# Physical Characteristics of Freeze-Dried Griseofulvin-Lipids Nanoparticles

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**We have attempted to prepare micronized drug particles and to maintain the micronized state long-term in order to improve the solubility of a practically insoluble drug, griseofulvin (GF). GF nanoparticles (GFNPs) prepared by high-pressure homogenization were micronized to about 45 nm (mean particle size). GFNPs were subjected to transmission electron microscopy (TEM) observation. The mean particle size remained at about 50 nm for 3 months at 25 °C. However, increased to about 300 nm in 6 months. After 5% (w/v) sugar was added, GFNP was freeze-dried and rehydrated. The mean particle size of the rehydrated GFNPs was about 160 nm when the sugar was a monosaccharide. In contrast, it was about 55—65 nm when the sugar was a disaccharide. The powder X-ray diffraction pattern of the monosaccharide-containing freeze-dried GFNPs revealed a crystal state of sugar. On the other hand, that of the disaccharide-containing freeze-dried GFNPs indicated an amorphous state of sugar. From this result, it is considered that the high viscosity of an amorphous sugar prevents GFNPs from aggregating through retardation of molecular movement. Freeze-dried GFNPs stored for six months at 25 °C showed a good re-dispersibility. After the rehydration the mean particle size of GFNPs was 55—65 nm. It was found that freeze-dried GFNPs containing sucrose, maltose or trehalose were stable for longer periods than GFNP suspensions.**

**Key words** nanoparticle; high-pressure homogenization; freeze-drying; rehydration; sugar

It is common practice to solubilize a poorly water-soluble drug in an aqueous medium in order to deliver it into the systemic circulation. There are several ways to improve the solubility of drugs including the use of surfactants[1,2] and co-grinding with water-soluble polymers.[3,4] The establishment of pharmaceutical technologies to micronize drug particles at the nano order level is useful to improve drug solubility.[5,6] Besides, micronizing drug particles into the nano-order range is expected to improve drug dissolution rate by increasing the specific surface area of the particles and deliver drugs to the circulation through payer's patches in the gastrointestinal tract.[7]

In this study, a practically insoluble drug, griseofulvin (GF), was used as a model. GF is an antibiotic and antifungal drug used in an oral dosage form. It is reported to have a very low solubility (15 $\mu$g/ml at 37 °C)[8] in water and hence low bioavailability and exhibit variable and incomplete absorption through the gastrointestinal tract (GIT).[8,9] Kraml *et al.* reported that a 0.5 g dose of micronized GF produced serum levels indistinguishable from those produced by a 1.0 g dose of non-micronized GF, and it was feasible to improve the low serum levels due to low absorption by administering micronized GF.[10] Additionally, the bioavailability of GF was reportedly improved as the specific surface area of the particles increased.[11] Therefore, absorption through GIT and bioavailability are expected to be enhanced by reducing the size of GF particles.

In general, it is difficult to micronize drug particles into the nanometer range under dry conditions. However, it is feasible to do so in water dispersions using a mechanical process.[12—14] In such conditions GF was micronized to a mean particle size of 200 nm.[15] In a study of absorption, different sized particles of Polystyrene microspheres (50—3000 nm) were administered to rats daily for ten days to investigate uptake across the gastrointestinal mucosa.[16] The fraction absorbed was 5% for particles of less than 1000 nm, 15% for particles of less than 500 nm and 26% for particles of less than 100 nm, demonstrating the fraction absorbed depended on particle size.

We have prepared fine particles of less than 100 nm using a negatively charged phospholipid (dicetyl phosphate: DCP) and high-pressure homogenizer.[17] However, DCP had high toxicity, and the suspension had low physicochemical stability at room temperature because of agglomeration in two weeks. Furthermore, it was difficult to maintain the mean particle size below 100 nm after freeze-drying/rehydration. Hence it is necessary to overcome these drawbacks for practical application.

In this study, we have used low-toxic and highly biodegradable negatively charged phospholipid (dimyristoyl phosphatidylglycerol: DMPG) to make micronized GF particles less than 100 nm in size, and attempted to maintain the micronized condition for at least half a year. Additionally, we have investigated the behavior of fine particles in suspension, and furthermore, prepared freeze-dried samples containing various sugars to investigate the physicochemical characteristics of the interaction between the sugars and particles.

## Experimental

**Materials** Hydrogenated soybean phosphatidylcholine (COATSOME® NC-21 (HSPC)) and dimyristoyl phosphatidylglycerol (COATSOME® MGLS-4040 (DMPG)) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Griseofulvin (JPXIV, GF) was provided by Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Ethanol, glucose, galactose, maltose and sucrose (reagent grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trehalose was provided by Fuji Sugar Manufacture Co., Ltd. (Tokyo, Japan).

The membrane filter (pore size: 100 nm) was purchased from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All reagents were used as is. Distilled water that had been treated by ion exchange was used.

**Preparation of Suspensions** Drug–Lipids Mixture: One gram of mixed lipids (HSPC : DMPG = 3 : 1 (molar ratio)) and 20 mg of GF were dissolved

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in 2 ml of ethanol and evaporated at 80 °C. The drug–lipids mixture was dispersed in 200 ml of distilled water and premixed with a Speed Stabilizer (10000 rpm; Kinematica Co. (Osaka, Japan)) for 10 min. This premixed suspension was homogenized using a high-pressure homogenizer (max pressure: 9.5 kg/cm$^2$) (Nanomizer, X form chamber; Tokushu Kika Kogyo Co. (Osaka, Japan)). The homogenization was performed for 5, 10, 20 and 40 cycles (cycle times: pass number).

**Measurement of Particle Size** Particle size distribution was analyzed with an electrophoretic light scattering photometer (ELS-8000; Otsuka Electronics Co., Ltd. (Tokyo, Japan)) at a fixed angle of 90° at room temperature. The particle size analysis data were evaluated using weight distribution. Particle size of prepared GF nanoparticle (GFNP) suspensions were measured just as they were.

**Turbidity of Suspensions** The turbidity of GFNP suspensions was measured using an absorption spectrophotometer (wavelength; 400 nm) (UV-150-02; Shimadzu Co. (Tokyo, Japan)) at room temperature.[18)]

**Quantitative Determination of Griseofulvin in Nanoparticles under 100 nm** Prepared GFNP suspensions were filtrated through a membrane filter (pore size 100 nm) to determine GF in nanoparticles under 100 nm. GF content was determined by high performance liquid chromatography (HPLC) (LC-9A; Shimadzu Co. (Tokyo, Japan)).

Experimental Procedures and Operational Procedures: One milliliter of GFNP suspensions was added to 5 ml of methanol and vortexed to dissolve GF and lipids. GF content of the methanol/water solution was measured employing the absolute calibration curve method by HPLC. The column was LiChroCART 250-4 (Merch Co. (New Jersey, U.S.A.)). The mobile phase was methanol/water (=5). The flow rate was 0.6 ml/min. The absorbance wavelength was 285 nm.

**Transmission Electron Microscopy (TEM)** Transmission electron microscopy was performed using an H-7100 (Hitachi, Ltd. (Tokyo, Japan)). Samples were stained with Tungsten stain solution.

**Freeze-Drying and Rehydration. Method A** Freeze-Drying: Two milliliters of the GFNP suspensions was collected into a vial, and 100 mg of sucrose, maltose, trehalose, glucose or galactose were added to the vial. Each vial was vortexed and the suspensions were frozen at −35 °C and stood for 24 h. The frozen sample was freeze-dried in a glass chamber for 24 h using a vacuum pump accompanied by a vapor condenser (−90 °C, $1.0\times10^{-3}$ Torr) (NEOCOOL; Yamato Scientific Co. (Tokyo, Japan)).

Rehydration: Two milliliters of distilled water was added to the vial and shaken by hand to rehydrate the freeze-dried sample. The mean particle size of rehydrated GFNP suspensions was determined by ELS.

**Method B** Freeze-Drying: Two milliliters of the GFNP suspensions was collected into a vial, and frozen at −35 °C and stood for 24 h. Freeze-drying procedures were performed as described above.

Rehydration: Two milliliters of 5% (w/v) of sucrose, maltose, trehalose, glucose or galactose solution was added to the vial and shaken to rehydrate the freeze-dried sample. The mean particle size of rehydrated GFNP suspensions was determined by ELS.

**Measurement of Powder X-Ray Diffraction** Powder X-ray diffraction (PXRD) was measured with a powder X-ray diffraction device (RAD-C, Rigaku Denki Co., Ltd. (Tokyo, Japan)) using CuK$\alpha$ radiation at 30 kV and 50 mA at room temperature. The scanning rate was 5 °/min, and diffraction angle (2$\theta$) was 2—30°.

**Stability Studies** 1) Stability of Rehydrated GFNP Suspensions: The GFNP suspensions containing a disaccharide (sucrose, maltose or trehalose) were freeze-dried and rehydrated immediately. Rehydrated GFNP suspensions were sealed and stored at 25 °C in the dark for 4 weeks. The mean particle size of GFNPs in the suspensions was determined by ELS.

2) Stability of Freeze-Dried GFNPs: The GFNP suspensions containing disaccharide were freeze-dried. Freeze-dried GFNPs were sealed and stored 25 °C in the dark and rehydrated after 1, 3 and 6 months. The mean particle size of GFNP in the rehydrated suspensions was determined by ELS.

## Results and Discussion

**Micronization by a High-Pressure Homogenizer and Physical Characteristics of Drug Particles** Figure 1 shows the influence of pass number during high-pressure homogenization on the mean particle size and the turbidity of suspensions. The particle size of the suspension decreased with an increase in the pass number, and mean particle size was about 45 nm at pass number 40. Turbidity was 1.121 at

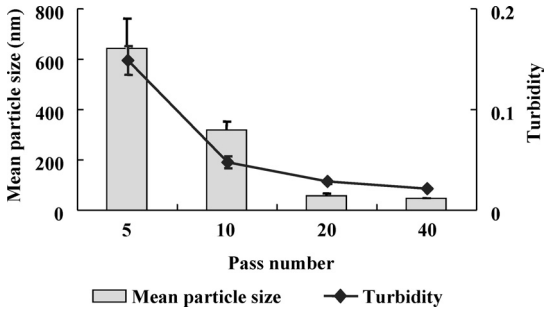


Fig. 1. Influence of Pass Number on the Mean Particle Size and the Turbidity of GF–Lipids Mixture Suspensions Prepared by High-Pressure Homogenization

Column shows the relationship between pass number and the mean particle size. Diamond shows the relationship between pass number and the turbidity. Each bar represents the mean±S.D. of 3 measurements.

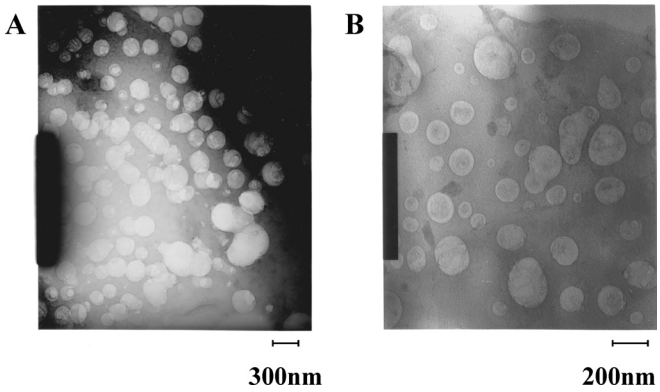


Fig. 2. TEM Photographs of Nanoparticles Prepared by High-Pressure Homogenization

Panel A: GFNPs, panel B: lipids nanoparticles.

pass number 0 and 0.021 at pass number 40. The turbidity decreased with an increase in the pass number, the same behavior as mean particle size. No significant difference was found at pass numbers 20 and 40 in mean particle size and turbidity. GF content of fine particulate suspensions filtrated through a membrane filter (pore size 100 nm) was 61.2± 2.7% and 91.3±1.5% to the initial strength at pass number 20 and 40, respectively. No much difference was observed in mean particle size between pass number 40 and pass number 20. However, a significant difference was detected in GF content. This might be caused by more uniform dispersion of GF with an increase of pass number. It was confirmed that 97.6±1.8% of the GF content was filtrated through a membrane filter of pore size 200 nm. From this result, it was indicated that more than 40 passes were required to micronize the majority of dispersed GF particles adequately. In this study, the particles in suspension obtained at pass number 40 are denoted as GFNPs.

Figure 2 shows TEM photographs of GFNPs (panel A) and lipids particles (HSPC : DMPG=3 : 1) without GF (panel B). An irregular spherical shape was observed inside the lipids particles in panel A. Therefore, it was suggested that solid GF was incorporated in the lipids particle. Figure 3 shows the stability of the GFNPs suspension. The mean particle size of the suspension remained 50 nm for 3 months at 25 °C. However, it increased to about 300 nm in 6 months. Because of the aggregation of GFNPs, the suspension be-

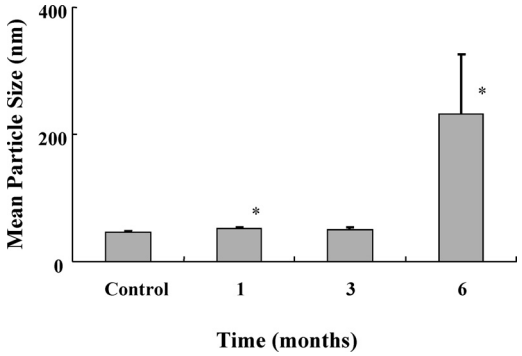


Fig. 3. Particle Size Stability of the GFNP Suspension at 25 °C

Column shows the relationship between time and the mean particle size. Control: mean particle size of GFNPs immediately after homogenization. Statistical analysis was Student's *t*-test. Significant differences from the mean particle size of control group was indicated by $*p<0.05$. Each bar represents the mean±S.D. of 3 measurements.

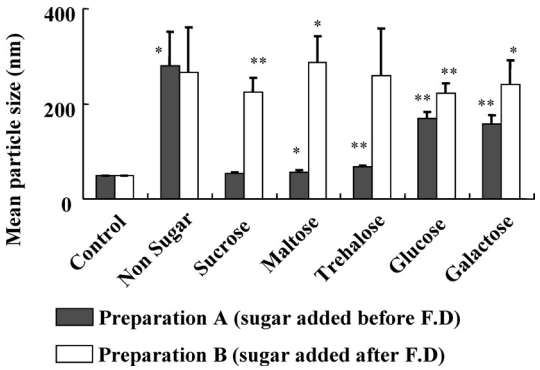


Fig. 4. Effect of Various Sugars on Mean Particle Size before/after Freeze-Drying and Rehydration of GFNPs

Closed column shows the mean particle size of rehydrated GFNPs. GFNPs with various sugars were freeze-dried and rehydrated in distilled water. Open column shows the mean particle size of rehydrated GFNPs. GFNPs without sugars were freeze-dried and rehydrated in distilled water with sugars. Control: mean particle size of GFNPs before freeze-drying. Statistical analysis was Student's *t*-test. Significant differences from the mean particle size of control group was indicated by $*p<0.05$, $**p<0.01$. Each bar represents the mean±S.D. of 3 measurements.

came opaque.

As described above, it is feasible to obtain particles under a mean size of 100 nm by high-pressure homogenization. However, the mean particle size of GFNPs increased with time. Consequently, to stabilize GFNPs under 100 nm for long-term storage, we have tried to adopt the freeze-drying method used to preserve fine particles of microemulsions[19] and nanosphere suspensions.[20] Konan *et al.* has reported that in the absence of lyoprotectant, freeze-dried and rehydrated particles aggregated, meanwhile, the addition of sugars prevented the particles from aggregating after freeze-drying/rehydration.[21—24] So we have also tried to use various sugars to inspect an influence of sugars to the mean particle size of GFNPs.

**Influence of Sugars on Particle Size in Rehydration of Freeze-Dried Nanoparticles** Closed columns in Fig. 4 show the mean particle size of rehydrated freeze-dried GFNPs prepared by the Method A. If no sugar was added, the mean particle size increased significantly (280 nm) after rehydration, and consequently aggregation was observed. When a monosaccharide (glucose or galactose) was added, the mean size was about 160 nm. The monosaccharide prevented aggregation, but was not very effective. In contrast, when a disaccharide (sucrose, maltose or trehalose) was added the mean size was about 55 nm, 55 nm and 65 nm, respectively. From these results, disaccharides can maintain the size of GFNPs after freeze-drying/rehydration. Open columns in Fig. 4 show the mean particle size of GFNP suspensions prepared by the Method B. It was confirmed that aggregation occurred in all rehydrated GFNP suspensions under these conditions. From this result, it is considered that sugar was needed during freeze-drying, not rehydration.

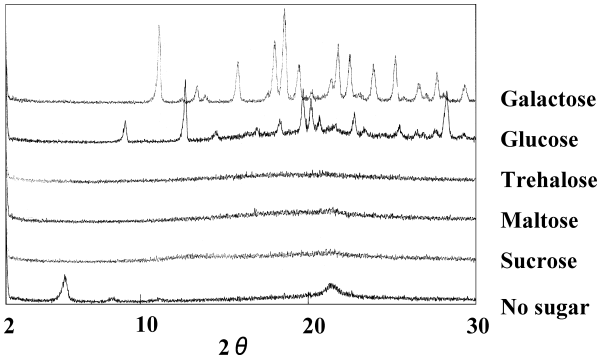


Fig. 5. Powder X-Ray Diffraction Patterns of Freeze-Dried GFNPs with Sugars

Samples were prepared by adding various sugars to GFNP suspensions and freeze-drying. Freeze-dried GFNPs were measured.

Figure 5 shows the PXRD pattern of sugar-containing freeze-dried GFNPs. Monosaccharide-containing freeze-dried GFNPs had sugar in a crystal state. On the other hand, disaccharide-containing freeze-dried GFNPs were in an amorphous state. The disaccharide in the amorphous state (as shown in Fig. 5) maintained a mean particle size of less than 100 nm after rehydration (as shown in Fig. 4), and the monosaccharide in the crystal state showed aggregation after rehydration. It is reported that the high viscosity of an amorphous sugar protects proteins from physical and chemical degradation through a retardation of molecular movement.[25] Consequently, we conclude that inhibition efficacy of GFNPs aggregation by adding disaccharide could be explained in the same way. Li *et al.* has proposed that during freezing, sugars have the ability to form a rigid sugar-glass or vitrified network structure in which liposomes can be embedded. This vitrified network preserves the integrity of the liposome.[26] From Fig. 4 and this report, sugars play an important role during freezing rather than rehydration. Li *et al.* has also reported that particles had a tendency to aggregate because a monosaccharide was less effective than a disaccharide in interacting with particles.[26] From this report, it was assumed that the mean particle size of monosaccharide-containing freeze-dried GFNPs are larger than that of disaccharide-containing freeze-dried GFNPs because of less effective interaction with the particles.

Figure 6 shows the mean particle size of GFNPs in the suspensions, which are prepared by rehydration of freeze-dried GFNPs and kept 4 weeks at 25 °C. The mean particle size of the sucrose or maltose-containing GFNP suspension remained at in about 55 nm, and the trehalose-containing GFNP suspension was about 65 nm in size for one month after rehydration. From this result, it was suggested that a dispersed state of rehydrated GFNPs was maintained for 1

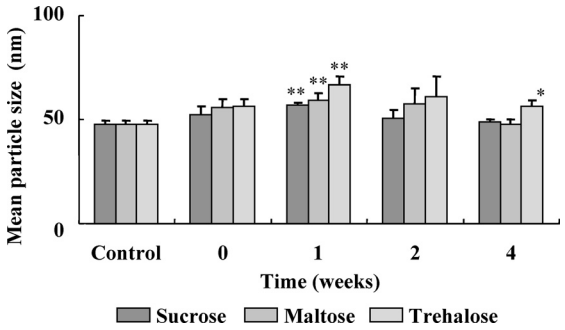


Fig. 6. The Mean Particle Size of Freeze-Dried and Rehydrated GFNP Suspension Stored for 4 Weeks at 25 °C

GFNP suspensions with disaccharides were freeze-dried and immediately rehydrated in distilled water. The rehydrated GFNP suspensions were stored for 4 weeks. Control: mean particle size of GFNPs before freeze-drying. Statistical analysis was Student's *t*-test. Significant differences from the mean particle size of 0 week group was indicated by $*p<0.05$, $**p<0.01$. Each bar represents the mean±S.D. of 3 measurements.

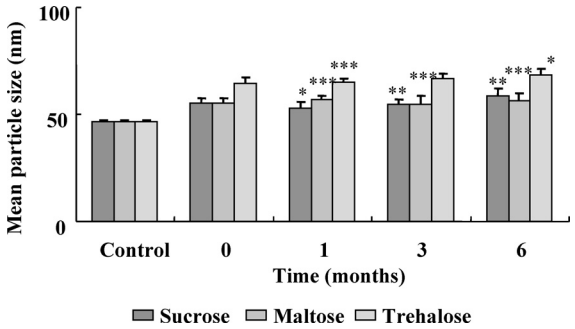


Fig. 7. Stability of Freeze-Dried GFNPs Stored at 25 °C

GFNP suspensions with disaccharides were freeze-dried and stored for 6 months. Each mean particle size was measured after rehydration by adding distilled water. Control: mean particle size of GFNPs before freeze-drying. Statistical analysis was Student's *t*-test. Significant differences from the mean particle size of 0 month group was indicated by $*p<0.05$, $**p<0.01$, $***p<0.001$. Each bar represents the mean±S.D. of 3 measurements.

month.

Figure 7 shows the mean particle size of GFNPs in the suspensions, which are prepared by rehydration of freeze-dried GFNPs stored at 25 °C and rehydrated after 1, 3, and 6 months. The freeze-dried GFNPs stored for six months showed good re-dispersibility on rehydration. Consequently, it is feasible to preserve freeze-dried GFNPs for 6 months at room temperature by adding a disaccharide.

## Conclusion

The results obtained can be summarized as follows.

(1) A mixture of GF and lipids (HSPC : DMPG=3 : 1) was micronized into about 45 nm (mean particle size) by high-pressure homogenization. The yield of GFNPs under 100 nm was 91.3±1.5%. TEM analysis showed an irregular spherical shape in the GFNPs.

(2) Although the GFNPs were maintained as fine particles in suspension for 3 months at 25 °C, the suspension became opaque gradually because of aggregation in 6 months.

(3) The mean particle size of rehydrated GFNPs was 55—65 nm when a disaccharide was added and the freeze-dried GFNPs were rehydrated. In addition, the mean particle size of the rehydrated GFNP suspension was maintained for 1 month.

(4) The X-ray pattern of monosaccharide (glucose or galactose)-containing freeze-dried GFNPs showed a crystalline state, while it showed an amorphous state when a disaccharide (sucrose, maltose or trehalose) was added.

(5) Rehydrated disaccharide (sucrose, maltose or trehalose)-containing and freeze-dried GFNPs stored for 6 months at 25 °C showed almost the same value for mean particle size as the GFNP suspension after high-pressure homogenization.

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