

ORIGINAL ARTICLE

The preparation and application of pulmonary surfactant nanoparticles as absorption enhancers in insulin dry powder delivery

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Abstract

Aim: To study the preparation and application of pulmonary surfactant (PS) nanoparticles as absorption enhancers in insulin dry powder delivery. PS is a complex mixture of mainly phospholipids and proteins. The composition leads to a unique spreading effect of the surfactants as well as spontaneous nanoparticles formation, which may be favorable characteristics of pulmonary drug delivery systems. **Method:** In this article, insulin has been a model drug, as an important drug in lowering blood glucose and as a classic protein drug. PS was extracted from pig lungs and was observed as nanoparticles morphology in water by transmission electron microscopy after preparation. Inhalation dry powders were formulated by PS nanoparticles, insulin, and other excipients. In addition, blood glucose level of rats was determined when the insulin dry powder of PS group and control group was delivered by inhalation route. **Result:** Compared with control group, scanning electron microscopy showed that the dry powders of PS group possess similar particle size and smoother surface. The spray-dried powders re-suspended possessed similar nanoparticle appearance compared with the original PS suspension. Animal studies showed that blood glucose level was effectively reduced when the insulin dry powder of PS group was delivered by inhalation route. Compared with subcutaneous injection group (4 U/kg), the relative pharmacological bioavailability of the insulin dry powders of control group (14 U/kg) and PS group (14 U/kg) was 19.97% and 32.57%, respectively. **Conclusion:** PS nanoparticles, as absorption enhancers used in dry powder inhalation, could evidently enhance the effect of insulin in lowering blood glucose.

Key words: Dry powder; insulin; nanoparticle; pulmonary delivery; pulmonary surfactant

Introduction

Pulmonary delivery is known to be an important new route of noninvasive systemic administration¹. A number of drugs that are poorly absorbed from enteral and other topical sites are well absorbed from lung because of the large surface area and high permeability of the alveolar epithelium. Efficient absorption from the lung results from unique anatomical features of the alveoli as well as from aerosol generation systems that successfully bypass particle filtering in upper airways. The alveoli provide a 100 m² surface for absorption, a 0.5 µm diffusion path to the bloodstream, and a dense capillary network that allows passage of 5 L of blood per minute. However, the bioavailability of some macromolecules including peptide and protein drugs, which are susceptible to

hydrolysis in lung tissues, from the pulmonary route, is still poor when compared with the injection route. As an important for lowering blood glucose and as a classic protein drug, insulin has been researched and developed far and wide. Exubera[®], an inhaled powder form of recombinant human insulin (rDNA) for the treatment of adult patients with type 1 and type 2 diabetes, was approved by FDA on January 27, 2006. But in October 2007, Pfizer announced that it would be discontinuing the production and sale of Exubera[®] because of poor sales. The drug failed to gain acceptance among patients and physicians because of three important reasons: (1) the heavy and large administration equipment², (2) poor bioavailability [about 10% compared with subcutaneous (SC)]³, (3) insufficient security data^{3,4}. Therefore, safety and high efficacy of preparation would be focal

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points of insulin pulmonary delivery. Pharmaceutical necessities such as absorption enhancers and protease inhibitors are usually required to enhance the pulmonary absorption of such macromolecular drugs. These absorption enhancers have been reported to include surfactants, bile salts, fatty acids, sodium hyaluronate, cyclodextrins, polyamines, liposomes and phospholipids, and chitosan and its derivatives^{5–11}.

Pulmonary surfactant (PS) is a complex mixture of phospholipids and proteins, whose main function is to reduce the surface tension at the alveolar air–liquid interface of lungs to avoid alveolar collapse at the end of expiration and to facilitate the work of breathing. This material is synthesized by type II pneumocytes, one of the cell types forming the alveolar epithelium, and follows a regulated exocytic pathway leading to secretion into the thin aqueous layer covering the alveoli. PS is absolutely required for lung breathing. The lack, deficiency or inactivation of this essential material is the cause of severe respiratory disorders, sometimes lethal, such as the neonatal respiratory distress syndrome¹², or the pulmonary dysfunction associated with the acute respiratory distress syndrome, a disease related with different processes such as injury, inflammation, or sepsis¹³. The recommended dosages of all kinds of PS products are various, from 50–200 mg.

The phospholipids composition of PS of different species was reviewed by Veldhuizen *et al.*¹⁴ In general terms, mammalian PSs are composed of approximately 80% (weight of total material) of phosphatidylcholine, about half of which is dipalmitoyl phosphatidylcholine (DPPC). The protein moiety of PS comprises four specific surfactant-associated proteins¹⁵. They can be classified into two groups, the hydrophilic surfactant proteins, SP-A and SP-D, and the hydrophobic surfactant proteins, SP-B and SP-C. Both SP-A and SP-D belong to the collectin family of proteins. They are multimeric proteins with collagen-like domains and globular calcium-dependent carbohydrate-binding domains. These structural characteristics provide them the ability to bind many different ligands such as calcium, sugars, or phospholipids molecules in a concerted manner and made them appropriate to play an important role in the innate immune system¹⁶. Both SP-B and SP-C are very small hydrophobic polypeptides resulting from the proteolytic processing of larger precursors along the exocytic pathway of PS in type II pneumocytes. Each of them accounts for no more than 1–1.5% of total surfactant weight; despite their relatively low abundance, they play critical roles in the formation and stabilization of PS films¹⁷. SP-B is known as a mainly α -helical protein (40–45%), containing four to five amphipathic helices that allow it to interact with and perturb phospholipids membranes¹⁸. In type II pneumocytes, SP-B processing occurs along the exocytic pathway of surfactant, and it is

not completed until assembled in phospholipids membranes together with the other phospholipids and proteins¹⁹. SP-C is an integral membrane protein, which consists of an extremely hydrophobic transmembrane domain and a 10–12 amino acid extramembrane segment located at the N-terminus of the mature peptide¹⁸. These peptides from the N-terminal tail of SP-C seem to adopt an amphipathic conformation, with strong tendency to partition into the interface of phospholipids membranes and monolayers²⁰.

There were several preliminary researches on PS as a drug delivery system^{21,22}. However, there are not reports for PS in insulin dry powder applied on pulmonary delivery. In this study, PS as a novel pulmonary absorption enhancer was developed. PS was extracted from tissue homogenate of pig lungs. Dry powders were prepared by spray-drying and formed of insulin, PS, and other excipients. The scanning electron microscope (SEM) of dry powder of control group and PS group was investigated. The nanoparticles morphological characterization of PS suspension and dry powder resuspended were observed by transmission electron microscope. Furthermore, to clarify the absorption enhancing effect of PS, the blood glucose level of rats administrated with dry powder was detected. The aims of this work were (1) to observe the morphological characterization of PS extraction suspension *in vitro*, (2) to study whether the PS nanoparticles morphology changed after formulation of spray-dried, (3) to compare the blood glucose lowering efficiency of control group and PS group dry powders for pulmonary delivery of insulin and highlight a possible enhancer of dry powder excipients on insulin absorption.

Materials and methods

Materials

Pure crystalline porcine insulin was purchased from Xuzhou Wanbang Bio-Chemical Co. Ltd. (No. 0706A15, Jiangsu, China), with a nominal activity of 28 IU/mg. Double-distilled water was used for all solutions and dilution. All the other reagents were of analytical grade.

Extraction of pulmonary surfactant

Pig lungs were triturated in a mixer and the tissue fragments were washed in a physiological saline solution. The mixture was filtered and centrifuged several times in the following steps: (1) subjected to preliminary centrifugation at $1000 \times g$ at 4°C for 10 minutes to eliminate cellular fragments; (2) the supernatant liquor was then centrifuged at $6000 \times g$ at 4°C for 30 minutes. After that the solid precipitate was resuspended with physiological

saline solution and recentrifuged in the same method. This procedure was repeated twice. (3) The solid precipitate was resuspended with 0.8 M sucrose solution and recentrifuged at $6000 \times g$ at 4°C for 30 minutes. This procedure was repeated twice. The raw surfactant (solid precipitate) was washed twice by double-distilled water and extracted with 2:1 chloroform/methyl alcohol (v/v)²³. The organic phase was removed by rotary evaporation to obtain a PS concentration. The concentration was lyophilized as solid extract.

Preparation of pulmonary surfactant suspensions

PS was dissolved in 2:1 chloroform/methyl alcohol (v/v). In film shaking method, phospholipids film was first formed at the bottom of a rotary vacuum evaporator. Double-distilled water was used to hydrate the thin film. The particle size of PS suspension was reduced by probe ultrasonic. The suspension with ice-water bath was suspended with ultrasonic power of 180 W, working 2 seconds and an interval of 3 seconds, for a group of 20 times, this procedure was repeated three times. Subsequently, the suspension was filtered through 0.45- μm filters (two passes).

Characterizations of pulmonary surfactant nanoparticle suspension

The size distribution (mean diameter and polydispersity index) and zeta potential of the nanoparticle dispersions were measured by dynamic light scattering and laser Doppler electrophoresis, respectively, on a Zetasizer 3000 HSA (Malvern Instruments, Bergen op Zoom, the Netherlands), which enabled the mass distribution of particle size as well as the electrophoretic mobility to be obtained. Sizes quoted are the Z average mean (dz) for the nanoparticle hydrodynamic diameter (nm). Calculation of zeta potential (mV) was done by the instrument (from electrophoretic mobility). All measurements were preformed at 25°C .

PS nanoparticles were analyzed on negative stain electron microscopy using a JEM-1230 electron microscopy (Joel, Japan). PS suspension and the suspension of dry powders resuspended was diluted at 1 mg/mL with double-distilled water. A drop of suspension (1 mg/mL) was applied to carbon-coated grids, and after 2 minutes, the excess was drawn off with filter paper; uranyl acetate aqueous solution was used as a staining agent. The excess was eliminated with distilled water and the sample was analyzed by transmission electron microscope.

Formulation of spray-dried powders

Control group: mannitol (720 mg) and threonine (360 mg) was dissolved in double-distilled water (60 mL). Insulin

(30 mg) was dissolved in pH 8.0 NaOH solution (40 mL). The insulin was then diluted with the mannitol-threonine solution.

PS group: The solution was prepared as that of control group. The PS suspension prepared was added slowly to the solution and whisked.

Solution and suspension were spray-dried using a BÜCHI Mini Spray Dryer B-191 (Buchi, Flawil, Switzerland). The inlet temperature was fixed at 110°C . The aspiration was set at 100%. The atomizing air flow rate was set at 600 L/hour and the pump ran at 70% of its capacity (100% corresponds to 1.5 L/hour and 70% to 1.05 L/hour). Spray-dried powders were collected via a cyclone. Samples were stored under vacuum in a desiccator to protect them from moisture.

SEM images and particle size distributions of dry powders

SEM photographs of control group and PS group insulin dry powders were taken by an SEM (S-3000; Hitachi, Tokyo, Japan). The samples were mounted on metal plate and sputtered with gold to a thickness of 10–30 nm under 6×10^{-2} mbar using an Ion Sputter (E-1010, Hitachi, Japan).

Approximately 5 mg of the spray-dried powders were suspended in acetone. The particle size distributions of the dry powders were measured by laser diffraction (particle size analysis (Mastersizer 2000; Malvern Instruments, Malvern, UK).

In vivo dry powder pulmonary administration study

Sprague–Dawley rats (180–220 g, Experimental Animal Center of China Pharmaceutical University, Nanjing, China) were used. The animals were allowed ad libitum access to a standard diet and water except wherever indicated.

The rats were fasted but allowed free access to water for 10 hours before the experiments. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (25 mg/kg). A dry 18# blunted needle was inserted by mouth and throat until the principal bifurcation. Powders were administered by endotracheal route via a needle-tubing-syringe arrangement²⁴. Most of all dry powders were inhaled autonomously by rats. The remaining dry powders were accessorially administered by syringe-injected air. When rats aspirated, 1 mL air was injected rapidly, this procedure was repeated three times. Blood was drawn from the tail. Glucose concentration in the blood was determined by a glucose meter (ACCU-CHEK® Active; Roche Diagnostics, Mannheim, Germany).

To calculate relative pharmacological bioavailability (PA) of the dry powders of control group and PS group, a group of SC injections was administered at the same

time. The control group and PS group were administered at 14 U/kg, relative to the dosage of 4 U/kg of the SC injection group. The relative PA of dry powders was calculated from the area above the curve (AAC) in contrast with SC injection²⁵.

$$PA = \frac{AAC_{inh} \times Dose_{sc}}{AAC_{sc} \times Dose_{inh}} \times 100.$$

Results and discussions

Particle size, zeta potential, morphology of PS suspension, and the suspension of dry powder resuspended

PS suspension was diluted at 1 mg/mL with double-distilled water. The Z-average mean size of PS nanoparticle was 138.9 nm and the polydispersity index was 0.315 (Table 1). The suspension of dry powders resuspended was diluted at 1 mg/mL with double-distilled water. To compare with PS suspension, the Z-average mean size of the suspension of dry powders resuspended was 106.6 nm and the polydispersity index was 0.345.

The zeta potential is the electrostatic potential at the hydrodynamic slip plane and is characterized as having an electrical double layer consisting of the Stern layer and the diffuse layer. The pH of PS nanoparticles suspension and the suspension of dry powders resuspended were 7.78 and 7.79, respectively. Nanoparticle suspension containing PS (1 mg/mL) had a negative surface (zeta potential: -31.5 ± 1.6 mV), indicating that the nanoparticle is steady in suspension. The suspension of dry powders resuspended (1 mg/mL) had a similar negative surface (zeta potential: -28.8 ± 2.8 mV).

Figure 1 showed the morphological characterization of PS suspension and the suspension of dry powder resuspended. In two cases, the presence of spherical-shaped nanoparticles was predominant. To compare with PS suspension, the nanoparticles of dry powders resuspended possessed similar conformation and particle size. This result indicated that the process of dry powders formulation had not mangled the morphological characterization of PS nanoparticles.

Table 1. Characterization of PS suspension and the suspension of dry powder resuspended.

	Mean size (nm)	Polydispersity index	pH	Zeta potential (mV)
PS suspension	138.9	0.315	7.78	-31.5 ± 1.6
Suspension of dry powder resuspended	106.6	0.345	7.79	-28.8 ± 2.8

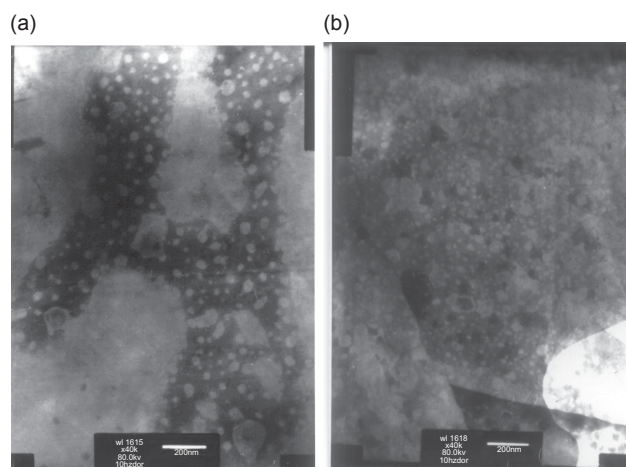


Figure 1. Transmission electron microphotographs of PS suspension. (a) PS suspension and (b) dry powders resuspended.

As has been elucidated in the foregoing, the surfactant protein comprises four specific surfactant-associated proteins. SP-B as the essential role of the protein to promote interfacial adsorption of surfactant has been demonstrated in many different in vitro assays. Some authors have suggested that SP-B and SP-C accelerate pulmonary adsorption by acting as a sort of catalysts that stabilize the high-energy intermediates required for the phospholipids to pass from the bilayers to the interface^{26,27}. Thus, PS extraction could form spherical-shaped nanoparticles easily in water with the two surfactant proteins.

The morphological characterization of dry powders

Formulated particles of control group were found to have a mean diameter of 1.79 μ m, with undersize diameters of d10% of 0.82 μ m, d50% of 1.79 μ m and d90% of 2.60 μ m. As compared with control group, PS group were found to have a mean diameter of 1.71 μ m with undersize diameters of d10% of 0.86 μ m, d50% of 1.71 μ m, and d90% of 2.50 μ m. The two groups showed similar particle size character (Table 2). The dry powders of PS group had a smoother surface than that of control group when visualized using an SEM (Figure 2).

It has been demonstrated that surface roughness related to the proportion of mannitol and amino acids

Table 2. Mean diameter of dry powders of control group and PS group.

Mean diameter (μ m)	Control group	PS group
d10%	0.82	0.86
d50%	1.79	1.71
d90%	2.60	2.50

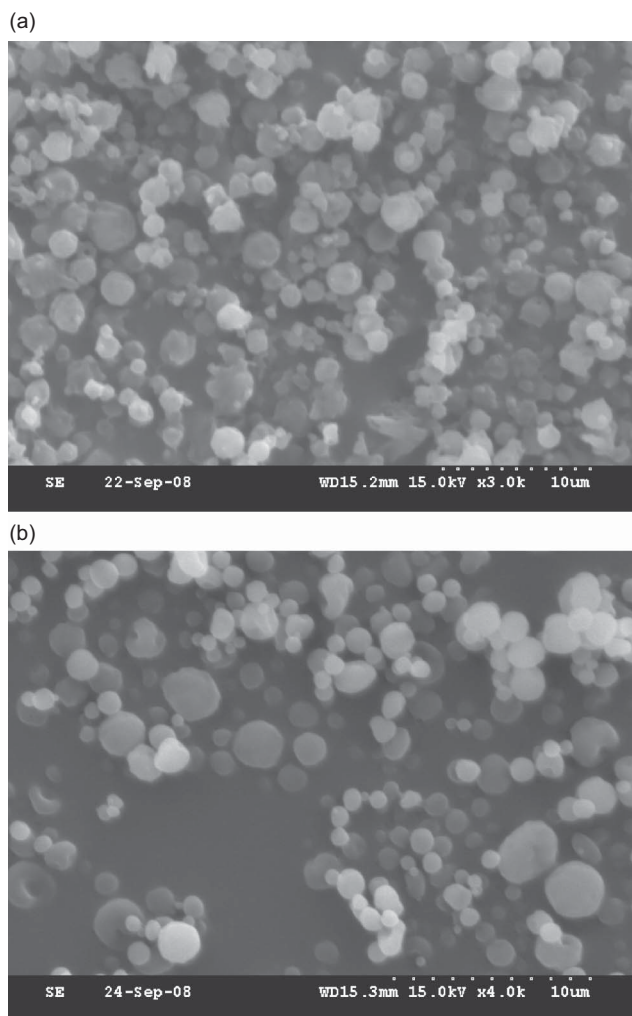


Figure 2. Scanning electron microphotographs of insulin dry powder. (a) Control group and (b) PS group.

in the formulations^{28,29}. As amino acid concentration was increasing, stickiness and cohesivity of the powders decreased. SEM images show that the increase in leucine concentration between 2% and 15% is correlated with an increase of surface roughness. When leucine percentage is above 15%, surface roughness does not seem to vary anymore³⁰. It has been reported that DPPC, the major lung surfactant, was added to the formulation prior to spray-drying^{31,32}. The influence of DPPC concentration has been evaluated: no effect on particle size has been observed for DPPC concentration up to 10%, but the use of this chemical considerably reduces particles solubility in water because of its high hydrophobicity. The granular appearance of the particle surface likely comes from threonine as particles only made of a sugar and DPPC had a smooth surface³³.

PS is a complex mixture of phospholipids and proteins, and its suspension possesses lower surface

tension. It has the tendency to go to liquid–air interface where it decreases the surface tension and consequently the size of the droplet detaching from the nozzle. As the low melting point of phospholipids, the dry powder could conform smoother surface in formulation of spray-dried.

Pulmonary pharmacodynamics study

Blood glucose level–time curve of inhaled insulin and insulin administered by SC injections were showed in Figure 3. The onset of the effect occurred coincided in rats receiving inhaled PS group (14 U/kg) compared with SC injection (4 U/kg). The control group were slightly slower than the other two groups. The t_{\max} values were 60, 60, and 90 minutes. Within 4 hours, blood glucose level of control group and SC group had basically returned to initial, and the corresponding blood glucose level of PS group was still about 80% of the initial value. The relative PA of control group and PS group was 19.97% and 32.57%, respectively, of that of SC-administered insulin. The AAC of PS group was 63.09% higher than that of control group.

The statistical analysis data of blood glucose level of control group and PS group at different time points are shown in Table 3. The data of two groups indicate that there is significant difference ($P < 0.05$) at 0.5, 1, 4 hours. The result suggests that the insulin dry powders of PS group could be faster and have longer effect on the lowering blood glucose level.

As mentioned above, compared with control group, PS group have significantly enhanced the effect of lowering blood glucose. The use of phospholipids has been suggested to provide sustained pulmonary release

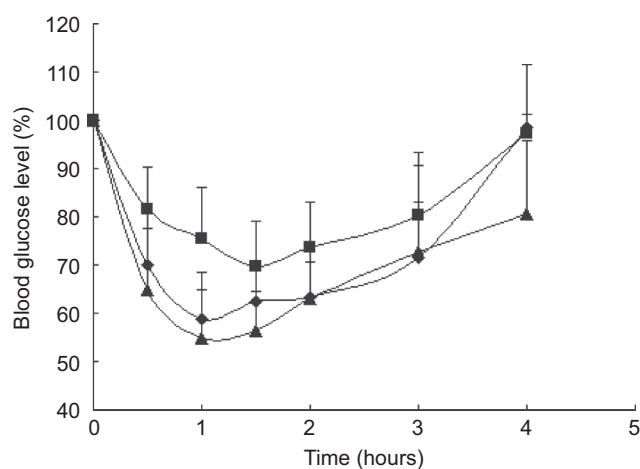


Figure 3. Blood glucose level–time curve of rats that received 4 U/kg SC free insulin (◆), 14 U/kg dry powders of control group (■), or 14 U/kg PS group (▲). Data are expressed as means ($n = 6$).

Table 3. Statistical significance probability of control group and PS group ($n = 6$).

Time (hour)	Control group	PS group	<i>P</i> value
0.5	-18.61 ± 9.34	-35.19 ± 13.46	0.0477
1	-24.68 ± 13.16	-45.21 ± 9.25	0.0187
1.5	-30.34 ± 13.74	-43.68 ± 8.28	0.110
2	-26.51 ± 9.78	-37.11 ± 7.14	0.074
3	-19.60 ± 15.50	-27.17 ± 14.00	0.264
4	-2.78 ± 3.85	-19.35 ± 15.25	0.041

for various drugs. Some authors' hypothesis is rather that the dry powder excipients and more particularly phospholipids might have membrane permeation enhancer properties. Exogenous phospholipids might alter the composition and/or organization of the surfactant mix lining the airway and/or alveolar epithelium and increase its permeability by mechanisms similar to those outlined for medium chain fatty acids and phospholipids^{34–37}. The essential role of the protein to promote interfacial adsorption of surfactant has been demonstrated in many different *in vitro* assays. Some authors have suggested that SP-B (and SP-C) accelerates adsorption by acting as a sort of catalyst that stabilizes the high-energy intermediates required for the phospholipids to pass from the bilayers to the interface^{38,39}.

In this study, as a complex mixture of phospholipids and proteins, PS nanoparticles applied dry powder delivery system and could evidently enhance the effect of lowering blood glucose of insulin.

Conclusions

There has been some research regarding PS as a drug delivery system in these years. The research mainly focused on preparing liquid suspension *in vitro*. Little research has been reported on PS applied to the dry powder inhalant. In this study, we have demonstrated three points: (1) PS could form stable phospholipids-protein complex nanoparticles in water. (2) The spray-dried powders resuspended possessed similar nanoparticles compared with the original PS suspension; the result suggested that the activity of surfactant was not mangled in the process of spray-drying. (3) The dry powder of PS group had a stronger and longer effect of lowering blood glucose relative to dry power of control group. It indicated that the PS nanoparticles can remarkably enhance the absorption of insulin in rat lung.

Therefore, PS nanoparticles showed a high potential to be applied to dry powder inhalant, and it could provide a clinically effective option as a new strategy for pulmonary macromolecule drug delivery.

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Declaration of interest: The authors report no conflicts of interest.

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