FORMULATION, CHARACTERIZATION AND *IN VITRO* BIOACCESSIBILITY EVALUATION OF MIXED SOY LECITHIN-BASED SQUALENE LIPOSOME-ENCAPSULATED QUERCETIN WITH ADDED PHYTOSTEROLS

By

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Liposomes, resembling biomembranes, are effective delivery systems for lipid bioactive compounds but face instability from various stressors. Understanding the impact of incorporated materials on membrane integrity is crucial. Squalene (SQ) conjugation to phospholipids enhances lipophilicity, while cholesterol (CH), β-sitosterol (βS), and stigmasterol (ST) modify membrane properties. However, quercetin's (QU) insolubility hampers absorption, necessitating novel nanovesicles for solubilization.

In the first part of the study, the effect of membrane composition and concentrations on the degradation of mixed soy lecithin (ML)-based liposomes was investigated. Liposomes were prepared using SQ, CH, and Tween 80 (TW80) to increase bilayer deformability. Liposome batches were prepared with and without SQ, and their zeta potential, particle size, and antioxidant activity against UV-induced ROS generation were evaluated. Results showed SQ association reduced size without extra energy input and improved stability and antioxidant activity. Additionally, different lipid compositions significantly influenced physical and chemical characteristics.

The second part focused on enhancing the photostability, integrity, and antioxidant capacity of ML-based liposomes by adjusting the formulation within the membrane. QU encapsulation in ML-based liposomes and the concentration-dependent solubility of QU were investigated. A combination of βS and ST was used to modulate ML bilayers, maintaining liposome function. Encapsulation efficiency measurements revealed effective QU encapsulation using specific concentrations of βS and ST. The

presence and type of phytosterols affected membrane integration characteristics and photostability.

In the subsequent part, the effects of environmental conditions on liposome stability and antioxidant capacity were evaluated. Liposomes stored at 4°C for 8 weeks showed decreased stability over time, but remained stable for the first 6 weeks. Differential scanning calorimetry results showed desirable interaction with liposome membrane models. X-ray diffraction patterns revealed variations in lattice distortion and structural disorder upon changing membrane composition.

Finally, the in vitro bio-accessibility of QU-loaded ML-based liposomes was examined. Different membrane stabilizers influenced QU bio-accessibility, correlating with entrapment efficacy. The phase transition temperature in liposomes changed with the addition of stabilizers, indicating enhanced membrane stability. This highlights the potential of well-designed ML-based liposomes to increase the stability and bio-accessibility of lipophilic bioactives like QU.

In conclusion, the presence of different lipid compositions significantly influenced the physicochemical characteristics of the liposomes, including entrapment efficacy and photodamage transformation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERUMUSAN, PENCIRIAN, DAN KESTABILAN LIPOSOM SQUALENE BERASASKAN LESITIN SOYA CAMPURAN BERKAPSUL KUERSETIN, DENGAN TAMBAHAN FITOSTEROL DAN BIO-KEBOLEHCAPAIANNYA MELALUI MODEL PENCERNAAN SECARA *IN VITRO*

Oleh

SAHAR PAKBATEN TOOPKANLOO

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Liposom, yang menyerupai biomembran, adalah sistem penghantaran yang berkesan bagi sebatian bioaktif lipid tetapi menghadapi ketidakstabilan daripada pelbagai tekanan. Memahami impak bahan yang dimasukkan ke atas integriti membran adalah penting. Konjugasi Squalene (SQ) kepada fosfolipid meningkatkan lipofilisiti, manakala kolesterol (CH), β-sitosterol (βS), dan stigmasterol (ST) memodifikasi sifat membran. Walau bagaimanapun, keengganan quercetin (QU) menghalang penyerapan, memerlukan nanovesikel baru untuk penyelesaian.

Dalam bahagian pertama kajian ini, kesan komposisi membran dan kepekatan terhadap penurunan liposom berasaskan lecithin soya campuran (ML) telah disiasat. Liposom disediakan menggunakan SQ, CH, dan Tween 80 (TW80) untuk meningkatkan kebolehlarasan dwilapisan. Set liposom disediakan dengan dan tanpa SQ, dan potensi zeta, saiz zarah, dan aktiviti antioksidan terhadap generasi ROS oleh UV dinilai. Keputusan menunjukkan pengurangan saiz dengan penggunaan SQ tanpa input tenaga tambahan dan peningkatan kestabilan dan aktiviti antioksidan. Selain itu, komposisi lipid yang berbeza secara signifikan mempengaruhi ciri-ciri fizikal dan kimia.

Bahagian kedua menumpukan kepada meningkatkan fotostabiliti, integriti, dan kapasiti antioksidan liposom ML dengan menyesuaikan formulasi dalam membran. Pengapalan QU dalam liposom ML dan kelarutan bergantung kepada kepekatan QU telah disiasat. Gabungan βS dan ST digunakan untuk mengubah suai dwilapisan ML,

mengekalkan fungsi liposom. Pengukuran kecekapan penjaringan mendedahkan pengepungan QU yang berkesan menggunakan kepekatan βS dan ST tertentu. Kehadiran dan jenis fitosterol mempengaruhi ciri-ciri integrasi membran dan fotostabiliti.

Dalam bahagian seterusnya, kesan keadaan alam sekitar terhadap kestabilan dan kapasiti antioksidan liposom dievaluasi. Liposom yang disimpan pada 4°C selama 8 minggu menunjukkan penurunan kestabilan dari semasa ke semasa, tetapi kekal stabil untuk 6 minggu pertama. Keputusan kalis pemindahan calorimetry menunjukkan interaksi yang diingini dengan model membran liposom. Corak kitaran sinar-X mendedahkan variasi dalam penyimpangan kisi dan gangguan struktur dengan perubahan dalam komposisi membran.

Akhirnya, bio-kebolehaksesan in vitro liposom berasaskan ML yang dimuatkan QU telah dikaji. Pengstabil membran yang berbeza mempengaruhi bio-kebolehaksesan QU, berkorelasi dengan kecekapan penjaringan. Suhu peralihan fasa dalam liposom berubah dengan penambahan pengstabil, menunjukkan peningkatan kestabilan membran. Ini menonjolkan potensi liposom berasaskan ML yang direka dengan baik untuk meningkatkan kestabilan dan bio-kebolehaksesan bioaktif lipofilik seperti QU.

Secara kesimpulannya, kehadiran komposisi lipid yang berbeza secara signifikan mempengaruhi ciri-ciri fisiko-kimia liposom, termasuk kecekapan penjaringan dan transformasi fotodamage.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AA	Antioxidant activity
ADI	Acceptable daily intake
ANOVA	Analysis of variance
βS	β-sitosterol
CH	Cholesterol
DLS	Dynamic light scattering
DPPH	1-diphenyl-2-picrylhydrazyl
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
FDA	Food and Drug Administration
FTIR	Fourier-transform infrared spectroscopy
GRAS	Generally recognized as safe
h	Hour
HLB	Hydrophilic-Lipophilic Balance
LUV	Large Unilamellar Vesicles
ML	Mixed lecithin
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PUFA	Polyunsaturated fatty acids
QU	Quercetin
R^2	Coefficient of determination
RSM	Response surface methodology
SQ	Squalene

ST	Stigmasterol
TEAC	Trolox equivalent antioxidant capacity
TEM	Transmission electron microscopy
T_m	Transition temperature
TW80	Tween 80 (Polyoxyethylene sorbitan mono-oleate)
UK	United Kingdom
USA	United States of America
UV	Ultra Violet
UV-VIS	Ultra Violet Visible
W/w	Weight per Weight
XRD	X-ray diffraction
ZP	Zeta potential

CHAPTER 1

INTRODUCTION

Nutraceuticals or bioactive molecules found in foods are biologically active molecules that are not only essential for maintaining normal human functions, but may enhance human health and wellbeing by inhibiting certain diseases or improving human performance (Gupta, 2016; Wildman & Kelley, 2007). They are a natural way to achieve therapeutic outcome with minimal or no side effects. Numerous different classes of nutraceuticals are found in both natural and processed foods including carotenoids, flavonoids, curcuminoids, phytosterols, and certain fatty acids (Gupta, 2016). Many of these nutraceuticals have the potential to act as anticancer agents, and may therefore be suitable for incorporation into functional or medical foods as means of preventing or treating certain types of cancer. The potential health benefits of nutraceuticals are often not realized because of their poor water solubility, chemical instability, adverse taste profile, and low oral bioavailability. Besides, they are subject to degradation resulting from exposure to environmental factors such as humidity, oxygen, heat, light and extreme pH values.

Nanoliposomes, or nanometric bilayer phospholipid vesicles, are considered as a promising encapsulation system for the nutraceutical industry. Nanoliposomes are colloidal structures formed via the input of energy using a right combination of phospholipids and other ingredients in an aqueous solution (Danaei et al., 2018). Protection of sensitive bioactive molecules, prolonging storage stability, high loading capacity, enhanced bioavailability, and sustained-release mechanism are among the advantages offered by nanoliposomes. Nanoliposomes can also be employed to encapsulate and transport more than one bioactive agent, hence providing a synergistic effect (Gowda et al., 2017). However, pre-formulation studies aimed at the selection of the most suitable type of encapsulating materials and the most effective complex preparation method is important in order to gain the maximum benefit of this encapsulation system. Therefore, for an efficient application of nanoliposomal carriers as food/nutraceutical delivery systems, it is necessary to obtain deeper insights into the solubilization site, stability and impact of incorporated materials on the integrity of bilayer membranes. Such properties of liposomes depend on numerous factors such as size and chemical composition of the vesicles, which can be easily manipulated by using different preparation techniques and modulating the composition of the lipid bilayer. Thus, an appropriate selection of lipid vehicles along with formulation protocol and composition variables can lead to a successful design of the liposomal systems. As is the case with liposomes, the main ingredients of nanoliposomes are phospholipid molecules. However, they may also contain other molecules such as other types of functional lipids, sterols and surfactants in their structure.

The phospholipid composition is of great importance to liposome formation, as it dictates the morphology and size of the resulting liposomes. Chain length and degree of saturation determine the phase transition temperature of phospholipids (the temperature at which there is a change from an ordered gel phase to a disordered liquid

crystalline phase), which in turn closely affects mobility and fluidity as well as packing style (Bowman et al, 2013). Soybean lecithin, also known as mixed lecithin, has not been widely investigated as a main component of liposomes. The use of mixed soy lecithin (ML) as non-synthetic mixed phospholipids for producing liposomes does not raise any food legislation concerns and provides nutritional value owing to the high polyunsaturated fatty acids (PUFA) composition (Laye, McClements &Weiss, 2008). However, liposomes from ML were found to be large in size, not stable, and have low entrapment efficiency. Also, it is easily oxidized and sensitive to heat and light, which restricts its application in industries.

Squalene (SQ), a long linear hydrophobic lipid, can be exploited because it may be able to bind to acyl chains of phospholipids, which represent a lipid complex having stability properties. According to Richens et al. (2015), oily nature substances such as SQ can modify the membrane dipole potential. Besides, SQ possesses antilipidemic, anticancer and antioxidant properties and is well tolerated intravenously or orally (Reddy & Couvreur, 2009) and has wide applications in the food and biomedical industries. However, there is a lack of exploration on SQ incorporation in liposomal formulations. Here, SQ is integrated with ML to form mixed liposomes to engineer the properties of the membrane of the liposomes by rationing the SQ with ML. Having said that, SQ is an entirely non-polar, low viscous lipid, which may affect the fluidity of the phospholipid membranes. Therefore, one possible mechanism to improve the stability and maintain the integrity of liposome membranes includes the incorporation of sterols (e.g. cholesterol (CH), and phytosterols) in the structure of the vesicles. Sterols are important components of most natural membranes, and the incorporation of sterols into liposome bilayers can result in major changes in the properties of these vesicles. Sterols are used in liposome structure in order to increase the stability of the bilayer vesicles by modulating the fluidity of the phospholipid bilayer. This contributes to the reduction in the permeability of the phospholipid membrane to solutes (Reineccius, 1995). Likewise, addition of non-ionic surfactants such as Tween 80 (TW80) to liposome compositions has been found to affect the hydrophilicity and fluidity of the liposome membranes (Tai et al., 2017). These types of surfactants can also help in the wetting and dispersion of the very hydrophobic molecules (like SQ) and enhance their solubility in the dispersion (Kesisoglou, Panmai & Wu, 2007).

Quercetin (QU) is introduced as a specific nutraceutical for chemopreventative agent due to its great antioxidant/anti-inflammatory property. Many studies suggest that QU exhibits anticancer activity against various forms of cancer (Boots, Haenen & Bast, 2008; Azuma, Ippoushi & Terao, 2010). Many *in vitro* and *in vivo* studies have shown the anticancer potential of QU against a variety of human cancers, such as cervical, breast, colon, prostate, liver and lung cancers (Wang et al., 2016). QU normally exhibits a poor oral bioavailability due to its low absorption. A nanoencapsulation approach has been shown to enhance the effects of QU in reducing the oxidative damage and attenuating inflammation in a mouse colitis model, presumably due to increased absorption (Guazelli et al., 2013). QU is able to interact and permeate lipid bilayer and such capacity is very important because there is a positive correlation between the ability to incorporate into membranes and antioxidant activity (Areias, Rego, Oliveira & Seabra, 2001; Oteiza, Erlejman, Verstraeten, Keen & Fraga, 2005).

Once incorporated into membranes, QU changes the biophysical parameters of the membrane such as its fluidity, cooperativity and the temperature of phase transition (Tsuchiya, 2010). Since the efficacy of QU is limited by its hydrophobicity, instability in physiological media, poor gastrointestinal absorption, and extensive xenobiotic metabolism in the intestines and liver, its encapsulation in a suitable delivery system may improve its oral bioavailability and ensure its protection from degradation, thus preventing premature release.

This study was designed to develop a stable ML-based liposomal formulation via an extrusion method for use in the food and nutraceutical industries. Furthermore, this research aimed to provide a better understanding of QU physicochemical properties, stability, and bioaccessibility in response to various factors. In this context, the objectives of the present study were as follows:

1. To assess the effect of applying ML, SQ, CH and TW80 under different combination ratios in forming stable liposomes;
2. To optimize and characterize the liposomal formulations by means of encapsulating QU, and examine the effect of three added compounds (quercetin, β-sitosterol and stigmasterol) on enhancing the membrane integrity of ML-based liposomes;
3. To investigate the effect of storage period (for 8 weeks at 4°C, and 4 weeks at 45°C) and temperature (30-110 °C) on the stability of the resulting liposomes; and
4. To determine the bioaccessibility of QU liposomes in an *in vitro* digestion model.

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