Preparation of Powdered Phospholipid Nanospheres by Fluidized-Bed Granulating in an Aqueous System with Sugars

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Phospholipid nanosphere dispersions incorporating a lipophilic drug were prepared by the heating method. Prepared dispersions were powdered by depositing them on sugar nuclei in a fluidized-bed granulator in an aqueous system. Resultant free-flowing powders, which were agglomerates of sugar with phospholipid, reproduced phospholipid nanosphere dispersions when rehydrated with water. Incorporation of a lipophilic drug, *e.g.*, tocopherol, into the nanospheres or increasing of phospholipid concentration in the formulation increased the sizes of rehydrated nanospheres compared to the original dispersion before powdering due to the fusion or aggregation of the nanospheres. Proper selection of sugar nuclei, decreasing the sizes of original phospholipid nanospheres for powdering and addition of polyhydric or sugar alcohols into the sprayed dispersion, however, significantly reduced the aggregation of rehydrated nanosphere. All procedures from the preparation of the original nanosphere dispersion to its powdering were performed in an aqueous system without use of any organic solvent. The procedures described here are recommended to produce stable powdered nanosphere which can be rehydrated to form phospholipid nanosphere dispersions when required.

Keywords phospholipid nanosphere; powdering; fluidized-bed granulating; aqueous system; sorbitol

Introduction

A stable dispersion system is necessary to apply phospholipids, *i.e.*, liposomes and lipid microspheres, to drug carriers. To this end, powdering of the system, *i.e.*, lyophilization[1,2] and preparation of proliposomes, has been extensively investigated. In the former technique, control of physical qualities of powdery products is difficult, and the products often reveal a hygroscopic property. The latter technique involves preparation of free-flowing granules which produce liposomal dispersions by hydration, by drying an organic solution of soybean phosphatidylcholine (PC) and a lipophilic drug employing an evaporator,[3,4] a fluidized-bed granulator[5,6] or a spray-drier.[7,8] These techniques, however, should allow removal of the residual organic solvents in the proliposomes. The liposomal suspensions obtained by hydration of proliposomes often aggregate to form larger vesicles, 200—1400 nm in diameter, which are unacceptable for injections.

In the present study, in an attempt to overcome these problems, we prepared powdered phospholipid drug carriers using a fluidized-bed agglomeration technique with an aqueous system.

Phospholipid dispersions containing tocopherol acetate as a model drug for powdering were prepared in an aqueous system by the heating method.[9] The resultant aqueous dispersions were sprayed on sugar nucleus particles in a fluidized-bed chamber.

Operating condition of the fluidized-bed granulater, *e.g.*, spray rate and spray pressure, and formulation parameters, *e.g.*, concentration of phospholipid, drug and additives in spray solution were investigated to avoid the aggregation of rehydrated lipid dispersion. Successful modifications in formulation were accomplished and a mechanism preventing the aggregation of phospholipid dispersions is discussed.

Experimental

Materials Phospholipid used was partially hydrogenated soybean PC (HyPC) which was supplied by Ajinomoto Co. (Tokyo, Japan). The iodine value and phase transition temperature of HyPC were 39.7 and 9.5 °C, respectively. The acid value was less than 3.0 mg-KOH/g. The acyl chain composition was 13.2% palmitate, 27.4% stearate, 59.2% oleate and 0.2% other. HyPC contains a few per cent of phosphatidylethanolamine and cholesterol.

Sugars used as nuclei with fluidized-bed granulating were D-sucrose, D-lactose, D-mannitol and D-glucose; all were purchased from Kishida Chemical Co. (Osaka, Japan). Tocopherol acetate (VE, Eisai Co., Tokyo, Japan) was used as a lipophilic drug for incorporation into phospholipid dispersed particles.

Preparation of Phospholipid Nanosphere Dispersion by Heating Method Phospholipid nanosphere dispersion was prepared by the heating method.[9] Phospholipid (1.5 mmol) with or without VE (1.5 mmol) was added to distilled water (50 ml) at 60 °C. The system was homogenized for 1 min at 15000 rpm by Physcotron NS-50 (Nition'i-Rikakikai Co., Chiba, Japan) and sonicated at 90W for 40 min with a probe type sonicator, UR-200P (Tomy Seiko Co., Tokyo, Japan). During these procedures, the system was maintained at 60 °C.

Powdering of Phospholipid Nanospheres by Fluidized-Bed Granulation Glycerin or D-sorbitol (0—40 w/v%) was added to the prepared phospholipid nanosphere dispersion and maintained at 60 °C. The mixture was sprayed on fluidized core particles in the granulating chamber, STREA-1 (Powrex Co., Osaka, Japan), at 5 ml/min. One hundred grams of sugar (53—250 μm) was used as the core material.

Granulating conditions were as follows: top spray mode; air flow rate, 70 m^3/h; spray pressure, 1 kg/cm^2; inlet and outlet air temperatures, 50 °C and 35—45 °C, respectively.

Physicochemical Properties of Powdered Phospholipid Nanospheres Diameters of powdered phospholipid nanospheres were measured by the sieving method. Mean diameters (D_{50}) were calculated with regression lines of the cumulative weight percentages of sieved fractions to log-normal distribution.

Scanning electron micrographs of original sugar particles and powdered nanospheres were taken with a T330A (JEOL Ltd., Tokyo, Japan).

Water content in the powdered nanospheres was measured by the Karl–Fisher method with a moisture meter, model MKA-3 (Kyoto Electronics Co., Kyoto, Japan).

Physicochemical Properties of Original and Rehydrated Nanosphere Dispersions The original and rehydrated phospholipid nanosphere dispersions were centrifuged at 3000 rpm for 10 min to remove the contaminants derived from the sonicator, and the diameters of phospholipid nanospheres in the supernatants were measured by photon correlation method with a Photal LPA-3000/3100 (Otsuka Electronics Co., Osaka, Japan) equipped with a 5 mW He–Ne laser (632.8 nm) and with a 1024-channel correlator. Powdered nanospheres (1 g) were rehydrated with water (10 ml) at 60 °C. Quasi-elastically scattered light from dispersed nanospheres was measured at 20 °C and the weight-averaged particle sizes of the whole dispersions were evaluated. It was confirmed that no

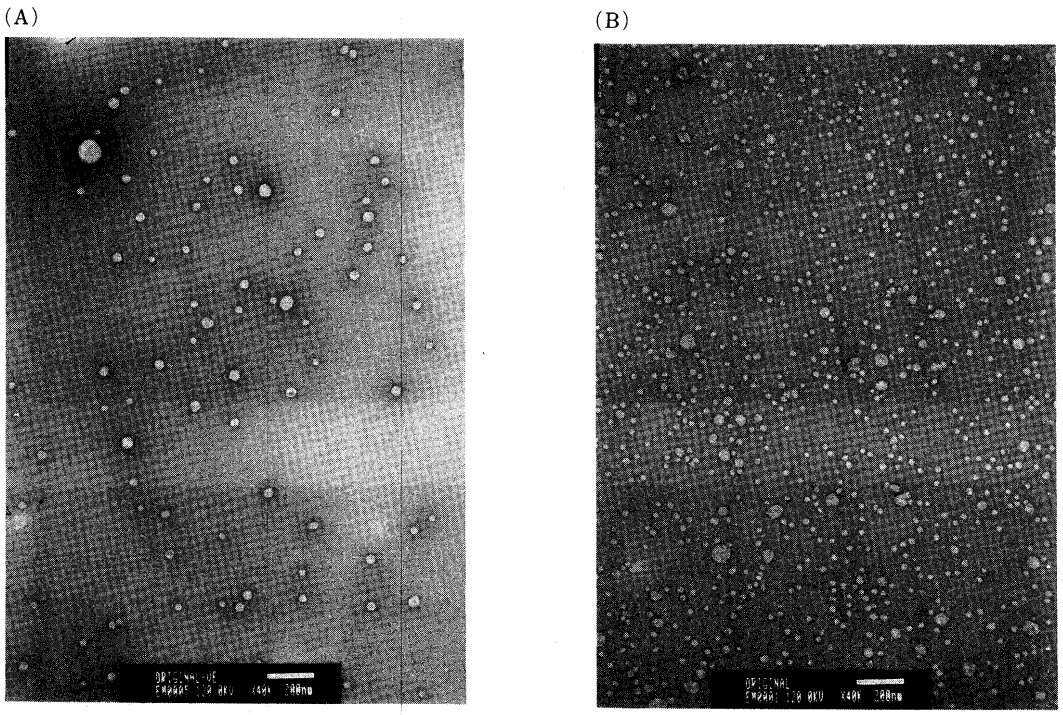

Fig. 1. Electron Micrographs of Phospholipid Nanosphere Dispersions Prepared with and without VE

(A) nanospheres prepared with VE, (B) nanospheres prepared without VE. Formulation for nanosphere dispersions; (A) HyPC, 30 mM; VE, 30 mM. (B) HyPC, 30 mM. White bar indicates 200 nm. The photographs were taken after one day-storage.

phospholipid was detectable in the precipitation after centrifugation.

Optical density of the dispersion per 3 mM phospholipid at 550 nm was measured as an index of turbidity,[10] and a significant correlation between particle diameters and optical densities of original and rehydrated nanosphere dispersions was observed.

Original and rehydrated nanosphere dispersions were observed with a transmission electron microscope, JEM-1200II (JEOL Ltd., Tokyo, Japan) after negative staining with 1% uranyl acetate solution. The supporting membrane was polyvinylformal coated with carbon.

Determination of PC and VE Contents in Powdered Phospholipid Nanospheres PC and VE contents in powdered phospholipid nanospheres were measured by high performance liquid chromatography (HPLC) with an LC-9A apparatus (Shimadzu Co., Kyoto, Japan).

Powdered nanospheres were dissolved in chloroform and eluted PC was detected spectrophotometrically at 205 nm. The column was a Shim-pack CLC-SIL (6.0 mm i.d. × 15 cm l., Shimadzu Co., Kyoto, Japan). The mobile phase was a mixture of 10 mM sodium phosphate buffer solution (pH 2.6) containing 100 mM sodium perchloride and acetonitrile with the volume ratio of 1 : 5. The flow rate was 1.0 ml/min.

VE content in powdered nanospheres dissolved in methanol was measured spectrophotometrically at 284 nm. The column was a TSK gel ODS-120T (4.5 mm i.d. × 7.5 cm l., Tosoh Co., Tokyo, Japan). The flow rate of the mobile phase, *viz.*, methanol, was 1.0 ml/min.

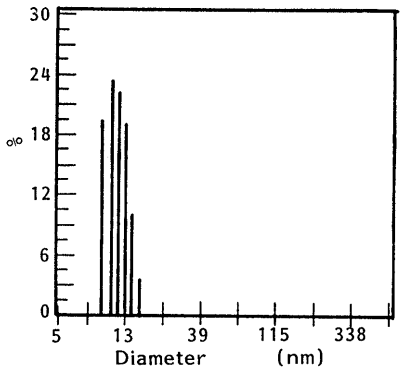

Fig. 2. Weight-Averaged Particle Size Distribution of Phospholipid Nanosphere Dispersion

Formulation for nanosphere dispersions; HyPC: 30 mM. Mean diameter was 11.8 nm.

Results and Discussion

Physicochemical Properties of Phospholipid Nanosphere Dispersion for Fluidized-Bed Granulating Figure 1 shows electron micrographs of phospholipid dispersions taken one day after preparation with HyPC, either with (A) or without VE (B).

The particle diameter of the dispersions observed in these photographs was in the range of 9—90 nm. Figure 2 shows the particle size distribution of phospholipid nanosphere dispersion measured immediately after preparation with HyPC without VE. Weight-averaged mean diameter was 11.8 nm. This value was much smaller than the diameter of the particles photographed in Fig. 1. This difference mighe be due to the aggregation and/or fusion of the particles during storage before the electron microphotographs were taken. The minimum diameter of a small unilamellar vesicle (SUV) is expected to be 20 nm, when calculated from the curvature of spheres of closely compacted PC molecules. The coexistence of o/w emulsion droplets and liposomal vesicles, *i.e.*, monolayers and bilayers of PC, has been reported[11,12] in a dispersion system of PC and a lipophilic substance. The phospholipid dispersions in Figs. 1 and 2 were assumed to be a mixture of o/w emulsions and liposomes. Therefore, the present system was termed phospholipid nanospheres.

It was found previously that the coexistence of small amounts of phosphatidylethanolamine and cholesterol with PC as found in HyPC was required to produce such small phospholipid dispersions.[13] The phospholipid nanosphere dispersions were thermodynamically unstable and their

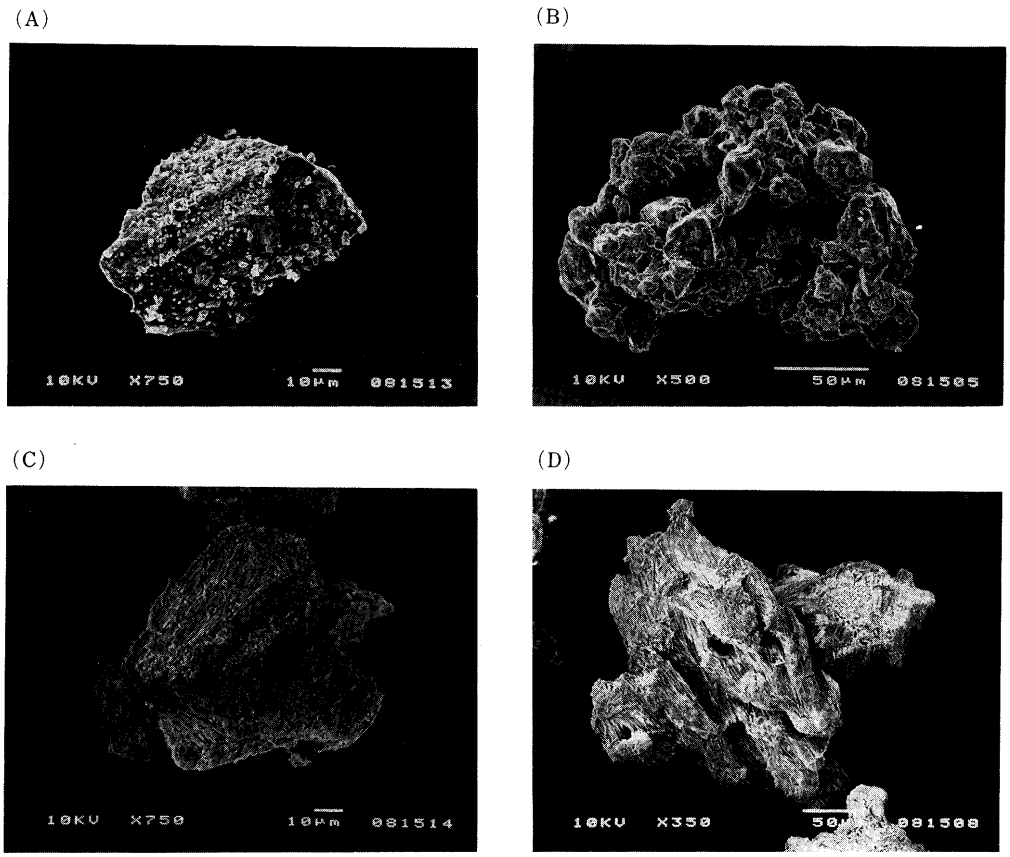

Fig. 3. Electron Micrographs of Original Sugars and Powdered Nanospheres

(A) original sucrose. (B) sucrose-based powdered nanosphere. (C) original sorbitol. (D) sorbitol-based powdered nanosphere.

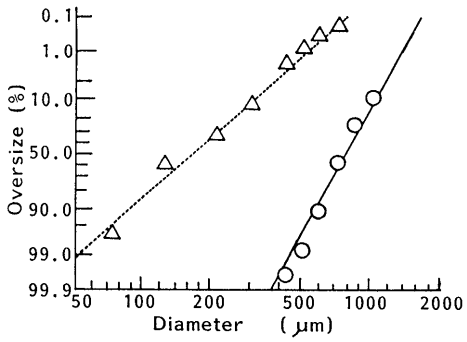

Fig. 4. Log-Normal Distribution of Sucrose and Sucrose-Based Powdered Nanospheres Prepared with a Fluidized-Bed Granulator

△, original sucrose; ○, powdered nanosphere. D_{50}: △, 163.6 μm; ○, 760.5 μm.

particle diameter increased during storage by aggregation or fusion of nanospheres.

Physicochemical Properties of Powdered Phospholipid Nanospheres and Rehydrated Phospholipid Nanosphere Dispersion Figure 3 shows scanning electron micrographs of the original sugars (A, C) and powdered nanospheres without VE prepared with sucrose and sorbitol (B and D, respectively) in a fluidized-bed granulator.

The powdered nanospheres were found to be agglomerates of sugar particles with phospholipid as shown in Fig. 3.

Figure 4 shows log-normal distribution of sucrose and sucrose-based powdered nanospheres.

Mean diameter of a powdered nanosphere was about five times that of the original sucrose. It is thus clear that sugar was agglomerated during powdering of phospholipid nanospheres by fluidized-bed granulating.

Figure 5 shows electron micrographs of phospholipid nanosphere dispersions obtained by rehydrating sorbitol-based fluidized-bed granulated products with distilled water. VE was incorporated in the nanospheres in Fig. 5A and not in those of Fig. 5B.

These photographs reveal that phospholipid nanospheres were reconstituted. However, comparing these photographs with those in Fig. 1, rehydrated dispersions consisted of more irregular and larger nanospheres than those of original dispersions caused by fusion or aggregation, which encouraged us to investigate a method of preventing the aggregation of nanospheres caused by powdering.

Establishment of Basic Operating Conditions of Fluidized-Bed Granulating To establish the basic operating conditions for the powdering of nanospheres, phospholipid nanospheres (50 ml, HyPC: 30 mM) without VE were powdered by fluidized-bed granulation under various conditions, *i.e.*, type of nucleus sugar (100 g), spray pressure and spray rate.

Table I shows particle diameters (d_w) and optical densities (OD_{550}) of original and rehydrated nanosphere dispersions with various sugars.

Rehydrated nanosphere dispersions had increased particle diameters and optical densities compared to those before powdering, with the sorbitol-based powdered nanospheres yielding the smallest rehydrated nanospheres. Sorbitol was therefore used as a core material for fluidized-bed granulating.

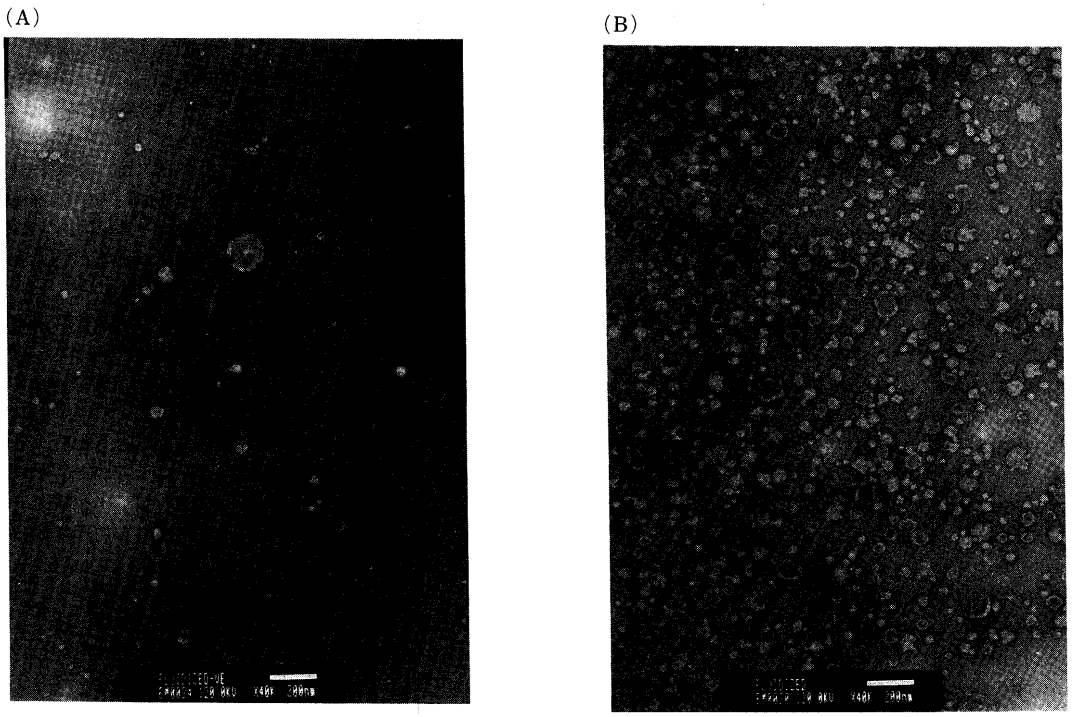

Fig. 5. Electron Micrographs of Rehydrated Nanosphere Dispersions with and without VE

(A) rehydrated nanosphere dispersion prepared with VE, (B) rehydrated nanosphere dispersion prepared without VE. Formulation for spray solution: (A) HyPC, 30 mM; VE, 30 mM. (B) HyPC, 30 mM. White bar indicates 200 nm. The photographs were taken after one day-storage.

TABLE I. Effects of Sugar on Mean Diameter and Optical Density of Rehydrated Nanosphere Dispersion

Sugar	Before powderization		After rehydration	
	d_w (nm)	OD_{550}	d_w (nm)	OD_{550}
Sucrose	8.9	0.012	1023.7	0.293
Mannitol	10.8	0.016	1028.7	0.206
Lactose	8.0	0.011	1045.7	0.458
Sorbitol	7.5	0.009	682.3	0.164

TABLE II. Effects of Spray Pressure of Fluidized-Bed Granulator on the Physical Properties of Powdered and Rehydrated Nanospheres

Spray pressure (kg/cm^2)	Powdered nanosphere		Rehydrated nanosphere	
	D_{50}[a] (μm)	Water content (%)	d_w[b] (nm)	OD_{550}[c]
1.0	235.9	0.29	682.3	0.164
1.5	196.3	0.27	523.3	0.147
2.0	177.9	0.23	710.8	0.160

a) D_{50} of original sorbitol was 139.6 μm. *b, c*) d_w and OD_{550} of original phospholipid nanosphere dispersion were 8.5 nm and 0.008, respectively.

Two hundred milliliters of original nanosphere dispersion was prepared and aliquots (50 ml) were powdered under various spray pressures at fluidized-bed granulating. Table II shows the effects of spray pressure with a fluidized-bed granulator on the physical properties of powdered nanospheres and rehydrated nanosphere dispersions.

Diameter of powdered nanosphere and its water content decreased with increase in spray pressure as expected. With higher spray pressure, the diameters of spray droplets decrease, so that fewer nucleus particles are captured by

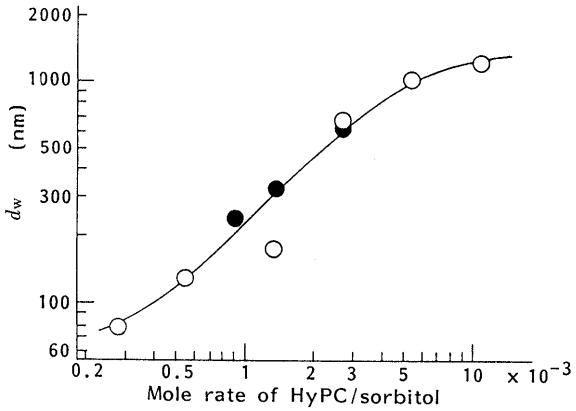

Fig. 6. Effects of Mole Rate of Phospholipid/Sorbitol Nuclei on Nanosphere Size of Rehydrated Dispersions

○, weight of sorbitol nuclei was fixed at 100 g; ●, concentration of phospholipid in original dispersion was fixed at 30 mM. Mean diameter of original nanosphere: ○, 14.5 nm; ●, 11.8 nm.

droplets and the evaporation rate of water from the droplets is accelerated. These effects produced smaller and less moistured powdered nanospheres.

Under the spray pressure of 1.0 kg/cm^2, the spray rate was varied at 1.0, 3.0 and 5.0 ml/min. The water content in powdered nanosphere increased from 0.20 to 0.30% with increase in spray rate, while the particle diameter and optical density of rehydrated nanosphere dispersion remained unchanged.

The spray pressure and rate for fluidized-bed granulation were therefore set at 1 kg/cm^2 and 5 ml/min, respectively.

Factors Controlling the Diameter of Rehydrated Nanosphere. a) Effects of HyPC Concentration in Powdered Phospholipid Nanospheres on Rehydrated Nanosphere Size Phopholipid nanosphere dispersion containing 120 mM

HyPC was prepared and aliquots of the dispersion were diluted to various concentrations. Resultant diluted dispersion (50 ml) was sprayed on sorbitol nuclei (100 g, *i.e.*, 549 mmol) in a fluidized-bed granulator. Nanosphere diameters of rehydrated dispersions are plotted with open circles in Fig. 6.

Two hundred milliliters of original phospholipid nanosphere dispersion (HyPC; 30 mM, *i.e.*, 1.5 mmol) was prepared and aliquots (50 ml) were sprayed on various amounts of sorbitol for powdering. Nanosphere diameters of rehydrated nanospheres are plotted with closed circles in Fig. 6, where, the abscissa shows mole ratio of HyPC/sorbitol in powdered nanospheres calculated from formulations.

Diluting the original nanosphere dispersion or increasing the amount of core sorbitol reduced the diameter of a rehydrated nanosphere. As all data regressed to the same curve in Fig. 6, the diameter of rehydrated phospholipid dispersion was shown to be controlled by the concentration of HyPC in powdered nanospheres, *i.e.*, thickness of deposited phospholipid nanospheres on the surface of sugar nucleus. When sugar particles were coated thickly by HyPC, the deposited nanosphere might remarkably aggregate or fuse on the surface of nucleus particles during drying.

b) Particle Diameter of Original Phospholipid Nanosphere Dispersion When the sonication was omitted, mean diameter of the resultant phospholipid dispersion without VE increased to 1234.6 nm. The aliquots of prepared phospholipid dispersion (HyPC: 120 mM) were diluted a certain number of times with water. Using such coarse dispersion, nanospheres were powdered, followed by their rehydration. The mean diameters of resultant nanosphere dispersions are plotted as a function of concentration of phospholipid in sprayed nanosphere dispersions in Fig. 7. Open circles shown as references in the figure represent the mean diameters of rehydrated nanospheres prepared from the original dispersions produced with homogenization and sonication.

When the concentration of phospholipid in spray solution was low, the mean diameter of rehydrated nanosphere dispersions depended strongly on the original nanosphere size, because the fusion of nanospheres deposited on the nucleus particle decreased. When the phospholipid concentration was high, the fusion of smaller nanospheres increased because of their larger specific surface areas. Nanospheres prepared without sonication consisted of multilamellar vesicles, which were destroyed by powdering. Therefore, the diameters of rehydrated nanosphere dispersions became smaller than the original ones.

Prevention of Fusion of Deposited Nanospheres on Nucleus Particle by Addition of Polyhydric Alcohol or Sugar Alcohol into Sprayed Dispersion at Fluidized-Bed Granulating It is well known that addition of sugar[14-16] or polyhydric alcohol[17] to a liposomal suspension prevents the vesicles from fusing during lyophilization. In the present system, sorbitol or glycerin was added into the original phospholipid nanosphere dispersion without VE and the system was powdered with fluidized-bed granulating.

Figure 8 shows the effects of sorbitol and glycerin in a sprayed solution on the diameter of rehydrated nanospheres. The increase in diameter size was remarkably reduced by these additives.

The diameter of rehydrated phospholipid nanosphere dispersion decreased to 86.3 nm with the addition of 4% *i.e.*, 2 g of sorbitol to sprayed solution at the granulation using 100 g sorbitol, whereas when 200 g sorbitol was granulated without additives in sprayed solution, the diameter of rehydrated nanosphere was 323.8 nm (Fig. 6). Thus, sorbitol dissolved in the system was more effective in preventing the fusion of nanospheres during drying than in powdered form as a core material. Addition of glycerin in sprayed solution was as effective as sorbitol in preventing fusion. Sorbitol or glycerin may be strongly adsorbed with hydrated water surrounding dispersed phospholipid nanospheres and may replace hydrated water during drying, thus protecting individual phospholipid nanospheres from fusing. Further, such additives molecularly dispersed into phospholipid particles in the powdered nanosphere may extensively promote the rehydration of phospholipid particles when redispersed in an aqueous medium.

Control of Diameter of Rehydrated Nanosphere Containing Lipophilic Drug Phospholipid nanosphere dispersions (50 ml) were prepared with HyPC (30 mM) and various concentrations of VE, and then were deposited on sorbitol

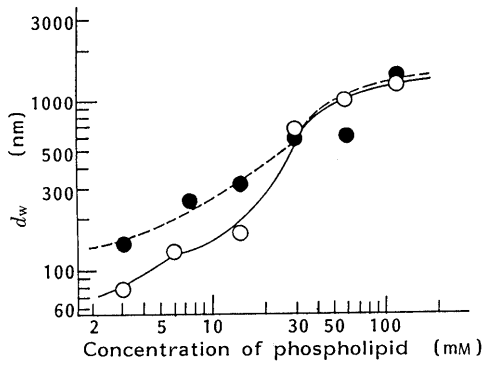

Fig. 7. Effects of Concentration of Phospholipid on Particle Size of Rehydrated Nanospheres

Preparation of original nanosphere dispersion before spraying: ○, homogenization and sonication; ●, homogenization. Mean diameter of original nanosphere before powdering: ○, 14.5 nm; ●, 1234.6 nm.

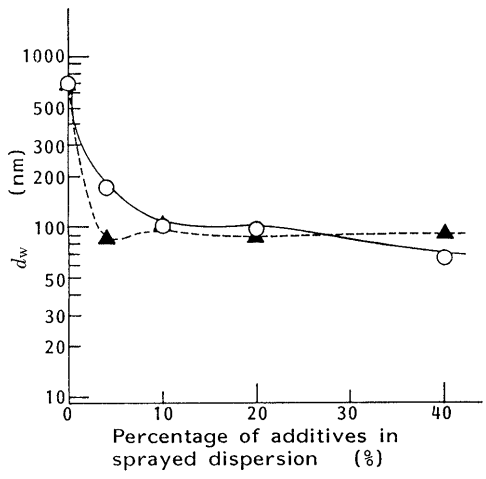

Fig. 8. Effects of Additives in Sprayed Dispersion on Mean Diameter of Rehydrated Nanospheres

Additives: ○, glycerin; ▲, sorbitol. Mean diameter of original nanospheres before spraying: 14.5 nm. Volume of originally prepared nanosphere dispersion (HyPC: 30 mM) was 250 ml.

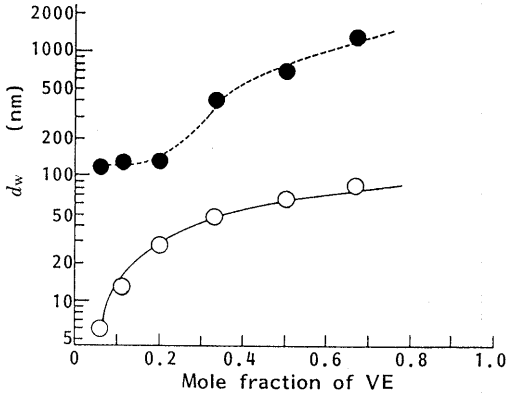

Fig. 9. Effect of Mole Fraction of VE on Mean Diameter of Phospholipid Nanosphere

○, original nanosphere before powdering; ●, rehydrated nanosphere.

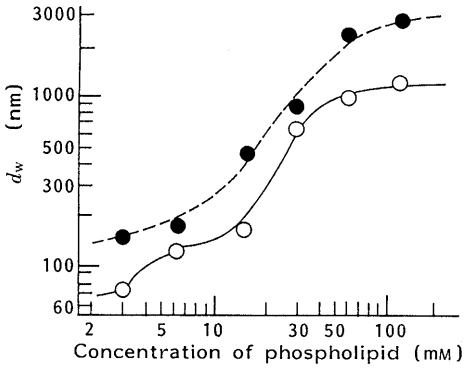

Fig. 10. Effects of Concentration of Phospholipid on Particle Size of Rehydrated Nanospheres with and without VE

●, with VE; ○, without VE. Mean diameter of original nanosphere before powdering: ●, 61.4 nm; ○, 14.5 nm.

nuclei (100 g) without additive, *e.g.* sorbitol of glycerin, by fluidized-bed granulation. Figure 9 shows plots of mole fractions of VE, *i.e.*, VE/(VE+HyPC) *vs.* nanosphere diameters of original and rehydrated dispersions.

Diameters of original and rehydrated nanospheres increased with increase in VE content, and rehydrated nanospheres were larger than original ones through all mole fractions of VE.

The present system consisted of mixtures of liposome and o/w emulsion. In liposome, VE is a component of vesicle membrane through hydrogen bonding to the polar headgroup of PC.[18] VE might also be entrapped as a core in an o/w emulsion droplet, and therefore, the nanosphere diameter increased with increase in VE content. Ishii *et al.* reported that the droplet size of o/w emulsion prepared with PC and soybean oil increased with increase in the volume of the oil.[19]

Content ratios of VE/HyPC in powdered nanospheres agreed well with the calculated values from the formulation. It was found that the powdered nanospheres stored at 5 °C for 1 month produced nanospheres having almost the same diameters as the dispersions prepared by rehydrating immediately after powdering of nanospheres.

The relationships between the concentration of phospholipid in original dispersion and the diameters of rehydrated nanosphere with and without VE are shown in Fig. 10, where mole ratio of HyPC:VE was fixed at 1:1. The concentration of HyPC in the originally prepared nanosphere dispersion was 120 mM and aliquots were diluted at certain times before spraying.

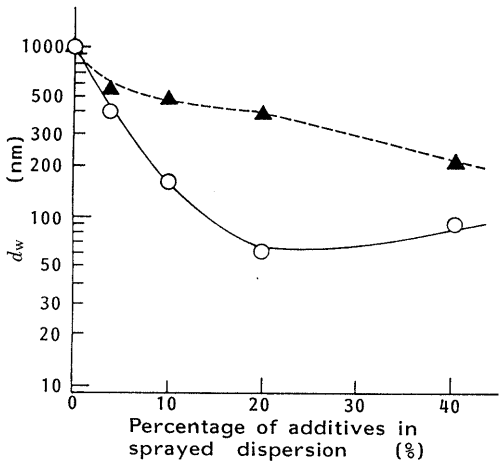

Fig. 11. Effects of Additives in Sprayed Solution on Mean Diameters of Rehydrated Nanospheres

Additives: ○, glycerin; ▲, sorbitol. Mean diameter of original nanosphere before powdering: 61.4 nm. Concentrations of VE and HyPC: 30 mM. Volume of originally prepared nanosphere dispersion was 250 ml.

Nanosphere diameters of rehydrated dispersion with and without VE increased with increase in the concentration of phospholipid. The aggregation of phospholipid nanospheres deposited on the nucleus particle enhanced with increasing phospholipid concentration during drying. Irrespective of phospholipid concentration, the diameter of rehydrated nanosphere increased with the incorporation of VE, because the diameter of the original phospholipid nanosphere increased with the incorporation of VE as shown in Fig. 10.

To prevent the fusion of VE-containing nanosphere during powdering, glycerin or sorbitol was added to the spray dispersion (Fig. 11).

The diameters of rehydrated nanospheres significantly reduced with increasing concentration of additive in the spray dispersion. Glycerin was the more effective of the two in preventing the aggregation of phospholipid nanospheres during powdering, retaining a liquid state throughout the powdering process, which keeps the phospholipid nanospheres in a more hydrated state than in coexistence with sorbitol. In addition, glycerin in powdered nanosphere effectively induces the rehydration of powdered nanosphere when redispersed in aqueous medium.

In conclusion, phospholipid nanospheres, capable of solubilizing lipophilic drugs, were successfully powdered using a fluidized-bed agglomeration technique with an aqueous system and without use of any organic solvent. The increase in diameter of rehydrated nanosphere caused by aggregation of nanospheres during drying was prevented by the addition of polyhydric or sugar alcohols to the sprayed solution. The present procedures might be suitable for production of stable nanosphere powders that can be rehydrated, when required, to easily regenerate phospholipid nanosphere dispersions without increasing their sizes.

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