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Preparation of liposomes using supercritical carbon dioxide technology: Effects of phospholipids and sterols

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ABSTRACT

Liposomes were prepared utilizing a supercritical carbon dioxide (SC-CO_2) process. A phospholipid suspension was first equilibrated with CO_2 at 300 bar and then depressurized at a constant pressure and rate. The effects of phospholipid concentration, phospholipid type, sterol concentration and sterol type on particle size, uniformity, zeta potential and morphology were investigated. With increasing soy lecithin concentration (5–30 mM) at 50 °C, the smallest particle size of liposomes (146.1 ± 0.8 nm) was obtained at 30 mM with the polydispersity index (PdI) of 0.398 ± 0.008. Increased phospholipid concentration was favorable for the formation of smaller size vesicles with higher uniformity. Longer chain length of fatty acids in pure phospholipids resulted in a larger particle size with more spherical shape while a phospholipid with unsaturated fatty acyl chains resulted in increased asymmetry. With elevated β-sitosterol concentration (10%–50%), particle size and PdI increased to 245.5 ± 7.14 nm and 0.514 ± 0.018, respectively, with decreased absolute zeta potential. 6-Ketocholestanol showed the smallest diameter and PdI of liposomes with the most spherical shape among all sterol types tested. Soy lecithin exhibited the highest stability of vesicular systems due to its highest absolute zeta potential (−58.3 ± 2.17 mV) among all the phospholipid types. The SC-CO_2 method demonstrated superior characteristics of liposomes over traditional thin film hydration method for a smaller size and PdI as well as enhanced intactness without leakage. It might offer a promising way to reduce usage of sterol in liposome formulations while eliminating organic solvent usage.

1. Introduction

Recently, the use of micro- and nano-encapsulation for the production of functional food ingredients has gained increasing attention (Sanguansri & Augustin, 2006). Liposomes have been shown to have a number of advantages as systems to encapsulate high value components. Encapsulation may improve the *in vivo* absorption of hydrophobic components, which are insoluble in the aqueous phase (Zvonar, Berginc, Kristl, & Gašperlin, 2010). Encapsulation can result in controlled release of flavor and antimicrobial compounds to improve food quality (Augustin & Hemar, 2009). Encapsulation can also enhance the stability and bioavailability of compounds. Within liposomes, bioactives can be protected from degradation induced by unfavorable conditions like light, thermal and pH treatments applied during food processing or storage (Fahr & Liu, 2007). In addition, they can also be isolated from undesirable interactions such as pH, hydrolysis, or enzymatic reactions in the gastrointestinal tract (Augustin & Sanguansri, 2012).

Liposomes are self-assembled spherical vesicles, composed of a phospholipid bilayer enclosing an aqueous core. A hydrophobic component can be incorporated within the bilayer membrane while a hydrophilic component can be entrapped within the aqueous core. Conventional preparation methods, including thin film hydration (TFH) (Bangham, Standish, & Watkins, 1965), ethanol injection (Batzri & Korn, 1973), reverse phase evaporation (Szoka & Papahadjopoulos, 1978) and detergent dialysis methods (Petri & Wagner, 1979) can be used to produce liposomes. However, they usually lead to organic solvent residues, high energy cost, heterogeneous size distribution and low reproducibility. Thus, finding a cost-effective alternative to prepare liposomes without these drawbacks is gaining interest. Supercritical carbon dioxide (SC-CO_2) technology offers a promising approach to replace current reliance on organic solvents. SC-CO_2 technology can be employed to process naturally derived materials in a unique manner: in the fluid state at conditions above the critical point of CO_2 (31.1 °C; 7.4 MPa). SC-CO_2 has solvating power similar to liquid organic solvents but offers additional advantages including tunability of density, high diffusivity, low interfacial tension and viscosity. It is an inert, inexpensive, non-toxic and environmentally friendly solvent. SC-CO_2 technology is also suitable for large scale production of materials in the food industry due to the relative ease of scale up.

In our previous study, a single step preparation via a modified SC-CO_2 process was developed (Zhao & Temelli, 2015), which took advantage of the different aspects of previously reported approaches (Elizondo et al., 2012; Otake et al., 2006) without the addition of organic solvent

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or surfactant. The effects of pressure, depressurization rate, depressurization protocol and temperature on liposome preparation were evaluated and 300 bar and depressurization at a constant rate of 60 bar/min and pressure were selected (Zhao & Temelli, 2015). To further elucidate this method, investigation of the effects of other parameters on liposome preparation is necessary. The phospholipid composition is of great importance to liposome formation. Each phospholipid type has an individual packing parameter (the geometry of amphiphiles), which can dictate the morphology and size of the resulting liposomes. The head group manipulates the surface charge of vesicles and influences the zeta potential and in turn the stability of liposomes (Seelig, MacDonald, & Scherer, 1987). 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 closely affects mobility and fluidity as well as packing style (Bowman, Ofner, Schott, & Perrie, 2013).

Sterols are usually added during conventional liposome preparation since they fill the gaps within bilayers and reduce leakage created by imperfect packing. The addition of sterol to liposomes can modulate membrane fluidity and promote stability of phospholipid bilayers (Chen, Han, Cai, & Tang, 2010). Even though cholesterol has been commonly used in traditional liposome formulations, the literature lacks information on the performance of phytosterols in liposomes. Phytosterols are plant-derived steroids that are similar in structure and function to cholesterol. It has been demonstrated that phytosterols can lower plasma cholesterol and triacylglyceride levels and hence reduce the risk of a number of diseases (Quilez, Garcia-Lorda, & Salas-Salvado, 2003). Thus, it is desirable to use phytosterols to replace cholesterol and to assess their function in liposome formation. Therefore, the objective of this study was to evaluate the effect of the compositional parameters (phospholipid concentration, phospholipid type, sterol concentration and sterol type) on the liposomal characteristics (particle size, uniformity, zeta potential and morphology). The properties of liposomes prepared using SC-CO_2 method were compared to those obtained by the traditional thin film hydration method.

2. Materials and methods

2.1. Materials

Soy lecithin purchased from Fisher Scientific (Ottawa, ON, Canada) was used for liposome preparation. Cholesterol (99%), β-sitosterol (97%), stigmasterol (95%) and 6-ketocholestanol (95%) were obtained from Sigma-Aldrich (Oakville, ON, Canada). 1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-3-phosphatidylcholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) with purity > 99% were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Liquid CO_2 (purity of 99.99%) supplied by Praxair Canada (Mississauga, ON, Canada) was used in all high pressure CO_2 processing. Water purified by Milli-Q® ultrapure water purification system (EMD Millipore, Billerica, MA, USA) was used in all experiments.

2.2. Preparation of phospholipid suspension

Phospholipid suspension was prepared weekly for liposome preparation. Phospholipid (1.33 g) was dispersed in 100 mL Milli-Q water at 65 °C with continuous agitation for 30 min. The final concentration of phospholipid was controlled at 20 mM. The crude suspension was sealed under nitrogen in light proof bottle, stored at 4 °C and used within one week.

2.3. Experimental apparatus

A schematic diagram of the apparatus (Phase equilibria apparatus, SITEC-Sieber Engineering AG, Maur/Zurich, Switzerland) used for liposome formation was provided in a separate study (Zhao & Temelli, 2015). The apparatus mainly consisted of a 10 mL high pressure view cell hooked up to an ISCO 250D syringe pump (Teledyne Isco, Lincoln, NE, USA). A heating jacket filled with circulating water connected to a water bath (Model 260, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to regulate the cell temperature. A micrometering valve installed at the lower level exit of the cell was used to depressurize and collect samples.

2.4. Preparation of liposomes using supercritical CO_2 process

The liposomes were produced in a batch mode. Phospholipid suspension (6 mL) was sealed inside the high pressure vessel and the vessel was gently flushed with CO_2 to remove the air trapped inside. The vessel was equilibrated until set temperature was reached and then pressurized with CO_2 up to the required pressure. A magnetic stirrer at the bottom of the cell was used for thorough mixing of the cell contents for 1 h to reach equilibrium. CO_2-expanded liquid phase was then depressurized through a micrometering valve. To achieve a specific depressurization rate, preliminary tests were performed and the specific position on the scale of the micrometering valve corresponding to a certain depressurization rate was recorded. This valve position was then applied during liposome preparation to achieve the desired depressurization rate. Liposomes were formed through depressurization of the CO_2-expanded liquid phospholipid suspension phase at constant depressurization rate and pressure. CO_2 was introduced into the cell from the top to maintain constant pressure while the liquid sample was being depressurized through the micrometering valve at a fixed rate. The liposome suspension was collected in a vial for further analysis. All of the experimental conditions employed were summarized in Table 1. The effect of phospholipid type was studied at 65 °C (the temperature above the phase transition temperatures of all the phospholipid types investigated) to guarantee liposome formation.

2.5. Preparation of liposomes using thin film hydration (TFH)

Liposomes were also prepared by the TFH method (Bangham et al., 1965). Soy lecithin (54.23 mg) was solubilized in chloroform (3 mL) with continuous agitation for 3 min using a magnetic stirrer. Then, chloroform was gradually removed under a gentle stream of nitrogen to form a homogeneous thin film of phospholipid at the round bottom of the flask. Milli-Q water (4 mL) was added and held at the same temperature as SC-CO_2 preparation in a water bath for 10 min for hydration of the thin film, followed by 1 min vortex agitation to thoroughly disperse the phospholipid film and form heterogeneous multilamellar vesicles. Vesicle suspension was then sonicated for 5 min using an ultrasonic sonication bath (Model FS30H, Fisher Scientific, Ottawa, ON, Canada) to form liposomes. The final concentration of soy lecithin in water suspension was controlled at 20 mM.

Table 1
Processing conditions of liposome preparation using supercritical CO_2.

Factors	Levels	Processing parameters
Phospholipid concentration (mM)	5, 10, 15, 20, 25, 30	300 bar, 60 bar/min at 40, 45, 50 °C
Phospholipid type	DOPC, DMPC, DPPC, DSPC, soy lecithin	65 °C, 300 bar and 60 bar/min
Molar concentration of sterol (%)	0, 10, 20, 30, 40, 50	50 °C, 300 bar and 60 bar/min
Sterol type	Cholesterol, β-sitosterol, stigmasterol, 6-ketocholestanol	50 °C, 300 bar and 60 bar/min