Preparation and Properties of Liposomes Composed of Various Phospholipids with Different Hydrophobic Chains Using a Supercritical Reverse Phase Evaporation Method

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Abstract: Liposomes were prepared by the supercritical reverse phase evaporation method developed in our laboratory using various phospholipids with different hydrocarbon chains. The effects of the length of alkyl chain and number of unsaturated bonds of phospholipids on the properties of liposomal membranes were examined through trapping efficiency measurements, transmission electron microscopic observations, and osmotic response measurements. The trapping efficiency for water-soluble drugs of liposomes prepared by our method was greatly higher than that of liposomes prepared by the conventional Bangham method. Liposomes prepared using unsaturated phospholipids showed a high trapping efficiency compared with those prepared using saturated phospholipids. In addition, the trapping efficiency of liposomes prepared using 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), a complex phospholipid with both saturated and unsaturated alkyl groups, had a value intermediate between 1- α -dipalmitoyl-phosphatidylcholine (DPPC), a saturated phospholipid, and 1- α -dioleoylphosphatidylcholine (DOPC), an unsaturated phospholipid. That is, the trapping efficiency of liposomes was dependent on the number of unsaturated bonds rather than the alkyl chain length of phospholipid molecule and it increased with increasing bulkiness of the molecule. The osmotic response was higher for liposomes prepared using unsaturated phospholipids than for those formed by saturated phospholipids.

Key words: liposome, supercritical carbon dioxide, reverse phase evaporation method, trapping efficiency, membrane property

1 INTRODUCTION

Liposomes are closed vesicles made of bimolecular membrane formed by self-assembling of various phospholipids, a major component of biomembranes, in water and frequently used as materials for the research on biomembrane model and artificial cells¹⁻⁴. Liposomes have attracted attention as a carrier for drug delivery systems (DDS) because their inner aqueous phase retains water-soluble substances while their bimolecular membrane allows oil-

soluble substances to dissolve in it^{5,6}. For instance, liposomes are used to reduce the side effects in targeting the cells in cancer treatment^{7,8} and to perform gene therapy using liposomes containing a required gene⁹. Also, the usefulness of liposomes as externally applied pharmaceuticals is very high, and when drug-containing liposomes are applied to the skin, they stay on the skin or between the horny layers for long time to release an effective amount of the drug, thereby enhancing the transdermal drug absorp-

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tion¹¹⁻¹³).

Yet, there exists a problem in putting liposomes to practical use that no efficient preparation method has been established. In particular, the use of organic solvents is undesirable in the preparation of liposomes to be used for medicines considering the unacceptable effects of the solvents on the human body and environment. Although various liposome preparation methods including the Bangham method¹⁴, organic solvent injection method^{15,16}, reverse phase evaporation method¹⁷, and freeze/thaw method¹⁸ have been proposed, almost all of these methods need the use of organic solvent in a large amount and fail to yield liposomes having a large inner aqueous phase without using organic solvent¹⁹. The freeze/thaw method has been proposed as the only method to allow the organic solvent-free preparation of liposomes with a large inner aqueous phase¹⁸, but this method requires repeats of freezing and thawing, and hence, consumes a lot of energy and time. Hence, the development is demanded of the organic solvent-free preparation method of liposomes with a high trapping efficiency, when liposomes are to be used in the fields of medicines and cosmetics²⁰.

In these circumstances, we have paid attention to the characteristics of supercritical fluids; that is, minute changes in temperature and pressure cause an appreciable change in the solubility of substances in the liquid, and the transfer rates of mass and heat are high in it since the fluid is weakly viscous and highly diffusible²¹⁻²⁶. In particular, supercritical carbon dioxide (scCO₂) attracts attention as an environment-friendly substitute solvent around normal temperature³²⁻³⁴, because its critical temperature and pressure are 31.1°C and 73.8 bar²⁷, respectively, and its properties as solvent are similar to those of nonpolar solvents such as hexane²⁸⁻³¹. We have so far developed the supercritical reverse phase evaporation method (scCO₂RPE method)³⁵, a new method for liposome preparation. With the aid of this new method, we have succeeded in preparing liposomes with a significantly high trapping efficiency for water-soluble drugs with ease and in one step without using toxic organic solvent³⁶⁻³⁹. Meanwhile, various kinds of phospholipids with different chemical structures are found in the natural world⁴⁰⁻⁴² and the effects are still unknown of the chemical structure of hydrophobic groups of these phospholipids on the formation and properties of liposomes prepared by the scCO₂RPE method.

The present work aims to prepare liposomes by the scCO₂RPE method using various phospholipids with different hydrophobic groups, investigate the effects of the number of unsaturated bonds and alkyl chain length on the liposomal membrane properties, and find the molecular structure of phospholipid most favorable to liposome preparation.

2 EXPERIMENTAL

2.1 Chemicals

The saturated phospholipids used were L- α -dilaurylphosphatidylcholine (DLPC, C12:0, > 99%), L- α -dimyristoylphosphatidylcholine (DMPC, C14:0, > 99%), L- α -dipalmitoylphosphatidylcholine (DPPC, C16:0, 99.6%), and L- α -distearoyl phosphatidylcholine (DSPC, C18:0, 99.6%). The complex phospholipid used was 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, C16:0-C18:1, 99.6%). In addition, L- α -dioleoylphosphatidylcholine (DOPC, C18:1, 99.1%) and L- α -dielucoylphosphatidylcholine (DEPC, C22:1, 99.5%) were used as the unsaturated phospholipids. All of these lipid samples were obtained from NOF Corp. Distilled water for injection (Ohtsuka Pharm. K.K.) was used as the solvent. The water-soluble substance to be trapped in liposomes was D(+)-glucose (purity 98.0%, Wako Pure Chemicals, K.K.).

2.2 Preparation of liposomes by supercritical reverse phase evaporation method (scCO₂ RPE method)

Figure 1 shows schematically the apparatus for liposome preparation used in this work. The apparatus consists of a pressure variable-type view cell, heater, electronic thermoregulator, pressure gauge, CO₂ screw pump, magnetic stirrer, liquid chromatography (LC) pump, and electronic balance. The inner volume and limiting pressure tightness are 50 cm³ and 300 bar, respectively. Given amounts of phospholipid (0.3 wt% to carbon dioxide) and ethanol (7.0 wt%) were sealed into the front part of the cell, into which CO₂ (11.87 g) was introduced. The temperature and pressure of the cell were raised to 60°C and 91.7 bar, respectively. CO₂ was then introduced into the back part of the cell and the front part was pressurized by pushing forward the piston to bring the pressure of the content to 200 bar suitable for liposome preparation. A glucose solution to be trapped in liposomes was forcibly introduced into the cell using the pump for LC (0.10 mL/min) and the cell content was thoroughly stirred. A uniform liposome suspension was obtained in the cell by removing CO₂ from the cell after a given amount of water was introduced in it. The stirring of the cell content was done with the magnetic stirrer.

2.3 Measurement of trapping efficiency

The trapping efficiency for water-soluble substance is given by the following equation:

$$\text{Trapping efficiency (\%)} = \frac{(\text{Amount of glucose trapped in liposomes})}{(\text{Total amount of glucose in the system})} \times 100 \quad (1)$$

Trapping efficiency measurements were performed by the glucose dialysis method. A liposome dispersion was placed in a semipermeable membrane bag (Viskase Co. Inc) and

Fig. 1 Apparatus for Preparation of Liposomes by scCO₂ RPE Method.

glucose untrapped in liposomes was removed by dialysis at 5°C. Ethanol was added to the liposome solution after dialysis to disrupt liposomes. The amount of glucose released from liposomes was determined by the mutarotase GOD method^{43,44}.

2.4 Osmotic response measurement

Bimolecular lipid membranes are highly permeable to water while they are impermeable to ions and neutral low molecular weight solutes such as urea⁴⁵. Liposomes exhibit osmotic response through their shrinkage or dilation to quickly compensate the difference created in solute concentration between the inside and outside of the bimolecular membrane. Since poorly osmotically responsive liposomes hardly keep their closed vesicular structure and collapse, the osmotic response is an important index for evaluating the drug release behavior and stability of liposomes. Volume change with time of liposomes caused by their osmotic response is given by the following equation:

$$-dV/dt = K_w S R T (c_i^o - c_i^i) \quad (2)$$

where dV/dt is the rate of volume change with time, K_w the permeability coefficient to water, S the area occupied by bimolecular membrane, R the gas constant, T the absolute temperature, c_i^i and c_i^o are the concentrations of membrane-impenetrable solute in the inner and outer aqueous phases of liposomes, respectively. When an osmotic shock is given by adding a hypertonic solution into the outer aqueous phase of liposomes, the movement of water occurs from the inner aqueous phase to the outer aqueous phase to produce a shrinkage of liposomes. This shrinking behavior of liposomes can be pursued and estimated by measuring changes with time in the absorbance of liposome dis-

persion. Thus, when a hypertonic solution is added to liposome solution, the absorbance increases with time and then levels off. Since the absorbance immediately after osmotic shock changes linearly with the volume of liposomes, the following equation holds.

$$-d(1/A)/dt\% = \kappa dV/dt = \kappa K_w S R T (c_i^o - c_i^i) \quad (3)$$

where $d(1/A)/dt\%$ is the shrinkage rate immediately after osmotic shock and calculated from $(\Delta A_t/\Delta t)(100A_0)$, the limiting shrinkage rate⁴⁷.

A 1 mM liposome dispersion obtained by diluting the prepared 10 mM dispersion was used for osmotic response measurements. The measurement was started when 0.1 mL of 2M glucose solution was added to 10 mL of the 1 mM liposome dispersion after the temperature attained a constant value (37°C). Turbidity changes were pursued by measuring the absorbance of the dispersion at a wavelength of 450 nm.

2.5 Transmission electron microscopic observation by freeze-replica method

Freeze-replicas of liposomes were prepared using a freeze-replica preparing apparatus (FR-7000A, Hitachi Science System, K.K.).

A liposome dispersion was rapidly frozen in liquid propane and the frozen dispersion was cleaved in the vacuum apparatus at -150°C . Platinum was evaporated on the cleaved section at 45° to shade its fine structure unevenly. Carbon was then evaporated on the platinum-evaporated section at 90° to reinforce the unevenly evaporated metal film and make its replica. The replica thus prepared was transferred onto a copper grid after being washed and used as the specimen for TEM observations with a transmission

electron microscope (JEM-1200EX, JEOL) at an acceleration voltage of 80 kV.

3 RESULTS AND DISCUSSION

3.1 Trapping efficiency for liposomes prepared by Bangham method

Figure 2 shows the trapping efficiencies of liposomes prepared by the conventional Bangham method using saturated phospholipids. Even though the trapping efficiency increased with increasing phospholipid concentration, the maximum efficiency was low and stayed at about 6%. The efficiency depended on the alkyl chain length of phospholipid molecule following the order: DSPC (C18:0) > DPPC (C16:0) > DMPC (C14:0) > DLPC (C12:0). In particular, the efficiency of DLPC was practically zero. It is known that only phospholipid molecules with alkyl chain longer than C₁₀ can form liposomes. The reason for the finding that DLPC liposomes have no trapping ability while the lipid can form liposomes would be due to their lack of the ability as barrier to prevent solute release into the outer phase⁴⁸⁾. The trapping efficiency of DSPC liposomes was high compared with that of DPPC liposomes. DSPC can form larger liposomes than DPPC, since the former has a larger critical packing parameter (CPP) than the latter⁴⁹⁾. The higher trapping efficiency for DSPC would reflect the above situation.

Figure 3 shows the trapping efficiencies of liposomes prepared by the conventional Bangham method using DOPC (C18:1), DEPC (C22:1), and POPC (C16:0-C18:1), all of them being unsaturated phospholipids. Note here that no

trapping efficiency value has been reported for POPC liposomes. The trapping efficiencies obtained for the liposomes were in the order: DOPC (C18:1) > POPC (C16:0-C18:1) > DEPC (C22:1). The trapping efficiencies of liposomes formed by DOPC and POPC were found to be appreciably higher than those of liposomes formed by DPPC and DSPC. This difference would arise from the difference in the molecular packing in bimolecular liposomal membrane between the saturated and unsaturated phospholipids. Bimolecular membranes for DOPC and POPC are in L_α phase (liquid crystal phase) at 5°C, a dialysis condition in trapping efficiency measurement, while the membranes for DPPC and DSPC are in L_β phase (gel phase)⁵⁰⁾. It is also known that the size and volume of liposomes in L_α phase are larger than those in L_β phase⁵¹⁾. Hence, the trapping efficiencies for liposomes formed by unsaturated phospholipids become higher than those for liposomes formed by the corresponding saturated phospholipids. In contrast, the trapping efficiency of DOPC liposomes is higher than that of DEPC liposomes, the former unsaturated lipid having shorter alkyl chains than the latter. When a comparison is made in terms of CPP, the trapping efficiency is expected to be larger for DEPC liposomes than for DOPC liposome, which is contrary to the experimental results. If, instead, a comparison is made in terms of phase transition temperature, the trapping efficiency is expected to be higher for DOPC liposomes than for DEPC liposomes, because the transition temperature of the former lipid membrane is -22°C while that of the latter is 13°C, indicating that the former is in a liquid crystal phase while the latter is in a gel phase at the dialysis temperature. This would be the reason for the higher trapping efficiency of DOPC lipo-

Fig. 2 Trapping Efficiency of Liposomes Prepared by Bangham Method Using Various Phospholipids with Saturated Alkyl Chains.

Fig. 3 Trapping Efficiency of Liposomes Prepared by Bangham Method Using Various Phospholipids with Unsaturated Alkyl Chains.

somes.

3.2 Trapping efficiency for liposomes prepared by scCO₂RPE method

3.2.1 Liposomes prepared using saturated phospholipids

Figure 4 shows the trapping efficiencies for saturated phospholipid liposomes. The trapping efficiency for liposomes prepared by the scCO₂RPE method was remarkably higher than that for those prepared by the Bangham method. The size and number of bimolecular layers of liposomes affect most their trapping efficiency among various factors⁵². The trapping efficiency for vesicles prepared using the same phospholipid concentration is known to increase depending on the number of lamellae in the order: small unilamellar vesicle (SUV) < multilamellar vesicle (MLV) < large unilamellar vesicle (LUV) and the efficiency of SUV is extremely smaller than that of LUV⁵². Because the scCO₂RPE method yields unilamellar liposomes with a large inner aqueous phase³⁵, their trapping efficiency is markedly higher than that of liposomes prepared by the Bangham method.

The trapping efficiency of liposomes prepared by the scCO₂RPE method was found to depend on the alkyl chain length of phospholipid. Thus, the trapping efficiency was in the order: DPPC (C16:0) > DSPC (C18:0) > DMPC (C14:0) > DLPC (C12:0), indicating a general trend that the efficiency increases with increasing alkyl chain length of phospholipid. Yet, a comparison of DPPC with DSPC showed an efficiency higher for DPPC liposomes than for DPPC liposomes, in contrast to the order for liposomes prepared by the Bangham method. As mentioned before, the liposome preparation by the scCO₂RPE method was performed at

60°C and the phase state of phospholipid greatly affects liposome formation. The temperature at which DPPC undergoes the phase transition from gel to liquid crystal is 41°C, while DSPC changes its phase from gel to liquid crystal at 58°C, suggesting an incomplete phase transition for the latter at the temperature of liposome preparation, thereby making the formation of phospholipid bimolecular membrane inefficient at the supercritical CO₂/water interface. This would be the reason why the trapping efficiency for DSPC liposomes was lower than that for DPPC liposomes.

3.2.2 Liposomes formed from unsaturated phospholipids

Liposomes were prepared by the scCO₂RPE method using unsaturated phospholipids and their trapping efficiency was examined, and the results are shown in Fig. 5. The scCO₂RPE method greatly improved the trapping efficiency as is clear from the comparison of the above results with the data for liposomes prepared by the Bangham method given in Fig. 3. In particular, liposomes prepared using DOPC exhibited an extremely high trapping efficiency giving the maximum value of about 40%. This would indicate that the scCO₂RPE method yields unilamellar liposomes as in the case of liposomes prepared using saturated phospholipids. Moreover, since DOPC undergoes the phase transition at -22°C⁵¹ and stays in the liquid crystal phase at the temperature during dialysis, the volume per molecule of the lipid is large to make the trapping efficiency of liposomes high.

The following order of trapping efficiency was found for liposomes prepared using DPPC, DSPC, DOPC, and POPC, all of which have alkyl groups of similar lengths: DOPC > POPC > DPPC > DSPC. Thus, the trapping efficiency for

Fig. 4 Trapping Efficiency of Liposomes Prepared by scCO₂ RPE Method Using Various Phospholipids with Saturated Alkyl Chains.

Fig. 5 Trapping Efficiency of Liposomes Prepared by scCO₂ RPE Method Using Various Phospholipids with Unsaturated Alkyl Chains.

POPC liposomes was intermediate between those for DOPC and DPPC liposomes. The molecular structure of POPC with an unsaturated alkyl chain is also intermediate between those of DOPC and DPPC. That is, a trend is found that the larger the number of unsaturated alkyl groups in phospholipid molecule is, the higher is the trapping efficiency of liposomes prepared using the lipid. Unsaturated alkyl groups make the packing of phospholipid molecules in liposomal bimolecular membrane loose and they bend at the position of double bond to take an open structure, thereby raising the value of CPP and the trapping efficiency through formation of large liposomes.

3.3 Effect of alkyl group on osmotic response of liposomes

Osmotic response measurements were performed to evaluate the effect of the alkyl group of phospholipid molecule on the molecular packing in liposomal bimolecular membrane. The measurements were carried out at 20°C. Figure 6 shows the relation between the shrinkage rate of liposomes caused by osmotic shock and the molecular structure of phospholipid. With regard to saturated phospholipids, the osmotic response was lower for the lipid with longer alkyl groups, suggesting its closer molecular packing in the bimolecular membrane. Also, liposomes prepared by the scCO₂RPE method were found to osmotically respond much more strongly than those prepared by the Bangham method. Osmotic response of liposomes was greatly dependent on their particle size and the number of lamellae and the following order was found for liposomes

formed from the same phospholipid: SUV > LUV > MLV⁵³. Namely, liposomes with a larger radius of curvature and unilamellar structure tend to osmotically respond to a greater extent⁵⁴⁻⁵⁶. This would reflect the difference in the membrane permeability to water in the inner aqueous phase due to the difference in membrane structure of liposomes prepared by different methods.

The osmotic response for liposomes prepared by the scCO₂RPE method was also stronger than that for those prepared by the Bangham method when unsaturated phospholipids were used. With respect to POPC, DOPC, and DEPC, the lipid with a larger number of unsaturated alkyl groups or that with a shorter unsaturated alkyl group showed a stronger osmotic response.

3.4 Direct observations of liposomal membrane with transmission electron microscope (TEM)

TEM observations were conducted to verify the relationship between the membrane structure and trapping efficiency of liposomes. Figure 7(a)-(e) shows typical TEM images of liposomes prepared using various saturated phospholipids. Figure 7(a) is a TEM image of DPPC liposomes prepared by the Bangham method, indicating a multilamellar structure as evidenced by the presence of several steps. Multilamellar liposomes with diameters of about 1000 nm were observed independent of the alkyl chain length of phospholipid when they were prepared by the Bangham method. In contrast, the scCO₂RPE method yielded liposomes with diameters of 150-600 nm for DLPC

Fig. 6 The Initial Shrinkage Rate after Osmotic Shock for Various Liposomes Prepared by Bangham and scCO₂ RPE Methods.

Fig. 7 Transmission Electron Microscope (TEM) Images of Liposomes of Phospholipids with Saturated Alkyl Chains.

(Fig. 7(b)) and DMPC (Fig. 7(c)) liposomes and 300-1200 nm for DPPC (Fig. 7(d)) and DSPC (Fig. 7(e)) liposomes. No step-like structure was observed in these TEM images, showing the formation of unilamellar liposomes with a large inner aqueous phase. That is, the high trapping efficiency of liposomes prepared by the scCO₂RPE method was verified by the formation of large unilamellar liposomes.

Figure 8 shows typical TEM images of liposomes prepared by the scCO₂RPE method using unsaturated phospholipids. The Bangham method yielded multilamellar liposomes with diameters of about 1000 nm (Fig. 8(a)) as in the above case where saturated phospholipids were used, while the scCO₂RPE method gave much more unilamellar liposomes with smaller sizes compared with the case of saturated phospholipids.

Concerning the difference between saturated DPPC and DSPC and unsaturated POPC and DOPC, all with a similar length of alkyl group, POPC (Fig. 8(b)) and DOPC (Fig. 8(c)) liposomes prepared by the scCO₂RPE method were found in a number predominantly larger than that for DPPC and DSPC liposomes in TEM images. This demonstrates that the scCO₂RPE method allows to form a large number of small unilamellar liposomes when phospholipids with a high CPP are used, showing a high trapping efficiency and osmotic response.

Fig. 8 Transmission Electron Microscope (TEM) Images of Liposomes of Phospholipids with Unsaturated Alkyl Chains.

4 CONCLUSIONS

Liposomes were prepared by the supercritical reverse phase evaporation (scCO₂RPE) method using various phospholipids, and the effects were examined of the lipid structure on the membrane properties of liposomes obtained. The membrane properties were found to strongly depend on the number and chain length of unsaturated alkyl groups in phospholipid molecule. The optimum alkyl chain length was C₁₆ or C₁₈ because if the chain length was shorter or longer, the liposome hardly retained water-soluble substances or the osmotic response of liposomal membrane lowered. Liposomes prepared using phospholipids with two unsaturated alkyl groups exhibited an extremely high trapping efficiency and osmotic response. The scCO₂RPE method was thus found to yield unilamellar liposomes efficiently.

References

1. Balon, K.; Riebesehl, B.U.; Mueller, B.W. *J. Pharm. Sci.* **88** (8), 802 (1999).
2. Colic, M.; Moese, D. *Colloids Surf. A* **154** (1-2), 167 (1999).
3. Montero, M.T.; Hernandez-Borrell, J.; Keough, K.M.W. *Langmuir* **14** (9), 2451 (1998).
4. Komatsu, H.; Okada, S. *Biochim. Biophys. Acta* **1237** (2), 165 (1995).
5. Kaneda, Y. *Adv. Drug Delivery Rev.* **43** (2-3), 197 (2000).
6. Marjan, J.; Xie, Z.; Devine, D.V. *Biochim. Biophys. Acta* **1192** (1), 35 (1994).
7. Hayashi, A.; Nakanishi, T.; Nakagawa, S.; Mayumi, T. *Drug Delivery Syst.* **15** (1), 49 (2000).
8. Hashida, M. *Farumashia* **34** (3), 235 (1998).
9. Nishikawa, M.; Huang, L. *Drug Delivery Syst.* **15** (2), 80 (2000).
10. Perugini, P.; Genta, I.; Pavanetto, F.; Conti, B.; Scalia, S.; Baruffini, A. *Int. J. Pharm.* **196** (1), 51 (2000).
11. El-Maghraby, G.M.M.; Williams, A.C.; Barry, B.W. *J. Pharm. Pharmacol.* **51** (10), 1123 (1999).
12. Waranuch, N.; Ramachandran, C.; Weiner, N. *J. Liposome Res.* **9** (1), 139 (1999).
13. van Kuijk-Meuwissen, M.E.M.J.; Mougin, L.; Junginger, H.E.; Bouwstra, J.A. *J. Controlled Release* **56** (1-3), 189 (1998).
14. Bangham, A.D.; Standish, M.M.; Watkins, J.C. *J. Mol. Biol.* **13**, 238 (1965).
15. Batzri, S.; Korn, E.D. *Biochim. Biophys. Acta* **298**, 1015 (1973).
16. Demer, D.; Bangham, A.D. *Biochim. Biophys. Acta* **443**, 629 (1976).
17. Szoka, Jr. F.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A* **75**, 4194 (1978).
18. Azaki, K.; Yoshida, M.; Kirino, Y. *Biochim. Biophys. Acta* **1021**, 21 (1990).
19. Patric, L.A.; Chen, L.; Walter, R.P.; Sharama, R.M.; Lawrence, T.B.; Theodore, F.T.; Andrew, S.J. *Biochim. Biophys. Acta* **1195**, 237 (1994).
20. Stephen, P.; Michael, G.; Patric, W. *J. Pharm. Pharmacol.* **43**, 154 (1991).
21. Schneider, G.M.; Stahl, E.; Wilke, G.E. *Extraction with Supercritical Gases*, Verlag Chemie, Deerfield Beach, Florida (1980).
22. Randall, I.G. *Sep. Sci. Technol.* **17**, 1 (1982).
23. Paulaitis, M.E.; Krukonis, V.J.; Krunik, R.T.; Reid, R.C. *Rev. Chem. Eng.* **1**, 179 (1983).
24. McHugh, M.A.; Krukonis, V. *Supercritical Fluid Extraction : Principles and Practice*, 2nd ed. Butterworth-Heinemann, Stoneham, M.A. (1993).
25. Stahl, E.; Quirin, K.W.; Gerard, D. *Dense Gas for Extraction and Refining*, Springer-Verlag, New York (1987).
26. Charpentier, B.A.; Sevenants, M.R. *ACS Symposium*

- Series 366 : Supercritical Fluid Extraction and Chromatography*, ACS, Washington, DC (1988).
27. Span, R.; Wagner, R. *J. Phys. Chem. Ref. Data* **25**, 1509 (1996).
 28. Drake, B.D.; Smith Jr., R.L. *J. Supercrit. Fluids* **3**, 162 (1990).
 29. Micjels, M.; Kleerekoper, L. *Physic.* VI, **586** (1939).
 30. Iwasaki, H.; Tarahashi, M. *J. Chem. Phys.* **74**, 1930 (1981).
 31. Vesovic, V.; Wakeham, W.A.; Colchowy, G.A.; Sengers, J.V.; Watson, J.T.; Millat, R.J. *J. Phys. Chem. Ref. Data* **19**, 763 (1990).
 32. Brennecke, J.F.; Chateaneuf, J.E. *Chem. Rev.* **99**, 433 (1999).
 33. Baiker, A. *Chem. Rev.* **99**, 453 (1999).
 34. Jessop, P.G.; Ikariya, T.; Noyori, R. *Chem. Rev.* **99**, 475 (1999).
 35. Otake, K.; Imura, T.; Sakai, H.; Abe, M. *Langmuir* **17**, 3898 (2001).
 36. Imura, T.; Otake, K.; Hashimoto, S.; Gotoh, T.; Yuasa, M.; Yokoyama, S.; Sakai, H.; Rathman, J. F.; Abe, M. *Colloids Surf. B: Biointerfaces* **27**, 133 (2002).
 37. Imura, T.; Gotoh, T.; Otake, K.; Yoda, S.; Takebayashi, Y.; Yokoyama, S.; Takebayashi, H.; Sakai, H.; M. Abe, *Langmuir* **19**, 2021 (2003).
 38. Imura, T. Gotoh, T.; Yoda, S.; Otake, K.; Takebayashi, K.; Yokosuka, M.; Sakai, H.; Abe, M. *Material Technol.* **21** (1), 30 (2003).
 39. Otake, K.; Imura, T.; Abe, M. *Hyomen* **40** (10), 368 (2002).
 40. Day, E.A.; Kwok, K.S.; Hark, K.S.; Ho, J.T.; Vail, W.J.; Bentz, J.; Nir, S. *Proc. Natl. Acad. Sci. U.S.A* **77**, 4026 (1980).
 41. Ohki, S.; Düzgünes, N.; Leonards, K. *Biochem.* **21**, 2127 (1982).
 42. Ohki, S.; Roy, S.; Ohshima, H.; Leonards, K. *Biochem.* **23**, 6126 (1984).
 43. Miwa, I.; Okuda, J.; Maeda, K.; Okuda, G. *Clin. Chim. Acta* **37**, 538 (1972).
 44. Okuda, J.; Miwa, I. *PNE* **17**, 216 (1972).
 45. Bangham, A.D.; De Gier, J.; Greville, G.D. *Chem. Phys. Lipids* **1**, 225 (1967).
 46. Kedem, O.; Katchalsky, A. *Biochim. Biophys. Acta* **27**, 229 (1958).
 47. De Gier, J.; Mandersloot, G.D.; van Deenen, L. *Biochim. Biophys. Acta* **150**, 666 (1968).
 48. Nojima, S.; Sunamoto, J.; Inoue, K., *Liposomes*, p.114, Nankodo Publish (1988).
 49. Ijeoma, F.U.; Suresh, P.V. *Int. J. Pharm.* **172**, 33 (1998).
 50. Komatsu, H.; Okada, S. *Biochim. Biophys. Acta* **1283**, 73 (1996).
 51. Komatsu, H.; Okada, S. *Chem. Phys. Lipids* **85**, 67 (1997).
 52. Perkins, W.P.; Minchey, S.R.; Ahl, P.L.; Janoff, A.S. *Chem. Phys. Lipids* **64**, 197 (1993).
 53. Yoshikawa, W.; Akutsu, H.; Kyogoku, Y.; Akamatsu, Y. *Biochim. Biophys. Acta* **821**, 277 (1985).
 54. Bisset, P.; Wolff, G.; Albrecht, A.M.; Tanaka, T.; Nakatani, Y.; Ourisson, G. *Biochim. Biophys. Res. Commun.* **11**, 320 (1983).
 55. Sun, S.T.; Milon, A.; Tanaka, T.; Ourisson, G.; Nakatani, Y. *Biochim. Biophys. Acta* **860**, 525 (1986).
 56. Hanz, E.; Cao, A.; Escaig, J.; Taillandier, E. *Biochim. Biophys. Acta* **862**, 349 (1986).