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Preparation of anthocyanin-loaded liposomes using an improved supercritical carbon dioxide method

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ABSTRACT

Anthocyanin-loaded liposomes were prepared via a single step supercritical carbon dioxide (SC-CO_2) process. Phospholipid/anthocyanin suspension equilibrated with CO_2 was depressurized at a constant pressure and rate. The effects of pressure, depressurization rate and temperature on the characteristics of liposomes were investigated. Liposomes obtained had a mean diameter of 160 ± 2 nm, polydispersity index of 0.26 ± 0.01, encapsulation efficiency of 52.2 ± 2.1% and zeta potential of −44.3 ± 2.9 mV. Elevated pressure and depressurization rate generated smaller particles with higher uniformity while high temperature led to reduced sphericity. The SC-CO_2 method produced liposomes with enhanced intactness, sphericity and uniformity compared to the thin film hydration method. This method offers the possibility to utilize dense phase CO_2 to process the phospholipid aggregates into nano/micro particles and control their characteristics via tuning of processing parameters. SC-CO_2 method shows promise in scalable production of liposomes loaded with a variety of bioactives, targeting food applications.

Industrial relevance: High quality anthocyanin-loaded liposomes were prepared using SC-CO_2. In this process, heterogenous phospholipid aggregates suspended in an aqueous medium are transformed into unilamellar and spherical liposomes with a narrow size distribution and other characteristics that can be regulated via tuning of the processing parameters (pressure, depressurization rate and temperature). The SC-CO_2 method resulted in superior particle characteristics over those prepared via the traditional thin film hydration method and also overcame some drawbacks like organic solvent residue and vesicle leakage associated with the traditional method. Anthocyanin encapsulated into liposomes can be protected from adverse external conditions with potential benefits in food and nutraceutical formulations for improved efficacy and health benefits. This improved SC–CO_2 process shows great promise for potential scale up of liposome production in the industry to encapsulate a variety of bioactives, targeting food and nutraceutical applications.

1. Introduction

Liposomes are phospholipid assemblies, which are being used as an encapsulation vehicle to deliver hydrophilic and hydrophobic ingredients (Cabrera et al., 2013). Liposomes offer unique features in terms of biocompatibility, amphiphilicity, non-toxicity and non-immunogenity (Bozzuto & Molinari, 2015) and are suitable for a number of versatile applications with respect to cell membrane models, reaction vessels, encapsulation media and delivery systems (Elizondo et al., 2012). With regard to encapsulation, drugs, nutraceuticals and bioactives can be protected from adverse external conditions and chemical reactions to improve their bioavailability and efficacy. In addition, the surface of liposomes can be modified with functional groups such as peptides or antibodies for targeted delivery of agents to specific sites (Cabrera et al., 2013). Liposomes are broadly applied in medical, genetic and cosmetic formulations and recently, have also drawn increasing attention in food and nutritional applications (Marsanasco, Márquez, Wagner, del V. Alonso, & Chiaramoni, 2011).

With all of the above advantages, development of an efficient, reproducible, environmentally friendly and readily scalable process to yield liposomes with desirable characteristics like small particle size, high homogeneity and high encapsulation efficiency is of great industrial interest (Cano-Sarabia et al., 2008). However, conventional methods like thin film hydration (TFH) rely on the use of organic solvents and suffer from limitations such as organic solvent residues or undesirable liposome characteristics in terms of their uniformity and encapsulation efficiency (Zhao & Temelli, 2015a). To improve liposomal characteristics, novel methods such as dual asymmetric centrifugation (Hirsch, Ziroli, Helm, & Massing, 2009), membrane contactor technology (Laouini, Jaafar-Maalej, Sfar, Charcosset, & Fessi, 2011), cross-flow filtration (Peschka, Purmann, & Schubert, 1998), freeze-drying double emulsion

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(Wang et al., 2006) and microfluidic mixing (Jahn, Vreeland, DeVoe, Locascio, & Gaitan, 2007) have been developed with significant progress; however, several drawbacks still remain. To overcome these drawbacks, supercritical carbon dioxide (SC-CO_2) technology offers a promising alternative to process amphiphilic aggregates into nanoforms with unique features (Zhao, Temelli, Curtis, & Chen, 2015). SC-CO_2 is a dense fluid at processing conditions above the critical point of CO2 (31.1 °C, 74 bar), which offers solvating power similar to liquid organic solvents but is inert, non-toxic and environmentally friendly. In addition, its tunability of density, high diffusivity, low interfacial tension and viscosity provides advantages, which cannot be easily achieved in traditional solvent-based methods. SC-CO_2 technology can also be scaled up for industrial production.

Several different SC-CO_2-based processes have been developed to produce liposomes with a focus on utilizing the high pressure CO_2 to achieve homogenization and assembly of phospholipids into micro-/nano-sized liposomes (Campardelli et al., 2016; Elizondo et al., 2012; Lesoin, Crampon, Boutin, & Badens, 2011; Otake et al., 2006). Among these methods, Cano-Sarabia et al. (2008) used the DELOS (depressurization of an expanded liquid organic solution) method and yielded unilamellar liposomes with a small particle size (167 nm) and high uniformity. However, this process involved the use of a surfactant and an organic solvent. Otake et al. (2006) utilized the ISCRPE (improved supercritical reverse phase evaporation) method and yielded liposomes with a large particle size (around 1.5 μm) but free of organic solvent/surfactant. To enhance the characteristics and safety level of liposomes, an improved SC-CO_2 process, combining the advantages of these two supercritical methods (Cano-Sarabia et al., 2008; Otake et al., 2006), was developed in our previous study (Zhao & Temelli, 2015a). The performance of unloaded liposomes was firstly studied and processing factors, including pressure, depressurization rate, depressurization protocol (Zhao & Temelli, 2015a), temperature and liposomal composition (Zhao et al., 2015) were assessed and optimized. Liposomes generated by this improved method displayed superior characteristics over those prepared by the traditional TFH method, including being free of organic solvent, small particle size, high homogeneity, enhanced vesicular intactness and storage stability (Zhao & Temelli, 2015a; Zhao et al., 2015). To further assess the effectiveness of this SC-CO_2 method, the next step is to encapsulate a hydrophilic compound into liposomes and compare their characteristics with those obtained by the traditional TFH method. Anthocyanin was selected as the model hydrophilic component for encapsulation into liposomes using this method.

Anthocyanin is a powerful flavonoid antioxidant, which gives the colors of red, blue and purple in different varieties of flowers, fruits and vegetables. Anthocyanin has received growing attention as a natural colorant in food, nutraceutical and cosmetic formulations not only due to its extensive range of colors but also its health benefits. Anthocyanin has been shown to offer health benefits, including reducing the risk of cardiovascular disease, control of obesity and diabetes as well as improvement of visual and brain functions (Tsuda, 2012). However, its high reactivity renders it susceptible to degradation due to factors such as light, heat, oxygen and relatively basic pH in the gastrointestinal tract. Thus, encapsulation of anthocyanin in liposomes is of great importance to protect anthocyanin from these adverse conditions and maintain its stability and efficacy. A small amount of cholesterol is incorporated into liposome membranes to improve the packing of phospholipids, reduce the membrane permeability and prevent the leakage of anthocyanins from the vesicles (Bozzuto & Molinari, 2015). The objective of this study was to evaluate the effects of pressure, depressurization rate, and temperature on the characteristics of liposomes, including particle size distribution, morphology, encapsulation efficiency, bioactive loading and stability. In addition, anthocyanin-loaded liposomes generated by the SC-CO_2 method were compared to those prepared by the traditional TFH method.

2. Materials and methods

2.1. Materials

Soy lecithin obtained from Fisher Scientific (Ottawa, ON, Canada) was used for liposome preparation. Anthocyanin (76%) from bilberry (*Vaccinium myrtillus*) was purchased from Hangzhou Ningsi Bio-tech (Hangzhou, China). Sodium citrate dehydrate (99%), citric acid monohydrate (99%), cholesterol (99%), ethanol (99.8%) and methanol (99.9%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 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 crude suspension

Crude lecithin/anthocyanin/cholesterol suspension was prepared fresh for liposome preparation. Anthocyanin from bilberry (*Vaccinium myrtillus*) (MW: 466 g/mol) at 10% (mole %) was dissolved in 100 mL sodium citrate buffer (pH 3.5) in a dark environment. Then, soy lecithin (1.33 g, MW: 677.92 g/mol) was dispersed into the anthocyanin solution with continuous agitation for 30 min using a magnetic stirrer at 1200 rpm. The final concentration of phospholipids was controlled at 20 mM. Cholesterol at 20% (mole % of the total moles of lecithin/anthocyanin/cholesterol) was slowly added and thoroughly mixed with the phospholipid/anthocyanin suspension at 1200 rpm in the dark for 1 h. The crude lecithin/anthocyanin/cholesterol suspension was stored under nitrogen and dark at 4 °C and used within one week.

2.3. Preparation of liposomes using the SC-CO_2 method

The experimental setup used for the liposome preparation was described previously (Zhao & Temelli, 2015a). Crude soy lecithin/anthocyanin/cholesterol suspension (6 mL) was sealed in a 10 mL high pressure vessel. CO_2 was used to gently flush the vessel to remove the air trapped inside. The cell was equilibrated until set temperature was reached and pressurized with CO_2 up to the required pressure. A magnetic stirrer at the bottom of the vessel was used for thorough mixing of the cell contents at 550 rpm for 1 h. CO_2-expanded soy lecithin/anthocyanin/cholesterol liquid mixture was depressurized at a constant pressure and rate. To maintain constant pressure, CO_2 was introduced into the cell from the top while the liquid phase was being depressurized from the bottom of the cell. To achieve a constant depressurization rate, preliminary trials were performed where each specific position on the scale of the micrometering valve corresponding to a certain depressurization rate was tested and confirmed in quadruplicates. The valve position was then applied in liposome preparation to achieve the desired depressurization rate. Liposomes were formed as CO_2-expanded suspension was depressurized. Different levels of pressure, depressurization rate and temperature were evaluated as summarized in Table 1.

2.4. Particle size distribution of liposomes

The particle size and size distribution of anthocyanin-loaded liposomes were determined using the Zetasizer Nano ZS instrument

Table 1
Processing conditions for liposome preparation using the SC-CO_2 method.

Factors	Levels	Processing parameters
Pressure (bar)	60, 80, 100, 150, 200, 250, 300	50 °C, 90 bar/min, 10% anthocyanin and 20% cholesterol
Depressurization rate (bar/min)	10, 20, 40, 60, 90, 120, 150, 200	50 °C, 300 bar, 10% anthocyanin and 20% cholesterol
Temperature (°C)	40, 45, 50, 55, 60, 65	300 bar, 90 bar/min, 10% anthocyanin and 20% cholesterol