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Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid

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Abstract

Solid lipid nanoparticles (SLNs) have gained attention as a colloidal drug carrier, particularly for drugs with limited solubility. The poor aqueous solubility of all-trans retinoic acid (ATRA) has been a limiting factor in its clinical use. This study was undertaken to overcome the solubility limitation of ATRA by loading in SLNs. The physicochemical characteristics of ATRA-loaded SLNs were investigated by particle size analysis, zeta potential measurement, thermal analysis and HPLC determination of ATRA content. The mean particle size of ATRA-loaded SLNs could be reduced (1) by mixing EggPC and Tween 80 as a surfactant and (2) by increasing the total surfactant amount. The smallest mean particle size of SLNs was obtained with 50 mg/g surfactant mixture composed of 54:46% (w/w) EggPC:Tween 80 (154.9 nm). The zeta potential of SLNs could be increased by mixing EggPC, Tween 80 and DSPE-PEG in the surfactant mixture. The zeta potential of SLNs prepared with 50 mg/g surfactant mixture composed of 48:6:46% (w/w) of EggPC:DSPE-PEG:Tween 80 was −38.18 mV. ATRA could be loaded at 2.4% (percentage of lipid matrix) on these SLNs without impairing their physical stability. After freeze-drying, the mean particle size and polydispersity index of ATRA-loaded SLNs were only slightly increased (181.8 vs. 265.2 nm, 0.173 vs. 0.200). Furthermore, no significant change was observed in the SLN-loaded concentration of ATRA and the zeta potential of SLNs after freeze-drying. Taken together, SLN formulation of ATRA with similar characteristics to those of parenteral emulsions could be obtained even after freeze-drying.

Keywords: All-trans retinoic acid; Solid lipid nanoparticle; Stability; Freeze-drying

1. Introduction

Recently, solid lipid nanoparticles (SLNs) have gained increasing attention as a promising colloidal carrier system, particularly for lipophilic drugs (Chen et al., 2001). SLNs are composed of a high melting point lipid as a solid core coated

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by surfactants such as lecithin. Thus, lipophilic drugs can be highly efficiently incorporated in the lipid core of SLNs. The solid core of SLNs, instead of fluid core of liposomes and emulsions, allows the prolonged and controlled release of drugs and may protect the incorporated drugs against chemical degradation. SLNs are less cytotoxic than polymeric nanoparticles. In fact, the toxicological acceptability of SLNs is similar to that of fat emulsions that have been proven to be non-toxic from parenteral nutrition studies. The production of SLNs is easier to scale up (Gohla and Dingler, 2001), compared with polymeric nanoparticles and liposomes. Thus, SLNs possess the combined advantages of polymeric nanoparticles, fat emulsions and liposomes (Muller et al., 2000; Schwarz and Mehnert, 1999). The additional advantage of SLNs may be the possibility of freeze-drying. Freeze-drying can be a promising way to increase chemical and physical stability of drugs and drug carriers over extended periods of time.

Several advantages of SLNs have driven numerous studies for the application of SLNs as dosage forms for topical (Wissing and Muller, 2001), peroral (Runge et al., 1996) and particularly for parenteral administrations (Yang et al., 1999). Studies have shown that the physicochemical characteristics and stability of drug-loaded SLNs are quite different from unloaded SLNs, but dependent on the properties of drugs. In this regard, the effect of formulation parameters on the physicochemical characteristics of drug-loaded SLNs should be investigated for each drug to get a SLN formulation optimal for that drug.

All-trans retinoic acid (ATRA) has been shown to exert anticancer activities in a variety types of cancer (Tallman et al., 1997). For the clinical use of ATRA in cancer treatment, the poor aqueous solubility of ATRA should be improved (Lin et al., 2000). The principal aim of this study was to prepare SLN formulation loaded with ATRA as a means to improve its aqueous solubility. The impacts of surfactant composition, drug loading and freeze-drying on the physicochemical characteristics of SLNs were investigated to get optimized formulation of ATRA.

2. Materials and methods

2.1. Materials

ATRA, tricaprin (TC) and MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetra-zolium bromide) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Egg phosphatidylcholine (EggPC) and distearoylphosphatidylethanolamine-*N*-poly(ethylene glycol) 2000 (DSPE-PEG) were provided by Avanti Polar Lipids (Alabasster, AL, USA). Tween 80 was purchased from ICI Americas (Wilmington, DE, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of SLN

SLNs were prepared by melt homogenization method with slight modification (Heiati et al., 1997). Briefly, 200 mg of TC, varying amount of ATRA, EggPC, Tween 80 and DSPE-PEG were mixed and dissolved in approximately 4 ml of tertiary butyl alcohol. After rapid freezing in liquid nitrogen tank, mixtures were dried in Ultra 35EL freezer dryer (Virtis, USA). Finely-dispersed cakes were obtained after overnight drying and then cakes were put in water bath at 50 °C. After a few minutes of incubation, lipid melts containing ATRA, TC and surfactants were obtained. Preheated (50 °C) water for injection was slowly added to the melts (2 g of final total weight) and sonicated in bath type sonicator for 30 min at 50 °C until crude and milky emulsions were obtained. These crude emulsions were homogenized for seven cycles at 60–70 °C and 100 MPa using a high pressure homogenizer (Emulsiflex EF-B3, Avestin Inc., Canada) wired with heating tape (Thermolyne, Barnstead International, USA). SLNs were produced by subsequent cooling of homogenized emulsions in liquid nitrogen. SLNs were then thawed at room temperature and stored at 4 °C.

When required, SLNs were further subjected to lyophilization. Our preliminary studies revealed that the reconstitution of freeze-dried SLNs was impossible when they were freeze-dried without addition of cryoprotectants. Therefore, for the

freeze-drying of SLNs, sucrose was added as a cryoprotectant to prevent the aggregation of SLNs (Joshi and Misra, 2001). After 1:1 dilution with 5% of sucrose as a cryoprotectant, SLNs were rapidly frozen in liquid nitrogen tank and then lyophilized in Ultra 35EL freezer dryer. Lyophilized SLNs were stored at 4 °C. Just prior to use, distilled water was added to the vial and the SLNs were redispersed by vortexing for 2–3 s.

2.3. Measurement of particle size and zeta potential

The mean particle size and polydispersity index (P.I.) of ATRA-loaded SLNs were determined by dynamic light scattering method using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at a fixed angle of 90 °C and at room temperature. Prior to measurement, SLN dispersions were diluted with filtered water. The system was used in the auto-measuring mode. The particle size analysis data was evaluated using volume distribution to detect even a few large particles. The P.I. is a measure of the distribution of nanoparticle population (Koppel, 1972).

The electrophoretic mobility of SLNs were determined using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan). The electrophoretic mobility was measured after dilution of samples with PBS buffer (pH 7.4) at room temperature ($n = 3$). The measured electophoretic mobility data was converted into zeta potential by using Helmholtz–Smoluchowski equation. The processing was done by the software included within the system.

2.4. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using VP-DSC Micro Calorimeter (Microcal TM Incorporated., Northampton, MA, USA). For DSC measurement, 80 μl of SLNs stored for 2 weeks at 4 °C were diluted with 1 ml of distilled water. A scan rate of 1 °C/min was employed in the 15–65 °C temperature range. The base line was adjusted by using distilled water as a reference.

2.5. Determination of concentration of ATRA loaded in SLNs

After homogenization, the hot ATRA-containing SLNs were immediately filtered through a 0.45 μm membrane filter to remove precipitated ATRA. The SLN fraction was dissolved in methanol and the amount of ATRA was directly determined by HPLC method (Wyss, 1990; Lin et al., 2000). The HPLC system consisted of mobile phase delivery pump (LC-10AS, Shimadzu, Japan), UV detector (SPD-10A, Shimadzu, Japan) and Chromatopac integrator (CR6-A). The C_{18} reverse phase column (Phenomenex Luna 5u C_{18} (25 cm × 4.6 mm 5u), Germany) was used. The eluent was 50.6: 24.4: 25.6 (v/v) mixture of acetonitrile: methanol: 2.5% aqueous ammonium acetate, and detected at 340 nm. The injection volume was 20 μl and the flow rate was 1.0 ml/min. Under these conditions, the linear calibration curve of ATRA was obtained in the range of 0.1–5 μg/ml ($r^2 > 0.999$).

3. Results and discussion

For the preparation of ATRA-loaded SLNs, TC was selected as a solid lipid component that will constitute the core of SLNs. Tween 80 and EggPC were chosen as components of surfactant mixture to stabilize SLNs because they are acceptable surfactants even in parenteral administration. DSPE-PEG was included as a steric stabilizer (Blume and Cevc, 1993).

Our preliminary thermal analysis to confirm the solidification of the lipid core in SLNs after cooling revealed that the cooling and crystallization of homogenized emulsions in liquid nitrogen was required to assure the solidification of cores of SLN. No melting peak was observed with SLNs after cooling at 4 °C even for a week. Previous studies, which reported the possibility of existence of supercooled melts depending on the preparation and storage condition, may explain our data (Westesen and Bunjes, 1995).

The mean particle size, P.I. and zeta potential of colloidal carriers are important characteristics of SLNs from which the stability of drug-loaded

SLNs can be predicted. We investigated the changes in those parameters by varying surfactant composition and drug loading to get stable SLN formulation of ATRA applicable for both parenteral and oral administration. We evaluated our data based on the reported values of fat emulsions for parenteral nutrition (200–400 nm of mean particle size, 0.050–0.125 of polydispersity index, −30 to −60 mV of zeta potential) (Muller and Heinemann, 1992).

Freeze-drying may increase the SLN stability by prevention of Ostwald ripening and hydrolysis reactions (Mehnert and Mader, 2001). Freeze-drying also offers possibilities for SLN incorporation into pellets, tablets or capsules. However, it is critical to prevent structural and functional damages of SLN in order to ensure the quality of freeze-dried product. Since only a few studies are currently available in the area of freeze-drying of SLNs, we first attempted to optimize the freeze-drying process in our preliminary study and then speculated the impact of freeze-drying on the physicochemical characteristics of SLNs with varying surfactant composition.

3.1. Effect of content of EggPC in the surfactant mixture

Since SLNs prepared with combination of surfactants generally tend to have smaller particle size and higher storage stability by preventing particle agglomeration more efficiently (Siekmann and Westesen, 1992; Olbrich and Muller, 1999), we prepared ATRA-loaded SLNs with surfactant mixture composed of varying ratio of Tween 80 and EggPC and investigated the effect of surfactant composition on the size and zeta potential of resultant SLNs. Indeed, the combination of Tween 80 and EggPC resulted in the reduction in the particle size of SLNs (Table 1). The mean particle sizes of SLNs prepared with 100% EggPC or 100% Tween 80 were 397.1 and 487.7 nm. Mixing of EggPC and Tween 80 reduced the particle size, resulting in 227.2 nm in SLNs with 46:54 weight ratio of Tween 80 and EggPC. The P.I. of SLNs were similarly low (0.180–0.208), suggesting the narrow size distribution in all the formulations.

The zeta potentials of SLNs were also affected by the combination ratio of EggPC and Tween 80 (Table 1). The zeta potential of SLNs prepared with 100% Tween 80 was very close to zero (−1 mV), corresponding to the region of limited flocculation. According to the increase in the content of EggPC, the negative value of zeta potential of SLNs linearly increased, reaching −22 mV in SLNs prepared with 100% Tween 80. A zeta

Table 1
Effect of content of Tween 80 in the surfactant mixture composed of EggPC and Tween 80 on the mean particle size, polydispersity index and zeta potential of resultant SLNs loaded with ATRA

Tween 80 in the surfactant mixture (%)	SLN without freeze-drying		SLN after freeze-drying		ζ potential (mV)
	Mean diameter (nm)	Polydispersity index	Mean diameter (nm)	Polydispersity index	
0	397.1	0.184	440.3	0.183	−22
20	364.6	0.196	452.1	0.123	−25
33	317.2	0.208	463.2	0.241	−23
46	227.2	0.193	329.5	0.215	−18
60	233.6	0.180	381.1	0.386	−19
80	301.9	0.180	477.6	0.346	−5
100	487.7	0.182	1459.7	0.542	−1

SLNs were prepared with 2 mg/g of ATRA and 30 mg/g of surfactant mixture composed of varying ratio of EggPC and Tween 80. Freeze-drying was performed in the presence of sucrose as described in the material and methods section. Numbers are the mean of three independent determinations.

potential range of −20 to −11 mV corresponds to the threshold of agglomeration in dispersions, according to the definition of Riddick (1968). Thus, the range of zeta potential obtained was not high enough for a sufficient electrostatic stabilization. However, since Tween 80 can provide additional steric stabilization of particles, we can still expect combined electrostatic and steric stabilization of our SLN formulations.

The combination of Tween 80 and EggPC also contributed to the reduction in the size of freeze-dried SLNs: The mean sizes of SLNs prepared with 54:46, 100:0 or 0:100 weight ratio of EggPC:Tween 80 were 329.5, 440.3 and 1459.7 nm, respectively. In SLNs prepared with 54:46 of EggPC:Tween 80, the mean particle size was only 1.1-fold increased by freeze-drying.

The P.I. of freeze-dried SLNs prepared with EggPC more than 54% were similar or slightly decreased compared with those without freeze-drying. However, the P.I. of SLNs after freeze-drying tended to increase when they were prepared with more than 46% of Tween 80. The P.I. of SLNs prepared with 100% Tween 80 was 3.0-fold increased (from 0.182 to 0.542), suggesting the heterogeneous distribution of freeze-dried particles. The structure of surface layer of SLNs is inevitably dependent on the composition of surfactants. The presence of bilayer, instead of monolayer, was observed in SLNs where phospholipids were used as surfactants (Heiati et al., 1996). In our study, the presence of EggPC may favourably alter the surface arrangement of surfactants not to be impaired by the freeze-drying process.

Taken together, (1) The smallest mean particle size of SLNs could be obtained by mixing EggPC and Tween 80 at the ratio of 54:46. (2) The zeta potential of SLNs increased according to the increase in the content of EggPC. (3) The P.I. of SLNs could be maintained low after freeze-drying when SLNs were prepared with more than 54% EggPC. From these results, the ratio of EggPC:Tween 80 was fixed at 54:46 in the subsequent studies.

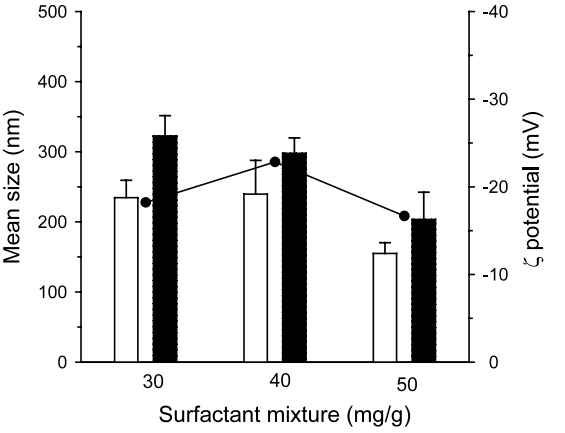

Fig. 1. Effect of surfactant amount on the mean particle size and zeta potential of ATRA-loaded SLNs before and after freeze-drying. SLNs were prepared with 30, 40 or 50 mg/g surfactant mixture composed of 54:46% (w/w) of EggPC:Tween 80. The particle size and zeta potential of non-freeze-dried SLNs (□) and freeze-dried SLNs (■) were also compared.

3.2. Effect of amount of surfactant mixture

The increase in the surfactant amount in colloidal dispersions may contribute to the reduction of mean particle size because of the surface-active properties of surfactants (Park et al., 1999). We examined whether the surfactant amount influenced those parameters in our SLN formulation.

The increase in the total amount of surfactant mixture from 30 to 50 mg/g reduced the mean particle size of SLNs (Fig. 1). The mean particle size of SLNs prepared with 50 surfactant mixture was 154.9 ± 15.5 nm, while that of SLNs with 30 mg/g surfactant mixture was 234 ± 24.9 nm. After freeze-drying, the impact of surfactant amount on the mean particle size was more evident. The mean particle size of SLN with 30, 40 and 50 mg/g surfactant mixture was 322, 297 and 203 nm, respectively.

The zeta potentials of SLNs were not much affected by the total amount of surfactant (−16 to −23 mV) (Fig. 1).

3.3. Effect of loading concentration of ATRA

We also investigated the impact of ATRA loading on the mean particle size, P.I. and zeta potential of SLNs before and after freeze-drying. The results were summarized in Table 2.

The loading concentration of ATRA linearly increased according to the increase in the initial concentration of ATRA from 1 to 3 mg/g (1–3% of lipid core). Regardless of the loading concentration of ATRA, the mean particle sizes of SLNs were similar and they were in the range of 145.1 and 167.2 nm (Table 2). Although the P.I. of SLNs increased with the increase in the loading concentration of ATRA, they were still below 0.150 at 2.84 mg/ml of ATRA loading concentration, indicating narrow particle distribution. The loading of ATRA also had little effect on the zeta potential of SLNs, suggesting that it did not impede the electrostatic repulsion among SLN particles.

After freeze-drying, the particle size of SLNs was slightly increased but similar regardless of loading concentration of ATRA (Table 2). Moreover, the P.I. of freeze-dried SLNs were similarly low, regardless of ATRA loading (0.168 and 0.189 in freeze-dried SLNs with 0 and 2.84 mg/ml ATRA). In many cases, drug loading in SLNs have shown to impair the stability of SLNs, particularly after freeze-drying (Schwarz and Mehnert, 1997). However, our data suggests that ATRA loading did not impair the stability of SLNs after freeze-drying. Taken together, these data demonstrate that ATRA loading in SLN formulations could be achieved with little adverse effect on the particle size and zeta potential.

3.4. Effect of inclusion of DSPE-PEG

We further investigated whether the inclusion of DSPE-PEG, which is known as a steric stabilizer, may affect the particle size and zeta potential of SLNs (Table 3). Inclusion of DSPE-PEG tended to increase the particle size (149.1, 168.0, 181.3 and 364.2 nm in case of SLNs prepared with 0, 6, 12 and 18% DSPE-PEG). The increase in the mean particle size of SLNs was approximately 2-fold by inclusion of 18% DSPE-PEG. Presumably it is due to the repulsion among hydrophilic polymers on the surface of SLNs.

The P.I. of SLNs also slightly increased according to the increased percent of DSPE-PEG, but were still below 0.2 even at the 18% of DSPE-PEG.

The negative values of zeta potential of SLNs were greatly increased by inclusion of DSPE-PEG. By inclusion of 6% DSPE-PEG, it was increased by 2-fold (from -17 to -35 mV). Higher inclusion of DSPE-PEG at above 6% further increased the zeta potential (-35, -39 and -46 mV by the inclusion of 6, 12 and 18% DSPE-PEG). These data indicate that the DSPE-PEG could also serve as an electrostatic stabilizer in our SLN formulation. This is in accordance with previous studies showing that PEG-PE contributes to the negative surface charge of PEG-grafted liposomes (Woodl et al., 1992).

After freeze-drying, the mean particle size and P.I. increased in every formulation. However, except SLNs prepared with 18% DSPE-PEG, the mean particle size of freeze-dried SLNs were still below 400 nm. The zeta potential of SLNs was not affected by freeze-drying, regardless of the percent of DSPE-PEG. Our data indicate that the inclusion of DSPE-PEG may be desirable in getting stable SLN formulation of ATRA even after freeze-drying. Considering the size and zeta potential of parenteral emulsions, inclusion of 6% DSPE-PEG is enough for that purpose.

3.5. Effect of surfactant composition on the ATRA loading capacity

Table 4 shows that the amount and composition of surfactant mixture affected the ATRA loading capacity of SLNs. The increased amount of surfactant mixture linearly increased the maximum loading concentration of ATRA: 2.09 ± 0.072, 2.25 ± 0.494 and 3.25 ± 0.353 mg/ml of ATRA could be loaded in SLNs prepared with 30, 40 and 50 mg/g of surfactant mixture (EggPC:Tween 80 = 54:46). The increased loading of ATRA by surfactant increase suggests that ATRA may be embedded in the surfactant layer rather than being incorporated in the innermost solid lipid core as shown in other drug-incorpo-

Table 2
Effect of loading concentration of ATRA on the particle size, polydispersity index and zeta potential of resultant SLNs with or without freeze-drying

Initial concentration of ATRA (mg/g)	Loaded concentration of ATRA (mg/ml)	SLNs without freeze-drying			SLNs after freeze-drying		
		Mean diameter (nm)	Polydispersity index	ζ potential (mV)	Mean diameter (nm)	Polydispersity index	ζ potential (mV)
0	–	167.2	0.054	−15	209.7	0.168	−17
1	0.902 ± 0.034	158.2	0.101	−23	212.9	0.184	−21
2	2.108 ± 0.058	145.1	0.116	−22	201.5	0.200	−21
3	2.838 ± 0.121	163.1	0.144	−17	211.9	0.189	−17

SLNs were obtained with varying amount of ATRA and 50 mg/g of surfactants composed of 54:46 w/w% of EggPC:Tween 80. Numbers are the mean of three independent determinations.

Table 3
The mean particle size, polydispersity index and zeta potential of SLN loaded with ATRA as a function of DSPE-PEG content in the surfactant mixture

DSPE-PEG in the surfactant mixture (%)	SLN without freeze-drying			SLN after freeze-drying		
	Mean diameter (nm)	Polydispersity index	ζ potential (mV)	Mean diameter (nm)	Polydispersity index	ζ potential (mV)
0	149.1	0.144	−17	211.9	0.189	−17
6	168.0	0.187	−35	229.0	0.207	−36
12	181.3	0.191	−39	311.2	0.223	−39
18	364.2	0.193	−46	432.7	0.249	−44

SLNs were prepared with 2.5 mg/g ATRA and 50 mg/g surfactant mixture (EggPC/DSPE-PEG/Tween 80). The weight ratios of phospholipid (EggPC and DSPE-PEG) and Tween 80 were fixed at 54:46 w/w% in all formulations. Numbers are the mean of three independent determinations.

rated SLNs (Heiati et al., 1997; Jenning and Gohla, 2001). However, the loading concentration of ATRA also slightly increased according to the increase in the TC amount (data not shown), suggesting that probably ATRA was partly incorporated in the solid lipid core and partly in the surfactant layer.

When the total amount of surfactants was fixed, the ATRA loading capacity was influenced by the composition of surfactant mixture. The loading capacity of SLNs prepared with 100% Tween 80 as surfactants was the lowest and it was only 0.809 ± 0.297 mg/ml. In contrast, the loading capacity of SLNs prepared with 54:46% of EggPC:Tween 80 increased up to 2.09 ± 0.072 mg/ml.

Although the inclusion of 6% DSPE-PEG to stabilize the SLNs decreased the ATRA loading capacity of SLNs, it was not critical (from 2.09 to 1.40 mg/ml in SLNs prepared with 30 mg/g of surfactants and from 3.25 to 2.66 mg/ml in SLNs prepared with 50 mg/g of surfactants). The decrease in the loading concentration of ATRA by inclusion of DSPE-PEG may be due to that the portions of ATRA embedded in the surfactant layers were expelled out by the inclusion of DSPE-PEG.

Table 4
Effect of amount and composition of surfactant mixture on the ATRA-loading capacity of SLNs

Composition of surfactant mixture (EggPC:Tween 80:DSPE-PEG) (% (w/w))	Concentration of ATRA loaded in SLNs (mg/ml)
30 mg of surfactant mixture	
100:0:0	1.93 ± 0.228
0:100:0	0.809 ± 0.297
54:46:0	2.09 ± 0.0720
48:46:6	1.40 ± 0.0700
40 mg of surfactant mixture	
54:46:0	2.25 ± 0.494
50 mg of surfactant mixture	
54:46:0	3.25 ± 0.353
48:46:6	2.66 ± 0.0304

SLNs were prepared with excess amount of ATRA (5 mg/g of ATRA) and surfactant mixture with varying composition ($n = 3$)

3.6. Characteristics of optimised SLN formulation of ATRA

From the optimization studies discussed above, SLN formulation of ATRA was prepared with 50 mg/g surfactant mixture composed of 48:46:6% (w/w) mixture of EggPC:Tween 80:DSPE-PEG and 3 mg/g of ATRA. For the comparison, ATRA-free SLNs were separately prepared and freeze-dried. As shown in Fig. 2, by optimization of formulation parameters, ATRA-free and ATRA-loaded SLNs could be produced with mean particle size similar to that of parenteral emulsions (200–400 nm) even after freeze-drying. The increase in the mean particle size of ATRA-free and -loaded SLNs by freeze-drying was only approximately 1.5-fold (165.9 ± 3.15 vs. 250.5 ± 68.77 nm in ATRA-free SLNs and 181.8 ± 21.5 vs. 265 ± 36.2 nm in ATRA-loaded SLNs). The P.I. also only slightly increased after freeze-drying (1.9- and 1.1-fold in ATRA-free and ATRA-loaded SLNs). Thus, the P.I. of freeze-dried SLN formulation of ATRA (0.200) were only slightly higher than the P.I. range of parenteral emulsions (0.050–0.125). The sizes of 95% of particles present in SLN dispersions was below 198.4 nm (ATRA-free) and 292.4 nm (ATRA-loaded) without freeze-drying and 539.1 nm (ATRA-free) and 566.1 (ATRA-loaded) after freeze-drying, far from the size limit that might block the capillaries (4–6 μm). The zeta potential value of SLNs loaded with ATRA was more than -30 mV, indicating the electrostatic repulsions among particles are enough for good stability. No significant change in the zeta potential values by freeze-drying process also demonstrates that the stability of SLNs was not impaired by the processing (-38 vs. -36 mV).

The loading concentration of ATRA was 2.36 ± 0.0464 mg/ml in SLNs prepared with 3 mg/g ATRA. The calculated loading efficacy is $78.7 \pm 1.55\%$. When ATRA-loaded SLN dispersions were dialyzed or ultrafiltrated to separate the portion of ATRA dissolved in the outer aqueous medium, no ATRA was detected in the aqueous medium. It indicates that more than $> 99\%$ of ATRA was incorporated in the SLNs, rather than being dissolved in outer phase. It is

(A)

Freeze-Drying	Mean size (nm)	P.I.	Diameter 95% (nm)	ζ poetntial (mV)	Incorporated ATRA (mg/ml)
ATRA-unloaded					
Before	165.9 ±3.15	0.063 ± 0.015	198.4	ND	–
After	250.5 ±68.77	0.122 ±0.040	539.1	ND	–
ATRA-loaded					
Before	181.8 ±21.5	0.173 ±0.053	292.4	- 38	2.36 ± 0.046
After	265.0 ±36.2	0.200 ± 0.019	566.1	- 36	2.27 ± 0.056

(B)

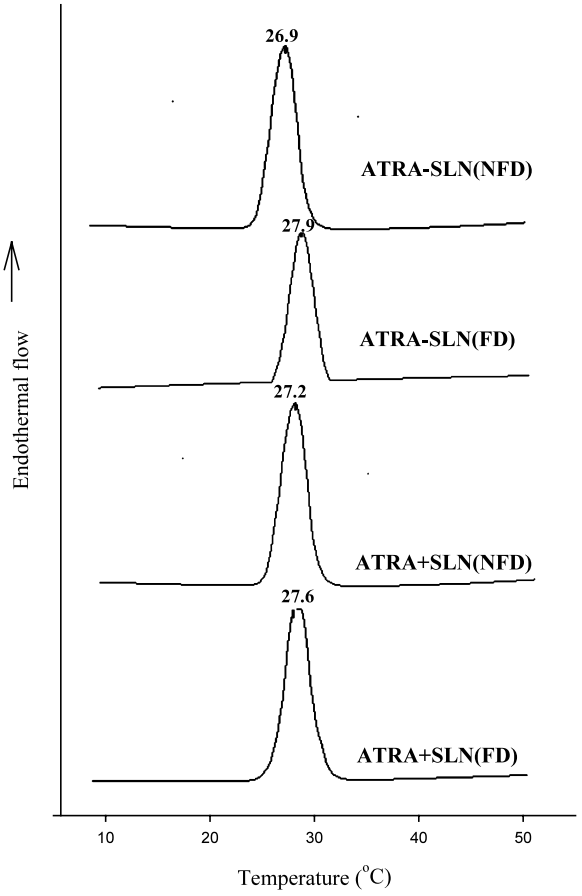

Fig. 2. The physicochemical characteristics of optimised SLN formulation. SLNs were prepared with 50 mg/g surfactant mixture composed of 48:6:46% (w/w) of EggPC:DSPE-PEG:Tween 80 with or without 3 mg/g of ATRA, and then freeze-dried as described in the material and method section. (A) Effect of ATRA loading and freeze-drying on the mean particle size, polydispersity index, zeta potential and ATRA-loading concentration of SLNs (B) DSC curves of SLNs prepared with or without ATRA. ATRA-free or ATRA-loaded SLNs were further subjected to freeze-drying (ATRA − SLN(FD) and ATRA + SLN(FD)) and then compared with the DSC curves of those without freeze- drying (ATRA − SLN(NFD) and ATRA + SLN(NFD)).

expected because the aqueous solubility of ATRA is nil (80 ng/ml) (Szuts and Harosi, 1991). After freeze-drying, the ATRA concentration incorporated in SLNs was 96.2% of that in SLNs before freeze-drying (2.36 ± 0.0464 vs. 2.27 ± 0.0560 mg/ml), suggesting that ATRA could be stably retained in the SLNs during lyophilization process.

In other studies, the mean particle size and the number of particles greater than four to six were substantially increased by freeze-drying, rendering the freeze-dried SLNs not suitable for intravenous administration (Cavalli et al., 1997; Schwarz and Mehnert, 1997). i.v.-injectable SLN formulation could be obtained in one study but additional heating and cooling step was required during lyophilization process (Zimmermann et al., 2000). In our study, ATRA-loaded SLNs could be freeze-dried without tedious heating and cooling step, and it was still i.v.-injectable with regard to the particle size after reconstitution. More promising results obtained in our study may be attributed to the molecular property of ATRA. One possible mechanism responsible for the substantial increase in the size of freeze-dried SLNs is the presence of free drugs dissolved in the dispersion medium, causing the reduction of zeta potential during the freezing process (Schwarz and Mehnert, 1997). The good quality of freeze-dried SLNs loaded with ATRA may be the relative absence of free ATRA in the dispersion medium, due to the nil solubility of ATRA in the aqueous medium and/or the strong interaction between ATRA and phospholipids such as EggPC (Parthasarathy et al., 1994).

Thermal analysis was performed to investigate the degree of crystallinity and the recrystalization behavior of SLNs (Siekmann and Westesen, 1992). Generally, the melting peak of lipid core in SLNs is observed at lower temperature than that of bulk lipid due to the nanocrystaline size of lipids in SLNs (Westesen and Bunjes, 1995). In our study, the melting peak of SLNs was also observed at a temperature 4–5 °C lower than the bulk TC (27 vs. 31 °C). The incorporation of ATRA did not greatly change the melting point (26.9 vs. 27.2 °C). After freeze-drying, the melting point of ATRA-free SLNs was 1°C higher than that before freeze-drying (from 26.9 to 27.9 °C), while that of ATRA-loaded SLNs was 0.4 °C increased by freeze-drying (from 27.2 to 27.6 °C). It may be caused by that some components such as surfactants were expelled out from SLNs during freeze-drying process. However, the height and sharpness of melting peaks were not significantly changed by freeze-drying, suggesting that the physical state of SLNs was not greatly affected by the freeze-drying process.

No change was observed in the mean particle size and P.I. of ATRA-loaded SLNs during storage at 4 °C for 90 days. On the other hand, the P.I. of freeze-dried SLNs was slightly but gradually increased during 4 weeks of storage without significant change in the mean particle size (data not shown). The changes in the packing material or storage condition may further improve the stability of freeze-dried SLNs (Freitas and Muller, 1999).

4. Conclusion

In this study, ATRA-loaded SLNs with desirable mean particle size, P.I. and zeta potential, similar to those of parenteral emulsions, could be obtained even after freeze-drying. The combination of nonionic surfactant, Tween 80, and lipid surfactants, EggPC and DSPE-PEG, contributed to the preparation of stable SLN formulation. The ATRA loading capacity of our optimised SLN formulation was 2.4% of lipid matrix and it was much higher than the value obtained in other study (less than 1%, Jenning and Gohla, 2001). ATRA-loaded SLNs may enhance the therapeutic efficacy of ATRA after oral or parenteral administration. SLN formulation in this study may also have a potential as a dosage form of various retinoid analogues as well as ATRA.

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