Development of highly stable nifedipine solid-lipid nanoparticles

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Abstract:

To improve the solubility of the drug nifedipine (NI), highly stabilized solid-lipid nanoparticles (SLNs) of nifedipine (NI-SLNs) were prepared by high pressure homogenization using two phospholipids, followed by lyophilization with individual sugar moieties (four monosaccharides and four disaccharides). The mean particle diameter, polydispersity index (PDI), zeta potential, drug loading, and the encapsulation efficiency of the NI-SLN suspension were determined to be 68.5 nm, 0.3, -62.1 mV, 2.7%, and 97.5%, respectively. In comparison with the NI-SLNs, the NI-SLNs lyophilized with trehalose (NI-SLN-Tre) showed a slight increase in the particle size from 68.5 to 107.7 nm, but the PDI decreased from 0.38 to 0.33, and no significant change in zeta potential was observed. Aqueous re-dispersibility study demonstrated that NI-SLNs lyophilized with trehalose had the maximum concentration (14.7 µg/ml) at 5 min, compared with lyophilized SLNs using other sugars; the use of other sugars also resulted in significant changes in the particle size, PDI, and zeta potential. A trehalose concentration of 2.5% w/v and a two-fold dilution of the SLN suspension were found to be the best conditions for lyophilization. Data from lyophilized SLNs using differential scanning calorimetry, powder X-ray diffraction, Fourier-transform infrared spectroscopy, and scanning electron microscopy indicated eventual transformation of NI-SLN-Tre from a crystalline to an amorphous state during the homogenization process. Finally, a stability study was performed with NI-SLN-Tre for up to 6 months at 30°C and 65% relative humidity, with no significant deterioration observed, suggesting that trehalose might be a useful cryoprotectant for NI-SLNs.

Keywords: Nifedipine, solid-lipid nanoparticle, trehalose, cryoprotectant.

1. Introduction

Recently, about 40% of molecules being developed by the pharmaceutical industry have been reported to be poorly water soluble, which limits their absorption in the gastrointestinal tract and reduces the overall bioavailability.[1,2] Most probably, many molecules have been rejected during the early stages of drug development because of this lack of water solubility. Therefore, the development of effective technologies and novel drug formulations for poorly water-soluble drugs is a mainstay of pharmaceutical research. Nanotechnology has revolutionized the field of drug delivery research. It offers some conventional delivery approaches, such as surface modification, complex formation, and use of colloidal lipid carriers for delivery to intestinal lymphatics.[3,4] In addition, polymeric nanoparticles, self-emulsifying delivery systems, liposomes, microemulsions, micellar solutions, and solid-lipid nanoparticles (SLNs) have been exploited as possible drug carriers for oral intestinal lymphatic delivery. Since 1991, SLNs have been investigated comprehensively for use in drug delivery through various administration routes. SLN-based systems possess the characteristics of conventional carriers, as well as some additional characteristics that prevent the drawbacks and reported for conventional systems.[5-7] SLNs have also been reported to be useful carriers for the successful delivery of peptides (e.g., insulin) and anticancer drugs (e.g., doxorubicin) through the oral intestinal route.[8,9] Recently, Li *et al.* studied the pharmacokinetic aspects of quercetin-loaded SLNs after oral administration and found that SLNs improved the oral bioavailability.[10]

In particular, many formulations of SLN-like nanocarriers have been investigated with nifedipine (NI). NI is a highly potent calcium-channel blocker, which is poorly soluble in water (≈20 µg/ml). Previous attempts at formulations of NI-nanocarriers have been reported, with mixed results. These approaches for the dissolution rate enhancement of NI include compaction with hydroxypropyl methyl cellulose (HPMC)[11]; co-grinding with HPMC[12], and bile salts[13]; and formation of co-precipitates or co-evaporates with mannitol[14], phosphatidylcholine esters[15], HPMC[16], chitosan derivatives[17], polyethylene glycols[18,19], and polyoxyethylene-polyoxypropylene copolymers[20]. Recently, a high pressure homogenization technique has been successfully employed to prepare solid-lipid nanoparticles of NI using only phospholipids such as HSPC (hydrogenated soybean

phosphatidylcholine) and DPPG (dipalmitoylphosphatidyl glycerol) as carriers to enhance the solubility of NI.[21] This formulation has the advantage that no emulsifier and no toxic organic solvents were used. However, the limitation of this formulation was the reduced stability of the SLN suspension under ambient conditions. The suspension was only found to be stable, without suffering from any particle aggregation, for up to 4 months at 4°C.[21] To overcome this limitation and to extend the storage life under ambient conditions, various sugars have been investigated as cryoprotectants. Sugars have proved to be very effective in preventing particle aggregation and inhibiting leakage of an active ingredient during the freeze drying of solid-lipid nanoparticles.[22] Ohshima *et al.* studied two monosaccharides (glucose and fructose) and two disaccharides (maltose and sucrose) as cryoprotectants, at arbitrary concentrations of 2% w/v, and found that disaccharides had higher cryoprotectant activity than monosaccharides.[21] From these results, we speculated that sugars, specifically disaccharides, can be used to make SLNs physically stable. Therefore, there is a need for investigation into the use of sugar moieties as cryoprotectants that may be highly effective at inhibiting NI-nanoparticle aggregation.

To develop NI-loaded lyophilized SLN (NI-SLN) formulations with high physical stability, SLNs were prepared by high pressure homogenization and lyophilized with monosaccharides and disaccharides. The particle size and shape of the NI-SLNs were studied using dynamic light scattering (DLS) and scanning electron microscopy (SEM). X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and differential scanning calorimetric (DSC) analysis were performed to detect changes in the crystal structure and any chemical interactions between components. The results of these studies are discussed herein.

2. Materials and Methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine [COATSOME® NC-21(HSPC)] and dipalmitoylphosphatidylglycerol [COATSOME® MGLS-6060 (DPPG)] were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Nifedipine (JPXIV, NI) was provided by Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Glucose, fructose, galactose, xylose, lactose, trehalose, maltose, and sucrose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The membrane filters (pore size: 0.20 and 0.45 µm) were purchased from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All reagents were of the highest grade commercially available and all solutions were prepared using de-ionized distilled water.

2.2. Preparation of NI-loaded solid-lipid nanoparticle (NI-SLN) suspension and lyophilized NI-SLNs

To prepare the NI-SLN suspension, 40 mg of NI and 1000 mg of lipids (HSPC: DPPG, 5:1 molar ratio) were added to a mortar and physically mixed for 5 min. The mixture was then co-ground by a roll mill (R3-1R, Kodaira Seisakusho Co., Ltd.). The grinding part consisted of three rollers and the rotating velocity ratios for each roller were fixed as 1:2.5:5.8. Grinding was carried out for 5 min. During grinding, most of the sample adhered to the rollers, but some partially fell down from the rollers. Therefore, the mill was stopped every 30 s to collect fallen material. The co-grinding cycle was repeated 10 times. The resultant roll mixture was dispersed in 200 ml of de-ionized distilled water and premixed using a Speed Stabilizer (10,000 rpm, Kinematica Co.) at 9000 rpm for 10 min, followed by high pressure homogenization (max pressure: 200 MPa, Nanomizer, NM2-L200-D10; Yoshida Kikaikogyo, Co.) with a pass cycle of 100.[23)] The suspension thus obtained was filtered through a 0.2 µm membrane filter and stored in a refrigerator at 4°C for further use.

To prepare lyophilized SLNs, 2 ml of the NI-SLN suspension was collected into vials, each containing 20 mg of glucose, fructose, galactose, xylose, lactose, maltose, trehalose, or sucrose. Each vial was frozen at −40°C for 3 h and then the frozen sample was freeze-dried in a glass chamber for

24 h using a vacuum pump accompanied by a vapor condenser (−20°C, 0.0225 Torr). Then, secondary drying was carried out at 20°C for 24 h.

2.3. Examination of the physico-chemical properties of the NI-SLN suspension and lyophilized NI-SLNs

(a) Mean particle size and zeta potential analysis

The mean particle size was measured by dynamic light scattering (Nano ZS, Malvern Instruments), and zeta potential was estimated on the basis of electrophoretic mobility under an electric field at room temperature. The particle sizes were analyzed based on weight distribution.

(b) Reconstitution

De-ionized distilled water (2 ml) filtered through a membrane filter (0.20 µm) was added to the vial and the vial was shaken by vortex agitation to rehydrate the freeze-dried sample. The mean particle size and zeta potential were determined by Nano ZS mentioned above.

(c) HPLC analytical method

An HPLC analytical method was developed for estimation of total drug content (TDC), encapsulation efficiency (EE), and drug loading (DL) of the NI-SLN suspension and lyophilized products, and for a re-dispersibility study of the lyophilized NI-SLNs. The analytical method used was reversed phase HPLC (RP-HPLC) (Shimadzu, Japan) in a binary mode (dual pump), with a UV detector at a wavelength of 236 nm. The HPLC column was reversed phase (Cadenza 5CD-C18, 4 mm ID×150 mm; Imtakt) at 40°C. The mobile phase of methanol: aqueous formic acid (0.2% v/v) at a ratio of 60:40 was delivered at a flow rate of 0.4 ml/min. The retention time of NI was found to be 6.1±0.1 min.

(d) Total drug content (TDC), encapsulation efficiency (EE), and drug loading (DL) determinations

TDC: NI-SLN suspensions (200 µL) were transferred to an eppendorf tube (1.5 ml). Exactly 800µL of methanol was added and the suspension was sonicated for 3 min, followed by centrifugation at 7000 rpm for 10 min. The supernatant was filtered through a 0.2µm syringe filter. The drug content was assayed by HPLC, as described above, using a calibration curve with at least three standard concentrations (10µg/mL, 50µg/mL, and 100µg/mL) of NI.

EE and DL: The EE and DL were determined indirectly by calculating the amount of free drug (NI) concentration in the dispersions, according to the following equations.[24,25)]

$$EE(\%) = \frac{(\text{Total drug content (TDC)} - \text{Free nifedipine}) \times 100}{\text{Total drug content (TDC)}}$$

$$DL(\%) = \frac{(\text{Total drug content} - \text{Free dissolved drug}) \times 100}{\text{Drug amount used} - \text{Free dissolved drug} + \text{Weight of the lipids}}$$

The free NI was separated by filter centrifugation, using an Ultracel-50K (Amicon®, Millipore Corporation, Ireland) centrifugal filter device. Briefly, 2.0 ml of SLN suspension was placed in the filter device and centrifuged at 12,000 rpm, for 30 min using an Eppendorf® centrifuge (Germany). After this, the encapsulated NI was retained in the filter device, while the free NI passed through the filter membrane and was available for quantification by RP-HPLC as described above. The experiments were performed in replicates (n = 3) and the EE and DL were calculated using the above equation.

(e) Aqueous re-dispersibility test

Aqueous re-dispersibility study of the lyophilized NI-SLNs was performed by mixing the freeze-dried nanoparticles equivalent to 290 µg of NI in 10 or 20 ml of water contained in a 50 ml beaker that was being continuously stirred magnetically at 100 rpm at room temperature. The analyte samples (0.5 mL each) were withdrawn at intervals of 1, 5, 10 and 20 min, followed by the replacement of an equal volume of water. The solution was then filtered through a membrane filter (0.45 µm) to remove some aggregates of NI-SLNs. The amount of NI dispersed in the solution was monitored using the HPLC method as described above. The concentration of NI was plotted against

time to derive the re-dispersibility profile.

2.4. Solid-state characterization of lyophilized NI-SLNs

(a) Characterization by DSC

Thermograms of the different samples (NI-SLNs lyophilized with fructose, galactose, glucose, xylose, lactose, maltose, sucrose, and trehalose) were obtained from DSC (Exstar, SII DSC7020). Lyophilized SLN samples, the freeze-dried lipid mixture, and free NI (3–5 mg) were placed in sealed standard aluminium pans and heated from 0 to 300°C, at a scanning rate of 10°C/min, under nitrogen purge, with an empty aluminium pan as reference.

(b) Characterization by PXRD

An X-ray diffractometer (RAD-C, Rigaku Denki Co., Ltd.) was used for the diffraction studies. The samples were exposed to Cu-Kα radiation (30 kV, 50 mA) and scanned from 2–40°, 2θ at a scanning rate of 5°/min. Samples used for XRD analysis were free NI, a physical mixture of the lipids alone, a ground mixture of NI and lipids, and the lyophilized products NI-SLN-Fru, NI-SLN-Gal, NI-SLN-Glu, NI-SLN-Xyl, NI-SLN-Lac, NI-SLN-Mal, NI-SLN-Suc, and NI-SLN-Tre.

(c) Characterization by SEM

A scanning electron microscope (SSX-500, Shimadzu, Japan) was used to obtain SEM micrographs of the lyophilized products and the respective sugars used for lyophilization, after coating with gold/palladium in a vacuum beforehand. An accelerating voltage of 15 kV was used.

(d) Characterization by FTIR

Lipid mixture alone (HSPC: DPPG, 5:1), the physical mixture of NI and lipid, NI-loaded ground mixture, and all the lyophilized products were analyzed by FTIR. In addition, to find differences in the spectra, subtractions of the spectrum of the lipid mixture alone from that of the NI-SLN physical mixture, and from that of the NI-SLN roll mill mixture, were calculated. The samples were measured

by the diffuse reflection method using an FTIR spectrometer (IR-Prestige 21, Shimadzu Co.).

2.5. Stability studies

The stability of lyophilized NI-SLN-Tre was investigated by measuring the particle size, zeta potential, and dissolved concentration of NI in water at 0 (initial) months and after storage at 30°C and 65% RH for 6 months.

2. 6. Statistics

Statistical analyses were performed using the Student t-test. A probability value of $P < 0.05$ was considered to indicate statistical significance.

3. Results and Discussion

3.1. The cryoprotectant efficiency of different sugars towards lyophilized NI-SLNs

The NI-SLN suspension had a particle size of 68.5 nm, and PDI, ZP, DC, EE, and DL values of 0.3, −62.1 mV, 145.0 µg/ml, 97.5%, and 2.7%, respectively. Previously, electrostatic stabilization of SLNs was reported to require a ZP ≥ 30 mV or ≤ −30 mV.[26)] It has also been reported that a ZP between 5 and 15 mV results in limited flocculation and a ZP between −5 and −15 mV leads to maximum flocculation.[26)] The highly negative value of the ZP of the NI-SLN suspension clearly suggests that the negatively charged phospholipid (DPPG) provides electrostatic stabilization of the SLNs. In addition, lyophilization of NI-SLNs with 2% w/v of sugars (glucose, fructose, maltose, and sucrose) has been reported to prevent aggregation of nanoparticles.[21)] Among these sugars, two disaccharide moieties showed rapid dispersibility. Selection of the appropriate sugar moiety for lyophilization depends on the dispersibility, keeping the particle size and ZP, as much as possible, unchanged. Four monosaccharides (fructose, galactose, glucose, and xylose) and four disaccharides (lactose, maltose, sucrose, and trehalose) at concentrations of 2.0% w/v were investigated to select the sugar/s with the highest cryoprotective activity for lyophilization; freeze-dried NI nanoparticles

without the presence of a sugar were used as a control. **Table 1** shows the PDI, particle diameter, and ZP, and **Fig.1** shows the re-dispersibility profile of the lyophilized samples. More negative values for the ZP were observed for SLNs with the disaccharides, because disaccharides have a negatively charged head group that stabilizes the lipid by getting absorbed on the surface and providing electrostatic stabilization of the particles.[27] Moreover, the re-dispersibility profiles indicated that the disaccharides had maximum cryoprotectant activity with the highest solubility of NI. The lowest ZP values result in the highest efficiency of protection against particle aggregation. The four disaccharides, trehalose, maltose, lactose, and sucrose, exhibited almost identical maximum concentrations at 5 min of 14.7, 14.6, 14.1, and 13.9 µg/ml, respectively (**Fig. 1**). However, maltose, lactose, and sucrose containing NI-SLNs had relatively higher particle diameters, PDI, and ZP values (174.9 nm, 0.7, and −56.4 mV; 109.3 nm, 0.5, and −51.1 mV; and 162.9 nm, 0.4, and −43.7 mV, respectively) than those observed for trehalose (107.7 nm, 0.3, and −58.6 mV). Therefore, trehalose appeared to be the best cryoprotectant for use in further studies.

3.2. Dilution effect of the NI-nanoparticle suspension in water for freeze drying (freeze thawing test)

The optimum concentration of the NI-SLN suspension for freeze drying was determined by a freeze thawing test using 2% trehalose as the cryoprotectant. The particle size (P_b) and PDI (D_b) of the nanoparticle suspension before freeze thawing were 53.2 nm and 0.3, respectively. The lowest particle diameter ratio, P_a/P_b (1.0) but highest PDI ratio D_a/D_b (2.3) were observed for a dilution ratio of 1:5 (suspension: water) **(Table 2**). In spite of having a moderately high value for P_a/P_b (1.4), the sample with a 1:1 dilution ratio showed the least PDI ratio (D_a/D_b) of 1.0. Guan *et al.* reported that a two-fold dilution of nanoliposomes, using trehalose as a cryoprotectant, shifted the P_a/P_b value from 1.9 to 1.2 and a value smaller than 2.0 is preferable for freeze drying purposes.[28] The smaller the P_a/P_b and D_a/D_b values, the better will be the freeze drying effect. In our study also, a dilution ratio of 1:1gave satisfactory results.

3.3. Effect of the concentration of trehalose on freeze drying

The effectiveness of various concentrations of trehalose on the freeze drying of NI-SLNs was examined (**Table 3)**. A mean particle size around 100 nm was observed for lyophilized nanoparticles containing 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% trehalose. The mean PDI of 0.39 and 0.38 were observed for concentrations of 2.5% and 3.0% w/v trehalose, respectively, suggesting that these might be effective concentrations of trehalose for cryoprotection during lyophilization. The lowest ZP (−59.1 mV) was observed for 2.5% trehalose, indicating that this concentration should have more efficacy in preventing particle aggregation than 3.0% trehalose (ZP = −45.9 mV).

3.4. Solid-state characterization

The thermal stability of the SLNs and the compatibility of the constituents in the formulation were analyzed by simultaneous DSC. DSC also gives an insight into the melting and re-crystalline behavior of crystalline materials like SLNs. Therefore, DSC experiments are useful to understand solid dispersions, such as solid solutions, simple eutectic mixtures, and, as in this case, the effect of lipid mixtures and cryoprotectant sugars on crystal ordering. The depression of melting point is determined in proportion to the curvature, 1/r, of a spherical nanoparticle, according to the Gibbs-Thomson equation.[29] **Fig.2** gives an overview of the melting process of intact NI, a freeze-dried mixture of lipids, and the lyophilized NI-SLNs. The endothermic (melting point) peak of the pure drug NI occurs at 176.9°C, whereas the DSC thermal curve for the lipid mixture shows endothermic peaks at 75.0, 86.4, and 131.4°C. The thermogram for lyophilized NI-SLNs without any sugar (control) showed an endothermic peak of drug melt at 76.5°C, which corresponds to the peaks of the lipids, probably because of the low concentration of NI compared with the lipids. After the use of fructose (mp 103.0°C), galactose (mp 167.0°C), glucose (mp 146.0°C), xylose (mp 144.0°C), lactose (mp 202.0°C), and sucrose (mp 186.0°C) as cryoprotectants for lyophilization, the NI-SLNs showed melting points for the SLNs at 133.1, 171.1, 135.5, 159.5, 152.0, and 190.0°C, respectively, that do not correspond with the melting points of the respective sugars. Whereas, NI-SLNs lyophilized with maltose and trehalose showed no peaks near to the melting point of the corresponding sugars (102 and 203°C**,** respectively), only peaks attributed to the lipid (at 57.9 and 60.0°C, respectively) were

278 observed, indicating the conversion of crystalline SLNs to an amorphous state.

279 As shown in **Fig. 2**, almost all the lyophilized SLNs showed melting peaks due to the lipids with
280 more or less intensity in the range 51.1–135.5°C, except for NI-SLN-Gal with a high intensity peak
281 at 171.1°C, corresponding to that of NI (176.9°C). This peak may be due to the transformation of NI
282 polymorphs from I to II.[30] However, no monosaccharide demonstrated efficiency in altering the
283 crystallinity of the SLNs. Among the disaccharide-containing SLNs, NI-SLN-Lac and NI-SLN-Suc
284 showed sharp melting peaks at 152 and 190°C, with a lower intensity than that observed with the
285 monosaccharides, which also demonstrates some crystalline nature for the SLNs. However, NI-SLN-
286 Mal and NI-SLN-Tre do not possess such peaks that indicate crystallinity, only very low intensity
287 peaks corresponding to lipids at 57.9 and 60.0°C, respectively.

288 The DSC thermograms of the four disaccharides show moderately less intensity peaks indicative
289 of crystallinity than those of the four monosaccharides, indicating the higher efficiency of the
290 disaccharides for protecting the SLNs from crystal formation. Among the disaccharides, only
291 trehalose and maltose could prevent crystal formation of the SLNs, as proved by the maximum
292 dissolved concentrations (14.7 and 14.6 µg/ml, respectively) (**Fig. 1**).

293 The PXRD pattern of NI (intact) exhibited sharp peaks with high intensity at 2θ scattered angles
294 8.1, 11.8, 16.2, 19.6, and 24.4° indicating the highly crystalline nature of NI (**Fig. 3**). Moderately
295 intense peaks were observed for the lipid mixture and the ground mixture of lipids and NI. Among
296 the NI-SLNs containing monosaccharides, high intensity peaks indicating crystallinity were observed
297 for NI-SLN-Gal (18.9, 22.1, 25.5, 28.6, and 36.7°), NI-SLN-Glu (19.9, 22.9, and 28.7°) and NI-
298 SLN-Xyl (17.6 and 35.0°). The presence of these peaks gives further proof of the crystalline nature
299 of NI-SLN-Gal, NI-SLN-Glu, and NI-SLN-Xyl. However, no significant peaks were observed for
300 NI-SLN-Fru indicating crystallinity of SLN, only peaks attributed to lipids (**Fig. 2**).

301 No peaks of significant intensity attributable to NI were found in NI-SLNs containing lactose,
302 maltose, sucrose, and trehalose. Thus, the PXRD pattern along with the re-dispersibility profile and
303 DSC thermograms provide further evidence that disaccharides might have the best cryoprotectant
304 properties for NI-SLNs.

The FTIR spectra (**Fig. 4**) of the physical mixture of lipids and the ground mixture of NI and lipids correspond with each other, but differ in intensities, because the spectrum of the lipids is strong due to its weight ratio being about 25 times higher than that of NI. The spectrum of the ground mixture was apparently different from that of NI in the range 2800–3000 cm^{-1}. The appearance of peaks outside the regions of C=O and N-H stretching vibrations suggests that partial structural changes or new interactions have occurred. It has been reported that roll mixing of poorly water-soluble drugs with carriers alters the drug from crystalline to the amorphous state and induces intermolecular interactions.[31)]

In the lyophilized NI-SLNs (without sugar), an interaction was observed in the range 3200–3400 cm^{-1} (**Fig. 4**). Subtraction of the spectrum of the freeze-dried sugar from that of the respective lyophilized NI-SLNs showed a common difference can be observed in the range 2800–3000 cm^{-1}, suggesting that an intermolecular interaction between the NI-SLN and the sugar has occurred, as indicated by O-H stretching. This stretching is due to the formation of a hydrogen bond between the O-H groups of the sugar moieties with the polar head group of the lipids, resulting in the cryoprotectant activity of the sugars in different order.

Figure 5 shows the SEM images for the ground mixture of NI and lipids, lyophilized NI-SLNs (without sugar), the free sugars, and the lyophilized NI-SLNs with those sugars. NI-SLN-Mal **(l)** and NI-SLN-Tre **(p)** have a morphology that is discrete and regular in shape. The smooth surface ensured the absence of drug on it; rather the drug is entrapped within the lipid matrix. Lyophilized NI-loaded SLNs containing trehalose **(p)** also appear to have a network-like structure that facilitates the penetration of water within the structure to allow quick re-dispersibility.

Taken together, the FTIR, DSC, PXRD, and SEM data indicate that both maltose and trehalose are highly efficient cryoprotectants for the lyophilization of NI-SLN suspension. However, trehalose has a mean particle diameter, and PDI and ZP values that are more conducive to cryoprotectant ability compared with maltose. Probably for this reason, Patist and Zoerb reported that trehalose is a more effective cryoprotectant than other disaccharides.[32)] In addition, trehalose has a very high T_g (glass transition temperature) and has the ability to form a dihydrate to maintain an elevated T_g in the

sample. Moreover, trehalose has a membrane-protecting effect, not only because of the formation of hydrogen bonds with the polar head groups of lipids, but also because trehalose can disrupt the tetrahedral hydrogen bond network of water and reduce the amount of freezable water. Thus, trehalose can be reported as one of the best cryoprotectant of all the disaccharides investigated for NI-SLNs and was used for further studies.

3.5. Stability Study

Upon storage at 30°C and 65% RH for 6 months, NI-SLN-Tre was found to be stable in terms of mean particle diameter, PDI and ZP (**Table 4**) and re-dispersibility rate (**Fig. 6**). No significant increase in particle size, PDI, or ZP was observed. The concentration profile of NI-SLN-Tre at 6 months showed drug release almost identical to the initial (0 month) values. The physical appearance of NI-SLN-Tre in aqueous medium was also found to be transparent as similar to the original. In addition, although DSC, PXRD, and FTIR analyses were performed to determine the physicochemical changes of SLNs after long-term storage, any significant changes were not observed (data not shown). Taken together, this study revealed that NI-SLN-Tre was more stable under ambient conditions than the NI-SLN suspension was at 4°C.

4. Conclusion

This study demonstrates that lyophilization of solid-lipid nanoparticles of water insoluble or poorly water-soluble drugs (such as nifedipine) with disaccharides can enhance the solubility of the drug. Nifedipine was chosen as a model drug in this study because this drug is one of extensively studied class II group drugs in accordance with biopharmaceutics classification system. As for an aqueous re-dispersibility test, maximum concentration of nifedipine was obtained with solid-lipid nanoparticles lyophilized with trehalose (14.7 µg/ml) at 5 min that was more than three times higher than that of without trehalose (4.6 µg/ml). The particle size and ZP can be controlled at around 100 nm and −60 mV, respectively. This is because nifedipine, during high pressure homogenization at

175 MPa with up to 100 pass cycles, is transformed from a crystalline to an amorphous state. Trehalose had a strong intermolecular interaction with the phospholipids, which resulted in trehalose performing as the best cryoprotectant to prevent the agglomeration of solid-lipid nanoparticles of nifedipine. Moreover, storage limitations could be overcome by using trehalose as the cryoprotectant for nifedipine solid-lipid nanoparticles. Previously, nifedipine solid-lipid nanoparticles in suspension could be stored at 4°C for up to 4 months. In our study, lyophilization with trehalose at a concentration of 2.5% w/v could successfully keep the nanoparticles physico-chemically constant for up to 6 months at 30°C and 65% RH. This also indicates that, under ambient conditions, the shelf life of nifedipine loaded solid-lipid nanoparticles may be increased by more than 6 months, although further study is needed. In addition, our recent study demonstrated that nifedipine solid-lipid nanoparticles just after preparation showed improved oral absorption rate in comparison to nifedipine itself[33)]; further *in vivo* pharmacokinetic study using lyophilized nifedipine solid-lipid nanoparticles with trehalose should also be conducted. In conclusion, nifedipine solid-lipid nanoparticles lyophilized with trehalose might provide effective drug delivery of nifedipine for the treatment of hypertension and angina pectoris.

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427 **Table 1. Effect of different sugars on the particle size, polydispersity index (PDI), and zeta**
428 **potential of nifedipine loaded solid-lipid nanoparticles.**

Sample	PDI, D_a	Particle size, P_a (nm)	Zeta Potential (mV)
NI-SLN (Control)	0.4	9.1×10^4	−29.0
NI-SLN-Glu	1.0	156.45	−38.0
NI-SLN-Fru	1.0	2.3×10^4	−35.8
NI-SLN-Gal	1.0	227.59	−34.5
NI-SLN-Xyl	0.6	2.1×10^3	−38.9
NI-SLN-Lac	0.5	109.3	−51.1
NI-SLN-Mal	0.7	174.9	−56.4
NI-SLN-Tre	0.3	107.7	−58.6
NI-SLN-Suc	0.4	162.9	−43.7

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Table 2. Effect of the concentration of the NI-SLN suspension on freeze drying (freeze thawing test).

Dilution ratio (NI-SLN suspension : water)	Particle size (nm) after freeze thawing, P_a	Particle diameter ratio, P_a/P_b	PDI after freeze thawing, D_a	PDI ratio, D_a/D_b
3:0	107.7	3.1	0.5	1.6
5:1	125.5	2.3	0.6	2.0
2:1	103.8	1.9	0.3	1.0
1:1	79.6	1.5	0.5	1.0
1:2	85.9	1.6	0.4	1.3
1:5	53.8	1.0	0.7	2.3

*PDI: Polydispersity index, P_b: Particle diameter before freeze thawing, D_b: PDI before freeze thawing.

435 **Table 3. Effect of the concentration of trehalose on freeze drying.**

Trehalose concentration (%w/v)	PDI	Particle Size (nm)	Zeta Potential (mV)
0	0.43±0.29	$7.32\pm1.26 \times 10^4$	−40.2±2.4
0.5	0.72+0.17	$5.8\pm1.25 \times 10^4$	−46.6±1.0
1.0	0.47±0.26	134.8±22.1**	−45.7±2.3
1.5	0.66±0.11	102.5±29.8**	−49.0±0.9
2.0	0.52±0.09	131.9±44.1**	−48.6±4.6*
2.5	0.39±0.04	111.7±9.6**	−59.1±3.9**
3.0	0.38±0.04	112.5±18.2**	−45.9±2.9

436 PDI: Polydispersity index. * $P < 0.05$, ** $P < 0.01$ versus each 0% value.

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439 **Table 4. Stability data [particle diameter, polydispersity index (PDI), and zeta potential (ZP)]**

440 **for lyophilized NI-SLNs with 2.5% trehalose.**

Time (month)	Particle size (nm)	PDI	Zeta Potential (mV)
0	111.7±9.6	0.39±0.04	−59.1±3.9
4	109.4±9.9	0.38±0.04	−59.9±3.5
6	104.5	0.3	−63.0

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Figure captions

Figure 1. Aqueous re-dispersibility profile of solid lipid nanoparticles using various sugars as cryoprotectants. NI-SLN (control): Freeze-dried SLNs with NI only; NI-SLN-Glu: freeze-dried NI-SLN with 2.0% glucose; NI-SLN-Fru: freeze-dried NI-SLN with 2.0% fructose; NI-SLN-Gal: freeze-dried NI-SLN with 2.0% galactose; NI-SLN-Xyl: freeze-dried NI-SLN with 2.0% xylose; NI-SLN-Lac: freeze-dried NI-SLN with 2.0% lactose; NI-SLN-Mal: freeze-dried NI-SLN with 2.0% maltose; NI-SLN-Tre: freeze-dried NI-SLN with 2.0% trehalose; NI-SLN-Suc: freeze-dried NI-SLN with 2.0% sucrose. The study was performed using 20 ml of water at room temperature. Since the NI in the SLN suspension is completely dissolved, the concentration should theoretically be 14.5µg/ml. This value was adopted for 100% dissolved NI. Each value represents mean ± SD (n=3).

Figure 2. DSC thermograms. Nifedipine; FD (freeze-dried) lipid mixture; NI-SLN (control): freeze-dried SLNs with NI only; NI-SLN-Glu: freeze-dried NI-SLN with 2.0% glucose; NI-SLN-Fru: freeze-dried NI-SLN with 2.0% fructose; NI-SLN-Gal: freeze-dried NI-SLN with 2.0% galactose; NI-SLN-Xyl: freeze-dried NI-SLN with 2.0% xylose; NI-SLN-Lac: freeze-dried NI-SLN with 2.0% lactose; NI-SLN-Mal: freeze-dried NI-SLN with 2.0% maltose; NI-SLN-Tre: freeze-dried NI-SLN with 2.0% trehalose; NI-SLN-Suc: freeze-dried NI-SLN with 2.0% sucrose.

Figure 3. Powder X-ray diffraction patterns. Nifedipine; lipid mixture; ground sample: ground mixture of NI and lipids; NI-SLN-Glu: freeze-dried NI-SLN with 2.0% glucose; NI-SLN-Fru: freeze-dried NI-SLN with 2.0% fructose; NI-SLN-Gal: freeze-dried NI-SLN with 2.0% galactose; NI-SLN-Xyl: freeze-dried NI-SLN with 2.0% xylose; NI-SLN-Lac: freeze-dried NI-SLN with 2.0% lactose; NI-SLN-Mal: freeze-dried NI-SLN with 2.0% maltose; NI-SLN-Tre: freeze-dried NI-SLN with 2.0% trehalose; NI-SLN-Suc: freeze-dried NI-SLN with 2.0% sucrose.

Figure 4. FTIR spectra. Nifedipine; lipid mixture; ground mixture of NI and lipids; NI-SLN

(without sugar): freeze-dried SLNs with NI only; freeze-dried fructose; NI-SLN-Fru: freeze-dried NI-SLN with 2.0% fructose; freeze-dried galactose; NI-SLN-Gal: freeze-dried NI-SLN with 2.0% galactose; freeze-dried glucose; NI-SLN-Glu: freeze-dried NI-SLN with 2.0% glucose; freeze-dried xylose; NI-SLN-Xyl: freeze-dried NI-SLN with 2.0% xylose; freeze-dried lactose; NI-SLN-Lac: freeze-dried NI-SLN with 2.0% lactose; freeze-dried maltose; NI-SLN-Mal: freeze-dried NI-SLN with 2.0% maltose; freeze-dried sucrose; NI-SLN-Suc: freeze-dried NI-SLN with 2.0% sucrose; freeze-dried trehalose; NI-SLN-Tre: freeze-dried NI-SLN with 2.0% trehalose.

Figure 5. SEM images.(a) Ground mixture of NI and lipids; (b) NI-SLN (control): freeze-dried SLNs with NI only; (c) Galactose; (d) NI-SLN-Gal: freeze-dried NI-SLN with 2.0% galactose; (e) Glucose; (f) NI-SLN-Glu: freeze-dried NI-SLN with 2.0% glucose;(g) Fructose; (h) Xylose; (i) Lactose; (j) NI-SLN-Lac: freeze-dried NI-SLN with 2.0% lactose; (k) Maltose; (l) NI-SLN-Mal: freeze-dried NI-SLN with 2.0% maltose; (m) Sucrose; (n) NI-SLN-Suc: freeze-dried NI-SLN with 2.0% sucrose; (o) Trehalose; (p) NI-SLN-Tre: freeze-dried NI-SLN with 2.0% trehalose.

Figure 6. Aqueous re-dispersibility profile of lyophilized NI-SLNs using 2.5% trehalose after 6months. This study was performed using 10 ml of water at room temperature. Each value represents the mean ± SD (n=3).