ELSEVIER

Colloids and Surfaces B: Biointerfaces 27 (2002) 133–140

COLLOIDS AND SURFACES B

www.elsevier.com/locate/colsurfb

Preparation and physicochemical properties of various soybean lecithin liposomes using supercritical reverse phase evaporation method

Tomohiro Imura [a], Katsuto Otake [b], Satoru Hashimoto [a], Toshihiro Gotoh [a], Makoto Yuasa [a,c], Shoko Yokoyama [d], Hideki Sakai [a,c], James F. Rathman [e], Masahiko Abe [a,c,*]

[a] *Faculty of Science and Technology, Tokyo University of Science, Yamazaki 2641, Noda, Chiba 278-8510, Japan*
[b] *National Institute of Advanced Industrial Science and Technology, Institute for Green Technology, Higashi 1-1, Tsukuba, Ibaraki 305-8510, Japan*
[c] *Institute of Colloid and Interface Science, Tokyo University of Science, Kagurazaka 1-3, Shinjuku-ku, Tokyo 162-0825, Japan*
[d] *Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105-8512, Japan*
[e] *The Ohio State University, Chemical Engineering Department, 140W. 19th Avenue, Columbus, OH 43210, USA*

Received 13 February 2002; accepted 12 April 2002

Abstract

Three kinds of soybean lecithin liposomes composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA), were prepared by using the previously developed supercritical reverse phase evaporation method (Langmuir 17 (2001) 3898). The effect of phospholipid composition on the formation of liposomes and their physicochemical properties were examined by means of trapping efficiency measurements, transmission electron microscopy, dynamic light scattering and zeta potential measurements. The trapping efficiency of liposomes for D-(+)-Glucose made of Lecinol S-10EX which contains approximately 95% PC is higher than that of Lecinol S-10 and SLP white SP which contain approximately 31% PC. However there is not any difference between the trapping efficiency of liposomes for D-(+)-Glucose made of Lecinol S-10 which has saturated hydrocarbons tails and that of liposomes made of SLP white SP which has unsaturated hydrocarbon chains. The electron micrographs of liposomes made of Lecinol S-10 and SLP white SP show small spherical liposomes with diameter of 0.1–0.25 μm, while that of Lecinol S-10EX shows large unilamellar liposomes (LUV) with diameter of 0.2–1.2 μm. These results clearly show that phospholipid structure of PC allows an efficient preparation of LUV and a high trapping efficiency for water-soluble substances. Liposomes made of Lecinol S-10 and SLP white SP remained well-dispersed for at least 14 days, while liposome suspension made of Lecinol S-10EX separated in two phase at 14 days due to aggregation and fusion of liposomes. The dispersibility of liposomes made of Lecinol S-10EX is lower than that of Lecinol S-10 and SLP white SP due to the smaller zeta potential of Lecinol S-10EX.

* Corresponding author. Fax: +81-471-24-8650
E-mail address: abemasa@ci.noda.sut.ac.jp (M. Abe).

0927-7765/02/$ - see front matter
PII: S0927-7765(02)00048-6

Keywords: Liposome; Supercritical carbon dioxide; Soybean lecithin; Trapping efficiency; Freeze fracture TEM

1. Introduction

Liposomes are colloidal structures formed by the self-assembly of amphiphilic lipid molecules in solution. Liposomes are self-enclosed and often spherical, with the lipid bilayer encapsulating an inner solution phase [1]. They have long been used as models for biological membranes because they have a structure and functions similar to those of biological membranes [2–5]. Recently, liposomes have proven to be promising carriers for drug delivery systems because they can retain water-soluble substances in the inner aqueous phase and oil-soluble substances in the bilayer walls [6,7]. It is easy to modify the liposomal surface with sugars such as galactose [8,9] and mannose [10], or with hydrophilic compounds such as polyethyleneglycol [11] and polyglycerols [12], for targeting liposomes to specific tissues or cells. Thus, liposomes have found applications, for example, in cancer treatments targeting specific cells with reduced side effects [13,14] and gene therapy using gene-trapping vesicles [15]. Liposomes are also widely used in the drug formulations and cosmetics.

Many methods for the preparation of liposomes have been reported such as the Bangham method [1], organic solvent injection [16,17], and reverse phase evaporation [18]. However, these methods require large amounts of organic solvents like chloroform, ether, freon, methylenechloride, and methanol that are harmful to the environment and human body, and very few methods have been developed that yield liposomes having a high trapping efficiency for water-soluble substances without using any organic solvent. When liposomes are used as drug carriers, use of organic solvents must be avoided as much as possible in their preparation because complete removal of the remaining organic solvent is required. All of these methods are not suitable for mass production of liposomes because they consist of many steps. Because of these reasons, few examples of the effective use of liposomes are known even though their potential advantages have long been widely recognized.

Supercritical fluids are non-condensable and highly dense at temperatures and pressures beyond their critical point. Supercritical fluids are highly functional solvents whose properties can be altered remarkably by varying temperature and pressure. Supercritical carbon dioxide ($scCO_2$), in particular, has attracted attention as an environmentally-friendly alternative solvent that can replace organic solvents because it has a low critical temperature ($T_c = 31\ ^\circ C$) and pressure ($P_c = 73.8$ bar), and because it is nontoxic, inflammable, and cheap [19–21].

We have previously developed a new method [22], the supercritical reverse phase evaporation method (scRPE method), for the preparation of liposomes in a single step using $scCO_2$ and ethanol. No other organic solvent is required. This method yields aqueous dispersions of large unilamellar liposomes (LUV) with diameters of 0.1–1.2 μm and a high trapping efficiency for water-soluble solutes. Although we believe that the scRPE method greatly contributes to moving liposomes technology toward practical use, little is known about the kinds of phospholipids that are best suited for this method and the stability and physicochemical properties of liposomes prepared by this method.

In this work, the scRPE method was used to prepare three kinds of soybean lecithin liposomes composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA). The effects of phospholipid structure on liposome formation and physicochemical properties were examined using trapping efficiency measurements, transmission electron microscopy, dynamic light scattering, and zeta potential measurements.

Table 1
Average composition of lecithins

Type of lecithin	PC (%)	PE (%)	PI (%)	PA (%)	Others (%)
Lecinol S-10EX (hydrogenated)	95	*	*	*	*
Lecinol S-10 (hydrogenated)	32	31	17	9	*
SLP white SP (unhydrogenated)	32	31	17	9	*

2. Experimental section

2.1. Materials

Three soybean Lecithins, Lecinol S-10EX, Lecinol S-10, and SLP white SP, were given by Nikko Chemicals Co., Ltd and used for liposome preparation as received. Average compositions in PC, PE, PI, and PA as given by the supplier are summarized in Table 1. Other components include carbohydrates and other phospholipids, but the exact composition was not provided by the supplier. Ethanol (99.8% pure) was purchased from Wako Pure Chemical Ind. and used as received. D-(+)-Glucose (98.0% pure) purchased from Showa Chemical Co. was used as supplied to examine the trapping efficiency of liposomes for a water-soluble substance. Water for injection (Ohtsuka Pharmaceutical Co.) was used as the solvent.

2.2. Liposome preparation by the scRPE method

Fig. 1 is a schematic of the experimental apparatus used for liposome preparation. After lecithin (0.036 g) and ethanol (0.85 g) were sealed in the view cell, CO_2 (12.15 g) was introduced into the cell, and the cell temperature was then raised to 60 °C, a temperature higher than the phase transition temperature (56 °C) of DSPC, while the pressure was maintained at 200 bar with observing inside the cell through the window. After 20 min for equilibration, an aqueous D-(+)-Glucose solution (0.2 mol/l) was slowly (0.05 ml/min) introduced into the cell via an HPLC pump until the desired amount of solution was attained. The pressure was then reduced to release CO_2, thereby giving a homogeneous liposome suspension. The contents of the cell were continuously mixed with a magnetic stirrer during the experiments. Detailed description of experimental procedure was described in the previous paper [22].

2.3. Trapping efficiency measurement

In the glucose trapping efficiency experiments, the liposome suspension was dialyzed against water using a cellophane tube (Viscase Scales Corp.) to remove the non-encapsulated glucose remaining in the dispersion medium. The liposomes inside the tube were then destroyed by addition of ethanol. The amount of glucose in the solution was determined by the mutarotase GOD method [23,24] using a spectrophotometer (MPS-2000, Shimazu).

2.4. Transmission electron microscope observation of liposomes

The liposome suspension was quickly frozen in liquid propane using a cryo-preparation apparatus (Leica EM CPC, Leica Co.). The frozen sample

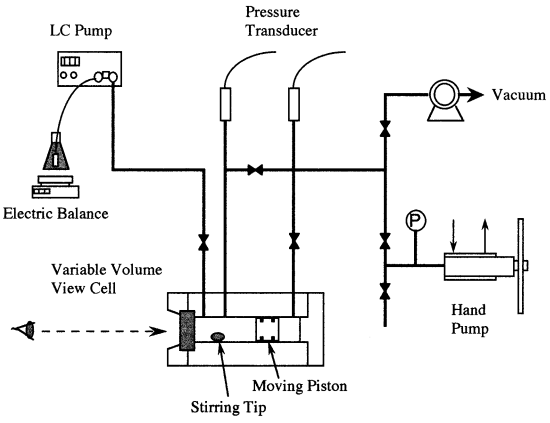

Fig. 1. Apparatus for preparation of liposome.

was fractured in a freeze-replica-making apparatus (FR-7000A, Hitachi Science Co.) at −150 °C. The fractured surface was replicated by evaporating platinum at an angle of 45°, followed by carbon at normal incidence to strengthen the replica. It was then placed on a copper grid mesh after washing with acetone and water and observed with a transmission electron microscope (JEM-1200EX, JEOL Co.).

2.5. Measurement of particle size of liposomes

The liposomes prepared by scRPE method were downsized by extrusion at 60 °C in a commercial extruder device (Lipex Biomembranes, Canada) equipped with a polycarbonate filter (200 nm) (Nucleopre, USA) in order to obtain liposomes of nominal size 200 nm. The particle size of liposomes was determined by dynamic light scattering. The size distribution was measured at 30 °C using a NICOMP 380ZLS (Particle Sizing System Co.). The light source was a diode pump solid state laser with a wavelength of 533 nm and the scattering angle was 90°.

2.6. Measurement of zeta potential of liposomes

The zeta potential of liposomes was measured with the laser Doppler method (heterodyne method) by using a NICOMP 380ZLS (Particle Sizing System Co.) at pH 6.5. This method gives the electrophoretic mobility and zeta potential of particles moving in an electric field based on the Doppler shift, a frequency shift of laser light scattered by the particles. The laser output power was 10 mW, the scattering angle was 19.8°, and the electric field strength was 5.0 V/cm.

3. Results and discussion

3.1. Change of phase appearance during the experiments

At first phase appearance of $scCO_2$/ethanol/lecithin mixtures were observed through the window before the water was introduced(200 bar, 60 °C). For the case of Lecinol S-10EX, very small amounts of lecithin gel were observed inside the cell. On the other hand for the case of Lecinol S10 and SLP white SP, precipitated lecithin powder remained at the bottom of cell. Montanari et al. [26] have reported extraction of phospholipids from soybeans with $scCO_2$/ethanol mixture. The composition of phospholipids extracted at 60 °C and 239 bar was 79.5% PC, 20.5% PE, 0.0% PI, and 0.0% PA. The precipitated observed in this study would be the PE, PI and PA that have lower solubility than PC. In the successive water adding process, while the amounts of introduced water were small, the inside of the cell remained transparent with no appearance of a water phase. With the further introduction of water gave a water phase at the cell bottom. The water phase was initially transparent and became turbid when more water was introduced with vigorous stirring. After the $scCO_2$ inside the cell was excluded, homogeneous liposome suspension were obtained inside the cell as reported previously [22].

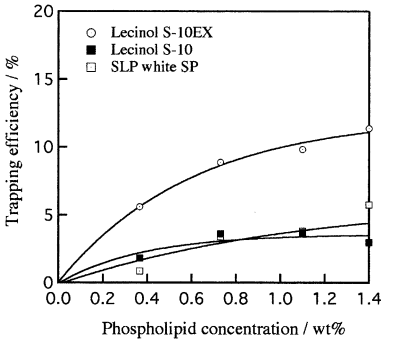

Fig. 2. The relationship between trapping efficiency and phospholipid concentration.

3.2. The effect of phospholipids composition on the trapping efficiency of liposomes

The relationship between trapping efficiency of liposomes for D-(+)-Glucose prepared by the scRPE method and phospholipids concentration is shown in Fig. 2. The abscissa represents the amount of phospholipids against the volume of added glucose solution, and the concentration of phospholipids becomes higher when the volume of glucose solution introduced into the cell is lower.

The trapping efficiency was calculated according to the equation.

$$\text{Trapping efficiency (\%)} = \frac{\text{glucose encapsulated in liposome}}{\text{glucose in liposomal solution}} \times 100 \quad (1)$$

The fact that trapping efficiencies of three kinds of soybean lecithin are not zero suggests that the liposomes are indeed formed. All of the trapping efficiencies increased with increasing phospholipids concentration. The ethanol added to the system constantly seems to be the reason the trapping efficiencies do not increase linearly. The trapping efficiency of liposomes for D-(+)-Glucose depends on the lecithin composition, and that made of Lecinol S-10EX which contains approximately 95% PC is higher than that of liposomes made of Lecinol S-10 and SLP white SP which contain approximately 32% PC. As described above the solubility of PC in $scCO_2$/ethanol is higher than that of PE, PI, and PA. It seems likely that higher solubility of PC in $scCO_2$/ethanol cause higher trapping efficiency of liposomes made of Lecinol S-10EX which contains approximately 95% PC. Of the lipid studied here, these results suggest that PC is the best for efficient preparation of liposomes with a high trapping efficiency for a water-soluble substance.

3.3. The effect of the phospholipids composition on the liposomes structure

The effect of the phospholipids composition on the liposomes structure was examined by freeze fracture TEM. Fig. 3 shows typical freeze fracture electron micrographs of liposomes before extrusion (phospholipid concentration 0.73 wt.%) prepared by the scRPE method. For the case of liposomes made of Lecinol S-10EX (Fig. 3(a)), large ellipsoidal LUV with diameter of 0.2–1.2 μm that are similar to those reported previously [22] were observed. The reason the liposomes have an ellipsoidal shape would be their low stability compared with spherical multilamellar liposomes (MLV). An increasing tendency was also found for the shape of liposomes to become spherical with decreasing liposome size. For the case of Lecinol S-10 and SLP white SP, spherical liposomes with diameter of 0.1–0.25 μm were observed. These liposomes are considerably smaller than those of Lecinol S-10EX and might be multilamellar. In general, the trapping efficiency of liposomes (more exactly, volume of their inner aqueous phase)

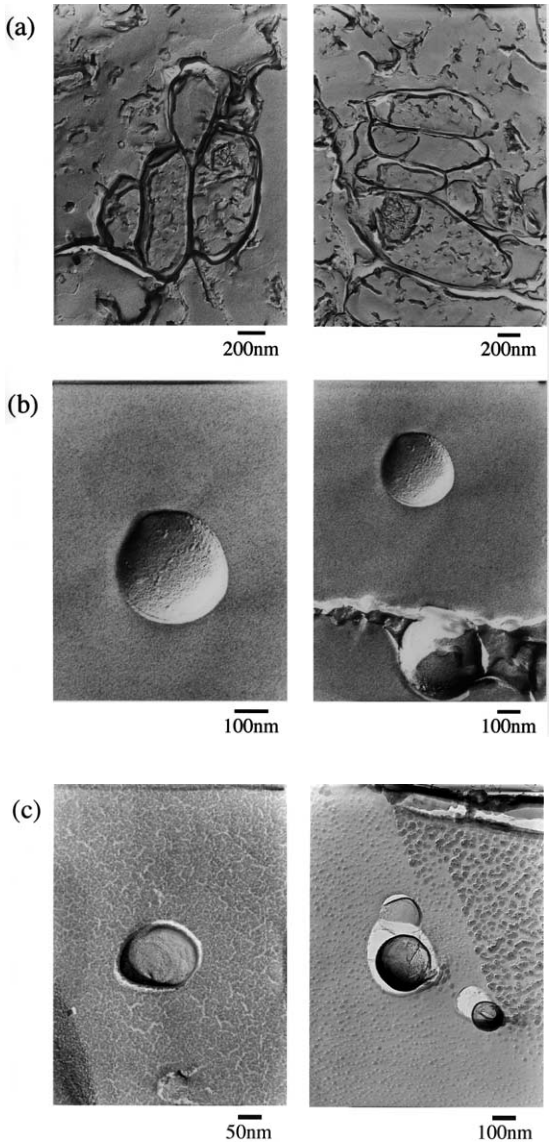

Fig. 3. Freeze fracture electron micrographs liposomes prepared by scRPE method. (a) Lecinol S-10EX; (b) Lecinol S-10; (c) SLP white SP.

depends on the physicochemical properties such as the number of bilayer shells within each of liposome and it is lower for those consisting of a bigger number of bilayers [25]. The trapping efficiencies of Lecinol S-10 and SLP white SP are well consistent with that of MLV made of L-α-Dipalmitoylphosphatidylcholine [22].

3.4. The effect of the phospholipids composition on the particle size of liposomes

The distribution of liposomal particle size is shown in Fig. 4. No significant changes in particle size and distribution of particle size of the liposomes made of Lecinol S-10EX, Lecinol S-10, and SLP white SP was found. On the other hand, the liposomes made of Lecinol S-10, SLP white SP required more nitrogen pressure (5 kgf/cm^2) to extrude through polycarbonate filter than those of Lecinol S-10EX (2 kgf/cm^2). These results suggest that phospholipid precipitates exist in the liposome suspension made of Lecinol S-10 and SLP white SP. As described above the solubility of PC in $scCO_2$/ethanol is higher than that of PE, PI, and PA. At the same time, Lecinol S-10EX which contains 95% PC exhibited higher trapping efficiency than the other two Lecithins. These facts indicate that the lower solubility of PE, PI, and PA for $scCO_2$/ethanol caused phospholipids precipitation in liposome suspension made of Lecinol S-10 and SLP white SP.

3.5. Effect of phospholipids composition on dispersibility of liposomes

The effect of phospholipid composition on the dispersibility of liposomes was examined by visual observation of liposome suspension. Fig. 5 illustrates stabilization results. Almost immediately after preparation all of the solutions were turbid, which is a characteristic of liposomal solution. Although the liposomes made of Lecinol S-10 and SLP white SP remained well-dispersed for at least 14 days, the liposome suspension made of Lecinol S-10EX exhibited significant phase separation at 14 days due to aggregation and fusion of liposomes. These results show that the dispersibility of liposomes made of Lecinol S-10 and SLP white SP

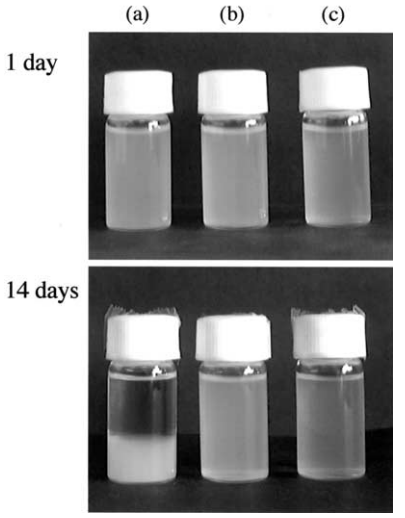

Fig. 5. Time dependence of liposomal solutions prepared by scRPE method. (a) Lecinol S-10EX; (b) Lecinol S-10; (c) SLP white SP.

is better than that of Lecinol S-10EX. In general, the dispersibility of liposomes depends on the physicochemical properties such as zeta potential and it is better for liposomes having higher zeta potential. Table 2 lists values of zeta potentials of liposomes prepared by scRPE method. The zeta potentials of liposomes made of Lecinol S-10 and SLP white SP are considerably higher than that of Lecinol S-10EX. PC is a zwitterionic lipid at the pH of the measurements (6.5), while PE, PI, and PA are negatively charged lipids whose presence in the liposomes enhanced the negative charge of the phosphate group. It seems likely that the dispersibility of liposomes made of Lecinol S-10EX is lower than that of Lecinol S-10 and SLP white SP due to the smaller zeta potential of Lecinol S-10EX.

4. Conclusions

We have succeeded in preparing liposomes made of Lecithins which are cheap and suitable for mass production of liposomes by using previously developed the scRPE method. The Lecinol S-

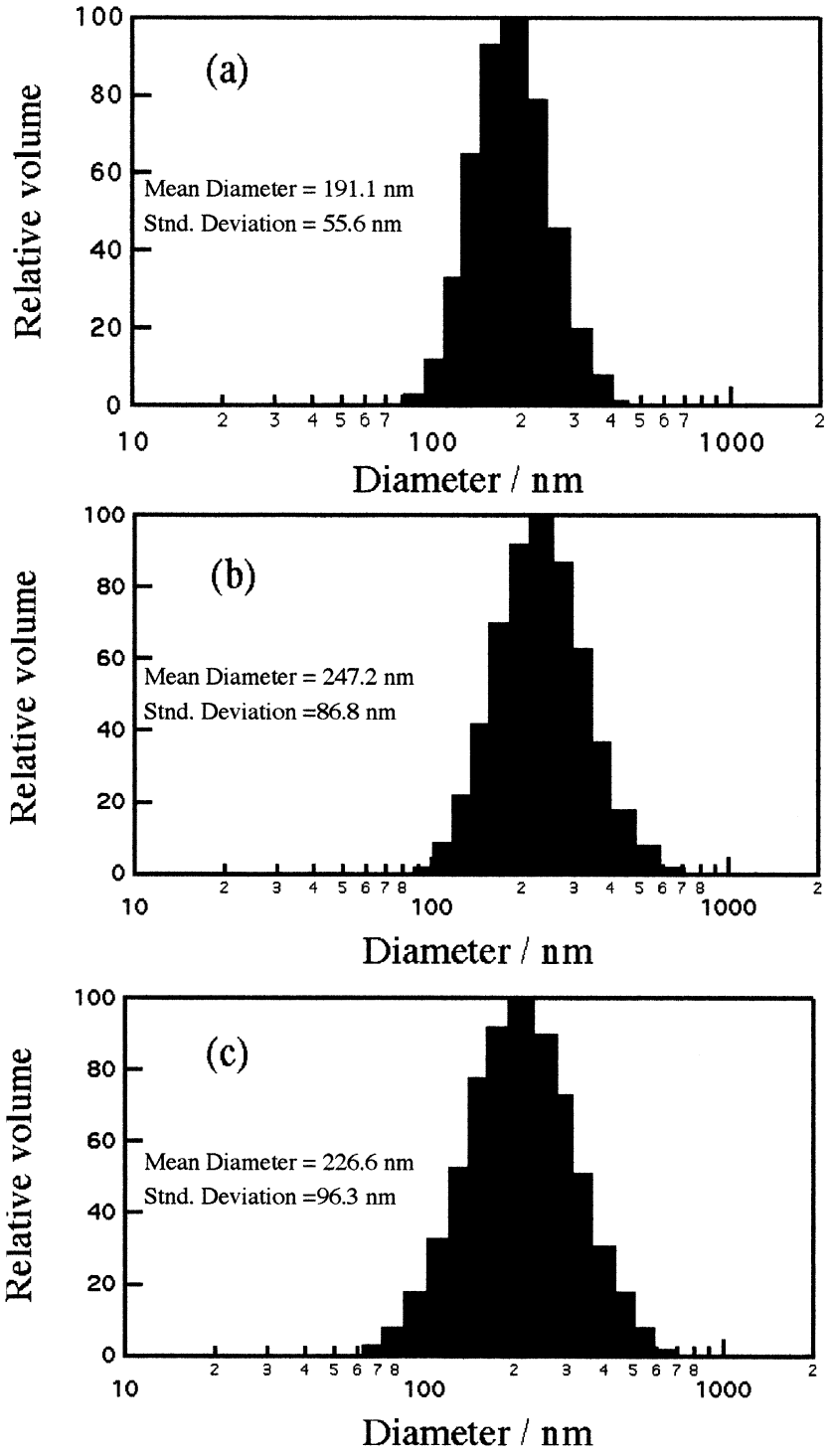

Fig. 4 Distribution of particle size of liposomes prepared by scRPE method. (a) Lecinol S-10EX; (b) Lecinol S-10; (c) SLP white SP.

Table 2
Values of zeta potential for liposomes prepared by scRPE method

Type of lecithin	Zeta potential, mV
Lecinol S-10EX	−4.7
Lecinol S-10	−22.8
SLP white SP	−24.3

10EX composed of 95% PC which is a zwitterionic lipid allowed us to prepare LUV with diameter of 0.2–1.2 μm. On the other hand, it seems likely that the liposome made of Lecinol S-10 and SLP white SP which are composed of 32% PC, 31% PE, 17% PI, and 9% PA are MLV with diameter of 0.1–0.25 μm. These results suggest that the structure of liposomes prepared by the scRPE method depends on the solubility of phospholipid in $scCO_2$/ethanol mixture. PC (zwitterionic lipid) is suitable for efficient preparation of liposomes with a high trapping efficiency than PE, PI, and PA (negatively charged lipids) for the present scRPE method.

References

[1] A.D. Bangham, M.M. Standish, J.C. Watkins, J. Mol. Biol. 13 (1965) 238–252.
[2] K. Balon, B.U. Riebesehl, B.W. Mueller, J. Pharm. Sci. 88 (8) (1999) 802–806.
[3] M. Colic, D. Morse, Colloids Surf. A 154 (1–2) (1999) 167–174.
[4] M.T. Montero, J. Hernandez-Borrell, K.M.W. Keough, Langmuir 14 (9) (1998) 2451–2454.
[5] H. Komatsu, S. Okada, Biochim. Biophys. Acta 1237 (2) (1995) 165–175.
[6] Y. Kaneda, Adv. Drug Delivery Rev. 43 (2–3) (2000) 197–205.
[7] J. Marjan, Z. Xie, D.V. Devine, Biochim. Biophys. Acta 1192 (1) (1994) 35–44.
[8] H. Yamauchi, H. Kikuchi, M. Sawada, M. Tomikawa, S. Hirota, J. Microencapsulation 11 (1994) 179–188.
[9] H. Yamauchi, H. Kikuchi, M. Sawada, M. Tomikawa, S. Hirota, J. Microencapsulation 11 (1994) 287–296.
[10] K. Yachi, H. Kikuchi, H. Yamauchi, S. Hirota, M. Tomikawa, J. Microencapsulation 12 (1995) 377–388.
[11] D. Lasic, F. Martin, Stealth Liposomes, CRC Press, Boca Raton, EL, 1995.
[12] K. Maruyama, S. Okuizumi, O. Ishida, H. Yamauchi, H. Kikuchi, M. Iwatsuru, J. Pharm. 111 (1994) 103–107.
[13] A. Hayashi, T. Nakanishi, S. Nakagawa, T. Mayumi, Drug Delivery Syst. 15 (1) (2000) 49–55.
[14] M. Hashida, Farumashia 34 (3) (1998) 235–239.
[15] M. Nishikawa, L. Huang, Drug Delivery Syst. 15 (2) (2000) 80–88.
[16] S. Batzri, E.D. Korn, Biochim. Biophys. Acta 298 (1973) 1015–1019.
[17] D. Demer, A.D. Bangham, Biochim. Biophys. Acta 443 (1976) 629–634.
[18] F. Szoka, Jr., D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA 75 (1978) 4194–4198.
[19] C.E. Bunker, H.W. Rollins, J.R. Gord, Y.-P. Sun, J. Org. Chem. 62 (21) (1997) 7324–7329.
[20] J.-H. Heo, S.-Y. Kim, H.-S. Kim, K.-P. Yoo, Biotechnol. Lett. 22 (12) (2000) 995–998.
[21] A.I. Cooper, J. Master. Chem. 10 (2) (2000) 207–234.
[22] K. Otake, T. Imura, H. Sakai, M. Abe, Langmuir 17 (2001) 3898–3901.
[23] I. Miwa, J. Okuda, K. Meada, G. Okuda, Clin. Chim. Acta 37 (1972) 538.
[24] J. Okuda, I. Miwa, PNE 17 (1972) 216.
[25] S. Vemuri, C.T. Rhodes, Pharmaceutica Acta Helvetiae 70 (1995) 95.
[26] L. Montanari, P. Fantozzi, M.J. Snyder, W.J. King, J. Supercrit. Fluids 14 (1999) 87–93.