College of Pharmacy, Shenyang Pharmaceutical University, China

Investigations on 5-fluorouracil solid lipid nanoparticles (SLN) prepared by hot homogenization

SHIRUI MAO, PU WANG, DIANZHOU BI

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Shirui Mao, Department of Pharmaceutics, College of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, 110016 Shenyang, China
maoshirui@vip.sina.com

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The purpose of this study was to investigate the effect of different formulation factors on the properties of solid lipid nanoparticles (SLN) prepared by a hot homogenization method. Using the particle size, physical stability constant (K_e) and ζ-potential as standards, the stability of SLNs was investigated as a function of phospholipid and poloxamer contents. It was demonstrated that the content of phospholipid had a significant influence on the ζ-potential, which increased considerably with increasing phospholipid content. However, the particle size increased remarkably when the phospholipid content was as high as 1.5% due to the increased viscosity. Poloxamer 188 exhibited no remarkable influence on particle size when the concentration was as low as 1.0%. The influence of the phospholipid and poloxamer content on the embedding ratios of drug substances was further studied using 5-fluorouracil (5-Fu) as a model drug. It was shown that the embedding ratio increased considerably with phospholipid content and independent of poloxamer content, implying that 5-Fu was incorporated into the phospholipid bilayer membrane.

1. Introduction

Since the beginning of the nineties, the attention of various research groups has been focused on solid lipid nanoparticles (SLN) (Mühlen et al. 1998; Müller et al. 1995; Müller et al. 2000). SLN are made from solid lipids, one can prepare them from emulsions for parenteral nutrition just by replacing the liquid lipid (oil) of the emulsion droplets with a solid lipid. The advantage of solid lipids is that the solid carrier matrix is stable against coalescence and should reduce the mobility of incorporated drugs thus preventing drug leakage from the carrier. Moreover, the solid core of SLN prolongs the release of the incorporated drugs. Due to their small size, SLN can be employed for the passive targeting of drugs and also as carrier systems not only for hydrophobic, but also for hydrophilic drugs. Generally, SLN consist of compositions that are biodegradable and non-toxic. Most have already been approved for parenteral nutrition formulations. Similar to emulsions, optimized SLN compositions possess a physical long-term stability of at least 3 years (Freitas et al. 1998). Alternatively, the SLN of liquid state can also be converted into a solid product by spray drying or lyophilization to avoid occurring instabilities (Müller et al. 1995). Drug loaded SLN systems are suitable for intravenous, oral or dermal application depending on the particle size.

However, drug leakage from the carriers is still often present in SLN due to polymorphic transitions or changes in the crystalline state of the lipids, especially for highly purified lipids such as tristearine, drug expulsion has been described (Bunjes et al. 1996). This led to a new, improved generation of lipid nanoparticles, the nanostructured lipid carriers (NLC), which are produced by controlled mixing of solid lipids with spatially incompatible liquid lipids, leading to special nanostructures with improved drug incorporation and release properties (Müller et al. 2002a, 2002b). On the other hand, in order to overcome the low loading ratios of hydrophilic drugs in SLN due to their poor solubility in the lipid, lipid-drug conjugates (LDC) were developed recently (Olbrich et al. 2002).

The two basic production methods of SLN are homogenization (Mühlen et al. 1998) and microemulsion (Gasco 1993; Boltril et al. 1995). SLN can be produced by high-pressure homogenization in a manner identical to that, which is used to generate parenteral o/w emulsions. This is a technique that has been used on a large scale ever since the fifties and is already well established in the pharmaceutical industry.

To date, several studies concerning the stability (Müller et al. 2000), biodistribution (Heiati et al. 1998; Siekmann et al. 1999) and optimization of SLN production parameters (Schwarz et al. 1995) have been undertaken. In addition, the effects of SLN composition on the stability and degree of drug incorporation are of particular importance. The primary objective of this study, therefore, was to investigate the effect of the most frequently used SLN components, stearic acid, phospholipid and poloxamer, and their content on the stability and drug incorporation ratio in different SLN formulations. 5-Fluorouracil (5-Fu) was selected as a model drug. A hot homogenization method was employed to generate the particles.

Table 1: Effect of phospholipid content on SLN properties

Phospholipid%[a]	0.5	1.0	1.5	2.0	2.5	3.0
ζ (mv)	−19.5 ± 3.6	−23.5 ± 3.8	−32.3 ± 3.2	−33.7 ± 8.6	−45 ± 8.6	−42.6 ± 8.7
Ke	0.004 ± 0.001	0.003 ± 0.001	0.176 ± 0.010	0.365 ± 0.085	0.108 ± 0.043	0.174 ± 0.037
Size (nm)	413.5 ± 5.6	383.3 ± 12.1	791.2 ± 30.2	1116.7 ± 90.3	702.5 ± 32.4	835.4 ± 34.6

[a] Calculated as percent of stearic acid mass
Results are presented as mean (n = 3) ± standard deviation

2. Investigations, results and discussion

2.1. Effect of phospholipid on the stability of SLN

The content of stearic acid was 3% (w/w), glycerin 2.5% and phospholipid was 0.5, 1.0, 1.5, 2.0, 2.5, 3.0% (w/w) (calculated as percent of stearic acid mass), respectively. SLNs were prepared according to the method described in the experimental part. Particle size, Ke and ζ-potential were determined after preparation (Table 1).
It was shown that the SLNs are negatively charged and ζ-potential increased with increasing phospholipid content. The following equation can be used to describe the correlation between ζ-potential and phospholipid content:

$$\mathrm{Lg}(-\zeta) = 0.1468\mathrm{C} + 1.2407\ (\mathrm{r} = 0.96,\ \mathrm{n} = 5) \qquad (1)$$

On the other hand, particle size and Ke increased considerably when the phospholipid content was higher than 1.0%. This is quite reasonable, as the viscosity of the lipid phase increases with phospholipid content, resulting in larger emulsion droplets and, therefore, larger particles and decreased stability. Moreover, the Ke value indicated that the stability of SLN is not only related to the ζ-potential but also particle size. A phospholipid content in the range of 0.5–1.0% is preferred.

2.2. Effect of poloxamer 188 on the stability of SLN

The ability of the amphiphilic poloxamer to act as a hydrophilic shield in drug carriers has been shown in many studies (Müller et al. 1995, 1997; Moghimi et al. 1995; Lenaerts et al. 1995). These results indicate that poloxamer is capable of sterically preventing the approach of destabilizing plasma components to the surface of the SLNs, thus, reducing their specific interaction with the phospholipid membrane and increasing the retention of the drug. In a study by Illum and Davis (1983), poloxamer was used as a coating material to prepare long-circulating NPs of polystyrene and poly(methyl methacrylate). A prolonged circulation time and reduction in liver uptake in rabbits were found for poloxamer-coated polystyrene NPs when compared to uncoated NPs of the same size. In the present study the influence of poloxamer coating of SLN was also investigated.
The content of stearic acid was 3%, phospholipid 1%, glycerin 2.5%, poloxamer 188 was 0.5, 1.0, 2.0, 2.5 and 3.0% (calculated as percent of stearic acid mass), respectively. SLN were prepared in the same manner. Particle size, Ke and ζ-potential were determined (Table 2).
Compared to the results in Table 1, no remarkable particle size changes were observed when poloxamer content was as low as 1.0%. However, the ζ-potentials increased about 32% after the addition of poloxamer 188. At first glance, this observation may seem unreasonable, because it contradicts the theory that the poloxamer adsorption layer shifts the shear plane away from the particle surface, that is to say, ζ-potential should decrease. However, this phenomenon was also observed by other researchers (Vandervoort et al. 2002). The addition of poloxamer in the preparation of PLGA nanoparticles increased the negative ζ-potential significantly. The mechanism has not been elucidated and requires further investigation. During the past two decades it has been established that, in addition to electrostatic and van der Waals forces, a number of other interactions could play an important role in colloid stability. The most popular among them include depletion interactions (due to the presence of soluble polymers or surfactant micelles in the dispersion medium) and steric interactions (due to polymer molecules at the droplet surface) (Petsev et al. 1995). Within the system under investigation, all of these interactions may occur.
In addition, Table 2 shows that there is no significant difference with respect to the ζ-potential values when the content of poloxamer is in the range of 0.5–3.0%. However, when the concentration of poloxamer was larger than 1.0%, a tendency to gelation was observed. This phenomenon was also reported by other groups (Freitas et al. 1998). This is not unexpected because poloxamer 188 itself has been used as a gelling agent (Frisbee et al. 1994). In this case, the content of poloxamer should be no more than 1.0% if a liquid state is preferred.

2.3. Factors influencing 5-Fu embedding ratio

2.3.1. Effect of the encapsulated drug on 5-Fu embedding ratio

SLNs were prepared using 5-Fu as a model drug. The content of stearic acid was 3%, phospholipid 1% and glycerin 2.5%. All other conditions, for example, the temperature of the aqueous phase, the duration of stirring and the volume of the ethanol were kept constant to minimize fluctuations. The amount of 5-Fu added was 3, 4, 5, 6, 7, 8, 9, 10% (calculated as percent of stearic acid mass, denoted as "encapsulated drug" in this paper), respectively. The drug-embedding ratio and drug-loading ratio were cal-

Table 2: Effect of poloxamer 188 on SLN properties

Poloxamer%[a]	0.5	1.0	1.5	2.0	2.5	3.0
ζ (mv)	31.1 ± 3.4	−40.0 ± 7.8	−30.7 ± 4.8	−30.6 ± 4.3	−25.6 ± 7.4	−28.5 ± 4.4
Ke	0.003 ± 0.001	0.010 ± 0.002	0.070 ± 0.010	0.000 ± 0.001	0.115 ± 0.023	0.080 ± 0.015
Size (nm)	381.2 ± 8.8	366.4 ± 8.4	546.3 ± 6.7	−*	−*	−*

[a] Calculated as percent of stearic acid mass
* not determined due to gelation
Results are presented as mean (n = 3) ± standard deviation

culated according to the following equations:

$$\text{Drug embedding ratio (\%)} = (A - B) \times 100/A \quad (2)$$

$$\text{Drug loading ratio(\%)} = (A - B) \times 100/C \quad (3)$$

where A is total amount of 5-Fu added to the SLN,
B is free amount of 5-Fu,
C is total amount of SLN.

The relationship between the encapsulated drug and drug embedding ratio (a), as well as that of the encapsulated drug and drug-loading ratio (b) are described in Fig. 1. It was observed that drug-embedding ratios decreased with increasing amount of encapsulated drug (Fig. 1). The highest value was achieved with 3% 5-Fu and levels remained stable when 4–7% 5-Fu was added. The drug loading ratios, on the other hand, which measure the amount of 5-Fu associated with the unit weight of nanoparticles, were nearly constant and independent of the amount of 5-Fu added to the formulation (Fig. 1). The mechanism was further investigated in the following section.

2.3.2. Effect of phospholipids on the 5-Fu embedding ratio

According to Müller et al. (2000), there are three drug incorporation models for SLN: the solid solution model, the core-shell model with a drug-enriched shell, and the core-shell model with a drug-enriched core. SLN produced by the hot homogenization technique are thought to belong to the class of the core-shell model with a drug-enriched shell. When cooling to room temperature, hydrophilic, encapsulated drugs are repartitioned to the shell from lipid solution. Normally, the lipid core of SLN is composed of stearic acid coated by a phospholipid bilayer membrane similar to that of liposomes. The hydrophilic drug is mainly found encapsulated within this phospholipid bilayer membrane. In this case, the content of the phospholipid is of extreme importance to the embedding ratio of a hydrophilic drug like 5-Fu. In order to verify our supposition about SLN structure characteristics, the content of the phospholipid on the drug-embedding ratio was investigated.

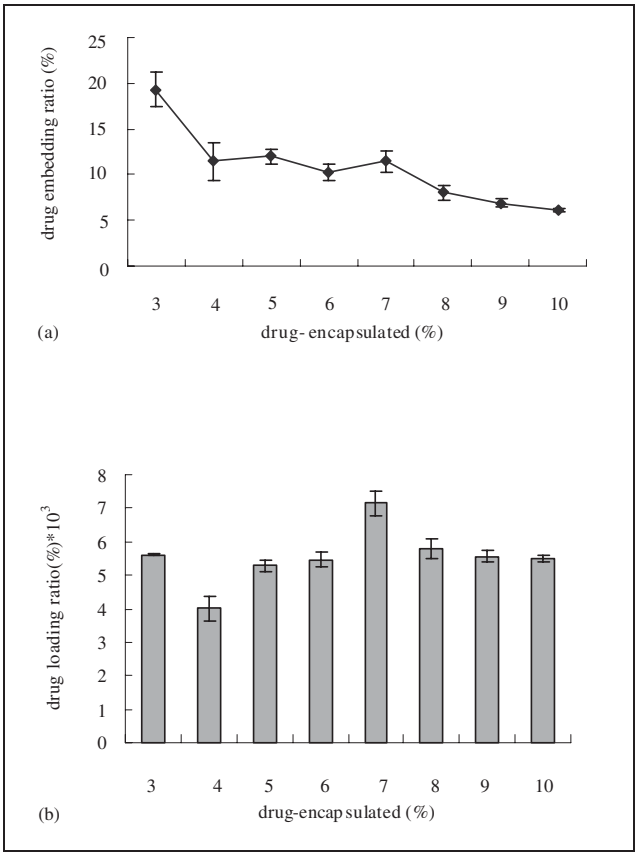

Fig. 1: The upper diagram (a) depicts 5-Fu embedding ratio as a function of the encapsulated drug (calculated as percent of stearic acid mass). The lower diagram (b) shows 5-Fu loading ratio as a function of the encapsulated drug (calculated as percent of stearic acid mass). Error bar indicates the standard deviation of three measurements

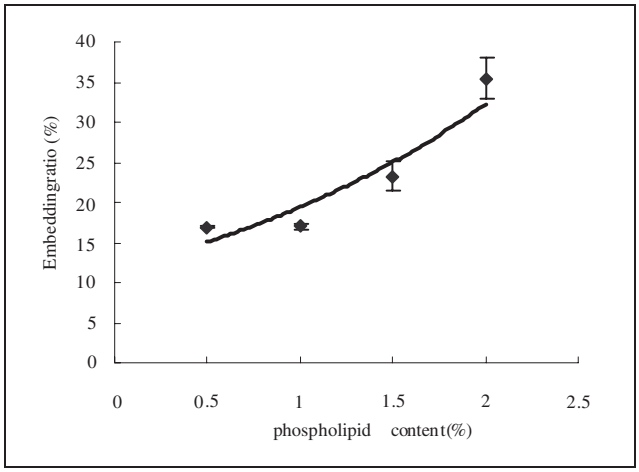

Fig. 2: Effect of phospholipid content on the embedding ratio of 5-Fu. Phospholipid content and the embedding ratio are calculated as a percent of stearic acid. Error bar indicates the standard deviation of three measurements

The content of stearic acid was 3% and the concentration of 5-Fu was also 3% (corresponding to the amount of stearic acid). The amount of phophilipid used was 0.5, 1.0, 1.5 and 2.0%, respectively. Figure 2 shows the embedding ratios as a function of phospholipid content. Increasing the phospholipid content from 0.5 to 2% in SLN resulted in a significant increase in the embedding ratio, approximately 20%. The relationship between embedding ratio and phospholipid content can be described with the following equation:

$$y = 11.686e^{0.5077x} \; (r = 0.94) \quad (4)$$

This result provides direct evidence that the actual location of drugs may not be within the solid core and 5-Fu is most probably incorporated into the phospholipid bilayer structure. Those results corroborate those reported by Heiati et al. (1996), who showed that multiple phospholipid bilayers surrounded a trilaurin core by means of freeze-fracture electron micrographs of SLNs. Additionally, these findings confirm our results described in part 2.3.1, which indicated that drug embedding ratios kept stable independent of the amount of 5-Fu encapsulated when phosphate content was constant.

2.3.3. Effect of poloxamer 188 on the 5-Fu embedding ratio

The content of stearic acid was 3%, phospholipid 1.5%, glycerin 2.5% and 5-Fu 3%. The amount of poloxamer 188 used was 0, 0.2, 0.4, 0.6, 0.8%, respectively. The SLNs were prepared in the same manner as described above. The embedding ratios were calculated and illustrated in Fig. 3. Incorporation of 5-Fu was independent of

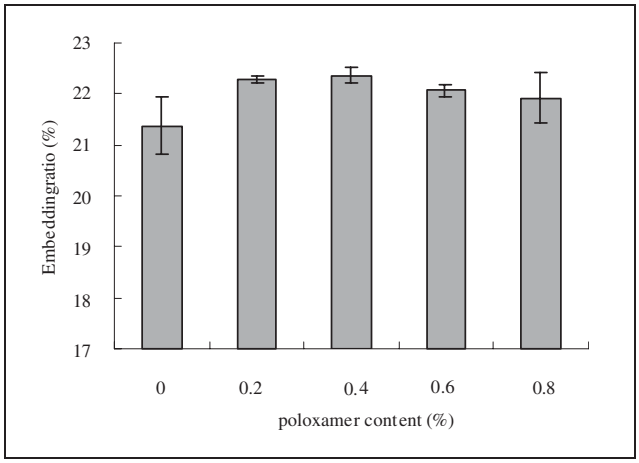

Fig. 3: Effect of poloxamer 188 content on the embedding ratio of 5-Fu. Poloxamer content and the embedding ratio are calculated as a percent of stearic acid. Error bar indicates the standard deviation of three measurements

the amount of poloxamer used. This is reasonable, as poloxamer only coats on the surface of SLN, with little interaction with the lipid shell, hence, it was not expected to have an effect on the drug incorporation.

In summary, this study demonstrated that the phospholipid content significantly influenced the ζ-potential and particle size of SLN. ζ-potentials and particle size increased considerably with increasing phospholipid content. SLN with small particle size could be obtained when the phospholipid content was in the range of 0.5–1.0%. The addition of poloxamer caused a further increase in ζ-potential and exhibited no remarkable influence on particle size when the concentration was as low as 1.0%. It should be noted, however, that poloxamer content should be no more than 1.5% in order to avoid gelation. The embedding ratio of 5-Fu increased significantly with increasing phospholipid content, indicating that the drug was probably incorporated into the phospholipid bilayer membrane, while the value was independent of the amount of poloxamer added.

3. Experimental

3.1. Materials

Stearic acid was purchased from Tianjin Baodi Chemical Cooperation (Tianjin, China). Pure soybean phospholipid (injection grade) was obtained from Shanghai First Oils and Fats Factory (Shanghai, China). Poloxamer 188 was purchased from Shenyang Pharmaceutical University (Shenyang, China). 5-Fu was obtained from Shanghai Hualian Pharmacia (Shanghai, China). Spehadex G-50 was from Pharmacia (Beijing, China). Glycerin was from Beijing First Reagent Factory (medical grade) (Beijing, China). Distilled water was filtered through a 0.45 μm micropore membrane before use. HEPES buffer used in the experiments was 10 mM at pH 7.4. All other chemicals were of analytical grade.

3.2. Preparation of blank SLN

In view of safety and biocompatibility, SLN were composed of biodegradable materials, i.e., stearic acid, phospholipid, poloxamer 188, glycerin and distilled water. Glycerin was added to the formulation in order to achieve isotonicity based on the composition of Intralipid®. Briefly, the phospholipid was dissolved in anhydrous ethanol and the stearic acid was melted at 70–80 °C in a water bath. Subsequently, the phospholipid solution was added to the stearic acid under constant magnetic stirring and stirred for a further 5 min to remove the ethanol. In the meantime, poloxamer 188 and glycerin were dissolved in water and heated to the same temperature as the lipid phase. The water phase was added to the lipid phase under high speed magnetic stirring and then emulsified under ultrasonication (Branson Sonifier 250) for 10 min. Finally, the suspension was pored into a beaker and stirred until it reached room temperature.

3.3. Physical stability constant (Ke) measurements

The physical stability constant (Ke) was first used to appraise the stability of an emulsion (Han et al. 1991). Because SLN suspensions show very similar physicochemical properties to emulsions, it was concluded that Ke could also be used to estimate the stability of SLN in an accelerated manner. The Ke was obtained using the following method: The SLN suspensions were centrifuged for 20 min at 2000 rpm/min and 0.3 ml supernatant was diluted to 50 ml. 0.3 ml SLN suspension was diluted to 50 ml without centrifugation as a control. The absorbance at 500 nm was determined spectrophotometrically. Ke was calculated according to the following equation:

$$Ke = |A_0 - A| \times 100\%/A_0 \quad (5)$$

where A_0 is the absorbance of the dilutent before centrifugation and A is the absorbance of the dilutent after centrifugation. According to Stokes' sedimentation law, $v = 2r^2(\rho_1-\rho_2)g/9\ \eta$, the particle size of SLN has a great effect on the stability. SLNs with smaller particle size are more stable.

3.4. Particle size measurements and ζ-potential

The mean particle size of the samples was determined using Zetasizer 3000 (Malvern Instruments, Germany) with detection at a 90 degree angle. Samples were diluted with distilled water to the appropriate concentration. The ζ-potential was measured using a BDL-B (Coulter Electronics Inc., Shanghai) instrument with the following conditions: current 0.7 mV, frequency range 500 Hz, temperature 20 °C, fluid refractive index 1.33, dielectric constant 78.3, conductivity 16.7 ms/cm, on time 2.5 s, off time 0.5 s, and sample run time 60 s. Before measuring, each sample was diluted ten times with distilled water pH 7.0. The ζ-potential was calculated from the electrophoretic mobility using the Helmholtz equation:

$$\zeta = K_t u/E \quad (6)$$

where K_t is a constant related to temperature, U is the electrophoretic mobility, E is the potential gradient, E=V/L, where V is the voltage between two electrodes, L is the distance between two electrodes.

3.5. Determination of the incorporated amount of 5-Fu

A reversed phase HPLC method was used to assay 5-Fu content as reported previously (Zhang et al. 2000). Mobile phase: methanol-water (30 : 70), flow rate: 1.0 ml/min, detection wavelength: 265 nm. In the concentration range of 2.43–19.44 μg/ml a good correlation between peak area and concentration was established. The regression equation was as follows:

$$A = 40.253C + 27.06r = 0.9997\ (n = 7) \quad (7)$$

The average absolute and relative recoveries for three different concentrations were 99.14% (RSD = 2.2%) and 99.17% (RSD = 2.0%) respectively.

Free 5-Fu was removed from the SLN preparations by gel permeation chromatography using Sephadex G-50 (30 × 1.2 column). Preliminary calibration of the column was carried-out using SLN and 5-Fu, the recovery ratio for 5-Fu was 100.7 ± 1.3%. SLN preparations (0.3 ml) were applied to the column and eluted with HEPES buffer (1.0 ml/min). Fractions of 2 ml sample containing SLNs were diluted with methanol to 10 ml. 20 μl of the sample was injected into the HPLC column and the peak area of 5-Fu was recorded. The content of 5-Fu was calculated according to the calibration curve. The percentage of 5-Fu incorporated in the SLNs was calculated relative to the content of the drug in the SLN suspension before gel permeation chromatography.

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