Novel Cubosome System Resistant to Lipid Removal by Serum Albumin

Makoto Uyama, Tetsurou Handa, and Minoru Nakano*

Introduction

Lipidic bicontinuous cubic phases are optically isotropic liquid crystalline phases with a three-dimensional network of water channels surrounded by lipid bilayers with a minimal surface. These cubic phases have attracted researchers' attention as soft materials for controlled release of pharmaceutically or cosmetically active agents. 1-Monoolein (MO, Fig. 1(A)) is a well-known cubic phase-forming lipid and it forms bicontinuous cubic phases in the presence of excess water.

We have previously reported that cubosomes consisting of MO and Pluronic F127 are revealed to be stable during storage or in blood have yet to be studied. 9–17) (Fig. 1(E)). Pluronic F127 reduces the cubic phase-forming lipids in the presence of stabilizers, typically or cosmetically active agents. 1-Monoolein (MO, Fig. 1(A)) is a well-known cubic phase-forming lipid and it forms bicontinuous cubic phases in the presence of excess water.

 properties suggest that the cubic phases and their dispersions in water, termed cubosomes (Fig. 2), are promising systems that can incorporate both hydrophilic and hydrophobic drugs. Cubosomes are formed by emulsifying the cubic phase-forming lipids in the presence of stabilizers, typically Pluronic F127 9–17) (Fig. 1(E)). Pluronic F127 reduces the surface tension between the aqueous and organic phase and thereby stabilize the nanoparticle suspension formation.

The interior of cubosomes is constituted of a bicontinuous cubic phase, whereas their water–particle interface is covered by vesicular structures, which are connected with the interior by interlamellar attachments. Properties and potential of cubosomes have been recently reviewed elsewhere. 20–22)

Although cubosomes have been proposed as novel carriers for drug delivery systems, important issues such as the stability of particles during storage or in blood have yet to be solved before clinical application. Leesajakul et al. revealed that cubosomes consisting of MO and Pluronic F127 are rapidly disintegrated after intravenous injection into rats and attributed this phenomenon to the uptake of MO by serum albumin, the most abundant protein in blood. 25) Instability of the MO-based cubosomes in plasma was also confirmed by the observation of the internal structural changes. 26) It is therefore important to reduce cubosome interaction with albumin to improve their stability in blood.

MacDonald and colleagues reported that 1,2-dioleoyl-sn-glycerol-3-hexylphosphocholine (hexyl-DOPC) and compared their interaction with bovine serum albumin (BSA), the most abundant protein in plasma, with that of conventional cubosome systems consisting of several bicontinuous cubic phase-forming lipids, including 1-monoolein (MO), 1-O-(5,9,13,17-tetramethyloctadecanoyl)erythritol (EROCO C22), or 1-O-(5,9,13,17-tetramethyloctadecyl)-β-D-xylopyranoside (β-XP). The average number of lipids bound to each BSA molecule was between 1.2–4.0 for MO, EROCO C22, and β-XP. On the other hand, hexyl-DOPC exhibited negligible binding to BSA. This result suggests that hexyl-DOPC, which was shown to resist removal from particles by BSA, can be used as a new lipid component of cubosomes, and has higher plasma stability than the other cubic phase-forming lipids.

Key words  bicontinuous cubic phase; monoolein; hexylphosphocholine; serum albumin

Experimental

MO was provided by NOF Corp. (Tokyo, Japan). EROCO C22 and β-XP were gifts from CytoPathfinder, Inc. (currently Farnex Inc., Yokohama, Japan), the synthesis of which has been previously described. 5,7,32) DOPC was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Pluronic F127 was supplied by BASF Japan Ltd. (Osaka, Japan). BSA (essentially fatty acid-free and essentially globulin-free, ≥99% purity) was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All the materials were used as received.

Synthesis of Alkylated DOPC 1,2-Dioleoyl-sn-glycero-3-hexylphosphocholine, 1,2-dioleoyl-sn-glycero-3-heptylphosphocholine, and 1,2-dioleoyl-sn-glycero-3-octylphosphocholine (hexyl-, heptyl-, and octyl-DOPC, respectively) were synthesized by a method modified from that previously reported 28,30). DOPC (0.79 g, 1.0 mmol) in CHCl3 was added to flasks and the solvent was evaporated and dried in vacuum. Tetra...
hydrofuran (4.0 mL) and 1-iodohexane, 1-iodoheptane, or 1-iodooctane (2.0 mmol) were added to these flasks, and the reaction mixture was stirred overnight at 50°C under argon. The solvent was evaporated and the crude product was purified using silica gel flash chromatography eluting with a gradient of 0–10% MeOH/CHCl₃ to afford pure product (50% yield): ¹H-NMR (CDCl₃, 400 MHz) δ (ppm): 5.34–5.27 (q, 4H), 5.26–5.20 (m, 1H), 4.58–4.00 (m, 10H), 3.56–3.49 (s, 9H), 2.34–2.27 (m, 4H), 2.01–1.94 (m, 8H), 1.69–1.62 (q, 2H), 1.60–1.54 (m, 4H), 1.30–1.20 (m, 38–42H), 0.90–0.81 (m, 9H); TLC Rₚ 0.67 (CHCl₃:MeOH:H₂O: 60:30:4); the exact mass calculated for [C₅₀H₉₇NO₈P]⁺ requires m/z 870.6952, observed 870.6955 (positive fast ion bombardment, FAB+), [C₅₁H₉₉NO₈P]⁺ requires m/z 884.7108, observed 884.7108 (FAB+), [C₅₂H₁₀₁NO₈P]⁺ requires m/z 898.7265, observed 898.7252 (FAB+).

Preparation of Nondispersed and Dispersed Phases Nondispersed lipid phases were prepared by hydrating lipids with an excess amount of phosphate buffer (50 mM phosphate, pH 7.4). For the preparation of dispersed phases (i.e., cubosomes), the cubic phase-forming lipids (MO, EROCO C22, β-XP, or hexyl-DOPC) were hydrated with the same buffer containing Pluronic F127 at a lipid-to-polymer weight ratio of 10:1. The mixtures were first roughly dispersed in two Hamilton syringes that were connected with each other by a luer lock adaptor (GL Sciences, Tokyo, Japan), and then extruded through a 200-nm pore size polycarbonate filter using a Lipo-soFast extruder (Avestin Inc., Ottawa, Canada). Particle size (mean hydrodynamic diameter) was measured by dynamic light scattering (DelsaMax CORE, Beckman Coulter Inc., Indianapolis, IN, U.S.A.).

Small-Angle X-Ray Diffraction The hexyl-DOPC/F127 dispersion or alkylated DOPC hydrated with phosphate buffer (50 mM phosphate, pH 7.4) was put into a thin-walled glass capillary (W. Müller, Berlin, Germany; 1.5 mm o.d., 1/100 mm wall thickness). Small-angle X-ray diffraction measurements were carried out as described previously.₃₃ Binding of Cubic Phase-Forming Lipids with BSA To determine the BSA binding properties of the cubic phase-forming lipids, excess amounts (approx. 0.1 g) of lipids were hydrated with 0.9 mL of phosphate buffer (50 mM phosphate, pH 7.4) containing 10 wt% BSA and incubated for 0.5 or 24 h at 37°C. After the incubation, the hydrated lipid phase was removed by filtration. From the resulting 200 µL of filtrate, 10 µL was withdrawn and used for protein quantification. The rest of the filtrate (190 µL) was freeze-dried and lipids were extracted by CHCl₃.

For the dispersed system, cubosome dispersion (1 mL) containing approx. 8 mM lipids was mixed with BSA (final concentration of 10 wt% (approx. 1.5 mM)) and incubated for 0.5 h at 37°C. Prior to ultracentrifugation, NaBr solution (1 mL) was added to adjust the density of the solution to approximately 1.2 g/cm³. Cubosomes were floated by ultracentrifugation at 452000 × g for 30 min at 25°C with a Himac CS100EX (Hitachi, Ltd., Tokyo, Japan), and the bottom (BSA) fraction (400 µL) was recovered. From the bottom fraction, 10 µL was withdrawn and used for protein quantification. The rest (390 µL) was freeze-dried and lipids were extracted by CHCl₃.

Among the lipids extracted by the methods described above, MO, EROCO C22, and β-XP were quantified using a TLC–hydrogen flame ionization detector (Iatroscan MK-5; Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine was used as an internal standard. Hexyl-DOPC was determined by phosphorus analysis as described previously.₃₅ BSA concentration was determined by the Lowry method.₃₆

Results and Discussion

Hexyl-DOPC Can Be a Component in the Formation of Cubic Phase and Cubosome Figure 3 shows the X-ray diffraction patterns of liquid crystalline phases formed in alkylated DOPC/buffer binary systems. Hexyl-DOPC showed diffraction peaks that correspond to the Pn3m space group (Fig. 3(a)). This is in good agreement with results reported by MacDonald and colleagues,₂₇–₃₁ indicating that hexyl-DOPC forms a bicontinuous cubic phase (C₀ phase, Fig. 2). Heptyl- and octyl-DOPC represented four and three peaks,
respectively, originating from the inverted hexagonal phase (Fig. 3(b) and (c)). Therefore, elongation of alkyl chains makes the spontaneous curvature more negative, resulting in a structural change from the bicontinuous cubic phase to the inverted hexagonal phase.

Although the phospholipid lamellar phase can disperse stably in the aqueous medium as vesicles, bicontinuous cubic phases cannot. Instead, Pluronic F127 has been reported to emulsify the cubic phases to form cubosomes. Hence, in this study, we dispersed the cubic phase with Pluronic F127 at a hexyl-DOPC:Pluronic F127 weight ratio of 10:1. Using a LiposoFast extruder, a dispersion with mean hydrodynamic diameter of approx. 220 nm was prepared. This preparation method does not need a high-pressure emulsifier, which is usually used in the preparation of cubosomes. This is advantageous in that a small amount (approx. 0.5 mL) of preparation volume is possible. The X-ray diffraction pattern of the dispersed system of the hexyl-DOPC/Pluronic F127 buffer system (Fig. 4), suggesting the formation of cubosomes with their interior consisting of the C3d phase. We did not observe diffraction of the Im3m space group (Cp phase); Pluronic F127 has been reported to affect the cubic phases of MO, β-XP, as well as EROCO C22, and change the diffraction from the Pn3m to the Im3m space group. The absence of the structural change implies low compatibility (i.e., little interaction) of hexyl-DOPC with Pluronic F127. Thus, Pluronic F127 does not disturb the structure of the hexyl-DOPC cubic phase but adsorbs exclusively on the particle surface to act as an emulsifier.

**BSA Does Not Bind Hexyl-DOPC**

It has been suggested that serum albumin binds MO and rapidly disintegrates MO-containing cubosomes. Hence, we investigated the interaction of BSA with several cubic phase-forming lipids, i.e., MO, EROCO C22, β-XP, and hexyl-DOPC. First, BSA was incubated with nondispersed cubic phases for 0.5 or 24 h at 37°C. As shown in Fig. 5(a), each BSA molecule bound approximately 3 molecules of MO, irrespective of the incubation time, sug-

---

**Fig. 3. X-Ray Diffraction Patterns of Nondispersed Systems Consisting of Hexyl-DOPC/buffer (a), Heptyl-DOPC/Buffer (b), or Octyl-DOPC/Buffer (c)**

The scattering vector \( q \) is given by \( q = \frac{4\pi \sin(\theta/2)}{\lambda} \), where \( \theta \) is the diffraction angle and \( \lambda \) is the X-ray wavelength.

**Fig. 4. X-Ray Diffraction Patterns from Nondispersed Hexyl-DOPC/Buffer System (a) and Dispersed Hexyl-DOPC/Pluronic F127/Buffer System (b)**

Note that (a) is identical to Fig. 3(a).

**Fig. 5. Lipid Binding Experiments**

(a) Cubic phases with excess amount of MO, EROCO C22, β-XP, or hexyl-DOPC were incubated with BSA (approx. 1.5 mM) for 0.5 or 24 h at 37°C, and the numbers of lipid molecules bound to BSA were quantified. “n.d.” represents “not detected.” (b) Cubosomes consisting of MO, EROCO C22, β-XP, or hexyl-DOPC (approx. 8 mM) were incubated with BSA (approx. 1.5 mM) for 0.5 h at 37°C, and the numbers of lipid molecules bound to BSA were quantified. (Color figure can be accessed in the online version.)
serum albumin (HSA) binds approximately two or three molecules of MO, EROCO C22, and Thumser et al. have shown that human serum albumin (HSA) binds approximately two or three molecules of MO at sites distinct from oleic acid. Our data are in good accordance with these results. Because EROCO C22 and β-XP have a branched alkyl chain, we expected that BSA does not bind these lipids. However, BSA still bound them, although the ratios of binding lipids (1.3–2.1) were smaller than that for MO. These results suggest that serum albumin is more or less able to bind single-chain lipids. On the contrary, BSA did not bind hexyl-DOPC. Hexyl-DOPC has double acyl chains and a short alkyl group, therefore BSA was deemed to have no ability to bind such a bulky lipid. Indeed, it has been shown that the binding affinity of BSA for double-chain lipids, such as 1,2-dioleoyl-3-(trimethylammonium)propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) is in the order of $10^8 \text{M}^{-1}$, whereas that of oleic acid is in the order of $10^3 \text{M}^{-1}$. 

We also evaluated the lipid binding properties of BSA using dispersed systems (cubosomes), and the results were nearly identical. From the binding data shown in Fig. 5(b), it is estimated that a large proportion (38–75%) of the single-chain lipids (MO, EROCO C22, and β-XP) in cubosomes was taken up by BSA. This suggests that Pluronic F127 on the surface of the particles cannot protect from the lipids from removal by BSA. On the other hand, BSA did not extract hexyl-DOPC from cubosomes. We also analyzed the particle size distribution of cubosomes consisting of hexyl-DOPC after incubation with BSA. Dynamic light scattering revealed that incubation with BSA tended to slightly increase the size of the cubosomes, with the appearance of new smaller (approx. 30-nm) particles (Fig. 6). This implies that the interaction of BSA can occur with the particles, in particular with Pluronic F127, which needs to be clarified further in the future.

**Conclusion**

X-Ray diffraction demonstrated that hexyl-DOPC forms a cubic phase, whereas heptyl-, octyl-DOPC forms an inverted hexagonal phase. We reported here for the first time that in addition to other cubic phase-forming lipids, hexyl-DOPC's cubic phase can be also dispersed by using Pluronic F127 to form cubosomes. We also demonstrated that BSA does not bind hexyl-DOPC at all, presumably because of its much bulkier structure relative to the other single-chain lipids. Hence, a cubosome system consisting of hexyl-DOPC can be a potential candidate for a drug delivery carrier. It is necessary to further clarify the interaction of hexyl-DOPC-containing cubosomes with other plasma components as well as their in vivo behavior, which will be described in a future paper.

**Acknowledgments** This study was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (JP17H02941). We thank Dres. Takeo Kawabata, Kyoto University, Takumi Furuta, Kyoto Pharmaceutical University, and Tomoyuki Yoshimura, Kanazawa University, for their assistance in synthesis of alkylated phospholipids.

**Conflict of Interest** The authors declare no conflict of interest.

**References**

1) Hyde S. T., Andersson S., Ericsson B., Larsson K., Z. Kristallogr., 168, 213–219 (1984).
2) Qin H., Caffrey M., Biomaterials, 21, 223–234 (2000).
3) Cioglosj J., Rathman J., Tomasko D., Walker H., Caffrey M., Chem. Phys. Lipids., 107, 191–220 (2000).
4) Ljusberg-Wahren H., Herslöf M., Larsson K., Chem. Phys. Lipids, 33, 211–214 (1983).
5) Yamashita J., Shiono M., Hato M., J. Phys. Chem. B, 112, 12286–12296 (2008).
6) Hato M., Minamikawa H., Salkar R. A., Matsutani S., Langmuir, 18, 3425–3429 (2002).
7) Hato M., Yamashita I., Kato T., Abe Y., Langmuir, 20, 11366–11373 (2004).
8) Barauskas J., Landth T., Langmuir, 19, 9562–9565 (2003).
9) Abraham T., Hato M., Hirai M., Colloids Surf. B Biointerfaces, 35, 107–117 (2004).
10) Abraham T., Hato M., Hirai M., Biotechnol. Prog., 21, 255–262 (2005).
11) de Campo L., Yaghmur A., Sagalowicz L., Leser M. E., Ljusberg-Wahren H., Glatter O., Langmuir, 20, 5254–5261 (2004).
12) Dong Y. D., Larson I., Hanley T., Boyd B. J., Langmuir, 22, 9512–9518 (2006).
13) Gustafsson J., Ljusberg-Wahren H., Almgren M., Larsson K., Langmuir, 12, 4611–4613 (1996).
14) Gustafsson J., Ljusberg-Wahren H., Almgren M., Larsson K., Langmuir, 13, 6064–6071 (1997).
15) Landth T., J. Phys. Chem., 98, 8452–8467 (1994).
16) Nakano M., Sugita A., Matsuoka H., Handa T., Langmuir, 17, 3917–3922 (2001).
17) Yaghmur A., de Campo L., Sagalowicz L., Leser M. E., Glatter O., Langmuir, 22, 9919–9927 (2006).
18) Snea R., Hari B. N. V., Devi D. R., Colloid and Interface Science Communications, 27, 49–59 (2018).
19) Demurtas D., Guichard P., Martiel I., Mezzenga R., Hebert C., Sagalowicz L., Nat. Commun., 6, 8915 (2015).
20) Barriga H. M. G., Holme M. N., Stevens M. M., Angew. Chem. Int. Ed., 58, 2958–2978 (2019).
21) Molly B. A., Prasanthi N. L., International Journal of Pharmacetical Sciences and Research, 10, 973–984 (2019).
22) Karami Z., Hamidi M., Drug Discov. Today, 21, 789–801 (2016).
23) Chountoulesi M., Pippa N., Pispas S., Chrysina E. D., Forys A., Trzebicka B., Demetzos C., Int. J. Pharm., 550, 57–70 (2018).
24) Akbar S., Anwar A., Ayish A., Elliott J. M., Squires A. M., Eur. J.
25) Leesajakul W., Nakano M., Taniguchi A., Handa T., Colloids Surf. B Biointerfaces, 34, 253–258 (2004).
26) Bode J. C., Kuntsche J., Funari S. S., Bunjes H., Int. J. Pharm., 448, 87–95 (2013).
27) Koynova R., Wang L., MacDonald R. C., Mol. Pharm., 5, 739–744 (2008).
28) MacDonald R. C., Rakhmanova V. A., Choi K. L., Rosenzweig H. S., Lahiri M. K., J. Pharm. Sci., 88, 896–904 (1999).
29) Rakhmanova V. A., McIntosh T. J., MacDonald R. C., Cell. Mol. Biol. Lett., 5, 51–65 (2000).
30) Rosenzweig H. S., Rakhmanova V. A., McIntosh T. J., MacDonald R. C., Bioconjug. Chem., 11, 306–313 (2000).
31) Koynova R., Tenchov B., MacDonald R. C., ACS Biomaterials Science & Engineering, 1, 130–138 (2015).
32) Minamikawa H., Murakami T., Hato M., Chem. Phys. Lipids, 72, 111–118 (1994).
33) Cheng A. H., Hummel B., Qiu H., Caffrey M., Chem. Phys. Lipids, 95, 11–21 (1998).
34) Nakano M., Nakatani Y., Sugita A., Kamo T., Natori T., Handa T., Langmuir, 19, 4604–4608 (2003).
35) Fiske C. H., Subbarow Y., J. Biol. Chem., 66, 375–400 (1925).
36) Lowry O., Rosebrough N., Farr A., Randall R., J. Biol. Chem., 193, 265–275 (1951).
37) Uyama M., Nakano M., Yamashita J., Handa T., Langmuir, 25, 4336–4338 (2009).
38) Duff S. M., Kalambur S., Boyle-Roden E., J. Nutr., 131, 774–778 (2001).
39) Thumser A. E. A., Buckland A. G., Wilton D. C., J. Lipid Res., 39, 1033–1038 (1998).
40) Charbonneau D. M., Tajmir-Riahi H. A., J. Phys. Chem. B, 114, 1148–1155 (2010).
41) van der Vusse G. J., Drug Metab. Pharmacokinet., 24, 300–307 (2009).