Production of Liposome from Sphingomyelin by Ultrasonic Device under Supercritical Carbon Dioxide

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ABSTRACT— ***Sphingomyelin is a type of sphingolipid found in animal cell membranes and can be exploited as an amphiphilic liposome component in the manufacture of liposomes capsule. Here, the fabrication of liposomes from sphingomyelin suspension solution via ultrasonic–supercritical carbon dioxide (CO_2) was studied. The experiments were conducted at temperatures of 40 – 60 ^oC and pressures of 10 – 20 MPa in batch process. As a starting material, sphingomyelin powder was dissolved and dispersed in distilled water. The TEM images indicated that the liposomes products were successfully formed in spherical and spherical–like shape morphologies with bimodal size at 91 – 220 nm and 396 – 955 nm. The liposomes products with smaller diameter were obtained when the experiments were conducted at higher operating pressure. The DLS measurement showed that the size distribution of liposomes products was increased with increasing operating temperature due to the aggregation. Based on the result, this process seems a powerful technique for organic solvent free liposome production technology from sphingomyelin solution for industrial purposes.***

Keywords— Liposomes, Supercritical CO_2, Sphingomyelin, Ultrasonic, Sphingophospholipids

1. INTRODUCTION

Generally, liposomes are defined as microscopic spherical vesicle in which an aqueous volume is entirely surrounded by a phospholipid membrane and this microscopic spherical vesicle consisted of at least one lipid bilayer. Due to the unique properties of liposomes, it became promising systems for drug delivery. Liposomes may involve a wide variety of hydrophilic and hydrophobic diagnostic or therapeutic agents, providing a larger drug payload per particle and protecting the encapsulated agents from metabolic processes [1–3]. The size of liposomes can range between 25 nm up to several micrometers. Based on the number of lamellae and diameter size, liposomes were classified into several types. They are small unilamellar visicles (SUV), medium–sized unilamellar vesicles (MUV), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV), oligolamellar vesicles (OLV), large multilamellar vesicles (LMV) and multivesicular vesicles (MVV). The range diameter for SUV and MUV is from 20 to 100 nm and 100 to 500 nm, respectively. The other types of LUV, GUV, OLV, LMV and MVV have a diameter size from few hundred nanometers to several micrometers [2]

Several parameters should be considered during the method selection for liposomes preparation, such as the physicochemical characteristics of the material to be entrapped, choice of liposomal ingredients, nature of the medium in which the lipid vesicles are to be dispersed, effective concentration of the entrapped substance, optimum size and shelf life of the vesicle, and batch–to–batch reproducibility [4,5]. In general, the methods for liposomes preparation were divided into bulk method, where liposomes are generated by transfer of phospholipids from an organic phase into an aqueous phase. The second method is film method, where lipid films are first deposited on a substrate and subsequently hydrated to result liposomes [6]. Due to the diameter size of liposomes is an important parameter to determine the circulation half–life of liposomes in drug delivery, the size optimization and the lamellarity of liposomes were typically subjected to the method selection for liposomes preparation.

Most of the preparation methods mentioned above use organic solvent. Remaining organic solvent in liposomes

may cause problem when they applied to medical field, cosmetics, or beverages. Supercritical CO_2 has been used as an alternative solvent in various processes such as decaffeination of green coffee bean and extraction from hops. Here, the preparation of liposomes from sphingomyelin which suspended in an aqueous media was carried out under pressurized carbon dioxide (CO_2) induced by ultrasound [7]. It has been known that CO_2 in supercritical conditions has been widely used as a solvent. CO_2 is a generally recognized as safe (GRAS) solvent and its critical temperature (T_c = 31.06 ^oC) and pressure (P_c = 7.38 MPa) are relatively low which helps in preventing thermal degradation during liposomes formation process [8,9]. The advantages of supercritical CO_2 in materials processing include that it is inert, relatively non–toxic, non–flammable, inexpensive, easily available, odorless, tasteless, and environment friendly. CO_2 is also easy to remove from products at atmospheric pressure, accordingly it does not contaminate products. In addition, when the supercritical CO_2 was applied on the liposomes preparation, the small size of liposomes products might be obtained [10–13]. There are several techniques to reduce the size of liposomes products, including sonication, extrusion, and homogenization. Of these, sonication technique, probe sonication or bath sonication, is a simple and a common technique to reduce the size of liposomes [6]. In this process, a very high energy was introduced into the lipid vesicles dispersion, as a result, the MLV are broken down into smaller SUV. In probe sonication process, the tip of a sonicator is directly introduced into the lipid vesicles dispersion, hence the potential of metal probe contamination may occur due to the metal probe will slough off and contaminate the solution during process [14]. Therefore, in this work, to avoid and to prevent the metal probe contamination, the bath sonication process was used for reducing size of liposomes products.

2. EXPERIMENTAL SECTION

2.1 Materials

Sphingomyelin ($C_{35}H_{75}N_2O_6P$) was obtained from NOF Corporation Japan. It was used without further purification. As one of sphingophospholipids, sphingomyelin is the most abundant sphingolipid and is important structural components of the myelin sheath. It may protect and coat surrounding the nerve fibers. In solution preparation, sphingomyelin was diluted in water distillate that produced by Auto Still WS 200, Yamato, Japan. The sphingomyelin concentration was 0.1 wt%. It should be noted that no sphingomyelin concentration variation was observed during the course of this work. Carbon dioxide (CO_2: 99%) was supplied by Sogo Kariya Sanso, Inc. Japan.

2.2 Ultrasonic – Supercritical CO_2 Apparatus

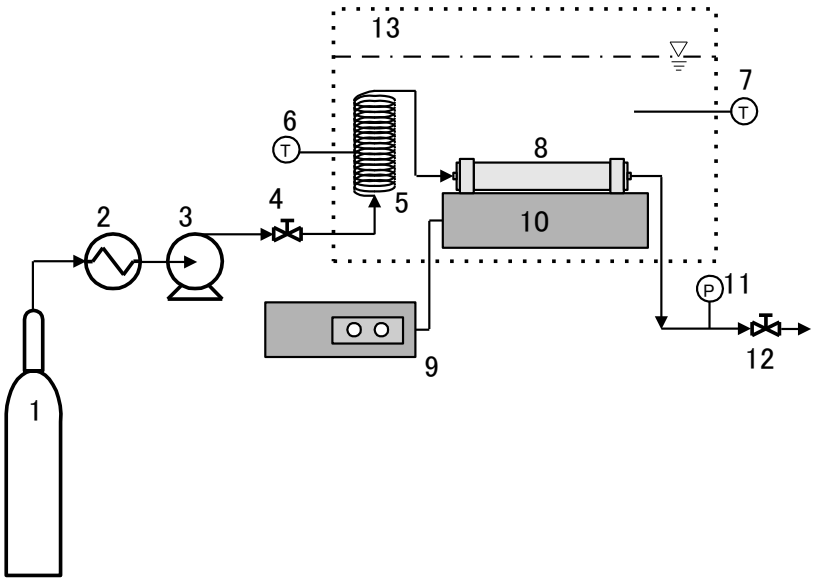

Figure 1: Experimental apparatus scheme: 1. CO_2 cylinder; 2. Chiller; 3. High pressure pump; 4. Needle valve; 5. SUS–316 pre–heater; 6, 7. Temperature monitor; 8. Reactor; 9. Ultrasonic controller; 10. Ultrasonic vibrator; 11. Pressure monitor; 12. BPR; 13. Water bath.

Figure 1 described the liposomes preparation apparatus scheme using ultrasonic – supercritical CO_2. The main apparatus was high pressures pump for CO_2 (PU–2086, Jasco, Japan), an ultrasonic device (Ultrasonic Multi Cleaner W–118, Honda Electronics Company, Japan), acrylic chamber equipped with electric heater, reactor (SUS–316, 80 ml), and back pressure regulator (BPR; AKICO, Tokyo, Japan). The coil preheater made of 1/6 inch stainless–steel tubing (SUS–316) with 300 cm length was placed in the heating chamber to introduce the CO_2 before entering to reactor. K–type thermocouples were attached in the preheater and placed in the water medium to monitor the temperature during

experiment. To monitor the liposomes generation pressures, the analog pressure gauge (GLT–21–25MPa, Migishita Seiki MFG. Co. Ltd., Japan) was assembled on the 1/16 inch stainless–steel tubing (SUS–316) and placed between reactor and BPR.

In this work, the liposomes generation from 0.1 wt% sphingomyelin suspension solution by using ultrasonic – supercritical CO_2 was carried out at temperatures of 40 – 50 ^oC and pressures of 10 – 20 MPa in batch process. The liposomes generation process can be explained briefly as follow. Initially, the power of heating chamber was switched on to heat the water medium including preheater to a desired temperature. Once the desired temperature was achieved, the stainless–steel reactor loaded by 60 ml sphingomyelin solution was immersed into the acrylic chamber. This reactor was equipped with removable threaded covers on both sides; these included stainless steel filters (0.1 – 1.0 μm). In order to remove the air, the reactor was purged by CO_2 gas and immediately sealed. Next, CO_2 was pumped into the liposomes preparation apparatus system via the 1/16 inch stainless–steel capillary tube at a desired pressure. A BPR was employed to keep a constant pressure during liposomes generation process. The time required to heat the reactor from room temperature to the desired temperature (the reactor temperature) was 5 – 8 min. Thereafter, ultrasonic was applied (28 kHz) in the liposomes preparation apparatus for 60 min at each experimental condition. After the process was completed, the CO_2 was slowly depressurized to atmospheric pressure. Then the solution products were collected in the bottles and stored in a refrigerator until further analysis.

To characterize the liposomes products, transmission electron microscopy (TEM) was carried out using negative staining method on a Hitachi H9000NAR microscope equipped with a cold field–emission gun. The acceleration voltage was 300 kV, and the TEM images were captured by CCD (charge–coupled device) camera. Prior to characterization, the liposomes products were diluted 10 times with water and then drop cast onto an elastic carbon–coated copper grid. It was stored in a desiccator overnight at room temperature to desorb atmospheric contaminants. The particle size distribution of the liposomes products was determined by dynamic light scattering analysis (DLS; Malvern Instruments, Malvern, England).

3. RESULTS AND DISCUSSION

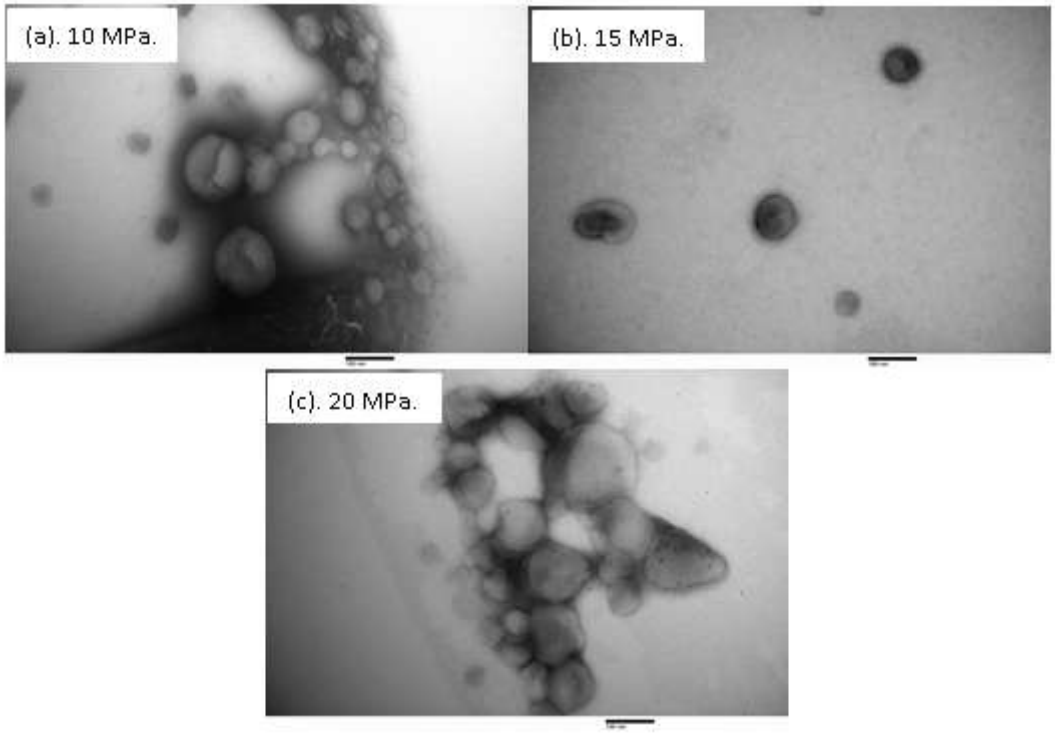

Figure 2: TEM images of liposomes products obtained at temperature of 40 ^oC with various CO_2 pressures.

Figure 2 shows the TEM images of the structural morphology of liposomes products generated under supercritical CO_2 at temperature of 40 ^oC with various pressures. It was well known that liposome preparation is simply understood and it can be produced from a various lipids and lipid mixtures, with phospholipids the most commonly used. The different preparations of liposomes were usually described by analyzing with TEM. As shown in this figure, many spherical and spherical–like particles morphologies of liposomes products were found at each experimental condition. At low CO_2 pressure (10 MPa), the spherical particles of liposomes products were obtained dominantly. Conversely, the liposomes products with spherical–like particles morphology were found prominently at higher CO_2 pressure (20 MPa).

It seems that the liposomes products with spherical shape morphologies were transformed into non–spherical shape morphologies with increasing CO_2 pressures. At these conditions, the CO_2 pressure as a driving force may press to change the liposome volume thereby changing the shape of liposomes products [15–17]. Choi *et al*. [16] informed that the osmotic pressure most likely led to the liposome morphological changes such as shrinkage, fission, swelling, and fusion of membranes. Hayashi *et al*. [17] reported that the liposome underwent deformation from a spherical shape to an ellipsoid shape and then to a lemon–like shape morphology when the pressure was applied on the liposomes solution. They informed that no changes were found in the morphology of liposomes before the application of pressure. On the contrary, the boundary between the central spherical part and the protruding parts led to obscure to result indefinite shapes of liposomes became with flabby membranes when the pressure was applied at 0.1 to 60 MPa. Judging from the results, it could be said that the morphologies of liposomes products seemed influence with changing operating pressure.

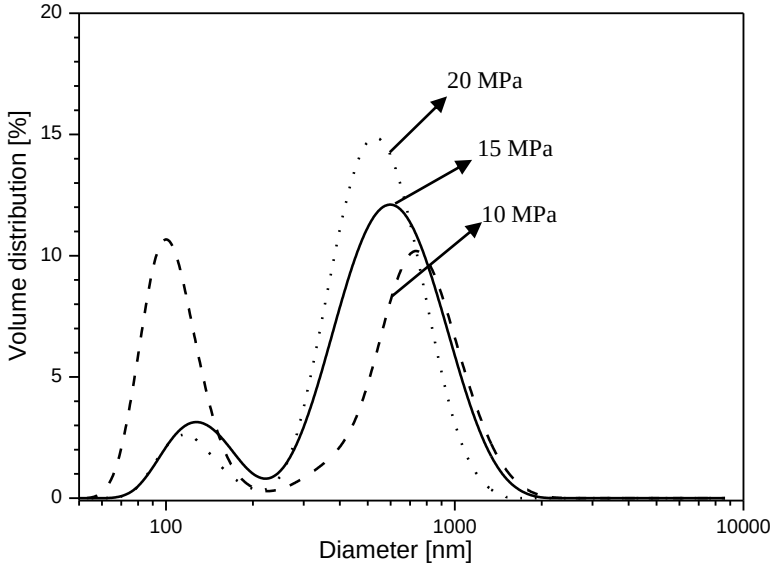

Figure 3: The liposomes products size distribution obtained at temperature of 40 ^oC with various CO_2 pressures.

Visually (see Figure 2), operating pressure did not seem to have a perceivable effect on the size of liposomes products. They seem to have a similar size at each operating condition. In order to understand the diameter size distribution of liposomes products, the sphingomyelin solution after treatment by ultrasonic – supercritical CO_2 was loaded in the cuvette and placed in the holder of DLS equipment. This analysis is common technique to quantify the small–molecule aggregates size by measuring the time–dependent fluctuation of scattering intensity of a coherent light source illuminating particles suspended in solution. Figure 3 shows the diameter size distribution of liposomes products generated at temperature of 40 ^oC and CO_2 pressures of 10, 15, and 20 MPa. The double–peaked (bimodal) diameter size distribution of liposomes products were found at each operating condition. Both peaks seem to approach at a smaller size around 91 – 122 nm and a larger size around 396 – 712 nm. It seems that the liposomes products in diameter ranges of 91 – 122 nm and 531 – 1106 nm were formed dominantly at 10 MPa CO_2 pressure. At 20 MPa CO_2 pressure, the diameter ranges of liposomes products were 105 – 122 nm and 396 – 712 nm. Thus, it could be said that although the CO_2 pressure did not give strong effect on the liposomes products size distribution, the narrower liposomes products size distributions were obtained with increasing CO_2 pressure at the same operating temperature. From this figure, it could be seen that the liposomes products also had smaller diameter when the experiments were conducted at higher operating pressure. In this work, the liposomes preparation under supercritical CO_2 without applied ultrasonic or by sonication process at room temperature did not perform. Hence, there was no comparison result between liposomes products formed by supercritical CO_2 without applied ultrasonic treatment or by the conventional sonication process and ultrasonic – supercritical CO_2 process. However, Karn *et al*. [13] informed that liposomes products prepared with supercritical CO_2 gave relatively much smaller and more homogenous in size. They observed that low CO_2 pressure (8 MPa) and low operating temperature (<35 ^oC) were insufficient conditions for producing liposomes. Similar results were also reported by Otake *et al*. [10] and Kadimi *et al*. [11] when they conducted experiments for preparation of liposomes using an improved supercritical reverse phase evaporation method and in vitro studies on liposomal amphotericin B obtained by supercritical CO_2–mediated process, respectively.

As informed above that the experimental temperature for liposomes preparation under ultrasonic – supercritical CO_2 were 40 to 60 ^oC. To reach the desired operating temperature, the reactor which has been filled by sphingomyelin solution was submerged in the acrylic chamber containing water medium. Due to the application of the ultrasound path–

length may give an effect on the temperature rise of water medium during experiments, the water from temperature–controlled bath was circulated by using water pump to maintain the desired temperature. Figure 4 shows the TEM images of liposomes products when the experiments were carried out at a constant pressure (20 MPa) with various operating temperatures from 40 to 60 ^{o}C. It has been known that the change of environmental temperature led to stimulate and cause shape transformations of liposomes [18–20]. This change may change the spontaneous curvature globally on the whole surface of the lipid vesicle. Jelger and Siewert [21] explained that the liposomes radius may increase with increasing the environment temperatures and hence the curvature difference between the two monolayers decreases. They also informed that at higher environment temperatures the effective volume of the tails increases due to the increased thermic motions, resulting the lipid vesicles effectively more inverted cone shaped. In other words, with increasing environment temperatures, the decreased volume of liposomes reduces and the spherical liposomes changes its shape. Zook and Vreeland [22] also informed that environment temperature may change the lipid vesicle size primarily as a result of its effect on the ratio of the membrane bending elasticity modulus to the line tension. However, as shown in Figure 4, the morphologies of liposomes products did not change obviously with increasing operating temperature. It seems that the morphologies of them were spherical and spherical–like shapes at each experiment. This phenomenon might be due to the physical properties of water media was not much change with increasing environment temperature. As a result, the similar morphologies of liposomes products were found at each experimental condition. At these environment temperatures, Zook and Vreeland [22] reported that the viscosity, free energy, and diffusion coefficients of water media which modulated by the changing environment temperatures may not have enough ability to give a sufficient effect for changing the liposomes products morphologies. Liu and Iglic [19] also informed that although the deformation of liposome morphology might occur by the changing environment temperatures, the shape of liposome morphology depended on the properties and the concentration of the components.

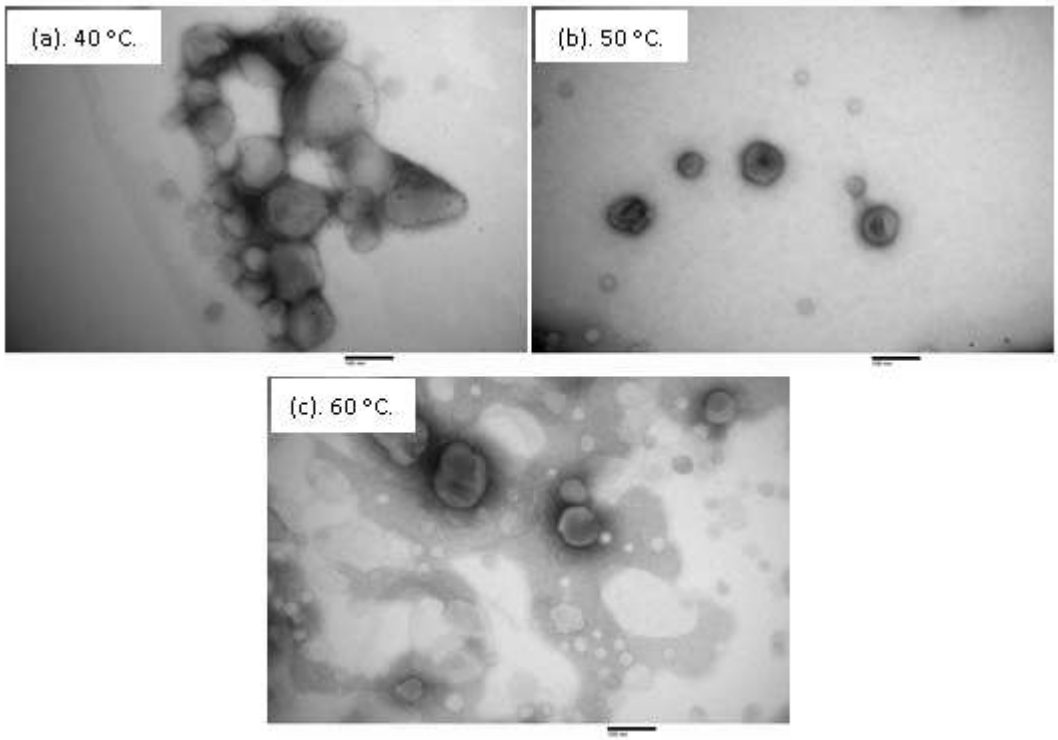

Figure 4: TEM images of liposomes products obtained at CO_2 pressure of 20 MPa with various operating temperatures.

Similar to the phenomenon in shape of liposome morphology, the changing environment temperatures will also affect to the size distribution of liposomes products [22]. Zook and Vreeland [22] explained that there were three ways for affecting temperature on the size of liposomes during process: first, by changing the free energy and the diffusion coefficients; second, by changing the medium viscosity; and third, by changing the membrane elasticity at or below the transition temperature and by changing the line tension. Figure 5 showed the size distribution of liposomes products obtained at 20 MPa CO_2 pressure with operating temperatures of 40 – 60 ^{o}C. It was well known that beside the decreasing medium density and viscosity contribute in the reduction process of liposome sizes, the higher operating temperature will also lead to in effective lipid vesicles dispersion and result the smaller size of liposomes [23]. However, as shown in Figure 5, the size distributions of liposomes products seem increased slightly with increasing operating temperature. At 40 ^{o}C, the liposomes products had diameter ranges of 105 – 122 nm and 396 – 712 nm. Their diameter

ranges increased to 164 – 220 nm and 615 – 955 nm when the operating temperature was increased to 60 ^{o}C at the same CO_2 pressure. It could be explained that the aggregation of liposomes products might be occur. The operating temperature was known as one of the factors that can affect the degree of aggregation liposomes during formation process [24,25]. Liposomes will be produced only at environment temperatures higher than the main transition temperature of the phospholipid which used as a starting material and has a significant effect on the size of liposomes products. The phase transition temperature ranges of all naturally occurring sphingomyelins is around 30 – 45 ^{o}C [26]. Unfortunately, the heat energy of the lipid vesicle membrane at high temperatures is sufficient to overcome the potential barrier of aggregation, consequently the liposomes products in the solution system tends towards aggregation. The aggregation process commonly was initiated at an environment temperature near to that for phase transition into hexagonal phase [24]. As a result, the size distribution of liposomes products obtained by DLS measurement is increased with increasing operating temperature range studied.

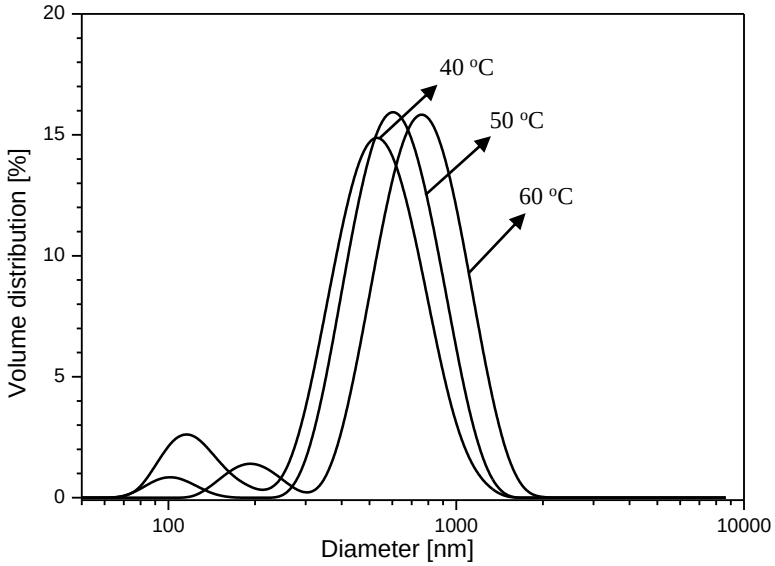

Figure 5: The liposomes products size distribution obtained at CO_2 pressure of 20 MPa with various operating temperatures.

4. CONCLUSIONS

The production of lipid vesicles from sphingomyelin solution via ultrasonic–supercritical CO_2 has been demonstrated. The experiments were carried out at temperatures of 40 – 60 ^{o}C and pressures of 10 – 20 MPa in batch process. The sphingomyelin powder dissolved in distillate water at 0.1 wt% was used as a starting material. At each experiment, the applied ultrasonic power was 28 kHz for 60 min. The TEM images showed that the liposomes products were successfully produced in spherical and spherical–like shape morphologies with size less than 1 μm. The liposomes products with smaller diameter were obtained when the experiments were conducted at higher operating pressure. The DLS analysis showed that the size distribution of liposomes products increased with increasing operating temperature due to the aggregation. Finally, it could be said that this process seems a powerful technique and to be an apt for liposome production from sphingomyelin solution.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

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