Preparation of Powdered Phospholipid Nanospheres by Spray Drying in an Aqueous System with Sugars

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Phospholipid nanosphere dispersion was prepared from phosphatidylcholine with or without vitamin E by the heating method. The dispersed particles had diameters of 8.6—150 nm. These nanosphere dispersions were powdered with sugars such as sucrose, lactose and mannitol by spray drying in an aqueous system. The resultant powders, except cofmulation with mannitol, consisted of spherical, homogenous and freely flowing particles, in which the sugar was amorphous. The powder yielded a nanosphere dispersion having almost the same particle size and optical density as the original dispersion, when rehydrated with water. The particle size of rehydrated nanospheres with vitamin E increased with the increasing amount of vitamin E cofomulated. A water-soluble drug, 5-fluorouracil, could be entrapped in the nanospheres by rehydrating the powdered nanospheres with an aqueous solution of the drug. All the steps from the preparation of the original nanosphere dispersion to spray drying were performed in an aqueous system without using any organic solvent. The procedures described here should be suitable for the production of stable powdered nanosphere which can be rehydrated to form phospholipid nanosphere dispersions as required.

Keywords phospholipid nanosphere; powdering; spray drying; aqueous system; sucrose

Introduction

Applications of phospholipids as drug carriers, i.e., in the form of liposomes and lipid microspheres, have been extensively investigated recently. To produce marketable aqueous phospholipid dispersions, several formulating problems, including fusion and aggregation in the system during storage, must be overcome. Further, a mass-production method which yields a product with reproducible and stable physicochemical properties must be developed.

Proliposomes have been developed by Payne et al.1,2 to avoid these problems. In their system, the phospholipid and drug are coated on a water-soluble carrier in an organic solvent system. They prepared free-flowing granules, which produced a liposomal suspension when hydrated. Alternatively, proliposomes have been prepared by coating core particles with an organic solution of soybean phosphatidylcholine (PC) by using a fluidized bed granulator3,4 or by spray drying an organic solution of PC and a lipophilic drug or dispersed core material.5,6 These techniques require the use of organic solvents, therefore the removal of residual solvents in the proliposome remains a problem to be solved. Furthermore, the liposomal suspensions obtained by hydration of proliposomes have large vesicle sizes of 200—1400 nm, which are unfavorable for injections.

In the present study, to overcome these problems, we prepared powdered phospholipid drug carriers by using an aqueous system. When rehydrated, the powdered carriers yielded PC dispersions that would be acceptable for dispensation. The phospholipid dispersions for powdering were prepared in an aqueous system by the heating method.7 The resultant aqueous dispersions were powdered by spray drying them directly; this method can minimize heat damage of the materials8 and produce spherical particles.

Sugars such as sucrose,9 trehalose,10,11 etc., used as cryoprotectants of liposomes during lyophilization, were added to the feed liquid to prevent fusion or aggregation of the phospholipid nanospheres during spray drying, as described.5,8 In order to find proper sugars that would allow regeneration of phospholipid dispersions having the same characteristics as the original ones before drying, physicochemical properties of the rehydrated phospholipid dispersions with various sugars were investigated.

Experimental

Materials

Phospholipids used were soybean PC, egg yolk PC and partially hydrogenated soybean PC (HyPC). Several kinds of soybean PC with different iodine values (I.V.) were used, i.e., Lecithin S-10EX (I.V.: 10.4), Lecithin S-30EX (I.V.: 28.8), Lecithin S-40EX (I.V.: 37.6), Lecithin S-50EX (I.V.: 51.3) and Epikuron 200 (I.V.: 94—99). The I. V. and phase transition temperature of HyPC are 39.7 and 9.5 °C, respectively. The acyl chain composition of HyPC was 13.2% palmitate, 27.4% stearate, 59.2% oleate and 0.2% others. HyPC contains a small percentage of phosphatidylethanolamine and cholesterol. The acid value of the phospholipid was less than 3.0mg-KOH/g. Soybean PC and egg yolk PC were supplied by Nikko Chemical Co. (Tokyo, Japan) and HyPC was supplied by Ajinomoto Co. (Tokyo, Japan).

Sugars used were d-sucrose, d-lactose, d-mannitol, d-sorbitol and d-glucose. These sugars were purchased from Kishida Chemical Co. (Osaka, Japan). Tocopherol acetate (VE, Eisai Co., Tokyo, Japan) and 5-fluorouracil (5-FU, Daiei-Koeki Co., Toyama, Japan) were used respectively as lipophilic and water-soluble drugs to be entrapped.

Preparation of Phospholipid Nanosphere Dispersion by Heating Method

Phospholipid nanosphere dispersion was prepared by the heating method. 5 Phospholipid (6.0mmol) with or without VE (6.0mmol) was added to distilled water (100ml) at 60 °C. The system was homogenized for 1 min at 15000rpm by a Physcotron NS-50 (Nittoh i-Rikakkai Co., Chiba, Japan) and sonicated at 90W for 30min with a probe-type sonicator, UR-200P (Tomy Seiko Co., Tokyo, Japan). Sugar (5g) was added to the dispersion. After further sonication (90W, 10min) and addition of sugar (95g), the volume of the dispersion was adjusted to 2000ml by the addition of water. During these procedures, the system was maintained at 60 °C.

When entrapping the water-soluble drug, 5-FU, in rehydrated nanospheres, total sonication time was altered to 20min to prepare slightly larger original phospholipid nanospheres before drying.

Powdering of Phospholipid Nanospheres by Spray Drying

The phospholipid nanosphere dispersion, maintained at 60 °C, was fed into a spray drier, model L-12 (Ohtkawara Kakohki Co., Yokohama, Japan), equipped with a rotating disk atomizer, spraying feed liquid into drying air. Spray drying conditions were as follows: spray rate, 30ml/min; rotational velocity of the atomizer, 15000rpm; inlet and outlet air temperatures, 140 °C and 100 °C, respectively.

Physicochemical Properties of Powdered Nanospheres

Powdered nanospheres were dispersed into dichloromethane, and their particle sizes were measured by using a laser-based time of transition system, Cis-1

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the powdered nanospheres with 10 ml of 0.5% 5-FU aqueous solution. Free drug and nanosphere fractions were separated by gel filtration, and the percentage of the drug entrapped in the nanospheres was calculated on the basis of spectrophotometric measurement of the drug content at 265 nm in each HPLC fraction. Five % Triton X-100 was used as a solubilizing agent. Gel filtration and HPLC were performed with an LC-9A apparatus (Shimadzu Co., Kyoto, Japan). The operating conditions of gel filtration were as follows: column, Shodex OHpak B-805 (8 mm i.d. x 500 mm l., Japan Spectroscopic Co., Tokyo, Japan); mobile phase, water. The conditions of HPLC were as follows: column, TSK gel ODS-120T (4.6 mm i.d. x 7.5 cm l., Tosoh Co., Tokyo, Japan); mobile phase, 0.01 M potassium dihydrogen phosphate solution; elution rate, 1.5 ml/min.

Results and Discussion

Physicochemical Properties of Phospholipid Nanosphere Dispersion for Spray Drying

Phospholipid dispersions were prepared with various phospholipids and sugars as listed in Table I, which shows the particle diameters ($d_w$) and optical densities (OD_{550}) of the dispersions.

Particle sizes and optical densities were remarkably small when the phospholipid dispersions were prepared with HyPC. Dispersions prepared with other phospholipids contained large particles more than 47 nm in diameter. Therefore, HyPC was employed as a phospholipid for the

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Sugar</th>
<th>$d_w$ (nm)</th>
<th>OD_{550}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecinol S-10EX</td>
<td>Sorbitol</td>
<td>51.1</td>
<td>0.044</td>
</tr>
<tr>
<td>Lecinol S-30EX</td>
<td>Sorbitol</td>
<td>50.1</td>
<td>0.043</td>
</tr>
<tr>
<td>Lecinol S-40EX</td>
<td>Sorbitol</td>
<td>48.8</td>
<td>0.037</td>
</tr>
<tr>
<td>Lecinol S-50EX</td>
<td>Sorbitol</td>
<td>47.4</td>
<td>0.025</td>
</tr>
<tr>
<td>Epikuron 200</td>
<td>Sorbitol</td>
<td>53.7</td>
<td>0.035</td>
</tr>
<tr>
<td>HyPC</td>
<td>Sorbitol</td>
<td>8.6</td>
<td>0.021</td>
</tr>
<tr>
<td>Egg yolk PC</td>
<td>Sucrose</td>
<td>134.9</td>
<td>0.907</td>
</tr>
<tr>
<td>Epikuron 200</td>
<td>Sucrose</td>
<td>55.8</td>
<td>0.072</td>
</tr>
<tr>
<td>HyPC</td>
<td>Sucrose</td>
<td>11.3</td>
<td>0.034</td>
</tr>
<tr>
<td>HyPC</td>
<td>—</td>
<td>18.2</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Fig. 1. Electron Micrographs of HyPC Nanosphere Dispersions Prepared with Sucrose, and with or without VE

(A) Phospholipid nanospheres prepared with VE. (B) Phospholipid nanospheres prepared without VE.
preparation of powdered nanospheres.

Although HyPC has a similar iodine value to Lecinol S-40EX, the dispersions prepared with HyPC were composed of much smaller particles (<20 nm) than those prepared with other phospholipids. It was found previously that a coexistence of small amounts of phosphatidyethanolamine and cholesterol in the phospholipid system, such as the present system with HyPC, was necessary to produce such small lipid particles.13)

A significant correlation between particle diameters and optical densities of the phospholipid dispersions was observed (Table I), i.e., the Spearman rank order correlation coefficient between them was 0.867 (> P<0.01). Therefore, in the present study, the physicochemical properties of the phospholipid dispersions were evaluated by measuring optical densities as well as particle diameters.

Figure 1 shows electron micrographs of phospholipid dispersions prepared with HyPC and sucrose, either with (A) or without (B), VE.

It was found that the particle diameter of the dispersions was in the range of 9–70 nm as observed in these photos. Photon correlation data showed the average diameters of HyPC dispersions with sorbitol and sucrose as being 8.6 and 11.3 nm, respectively (Table I); the optical densities at 550 nm were 0.021 and 0.034, respectively.

The minimum diameter of small unilamellar vesicle (SUV) is expected to be 20 nm, when calculated from the curvature of spheres of closely compacted PC molecules. In Fig. 1, the dispersed particles have a spherical shape, but fractured forms are not seen. In a dispersion system of PC and a lipophilic substance, the coexistence of oil-in-water (o/w) emulsion droplets and liposomal vesicles, i.e., monolayers and bilayers of PC, has been reported.14,15) The phospholipid dispersions in the present study were considered to be a mixture of o/w emulsions and liposomes. Therefore, the present dispersed particles were termed phospholipid nanospheres.

Figure 2 shows the changes in optical density of HyPC nanosphere dispersions stored at room temperature and shielded from light with aluminum foil. The phospholipid nanosphere dispersions were thermodynamically unstable and their optical densities increase remarkably after 10 d due to aggregation of fusion.

To overcome this problem, powdered phospholipid dispersions were prepared which could reproduce the original aqueous dispersion system upon rehydration.

**Physicochemical Properties of Powdered Nanospheres Prepared by Spray Drying**

Figure 3 shows scanning electron micrographs of the original sucrose (A) and powdered nanospheres without VE prepared with sucrose (B). Spray dried nanospheres were fine, spherical particles.

Table II shows the mean diameters (D50) and water content of powdered nanospheres prepared with various sugars with and without VE. Particle size and water content depended on the kind of sugar. Fine particles in the size range of 10–30 μm were obtained by spray drying HyPC nanospheres prepared with sucrose, lactose or mannitol. Yields of the powdery products were 30–43%. Those with a water contents of less than 3.2% (Table II) were acceptable for pharmaceutical processing since the products were free-flowing. Water content of the spray dried nanospheres containing lactose or sucrose was 2–3%, but that of the mannitol-containing products was only 0.15–0.2%. The spray-dried nanospheres containing glucose or sorbitol adhered to the inner wall of the drier because of their

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Vitamin E</th>
<th>D50 (μm)</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>13.6</td>
<td>2.86</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>27.1</td>
<td>3.03</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>25.5</td>
<td>0.20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>18.9</td>
<td>1.96</td>
</tr>
<tr>
<td>Lactose</td>
<td>−</td>
<td>29.9</td>
<td>3.17</td>
</tr>
<tr>
<td>Mannitol</td>
<td>−</td>
<td>21.9</td>
<td>0.15</td>
</tr>
</tbody>
</table>

a) +, contained; −, not contained.

Fig. 2. Changes in Optical Density of HyPC Nanosphere Dispersions during Storage

○, sugar was not added; □, sorbitol was added.

Fig. 3. Electron Micrographs of Original Sucrose (A) and Sucrose-Based Powdered Nanospheres (B)
Hydroscopic properties of the spray dried products were investigated, as shown in Fig. 4. The original sugars were in crystalline form. Sucrose, lactose and sorbitol in powdered nanospheres became amorphous on spray drying, but mannitol remained in crystalline form.

Physicochemical Properties of Nanosphere Dispersions Obtained by Rehydration of Powdered Nanospheres and Factors Influencing the Properties  

Electron Micrographs of Rehydrated Nanosphere Dispersions  

Figure 5 shows electron micrographs of phospholipid nanosphere dispersions obtained by rehydrating spray dried products with distilled water. VE was incorporated in the nanospheres in Fig. 5A and not in those of Fig. 5B.

Comparing these photos with those in Fig. 1, little difference in the size or morphology of rehydrated dispersions from those of the original nonsphere dispersions before drying can be seen. It is noteworthy that all processes, from the preparation of the original nanosphere dispersion by the heating method, through powdering by spray drying, to rehydration of powdered nanospheres, were successfully conducted in an aqueous system.

Factors influencing the particle diameters of rehydrated nanosphere dispersions were investigated as follows.

Effects of Kind and Amount of Sugar Added in the Formulation  

Phospholipid nanospheres were prepared with various sugars. Table III shows the particle sizes and optical densities of the original and rehydrated phospholipid nanosphere dispersions. Particle diameters of the original nanosphere dispersions with and without VE were 65–106 and 8.6–19 nm, respectively. Optical densities of original nanosphere dispersions with VE were higher than those in the absence of the drug.

Particle diameters of rehydrated nanospheres were almost the same as those of the original nanospheres before spray drying when dispersions for spray drying were prepared with sucrose or lactose. Mannitol failed to yield reproducible

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Vitamin E</th>
<th>Before spraying</th>
<th>After rehydration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$d_s$ (nm)</td>
<td>OD$_{350}$</td>
</tr>
<tr>
<td>Sucrose</td>
<td>—</td>
<td>11.3</td>
<td>0.034</td>
</tr>
<tr>
<td>Lactose</td>
<td>—</td>
<td>19.3</td>
<td>0.050</td>
</tr>
<tr>
<td>Mannitol</td>
<td>—</td>
<td>10.0</td>
<td>0.022</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>—</td>
<td>8.6</td>
<td>0.021</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.0 mM</td>
<td>64.8</td>
<td>0.695</td>
</tr>
<tr>
<td>Lactose</td>
<td>3.0 mM</td>
<td>106.1</td>
<td>0.835</td>
</tr>
<tr>
<td>Mannitol</td>
<td>3.0 mM</td>
<td>66.5</td>
<td>0.866</td>
</tr>
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</table>

Fig. 4. X-Ray Diffraction Patterns of Original Sugars and Sugar-Based Powdered Nanospheres  

(A) Sucrose. (B) Lactose. (C) Mannitol. (D) Sorbitol. Upper plots, original sugars; lower plots, powdered nanospheres with sugars.

Fig. 5. Electron Micrographs of Rehydrated HyPC Nanosphere Dispersions with Sucrose, and with or without VE  

(A) Rehydrated nanosphere dispersion prepared with VE. (B) Rehydrated nanosphere dispersion prepared without VE.
small dispersed particles after drying and rehydrating. Some powdery sorbitol products were recovered after spray drying, producing small nanospheres as well as spray dried powder with sucrose or lactose on rehydration.

The diameters of rehydrated nanosphere dispersions (Table III) depended on the residual water content in the powdered nanospheres and on the physical form of sugar in the powdered nanospheres (Table II and Fig. 4). Higher crystallinity of the powdered nanospheres with mannitol, as shown in Fig. 4, indicates a strong mutual interaction of the mannitol molecules. Thus during drying, the sugar molecules might be preferentially separated in the spray droplet of nanosphere dispersions, which might induce the aggregation or fusion of lipid nanospheres. Such aggregation might increase the particle size of rehydrated nanospheres with mannitol. On the other hand, sucrose, lactose, and sorbitol were an amorphous state in the powdered nanospheres, so the sugar molecules could be finely dispersed around the solidified phospholipid nanospheres. The intermolecular interaction of these sugars are not so strong that they might bind to the polar headgroup of phospholipid and water during powdering. Consequently, these sugars did not recrystallize, but became amorphous in the powdered form. Higher water contents in the powdered nanospheres with such sugars supported this speculation. Such sugar molecules, bound with water around the hydrated lipid nanospheres, could prevent the aggregation of the nanospheres during spray drying. Therefore, the diameters of the rehydrated nanospheres with these sugars were almost the same as those of the original nanospheres before powdering.

The diameters of nanospheres without VE tended to increase after spray drying and rehydration even if sucrose, lactose or sorbitol was used (Table III). Nanospheres without VE were considered to be a mixture of liposomes and w/o emulsion droplets as described above. It has been reported that sugars more effectively prevent the damage of vesicles during lyophilization when they are present inside the liposomal membranes than outside. In the present system, sugars protected only the outer surfaces of the nanospheres during dehydration, even if the dispersions were liposomes or o/w emulsion droplets. Therefore, a significant increase in the diameter of a nanosphere without VE was observed after rehydration. Whereas, nanospheres with VE were considered to be o/w emulsion droplets. A significant increase in nanosphere diameter with VE could not be detected after rehydration.

Nanospheres with various amounts of sucrose were prepared and spray-dried. Table IV shows the particle sizes and optical densities of the original and rehydrated nanosphere dispersions. A concentration of sugar of more than 5% had no significant further effect on the physicochemical properties of rehydrated nanosphere dispersions.

c) Effects of Rehydration Temperature VE-containing powdered nanospheres were prepared with various sugars and rehydrated at various temperatures in the range of 20–90 °C. Figure 6 shows the particle diameters and optical densities of the rehydrated dispersions.

Rehydration temperature in this experiment did not affect the particle diameter of rehydrated nanospheres. As the phase transition temperature of HyPC is 9.5 °C, the lipid is in a liquid crystalline state and VE is able to move laterally in the PC membrane. Therefore, rehydrated nanospheres presumably had similar structures over this temperature range.

d) Effects of the Amount of Lipophilic Drug Incorporated Phospholipid nanosphere dispersions were prepared with various amounts of VE and then spray-dried. The volume of original dispersion sprayed and the contents of sucrose and HyPC in the formulation were fixed at 2000 ml, 100 g and 6 mmol, respectively. Figure 7 shows the

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**Table IV. Effect of Concentration of Sucrose in Sprayed Solution on Particle Size of Rehydrated HyPC Nanospheres**

<table>
<thead>
<tr>
<th>Sucrose concentration (%)</th>
<th>Before spraying</th>
<th>After rehydration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$d_4$ (nm)</td>
<td>$d_5$ (nm)</td>
</tr>
<tr>
<td>5</td>
<td>11.3</td>
<td>17.8</td>
</tr>
<tr>
<td>10</td>
<td>10.7</td>
<td>19.6</td>
</tr>
<tr>
<td>20</td>
<td>9.8</td>
<td>18.5</td>
</tr>
</tbody>
</table>

**Table V. HyPC and Vitamin E Content in Sucrose-Containing Powdered HyPC Nanospheres**

<table>
<thead>
<tr>
<th>Mole fraction of vitamin E</th>
<th>Percentage in formulation (%)</th>
<th>Determined percentage in powdered nanosphere (%)</th>
<th>B/A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HyPC (A)</td>
<td>Vitamin E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>4.10</td>
<td>2.70</td>
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</tr>
<tr>
<td>0.333</td>
<td>4.16</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.21</td>
<td>3.45</td>
<td>81.9</td>
</tr>
</tbody>
</table>

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![Fig. 6. Effect of Rehydration Temperature on Mean Diameters and Optical Densities of Rehydrated HyPC Nanosphere Dispersions with VE](image1)

(A) Mean diameter. (B) Optical density. ○, lactose; ●, sucrose; □, mannitol.

![Fig. 7. Effect of Mole Fraction of VE on Mean Diameter of HyPC Nanospheres with Sucrose](image2)

○, original nanospheres before spray drying; ●, rehydrated nanospheres.
nanosphere diameters of the original and rehydrated dispersions vs. mole fractions of VE, i.e., VE/(HyPC+VE).
Particle diameters of the original and rehydrated dispersions increased with an increasing VE content. The rehydrated nanospheres had only a slightly increased diameter compared with the original nanospheres before drying, irrespective of the mole fraction of VE.

In the present system, HyPC dispersion with VE might more likely be o/w emulsion rather than liposome. Ishii et al. reported that the droplet size of o/w emulsion prepared with PC and soybean oil increased with increasing the volume of oil.18 This would explain why the nanosphere diameter increased with an increase in VE content.

The contents of HyPC and VE in the powdered nanospheres are given in Table V, and refer to those employed in the formulation.

Though HyPC content in the powdered nanospheres was decreased by spray drying in the absence of VE, the decrease was prevented by coformulating VE. This might be due to the phospholipid-antioxidant role of VE.19 It has been reported that radicals and superoxide anions are produced during sonication20 and spray drying.21 Phospholipid tends to be oxidized by radicals.20 VE produces its antioxidant effect by trapping radicals and superoxide anions.17,22 It has been reported that sucrose has an antioxidant effect during spray drying of hemoglobin.21 Therefore, oxidation of phospholipid is considered to have been prevented by the synergistic effects of VE and sucrose.

The powdered nanospheres (Fig. 7) stored at 5°C for 4 months produced nanosphere having almost the same diameters as the dispersions prepared by rehydration immediately after spray drying.

Entrainment of a Water-Soluble Drug in Rehydrated Nanospheres A water-soluble drug, 5-FU, was entrapped in nanospheres by rehydrating powdered nanospheres with 0.5% aqueous drug solution. Table VI shows the percentages of 5-FU entrapped in the rehydrated nanospheres and the captured volume per unit weight of phospholipid.

The values of entrapped percentage were very small. The mannitol-based nanospheres showed a somewhat larger value than those of other nanospheres due to the larger diameter of the particles produced after rehydration. Sucrose- or lactose-based nanospheres had very small entrapping capacities for 5-FU, but the values are considered to be reasonable since the captured volume, i.e., 0.5 µl/mg, coincides with the literature value for SUV.23

Conclusions
We have established a method to prepare powdered nanospheres that can be rehydrated to regenerate stable nanospheres having diameters of 8–150 nm in an aqueous system without using any organic solvent. The procedures are as follows: preparation of original nanosphere dispersion by the heating method, powdering by spray drying, and rehydration of powdered nanospheres before use. Coformulation of suitable sugars, e.g., sucrose and lactose, in the HyPC system was most important to reproduce the original nanosphere dispersions. Those sugars were converted to an amorphous form in the powdered nanospheres during the drying process. These procedures should be suitable for the production of a stable powdered nanosphere that can be rehydrated as required to generate phospholipid nanosphere dispersions having similar characteristics to those of the original dispersions before powdering.

Acknowledgement We thank Dr. Kazushige Ogawa (JEO Lid., Tokyo, Japan) for taking negative-stain electron micrographs of phospholipid dispersions, and Koichi Matsumoto and Kazuhiko Takahashi (Nihon Surfactant Kohyo Co., Tokyo, Japan) for providing phospholipids.

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