurements were via Siemens DCA Vantage Analyser (Siemens Healthcare Diagnostics, New York, USA). Plasma cholesterol and triglycerides were assessed via enzymatic assays (Randox Laboratories, Crumlin, UK)[75], while NEFAs were assessed with NEFA-C (ASC-ACOD method, Osaka, Japan)[76]. Visceral fat depositions were examined visually at the end of experiment, while plasma cytokines were assessed using cytokine bead array kit (BD Biosciences, California, USA) via Attune Acoustic Focusing Flow Cytometer (Life Technologies, Carlsbad, California, USA) as per our established methods[18,20,77].

Statistical analysis. Values are expressed as means ± standard error of the mean. Statistical measurements were carried out using parametric/non-parametric analysis or using a one way ANOVA and a Tuckey post-hoc, as appropriate. GraphPad Prism Version X8.2 (GraphPad, USA) was utilised for p value analyses.

Received: 14 June 2019; Accepted: 23 September 2019;
Published online: 09 January 2020

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Acknowledgements

The NIT-1 cells were a generous donation from Professor Grant Morahan at the University of Western Australia. The C2C12 cells were a generous donation from Professor Deirdre Coombe at the School of Pharmacy and Biomedical Sciences, Curtin University. The work is partially supported by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 872370 and Ministry of Education, science and Technological Development of the Republic of Serbia No. 41012.

Author contributions

The study was designed by Armin Mooranian, Hani Al-Salami, Ryu Takechi, John Mamo, Virginie Lam, Gerald F Watts and Hesh Al-Sallami; design, analysis and interpretation of research data was done by Armin Mooranian, Susbin Raj Wagle, Bozica Kovacevic, Ryu Takechi, John Mamo, Virginie Lam, Momir Mikov, Svetlana Golocorbin –Kon, Goran Stajonavoic and Hani Al- Salami; significant contribution in the data interpretation and presentation of quality figures and tables was done by all authors and the final version of manuscript was revised and approved by all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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Declaration of interest: Al-Salami H has been and is currently receiving of funding from Beijing Nat-Med Biotechnology Co., Ltd. and affiliated companies.

Appendix B

Contribution of thesis author to publications

I, Susbin Raj Wagle, as the author of this thesis, contributed to the following publications as outlined below. All the co-authors signed authorship forms approved by Curtin University and the contributions of every co-author (including myself) are available upon the request from the primary supervisor of this thesis Dr. Hani Al-Salami.

Publication one (chapter 1):

Complete literature search, analysis, drafting, editing, and reviewing of the manuscript for the publication.

- Analysis and interpretation of research data.
- Drafting significant parts of the work or critically revising it, which contributes to the interpretation.
- A significant contribution to data interpretation and presentation resulting in significant improvement of quality.
- Provide data or analytical skills, which add significantly to the design, quality, and readability of the work

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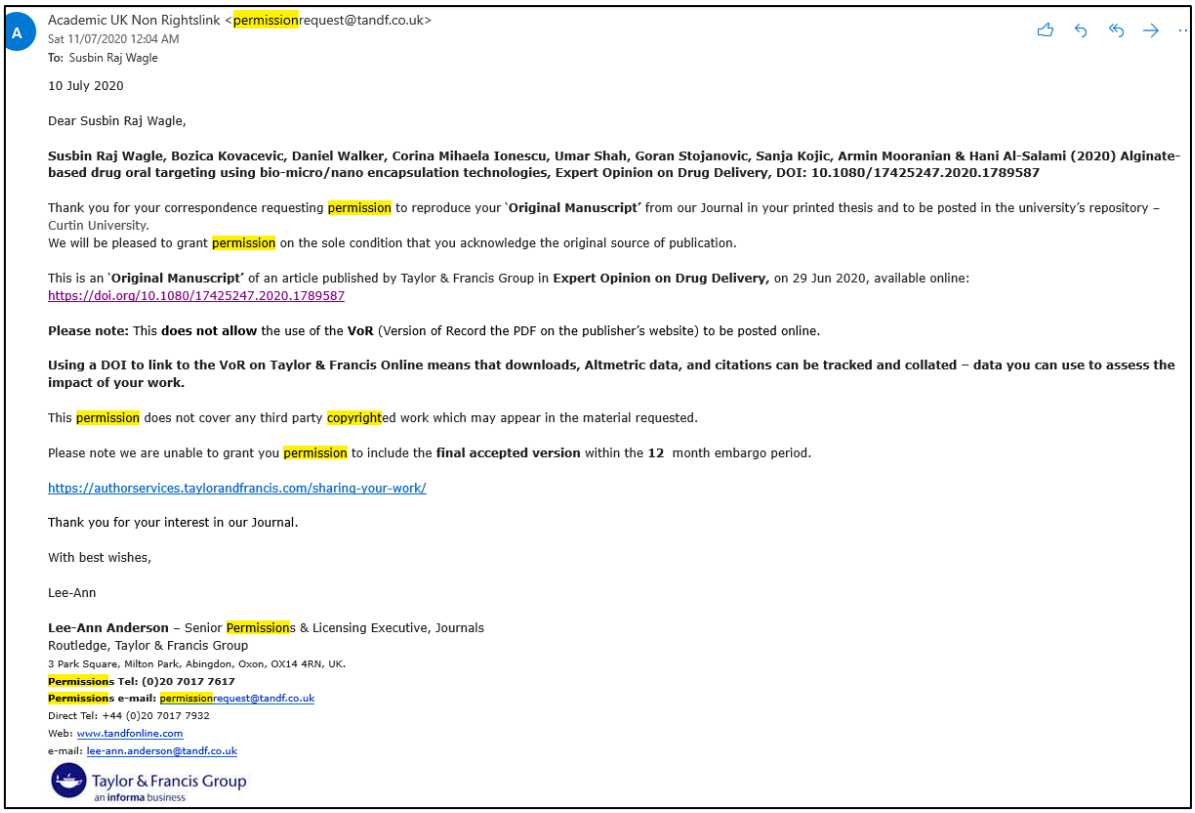

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