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Research paper

Physical properties of griseofulvin-lipid nanoparticles in suspension and their novel interaction mechanism with saccharide during freeze-drying

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ABSTRACT

Size reduction of drug particles to the nanoscale is important in improving the dissolution rate of poorly water-soluble drugs. The aim of this study was to investigate the physicochemical properties of griseofulvin (GF)-lipid nanoparticles and the interactions between GF-lipid nanoparticles and various saccharides during freeze-drying. The phase transition temperature of the GF-lipid nanoparticle suspension was 56.8 °C, whereas that of the lipid nanoparticle suspension alone was 57.9 °C, indicating that the GF crystals were incorporated into the lipid phase. The mean particle size of a rehydrated suspension of xylose-containing freeze-dried GF-lipid nanoparticles was about 220 nm. However, the mean particle size on the rehydration of nanoparticles containing mannose (monosaccharide), fructose (disaccharide), lactose (disaccharide), or raffinose (trisaccharide) was about 60 nm, suggesting that these saccharides prevented aggregation during the freeze-drying process. Powder X-ray diffraction revealed that xylose existed in the crystalline state in the freeze-dried nanoparticles, whereas the other saccharides existed in amorphous states. Thus, the crystallization of the saccharide was found to be strongly correlated with the aggregation property of the nanoparticles. In the case of freeze-dried xylose, the nanoparticles were squeezed out as the saccharine crystal lattice arranged itself regularly. Then, the ejected nanoparticles were aggregated. In contrast, in the case of the other freeze-dried saccharide, the saccharide remained incorporated with the GF-lipid nanoparticles because its crystal lattice was arranged irregularly. Thus, the particle size was maintained.

1. Introduction

It has been estimated that about 40% of newly developed pharmaceutical compounds are poorly water-soluble [1]. Administration of such drugs presents a significant challenge because of their low bioavailability, unpredictable toxicity, and irregular absorption in the gastrointestinal tract (GIT). The dissolution rate limits bioavailability in the case of lipophilic drugs with high permeability through biomembranes [2]. Size reduction of poorly water-soluble drugs into nanoparticles represents a very effective approach [3–6] to drug solubilization because it does not require the use of specific solubilizing excipients and, in addition, may be suitable not only for oral delivery but also for administration by inhalation [7], injection [8], and topical application [9]. Production of nanoparticulate powders or suspensions is a relatively new area of pharmaceutical technology and, therefore, requires a careful assessment of product consistency as well as process robustness and scalability [10].

Size reduction of drug particles to the nanometer range is expected to improve the dissolution rate by increasing the specific surface area of the particles, allowing them to enter the systemic circulation through the Peyer's patches in the GIT [11]. In this study, a practically insoluble drug, griseofulvin (GF), was used as a model. GF is an antibiotic and antifungal drug administered predominantly in oral dosages. It has very low solubility in water (15 μg/mL at 37 °C) and hence low bioavailability [12].

We have reported that a nanoparticle suspension with a mean particle size of less than 100 nm can be prepared using high-pressure homogenization [13]. The physicochemical properties of these nanoparticles are characterized in the present report. In addition, we present a method by which these nanoparticles, containing an appropriate lyoprotectant, can be freeze-dried and rehydrated without the loss of the nanoparticulate state [14].

Freeze-drying method is used for preservation of foods. In particular, many studies about preservation of protein have been reported [15–18]. Many studies about the preservation of liposome have been also reported [19–21]. Liposomes can be stored

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effectively by freezing using a wide variety of cryoprotectants, including dimethylsulfoxide, glycerol, quaternary amines, and carbohydrates. The requirements for drying are much more stringent, and only disaccharide seems to be effective [22]. The aggregation of additive-free nanoparticles increases when they are freeze-dried and rehydrated. However, the addition of a saccharide as a lyoprotectant can prevent this aggregation after rehydration [23,24]. Crowe et al. explained this as being the result of hydrogen bonding between the saccharide and the surfaces of the liposomes, forming a steric barrier that prevents aggregation [25]. The mechanism by which saccharide stabilizes liposomes during drying involves the formation of a glassy state by the saccharide as well as a direct interaction between the saccharide and the phospholipid head groups [26]. However, these explanations appear to be insufficient. In addition to his theory, we propose a novel hypothesis about a mechanism of freeze-drying between the saccharide and the nanoparticles. As a result of X-ray measurement, it has been reported that saccharide has high crystallinity in the group aggregated nanoparticles and is amorphous state in the group preserved their particle size [14]. These relationships were already known before. However, these reasons have not been mentioned. Therefore, we had tried to elucidate these mechanisms. We have proceeded with study while comparing results of X-ray pattern and mean particle size. The physicochemical interactions between the GF-lipid nanoparticles and various saccharides in freeze-dried samples are discussed in this paper.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (HSPC; COATSOME® NC-21) and dimyristoyl phosphatidylglycerol (DMPG; COATSOME® MGLS-4040) were purchased from Nippon Oil and Fats Co., Ltd., GF (JPXIV) was provided by Nippon Fine Chemical Co., Ltd., Ethanol, maltose, sucrose, xylose, mannose, fructose, lactose, and raffinose (reagent grade) were purchased from Wako Pure Chemical Industries Ltd. Trehalose was provided by Fuji Nihon Seito Co. The reagents were used as supplied. Purified water treated by ion exchange was used.

2.2. Methods

2.2.1. Preparation of GF-lipid (HSPC–DMPG) nanoparticle suspension

GF (20 mg) and lipid (1000 mg; HSPC:DMPG = 3:1 M ratio) were dissolved in 2 ml of ethanol in an 80 °C water bath, and the ethanol was evaporated away. The GF-lipid (HSPC–DMPG) mixture was dispersed in 200 ml of purified water and premixed using a Speed Stabilizer (max speed, 12,000 r/min; Kinematica Co.) at 9000 r/min for 10 min. This premixed suspension was transferred to a high-pressure homogenizer (max pressure, 9.5 kg/cm^2; Nanomizer, X-form chamber; Tokushu Kika Kogyo Co.) to afford a nanoparticle suspension; this was used as the measurement sample in the following.

2.2.2. Nanoparticle size measurement

The mean particle sizes were measured at room temperature using an electrophoretic light-scattering photometer (ELS; ELS-8000, Otsuka Electronics Co., Ltd.) at a fixed angle of 90°. The particle sizes were analyzed on the basis of the weight distribution of the nanoparticle suspension. The nanoparticle suspension was analyzed without dilution.

2.2.3. Thermal analysis of nanoparticle suspension

A lipid nanoparticle suspension prepared by high-pressure homogenization and the GF-lipid nanoparticle suspension was analyzed by DDSC. Each sample was poured into an aluminum container (AL70-CAPSULE, Seiko Instruments Inc.) and sealed. The programmed heating rate was 1 °C/min, and the temperature range was 40–100 °C.

2.2.4. Powder X-ray diffraction

The samples prepared were subjected to powder X-ray diffraction with CuKa radiation at 40 kV, 30 mA, and room temperature using a powder X-ray diffractometer (RAD-C, Rigaku Denki Co., Ltd.). The scanning rate was 5°/min, and the diffraction angle (2θ) was 2°–30°.

2.2.5. Measurement of freeze-dried GF-lipid nanoparticles

Sucrose, xylose, mannose, fructose, and lactose were added to the GF-lipid nanoparticle suspension as lyoprotectants. The freeze-dried nanoparticles containing the various saccharides were subjected to powder X-ray diffraction.

2.2.6. Freeze-drying and rehydration of GF-lipid nanoparticles

The GF-lipid nanoparticle suspension (2 mL) was collected in separate vials to which 100 mg of sucrose, maltose, trehalose, glucose, and galactose was added to the vials. Each vial was vortexed, and the suspensions were frozen at −35 °C and left standing for 24 h. The frozen samples were freeze-dried in a glass chamber for 24 h using a vacuum pump and vapor condenser (−90 °C, 1.0×10^{-3} Torr; NEOCOOL, Yamato Scientific Co., Tokyo, Japan). Purified water (2 mL) was added to the vials, and they were then shaken by hand to rehydrate the freeze-dried samples. The mean particle size of the rehydrated GF-lipid nanoparticle suspension was analyzed by using the ELS.

2.2.7. Effect of freezing rate on the freeze-drying of GF-lipid nanoparticle suspension

Sucrose, maltose, and trehalose were added in the GF-lipid nanoparticle suspension. The mixed suspensions were frozen by the following processes: (a) −4 °C in a cold store, 48 h; (b) −35 °C in a deep freezer, 24 h; (c) −70 °C in methanol with frozen carbon dioxide, 2 min; and (d) −200 °C in liquid nitrogen container, 1 min. The four types of the frozen samples were lyophilized for 24 h.

2.2.8. Deterioration stabilities of freeze-dried GF-lipid nanoparticles containing sucrose

Powder X-ray diffraction patterns were acquired after leaving sucrose-containing freeze-dried GF-lipid nanoparticles exposed to the atmosphere at 25 °C, RH 40%, for 2, 4, 6, and 8 h.

Additionally, the freeze-dried GF-lipid nanoparticles containing sucrose were rehydrated after being left under similar conditions for 2, 4, 6, and 8 h. The mean particle size of the obtained rehydrated GF-lipid nanoparticle suspension was measured by using DLS.

3. Results and discussion

3.1. Physical properties of GF-lipid nanoparticles

In our previous studies, we succeeded in preparing nanoparticles of GF, a poorly water-soluble drug, with high-pressure homogenization and also succeeded in preserving the stability for 6 months. The present study discusses the physical properties of the prepared nanoparticles and the freeze-dried products. The results indicate that the preparation of lipids nanoparticle by

high-pressure homogenization is applicable to other poorly water-soluble drugs.

It is very important that particulate property is searched to prevent nanoparticle from aggregation. Thus far, many preparing methods of the nanoparticle by high-pressure homogenization have been reported, but the particle state of drug and lipid was poorly studied. In previous report, the GF-lipid nanoparticles were observed only with a TEM photograph, but the physical property was not searched in detail. Therefore, the thermal analysis of the GF-lipid nanoparticle suspension was performed to investigate the relationship between a lipid nanoparticle and a GF.

Fig. 1 shows a DDSC thermograph of the nanoparticle suspension prepared by high-pressure homogenization. The phase transition temperature of the GF-lipid nanoparticle suspension was 56.8 °C, whereas that of the suspension of lipid nanoparticles alone was 57.9 °C. Hwang et al. reported that the phase transition temperature is decreased on the incorporation of drugs into a lipid layer because of the destabilization of the packing of the hydrocarbon chains [27–29]. Taken together, the phase transition temperature of the GF-lipid nanoparticle appears to be decreased because of the incorporation of GF crystals into a lipid layer. A physical characteristic of the GF-lipid nanoparticles prepared by high-pressure homogenization is the state that GF was incorporated in lipids.

3.2. Physical properties of freeze-dried GF-lipid nanoparticles

3.2.1. Relationship between freezing rate and particle size

We have previously reported on the particulate state after the rehydration of freeze-dried GF-lipid nanoparticles containing various saccharides. In that study, the mean particle size of the GF-lipid nanoparticles after rehydration was found to be about 160 nm when monosaccharides such as glucose and galactose were used as additives. However, the mean particle size was only about 60 nm when disaccharides such as sucrose, maltose, and trehalose were used.

Guzman et al. have reported that the particle size of freeze-dried polymer nanoparticles frozen at various rate was nearly the same as the initial ones [30]. We have studied in GF-lipid nanoparticles. In this study, the effects of various freezing conditions on the mean particle size of GF-lipid nanoparticles were investigated. Fig. 2 shows the mean particle size of the rehydrated GF-lipid nanoparticles containing sucrose, maltose, and trehalose that were freeze-dried at various temperatures. The mean particle size increased to 110–130 nm on freezing at −70 and −200 °C. And at −4 °C, though the mean particle size was below 100 nm, it tended to increase beyond the original value. However, freezing at −35 °C produced the size closest to that before freeze-drying, indicating

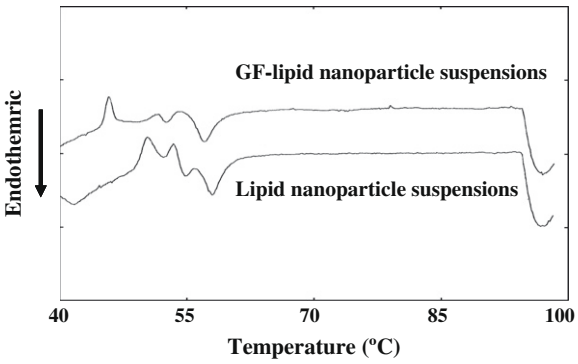

Fig. 1. DDSC curves of main components in GF-lipid nanoparticle suspension. Top, GF-lipid (HSPC:DMPG = 3:1) nanoparticle suspension prepared by high-pressure homogenization and bottom, lipid (HSPC:DMPG = 3:1) nanoparticle suspension prepared by high-pressure homogenization.

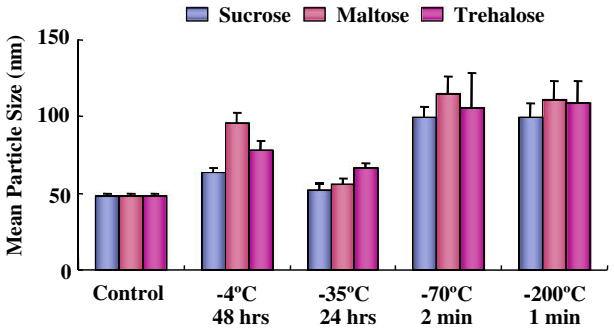

Fig. 2. Effect of various freezing methods on the mean particle size of GF-lipid nanoparticle suspension. The freeze-dried nanoparticles were rehydrated in purified water. Each bar represents the mean ± SD of three determinations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that the nanoparticle size was maintained. The reason why the mean particle size increased at −4 °C was probably due to the slow-freezing speed of the GF-lipid nanoparticle suspension, which caused the water molecules alone to begin to freeze, resulting in enhanced nanoparticle aggregation as the concentration of the suspension increased in the unfrozen portions. The reason for the increase at −70 and −200 °C is considered to be that parts of the intralipid aqueous phase were refrigerated rapidly, causing small ice nucleation and thereby an increase in the volume of the aqueous phase, which resulted in the GF-lipid nanoparticles being damaged. Although the addition of disaccharide was found to be adequate during freeze-drying, aggregation appeared to be promoted at both overly high and overly slow cooling rates. Consequently, maintaining the appropriate cooling temperature and speed were considered to be essential to prevent the aggregation of the nanoparticles.

3.2.2. Relationship between GF-lipid nanoparticles and saccharide during freeze-drying

We previously reported that the particle size increased with glucose and galactose (monosaccharide) as the additive, but not with sucrose, maltose, and trehalose (disaccharide). The protective effect of carbohydrates during drying of liposomes is based on a narrow balance between the interaction between the sugar and the lipid and glass-forming properties of the carbohydrate. A direct interaction between the sugar and phospholipid head groups is pivotal to prevent leakage through the bilayers, whereas vesicle fusion can be prevented by the formation of a stable glassy state [26]. Li et al. has reported that the particle size was maintained because the hydrogen bonding with the disaccharide was sufficient to prevent aggregation, whereas it was insufficient with the monosaccharide [31]. However, it was revealed that a part of monosaccharide was able to maintain particle size in an investigational progress process. Kind of saccharide such as monosaccharide or disaccharide appears to have no relation to an aggregation of nanoparticles. Therefore, we further investigated with the use of monosaccharide, disaccharide, and trisaccharide to find the detailed mechanism behind this aggregation control by saccharide for nanoparticles. Fig. 3 shows the mean particle size of the rehydrated GF-lipid nanoparticles that were freeze-dried with various saccharides added at 5% (w/v). With xylose, the mean particle size on rehydration was about 220 nm. With mannose, fructose, lactose, and raffinose, the mean particle size on rehydration was about 60 nm, suggesting that these saccharides prevented aggregation. Here, the fact that raffinose maintained particle size of the nanoparticles during freeze-drying and rehydration is reported for the first time. Raffinose has never previously been shown to have such an effect.

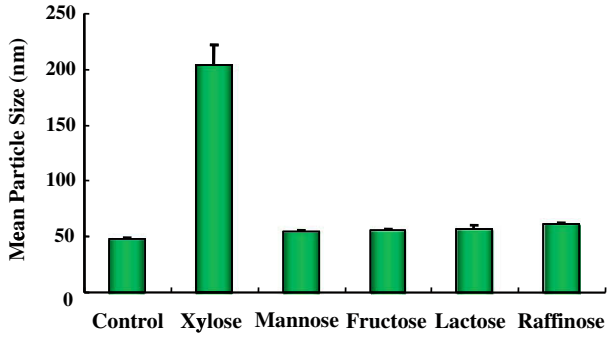

Fig. 3. Effect of various saccharides on the mean particle size of the GF-lipid nanoparticle suspension prepared by freeze-drying and rehydration in purified water. Control represents the mean particle size of GF-lipid nanoparticles prepared by high-pressure homogenization. Each bar represents the mean ± SD of three determinations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Because particle size of the nanoparticles after rehydration was different in equal monosaccharide, it is impossible to explain only by the distinction of monosaccharide, disaccharide, and trisaccharide. Next, we discuss the state of the saccharide during freeze-drying to find more precise mechanism.

The physical properties of the freeze-dried GF-lipid nanoparticles containing various saccharides were determined by powder X-ray diffraction (Fig. 4). Specific diffraction peaks of xylose appeared, suggesting that it existed in the crystalline state. In contrast, mannose, fructose, lactose, and raffinose produced halo patterns, suggesting that they existed in an amorphous state.

Imamura et al. reported that the high viscosity of an amorphous sugar protects proteins from physical and chemical degradation through a retardation of molecular movement [32]. Thus, the freeze-dried GF-lipid nanoparticles containing mannose, fructose, lactose, and raffinose in the amorphous state preserved their mean particle size. Meanwhile, the freeze-dried GF-lipid nanoparticles containing xylose had high crystallinity and, therefore, aggregated after rehydration. These results correspond well to those reported by Imamura et al., suggesting that a similar phenomenon may be responsible here. Wendell et al. also reported that as drying continues, the concentrated solution will transform into a syrup, then into a viscoelastic rubber, and finally into a stable glass [33]. In addition to these phenomena, the following hypotheses were proposed: the xylose solution did not transform into a glass during freeze-drying, producing the GF-lipid nanoparticles to be squeezed out as the saccharine crystal lattice arranged itself regularly. Consequently, the probability of the nanoparticles to approach each other and aggregate was increased (Fig. 5a). In contrast, with, for example, lactose, the saccharide remained incorporated with the GF-lipid nanoparticle because its crystal lattice was arranged irregularly, thus maintaining the particle size (Fig. 5b).

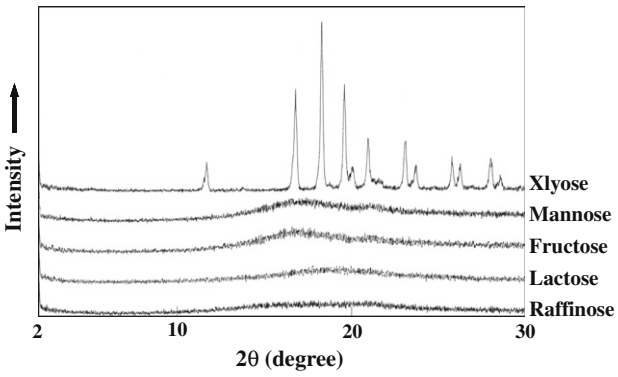

Fig. 4. Powder X-ray diffraction patterns of freeze-dried GF-lipid nanoparticles with various saccharides. The freeze-dried samples were analyzed.

3.3. Behavior of exposed freeze-dried sucrose and GF-lipid nanoparticle

When freeze-dried nanoparticles are stored long term, the mean particle size of the nanoparticles gradually increases. By this step, it is examined that the mechanism increasing the mean particle size of the nanoparticles. An aggregation of the nanoparticles appears to be associated with influence of moisture as described earlier. Hence, freeze-dried, sucrose-containing GF-lipid nanoparticles were exposed under condition at RH 40% and 25 °C, and particulate behavior was observed. Because sucrose has higher absorbency than other saccharide, it has the advantage that particulate behavior can monitor in a short time.

Fig. 6 shows the time course of the powder X-ray diffraction patterns of the freeze-dried GF-lipid nanoparticles containing sucrose at 25 °C and RH 40%. The amorphous state remains up to 2 h, but slight crystallization was observed at 4 and 6 h. Further, crystalline growth was observed at 8 h. The X-ray diffraction peaks of the nanoparticles correspond to those of intact sucrose crystals, thus clearly demonstrating that the observed crystalline growth was that of sucrose.

These phenomena can be explained as follows: the physical properties of freeze-dried sucrose transformed due to the adsorp-

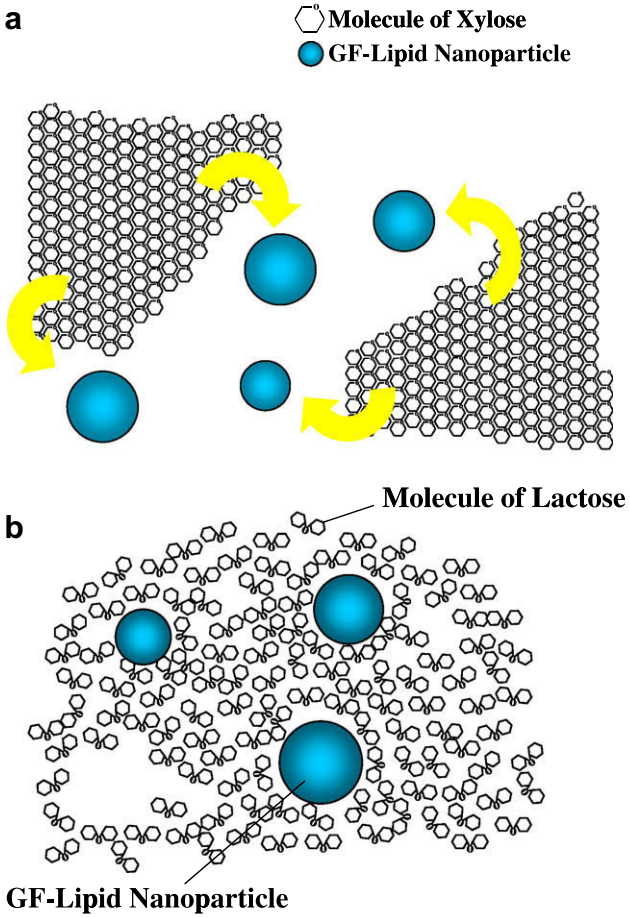

Fig. 5. (a) Behavior of xylose molecules and GF-lipid nanoparticles during freeze-drying. Molecules of xylose are arranged regularly during freeze-drying, producing the GF-lipid nanoparticles to be squeezed out of the lattice. Arrows represent trace of GF-lipid nanoparticles. (b) Diagram showing arrangement of lactose molecules and GF-lipid nanoparticles during freeze-drying. Molecules of lactose are arranged irregularly allowing the GF-lipid nanoparticles to exist within the molecular bulk of lactose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

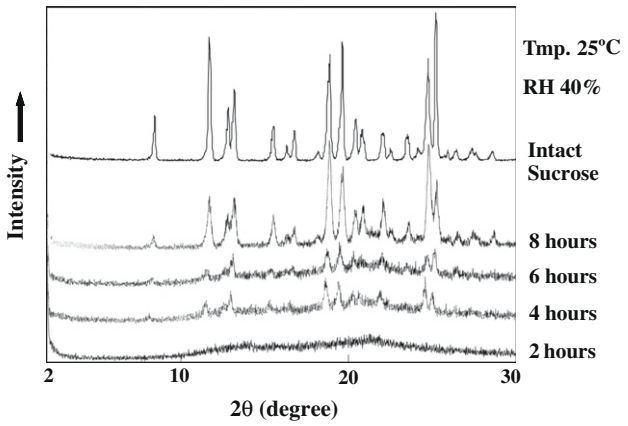

Fig. 6. Changes in powder X-ray diffraction patterns of freeze-dried, sucrose-containing GF-lipid nanoparticles with time. Freeze-dried samples were analyzed at RH 40% and 25 °C.

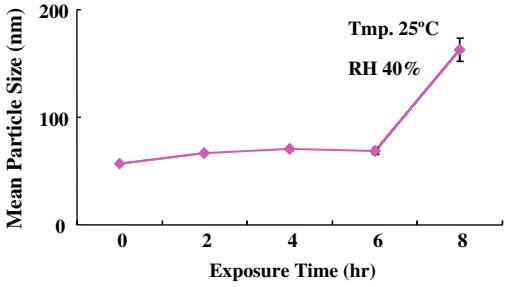

Fig. 7. Changes in the mean particle size of freeze-dried GF-lipid nanoparticles containing sucrose with time. Freeze-dried samples were analyzed at RH 40% and 25 °C. Bars represent the mean ± SD of three determinations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tion of moisture, resulting in crystallization. Fig. 7 shows the time course of the mean particle size of the GF-lipid nanoparticles after rehydration under same conditions as that in the case of Fig. 6. When no crystal transformation was observed (at 4 and 6 h in Fig. 6), the mean particle size of the rehydrated suspension was about 60 nm. When crystalline growth was observed (at 8 h in Fig. 6), the mean particle size of the rehydrated suspension increased to 150 nm, showing an enhancement of aggregation. This suggests that the amorphous sucrose incorporated in the GF-lipid nanoparticles is recrystallized by the adsorption of moisture from the atmosphere. The crystal lattice of sucrose is then rearranged in an orderly fashion, producing the GF-lipid nanoparticles to be squeezed out. Thus, Figs. 5–7 show that the crystallization of the saccharide additive is strongly correlated with the aggregation of the GF-lipid nanoparticles.

4. Conclusions

The results are summarized as follows.

(1) The DDSC thermogram of the nanoparticle suspension prepared by high-pressure homogenization shows that the phase transition temperatures of the GF-lipid nanoparticles and lipid nanoparticles were 56.8 and 57.9 °C, respectively, suggesting that the GF crystals were incorporated into the lipid phase.
(2) The mean particle size of the freeze-dried GF-lipid nanoparticles after rehydration was about 50–60 nm; the lowest particle size was achieved with sucrose, maltose, and trehalose as the additive and refrigeration at −35 °C and for 24 h.
(3) The mean particle size of the rehydrated GF-lipid nanoparticles containing xylose was about 220 nm, whereas that of the nanoparticles containing mannose, fructose, lactose, and raffinose was about 60 nm, suggesting that they prevent aggregation.
(4) The powder X-ray diffraction patterns of the freeze-dried xylose-containing GF-lipid nanoparticles suggested high crystallinity, while those of the freeze-dried mannose-, fructose-, lactose-, and raffinose-containing nanoparticles suggested that they existed in an amorphous state.
(5) The time course of the freeze-dried GF-lipid nanoparticles containing sucrose analyzed by powder X-ray diffraction indicated that crystallization occurred at 8 h. In addition, the mean particle size after rehydration was 150 nm, strongly suggesting that the state of crystallinity was correlated with the particle size of the GF-lipid nanoparticles.

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