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Injectable nimodipine-loaded nanoliposomes: Preparation, lyophilization and characteristics

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ABSTRACT

The main purpose of this study was to prepare nimodipine-loaded nanoliposomes for injection and evaluate their characteristics after lyophilization. Nimodipine-loaded nanoliposomes were prepared by the emulsion-ultrasonic method with sodium cholesterol sulfate (SCS) as the regulator and then lyophilized by adding different cryoprotectants. SCS was used as a blender of regulator and surfactant and helped to prepare smaller liposomes due to the steric hindrance of the sulfate group. The results showed that nimodipine-loaded nanoliposomes with a 20:1 of egg yolk lecithin PL-100M vs. SCS ratio had a particle size of 86.8 ± 42.007 nm, a zeta potential of −13.94 mV and an entrapment efficiency (EE) of 94.34% and could be stored for 12 days at 25 °C. Because of the good bulking effect of mannitol and the preservative effect of trehalose, they were used to obtain suitable lyophilized nanoliposomes. The lyophiles containing 10% mannitol and 20% trehalose had a good appearance and a slightly altered particle size after rehydration. In addition, the lyophilized products were characterized by differential scanning calorimetry, X-ray diffraction and scanning electron microscopy, which confirmed the morphous state of trehalose, mannitol and the mixture. Trehalose could inhibit mannitol crystallization to some extent. The drug release from nanoliposomes before and after lyophilization in pH 7.4 phosphate buffer containing 30% ethanol was also examined and both profiles were found to fit the Viswanathan equation. This means that the drug release was controlled by the pore diffusion resistance.

1. Introduction

Nimodipine (NMD) is a dihydropyridine calcium antagonist which has been shown to dilate cerebral arterioles and increase cerebral blood flow in animals and humans. It has potential applications in the treatment of a range of cerebrovascular disorders (Langley and Sorkin, 1989). NMD has been prepared as a variety of solid preparations, such as tablets and capsules, but its high first-pass effect in liver has limited its clinical usefulness (Blardi et al., 2002). Due to the poor water-solubility and lower bioavailability for oral administration, in clinic situations an injection of nimodipine ethanolic solution is usually used, but it has drawbacks including poor compliance and phlebitis caused by ethanolic solution and crystallization caused by the poor water-solubility of nimodipine (Xiang et al., 2009). In recent years, nano-preparations have been studied to develop a better tolerated injectable nimodipine formulation such as NMD-loaded nanosuspensions (Xiong et al., 2008) and nanospheres (Hu et al., 2008), however the preparation of NMD-loaded nanosuspensions is time-consuming and the solid lipid in the nanospheres could result in gelatinization. Nanoliposomes, which are microscopic vesicles composed of phospholipid bilayers entrapping one or more aqueous compartments (Mozafari et al., 2008), are ideal systems to overcome the above drawbacks.

There are many preparation methods for liposomes, including thin-film dispersion, reverse-phase evaporation, double emulsion, multiple emulsions, injection and freeze-drying (Meure et al., 2008). However, the most significant disadvantages of conventional preparation methods generally include being of a complex nature, time consuming, difficult to scale-up, large vesicles with no control of particle size, and the presence of undesirable solvent residues (Meure et al., 2008; Fan et al., 2008). An emulsification and ultrasonic method is usually used to prepare lipid nanoparticles (Fang et al., 2008; Liu and Wu, 2010) and, since it is time-saving, convenient and allows control of the particle size, it was used in this study to prepare NMD-nanoliposomes. Because of the lipophilicity of nimodipine, it can be incorporated in the bilayer within the lipid phase of the liposomes (Meisnera and Mezei, 1995; Wiechens et al., 1999). Microdialysis (Li et al., 2009, 2010; Liu et al., 2009) is a convenient, precise, and reproducible method and it was selected for measuring the liposomal free drug concentration.

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Cholesterol (CH) is found in many biological membranes and is the main sterol in animals. Other steroids, such as cholesterol sulfate (CS), have also been detected in substantial amounts in various tissues, such as the heads of spermatozoa and the stratum corneum. The hydrated sulfate group is much more bulky than the hydroxyl group, so CS can act as a spacer; because of this steric hindrance, the DMPC (dimyristoyl-phosphatidylcholine) acyl chains are more distant from the CS fused ring system than from the CH structure (Faure et al., 1996). Cholesterol, which is incorporated into the lipid bilayer and reduces any bilayer packing defects, is usually used as a membrane regulator and stabilizer when preparing liposomes, but few reports have described SCS. In this article, sodium cholesterol sulfate (SCS) was used to prepare liposomes instead of CH.

Lyophilization has been demonstrated to be an effective way of preparing liposomal formulations, as it overcomes most of the stability problems associated with liposomes, like chemical instability (of phospholipids as well as drugs), leaching, fusion, and aggregation (Gulati et al., 1998; Chen et al., 2010a, 2010b). However, the process of lyophilization can damage the liposome integrity and also markedly reduce the encapsulation efficiency (Glavas-Dodov et al., 2005). Good lyophilized liposomes have a cake-shaped appearance without shrinkage and spraying and offer good particle protection. In view of this, sugars, which are well-known cryoprotectants, were added to obtain optimal lyophilized liposomes (Li et al., 2000).

The aim of this article was to prepare NMD-loaded nanoliposomes and study their lyophilization properties. The effects of SCS, instead of CH, as a stabilizer and surfactant on liposomes were investigated. To overcome the short storage life, NMD-loaded nanoliposomes were freeze-dried. Furthermore, lyophilized NMD-loaded nanoliposomes were studied by DSC, X-ray and SEM and the comparative drug release from liposomes before lyophilization and after rehydration was also investigated.

2. Materials and methods

2.1. Materials

Nimodipine (NMD) was obtained from the Central Pharmaceutical Co., Ltd. (Tianjin, China). Lipoid E-80 (PC >80%), Lipoid S-75 (PC >75%) and hydrogenated soy lecithin Lipoid S PC-3 (PC >98%) were purchased from Lipoid (Ludwigshafen, Germany). Egg yolk lecithin PL-100M (PC >80%) was purchased from Q.P. Corporation (Tokyo, Japan). Sodium cholesterol sulfate (SCS) was purchased from Hubei Saibo Pharmaceutical Co., Ltd. (Wuhan, Hubei). Tween-80 was purchased from Shenyu Medicine and Chemical Industry Company (Shanghai, China). Pluronic F68 was obtained from BASF AG (Ludwigshafen, Germany). Trehalose dehydrate was purchased from Sinozyme Biotechnology Co., Ltd. (Nanning, China). Mannitol, maltose, glucose, lactose and sucrose were purchased from Tianjin Bodi Chemicals Co., Ltd. (Tianjin, China). All other chemicals were of analytical or chromatographic grade.

2.2. Preparation of nimodipine nanoliposomes

Nimodipine nanoliposomes were prepared by a modified emulsification and ultrasonic method. Nimodipine was dissolved in anhydrous ethanol to obtain a concentration of 2 mg/mL NMD ethanolic solution. The lipid phase containing 2% (w/v) phospholipids, 0.1% (w/v) SCS, and 2 mL 2 mg/mL NMD ethanolic solution was heated in a water bath at 60 °C. Pluronic F68 or Tween 80 was dissolved in 10 mL pH 6.5 PBS at 60 °C as the aqueous phase. When lecithin and SCS were completely dissolved in NMD ethanolic solution, the aqueous phase was dropped into the lipid phase under magnetic stirring. The obtained primary emulsion was stirred for another 10 min, and then it was ultrasonicated using probe sonication (Sonics & Material Vibra Cell, 750 W, 20 kHz) at 30% amplification for 10 min with a 3-s pulse-on period and a 1-s pulse-off period. The final nanoliposomes were obtained after being cooled in an ice bath, diluted to 20 mL and passed through a 0.22 μm filter membrane.

2.3. Lyophilization

2 mL NMD-nanoliposomes in a vial were rapidly lyophilized using a lab freeze-dryer (Advantage; Virtis, USA). The freeze-drying was conducted as follows: the pre-freezing temperature was −45 °C for 5 h; then, the shelf temperature was raised to −20 °C for 14 h; secondary drying was at 25 °C for 3 h. Finally, the vials were sealed with rubber caps.

2.4. Reconstitution

When needed, lyophilized nanoliposomes were immediately rehydrated with water to the original volume.

2.5. Determination of particle size and zeta potential

The intensity-weighed mean particle size and polydispersity index (PI) of the NMD-nanoliposomes were measured by photon correlation spectroscopy (dynamic light scattering) using a Nicomp™ 380 submicron particle sizer (Particle Sizing System, Santa Barbara, CA, USA) at 25 °C. Prior to measurement, samples were diluted with double-distilled water. The zeta potential (ζ) of the NMD-nanoliposomes was also determined using the Nicomp™ 380 with electrophoretic light scattering.

2.6. Drug content and entrapment efficiency (EE)

NMD-nanoliposomes (1 mL) were transferred to a 10-mL volumetric flask with a suitable amount of ethanol. After ultrasonic emulsion rupture, the solution was diluted to 10 mL and then passed through a 0.22 μm membrane filter. The drug content in nanoliposomes was assayed by high-performance liquid chromatography (HPLC).

The EE of NMD-nanoliposomes was evaluated by the microdialysis method. The microdialysis probe was inserted into glass vials filled with NMD-nanoliposomes at room temperature. The flow rate of the dialysate was set at 4 μL/min. After a 30-min equilibration period, the dialysate from the NMD-nanoliposomes was collected at 20 min intervals and analyzed by HPLC. Also, before the EE of the NMD-nanoliposomes was measured, the probe recovery was evaluated (Li et al., 2009).

Chromatographic conditions: Diamonsil C18 column (5 μm, 200 mm × 4.6 mm); mobile phase methanol–acetonitrile–water (35:35:30); flow rate 1 mL/min; UV detector wavelength 235 nm. The peak area response to the concentration of NMD was linear over the range 0.02–40.24 μg/mL (r = 0.9999).

2.7. Thermal analysis

DSC analysis was employed using a differential scanning calorimeter (DSC-60WS; Shimadzu, Kyoto, Japan). Samples were heated from 25 °C to 300 °C at a scan rate of 10 °C/min under a nitrogen purge.

2.8. X-ray diffraction

XRD measurements were performed using a type D/Max-2400 diffractometer (Rigaku Instrument, Tokyo, Japan). Samples were subjected to CuKα radiation under 56 kV and 182 mA over the

Table 1
Effect of different ratios of SCS/cholesterol and Lipoid E-80 on the NMD-loaded liposomes.

	Appearance	PSD (nm)	PI	Zeta potential (mV)
SCS: Lipoid E-80[a]				
1:5	Yellow transparence	39.4 ± 24.763	0.394	−26.48
1:8	Light yellow transparence	61.0 ± 35.807	0.345	−17.14
1:10	Light yellow translucence	106.4 ± 55.515	0.272	−20.64
1:20	Light blue translucence	119.6 ± 49.054	0.168	−18.61
No SCS	Opalescence	156.5 ± 59.171	0.143	−3.29
Cholesterol: Lipoid E-80[a]				
1:2	Opalescence	138.5 ± 46.942	0.115	−9.77
1:4	Light yellow transparence	85.7 ± 38.892	0.206	−6.18
1:5	Light yellow translucence	100.6 ± 41.952	0.174	−8.48
1:8	Light yellow translucence	83.2 ± 42.255	0.258	−5.49

[a] In all the formulations the concentration of Lipoid E-80 was fixed at 2%.

2-theta range from 3° to 45° in increments of 4°/min every 0.04°.

2.9. Transmission electron microscopy (TEM)

The morphological observations of NMD-nanoliposomes before lyophilization and after rehydratation were performed using an electronic transmission microscope (JEM-100CX/II, JEOL Ltd., Japan). The samples were diluted with purified water, placed on a copper grid coated with carbon film and air dried. Then, they were stained with 2% phosphotungstic acid. Finally, the samples were air dried prior to placing them in the TEM instrument.

2.10. Scanning electron microscopy (SEM)

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

2.11. In vitro release study

The drug release from the NMD-loaded nanoliposomes before and after lyophilization was performed in pH 7.4 phosphate buffer (PBS) containing 30% ethanol. A dialysis bag (14,000 Da) method was used. For this, 2 mL NMD-loaded proliposomes and reconstituted liposomes were transferred to the dialysis bag with the two ends fixed by clamps. Then, the bags were placed in 50 mL conical flasks and 40 mL release medium was added and the conical flasks were placed in a water bath (ZHWY-110X30; Zhicheng, Shanghai, China) at 100 rpm and 37 °C. At predetermined sampling times, 100 μL medium was drawn and replaced the same volume of fresh medium. The concentration of NMD in the dissolution medium was determined by HPLC.

3. Results and discussion

3.1. Formulation development of NMD-nanoliposomes

The ultrasonic method combined with emulsion was applied for preparing the nanoliposomes. Traditionally, cholesterol is used as a regulator in phospholipid membranes to prepare liposomes. Also, the EE could be changed by varying the ratios of cholesterol and phospholipids. However, in this study, SCS derived from cholesterol was used to prepare liposomes. The effect of the SCS concentration on the physical properties of the liposomes was evaluated by using different ratios of SCS to phospholipids (Lipoid E-80) while maintaining the concentration of Lipoid E-80 at 2% (w/v). As shown in Table 1, as the ratio increased, the size of the liposomes decreased, and the value of PI and the absolute value of the zeta potential increased. Also, liposomes with no SCS exhibited a relatively poor appearance with a larger size and low zeta potential. To obtain good stable nanoliposomes with a small particle size, narrow size distribution and higher zeta potential, the formulation with a 1:20 ratio of SCS to Lipoid E-80 was used. Different ratios of cholesterol to Lipoid E-80 were studied as shown in Table 1, and the formulation with cholesterol to Lipoid E-80 ratio of 1:5 was acceptable with a mean particle size of 100.6 ± 41.956 nm and a zeta potential of −8.48 mV. Using a 1:5 ratio of SCS to Lipoid E-80, the particle size was 39.4 ± 24.763 nm and the zeta potential was −26.48 mV. In other words, using the same proportion of SCS can produce much smaller sized particles than cholesterol, perhaps because the hydrated sulfate group of SCS is much more bulky than the hydroxyl group in CH which is described in Fig. 1. As it is reported (Faure et al., 1996) that CS can act as a spacer and due to this steric hindrance, the phospholipids chains would be more distant from the CS fused ring system than from CH. Also, owing to the sulfate groups, SCS could increase the absolute value of the zeta potential and a greater electrostatic repulsion between the nanoliposomes could also inhibit the vesicles fusing into larger ones. In summary, SCS is a singular blend of surfactant and regulator.

It has been reported that the addition of non-ionic surfactants leads to a decrease in the particle size and at the same time the steric stability of these emulsifiers. Also, the combination of surfactants would not only increase the stability of the system and particle morphous shape, but also decrease the particle size because

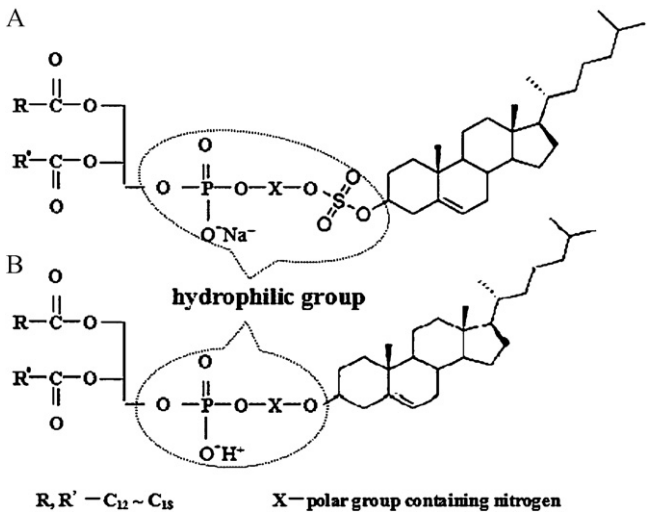

Fig. 1. Schematic diagrams of a combination of (A) phospholipids and SCS; (B) phospholipids and CH.

Table 2
Formulations of NMD-loaded liposomes.

Formulations	Lipoid E-80 (%)	SCS (%)	NMD (mg)	Pluronic F68 (%)	Tween 80 (%)
F1	2	0.1	4	0.1	–
F2	2	0.1	4	0.2	–
F3	2	0.1	4	0.3	–
F4	2	0.1	4	0.5	–
F5	2	0.1	4	–	0.1
F6	2	0.1	4	–	0.2
F7	2	0.1	4	–	0.3

Table 3
Physical characteristics of NMD-loaded liposomes in F1–F7.

Formulations	PSD (nm)	PI	EE (%)
F1	103.3 ± 45.443	0.194	97.68
F2	102.6 ± 44.015	0.184	97.91
F3	104.8 ± 45.501	0.188	97.68
F4	100.4 ± 47.503	0.224	96.52
F5	72.6 ± 37.536	0.267	97.83
F6	72.1 ± 37.876	0.276	98.42
F7	69.5 ± 36.842	0.281	98.34

of a synergistic effect (Han et al., 2008). Based on the above factors, apart from SCS, Pluronic F68 and Tween 80 were chosen to optimize the formulations. Table 2 shows the formulations of liposomes and Table 3 shows the resultant characteristics of liposomes listed in Table 2. Overall, Tween 80 and Pluronic F68 both reduced the particle size compared with liposomes without surfactant (119.6 ± 49.054 nm). Tween 80 produced more emulsification than Pluronic F68, but it also produced a higher PI and shorter storage life (<5 days for Tween 80 vs. 7 days for Pluronic F68 at 25 °C). Different proportions of Pluronic F68 (w/v) were investigated and it was found that liposomes in F2 had a small size, a lower PI value and a 7-day storage life. The relatively stable formulations F1–F3 may be due to both a low PI value and the additional steric stabilization effect of Pluronic F68 helping avoid aggregation of the nanoparticles in the colloidal system (Fan et al., 2008; Han et al., 2008).

For liposomes, besides regulators and surfactants, the type of phospholipids can markedly affect the chemical and physical properties of liposomes. Phospholipids are liable to oxidation so, according to the Chinese Pharmacopoeia 2010, the oxidation index (OI) of phospholipids should be lower than 0.2 and this was also determined. The determination was carried out as follows: a suitable amount of phospholipids was dissolved in absolute ethanol and then the phospholipid alcoholic solution was examined by UV spectrometry at 233 nm and 215 nm. The OI was calculated from the equation: $OI = A_{233\,nm}/A_{215\,nm}$. The oxidation index (OI) of phospholipids and characteristics of nanoliposomes with different phospholipids are shown in Table 4. It can be seen that the OI of Lipoid E-80 and PL-100M were both lower than 0.2, but the stability of the nanoliposomes containing Lipoid E-80 was inferior to PL-100M. In addition, nanoliposomes containing PL-100M had a relatively low PI and high EE. Therefore, based on an overall consideration of various factors, PL-100M was the optimum phospholipid for the preparation of NMD-loaded nanoliposomes.

Table 5
Effect of different cryoprotectants on the characteristics of NMD-loaded nanoliposomes in the freeze-thawing test.

Cryoprotectant[a]	Lyophilized appearance[b]	Thawed appearance[c]	P_a/P_b
None	▲	★	–
Glucose	▲	☆	1.94
Sucrose	▲	☆	1.69
Sodium chloride	▲	★	–
Maltose	△	☆	2.24
Lactose	△	★	–
Trehalose	△	☆	2.43
Mannitol	△△	☆	4.41

[a] The concentration of cryoprotectants was fixed at 10%.
[b] ▲ Non-cake, shrink critically, sprayed or loose; △ slightly shrink; △△ cake and compact.
[c] ★ Aggregated; ☆ translucent.

3.2. Lyophilization

As described earlier, NMD-loaded nanoliposomes were stable for 12 days. The stability problems can be overcome by lyophilization in which the final liposome product is freeze-dried with cryoprotectants and is reconstituted with vehicle immediately prior to administration. Lyophilization increases the shelf-life of the finished product by preserving it in a relatively more stable dry state (Sharma and Sharma, 1997). The effect of different cryoprotectants on NMD-loaded nanoliposomes is shown in Table 5. P_a and P_b respectively represented the mean diameter of NMB-nanoliposomes after and before freeze-thawing. The mean diameter ratio (P_a/P_b) was calculated to evaluate the effect of the cryoprotectants (Li et al., 2009). All lyophilized products mentioned in this article were redispersed with purified water by manual shaking. For glucose, sucrose, and sodium chloride, the appearance of the freeze-dried products was poor, and for lactose the thawed appearance was poor due to aggregation. Comparatively, in the presence of maltose, trehalose and mannitol, both lyophilized and thawed forms were better. Although the lyophilized appearance of samples containing mannitol was excellent, the particle size of the reconstituted samples was significantly changed, which showed that mannitol has good cake-supporting properties. Also, lactose, sucrose, maltose and trehalose had a particle size-protecting effect. Because of the advantages and disadvantages of the different cryoprotectants, mixed cryoprotectants were used. Considering that glucose and sucrose are highly hygroscopic, different ratios of maltose and trehalose vs. mannitol were investigated in Table 6. When 10% mannitol combined with 20% trehalose was used, the

Table 4
Effect of phospholipids on the characteristics of NMD-loaded nanoliposomes.

Phospholipids[a]	OI	PSD (nm)	PI	EE (%)	Zeta potential (mV)	Storage (25 °C)
Lipoid E-80	0.19	82.4 ± 41.183	0.250	94.58	−13.53	>7 days
Lipoid S-75	0.27	76.9 ± 38.350	0.249	91.46	−18.38	>12 days
Lipoid S PC-3	0.27	82.4 ± 39.707	0.232	85.58	−11.70	>12 days
PL-100M	0.16	86.8 ± 42.007	0.234	94.34	−13.94	>12 days

[a] All the formulations were composed of 2% phospholipids, 0.1% SCS, 4 mg NMD and 0.2% Pluronic F68.

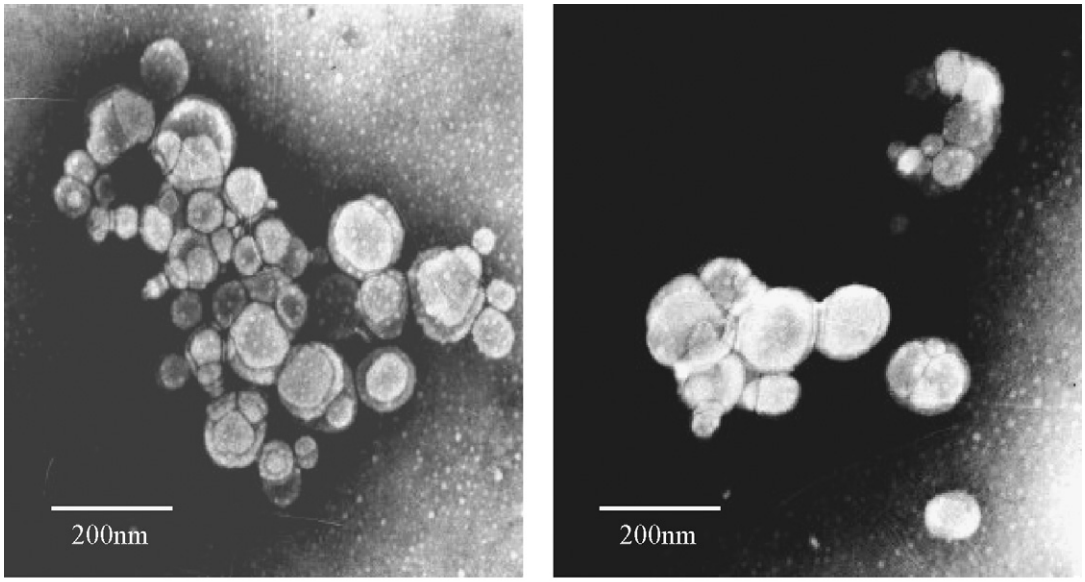

Fig. 2. Transmission electron microscope (TEM) photographs of NMD-liposomes before freeze-drying (A) and after rehydration (B).

lyophilized samples had a good appearance and a relatively small changed size with P_a/P_b 1.96.

Mannitol had a bulking effect as it trended to crystallize to facilitate the formation of elegant product cakes and its high eutectic melting temperature with ice (−1.5 °C) enabled primary drying at relatively high temperatures (Telang et al., 2003); however, there was no preservation on membranes since mannitol encapsulated in liposomes has been reported to be localized in the aqueous core and there is no evidence of an interaction with the lipid head groups (El Maghraby et al., 2005). Trehalose has a very high *Tg* (glass transition temperature) and has the ability to form a dihydrate to maintain an elevated *Tg* in the sample. Furthermore, trehalose has a membrane-protecting effect not only because of the formation of hydrogen bonds with the polar headgroups of lipids, but also because of disruption the tetrahedral hydrogen bond network of water and reducing the amount of freezable water (Patist and Zoerb, 2005). When mannitol and trehalose were used together, crystalline mannitol provided a robust matrix during lyophilization, which could endure "micro-collapse" of the amorphous trehalose without compromising the quality of the final lyophiles (Wang et al., 2004). That is why trehalose combined with mannitol can provide satisfactory lyophiles.

Also, when NMD nanoliposomes were diluted two-fold beforehand and then 10% mannitol and 20% trehalose were added, the particle size was slightly changed with a P_a/P_b of 1.21. Fig. 2 shows the TEM photographs of NMD-loaded liposomes before lyophilization and after rehydration. They were roughly spherical in shape and the particle size before and after lyophilization did not change significantly. For that reason, the diluted liposomes containing 10% mannitol and 20% trehalose as cryoprotectants were found to be the optimal formulation for freeze drying, and the resulting samples were further studied.

Table 6
Effect of mixed cryoprotectants on the NMD-loaded nanoliposomes in the freeze-thawing test.

Mixed cryoprotectant		Lyophilized appearance[a]	P_a/P_b
Mannitol(%) + maltose (%)	5 + 5	△△	2.56
	5 + 10	△	1.7
	5 + 16	△	1.51
	5 + 20	▲	1.28
	10 + 5	△△	4.40
	10 + 10	△△	3.49
	10 + 15	△	2.68
	10 + 20	△	1.80
Mannitol(%) + trehalose (%)	5 + 5	△	2.68
	5 + 10	△	1.89
	5 + 16	▲	1.59
	5 + 20	△	1.42
	10 + 5	△△	3.98
	10 + 10	△△	3.17
	10 + 15	△△	2.48
	10 + 20	△△	1.96

[a] ▲ Non- cake or shrink critically; △ slightly shrink; △△ cake and compact.

3.3. Differential scanning calorimetry

As shown in Fig. 3, the NMD (a) after heating had a sharp peak at 128.03 °C. At the same position of 128.03 °C in the DSC thermograms (b–e), no peak was present, but the physical mixture thermogram b should have an NMD peak. One reason for this might be that the concentration of NMD loaded in the nanoliposomes was too low to be determined, and another might be that during heating NMD reacted with phospholipids and formed a

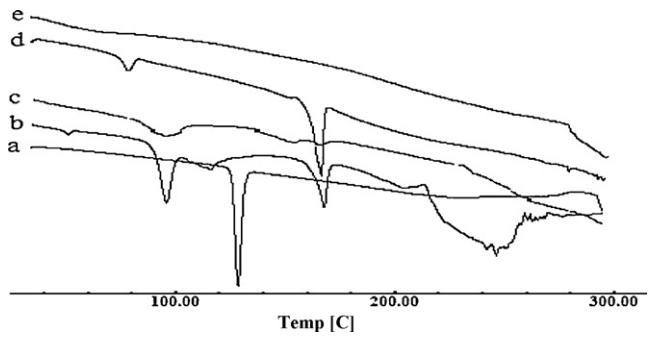

Fig. 3. DSC thermograms: (a) nimodipine; (b) physical mixture; (c) lyophilized NMD-nanoliposomes containing 10% mannitol and 20% trehalose; (d) lyophilized NMD-nanoliposomes containing 10% mannitol; (e) lyophilized NMD-nanoliposomes containing 10% trehalose.

complex. In addition, the DSC thermograms of lyophilized NMD-nanoliposome containing 10% mannitol (d) had a peak at 165.43 °C and the DSC thermogram of lyophilized NMD-nanoliposomes containing 10% trehalose (e) had no peak. Interestingly when 10% mannitol combined with 20% trehalose was used, the large sharp peak at around 165.43 °C disappeared. In other words, freeze-dried NMD-nanoliposomes containing mannitol (Yu et al., 1999; Cavatur et al., 2002) were present in a crystal state, freeze-dried NMD-nanoliposomes containing trehalose were in an amorphous form, but lyophilized NMD-nanoliposomes containing 10% mannitol and 20% trehalose appeared to be in a much less ordered sate. To investigate this phenomenon, X-ray diffraction was carried out.

3.4. X-ray diffraction

As for DSC, different samples were tested by X-ray diffraction as shown in Fig. 4. There was a less crystalline for lyophilized NMD-nanoliposomes containing 10% mannitol and 20% trehalose, a marked crystalline form in liposomes containing 10% mannitol and an amorphous form for liposomes containing trehalose, which was consistent with the results established by DSC. Furthermore, no characteristic peaks of NMD (a) appeared in the X-ray diffraction patterns of freeze-dried NMD-loaded samples, which also suggested that NMD was successfully loaded in nanoliposomes.

3.5. Scanning electron microscopy (SEM)

The internal structure of lyophilized samples containing mannitol, trehalose or the mixture was scanned by SEM as shown in Fig. 5 with a 500-fold magnification. The forms of the lyophilized samples containing different kinds of cryoprotectants were examined visually. Fig. 5A is the SEM photograph of lyophilized NMD-nanoliposomes containing 10% mannitol, and shows a chrysanthemum-like crystal. Lyophilized NMD-loaded nanoliposomes containing 10% trehalose (Fig. 5B) appeared to have a net structure. In Fig. 5C, few crystals were found. All these findings further confirmed the results of DSC and X-ray diffraction. Mannitol could form crystals (Yu et al., 1999; Cavatur et al., 2002) after freeze-drying, and this might damage the liposome structure. Trehalose (Chen et al., 2010a, 2010b; Christensen et al., 2007) could protect the liposomes because its amorphous form could maintain individual vesicles in a state of low molecular mobility and reduce

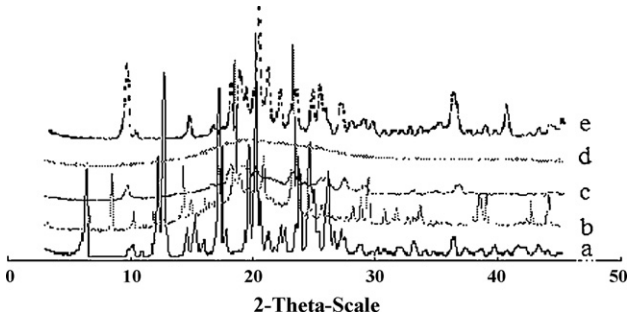

Fig. 4. X-ray diffraction patterns: (a) nimodipine; (b) physical mixture; (c) lyophilized NMD-nanoliposomes containing 10% mannitol and 20% trehalose; (d) lyophilized NMD-nanoliposomes containing 10% trehalose; (e) lyophilized NMD-nanoliposomes containing 10% mannitol.

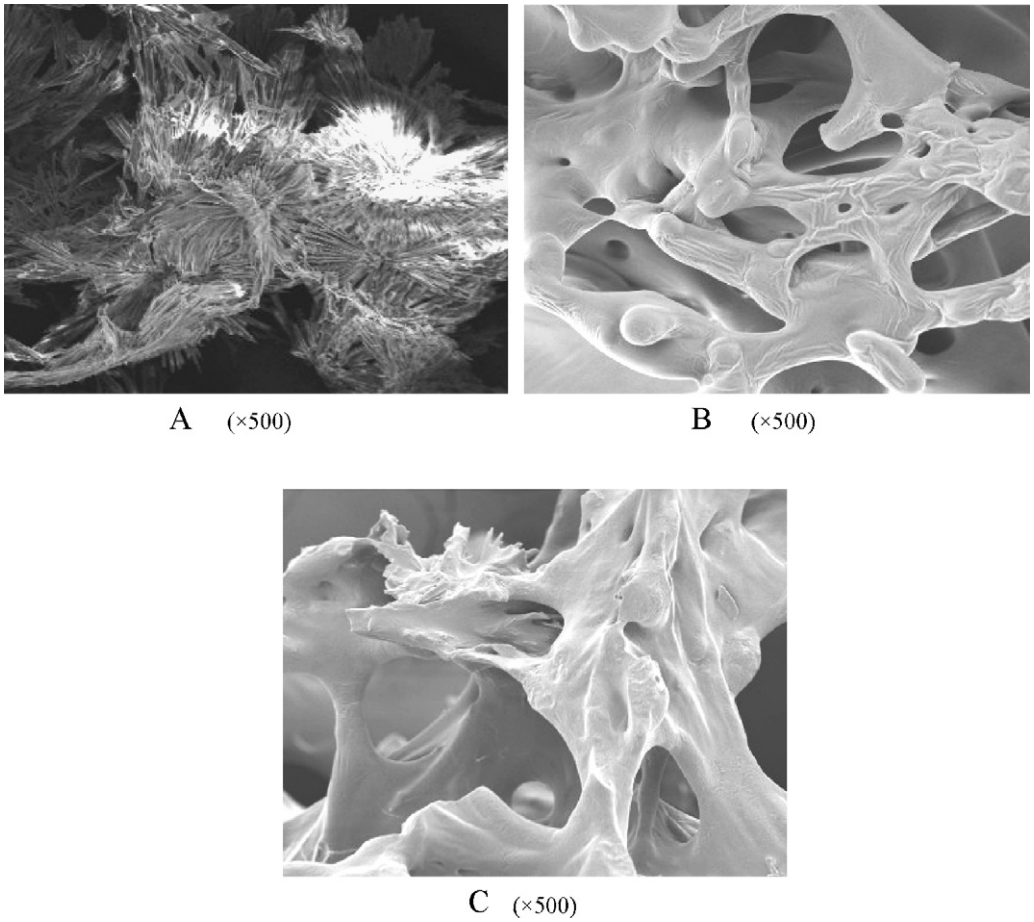

Fig. 5. SEM photographs: (A) lyophilized NMD-loaded nanoliposomes containing 10% mannitol; (B) lyophilized NMD-loaded nanoliposomes containing 10% trehalose; (C) lyophilized NMD-loaded nanoliposomes containing 10% mannitol and 20% trehalose.

Table 7
Regressing results for the drug release from NMD-loaded nanoliposomes before and after lyophilization.

Model		Zero order	First order	Higuchi	Viswanathan	Weibull
Before lyophilization	*k*	9.9111	−0.2390	32.372	0.5227	0.6219
	r	0.9060	0.9690	0.9839	0.9879	0.9683
After lyophilization	*k*	10.04	−0.2869	33.539	0.6265	0.7372
	r	0.8777	0.9785	0.9749	0.9961	0.9762

the surface tension of the vesicles during freeze-drying (Miyajima, 1997). Another reason is that trehalose participates in the network of hydrogen bonds between the phospholipid polar heads and, thus, replaces the water of hydration at the membrane–fluid interface and maintains the headgroups at their hydrated position (Wang et al., 2004). In this experiment, if trehalose alone was added to the NMD-loaded nanoliposomes, the lyophilized products would shrink to some degree. Also, if mannitol alone was added, the particle size would change significantly. Therefore, they were used together as cryoprotectants. As shown by the SEM photographs, DSC and X-ray results, after combination of mannitol and trehalose, the crystal structure formed by mannitol was improved and no notable crystals appeared, which indicated that trehalose could inhibit mannitol forming crystals during freeze drying consistent with reported findings (Pyne et al., 2002). In the presence of both mannitol and trehalose, the final lyophilized products would have a cake-shape, and be compact, and easy to reconstitute with only a slightly altered particle size.

3.6. *In vitro release*

Fig. 6 describes the drug release profiles of NMD-loaded nanoliposomes before and after lyophilization. It can be seen that NMD in lyophilized nanoliposomes is released faster than that in nanoliposomes without lyophilization. One of the reasons for this may be attributed to the greater particle size of the lyophilized products. During lyophilization, part of the liposomes with a small particle size fused and formed liposomes with a bigger particle size, which might lead to drug leakage after rehydration. On the other hand, since mannitol and trehalose were added as cryoprotectants, the lyophilized products were still characterized by a less ordered state although no crystals were present, as illustrated before. However, even small crystals can rupture the phospholipid bilayers which result in fast release after lyophilization. In order to evaluate the release mechanism, the release profiles were fitted to different models including zero-order, first-order, Higuchi (Higuchi, 1963), Viswanathan (Bhaskar et al., 1986) and Weibull distributions (Weibull and Sweden, 1951). Regression results in Table 7 indicated that the drug release from NMD-loaded nanoliposomes before and after lyophilization most closely fitted the Viswanathan equation with *r* values of 0.9879 and 0.9961 respectively. NMD is a water-insoluble drug, so it was located between phospholipid bilayers. During release, drug diffused from the phospholipid bilayers into the medium. Hence, the drug release was suspected to be controlled by the pore diffusion resistance (also called 'particle diffusion control') (Bhaskar et al., 1986).

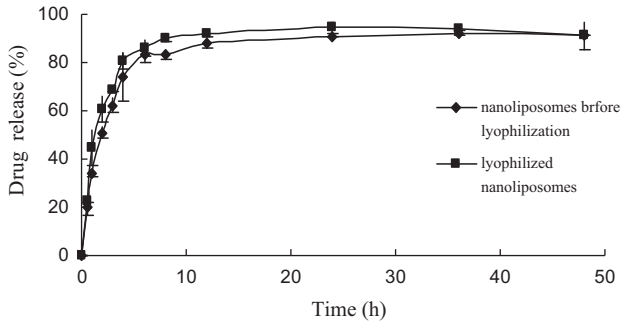

Fig. 6. The drug release of NMD-loaded nanoliposomes before and after lyophilization in pH 7.4 PBS containing 30% ethanol ($n = 3$).

4. Conclusion

Nimodipine-loaded nanoliposomes for injection were successfully prepared by the emulsion-ultrasonic method with SCS as the regulator and surfactant instead of CH. SCS was superior to CH because of its bulky sulfate group, which helped prevent liposomes fusing and allowed the preparation of much smaller liposomes. With a PL-100M vs. SCS ratio of 20:1, NMD-loaded nanoliposomes had a satisfactory state with a particle size of 86.8 ± 42.007 nm, a zeta potential of −13.94 mV, an entrapment efficiency (EE) of 94.34% and a 12-day storage life.

Lyophilized NMD-nanoliposomes were investigated by adding different cryoprotectants. Due to the bulking effect of mannitol and the membrane-protecting effect of trehalose, they were used together. Satisfactory freeze-dried products were obtained by using a mixture of 10% mannitol and 20% trehalose. Using differential scanning calorimetry, X-ray diffraction and scanning electron microscopy, it was found that trehalose could partially prevent mannitol forming crystals.

The drug release from nanoliposomes before and after lyophilization in pH 7.4 PBS containing 30% ethanol was tested. Both release profiles fitted the Viswanathan equation, which showed that the drug release may be controlled by the pore diffusion resistance.

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