*Drug Development and Industrial Pharmacy*, 2011, 37(2): 139–148

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI:10.3109/03639045.2010.497151

informa
healthcare

**ORIGINAL ARTICLE**

# The influence of lipid characteristics on the formation, in vitro release, and in vivo absorption of protein-loaded SLN prepared by the double emulsion process

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**Abstract**

*Purpose*: To study the influence of lipid characteristics on the formation, in vitro release, and in vivo absorption of solid lipid nanoparticles (SLN) prepared by the double emulsion method. *Methods*: Stearic acid (SA), octadecyl alcohol (OA), cetyl palmitate (CP), glyceryl monostearate (GM), glyceryl palmitostearate (GP), glyceryl tripalmitate (GT), and glyceryl behenate (GB) were selected as the representatives of different kinds of lipids, insulin and thymopentin (TP5) were selected as the model protein drugs. Before preparation, the contact angles between water and lipids were determined to investigate their hydrophobicity. The influence of lipid hydrophobicity or lipid solution viscosity on the preparation of primary emulsion, double emulsion, and SLN were studied by evaluating the particle size, state, and stability of the systems. CP-SLN, GT-SLN, and GP-SLN were selected to be loaded with insulin and TP5 for the in vitro release and in vivo absorption examination. After oral administration to diabetic rats, the pharmacological availability (PA) of insulin-CP-SLN, insulin-GP-SLN, and insulin-GT-SLN were determined. *Results*: The hydrophobicity order of the lipids was GM<GP<GT<GB<SA<OA<CP. SLNs could be prepared successfully by CP, GT, and GP, and their particle size was 447.5 ± 50.8, 444.8 ± 72.5, and 213.7 38.4 nm, respectively. All of the three SLNs exhibited burst release, and the percentage insulin released in 4 hours from these three SLNs were 76.37%, 45.36%, and 33.28%, respectively, and the corresponding TP5 release percentages were 75.72%, 56.89%, and 47.43%. Particle sizes increased significantly for CP-SLN and GP-SLN after a 24 hours release study in simulated gastrointestinal fluid. The PA of insulin-CP-SLN, insulin-GT-SLN, and insulin-GP-SLN were 2.92%, 3.44%, and 4.53%, respectively. *Conclusions*: This study suggested that GP with a suitable hydrophobicity, relatively lower burst release, and higher PA was the most promising lipid material of SLN for oral delivery of proteins.

**Key words:** Double emulsion, hydrophobicity, insulin, lipid, SLN, thymopentin

## Introduction

Solid lipid nanoparticle (SLN) is a widely used colloidal dispersion system for controlled release[1,2], topical drug delivery[3–5], gene delivery[6,7], and bioavailability improvement[8–10]. The application of SLN to oral protein delivery is a promising field and has drawn great attention in recent years. Different methods have been employed to prepare SLN, including emulsion solvent diffusion, micro-emulsification, and high-pressure homogenization. Obviously, the SLN production is based on solidified emulsion (dispersed phase) technologies. Therefore, because of their hydrophilic nature most proteins are expected to be poorly microencapsulated into the hydrophobic matrix of SLN[11].

To solve this problem, double emulsion and solvent-evaporation approach, which is a conventional method, for the preparation of PLGA nanoparticle[12–15] has been employed by some authors[16,17]. In this process, hydrophilic proteins are first dissolved in the inner water phase, whereas lipids are dissolved in organic solvents. The water phase is then dispersed in the organic phase to form a primary emulsion (w/o), and then this emulsion is dispersed further in the external water phase to form a double emulsion (w/o/w). Finally, the solvent is

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*(Received 22 Mar 2010; accepted 25 May 2010)*

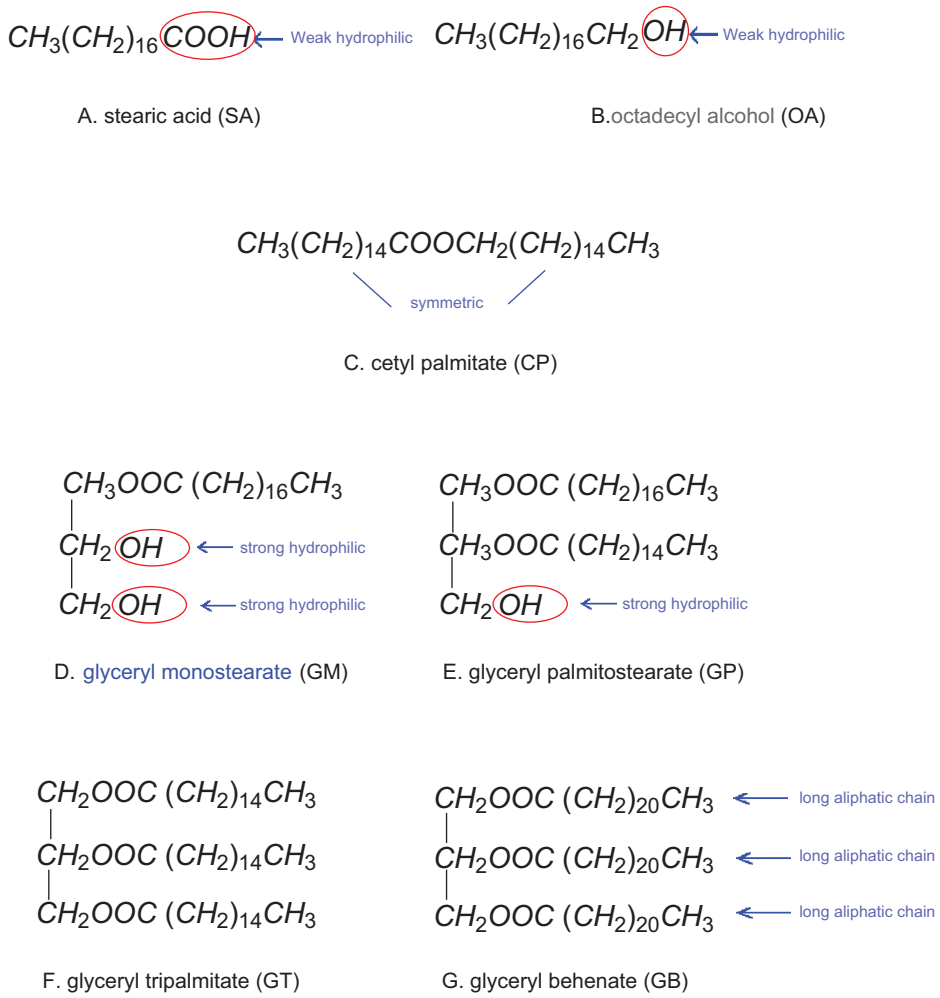


Figure 1. Structure formulas of lipids.

evaporated from the double emulsion and SLNs are obtained loaded with proteins.

During this process, the system is nonhomogeneous and changes from two phase (water-in-oil) to three phase (water-in-oil-in-water) and then into two phase (solid-in-water) again. The characteristic of lipid of the oil phase may have marked effects on the primary emulsion, double emulsion, and solvent-evaporation process. With the development of lipid materials, more choices are available for SLN. However, how the properties of lipids influence these processes has not been systematically investigated before, especially concerning the effect of the relative hydrophobicity of lipids. Similarly, the in vitro release and in vivo absorption of SLN made of different lipids have not yet been compared comprehensively. Therefore, the aim of this study was to investigate the influence of the lipid characteristics on the formation, in vitro release, and in vivo absorption of SLN prepared by the double emulsion method.

Seven commonly used lipids were selected for this research. Stearic acid (SA), octadecyl alcohol (OA), cetyl palmitate (CP), glyceryl monostearate (GM), and glyceryl palmitostearate (GP) were selected as the fatty acid, fatty alcohol, fatty acid ester, monoglyceride, and diglyceride, respectively. Glyceryl tripalmitate (GT) and glyceryl behenate (GB) were selected as triglycerides. The structures of the lipids were illustrated in Figure 1. Insulin and thymopentin (TP5) were selected as two model protein drugs.

## Materials and methods

### Materials

TP5 and insulin were bought from Shanghai Yihao Pharm (Shanghai, China) and Jiangsu Wanbang Pharm (Xuzhou, Jiangsu, China), respectively. SA, OA, and GM were bought from Tianjin Bodi Chemical (Tianjin, China); GP (PRECIROL® ATO 5) and GB (COMPRITOL® 888 ATO) were supplied by Gattefossé (Leon, France). CP and GT were bought from Alading Chemical (Shanghai, China). Phospholipid and solutol HS15 (PEG2000-steric) were supplied by Degussa and BASF (Ludwrigshafen, Germany), respectively. Other reagents were supplied by Yuwang Chemical (Yuzhou, China).

## Lipid characteristics

### *Lipid contact angles*

When the contact angle instrument was used, water climbed for only a little distance because of the strong lipophilicity of the lipids filled in the capillary and the experimental errors were too large to be evaluated. Therefore, the contact angle was tested by goniometry[18]. In brief, different lipids were heated to 80°C until melted and poured on to the surfaces of smooth glass plates. The plates were placed horizontally and cooled to room temperature. After the formation of lipid layers on the plates, water was dropped on to the surface of the lipid layers. Images of the interfaces were taken and the contact angles were measured by a goniometer.

The contact angle between dichloromethane (DCM) and water was also determined like the method explained above. Because the contact angle of DCM and water was not easy to be measured directly, DCM was dropped on the surface of ice. Water was frozen at –20°C for 12 hours to form a flat ice block and the surface of which was polished until smooth. Then, one DCM drop was dripped on to the surface of the ice block and the contact angle was determined.

### *Dissolving time of lipids in DCM*

Although most lipids have good solubility in DCM, their dissolving times are different which reflects the different miscibilities of the lipids with DCM. To determine this difference, the lipid dissolving time was examined. About 0.04 g lipid was dissolved in 1 mL DCM by shaking and the dissolving time was recorded. For GM and GB, 0.4 g lipid was dissolved in 10 mL DCM, the heating reflux was applied because they could not dissolve completely at room temperature.

### *The viscosity of lipid DCM solutions*

A sample of lipid (2 g) was dissolved in 50 mL DCM, and the viscosity of the lipid DCM solutions was determined with a viscosity meter (NDJ-8S, Shanghai Scientific Instrument, Shanghai, China).

## Preparation of SLN

### *Preparation of blank SLN*

SLN was prepared by the double emulsion combined with solvent-evaporation method. A sample of lipid (0.04 g) and lecithin (0.01 g) was dissolved in 0.5 mL DCM and then 0.2 mL water was added to form a primary emulsion (w/o) by the ultrasonic method at 400 W for 0.7 minutes. After the addition of 3 mL 2% solutol HS15, this system was further emulsified by ultrasonication at 400 W for 0.3 minutes to form a double emulsion (w/o/w). The double emulsion was then diluted with 7 mL 0.2% solutol HS15, and the organic solvent was removed using a rotary evaporator at 37°C for 0.5 hour.

### *Preparation of insulin-SLN and TP5-SLN*

The preparation of insulin-SLN and TP5-SLN was similar to the method used for the blank SLN apart from the fact that at the formation of the primary emulsion step, water was replaced by 0.5% insulin solution (0.01 M HCl) and 5% TP5 solution, respectively.

## Investigation of lipid performances at different stages

### *Primary emulsion stage*

The size of the primary emulsion droplets and state of the system were determined to evaluate the influence of lipids on the primary emulsion. The stability of primary emulsion was also evaluated in terms of the separation time following the placement of it in an ice bath.

### *Double emulsion stage*

The size of the droplets, system state, and separation time were also examined to evaluate the double emulsion as described above. In the preparation of GB double emulsion, gelatinization occurred, and a glass rod was dipped into the semi-solid double emulsion and the image was taken as shown in Figure 3. To study the mechanism of this gelatinization, the ultrasonic power was set at 400 W, the GB double emulsion was prepared again, and the system viscosity and practical power were recorded at different times.

### *Solvent-evaporation process*

On evaporating the solvent, the composition of the organic phase (lipid DCM solution) changed and the ratio of lipid to DCM (lipid concentration) increased gradually. The hydrophobicity of the organic phase depended increasingly on the lipid. To imitate the change in hydrophobicity during this process, DCM with the lipid concentrations of 4%, 8%, and 20% were dropped onto the surface of ice and the contact angles were determined.

## Particle size and zeta potential

The particle size and zeta potential were measured using a NICOMPTM 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). The mean particle size and distribution were measured by a photon correlation spectroscopy (dynamic light scattering), and the zeta potential was determined based on an electrophoretic light scattering technique.

## Entrapment efficiency determination

An ultrafiltration device with a molecular weight cut-off 10,000 was used to determine the entrapment efficiency (EE) of insulin-SLN and TP5-SLN. The EE was calculated by the following formula:

$$\mathrm{EE}\ (\%) = \frac{M_{\mathrm{total}} - M_{\mathrm{free}}}{M_{\mathrm{total}}} \times 100. \qquad (1)$$

The drug content in the ultrafiltration solution was determined by high-performance liquid chromatography (HPLC) method with the following conditions according to the United States Pharmacopeia (USP) and the literature[19]:

Insulin HPLC conditions: C18, 250 mm × 4 mm, 5 μm column; mobile phase=76% water phase (0.1 M sodium phosphate with the pH of 2.3) and 24% acetonitrile; flow rate = 1.0 mL/min; $\lambda = 214$ nm.

TP5 HPLC conditions: C18, 250 mm × 4 mm, 5 μm column; mobile phase = 93% PBS (0.02 M, pH 7.0) and 7% acetonitrile; flow rate =1.0 mL/min; $\lambda = 275$ nm.

### Particle morphology

Particle morphology was determined by transmission electron microscopy with a negative staining technique. Samples were diluted with water at the ratio of 1:3 and stained with 2% (w/v) phosphotungstic acid for 3 minutes and placed on a copper with film for observation. The operating voltage was 60 kV (TEM-1200 EX, Tokyo, Japan).

### In vitro drug release

Drug-loaded CP-SLN, GP-SLN, and GT-SLN were selected to test their in vitro drug release behavior. The experiments were performed as follows. SLN suspensions were subjected to ultracentrifugation (70,000 × *g*) for 2 hours at 4°C to remove the unloaded drugs. The final SLNs with 10 mg TP5 and 1 mg insulin were subjected to release testing, with shaking at 37°C and 100 rpm. The release time was 24 hours and the medium pH value was changed from 1.2 to 6.8 at 2 hours to simulate the in vivo condition. In brief, for insulin-SLNs, the first 2-hour release was performed in 7.5 mL 0.1 M HCl and then the pH of the medium was adjusted to 6.8 by the addition of 2.5 mL 0.2 M sodium phosphate for the next release test until 24 hours. Samples of the medium (0.2 mL) were withdrawn and replaced with fresh medium at 2, 4, 6, 10, 16, and 24 hours. The medium collected was diluted with 0.2 mL 0.1 M HCl and vortex-mixed for 10 minutes to wash off any insulin that might be absorbed on the surface of the SLN. The drug content in the release medium was determined by ultrafiltration (molecular cut-off 10,000).

Because TP5 was not stable in 0.1 M HCl, the release experiment was modified. TP5-SLN was divided into two groups. These two groups were dispersed in 7.5 mL 0.1 M HCl for 2-hour release. Then, for one group, the TP5 remaining in the SLN was determined, and the drug released was calculated by subtraction of the drug remaining. For the other group, 2.5 mL 0.2 M sodium phosphate was added to the release medium for further continuous release. Samples were collected as before except that they were diluted with water.

To evaluate the stability of SLN in a simulated gastrointestinal environment, the particle size and zeta potential of the SLN were tested after 24-hour release.

### In vivo evaluation

Insulin-loaded CP-SLN, GP-SLN, and GT-SLN were subjected to pharmacodynamical studies. Thirty streptozotocin-induced male diabetic Sprague–Dawley rats (200 ± 20 g, provided by Shenyang Pharmaceutical University) were fasted overnight and divided into five groups. Groups 1, 2, 3, and 4 were given insulin solution (50 IU/kg), insulin-CP-SLN (50 IU/kg), insulin-GP-SLN (50 IU/kg), and insulin-GT-SLN (50 IU/kg), respectively, by gavage. Group 5 was injected subcutaneously with insulin solution (2.5 IU/kg). Blood samples were taken from the orbital vein of the rats at 0, 1, 2, 4, 6, 8, 10, 12, 16, and 24 hours after administration. Plasma was separated by centrifugation at 8,000 × *g* rpm for 15 minutes and the plasma glucose level was determined by the glucose oxidase method (GOD kit, Beijing Bei Hua Kang Tai, Beijing, China). Plasma glucose levels were plotted against time. The pharmacological availability (PA) of different insulin-loaded SLNs was determined according to the following equation:

$$\mathrm{PA} = \frac{\mathrm{AAC}_{\mathrm{oral}} \times \mathrm{Dose}_{\mathrm{s.c.}}}{\mathrm{AAC}_{\mathrm{s.c}} \times \mathrm{Dose}_{\mathrm{oral}}} \times 100\%. \quad (2)$$

The area above the curve (AAC) below the 100% cut-off line was determined using the trapezoidal method.

### Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS software. The different formulations were compared by analysis of variance (ANOVA), and differences were considered significant for *P*-values <0.05 in the pharmacology study.

## Result and discussion

### Lipid characteristics

Lipids play an important role in the formation of SLNs and small differences in their characteristics may result in significant differences in the preparation process. The lipid should be very lipophilic to dissolve in the hydrophobic organic solvent completely; also, the degree of hydrophilicity is also needed to decrease the interfacial tension between the oil and water phases. To ensure the delivery of ultrasonic energy, the viscosity of the lipid solution should not be too high. Therefore, the lipid contact angle with water was measured as an indicator of lipid lipophilicity.

It can be seen from Figure 2 that the order of the contact angle with water was GM<DCM<GP<GT<GB<SA<OA<CP. There were significant differences between the contact angles of DCM with those of lipids ($P < 0.05$ for GM and $P < 0.01$ for other lipids) except GP (no significant difference). The contact angle is in direct proportion to

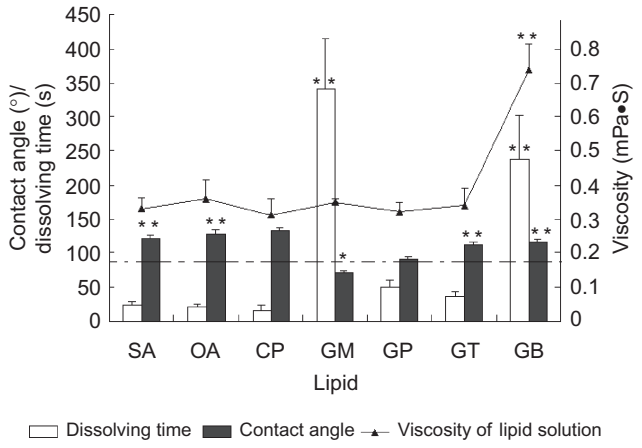


Figure 2. Characteristics of different lipids ($n$ = 3). (The dotted line was the value of DCM contact with ice *$P$ < 0.05, **$P$ < 0.0).

the hydrophobicity and, therefore, the hydrophilicity difference between lipids and DCM was of the order GP<GM<GT<GB<SA<OA<CP. The results were related to the structures of lipids. As shown in Figure 1, GM and GP had two and one glycerol hydroxyl group, respectively, which conferred a degree of hydrophilicity. So, the contact angles of GM and GP were relatively low. There is one carboxyl at the end of SA and a hydroxyl group at the end of OA; however, the hydrophilicity of these two lipids was still low and the contact angles were 121° and 127°, respectively. CP had the highest contact angle and a resulting high lipophilicity which may be due to its symmetric structure. All the lipid samples (0.04 g) dissolved in 1 mL DCM completely at different times except for GM, which could only dissolve after being heated because of its high hydrophilicity. In general, the dissolving time of glycerides was relatively longer than that of the other lipids.

After the addition of lipids, the viscosity of DCM increased slightly, suggesting that energy could be transferred easily to these systems. However, the viscosity of GB-DCM solution was increased significantly ($P$ < 0.01), which may be due to its relatively long aliphatic chains. Because of the relatively high viscosity, the dissolving time of GB in DCM was longer among the glycerides.

## Effects of lipid characteristics on the formation of SLNs

### *Primary emulsion stage*

The characteristics of the primary emulsion are very important for double emulsions and, therefore, the size of the droplets and separation time were investigated (Table 1).

All seven lipids formed yellow-white emulsion and the size of the droplets was below 100 nm except for GM, which had the drop size of 1554.8 ± 389.7 nm. Obviously, the drop size of double emulsion is larger than that of the primary emulsion and so it can be inferred that GM could not form a double emulsion at a nanoscale level. The large drop size of GM was because of its strong

Table 1. State, size of droplets, and separation time of the primary emulsion ($n$ = 3).

| Lipid | State | Size of droplets (nm) | Separation time (minutes) |
|---|---|---|---|
| SA | Yellow-white emulsion | 31.9 ± 19.1 | 4.4 ± 0.9 |
| OA | Yellow-white emulsion | 50.9 ± 10.6 | 3.5 ± 0.7 |
| CP | Yellow-white emulsion | — | <1 |
| GM | Turbid | 1554.8 ± 389.7 | 1.2 ± 0.4 |
| GP | Yellow-white emulsion | 56.4 ± 18.4 | >60 |
| GT | Yellow-white emulsion | 64.4 ± 38.8 | 10.5 ± 2.4 |
| GB | Yellow-white emulsion | 75.86 ± 39.40 | 15.1 ± 3.1 |

hydrophilicity and low solubility in DCM. As GM could not dissolve completely in DCM at room temperature, it tended to escape from DCM while its hydrophilicity was not strong enough to help it dissolve in water. As a result, GM was unable to exist stably in the water–oil system and so a disturbance was created at the water–oil interface that led to coalescence of the liquid drops. CP was strongly lipophilic and, after adding it, the hydrophobicity of DCM and the interfacial tension increased. This led to instability of the CP primary emulsion and layering took place during the particle size testing.

The separation time of different primary emulsions had an opposite order to the hydrophilicity differences between lipids and DCM except for GM and GB. GP emulsion was the most stable and did not separate within 1 hour. Although the hydrophobicity difference was greater than that of GT, the GB emulsion was much more stable than GT, because the GB emulsion had a relatively viscous dispersed phase, which slowed the separation of the system to 15 minutes.

### *Double emulsion stage*

During the preparation of the double emulsion, four different phenomena were observed as follows (Table 2) :

1. SA, CP, GP, and GT formed a white emulsion with a droplet size of a few hundred nanometers;
2. OA could still form a white emulsion while the drop size was about 2.5 μm;
3. GM double emulsion was a turbid system and a heavy precipitate was suspended in it;

Table 2. State, size of droplets, and separation time of the double emulsion ($n$ = 3).

| Lipid | State | Size of droplets (nm) | separation time (minutes) |
|---|---|---|---|
| SA | White emulsion | 476.7 ± 47.9 | 9.2 ± 2.8 |
| OA | White emulsion | 2651.8 ± 203.5 | 26.5 ± 3.6 |
| CP | White emulsion | 334.7 ± 13.7 | 4.9 ± 1.3 |
| GM | Turbidity | >10,000 | 40.2 ± 5.7 |
| GP | White emulsion | 366.4 ± 38.4 | 53.2 ± 4.0 |
| GT | White emulsion | 224.2 ± 22.9 | 14.5 ± 4.1 |
| GB | White semi-solid | Gelatinizing | >60 |

4. GB did not form a double emulsion. After addition of the external water phase, a primary emulsion settled at the bottom of the ultrasonic tube. On ultrasonication, the oil–water interface was disturbed and the external water phase entered the primary emulsion slowly. After 0.2 minute, the system changed from a liquid to a viscous semi-solid which could not flow even if the ultrasonic tube was inverted, and this was called gelatinizing here.

It can be seen from Figure 3 that this system was very sticky so that only violent shaking could produce drops from it. This phenomenon may be due to the viscosity of GB primary emulsion. When it was mixed with the external water phase, the viscosity prevented the delivery of crushing energy and the strong blasting effect generated by ultrasonication was converted to a weak vibration which did not provide enough energy for the organic phase to be removed from the bulk to form single drops in the external water phase. Therefore, the system became an interpenetrating network and the viscosity increased greatly.

To prove this speculation, the practical ultrasonic power and system viscosity were tested when 400 W ultrasonication was used to prepare the double emulsion. As can be seen from Figure 4, the practical ultrasonic power decreased with the increase in the system viscosity during this process, indicating that the ultrasonic power was attenuated in the presence of a high viscosity.

SA, CP, GP, and GT were selected for the next experiment.

### *Solvent-evaporation process*

As can be seen in Figure 5, there was no significant discrepancy between the contact angles of different lipid solutions at 4% concentration; this was because of the

Figure 3. A glass rod was dipped into the gelatinized GB emulsion and shaken violently.

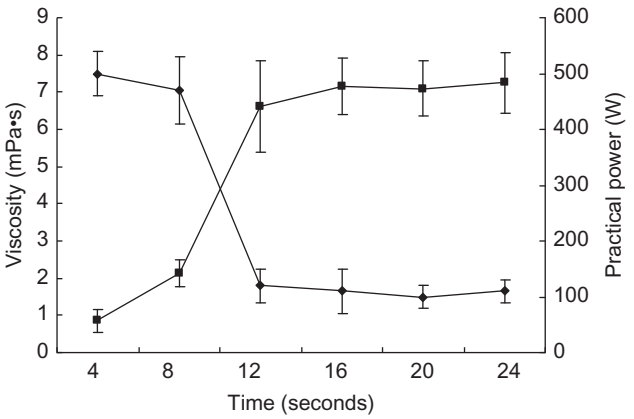


Figure 4. Viscosity and practical power changes during the double emulsion preparation process using ultrasonication at 400 W for GB (■, system viscosity; ◆, practical power; $n = 3$).

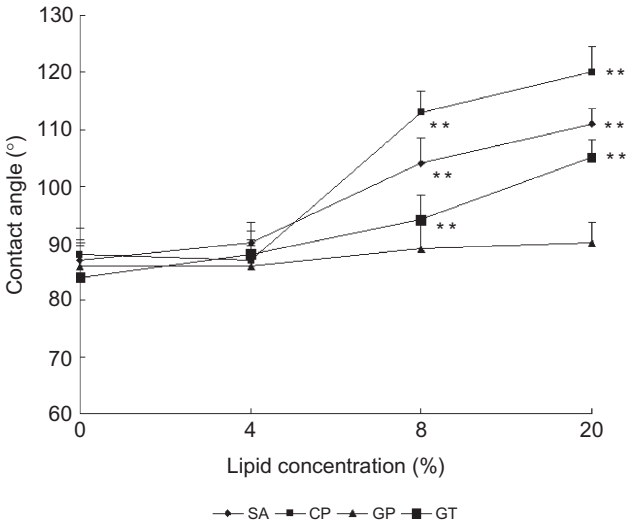


Figure 5. Contact angles of lipid DCM solutions with different concentrations (**$P < 0.01$, $n = 3$).

fact that this concentration was too low to alter the whole hydrophobicity of the organic phase. As the DCM volume declines, various changes appeared based on the hydrophobicity of the lipids. The contact angle of the GP solution did not change much because GP had the similar contact angle with DCM. As far as GT, CP, and SA at a concentration of 20% were concerned, the contact angle increased to 105°, 111°, and 120°, respectively, indicating the increase in hydrophobicity of the lipid solutions during the evaporation process. The surfactants used to stabilize the interface of DCM solution and external water in the double emulsion stage might not be enough to stabilize this interface during the DCM evaporation process, so aggregation could take place because of the interfacial tension. This prediction was proved by the high number of aggregates which formed during the solvent-evaporation process of SA. However, the increased hydrophobicity in the CP or GT system did not

result in the production of aggregates. Therefore, GP, CP, and GT were selected for subsequent study.

### *Characteristics of SLNs*

After evaporation, the double emulsions changed into nattier blue colloidal systems. The particle sizes and zeta potentials of the SLNs were listed in Table 3. GP-SLN had the smallest particle size of 216.8 ± 30.9 nm. The morphology of GP-SLN was investigated by TEM. From Figure 6, it can be seen that GP-SLNs were spherical with a particle size of about 200 nm and no liposome-like structure was found in this system, which implied that the phospholipid was coated as the surfactant on the surface of SLN to reduce the interfacial tension.

When the particle sizes of different double emulsions were compared with those of the corresponding SLNs, it was found that SLNs were larger than the double emulsion drops for CP and GT.

Generally speaking, there are two ways for the double emulsion drops to evaporate, one is the pure evaporation and the other is evaporation accompanied by coalescence (Figure 7). These two ways may result in different changes in particle sizes before and after evaporation. In the pure evaporation process, the difference between emulsion drops and SLNs was the particle composition. The former were a lipid DCM solution, whereas the latter were lipid crystals. To compare the size of these two particles, DCM solutions of different lipids were evaporated in vacuum and all the liquid crystals were smaller than those in the liquid solutions, indicating that the particle size of SLNs was smaller than emulsion drops in the pure evaporation process. However, SLNs had been coalesced and combined during the evaporation accompanied by coalescence process and the final size could be higher than that of the liquid drops.

Table 3. EE, particle size, and zeta potential of SLNs ($n = 3$).

| Lipid | Particle size (nm) | Zeta potential (mV) | EE (%) | |
|---|---|---|---|---|
| | | | Insulin | TP5 |
| CP | 440.9 ± 79.2 | −11.79 ± 0.88 | 16.74 ± 5.27 | 8.67 ± 2.71 |
| GP | 216.8 ± 30.9 | −14.74 ± 1.10 | 34.23 ± 3.19 | 22.77 ± 4.25 |
| GT | 445.6 ± 71.0 | −16.63 ± 1.24 | 25.37 ± 3.77 | 15.86 ± 3.19 |

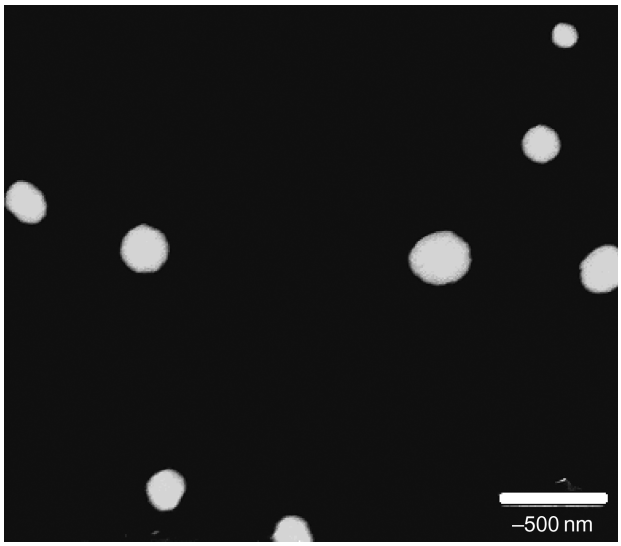


Figure 6. TEM image of SLN prepared with GP.

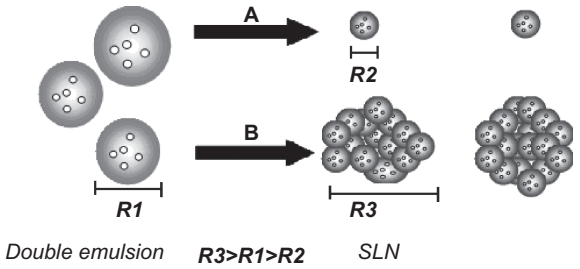


Figure 7. Two evaporation mechanisms: (a) pure evaporation and (b) evaporation accompanied by coalescence.

Therefore, it could be concluded that coalescence took place during the evaporation process of CP and GT. This result was consistent with the increased hydrophobicity of CP and GT organic phase as discussed before. Although no evident aggregation could be observed during the DCM evaporation process, surfactants could not satisfy the increased interfacial tensions of the CP and GT systems and, finally, particles accumulated. For the GP system, no dramatic change in hydrophobicity took place during the formation of SLN and the particles did not aggregate.

SLNs of CP, GP, and PT had a similar zeta potential of −15 mV approximately, indicating that the charge comes mainly from the phospholipid coating at the surface of the SLNs.

Insulin and TP5 were loaded into CP-SLN, GP-SLN, and GT-SLN, respectively. The particle size, zeta potential and EE were presented in Figures 8 and 9 and Table 3. Compared with TP5-SLNs, insulin-SLNs had a relatively higher EE, a larger particle size, and a lower zeta potential. This result was because of their different solubilities in water. Although both proteins were

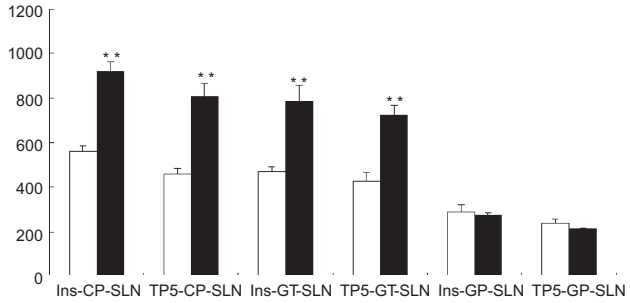


Figure 8. Particle sizes of insulin (Ins) or TP5-loaded CP-SLN, GT-SLN, and GP-SLN and the size changes after 24-hour release in simulated gastrointestinal fluid (□, particle size before release; ■ particle size after release; $^{**}P < 0.01$; $n = 3$).

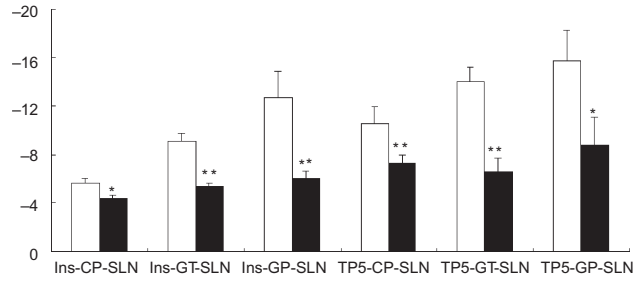


Figure 9. Zeta potentials of insulin (Ins) or TP5-loaded CP-SLN, GT-SLN, and GP-SLN and the size changes after 24-hour release in simulated gastrointestinal fluid (□, zeta potential before release; ■, zeta potential after release; $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$).

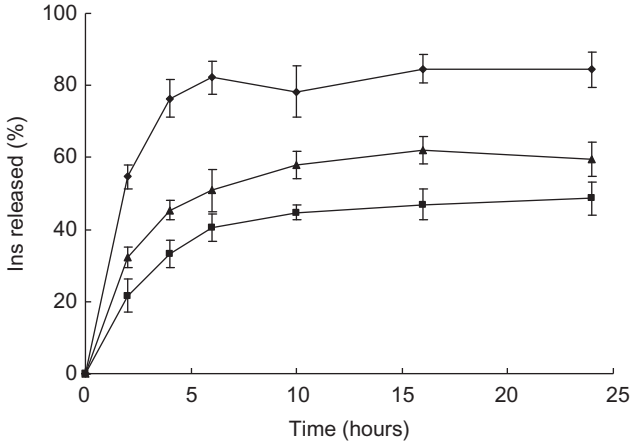


Figure 10. Release profiles of insulin (Ins) from different SLNs in simulated gastrointestinal fluid (◆, Ins-CP-SLN; ▲, Ins-GT-SLN; ■, Ins-GP-SLN, $n = 6$).

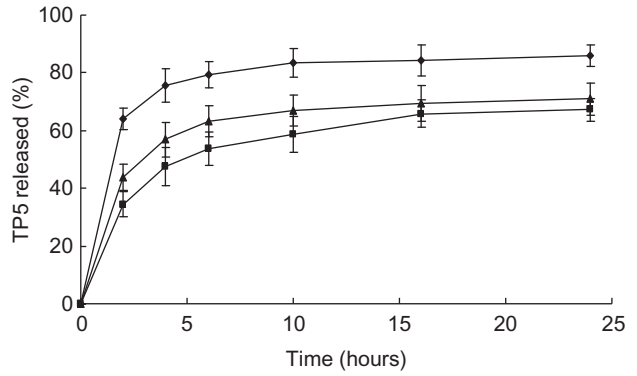


Figure 11. Release profiles of TP5 from different SLNs in simulated gastrointestinal fluid (◆, Ins-CP-SLN; ▲, Ins-GT-SLN; ■, Ins-GP-SLN, $n = 6$).

hydrophilic, TP5 had a much higher solubility of more than 1 g in 1 mL of water, whereas insulin had relatively lower solubility in water and 0.01 M HCl was used to dissolve it completely. During the ultrasonication and solvent-evaporation process, TP5 leaked more easily into the external water phase resulting in a lower EE. During the preparation of insulin-SLN, a fraction of the HCl was transferred to the particle's surface and the charge coming from the phospholipid was partly neutralized, as can be seen from the relatively lower zeta potential of insulin-SLNs compared with the corresponding TP5-SLN containing the same lipids. Although the EE values were different, the EE order of the drugs was the same, that is, CT<GT<GP.

## In vitro release

Because of the absorption properties of protein, the drug released into the medium may be reabsorbed on the surfactant layer of SLN. To avoid this, the release mediums for testing were diluted and vortex washing was carried out. In the insulin release study, SLNs exhibited burst release to different extents and sustained-release at the later stage (Figures 10 and 11). After 4-hour release, the percentage of drug released were 76.37%, 45.36%, and 33.28% for insulin-CP-SLN, insulin-GT-SLN, and insulin-GP-SLN, respectively. The sequence of burst release was insulin-CP-SLN>Insulin-GT-SLN>Insulin-GP-SLN. Similar release profiles were obtained for TP5-SLNs. The corresponding release percentages of TP5 at 4 hours were 75.72%, 56.89%, and 47.43% for TP5-CP-SLN, TP5-GT-SLN, and TP5-GP-SLN, respectively. The sequence of burst release for the three TP5-SLNs was the same as that for insulin-loaded SLNs.

SLN is used as a promising protein and peptide oral delivery system because it can protect drugs from the degradation caused by gastric acid and protease enzymes in the gastrointestinal tract and the drugs can then be absorbed by endocytosis of M cells in Payer's patches. As a common phenomenon, proteins will be released from SLN before they reach the absorption region and only the drug which is still at the inside of the preparation can be absorbed with SLN. Therefore, burst release is a serious problem for this process. In SLNs prepared by the double emulsion method, hydrophilic protein is distributed partly on the surface of particles and burst release is easy. The percentage release was related to the lipid hydrophilicity. GP had a relatively lower hydrophilicity and was compatible with proteins to some extent. So, very little protein migrated on the surface of GP-SLN during the evaporation process during SLN formation. However, a great deal of protein can distribute to the outer region of CP-SLN, leading to marked burst release because of the higher lipid hydrophilicity. This explanation can be proved by the zeta potential results in Figure 9. Insulin is very soluble in 0.01 M HCl and would move together with HCl inside SLN or on the surface of SLN. If HCl migrated to the particle surface, its charge was reduced. Therefore, the zeta potential is an index of the fraction of insulin distribution in SLN. It can be inferred that the surface–drug ratio had consequent order of CP>GT>GP.

As can be seen from Figures 8 and 9, GP-SLN was stable in a simulated pH gradient, the electrolyte

concentration used and the temperature in the drug release test, and the particle size was reduced slightly ($P > 0.05$) because of the insignificant erosion of the lipid material. As for CP-SLN and GT-SLN, the particle size increased greatly ($P < 0.01$) to about 800 nm, indicating that marked aggregation took place during long-time contact with simulated gastrointestinal fluid. The zeta potentials of the three SLNs were all reduced markedly ($P<0.05$ or 0.01) to about −6 mV. Interface stabilizing of SLNs was produced by solutol HS15 and phospholipid, the former was a nonionic polymer surfactant, whereas the latter was a zwitterionic surfactant. The interface stabilizing mechanism of solutol HS15 involved space repulsion which would not be influenced by the pH gradient and the electrolyte concentration in simulated gastrointestinal fluid. The stabilizing mechanism of phospholipid was electrostatic repulsion and this mechanism was destroyed by the low pH and high ion concentration in the experiment as can be seen from the decreasing zeta potential. So, it is reasonable to infer that the stability of GP-SLN was less dependent on the phospholipid because GP was less hydrophobic compared with CP and GT.

### In vivo evaluation

To investigate the influence of lipid characteristics on the absorption of SLN, insulin-CP-SLN, insulin-GT-SLN, and insulin-GP-SLN were administrated orally to diabetic rats. Blood glucose levels and PAs were determined to evaluate the absorption of these three insulin preparations. As can be seen from Figure 12, blood glucose levels decreased after the administration of SLNs. The PAs of insulin-CP-SLN, insulin-GT-SLN, and insulin-GP-SLN were 2.92%, 3.44%, and 4.53%, respectively.

There are two factors determining the PA of insulin, one is the absorption of SLN itself, the other is the residual drugs in SLN before the preparation reaches the absorption sites. It has been reported that the particle size, zeta potential, and hydrophobicity influence the absorption of nanoparticles in the gastrointestinal tract[20]. Particles with a small size, positive charge, and suitable hydrophobicity will be absorbed extensively in the gastrointestinal tract.

All three SLNs were negatively charged and the effect of charge can be neglected. As discussed before, the particle size of GT-SLN and CP-SLN increased after long-time contact with a simulated gastrointestinal environment. The differences in PA values were because of the complex effects of drug burst release, particle size, and hydrophobicity. Compared with GT-SLN and CP-SLN, although GP-SLN had a relatively lower hydrophobicity, its smaller particle size and lower bust release promoted the absorption of insulin.

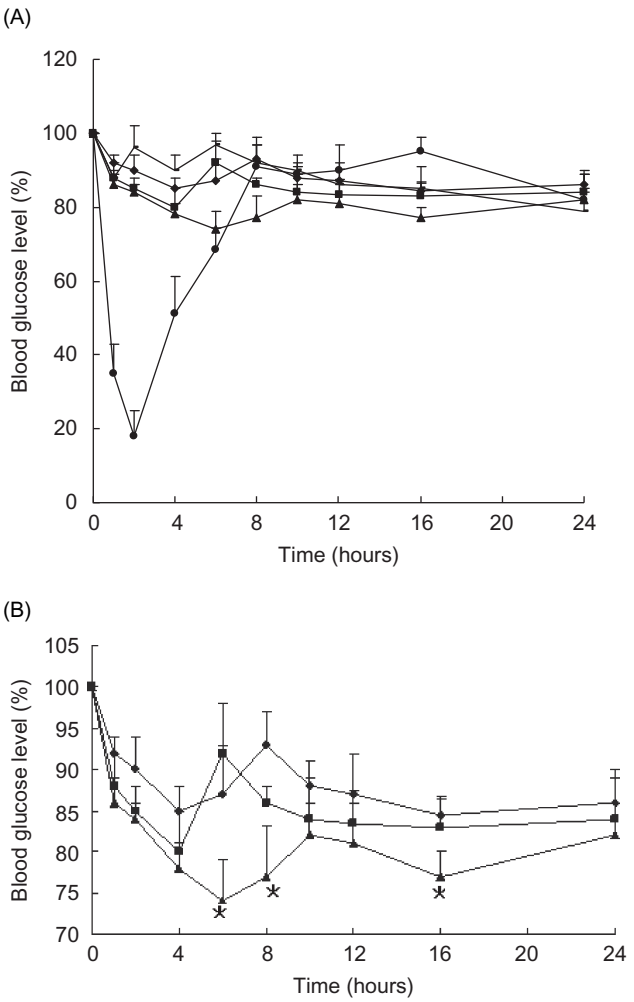


Figure 12. Percentage reduction in plasma glucose concentration in diabetic rats after oral administration of insulin-CP-SLN (◆), insulin-GT-SLN (■), insulin-GP-SLN (▲) 50 IU/kg, insulin solution (-) 50 IU/kg, subcutaneous injection of insulin(●) 2.5 IU/kg ($n = 6$). (a) The full diagram; (b) diagram locally enlarged for insulin-CP-SLN (◆), insulin-GT-SLN (■), insulin-GP-SLN (▲). (*) Statistically significant differences from blood glucose level of GT-SLN ($P < 0.05$).

## Conclusion

The influence of lipid characteristics on the formation, in vitro drug release, and in vivo absorption of SLN prepared by the double emulsion process was investigated in this research. The viscosity of the lipid solution influenced the transfer of ultrasonic energy and, therefore, double emulsion formation. The difference in hydrophobicity between lipid and solvent had significant effects on the formation of the primary emulsion, double emulsion, and the final evaporation process. The system described above would be unstable if the difference is too tremendous. CP, GT, and GP can be used to prepare SLNs and the particle sizes were 447.5 ± 50.8, 213.7 ± 38.4, and 444.8 ± 72.5 nm, respectively. Besides, hydrophobicity influences the distribution of proteins in SLN. The relatively high hydrophobicity of CP resulted in high burst release of more than 70% for insulin and TP5 within 4 hours. CP-SLN and GT-SLN were not stable in simulated gastrointestinal fluid and the particle size increased markedly. In the in vivo study, the PA of

insulin-CP-SLN, insulin-GT-SLN, and insulin-GP-SLN were 2.92%, 3.44%, and 4.53%, respectively. GP-SLN demonstrated lower burst release, and a stable particle size, together with a relatively high PA. In general, the hydrophobicity of the lipid played an important role in the preparation, release, and absorption of SLN. GP was the most promising lipid material for the preparation, in vitro release, and in vivo drug absorption because of its appropriate lipid characteristics.

## Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of this paper.

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