Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 356 (2008) 333–344
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Solid lipid nanoparticles for pulmonary delivery of insulin

Jie Liu [a], Tao Gong [a], Hualin Fu [a,b], Changguang Wang [a,c], Xiuli Wang [a], Qian Chen [a], Qin Zhang [a], Qin He [a], Zhirong Zhang [a,*]

[a] *Key Laboratory of Drug Targeting, Ministry of Education, Sichuan University, No. 17, Section 3, Southern Renmin Road, Chengdu 610041, PR China*
[b] *Animal Science and Technology College, Sichuan Agricultural University, Ya'an 625000, PR China*
[c] *Department of Pharmaceutics, School of Pharmaceutical Science, Shenyang Pharmaceutical University, Shenyang 110016, PR China*

Received 2 October 2007; received in revised form 6 December 2007; accepted 7 January 2008
Available online 16 January 2008

Abstract

Growing attention has been given to the potential of pulmonary route as an alternative for non-invasive systemic delivery of therapeutic agents. In this study, novel nebulizer-compatible solid lipid nanoparticles (SLNs) for pulmonary drug delivery of insulin were developed by reverse micelle-double emulsion method. The influences of the amount of sodium cholate (SC) and soybean phosphatidylcholine (SPC) on the deposition properties of the nanoparticles were investigated. Under optimal conditions, the entrapment delivery (ED), respirable fraction (RF) and nebulization efficiency (NE) of SLNs could reach 96.53, 82.11 and 63.28%, respectively, and Ins–SLNs remained stable during nebulization. Fasting plasma glucose level was reduced to 39.41% and insulin level was increased to approximately 170 μIU/ml 4 h after pulmonary administration of 20 IU/kg Ins–SLNs. A pharmacological bioavailability of 24.33% and a relative bioavailability of 22.33% were obtained using subcutaneous injection as a reference. Incorporating fluorescent-labelled insulin into SLNs, we found that the SLNs were effectively and homogeneously distributed in the lung alveoli. These findings suggested that SLNs could be used as a potential carrier for pulmonary delivery of insulin by improving both *in vitro* and *in vivo* stability as well as prolonging hypoglycemic effect, which inevitably resulted in enhanced bioavailability.

Keywords: Solid lipid nanoparticles; Insulin; Nebulization; Hypoglycemic effect; Bioavailability

1. Introduction

Recently, pulmonary route, as an alternative non-invasive approach for both local and systemic drug delivery has received more and more attention (Scheuch et al., 2006). Since lots of advantages such as large absorptive area, extensive vasculature, easily permeable membrane, low extracellular and intracellular enzyme activity (Malcolmson and Embleton, 1998; Hussain et al., 2004; Patton et al., 2004) were associated with this route, pulmonary delivery of drugs becomes a new hotspot, particularly for peptides and proteins (Hussain et al., 2004; Malik et al., 2007; Kumar et al., 2006; Adjei and Gupta, 1994).

However, for successful development of pulmonary drug delivery systems, several unique challenges still remain, and the major challenge is how to formulate drugs into inhalable forms with sufficient stability and appropriate size (Dahab et al., 2001; Dailey et al., 2003). Inhalation devices as well as the physicochemical characteristics of the formulation could influence aerodynamic size of the particles and ultimately affect the site of aerosol deposition.

There are a variety of inhalation devices on the market currently, however, no one could meet all the requirements for optimal pulmonary drug delivery. Although metered-dose inhalers (MDI) and dry powder inhalers (DPI) enjoy most popularity among the patients with chronic disease such as chronic obstructive pulmonary disease (COPD), the particle sizes generated from these devices are very large usually, which might not be suitable for efficient deposition due to inertial impaction in the upper respiratory tract. Advanced nebulizers, generating a mist of small particles which could penetrate the lung regions readily, could be better fit for pulmonary delivery of drugs (Gupta and Hickey, 1991; Roche and Huchon, 2000).

In addition to inhalation devices, drug carriers are equally important for effectiveness of respiratory delivery. To construct an ideal pulmonary drug delivery system, drug carriers with suitable properties are required. Drug carriers with average size in

* Corresponding author. Tel.: +86 28 85501566; fax: +86 28 85501615.
E-mail address: zrzzl@vip.sina.com (Z. Zhang).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.01.008

nanometer range such as liposomes (Huang and Wang, 2006; Karathanasis et al., 2005) and nanoparticles (Kawashima et al., 1999; Zhang et al., 2001) exhibit some well-defined and delicate characteristics, which have created an attractive and efficient approach for pulmonary delivery of drugs especially for proteins with higher bioavailability, controlled release properties and enzymatic tolerance (Chattopadhyay et al., 2007). Solid lipid nanoparticles (SLNs), emerged as a promising non-toxic nanocarrier for drugs in 1990s, the superior physicochemical characteristics of which make them more suitable as carrier for pulmonary drug delivery than their predecessors, such as small size, biocompatible composition and deep-lung deposition ability. Dual effect of prolonged drug release and rapid drug transport could be achieved by means of particulate systems including SLNs (Pandey and Khuller, 2005; Videira et al., 2002). However, the utility of the lung for drug delivery using particulate carriers is not fully appreciated. Most published data were limited to *in vitro* characterization of the particulate systems for pulmonary delivery, and most of the reports were focused on treatment of local diseases, few reports have been published concerning pulmonary applications of SLNs as systemic delivery carriers for macromolecular drugs such as proteins or genes (Rudolph et al., 2004; Almeida and Souto, 2007).

In this study, novel nebulizer-compatible Ins–SLNs for pulmonary delivery were prepared by reverse micelle-double emulsion method as previously reported by our group (Liu et al., 2007) and the influences of formulation parameters on deposition behaviours were also investigated carefully. Both *in vitro* and *in vivo* studies demonstrated that SLNs based on mixed micelles appeared to be a good candidate for pulmonary delivery of insulin.

2. Materials and methods

2.1. Materials

Pure crystalline porcine insulin was purchased from Xuzhou Wanbang Bio-Chemical Co. Ltd. (No. 0312A02, Jiangsu, China), with a nominal activity of 28 IU mg^{-1}. Stearic acid (obtained from Shanghai Chemical Reagent Co. Ltd., China) and palmitic acid (obtained from Chengdu Kelong Chemical Plant, China) were used as lipidic materials of SLNs. Soybean phosphatidylcholine was from Shanghai Taiwei Pharmaceutical Co. Ltd., China and sodium cholate was supplied by Beijing Aoboxing Biotechnologies Co. Ltd., China. Streptozotocin (STZ), sodium pentobarbital and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO, USA). Double distilled water was used for all solutions and dilution. All the other reagents were of analytical grade and used without further purification except those for HPLC assay, which were of HPLC grade.

2.2. Preparation of Ins–SLNs

Ins–SLNs were prepared using the procedure developed previously by our group (Liu et al., 2007) and optimized by central composition design using nebulization parameters as estimated indices, which will be reported elsewhere. Briefly, 200 μl of insulin solution (5 mg insulin dissolved in 1 ml 0.01 M hydrochloric acid) with a certain amount of sodium cholate (inner aqueous phase) were added to a 1 ml ethyl acetate solution containing stearic acid and palmitic acid (1:1, w/w) and soybean phosphatidylcholine (oily phase). This mixture was dispersed with an ultrasonic probe (JY92-II ultrasonic processor, Ningbo Scientz Biotechnology Co. Ltd., China) for 15 s at 40 W leading to a W/O emulsion. A double W/O/W emulsion was formed after addition of 4 ml 0.1% Poloxamer 188 solution (outer aqueous phase) to the previous W/O emulsion followed by sonication for 15 s at 80 W. This double emulsion was then diluted to 10 ml with a 0.1% Poloxamer 188 solution. The solvent was evaporated for 3 h in a rotary evaporator (Büchi, R-144 rotavaporator, Switzerland) at 25 °C.

2.3. Deposition study

PariBoy air-jet nebulizer with compressed air using an Inhalier Boy compressor (Type 37.00, Pari-Werk, Starnberg, Germany) was used for all nebulization experiments.

Fig. 1. The prototype of twin-stage impinger (TSI).

Aiming to assess the deposition of Ins–SLNs from the nebulizer, a home-made twin-stage impinger (TSI, Apparatus A; European Pharmacopeia, 2004) was used. Three collection stages were considered (Mendes et al., 2007). The upper stages (stages 0 and 1) represents the upper airways, and the lower stage (stage 2) represents the lower respiratory airways with a cut-off aerodynamic diameter of 6.4 μm at 60 l/min (Hallworth and Westmorel, 1987). For the consideration of solubility of insulin, 0.01 M HCl was used as the collection liquid, of which 7 and 30 ml were placed in stages 1 and 2 of the TSI, respectively. The air flow was drawn through the TSI using a vacuum pump (Zhejiang HuangYan Liming Co. Ltd., China) and the air flow rate was measured by a calibrated flow meter with a maximum flow rate of 100 l/min (LZM-15Z, Yuyao KingTai Instrument Co. Ltd., China).

Each TSI stage was rinsed with 0.01 M HCl, the liquid was collected and the volume was adjusted to 1, 10 and 50 ml for stages 0, 1 and 2, respectively (Fig. 1). Running time was adapted accordingly until the nebulizer was operated to "dryness", i.e. 30 s after aerosols completely ceased. In practice, Ins–SLNs (8 ml, 100 μg/ml) were placed in nebulizer with the device mouthpiece directed into the throat of the TSI. Three TSI replicates were performed for each sample. The nebulization efficiency (NE) (Desai et al., 2002), respirable fraction (RF) (Matilainen et al., 2006) and entrapment delivery (ED) (Elhissi et al., 2007) were employed as parameters to evaluate the aerodynamic behaviour of the nanosuspension which could be calculated as follows:

$$\text{Nebulization efficiency (\%)} = \frac{\text{Aerosolized drug mass}}{\text{Drug mass loaded in nebulizer}} \times 100 \quad (1)$$

$$\text{Respirable fraction (\%)} = \frac{\text{Drug mass deposited in stage 2}}{\text{Drug mass loaded in nebulizer}} \times 100 \quad (2)$$

$$\text{Entrapment delivery (\%)} = \frac{\text{Drug mass entrapped in SLNs deposited in stage 2}}{\text{Drug mass deposited in stage 2}} \times 100 \quad (3)$$

Insulin was determined by a validated high performance liquid chromatography (HPLC) assay described in a previous study (Liu et al., 2007).

2.4. Determination of size and morphology of SLNs delivered to TSI

When nebulizer was operated to "dryness", samples from nebulizer reservoir and different stages of the TSI were collected for particle size analysis using photon correlation spectroscopy (PCS) (Malvern zetasizer Nano ZS90, Malvern instruments Ltd., UK) or for morphology study using SEM. In this case, distilled water was used as collection fluid instead of 0.01 M HCl.

2.5. Sample stability during nebulization

The stability of Ins–SLNs expressed by the concentration of insulin as well as the size changes of the Ins–SLNs solution remained in the nebulizer reservoir during nebulization course were investigated. An aliquot of Ins–SLNs was withdrawn at scheduled time intervals for HPLC and PCS analysis, respectively. The output was measured by the weight loss of the nebulizer before and after nebulization at predetermined time points. Each experiment was performed in triplicate.

2.6. Cell culture and MTT-assay

The human lung cancer cell line, A549, was kindly donated by the National Key Laboratory of Biotherapy of Human Disease (Sichuan University, Chengdu, China). A549 cells were grown as a monolayer culture in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/ml) and penicillin G (100 U/ml). The cells were grown in 75 ml flasks at 37 °C in an atmosphere of 5% CO_2 and subcultured two to three times per week. At the beginning of the experiments, cells in exponential growth phase were removed from the flasks with 0.25% trypsin solution.

Cytotoxicity was determined using MTT-assay as an indicator of the viability of the cells. Cells were seeded in 96 wells/plate (1×10^4 cells/well) and allowed to attach to the flask for 24 h, seeding medium was removed and replaced by fresh culture medium. Cells were maintained for 3 days in medium supplemented with increasing concentrations of insulin solution, free-SLNs, Ins–SLNs as well as surfactant solution (F68 solution). The concentration of insulin solution and Ins–SLNs varied from 0.1 to 10 μg/ml (according to insulin concentration), and the concentration of lipid (free SLNs) and surfactant solution varied from 1 to 10 and 1 to 100 μg/ml, respectively.

After continual incubation for 72 h, the test medium was removed, 20 μl of 5 mg/ml MTT stock in PBS (pH 7.4) was added to each well, and the plate was incubated for 4 h. The solution was then discarded, 200 μl of DMSO was added to each well. After horizontal shaking for 15 min, the absorbance of the solution in each well was measured at a wavelength of 570 nm.

2.7. Animal studies

Male Sprague–Dawley rats weighing 250 ± 20 g were supplied by Experimental Animal Center of Sichuan University (protocol number for animal study: CSDGZ-10). The rats were housed at a room temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%. The animals were allowed *ad libitum* access to a standard diet and water except wherever indicated. Animals were used and treated as prescribed in the 'Guide for the care and the use of the laboratory animals' (NIH Publication No. 92-93, revised 1985) and all the animal studies were approved and supervised by Animal Ethics Committee of Sichuan University.

Fig. 2. "Nose-only" inhalation apparatus used for administering inhalable Ins–SLNs to rat.

2.7.1. Induction of diabetes

Animals were fasted for 14 h but allowed free access to water before an experiment. Streptozotocin was administrated intraperitoneally at a dose of 65 mg/kg in 0.1 M sterile citrate buffer (pH 4.4) and the animals were housed individually after then. Blood was withdrawn from caudal vein into heparinized centrifuge tubes and centrifuged at 4500 rpm for 10 min to separate the plasma. Plasma glucose level was determined using glucose estimation kit (Shanghai Rongsheng Biological Technology Co. Ltd., China) with a Cary 100 Conc UV–vis spectrophotometer (Varian, Palo Alto, USA). Animals with blood glucose level higher than 300 mg/dl (Çilek et al., 2005), which confirming the induction of diabetes, were selected for the subsequent study.

2.7.2. Pharmacodynamic and pharmacokinetic studies

Diabetic rats were randomly divided into five groups, 6 for each group. All animals including control group were fasted overnight prior to experiments. A home-made whole-body inhaled exposure chamber (20 cm × 10 cm × 10 cm) was employed for drug delivery, which was shown in Fig. 2. During the nebulization process, the rats were exposed to the aerosol cloud in a nose-only manner and could be restrained by movable baffle. The upstream of the chamber was connected to the jet nebulizer by sealing them by the parafilm®, and the downstream of chamber was open to the air through a distal hole to maintain a continuous air flow through the whole system. Ins–SLNs (20 IU/kg), plain insulin PBS solution (pH 7.4) plus blank SLNs (20 IU/kg), insulin PBS solution (pH 7.4, 20 IU/kg) or the PBS were delivered to the chamber to form a forced-inhalation to the rats for 30 min, using PariBoy air-jet nebulizer. Additional experimental group included subcutaneous administration of 1 IU/kg plain insulin PBS (1 IU/kg, pH 7.4) was employed as reference.

Animals were fasted but allowed free access to water. Blood samples were collected at scheduled time intervals, and plasma glucose levels were measured as described previously while the plasma insulin concentration was detected by radioimmunoassay (RIA kit, Beijing Puer Weiye Biotechnology Co. Ltd., Beijing, China) according to the manufacturer's instruction.

The plasma glucose concentration of each rat before administration ('0' h) was taken as the baseline level, and the changes in plasma glucose concentration (percentage of baseline level) as well as plasma insulin concentration at different time points after administration were plotted versus time. Then the area above the hypoglycemic curve versus time profile (AAC) and the area under the plasma insulin concentration–time curve (AUC) were calculated with the linear trapezoidal method. The pharmacological bioavailability (PA%) (Morishita et al., 2006) and relative bioavailability (F_R) (Damgé et al., 2007) of each formulation after administration were calculated according to the following equations:

$$\mathrm{PA} = \frac{\mathrm{AAC\ \ neb} \times \mathrm{Dose\ \ sc}}{\mathrm{AAC\ \ sc} \times \mathrm{Dose\ \ neb}} \times 100 \quad (4)$$

$$F_R = \frac{\mathrm{AUC\ \ neb} \times \mathrm{Dose\ \ sc}}{\mathrm{AUC\ \ sc} \times \mathrm{Dose\ \ neb}} \times 100 \quad (5)$$

2.8. Fluorescence microscopy studies

2.8.1. Fluorescence labeling of insulin with FITC

FITC labeled insulin was employed to study the fate of nanoparticles in the lung after pulmonary administration of SLNs. The labeling of insulin was conducted according to a reported method with some modifications (Li et al., 2007). Briefly, insulin was dissolved in 0.1 M phosphate buffer solution (PBS, pH 7.1) containing 0.2 mM EDTA, FITC solution (5 mg/ml) in acetone was added dropwise under magnetic stirring. The reaction was stirred at 4 °C for 20 h avoiding of light, and the pH of resultant solution was adjusted to 4.5 with HCl. After then the solution was dialyzed against distilled water and lyophilized. The obtained FITC–Ins was kept at −20 °C away from light until further use.

2.8.2. Preparation of FITC–Ins-loaded SLNs

The preparation course of FITC–Ins-loaded SLNs was almost the same as that of Ins–SLNs, except for small modifications. The inner phase was replaced by PBS (pH 7.4) due to the poor solubility of FITC–Ins in 0.01 M HCl.

2.8.3. Study of intrapulmonary fate of FITC–Ins–SLNs

Animals received pulmonary administration of FITC–Ins solutions and FITC–Ins–SLNs *via* nebulization (5 mg/kg) as described in Section 2.7.2 ($n = 6$). Rats were sacrificed at 0.5, 1 h after pulmonary administration of formulations, the lungs were harvested and rinsed twice with PBS (pH 7.4). Tissues were frozen prior to tissue sectioning using a Leica CM 1800 Cryostat (Leica Inst., Nussloch, Germany) and visualized with a fluorescence inverted microscope (Axiovert 40CFL, Carl Zeiss, Oberkochem, Germany) at 200× magnification, a video capture system (AxioVision Release 4.1, Carl Zeiss, Oberkochem, Germany) was used to acquire digital images. Photos (4 photoes/rat/time point) were further analyzed using Image Pro plus 4.5 (Media Cybernetics, USA) to manifest the differences between samples.

2.9. Data analysis

Data were expressed as mean ± S.D. Statistical tests were performed using single factor analysis of variance (ANOVA). Statistical significance was determined using the Student's unpaired *t*-test.

3. Results

All nebulization experiments were completed using a PariBoy air-jet nebulizer with compressed air using an InhalierBoy^{TM} compressor (Type 37.00, with a compressor pressure of 1.3 bar and inspiration flow rate of 12 l/min). The total output of the nebulizer in the manufacturer's manual was 460 mg/min, with a residual volume of 1 g, and mass median diameter (MMD) of the mist was 4.1 μm.

3.1. Influence of formulation parameters on nebulization behaviour of Ins–SLNs

There are several different parameters which determine the therapeutic efficacy of aerosol formulations. The design and variation of nebulizers, operating conditions (e.g. flow rate) and ancillary equipment are important (Dalby and Tiano, 1993). In addition, drug formulation is also a critical factor affecting aerosol nebulization efficiency and aerodynamic properties of drug loaded vehicles (Waldrep et al., 1993).

Preliminary studies were carried out to seek the relationship between formulation parameters and nebulization behaviour of Ins–SLNs. Because nebulization can lead to exhalation and leakage of drug, it is important to determine drug output and nebulization of the encapsulated drug simultaneously, which are called nebulization efficiency (NE) encapsulated delivery (ED), respectively. Respirable fraction (RF), which represents efficient deposition dose of the drug and predicts *in vivo* fate, was also estimated.

Therefore, various formulations containing different amount of SC and SPC were prepared and compared in terms of ED, RF and NE.

3.1.1. Influence of sodium cholate

Fig. 3A revealed the influence of the amount of SC on the entrapment efficiency and deposition properties of Ins–SLNs. With the increase of SC to 10 mg, entrapment efficiency of Ins–SLNs increased dramatically, which resulted in enhanced entrapment delivery of insulin. However, further increase did not have much influence on the entrapment delivery because the entrapment efficiency retained at a relatively constant level when 10 mg of SC was employed and further increase in SC amount might also lead to the leakage of Ins–SLNs during nebulization.

Most recently, Zaru et al. have found that the flexibility or rigidity of vehicles might affect the nebulization behaviour of liposomes and rigidity seemed to have negative effect on nebulization efficiency of liposomes (Zaru et al., 2007). This fact might be used to explain why the increase in SC amount led to the increase of RF and NE of Ins–SLNs. The rigidity of SLNs was reduced significantly by SC, which was employed as an edge activator in deformable liposomes (Zaru et al., 2007; Nguyen and Bouwstra, 2005; Cevc, 1996). With a high radius of curvature, SC could destabilize lipid bilayers of the vesicles and increase deformability (decrease rigidity) of SLNs, which might in turn increase RF and NE of Ins–SLNs after nebulization. Another explanation for this phenomenon was that the reduction of surface tension caused by anionic surfactants might associate with an increased aerosol output without affecting the aerosol particle size, this tendency also appeared in some previous published reports (McCallion et al., 1995, 1996).

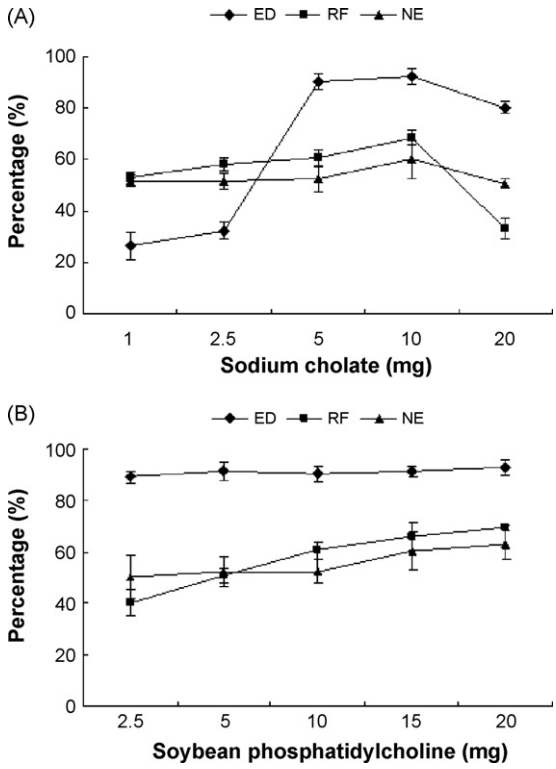

Fig. 3. Influence of the amount of SC (A) and SPC (B) on the efficient delivery (ED), respirable fraction (RF) and nebulization efficiency (NE) of Ins–SLNs.

However, an obvious decline in the RF of nebulized Ins–SLNs could be observed with 20 mg of SC, probably due to the fact that the negative effect of increased viscosity exceeded the positive effect of decreased rigidity, and strong aggregation of SLNs was observed at high amount of SC in our preliminary study. Furthermore, increased density and viscosity of solution could lead to the reduction of RF and NE. As in a jet-nebulizer, gas flow from the compressor transfers its momentum to the liquid surrounding to pass a very narrow hole, after then the liquid is drawn out and collapsed into droplets under influence of surface tension. And it is more difficult to form droplets from solutions with higher viscosity.

3.1.2. Influence of soybean phosphatidylcholine

In Fig. 3B, the RF and NE values of the Ins–SLNs subjected to nebulization were presented. In most cases, NE% was about 50% of initial concentration of particles placed in the nebulizer, and with increase in the amount of SPC, both RF and NE increased slightly. This result suggested that SPC could facilitate the nebu-

lization of Ins–SLNs, which agreed well with previous findings (Waldrep et al., 1993). Nebulization efficiency of drug-carrier increased when lipid with low gel to liquid phase transition temperatures (Tc) was used to formulate the liposomes. As the Tc of soybean phosphatidylcholine is relatively lower than solid lipid, increase in SPC fraction in lipid composition could reduce the Tc of nanoparticles and thus maximum RF and NE could be achieved.

3.1.3. Optimization of aerosolized Ins–SLNs

Based on single factor experiments, the formulation was optimized in terms of formulation properties and nebulization behaviour using average size, entrapment efficiency, zeta-potential, respirable fraction as well as nebulization efficiency as indices by central composition design (CCD) (data not shown). ED was not chosen as an index because it was above 90% under all the selected conditions. According to the results of CCD, three-dimensional (3D) contour plots were obtained to describe the effects of amounts of SC and SPC on the RF and NE of Ins–SLNs, which could also be used for further screening of the optimal formulation and technique (Sun and Zhang, 2004). All the spots inside the region with red color in Fig. 4 represented

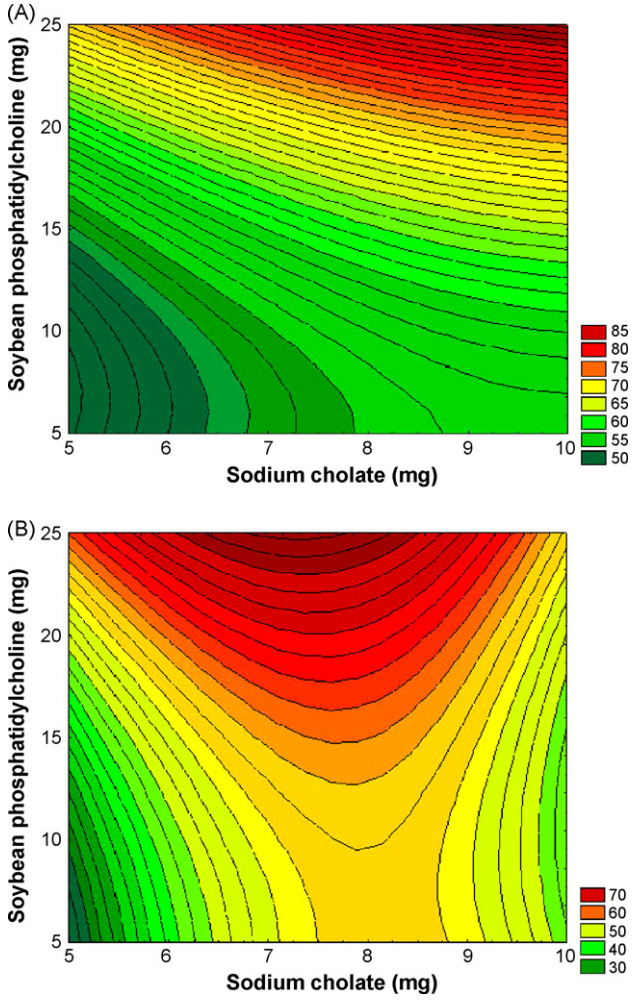

Fig. 4. 3D-contour plots to describe the effects of amount of SC and SPC on the respirable fraction (RF) (A) and nebulization efficiency (NE) (B) of Ins–SLNs.

that Ins–SLNs with higher RF and NE could be obtained under given amounts of SPC and SC.

It could be seen from Fig. 4A, SPC amount ranged from 22 to 25 mg and the SC amount in the range of 8.5–10 mg would result in relatively high RF, which was in good accordance with the results of single factor test. While in Fig. 4B, SPC amount seemed to play a major role in influencing the NE of Ins–SLNs, high NE could be achieved only when SPC amount ranged from 17.5 to 25 mg, which might be attributed to the reduction in rigidity by SPC. However, further increase in SPC amount did not lead to incremental change in NE, the possible reason might be that the surface tension of the whole system reached minimum at the critical micelle concentration (CMC) of SPC, thus no increase in NE could be observed. The EE, DL, RF, NE and ED of SLNs after optimized by CCD could reach 97.72 ± 0.54, 2.17 ± 0.12, 82.11 ± 2.65, 63.28 ± 2.83 and $96.53 \pm 1.05\%$, respectively.

3.2. Size and morphology of Ins–SLNs after nebulization

Air-jet nebulization may result in reduction in liposome size due to disruption of nanocarriers during nebulization (and continual reflux) by the shear forces generated by extrusion through the jet orifice (Taylor et al., 1990; Niven et al., 1991). In our study, compared with SLNs before nebulization, a slight decrease in the measured average size of Ins–SLNs collected from the stages 1 and 2 could be observed. The size and PDI of Ins–SLNs deposited on various stages (stages 0, 1 and 2) detected by PCS were 109.1 ± 2.0, 88.2 ± 1.8 and 97.8 ± 3.2 nm, 0.115 ± 0.232, 0.152 ± 0.113, 0.133 ± 0.158, respectively, while the size and PDI of original Ins–SLNs were 114.7 ± 2.1 nm and 0.105 ± 0.132. Fig. 5 shows the morphology of Ins–SLNs collected in each stage of TSIs. No obvious aggregation, cleavage or collapse of Ins–SLNs which usually occur in the case of liposomal aerosols were observed. However, the size distribution of Ins–SLNs in the mist seemed to be wider after nebulization and the average diameter of Ins–SLNs deposited in the stages 1 and 2 was smaller than initial size of Ins–SLNs, particularly in the stage 1, which was in accordance with the PCS measurements.

3.3. Stability of Ins–SLNs during nebulization

In some previous studies, air-jet nebulization has a detrimental effect on the physical stability of liposomes, resulting in loss of the entrapped hydrophilic drugs (Taylor et al., 1990; Niven et al., 1991). However, SLNs exhibited excellent protective effect for insulin against degradation or leakage from nanospheres. Fig. 6A depicted the average size and PDI of remained Ins–SLNs during nebulization and no significant changes could be observed. As shown in Fig. 6B, there were minimal differences between the starting size of Ins–SLNs and nanoparticles remained in reservoir. These results suggested that Ins–SLNs were relatively stable during nebulization *via* jet nebulizer. And a slight increase in residual concentration of Ins–SLNs versus time could be observed in Fig. 6B, which might be

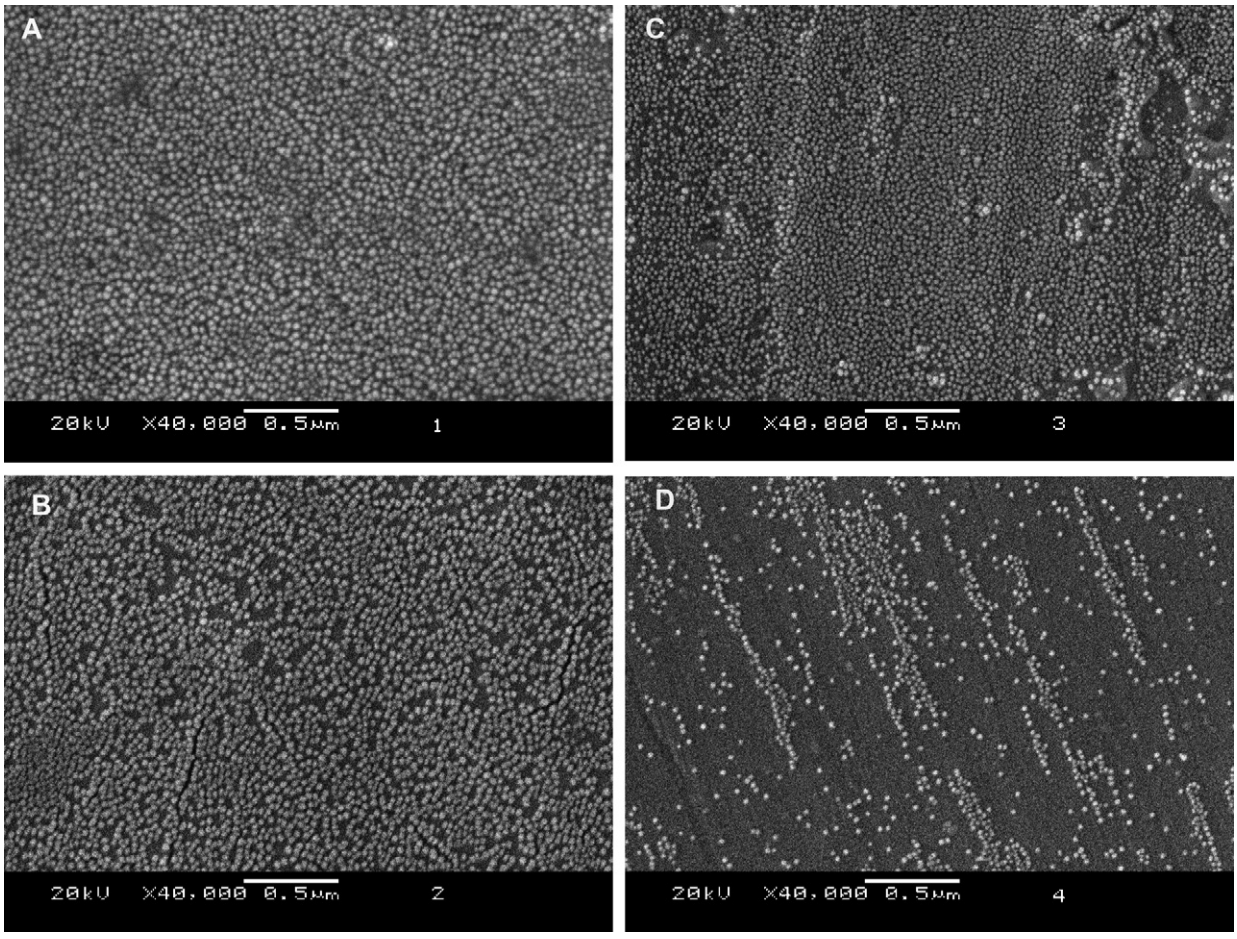

Fig. 5. Scanning electron micrographs of Ins–SLNs: (A) before nebulization (B) collected in stage 0 (C) collected in stage 1 (D) collected in stage 2, scale bar: 0.5 μm.

attributed to the well-known "concentration effect" of nebulizers. Evaporation of solvent during nebulization could lead to the increase in concentration of drug solution (Callaghan and Barry, 1997). The output of nebulizer was relatively stable during the whole nebulization course, however, the nebulization rate was much lower than the technique parameter provided in the manufacturer's book, which might be due to the different aerodynamic properties between 0.9% NaCl solution and Ins–SLNs.

3.4. Cytotoxicity

The cytotoxic effects of the Ins, Ins–SLNs, free SLNs as well as surfactant used in the preparation procedure on the A549 cells were studied by the MTT-assay. A549 cell line, which has been widely used in various kinds of studies for representation of the structural and biochemical characteristics of human type II cells (Chao et al., 2007), was employed for toxicity study in our experiment. The results of the MTT-assay, as a measure of metabolic response of the cells following 72 h exposure to different environment, were shown in Fig. 7. Following 72 h treatment of Ins, Ins–SLNs, free SLNs, the cell viability of A549 was over 95%. This result indicated that the SLNs had almost no or negligible cytotoxicity. In the case of surfactant (F68 solution), very low cytotoxicity could be observed due to its relatively low applied concentration.

3.5. The hypoglycemic effect

Previously, the effectiveness of nose-only exposure method for pulmonary absorption of insulin solution was demonstrated in the rat model (Kanaoka et al., 1999). *In vivo* pulmonary delivery potential of insulin loaded SLNs was assessed by measuring blood glucose levels. The time courses of glucose concentration in blood after intrapulmonary administration of Ins–SLNs, physical mixture of insulin PBS and free SLNs, insulin PBS (20 IU/kg) as well as PBS and subcutaneous administration of insulin PBS were shown in Fig. 8, and the pharmacodynamic parameters were listed in Table 1.

As shown in Fig. 8A and Table 1, significant difference in plasma glucose reduction (percentage relative to the initial value) between Ins-PBS and Ins–SLNs group could be observed at all the time points ($p < 0.05$), except 0.5 h after administration. As the threshold of optimal hypoglycemic effect, 70% level of initial glucose was employed as the parameter to estimate long-acting property (Park et al., 2007), and the time spans of pulmonary administration of Ins–SLNs or physical mixture, 17

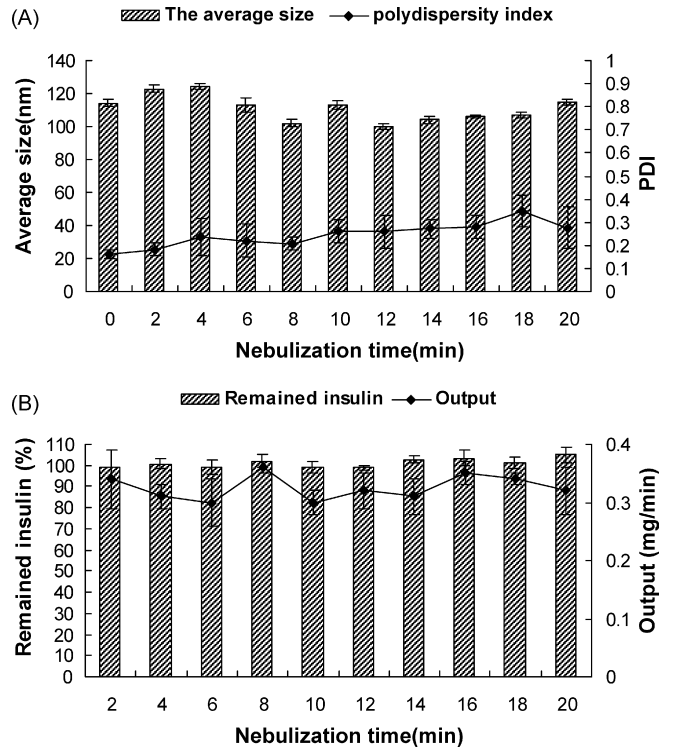

Fig. 6. Changes in average size and polydispersity index (A), concentration and output of Ins–SLNs (B) during nebulization ($n = 3$).

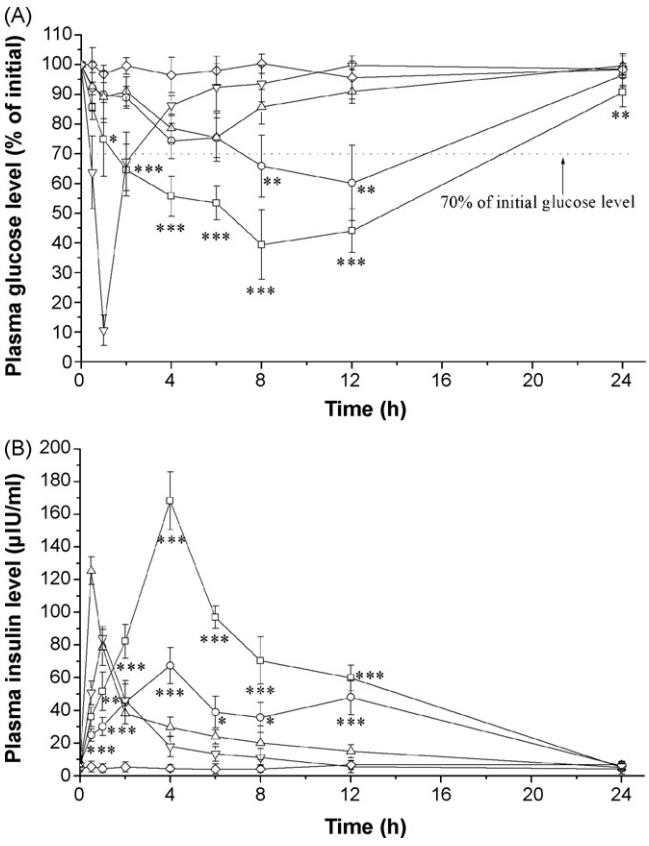

Fig. 8. Plasma glucose–time profile (A) and plasma insulin–time profile (B) following subcutaneous injection of 1 IU/kg insulin PBS (▽–▽), nebulization of PBS (◇–◇), 20 IU/kg physical mixture of insulin PBS and blank SLNs (◯–◯), 20 IU/kg Ins–SLNs (□–□), 20 IU/kg insulin PBS (△–△).

and 8.25 h, respectively, were much longer than Ins-PBS solution (1.75 h).

Blank SLNs showed either negligible or insignificant hypoglycemic activities, while the subcutaneous injection (s.c.) group were characterized by an acute and relatively short hypoglycemic effect 1 h after administration. Compared with s.c. group, a rapid and sustained hypoglycemic effect was found in rats after forced-inhalation of Ins–SLNs (20 IU/kg), a minimum blood glucose concentration (%MBGC) of $39.41 \pm 11.71\%$ was reached within 8 h and this continued to 12 h, and the plasma glucose levels returned to basal levels during 12–24 h. For the control group, the glucose levels were slightly reduced, to a nadir of $75.53 \pm 8.10\%$ of initial glucose level. These results demonstrated a strikingly higher effectiveness of Ins–SLNs, nev-

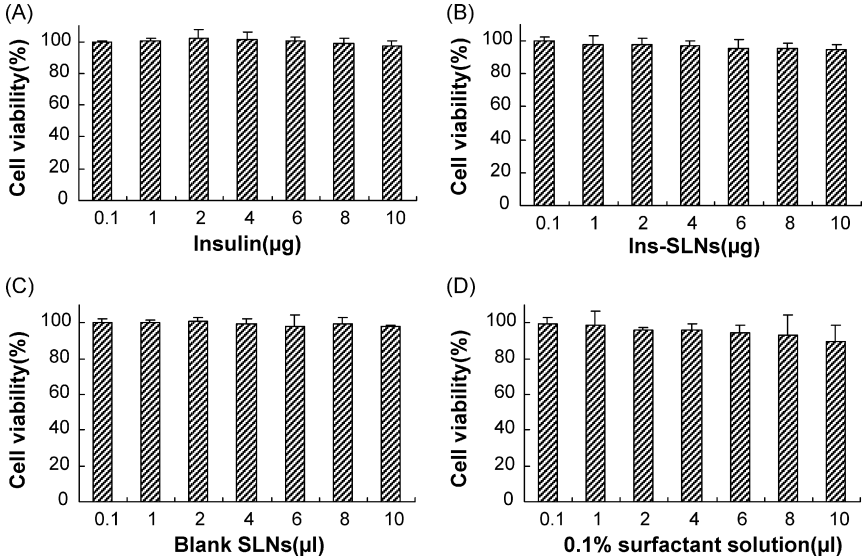

Fig. 7. Cytotoxicity of insulin (A), Ins–SLNs (B), blank SLNs (C) and 0.1% surfactant solution (F68 solution) (D) in A549 cells by MTT-assay. The concentrations of blank SLNs and surfactant solution were equal to the concentration in corresponding drug-loading nanoparticles.

Table 1
Pharmacodynamic and pharmacokinetic parameters after s.c. and pulmonary administration of insulin formulations in diabetes fasting rats ($n = 6$)

	Ins–SLNs (neb.)	Physical mixture (neb.)	Insulin PBS (neb.)	Insulin PBS (s.c.)
Dose (IU/kg)	20	20	20	1
%MBGC[a]	$39.41 \pm 11.71^{***,\Delta}$	$60.14 \pm 12.65^{*}$	75.53 ± 8.10	10.57 ± 5.10
T%MBGC[b](h)	8	12	4	1
Time[c] (h)	1.5–18.5	7.25–15.5	–	0.5–2.25
$AAC_{0-24\,h}$(%·h)	945.5 ± 131.7	$570.74 \pm 89.$	234.4 ± 53.3	194.4 ± 19.9
PA (%)	$24.33 \pm 3.39^{***,\Delta\Delta\Delta}$	$14.69 \pm 2.32^{***}$	6.03 ± 1.37	100
C_{max} (μIU/ml)	$168.27 \pm 17.67^{***,\Delta\Delta\Delta}$	67.46 ± 11.12	125.43 ± 8.41	84.20 ± 7.09
T_{max} (h)	4	4	0.5	1
MRT (h)	7.70 ± 0.36	$8.95 \pm 0.56^{***}$	7.40 ± 0.46	5.90 ± 1.75
AUC (μIU/ml h)	$1427.5 \pm 106.1^{***,\Delta\Delta\Delta}$	$838.1 \pm 118.2^{***}$	$453.\ 8 \pm 57.9$	319.7 ± 41.3
F_R(%)	$22.33 \pm 1.66^{***,\Delta\Delta\Delta}$	$13.10 \pm 1.85^{***}$	7.10 ± 0.91	100

Each value represents the mean ± S.D. ($n = 6$). Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 20 IU/kg insulin PBS (neb.); $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ and $^{\Delta\Delta\Delta}p < 0.001$, vs. 20 IU/kg physical mixture of insulin PBS and blank SLNs (neb.).

[a] %MBGC: the percent of minimum blood glucose concentration.

[b] T%MBGC: the time required to attain %MBGC.

[c] Time: the time during which less than 70% of blood glucose is held.

ertheless, coadministering insulin and free SLNs also showed enhancement of insulin adsorption, which exhibited a first reduction to 74.33 ± 5.92% in the initial phase (4 h) and a second nadir of 60.14 ± 12.65% (12 h). The possible reason for this fact might be a 'second phase' release of insulin induced by the dissociation of insulin absorbed on the surface of SLNs.

In the presence of SLNs, the pharmacological bioavailability of insulin in lungs was remarkably enhanced. Either pulmonary delivery of Ins–SLNs or coadministration of Ins-PBS and blank SLNs exhibit a relatively high pharmacological bioavailability, 24.33 ± 3.39% and 14.69 ± 2.32%, respectively, with statistical significance compared with Ins-PBS (Table 1).

3.6. Pharmacokinetic studies

The absorption of insulin was detected by measuring plasma insulin concentration. The kinetics of insulin absorption was illustrated in Fig. 8B and the pharmacokinetic parameters were

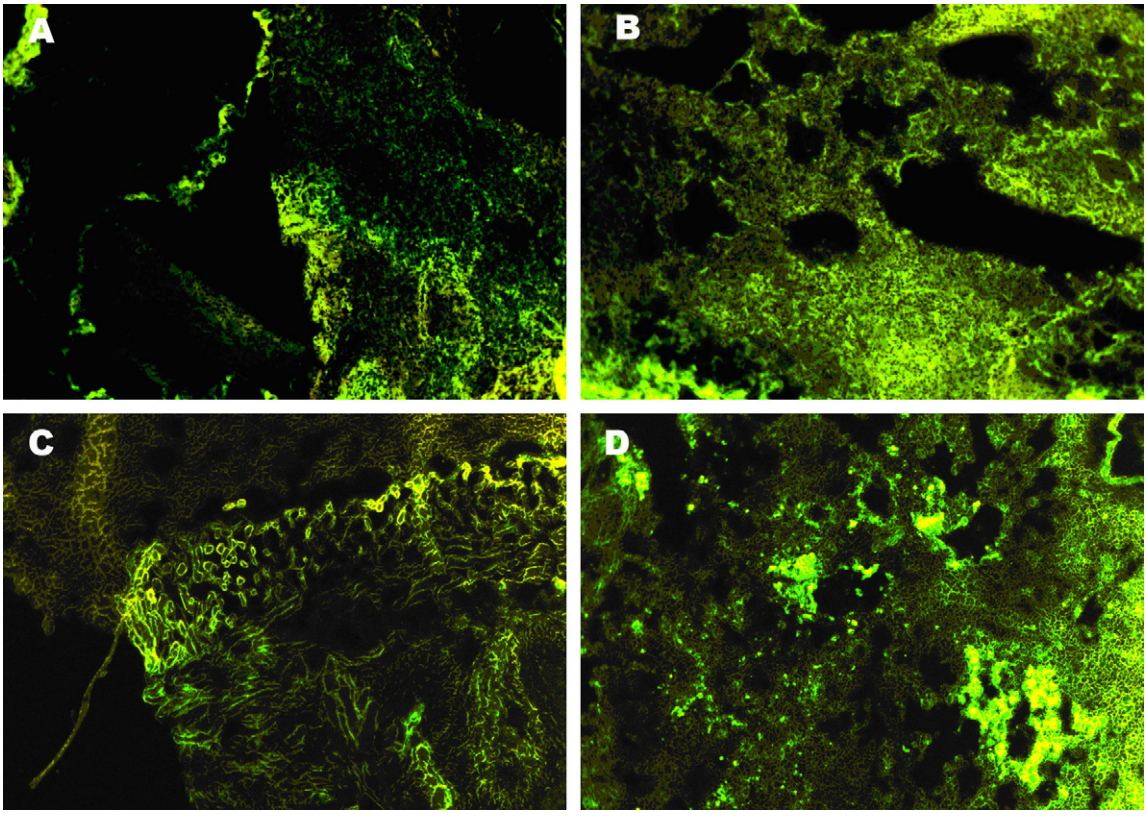

Fig. 9. Fluorescence micrographs of the cryosections of rat lung (A) 30 min and (C) 1 h after nebulization of FITC–Ins–SLNs; (B) 30 min and (D) 1 h after nebulization of FITC–Ins-PBS. Original magnification 200×.

listed in Table 1. Plasma insulin concentration raised dramatically after administration of Ins–SLNs and reached a maximum value close to 170 μIU/ml within 4 h, which was significantly higher than any other groups. Coadministration of insulin and free vesicles exhibited a similar absorption pattern at 4 h with a peak value of 67.46 ± 11.12 μIU/ml, but another crest value could be observed at 12 h, which was in accordance with the result of glucose measurement. Administration of plain insulin solution showed a rapid increase in plasma insulin level, however, the absorption effect was transient followed by a stiff drop.

A very significant difference ($p < 0.001$) in F_R was noted between insulin alone in PBS and Ins–SLNs, a similar difference also existed between insulin in PBS and insulin combined with PBS. Nevertheless, significant difference ($p < 0.001$) was found between SLNs entrapped insulin and insulin with blank SLNs.

A delay of drug absorption after pulmonary administration of either Ins–SLNs or insulin with blank SLNs was shown in plasma insulin concentration profile, the MRT was approximately 8 and 9 h, respectively. The long mean absorption time (MAT) may imply that insulin clearance in lungs is slow, probably due to tissue binding.

3.7. Fate of FITC–Ins–SLNs in the lung

The local fate of FITC–Ins–SLNs in the alveolar region of rats was visualized by fluorescence microscopy after nebulization of a solution of FITC–Ins–SLNs, and FITC–Ins PBS was adopted as control. Thirty minutes after delivery, both FITC–Ins and SLNs appeared to homogeneously spread on alveolar surfaces. And points with strong green fluorescence could be seen in Fig. 9A and B, with light density of 7834.76 ± 14.78 and 6313.5 ± 27.92 ODS unit, indicating that either protein or carrier diffused extensively in the alveolar tissue, while the intensity of fluorescence of FITC-labelled insulin solution was stronger than FITC–Ins-loaded SLNs, which might be attributed to the extremely high entrapment efficiency and shell–core structure of FITC–Ins–SLNs.

One hour later, the intensity of the green fluorescence decreased, from 6313.5 ± 27.92 to 939.95 ± 14.90 ODS unit, but FITC–Ins–SLNs remained clearly visible in the interstitium (Fig. 9C). In contrast, rather than occupy extracellular fluid spaces within the interstitium, obvious aggregations of fluorescent points could be observed in FITC–Ins treated group (Fig. 9C and D), with light density of 2917.04 ± 18.64 ODS, which might be due to association of FITC–Ins with fiber network and possible interaction with parenchymal septal fibers.

These results suggested that aggregations of FITC–Ins formed in the lung after pulmonary administration might hinder the effective transport to systemic circulation.

Further, continuous presentation of protein in the airspaces might be more susceptible to local degradation processes which might lead to reduction in bioavailability. Therefore, SLNs could enhance the *in vivo* stability of proteins by facilitating the transmembrane transport as well as preventing the formation of protein aggregates.

4. Discussion

During last a few decades, liposomes have been extensively employed as carriers for pulmonary delivery of polypeptides and proteins (Desai et al., 2002; Johannson et al., 2002). However, the stability concerns and the leakage problems during inhalation restricted their further applications. In order to overcome aforementioned problems, polymeric nanoparticles were also used for pulmonary delivery of insulin, nevertheless, cytotoxicity of the polymers is a great concern and an often discussed aspect. Therefore, increasing attention has been focused on SLNs, which might combine the merits of liposomes and nanoparticles and exhibit as a potential carrier for pulmonary delivery. Some promising results were obtained by SLNs-mediated pulmonary delivery for the treatment of local diseases, however, to the best of our knowledge, SLNs have seldom been used for systemic delivery of drugs *via* pulmonary route.

In the present study, SC and SPC were employed in the preparation of Ins–SLNs for several considerations: (1) they could facilitate the preparation of insulin–SLNs with a satisfactorily high entrapment efficiency and drug loading rate through formation of a reverse-micelle complex (which was explained in details in our previous report (Liu et al., 2007)); (2) as mentioned above, with the increase in the amount of SC and SPC, the rigidity of the nanoparticles could be reduced with SC as a deformation agent and SPC to decrease Tc, which resulted in the improvement of both *in vitro* and *in vivo* deposition; (3) both SC and SPC have been commonly employed as absorption enhancers in pulmonary drug delivery, and a significant increase in bioavailability of the inhaled proteins was observed in some published literatures (Johannson et al., 2002; Heinemann et al., 2000; Li and Mitra, 1996; Sakagami, 2006; Liu et al., 1993). Despite of the potential clinical disadvantage of sodium cholate, the applied concentration of SC (2.2 mM) was considerably lower than that in some previous studies (Johannson et al., 2002), and no obvious cytotoxicity was observed. However, the safety and feasibility for chronic application need further investigation.

For investigating the *in vivo* deposition of the drug after pulmonary administration, some sophisticated lung-dosing methods, such as forced instillation, microspray, nebulization and aerosol puff have been employed in past studies. And among these approaches, forced instillation and nebulization are principal lung-dosing methods used to study lung absorption and disposition of inhaled candidates in small rodents. Intratracheal instillation is technically simple and quick, requiring only small amounts of drugs and enabling accurate dosing, however, it is important to note that minor methodological differences in this technique might cause substantial variations in the lung-regional distribution and thereby, in the systemic drug profiles (Eljamal et al., 1996). Colthorpe's finding demonstrated that a preferential central deposition was evident following intratracheal instillation administration, which resulted in greater extent of insulin removal by mucociliary clearance than nebulization (Colthorpe et al., 1992). Neither anesthesia nor surgery is required, nose-only aerosol exposure, a common method in

inhalation toxicology (Eljamal et al., 1996), which could achieve deep-lung delivery in a physiologically natural fashion has been employed successfully for the kinetic assessment of lung absorption in some previous reports (Huang and Wang, 2006; Colthorpe et al., 1992; Choi et al., 2001), therefore, it was used as pulmonary administration method in our study. Furthermore, as a non-invasive approach, this administration pattern did not affect the measurement of plasma glucose level, which might provide more accurate pharmacodynamic results than other invasive strategies. However, the bioavailability was usually lower than that obtained by forced instillation. The aerosol delivery of insulin involves some losses of hormone that do not occur with injection. Liquid nebulizers are less efficient, for example, Laube et al. (1993) measured a nebulizer efficiency of 27% in one of their insulin studies and the bioavailability was corrected in a published report (Sakr, 1992). However, the bioavailability was not corrected in our study for two reasons, on the one hand, the same administration method was adopted in both positive control groups (insulin PBS and insulin plus blank SLNs) and reference group (insulin PBS *via* s.c.), on the other hand, it was difficult to eliminate the drug loss in the device and in the upper respiratory tract of the rats.

In our study, both pharmacodynamic response and pharmacokinetic analysis were used to investigate the absorption of insulin from different formulations. The merits of using pharmacodynamic response data stem from the ease of plasma glucose determination. In addition, this parameter is most relevant to therapeutic effects. However, pharmacological bioavailability calculations could not replace the pharmacokinetic studies (Li and Mitra, 1994). Because the fasting plasma glucose levels in the Ins–SLNs group did not return to the initial level after 24 h, which might be caused by dual effects of hunger and hypoglycemic agents and this problem has also been encountered in some other studies (Suzuki et al., 1998; Morçöl et al., 2004; Cui et al., 2006), thus a pharmacokinetic/pharmacodynamic (PK/PD) analysis was necessary for accurate determination of bioavailability of the formulations. The PK and PD results were in good consistency with a PA slightly higher than F_R, which might be attributed to the synergetic effect of anti-hyperglycemic agent and starvation. And it is worth to mentioning that, the *in vivo* findings were a little bit inconsistent with a previous report on pulmonary uptake of insulin liposomes. A similar insulin uptake and pharmacodynamic response were obtained after administration of non-liposomally and liposomally entrapped insulin in this study, however, it is not the case in our study. This discrepancy might be caused by the different lipid concentration and stabilization effects of two types of nanocarriers in two studies.

5. Conclusions

In this study, Ins–SLNs with desired deposition properties for pulmonary delivery were developed and optimized. The results of stability tests of Ins–SLNs including measurement of the size, morphology of nanospheres as well as the insulin concentration before and after nebulization demonstrated good tolerance to the nebulization course. No obvious cytotoxicity was found by MTT-assay, which suggested the safety of SLNs for pulmonary delivery at cell level. Fasting plasma glucose level in rats could be effectively reduced after pulmonary delivery of insulin using SLNs as carrier. Nanocarriers were homogeneously distributed in the lung alveoli and a prolonged release of insulin was observed in both plasma insulin and glucose profiles. A pharmacological bioavailability of 24.33% and a relative bioavailability of 22.33% were obtained after inhalation of Ins–SLNs, which were almost 4-folds higher than the control. Our results indicated that SLNs could be successfully applied as pulmonary carrier for insulin, which might provide valuable solutions to the currently unmet medical needs for systemic delivery of proteins.

Acknowledgements

The authors would like to thank Prof. Fuqiang Wen and Dr. Dan Xu for providing us the jet nebulizer to carry out the work and we would also like to extend our gratitude to Mr. Cong Chen for his kindly help and great efforts in preparation of TSI. This work was funded by the National Basic Research Program of China (973 Program, No. 2007CB935801).

References

Adjei, A., Gupta, P., 1994. Pulmonary delivery of therapeutic peptides and proteins. J. Control. Release 29, 361–373.

Almeida, A.J., Souto, E., 2007. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Adv. Drug Deliv. Rev. 59, 478–490.

Callaghan, C., O'Barry, P.W., 1997. The science of nebulised drug delivery. Thorax 52, S31–S44.

Cevc, G., 1996. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 13, 257–388.

Chao, Y.C., Chang, S.F., Lu, S.C., Hwang, T.C., Hsieh, W.H., Liaw, J.H., 2007. Ethanol enhanced in vivo gene delivery with non-ionic polymeric micelles inhalation. J. Control. Release 118, 105–117.

Chattopadhyay, P., Shekunov, B.Y., Yim, D., Cipolla, D., Boyd, B., Farr, S., 2007. Production of solid lipid nanoparticle suspensions using supercritical fluid extraction of emulsions (SFEE) for pulmonary delivery using the AERx system. Adv. Drug Deliv. Rev. 59, 444–453.

Choi, W.S., Murthy, G.G.K., Edwards, D.A., Langer, R., Klibanov, A.M., 2001. Inhalation delivery of proteins from ethanol suspensions. Proc. Natl. Acad. Sci. 98, 11103–11107.

Çilek, A., Çelebi, N., Tırnaksız, F., Tay, A., 2005. A lecithin-based microemulsion of rh-insulin with aprotinin for oral administration: investigation of hypoglycemic effects in non-diabetic and STZ-induced diabetic rats. Int. J. Pharm. 298, 176–185.

Colthorpe, P., Farr, S.J., Taylor, G., Smith, I.J., Wyatt, D., 1992. The pharmacokinetics of pulmonary-delivered insulin: a comparison of intratracheal and aerosol administration to the rabbit. Pharm. Res. 9, 764–768.

Cui, F.D., Shi, K., Zhang, L.Q., Tao, A.J., Kawashima, Y., 2006. Biodegradable nanoparticles loaded with insulin–phospholipid complex for oral delivery: preparation, in vitro characterization and in vivo evaluation. J. Control. Release 114, 242–250.

Dahab, R.A., Schäfer, U.F., Lehr, C.M., 2001. Lectin-functionalized liposomes for pulmonary drug delivery: effect of nebulization on stability and bioadhesion. Eur. J. Pharm. Sci. 14, 37–46.

Dalby, R.N., Tiano, S.L., 1993. Pitfalls and opportunities in the inertial sizing and output testing of nebulizers. Pharm. Technol. 17, 144–156.

Dailey, L.A., Schmehl, T., Gessler, T., Wittmar, M., Grimminger, F., Seeger, W., Kissel, T., 2003. Nebulization of biodegradable nanoparticles: impact of nebulizer technology and nanoparticle characteristics on aerosol features. J. Control. Release 86, 131–144.

Damgé, C., Maincent, P., Ubrich, N., 2007. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. J. Control. Release 117, 163–170.

Desai, T.R., Hancock, R.E.W., Finlay, W.H., 2002. A facile method of delivery of liposomes by nebulization. J. Control. Release 84, 69–78.

Elhissi, A.M.A., Faizi, M., Naji, W.F., Gill, H.S., Taylor, K.M.G., 2007. Physical stability and aerosol properties of liposomes delivered using an air-jet nebulizer and a novel micropump device with large mesh apertures. Int. J. Pharm. 334, 62–70.

Eljamal, M., Nagarajan, S., Patton, J.S., 1996. In situ and in vivo methods for pulmonary delivery. In: Borchardt, R.T., Smith, P.L., Wilson, G. (Eds.), Models for Assessing Drug Absorption and Metabolism. Plenum Press, New York, NY, pp. 361–374 (Chapter 19).

European Pharmacopeia, 2004. Council of Europe, 5th ed. Strasbourg, France, pp. 2799–2811.

Gupta, P.K., Hickey, A.J., 1991. Contemporary approaches in aerosolized drug delivery to the lung. J. Control. Release 17, 129–148.

Hallworth, G.W., Westmorel, D.G., 1987. The twin impinger: a simple devise for assessing the delivery of drugs from metered dose pressurized aerosol inhalers. J. Pharm. Pharmacol. 39, 966–972.

Heinemann, L., Klappoth, W., Rave, K., Hompesch, B., Linkeschowa, R., Heis, T., 2000. Intra-individual variability of metabolic effect of inhaled insulin together with an absorption enhancer. Diabetes Care 23, 1343–1347.

Huang, Y.Y., Wang, C.H., 2006. Pulmonary delivery of insulin by liposomal carriers. J. Control. Release 113, 9–14.

Hussain, A., Arnold, J.J., Khan, M.A., Ahsan, F., 2004. Absorption enhancers in pulmonary protein delivery. J. Control. Release 94, 15–24.

Johannson, F., Hjertberg, E., Eirefelt, S., Tronde, A., Bengtsson, U.H., 2002. Mechanisms for absorption enhancement of inhaled insulin by sodium taurocholate. Eur. J. Pharm. Sci. 17, 63–71.

Kanaoka, E., Nagata, S., Hirano, K., 1999. Stabilization of aerosolized IFN-γ by liposomes. Int. J. Pharm. 188, 165–172.

Karathanasis, E., Ayyagari, A.L., Bhavane, R., Bellamkonda, R.V., Annapragada, A.V., 2005. Preparation of in vivo cleavable agglomerated liposomes suitable for modulated pulmonary drug delivery. J. Control. Release 103, 159–175.

Kawashima, Y., Yamamoto, H., Takeuchi, H., Fujioka, S., Hino, T., 1999. Pulmonary delivery of insulin with nebulized DL-lactide/glycolide copolymer (PLGA) nanospheres to prolong hypoglycemic effect. J. Control. Release 62, 279–287.

Kumar, T.R., Soppimath, K., Nachaegari, S.K., 2006. Novel delivery technologies for protein and peptide therapeutics. Curr. Pharm. Biotechnol. 7, 261–276.

Laube, B.L., Georgopoulus, A., Adams, G.K., 1993. Preliminary study of the efficacy of insulin aerosol delivered by oral inhalation in diabetic patients. J. Am. Med. Assoc. 269, 2106–2107.

Li, M.G., Lu, W.L., Wang, J.C., Zhang, X., Wang, X.Q., Zheng, A.P., Zhang, Q., 2007. Distribution, transition, adhesion and release of insulin loaded nanoparticles in the gut of rats. Int. J. Pharm. 329, 182–191.

Li, Y.P., Mitra, A.K., 1994. A simple method of correlating pharmacodynamic equivalence with absolute bioavailability following noninvasive delivery of insulin. Pharm. Res. 11, 1505–1508.

Li, Y.P., Mitra, A.K., 1996. Effects of phospholipids chain length, concentration, charge, and vesicle size on pulmonary insulin absorption. Pharm. Res. 13, 76–79.

Liu, F., Shao, Z., Kildsig, D.O., Mitra, A.K., 1993. Pulmonary delivery of free and liposomal insulin. Pharm. Res. 10, 228–232.

Liu, J., Gong, T., Wang, C.G., Zhong, Z.R., Zhang, Z.R., 2007. Solid lipid nanoparticles loaded with insulin by sodium cholate-phosphatidylcholine-based mixed micelles: preparation and characterization. Int. J. Pharm. 340, 153–162.

Malcolmson, R.J., Embleton, J.K., 1998. Dry powder formulations for pulmonary delivery. Pharm. Sci. Technol. Today 1, 298–394.

Malik, D.K., Baboota, S., Ahuja, A., Hasan, S., Ali, J., 2007. Recent advances in protein and peptide drug delivery systems. Curr. Drug Deliv. 4, 141–151.

Matilainen, L., Järvinen, K., Toropainen, T., Näsi, E., Auriola, S., Järvinen, T., Jarho, P., 2006. In vitro evaluation of the effect of cyclodextrin complexation on pulmonary deposition of a peptide, cyclosporin A. Int. J. Pharm. 318, 41–48.

McCallion, O.N.M., Taylor, K.M.G., Thomas, M., Taylor, A.J., 1995. Nebulization of fluids of different physicochemical properties with air-jet and ultrasonic nebulizers. Pharm. Res. 12, 1682–1688.

McCallion, O.N.M., Taylor, K.M.G., Thomas, M., Taylor, A.J., 1996. The influence of surface tension on aerosols produced by medical nebulisers. Int. J. Pharm. 129, 123–136.

Mendes, P.J., Pinto, J.F., Sousa, J.M.M., 2007. A non-dimensional functional relationship for the fine particle fraction produced by dry powder inhalers. Aerosol Sci. 38, 612–624.

Morçöl, T., Nagappan, P., Nerenbaum, L., Mitchell, A., Bell, S.J.D., 2004. Calcium phosphate–PEG–insulin–casein (CAPIC) particles as oral delivery systems for insulin. Int. J. Pharm. 277, 91–97.

Morishita, M., Goto, T., Nakamura, K., Lowman, A.M., Takayama, K., Peppas, N.A., 2006. Novel oral insulin delivery systems based on complexation polymer hydrogels: single and multiple administration studies in types 1 and 2 diabetic rats. J. Control. Release 110, 587–594.

Nguyen, H., Bouwstra, J.A., 2005. Vesicles as tool for transdermal and dermal delivery. Drug Discov. Today: Technol. 2, 67–74.

Niven, R.W., Speer, M., Schreier, H., 1991. Nebulization of liposomes. II. The effects of size and modeling of solute release profiles. Pharm. Res. 8, 217–221.

Pandey, R., Khuller, G.K., 2005. Solid lipid particle-based inhalable sustained drug delivery against experimental tuberculosis. Tuberculosis 85, 227–234.

Park, S.H., Kwon, J.H., Lim, S.H., Park, H.W., Kim, C.W., 2007. Characterization of human insulin microcrystals and their absorption enhancement by protease inhibitors in rat lungs. Int. J. Pharm. 339, 205–212.

Patton, J.S., Fishburn, C.S., Weers, J.A., 2004. The lungs as a portal of entry for systemic drug delivery. Proc. Am. Thorac. Soc. 1, 338–344.

Roche, N., Huchon, G.J., 2000. Rationale for the choice of an aerosol delivery system. J. Aerosol Med. 13, 393–404.

Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Müller, R.H., Rosenecker, J., 2004. Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. Pharm. Res. 21, 1662–1669.

Sakagami, M., 2006. In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. Adv. Drug Deliv. Rev. 58, 1030–1060.

Sakr, F.M., 1992. A new approach for insulin delivery via the pulmonary route: design and pharmacokinetics in non-diabetic rabbits. Int. J. Pharm. 86, 1–7.

Scheuch, G., Kohlhaeufl, M.J., Siekeier, P.B.R., 2006. Clinical perspectives on pulmonary systemic and macromolecular delivery. Adv. Drug Deliv. Rev. 58, 996–1008.

Sun, X., Zhang, Z.R., 2004. Optimizing the novel formulation of liposome–polycation–DNA complexes (LPD) by central composite design. Arch. Pharm. Res. 27, 797–805.

Suzuki, A., Morishita, M., Kajita, M., 1998. Enhanced colonic and rectal absorption of insulin using the multiple emulsion containing eicosapentaenoic acid and docosahexaenoic acid. J. Pharm. Sci. 87, 1196–1202.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990. The stability of liposomes to nebulisation. Int. J. Pharm. 58, 57–61.

Videira, M.A., Botelho, M.F., Santos, A.C., Gouveia, L.F., de Lima, J.J., Almeida, A.J., 2002. Lymphatic uptake of pulmonary delivered solid lipid nanoparticles. J. Drug Target. 10, 607–613.

Waldrep, J.C., Scherer, P.W., Keyhani, K., Knight, V., 1993. Cyclosporin A liposome aerosol: particle size and calculated respiratory deposition. Int. J. Pharm. 97, 205–212.

Zaru, M., Mourtas, S., Klepetsanis, P., Fadda, A.M., Antimisiaris, S.G., 2007. Liposomes for drug delivery to the lungs by nebulization. Eur. J. Pharm. Biopharm. 67, 655–666.

Zhang, Q., Shen, Z.C., Nagai, T., 2001. Prolonged hypoglycemic effect of insulin-loaded polybutylcyanoacrylate nanoparticles after pulmonary administration to normal rats. Int. J. Pharm. 218, 75–80.