Preparation and Characterization of Liposomal Nanovesicles Using Supercritical Carbon Dioxide Expansion-Depressurization of Phospholipid Suspension (SEDPS)

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ABSTRACT

Liposomal nanovesicles (LN) were produced utilizing the supercritical carbon dioxide expansion-depressurization of phospholipid suspension (SEDPS) process via two depressurization protocols. The effects of pressure (60–300 bar), depressurization rate (10–120 bar/min) and depressurization protocol on the vesicle size, polydispersity index (PdI), morphology and storage stability of LN were investigated. The volumetric expansion of water in equilibrium with high pressure CO_2 (1–300 bar) at temperatures of 40, 50, 60 ℃ was also evaluated. Particle size of LN decreased at elevated pressures for depressurization protocols (I) and (II). Transmission electron microscopy images displayed unilamellar spherical or near-spherical nanovesicles and higher pressure could be favorable for the formation of more symmetrical and spherical particle. LN remained stable for up to 8 and 6 weeks ($\leq$5% increase in size) for depressurization protocols (I) and (II), respectively. The relative volumetric expansion of water in equilibrium with high pressure CO_2 was up to 14.7%, 12.3% and 10.2% at 40 ℃, 50 ℃ and 60 ℃, respectively.

INTRODUCTION

Liposomal nanovesicles (LN) are unilamellar structures composed of amphipathic phospholipids surrounding aqueous compartments. Due to the amphipathicity of its structure, LN allow encapsulation of hydrophilic and hydrophobic compounds. Hydrophilic components are entrapped within the aqueous core whereas lipophilic materials are incorporated into the phospholipid bilayer [1]. Through encapsulation, drugs, nutraceuticals and bioactives can be protected from unfavorable conditions such as pH, hydrolysis or enzymatic attack in the gastrointestinal tract to improve their stability and bioavailability [2]. Plus, the dispersibility of poorly water soluble components is enhanced. LN show great potential in pharmaceutical industry and have drawn much attention for food and nutritional applications [3].

Although considered promising for a number of applications, LN confronts various challenges in their preparation. Conventional preparation methods of LN, including thin film hydration (TFH), ethanol injection and reverse phase evaporation usually lead to organic solvent residues, high energy cost, heterogeneous size distribution and low reproducibility. Present supercritical carbon dioxide preparation methods for LN preparation include the rapid expansion of supercritical solution into a liquid solvent (RESOLV), supercritical reverse phase evaporation (SCRPE) and depressurization of expanded liquid organic solution (DELOS). Some of these techniques involve poor solubility of phospholipids in CO_2, high CO_2 consumption, relatively large size of LN and the use of surfactant and organic solvent. Thus, an alternative method, which can yield high quality LN and overcome these drawbacks is needed. In this study, a single step process via SC-CO_2 expansion-depressurization of phospholipid suspension (SEDPS), which is free of surfactants other than phospholipids and

organic solvents was developed taking advantage of some of the features of previously reported methods [4,5]. The suspension of phospholipids in water was expanded and equilibrated with high pressure CO_2 and depressurized from liquid phase at a controlled depressurization rate. The objectives of this study were to investigate the preparation of LN using the SEDPS process and to evaluate the effects of pressure, depressurization rate and depressurization protocol on the characteristic parameters including vesicle size, polydispersity index (PdI), morphology and storage stability of liposomes.

MATERIALS AND METHODS

A schematic flow diagram of the system used for volumetric expansion and LN preparation was shown in Figure 1. A supercritical system with a 10 mL view cell equipped with two sapphire windows was used. A heating jacket connected to a circulating water bath was used to regulate the temperature and an ISCO syringe pump was used to pump CO_2 and regulate the pressure. An on/off valve and a micrometering valve installed at the upper and lower levels of the cell, respectively, were used to depressurize and collect samples. A microscope with a video camera is used to observe and record all the visual changes inside the cell from the window. For the determination of volumetric expansion, a stainless steel frame equipped with a microscopic glass scale was placed in the cell. A calibration curve of reading on the glass scale versus liquid volume was established by quantifying the level of different volumes of water placed into the cell. Pure water or phospholipid suspension was pressurized with CO_2 and mixed with a magnetic stirrer for 2 h to reach equilibrium. The readings of expanded liquid volume were recorded and microscopic images were captured by the video camera. For the LN preparation, soy lecithin dispersed in water (20 mM) was sealed in the high pressure vessel and pressurized with CO_2 until desired pressure was reached. After mixing for 1 h, CO_2 expanded suspension was depressurized at a fixed depressurization rate. Phospholipid suspension was depressurized from liquid phase: (I) at a constant depressurization rate and (II) at constant pressure by adding more CO_2 into the vessel and depressurization rate. LN were formed through depressurization. The size distribution was measured by dynamic light scattering while the morphology was evaluated by transmission electron microscopy [4]. The storage stability of LN was assessed by storing liposome suspensions under nitrogen in the dark at ambient conditions (25 ℃) for up to 10 weeks and evaluating particle size distribution weekly. Liposome images were taken by a digital camera and the size distribution was measured as above. All the experiments were run in three replicates for the statistical analysis.

RESULTS

The relative volumetric expansions (ratio of volume difference after expansion to the original volume of liquid) of water and phospholipid suspensions, were determined. Figure 2 showed that volume of water increased with pressure and then remained constant where relative volume expansion was up to 14.7%, 12.3% and 10.2% at 40 ℃, 50 ℃ and 60 ℃, respectively. The volume elevation was attributed to the solubilization of CO_2 in water under high pressure and higher pressure favored elevated solubility of CO_2 in water [6]. At higher temperature, the relative volumetric expansion was reduced since solubility of CO_2 in water decreased with an increase in temperature. The volumetric expansion of phospholipid suspension in equilibrium with CO_2 was also evaluated. However, it was difficult to acquire accurate readings from the glass scale in images captured by the video camera, since the phospholipid suspension scattered light and allowed limited amount of light to pass through the microscope.

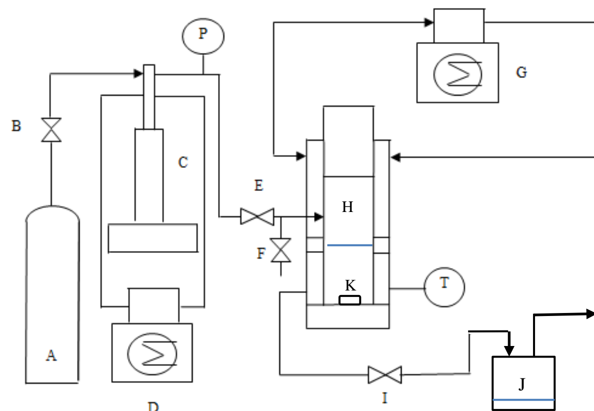

Figure 1: Schematic flow diagram of SEDPS system. (A) CO_2 tank; (B) (E) (F) on/off valves; (C) ISCO syringe pump; (D) refrigeration bath; (G) water bath; (H) high pressure view cell; (I) micrometering valve; (J) sample vial; (K) magnetic stirrer; (P) pressure gauge; (T) thermocouple.

The preparation of LN utilizing SEDPS was investigated. The effect of pressure on two size distribution parameters, particle size and PdI, was presented in Figures 3 and 4 for depressurization protocols (I) and (II), respectively. With an increase in pressure, particle size was reduced for both depressurization protocols. For protocol (I), with an increase in pressure from 60 to 300 bar, liposome size was reduced from 326.2±3.8 nm to 264.7±3.5 nm. For protocol (II), liposome size was reduced from 306.6±9 nm to 213.7±3 nm. The effect of depressurization rate on the vesicle size was presented in Figure 5. For protocol (I), LN size was reduced from 343.1±6.53 nm to 287.6±2.11 nm with an increase in depressurization rate from 10 to 40 bar/min and then remained constant. For protocol (II), as the depressurization rate increased, particle size of LN remained constant without a significant change. The liposome formation may be attributed to the "dispersing effect" of CO_2 release upon depressurization. At ambient conditions, phospholipid molecules were present primarily in the form of bilayer or curvature when dispersed in water [7]. After pressurization and reaching equilibrium with CO_2, carbon dioxide molecules were solubilized in water and partially existed in between the hydrophobic fatty acyl chains of phospholipids through dipole-induced dipole and Van der Waals interactions. Upon depressurization, CO_2 molecules were rapidly released from between the fatty acyl chains, breaking up the curvatures of phospholipids into scattered phospholipid molecules, which would form an instantaneous water dispersion of discrete phospholipids. Due to the hydrophobic interactions, separated phospholipid molecules would aggregate into liposomes spontaneously. Increased pressure may be favorable for better dispersion of phospholipids and less phospholipids per unit volume of aggregates may result in a smaller size of LN. In contrast, depressurization rate did not affect the size of LN in a significant manner. This indicated that the formation of LN may primarily depend on the amount of CO_2 solubilized in the aqueous phase and interacting with the fatty acyl chains of phospholipids instead of the release rate. The PdI of LN was also evaluated. For protocol (I), with an increase in pressure the PdI ranged from 0.455 to 0.520, whereas for protocol (II), PdI varied from 0.417 to 0.484. The PdI data indicated that the SEDPS method and the conditions employed yielded heterogeneous size distribution of liposomes and the uniformity of liposomes should be further improved. Since the pressure in the vessel was maintained as constant with the addition of more CO_2 during the depressurization of protocol

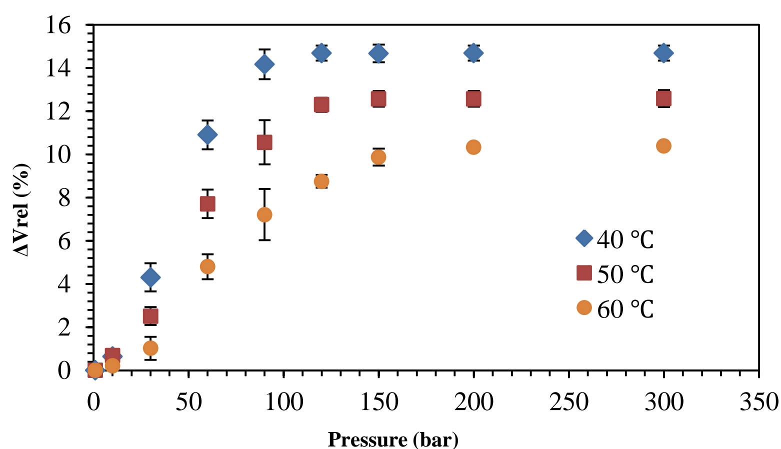

Figure 2: Relative volumetric expansion of pure water in equilibrium with CO_2 as a function of pressure at different temperatures.

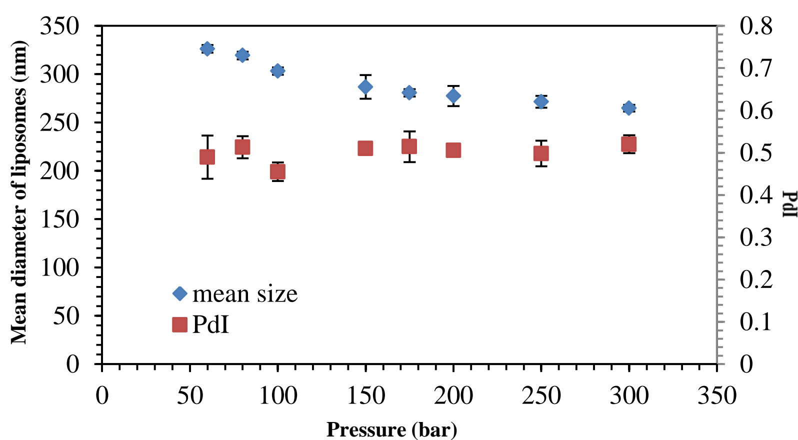

Figure 3: Effect of pressure on mean particle size and polydispersity index (PdI) of LN using the depressurization protocol (I) at 40 ℃ and 60 bar/min.

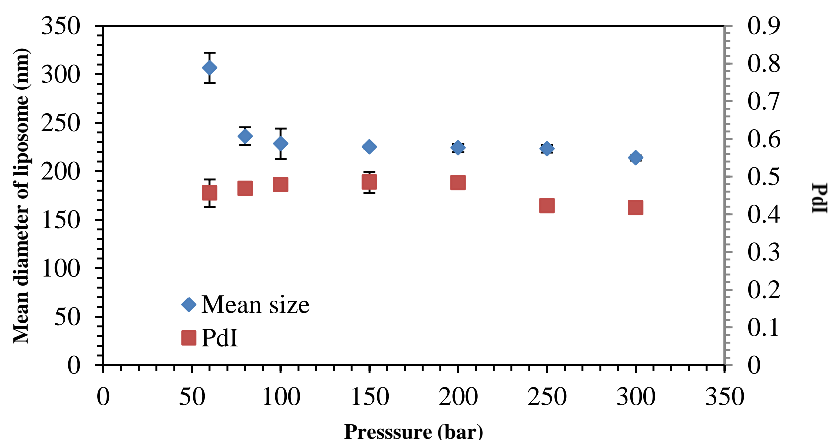

Figure 4: Effect of pressure on mean particle size and polydispersity index (PdI) of LN using the depressurization protocol (II) at 40 ℃ and 60 bar/min.

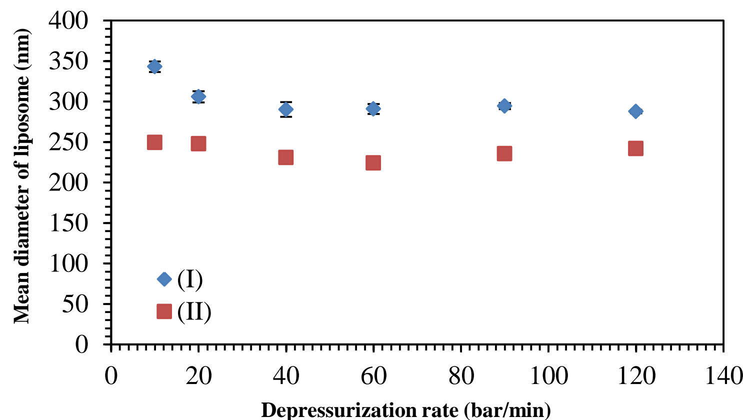

Figure 5: Effect of depressurization rate on mean particle size of LN using depressurization protocols (I) and (II) at 40 ℃ and 200 bar.

(II), more CO_2 was present in the bulk soy lecithin suspension to break up the phospholipid aggregates and form smaller particle size of LN (Fig. 5). In summary, protocol (II) showed superior formation of LN over protocol (I), where there was a continuous drop in the pressure of the suspension during the depressurization step.

The morphology of LN at different pressures was investigated using transmission electron microscopy (TEM). Figure 6 showed that unilamellar spherical or near-spherical nanovesicles were obtained. Higher pressure could be favorable for the formation of more symmetrical and spherical shape, whereas low pressure may be not sufficient to separate phospholipid molecules thoroughly. Due to elevated stress on the specific sites of the phospholipid membrane, asymmetric vesicles exhibited reduced stability and readily underwent rupture and leakage. Therefore, relatively higher pressure may be required to achieve a spherical vesicle. For both protocols (I) and (II), at least 100 bar was required to form spherical vesicles of LN. The storage stability of LN was evaluated based on the size distribution measured weekly for up to 10 weeks (data not shown). LN remained stable for up to 8 and 6 weeks ($\leq$5% increase in size) for protocols (I) and (II), respectively. LN prepared with both depressurization protocols presented good storage stability without the addition of any surfactant or stabilizer into the samples. High pressure CO_2 effectively dispersed phospholipid molecules and favored the formation of LN and rapid cooling effect caused by the depressurization of CO_2 stabilized the LN over longer storage. This indicated that LN with high stability could be produced by SEDPS method, which was an advantage brought about by SEDPS in contrast with conventional preparation methods.

CONCLUSIONS

The findings of this study demonstrated that SEDPS method yielded spherical LN with a relatively small mean diameter and high stability over long-term storage. High pressure was in favor of the formation of a smaller and spherical liposomes while high depressurization rate did not affect the LN formation in a significant manner. Protocol (II) was superior for preparation of LN over protocol (I). LN produced by SEDPS had a heterogeneous size distribution and the uniformity of LN should be improved. SEDPS is a simple, reproducible and organic solvent-free method to prepare LN. It shows great potential for encapsulation of bioactives in LN with promising applications for functional foods and nutraceutical supplements for enhanced nutritional and health benefits.

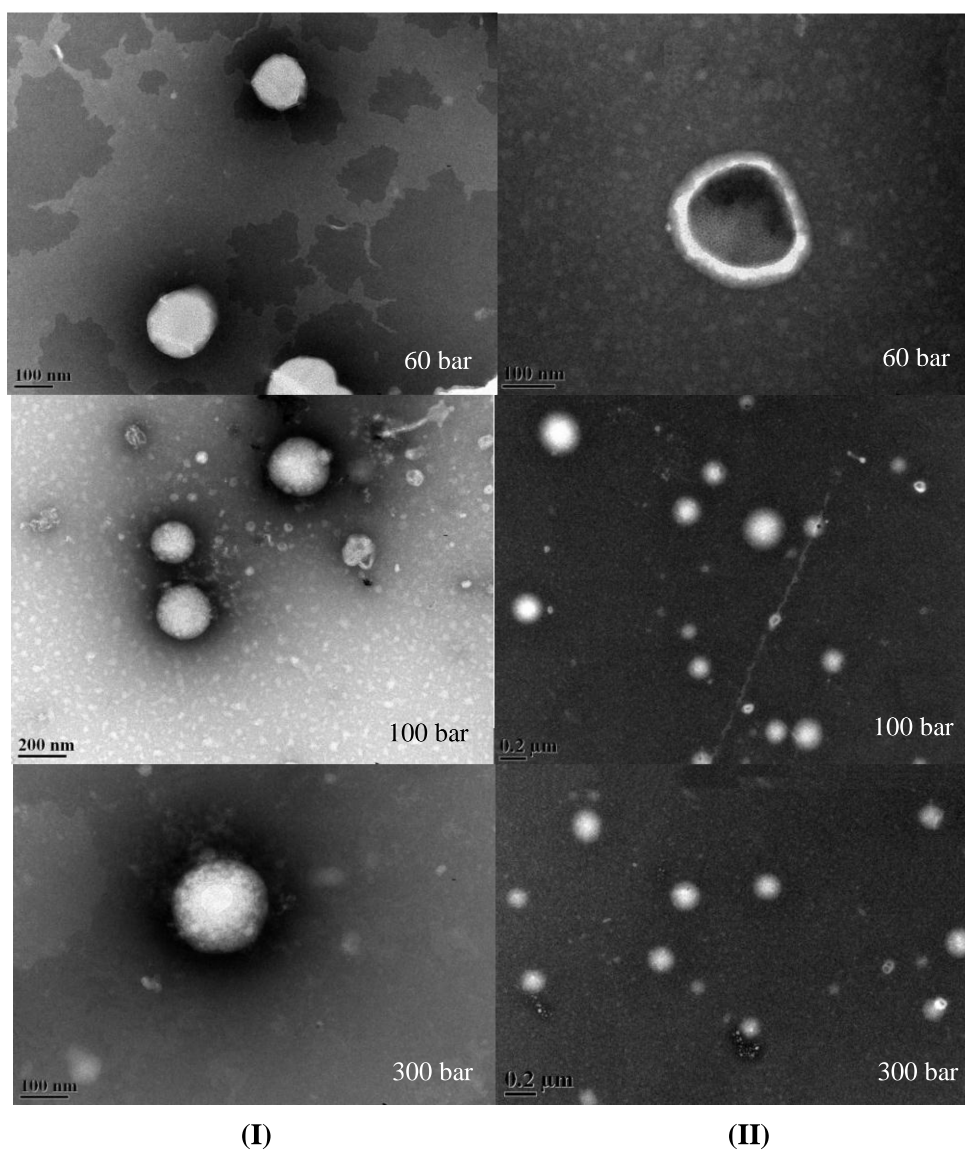

Figure 6: Transmission electron microscopy images of LN at different pressures obtained via depressurization protocol (I) and (II).

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