Preparation of Gel-Core-Solid Lipid Nanoparticle: A Novel Way to Improve the Encapsulation of Protein and Peptide

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Using thymopentin and insulin as the model protein–drugs, novel Gel-Core-solid lipid nanoparticle (SLN) with the hydrogel core and lipid shell were prepared by double emulsion and thermal sensitive gel technology, with the intention to improve the entrapment efficiency. Pluronic F127 and Glyceryl palmitostearate were selected as hydrogel material and lipid material, respectively. The particle sizes and zeta-potential were characterized by dynamic light scattering and electrophoretic light scattering. Transmission electron microscopy (TEM) was employed to investigate the structure of this Gel-Core-SLN. The Gel-Core-SLN was successfully prepared and the particle size was 305.2 nm with zeta potential of −17.15 mV. Observations by TEM confirmed that most solidified hydrogel particles were dispersed in the central of Gel-Core-SLN as a form of single core, which effectively prevented the diffusion of proteins to the external water phase during preparation process. The entrapment efficiency of thymopentin-loaded Gel-Core-SLN and insulin-loaded Gel-Core-SLN were 61.97% and 57.36%, respectively. Both the two drug-loaded Gel-Core-SLNs showed relatively low burst release. The pharmacological availability of insulin-loaded Gel-Core-SLN was 6.02%. It was suggested that the Gel-Core-SLN could be a promising drug delivery system with the outstanding encapsulation efficiency, low burst release and relatively high pharmacological availability.

Key words solid lipid nanoparticle; thermal sensitive gel; double emulsion; entrapment efficiency

It is well known that the bioavailability of protein and peptide drugs after oral administration is very low because of their instability in the gastrointestinal tract and low permeability through the intestinal mucosa.[1)] Lots of methods have been employed to solve this problem, including Chemical modification[2)] addition of enzyme inhibitor[3,4)] or absorption enhancer,[4,5)] conjugation with receptor-recognizable ligand,[6—8)] using particulate delivery carrier systems,[9,10)] and so on. Nanoparticle is the most commonly used method because it can protect drug from the attack of gastric acid or pancreatic enzyme and then promote drug absorbed by endocytosis[11)] or other mechanisms.

Solid lipid nanoparticle (SLN), composed of physiological compatible lipid, has been used successfully to improve the bioavailability[12—14)] of protein and peptide. Given that SLN has solid structure with strong hydrophobicity, gastric acid and protease enzyme can not penetrate into it and the drug loaded is protected greatly. Compared with poly(latic-co-glycolic acid) (PLGA) nanoparticle, the degradation products of SLN are weaker acids which have little effect on the stability of protein and peptides. However, due to their hydrophilic nature most proteins are poorly encapsulated into the hydrophobic matrix of SLN, tending to partition in the water phase during the preparation process.[15)] Therefore, the drug loading capacities are extremely low.

This research aimed to develop protein loaded SLN with high entrapment efficiency (EE). For this purpose, hydrogel is selected as carrier for protein, due to its hydrophilic nature, to achieve high entrapment of protein. Moreover, it has been reported that hydrogen bonds are formed between hydrogel and protein. If hydrogel is implanted into SLN, its compatibility with protein can be utilized to improve the EE of SLN. But, how to implant the hydrogel? In fact, hydrogel has been embodied into liposome through various ways to improve its stability.[16)] In brief, the sol was firstly incorporated into liposome during its preparation. And then radiation, ion and pH value have been utilized to change the sol into gel. However, these methods cannot be applied to implant hydrogel into protein loaded SLN because they will lead the degradation of protein and hence mild method is need to be developed. This

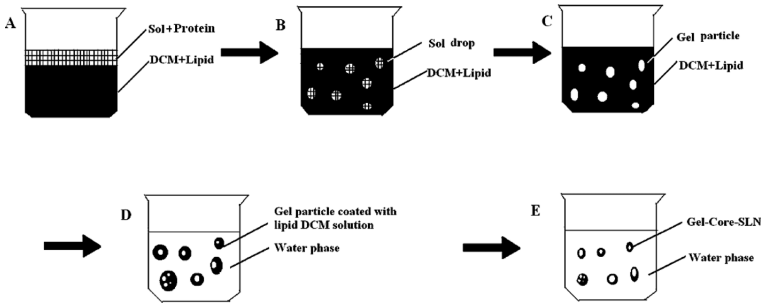

Fig. 1. Preparation Procedure of Gel-Core-SLN

(A) Water phase containing thermal–sensitive hydrogel material and peptide was added to oil phase containing lipid. The upper layer was water phase while the under layer was oil phase; (B) after the ultrasonic in ice bath, primary emulsion was formed. The disperse phase was sol drop; (C) sol drop changed into gel particle as temperature increased; (D) the gel-in-oil system was added to external water phase and was supersonic again to achieve solid gel-in-oil-in-water system; (E) after evaporating the oil, lipid deposited at the surface of gel particle and the Gel-Core-SLN was then formed.

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implanting process likely can be realized by the combination of sol–gel technique and double emulsion method.

Based on the consideration above, the preparation process of this SLN with a hydrogel core and lipid shell are hypothesized as follows: firstly, the protein is dissolved into liquid phase which contains hydrogel materials and this solution is emulsified to the oil phase which contains lipid; secondly based on the thermosensitive nature of hydrogel, this water/oil emulsion is transformed to a gel/oil with a solid core; then, external water phase is added and a gel/oil/water system is formed with ultrasonic technique; finally, organic solvent is evaporated and the gel-in-lipid structure is formed. This preparation process is illustrated with Fig. 1. With this structure, protein is expected to exist in gel core with high EE and be protected by the lipid shell. This Gel-Core-SLN system combines the beneficial properties of hydrogel and SLN for oral protein delivery.

The objectives of the present work were: (i) to investigate the preparation feasibility of Gel-Core-SLN based on the study of formulation and preparation parameters (ii) to examine the EE and morphology of the Gel-Core-SLN. (iii) to evaluate its potential advantages *in vitro* and *in vivo*. Therefore thymopentin and insulin were selected as the model drugs of peptide and protein. Different hydrogels, lipids and preparation conditions were studied in details.

Experimental

Materials Thymopentin (TP) and insulin (INS) was bought from Shanghai Yihao Pharm (China) and Jiangsu Wanbang Pharm (China) respectively. Pluronic F127 and Solutol HS 15 (Polyglycol ester of 12-hydroxystearic acid) was kindly provided by BASF (BASF Shanghai, China). Glyceryl palmitostearate (PRECIROL® ATO 5) was supplied by Gaffasi Company (Gaffasi Shanghai, China). polyethylene glycol (PEG)-PLGA-PEG was provided by Shenyang Pharmaceutical University (China). Cetyl palmitate, Glyceryl tripalmitate, Chitosan (Mw 20000), Hydroxypropyl cellulose (HPC LF PH), Hydroxypropyl methyl cellulose (HPMC K15M) were obtained from Alading chemical (China), Jinke Chemical (China), Ashland Aqualon Functional Ingredients (U.S.A.) and Colorcon Company (China) respectively.

Methods. Preparation of Gel-Core-SLN and Conventional SLN Gel-Core-SLNs with different lipid materials were prepared by the combination of double emulsion and solvent-evaporation method and thermal–sensitive hydrogel technique. In brief, 5% TP or 0.5% INS was dissolved in 45% Pluronic F127 aqueous solution or 0.01 M HCl solution respectively at room temperature. 0.2 ml of this solution was dropped into 1 ml dichloromethane (DCM) which contained 0.04 g lipid and 0.01 g phospholipid and a dual solvent system was formed. This system was ultrasoniced for 1.0 min at 500 W to make water-in-oil drops, and then additional 0.5 min ultrasonic was utilized to improve the temperature of the inner water by the ultrasonic heat effect. When temperature reached the sol–gel transition point, water drop changed into solid gel, and this system can be called as solid gel-in-oil. After this change, 3 ml 2% Solutol HS 15 (Polyglycol ester of 12-hydroxystearic acid) solution (preheated to 35 °C) was added to the solid gel-in-oil system and 0.3 min 600 W ultrasonic was performed to prepare the solid gel-in-oil-in-water system. This solution was diluted with 7 ml 0.2% Solutol HS 15 solution (preheated to 35 °C) and then rotary evaporated at 35 °C. After the evaporation of dichloromethane, lipid deposited as solid lipid nanoparticle with gel core and the solid gel-in-lipid-in-water nano-dispersion colloid system was finally formed. Conventional SLNs were prepared by the same procedure except that there was no Pluronic F127 the inner water phase.

Selection of Gel Material To select the gel material, four commonly used reverse phase thermal-sensitive gels *i.e.* Pluronic F127, chitosan, PEG-PLGA-PEG, HPC, and HPMC were prepared as follows.

Pluronic F127, PEG-PLGA-PEG, HPC, and HPMC were dissolved in water at 4 °C with the concentration of 45%, 20%, 20%, and 10% respectively. Chitosan of 0.08 g was dissolved in 4 ml 0.1 M HCl, glycerophosphate of 1.4 g was disolved in 2 ml water and then the glycerophosphate solution was added to chitosan solution. All of the gel solutions were heated at 35 °C in water bath to form solid gel. The gel morphology and gelation time were studied.

To select the concentration, Pluronic F127 solutions of 10%, 20%, 30% and 45% were heated at 35 °C and the gel flowability was observed by inverting the test tube.

Selection of Lipid Glyceryl palmitostearate, glyceryl tripalmitate, and cetyl palmitate were utilized as the lipid materials to prepare Gel-Core-SLN. The particle size, zeta potential and entrapment efficiency were tested.

To determine the hydrophilic of different lipid, Nuclear Magnetic Resonance (NMR) technique (300 MHz, Bruker, German) was carried out and the proportion of hydrophilic protons in the total number of protons (R) was calculated according the following equation[17]:

$$R = \frac{\sum H_{(W)}}{\sum H_{(W)} + \sum H_{(O)}} \quad (1)$$

$\sum H_{(W)}$ is the height sum of hydrophilic protons, $\sum H_{(O)}$ is the height sum of lipophilic protons.

Particle Size and Zeta Potential The particle size and zeta potential of Gel-Core-SLN were measured using a ZetaPotential/Particle Sizer (NICOMPTM 380, U.S.A.). The mean particle size and distribution were measured based on photon correlation spectroscopy (dynamic light scattering, DLS) technique. The zeta potential was determined based on an electrophoretic light scattering (ELS) technique.

Entrapment Efficiency Ultrafiltration method was used to evaluate the entrapment efficiency (EE). The suspension of Gel-Core-SLN or conventional SLN was added into a 0.5 ml ultrafiltration device tube (100000 cut off MW, Millipore), and then centrifuged for 5 min at 2000 rpm, the drug content of the filtrate was determined by HPLC.

The EE was calculated by the following equation:

$$EE\ (\%) = \frac{M_{total} - M_{free}}{M_{total}} \times 100 \quad (2)$$

Where: M_{total} is the mass of total drug, M_{free} is the mass of free drug.

HPLC condition for TP was: C18, 250 mm×4 mm, 5 μm column; mobile phase=93% phosphate buffer solution (PBS) (0.02 M, pH 7.0) and 7% acetonitrile; flow rate=1.0 ml/min; λ=275 nm. HPLC condition for INS was: C18, 250 mm×4 mm, 5 μm column; mobile phase=74% water phase (0.2 M sodium phosphate with the pH of 2.3) and 26% acetonitrile; flow rate=1.0 ml/min; λ=214 nm.

Transmission Electron Microscopy (TEM) Tecnai G2 F30 TEM (Philips-FEI, Holland) with resolution of 0.2 nm was employed to study the structure of nanoparticle. The working voltage was 300 kV and all tests were operated at bright field. For imperfect Gel-Core-SLN, TEM were tested immediately after they were prepared to avoid the dissolution of Pluronic F127.

Stabilities of Gel-Core-SLNs The Gel-Core-SLN suspensions were subjected to ultracentrifugation for 2 h at 4 °C to remove the unloaded drugs. After the addition of water containing 7.5% trehalose and 2.5% mannitol, Gel-Core-SLN was lyophilized. After 6 months storage at 4 °C, the particle size, EE and *in vitro* drug release were determined to evaluate its stability.

Stabilities of Proteins The preparation-stability and 6-months-stability of TP and INS were evaluated by HPLC method and mouse blood glucose method respectively. For TP, the Gel-Core-SLN was dissolved in water-DCM (2/1, v/v) solution, then vortex mixed for 5 min to dissolve the lipid and extract the TP into water. After centrifugalization, the supernatant was examined by HPLC.

INS for activity test was extracted from Gel-Core-SLN as the same process. Forty mice (18—22 g, provided by Shenyang Pharmaceutical University) were divided into four groups. The following INS solutions were administrated to mouse (group1 to group 4): 0.05 IU INS standard solution, 0.1 IU INS standard solution, 0.05 IU INS solution from Gel-Core-SLN, 0.1 U INS solution from Gel-Core-SLN. Blood samples were taken from the orbital of mouse at 40 min after administration. Plasma was separated by centrifugation at 6000 rpm for 15 min and the plasma glucose level was determined by glucose oxidase method (GOD kit, Beijing Bei Hua Kang Tai, China). After 4 h, INS solutions were cross administrated to the mouse as following: from group1 to group 4, 0.1 IU INS standard solution, 0.05 IU INS standard solution, 0.1 IU INS solution from Gel-Core-SLN, 0.05 IU INS solution from Gel-Core-SLN. The plasma glucose level was determined at 40 min after administration. Quantitative response parallel line assay was utilized to determine the potency of INS.

***In Vitro* Drug Release** The *in vitro* drug release behavior of TP-Gel-

Table 1. The Gel Morphology and Gelation Time of Different Gel Materials (n=3)

Gel material	Pluronic F127	Chitosan	PEG-PLGA-PEG	HPC	HPMC
Gel state	Transparent solid	Solid surrounded by water	Transparent solid	White solid	Solid with expansion
Gelation time (min)	1.2±0.3	2.3±0.4	4.3±0.7	5.6±0.6	3.5±0.4

Core-SLN and INS-Gel-Core-SLN were studied and compared with the conventional TP-SLN and INS-SLN. The experiments were performed as follows. SLN freeze-dried powders with 10 mg TP5 or 1 mg INS was subjected to release testing (in sink condition) with shaking at 37 °C and 100 rpm. The release time was 24 h and the medium pH value was changed from 1.2 to 6.8 at 2 h to simulate the *in vivo* condition. In brief, for INS-SLNs, the first 2 h 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 h. 0.2 ml medium was withdrawn at 2, 4, 6, 10, 16, 24 h and fresh medium was added. The medium fetched was diluted with 0.2 ml 0.1 M HCl and vortex mixed for 10 min to wash off any INS that might be absorbed on the surface of SLN. The drug content in the release medium was determined by the ultrafiltration method mentioned above.

It has been reported that TP5 was unstable in 0.1 M HCl, the release condition should be modified as follows: TP5-SLN was divided into two groups. These two groups were dispersed in 7.5 ml 0.1 M HCl for 2 h. Then, one group was stopped release and the TP5 remaining in the SLN was determined, the drug released was calculated by the subtraction of drug remaining. The other group was added 2.5 ml 0.2 M sodium phosphate for the following release test. Samples were collected at the same time points and diluted with water.

***In Vivo* Evaluation** INS-loaded Gel-Core-SLN and conventional SLN were subjected for 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 (six rats in each group). Group 1, 2, 3 and 4 were given by gavage INS solution (50 IU/kg), conventional INS-SLN (50 IU/kg), INS-Gel-Core-SLN (50 IU/kg), and 6-months-stored INS-Gel-Core-SLN respectively. Group 5 was injected subcutaneously with INS solution (2.5 IU/kg). Blood samples were taken from the vein of rats at 0, 1, 2, 4, 6, 8, 10, 12, 16, 24 h after administration. Plasma was separated by centrifugation for 15 min at 6000 rpm and the plasma glucose level was determined by glucose oxidase method (GOD kit, Beijing Bei Hua Kang Tai, China). Plasma glucose levels were plotted against time. Pharmacological availability (PA) of different INS-loaded SLN was determined according to the following equation.

$$\mathrm{PA} = \frac{AAC_{\mathrm{oral}} \times \mathrm{Dose}_{\mathrm{s.c.}}}{AAC_{\mathrm{s.c.}} \times \mathrm{Dose}_{\mathrm{oral}}} \times 100\% \quad (3)$$

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 (S.D.). Statistical analysis was performed with SPSS. The different formulations were compared by analysis of variance (ANOVA), and differences were considered significant for p values <0.05 in the pharmacology study.

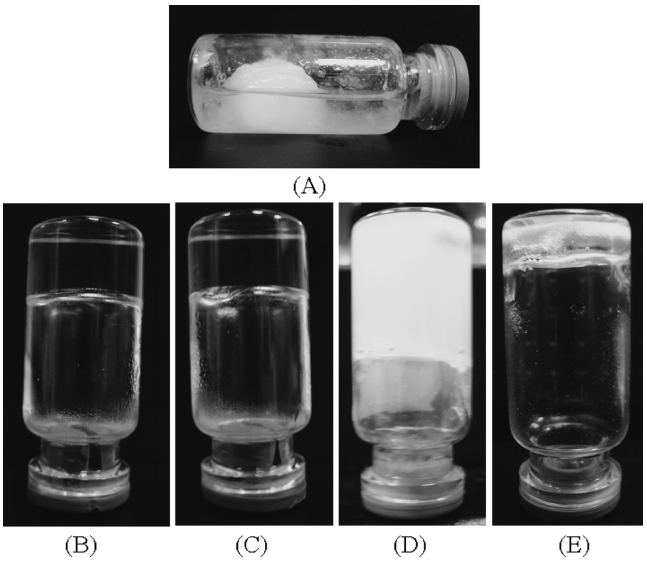


Fig. 2. Photoes of the Gel Morphology

(A) Chitosan gel, (B) pluronic F127 gel, (C) PEG-PLGA-PEG gel, (D) HPC gel, (E) HPMC gel.

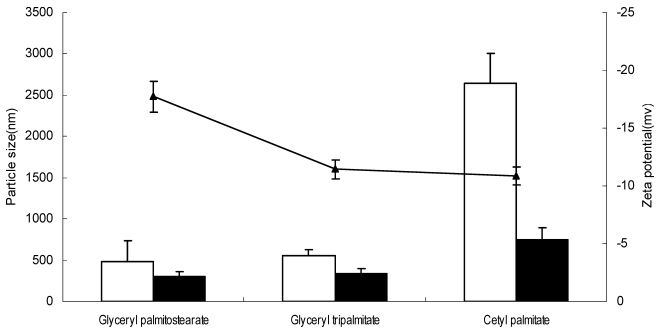


Fig. 3. Particle Size and Zeta Potential of Double Emulsion Containing Gel Cores Prepared by Different Lipids and the Corresponding Gel-Core-SLN

(■) Particle size of Gel-Core-SLN, (▲) Zeta potential of Gel-Core-SLN (n=3).

Results and Discussion

Selection of Thermal Sensitive Gel The gel morphology and gelation time of Pluronic F127, chitosan, PEG-PLGA-PEG, HPC and HPMC were listed in Table 1 and Fig. 2.

Gel state represented the distribution of water in gel polymer after the system had changed from liquid sol to solid or semi-solid gel. As can be seen from Table 1, Pluronic F127, PEG-PLGA-PEG and HPMC gel were all uniform system with transparent appearance, except that HPMC swelled greatly indicating which was not able to prepare gel core with small size. After heated to 35 °C, chitosan gel was divided into two parts, one was solid gel core in the central of the system, the other was water surrounding the solid gel. The separation of solid and liquid went against the encapsulation of hydrophic protein or peptide because some drug would distribute in water surrounding the gel and could be easily drawn out of the inner core in the following ultrasonic and evaporation process.

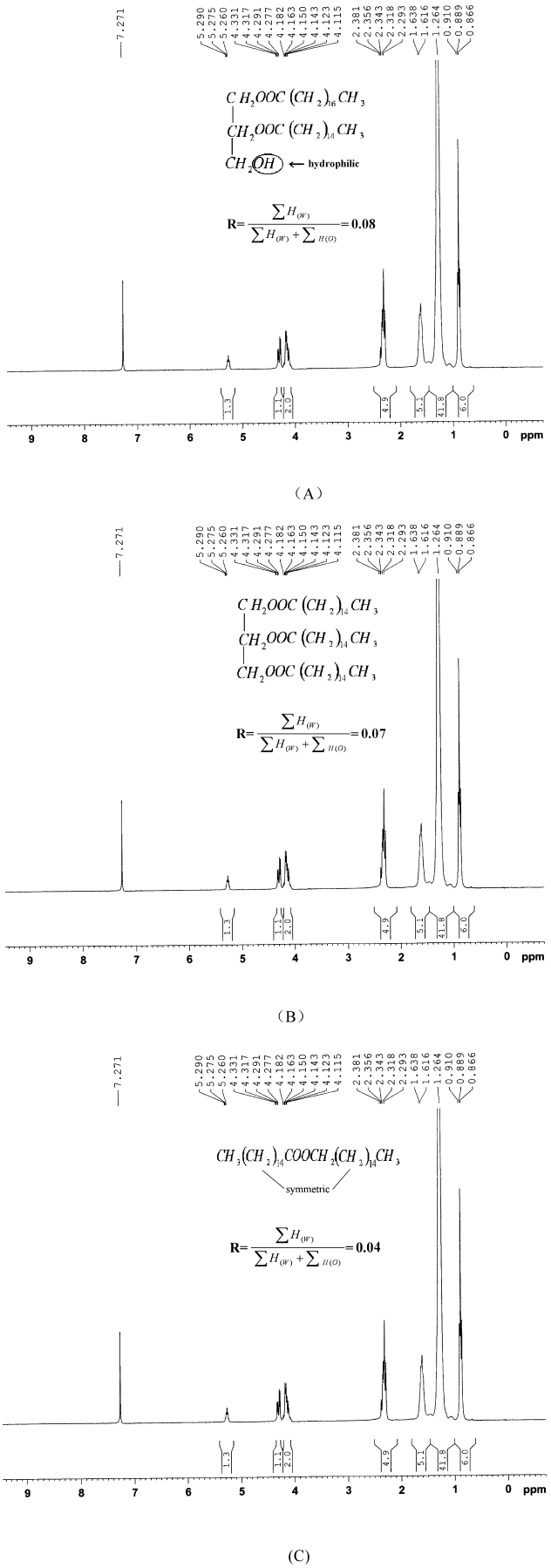
For the preparation of protein and peptide nanoparticles, the operation time should be as short as possible because they were not stable with long term contact with oil–water interface. So, rapid sol–gel change was needed in this experiment. Although the gelation time could be reduced by the increasing of temperature, the temperature was set at 35 °C to screen the best gel material to avoid the following thermal degradation. The results showed the order of gelation times of all the materials studied is Pluronic F127<chitosan< HPMC<PEG-PLGA-PEG<HPC.

Pluronic F127, which showed the excellent gel state with

the shortest gelation time, was selected as the gel material in this research. The application of gel in inner phase was to dissolve hydrophic drugs and prevented their diffusion to external part by binding drugs in solid gel. The flowability of gel was important to the ideal EE. Ideal gel should be complete solid and can stay stably in inner phase during the ultrasonic or evaporation process. Concentration of Pluronic F127 was studied in this experiment and the gel still had little flowability at the concentration below 45%. However, it has been reported that F127 with concentration more than 20% can form gel at high temperature. Here, with further increased concentration above 45%, the stable gel could formed and did not move even by violent shaking. The concentration was set at 45% in the following experiment.

Selection of Lipid The basic process for the preparation of Gel-Core-SLN is the combination of double emulsion and solvent evaporation method with thermal sensitive gel technique. The former process has some requirements to lipid materials. From our preliminary research, Glyceryl palmitostearate, Glyceryl tripalmitate and Cetyl palmitate could be used to prepare solid lipid particles at nano-size. These three lipids were employed in the preparation of Gel-Core-SLN here. Double emulsion and Gel-Core-SLN particle size, zeta potential and EE were investigated (Figs. 3, 5, 6).

The particle sizes of Glyceryl palmitostearate, Glyceryl tripalmitate and Cetyl palmitate Gel-Core-SLN were 305.2, 336.9 and 745.2 nm, respectively. The corresponding double emulsion containing gel core had the same particle size order. With the same gel core, emulsifier and procedure, what resulted in these particle size differences? As for double emulsion, the drop size depended on not only emulsifier but also the compatibility of organic phase with water. In this experiment, the organic phase was not simple DCM but a mixture of DCM and lipid. The compatibility of organic phase with water, in other words, the hydrophilicity of organic was the sum of DCM and lipid. The hydrophilicity of lipid can be expressed as the proportion of hydrophic protons in the total number of protons (R). The R value tested by NMR technique was shown in Fig. 4 and its sequence was Glyceryl palmitostearate>Glyceryl tripalmitate>Cetyl palmitate. Glyceryl palmitostearate has the highest R value and hydrophilicity because there is one hydrophilic hydroxyl in its molecule structure. Therefore, the Glyceryl tripalmitate double emulsion system had the smallest interface tense and drop size. In Glyceryl tripalmitate molecule, three hydroxyl were all esterificated by palmic acid and the hydrophilicities were decreased while interface tense and drop size were increased. Cetyl palmitate had the most symmetrical structure and lowest R value which led the increasing of double emulsion size till 2647 nm. With the evaporation of DCM, the lipid ratio increasing in organic phase and the system particle size depended more and more on the hydrophilicity of lipid and the final Gel-Core-SLN particle size order was the same as double emulsion. The hydrophilicity advantage of Glyceryl palmitostearate was reflected in high particle zeta potential. If lipid is strong hydrophobic, it will aggregate together in evaporation process and phospholipid will coat on the surface of particle, the main charge source, will then be embodied inside the aggregates. This process leads to the decreasing of zeta potential. It can be concluded that there was relatively few aggregation in Glyceryl palmitostearate than in



(A)

(B)

(C)

Fig. 4. NMR Diagrams of Glyceryl Palmitostearate (A), Glyceryl Tripalmitate (B), Cetyl Palmitate (C)

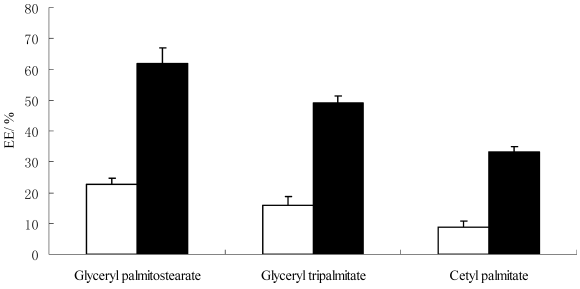


Fig. 5. Entrapment Efficiency of Conventional TP-SLN and TP-Gel-Core-SLN Made from Three Lipids

(□) Conventional SLN, (■) Gel-Core-SLN (n=3).

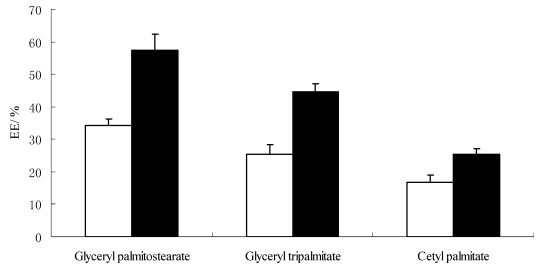


Fig. 6. Entrapment Efficiency of Conventional INS-SLN and INS-Gel-Core-SLN Made from Three Lipids

(□) Conventional SLN, (■) Gel-Core-SLN (n=3).

Glyceryl tripalmitate and Cetyl palmitate Gel-Core-SLN.

The EEs of TP and INS loaded nanoparticles were shown in Figs. 5 and 6. For TP, the EE values of Glyceryl palmitostearate, Glyceryl tripalmitate and Cetyl palmitate Gel-Core-SLN were 61.97%, 49.08% and 33.3% respectively, which were 2.7, 3.1 and 3.8 times of corresponding conventional SLN. For the INS-loaded Gel-Core-SLN, the EE values of the three Gel-Core-SLNs were 57.36%, 43.31% and 25.47%, which were 1.7, 1.8 and 1.5 times of corresponding conventional SLNs. Compared with conventional solid lipid nanoparticle (SLN), nanoparticle with thermal sensitive gel core prevented the diffusion of TP or INS from inside to outside, therefore, the entrapment efficiency was greatly improved. Glyceryl palmitostearate Gel-Core-SLN was selected for the next study because of its small particle size and high EE.

Primary Emulsion Time To prepare the Gel-Core-SLN with small particle size and high EE, the gel core should be liquid state in primary stage and changed afterwards into solid state. Heating primary emulsion could achieve this change while the gel core might have some degree of aggregation during this process if there was no dispersion operation. However, to assure the continuity of the process, no additional heating or dispersing was administrated. All the roles of gel smashing, primary heating and particle dispersing were played by ultrasonic through its blasting and heating effects. In brief, compared with conventional ultrasonic, no ice bath was used, temperature was kept increasing accompanied by 500 W ultrasonic during the primary emulsion stage until the formation of solid gel core emulsion. To monitor the temperature changes, the temperature probe was tied on the ultrasonic tube and the temperature change was recorded during the primary emulsion stage.

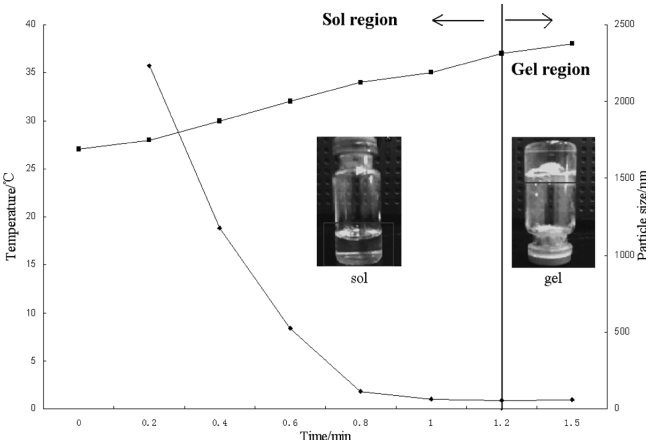


Fig. 7. Primary Emulsion Temperature and Particle Size Changed with Ultrasonic Time

(■) System temperature, (◆) gel particle size. The left side in the diagram is sol region and right side is gel region.

As was shown in Fig. 7, system temperature had a linear correlation with time and the linear equation was:

$$T=7.7589\,t+27\ (r=0.9938) \quad (4)$$

T, system temperature; t, ultrasonic time.

To observe the gel state in the ultrasonic process clearly, F127 gel solution was heated at water bath according to Eq. 4. The gel solution maintained sol state within 1.2 min (see left picture in Fig. 7), after that it became sticky gradually until turned into solid at 1.5 min (see right picture in Fig. 7). Because ultrasonic has more rapid and uniform heat delivery, it can be referred that F127 gel solution had completed the state change from sol to gel within 1.5 min, which can be proved by the gel core size change.

The gel size change curve can be divided into two regions. Dramatically size decreasing can be seen in Fig. 7 before 1 min and then the size kept at about 60 nm with a little change in the following 0.5 min. According to our previous study, toughness solid gel can not be crushed through ultrasonic. The stable particle size in the following 0.5 min reflected the formation of solid gel core.

Double Emulsion Time Ultrasonic time was an important factor to the structure of the nanoparticle. Based on the previous study, ultrasonic time for primary emulsion was set at 1.5 min, and for second emulsification, the ultrasonic times of 0.5 min and 1.5 min were employed to study their influence on particle morphology.

When second ultrasonic time was prolonged to 1.5 min, the nanoparticle partly lost the lipid coating and changed to naked cores with staccato shells in TEM pictures (Fig. 9D). The black cores (gel particles) could not exist for a long time and disappeared gradually as the dissolving of F127.

Solvent Evaporation Temperature Different solvent-evaporation temperatures of 27 °C, 31 °C, 36 °C and 40 °C were also investigated and the EE was compared to study their effect on encapsulation efficiency (Fig. 8). The evaporation temperature of double emulsion dictated the state of the gel-core. At low temperature, with the evaporation of solvent, the liquid sol will leak from inner core and result in the decrease of EE. Therefore, suitable temperature should be kept in solvent evaporation process. The EE increased as

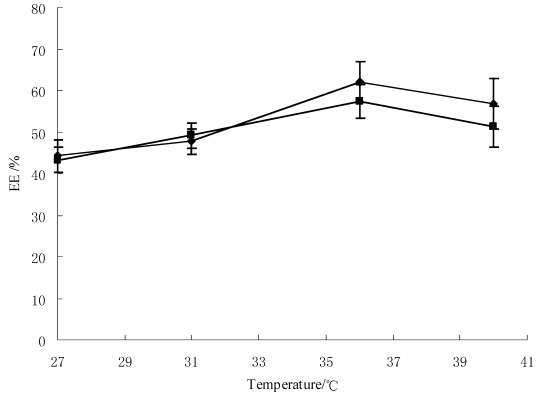


Fig. 8. EE Changes at Different Solvent-Evaporation Temperatures

(▲) EEs of TP-Gel-Core-SLNs, (■) EEs of INS-Gel-Core-SLNs ($n=3$).

temperature ascending before sol–gel changing temperature, which can be explained that more gels kept in solid state and prevented the diffusion of drugs. When the temperature went up to the boiling point of DCM, the EE decreased. The double emulsion boiled violently at 40 °C, which was a great impact to gel cores and that gel cores could not be stayed stably even if they were in solid state and the EE decreased finally.

Structure of Gel-Core-SLN To study the internal structure of this nanoparticle, the gel and solid lipid should be labeled respectively. We added hydrophilic fluorescein isothiocyanate (FITC) to F127 solution and dissolve lipophilic Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) to DCM contained Glyceryl palmitostearate before preparation, after dialysis the unloaded fluorescent dye, the final nanoparticle was observed by confocal laser scanning microscopy (CLSM). Unfortunately, the flowing nanoparticles were too small and it was difficult to distinguish the meticulous structure.

When TEM technology and negative staining method were applied, Gel-Core-SLN appeared as a homogeneous particle seen in Fig. 9. This phenomenon showed that as organic material, Glyceryl palmitostearate and F127 had the similar response to electron beam, so we could not tell the difference. Some strategy was needed to break the homogeneous of gel and solid lipid. In this study, we added sodium phosphotungstate, a hydrophilic heavy metal salt, to label the gel core, and the bulk solution was negative staining with phosphato-tungstic acid. In the TEM photos, the new type of nonaparticles were irregular spheres with one or few gel core (Figs. 9B, C, the black region inside was phosphato-tungstic acid labeled gel) while the common particles were an uniform regular spheres with little phosphato-tungstic acid distributed on the surface of solid lipid (Fig. 9A, the gray region on the surface of nanoparticle was phosphato-tungstic acid). Because phosphato-tungstic acid had the similar hydrophilic character with proteins, its distribution reflexed the possible position of proteins in this nanoparticle.

The formation of the hydrophilic gel core inside the lipophilic solid lipid nanoparticle was a key process to the structure. However, these nanoparticles were thermodynamically unstable system due to large interfacial free energy between gel and lipid and the solid gels prone to enter the bulk water lipid even if lipid had some hydrophilicity. Two mechanisms may promote the formation of this structure, one is the

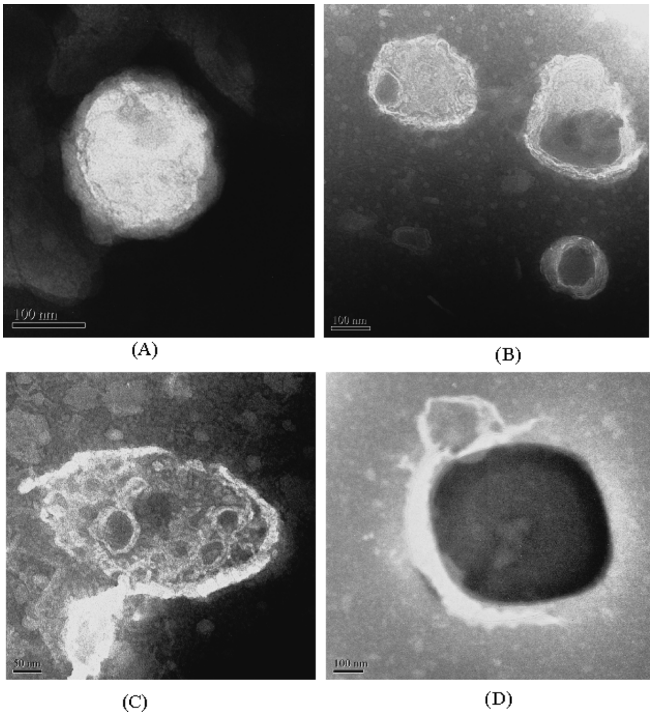


Fig. 9. TEM of the Glyceryl Palmitostearate Nanoparticles

(A) Conventional SLN, (B) SLN with single gel core, (C) SLN with multiple cores, (D) imperfect Gel-Core-SLN.

passive encapsulation action by solid lipid and the other is the interface stabilization effect by phospholipid. In the experiment, gel particles were firstly entrapped into DCM drops containing Glyceryl palmitostearate (Fig. 1). As solids, they had little fluidity. However, gel particles still had the trend to leak out and combined together during ultrasonic and evaporation process if no surfactant (water in oil emulsifier) was used to decrease the interfacial tense between gel and lipid. HLB value is widely used to classify the type of surfactant and most o/w emulsifiers have the value of 2—8 while w/o emulsifiers have the value of 6—16.[18] Phospholipid, a natural emulsifier with the HLB value of about 7, has double emulsification effect and is utilized as emulsifier in both o/w[19—24] and w/o emulsion.[25] From Fig. 9B, we can see that phospholipid bilayers distributed in both the surface of gel core and solid lipid nanoparticle, indicating it stabilized both the two interfaces.

Stabilities of Gel-Core-SLNs The particle size and zeta potential of TP-Gel-Core-SLN and INS-Gel-Core-SLN were similar to the blank Gel-Core-SLN. After 6 months storage, the particle size increased slightly to 363.7 nm and 391.4 nm respectively (Fig. 10). However, there was no significant change in EE and zeta potential (data not shown). The drug release and *in vivo* performance were described in the following section.

Stabilities of Proteins During the preparation and storage of Gel-Core-SLN, ultrasonic, heating, contacting with DCM and other factors might have some influences to the activities of TP and INS. Because TP was a peptide composed with only five amino acids, its activity depended mainly on its chemical structure. Therefore, HPLC method was used to test its stability. No degradation peak was observed (Fig. 11), and the percentage of practical drug content to the theory

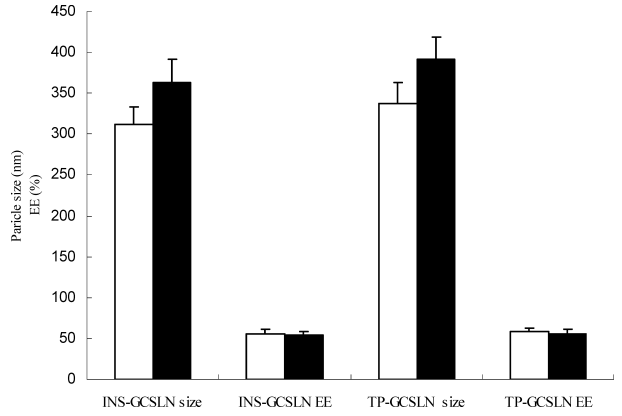


Fig. 10. The Particle Size and EE of Gel-Core-SLN after Stored for 6 Months

(□) 0 months, (■) 6 months (n=3).

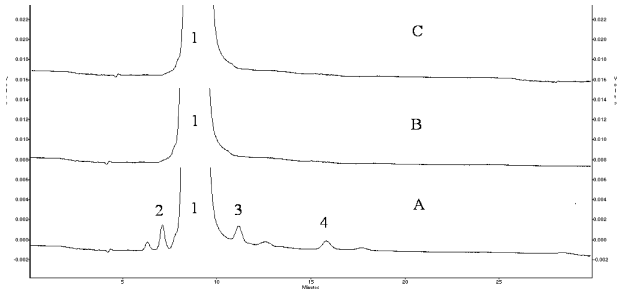


Fig. 11. Investigation of TP Degradation Products after the Preparation of Gel-Core-SLN or Storage for 6 Months by HPLC

(A) Chromatogram of TP degradation products prepared by long term ultrosonic and heating, (B) chromatogram of TP from Gel-Core-SLN freshly prepared, (C) chromatogram of TP from Gel-Core-SLN stored for 6 months, (1) TP, (2) degradation product, (3) degradation product, (4) degradation product.

content was 97.13% after the preparation. INS was a protein with 51 amino acids, its activity was influenced greatly by its spatial structure. In this study, the activity of INS from Gel-Core-SLN was determined by mouse blood method. After the administration of high dose and low dose INS from Gel-Core-SLN, mouse exhibited the same hypoglycemic effect as INS standard solution, the potency of INS was 25.12 U/mg. After a 6-month-storage, the drug content of TP-Gel-Core-SLN was 96.77% compared with initial drug content. The potency of INS from Gel-Core-SLN was 24.58 U/mg. Both TP and INS showed good stabilities.

***In Vitro* Release** SLN has been used as a promising protein and peptide oral delivery system because it can protect drugs from the degradations of gastric acid and protease enzyme in gastrointestinal tract and then be absorbed by the endocytosis of M cell in Pyer's patches. As a common phenomenon, proteins will release from SLN before it reaches the absorption region and only drug still at the inside of the preparation can be absorbed along with SLN. Therefore, burst release is a big problem for this process.

As can be seen from Figs. 12 and 13, significant burst releases took place during the release study of conventional INS-SLN and TP5-SLN. After 4-h release, the percentage of drug released from the two SLNs were 33.28% and 47.43% respectively. Interestingly, the burst releases decreased greatly with the Gel-Core-SLNs and the corresponding drug

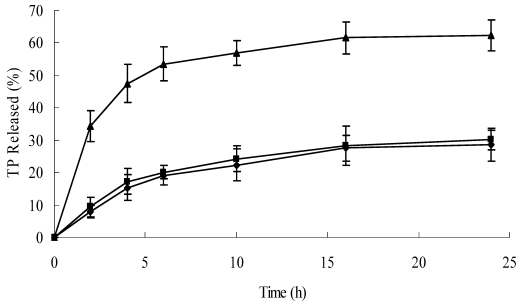


Fig. 12. TP Release Percentage from Different SLNs in Simulated Gastrointestinal Fluid

(▲) Conventional TP-SLN, (◆) TP-Gel-Core-SLN, (■) TP-Gel-Core-SLN stored for 6 months (n=6).

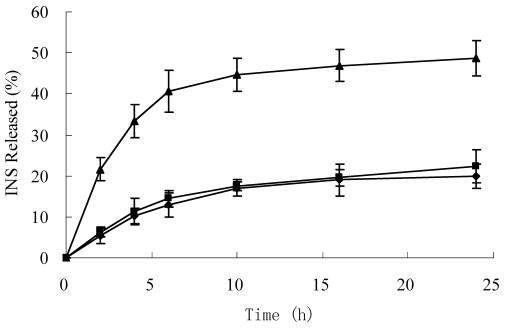


Fig. 13. INS Release Percentage from Different SLNs in Simulated Gastrointestinal Fluid

(▲) Conventional INS-SLN, (◆) INS-Gel-Core-SLN, (■) INS-Gel-Core-SLN stored for 6 months (n=6).

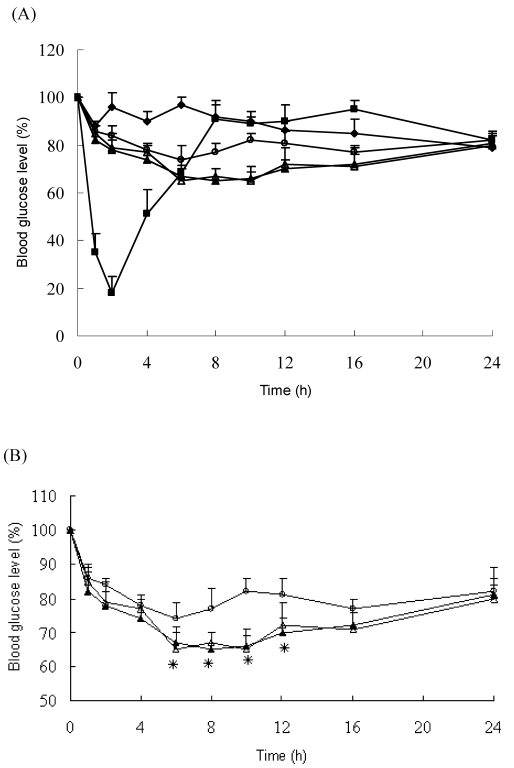


Fig. 14. Percentage Reduction of Plasma Glucose Concentration in Diabetic Rats after Oral Administration of Different INS Loaded SLNs

(○) Conventional INS-SLN 50 IU/kg, (▲) INS-Gel-Core-SLN 50 IU/kg, (△) INS-Gel-Core-SLN stored for 6 months, (◆) INS solution 50 IU/kg, (■) subcutaneous injection of insulin 2.5 IU/Kg (n=6). (A) The full diagram, (B) diagram locally enlarged for SLNs, * statistically significant differences between Gel-Core-SLN and conventional IN-SLN (p<0.05).

release percentage of INS and TP5 were 10.17% and 15.43%. This difference was due to the different material and structure between conventional SLN and Gel-Core-SLN. The conventional SLN composed of lipid material was a homogeneous hydrophobic structure and the hydrophilic proteins distributed mainly on its surface layer. During the release experiment, the drug release quickly and led to burst release. The Gel-Core-SLN had a perfect combination of hydrogel core and lipid shell, the core accommodated the proteins while the shell prevented the penetration of water. Therefore, the burst release was decreased dramatically.

After the 6 months storage, the drug release percentage from TP-Gel-Core-SLN and INS-Gel-Core-SLN were slightly increased, the 4 h release percentages were 14.37% and 17.92% respectively. However, these changes had no significant difference.

***In Vivo* Evaluation** As can be seen above, TP-Gel-Core-SLN and INS-Gel-Core-SLN had the similar advantages in EE and *in vitro* release compared with the corresponding conventional SLNs, therefore, INS-Gel-Core-SLN was selected as the representative for *in vivo* study. Figure 14 illustrated changes in blood glucose level after oral administration of INS-Gel-Core-SLN and INS conventional SLN. Both the two SLNs exhibited obvious hypoglycemic effect compared with INS solution oral administration ($p<0.05$). As far as the two SLNs were concerned, the Gel-Core-SLN had much more significant hypoglycemic effect, especially during 8 to 12 h ($p<0.05$).The PA values of conventional SLN and Gel-Core-SLN were 4.53% and 6.02% respectively.

Obviously, after INS was incorporated into this novel Gel-Core-SLN, its PA was improved. This might be due to the relatively high EE, low burst release and therefore the more drugs would remain in Gel-Core-SLN. Although SLN can be absorbed by intestinal cell, the number absorbed was limited. So, improving the drug content in SLN before absorption could result in the increasing of the PA.

The PA of INS-Gel-Core-SLN stored for 6 months was 5.98%, similar with the initial preparation. Although slight increasing in particle size and drug release, the *in vivo* performance of Gel-Core-SLN was almost the same, suggesting that it was a stable dosage form for the oral protein delivery system.

Conclusion

Thermo sensitive gel loading TP and INS were implanted successfully into SLN by double emulsion technology in this research. Pluronic F127 was selected as the gel material with the shortest gelation time and excellent gel state. The Gel-Core-SLN made from Glyceryl palmitostearate has the particle size of 305.2 nm and zeta potential of −17.74 mV. Most particles had a single gel core and the EE was improved to be 61.97% and 57.36% for TP and INS respectively. Blasting and heating effects of ultrasonic can be utilized to crushing gel size and complete the sol to gel change at the primary emulsion stage. At the double emulsion stage, excessively long ultrasonic time resulted in the loss of lipid from the surface of gel core and the imperfect structure of Gel-Core-SLN. The EE increased with the ascending of solvent evaporation temperature while it would decrease once the temperature was over DCM boiling point. During the preparation of drug-loaded Gel-Core-SLN, the activities of TP and INS were not affected. Compared with the conventional SLN, Gel-Core-SLN inhibited the drug burst release dramatically. The release percentages of TP and INS at 4 h were 10.17% and 15.43% respectively. After orally administration to diabetic rats, INS-Gel-Core-SLN improved the PA from 4.53 to 6.02%. The Gel-Core-SLN kept stable during the 6-month-storage at 4 °C. This study developed a feasible preparation method for protein loaded SLN with gel core–lipid shell structure, high encapsulation efficiency, low burst release and high bioavailability.

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