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RESEARCH PAPER

Inhalable Microparticles as Carriers for Pulmonary Delivery of Thymopentin-Loaded Solid Lipid Nanoparticles

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ABSTRACT

Purpose Microparticles containing solid lipid nanoparticles (SLNs) are receiving increased attention as carriers for the lung delivery of the SLNs. Thus, we aim to prepare the hybrid microparticles and thoroughly evaluate their feasibility for the pulmonary drug delivery.

Methods The microparticles were prepared by co-spray-drying the thymopentin (TP5)-loaded SLNs with bulking agents. Thereafter, we systematically estimated the potential of the microparticles as the carriers for the pulmonary delivery of the SLNs, including the investigations of their characteristics, aerodynamic properties, pharmacokinetics and pharmacodynamics.

Results The spherical and hollow microparticles presented a size of 4.1 ± 0.1 μm and a low tap density of 0.175 ± 0.02 g/cm^3. In addition, the microparticles showed a high aerosolization efficiency (emitted dose of $98.0\% \pm 1.23\%$ and respirable fraction of $51.07\% \pm 1.21\%$). Furthermore, the SLNs could be easily recovered from the microparticles without essential changes on their characteristics and the drug release behavior. The pharmacokinetic and pharmacodynamic studies suggested that, compared to i.v. TP5 solution, the bioavailability and therapeutic efficacy of TP5 were remarkably strengthened after the pulmonary administration of the microparticles.

Conclusions Taken together, we believe the microparticles were suitable for inhalation and possesed an ample potential for the pulmonary delivery of the SLNs.

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KEY WORDS pulmonary delivery · microparticles · solid lipid nanoparticles · spray-drying · thymopentin

ABBREVIATIONS

AUC	area under the curve
CLSM	confocal laser scanning microscope
ED	emitted dose
EE%	encapsulation efficacy
DPI	dry powder inhaler
FITC	fluorescein isothiocyanate
HPLC	high performance liquid chromatography
i.v.	intravenous injection
mAb	monoclonal Antibodies
MRT	mean residence time
PBS	phosphate buffer
PDI	polydispersity index
RD	respirable dose
RF	respirable fraction
SD	standard deviation
SEM	scanning electron microscopy
SLNs	solid lipid nanoparticles
SOD	superoxide dismutase
TP5	thymopentin
TSI	twin stage impinger
W/O	water in oil
W/O/W	water in oil in water

INTRODUCTION

Thymopentin (TP5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr) which corresponds to the residues 32–36 of the 49 amino acid thymopoietin. This peptide could reproduce the bioactivity of the thymopoietin and is, therefore, considered to be the active sequence (1). As an immunomodulator, TP5 has

been successfully used to treat a variety of diseases, including primary and secondary immune deficiency, autoimmune diseases, infections and cancers (2,3). However, because of the extensive metabolism in the gastrointestinal tract and the short half-life (≤30 s) in plasma (4), a once daily intravenous or intramuscular injection of TP5 is necessary. Moreover, a course of the TP5 treatment usually lasts one to six months, which brings great suffering and inconvenience to the patients.

In the search for needle-free drug delivery, the pulmonary administration route is receiving increased attention as a noninvasive alternative for systemic drug delivery, owing to the unique physiological features of the lungs. These features involve the large alveolar surface area, extensive vascularization, low thickness epithelial barrier and relatively low proteolytic activity, as well as the avoidance of first-pass hepatic metabolism (5). So far, several protein-based drugs, such as insulin, human growth hormone, calcitonin and deslorelin, have been reported to reach the systemic circulation following aerosol administration (6). Dry powder inhalers (DPIs) have recently become a subject of active research with FDA approval of the use of insulin DPIs (Exubera, Pfizer) to treat patients with type 1 and type 2 diabetes (7). Although it was later withdrawn in October 2007 for reasons other than safety or efficacy (8), the FDA approval of Exubera provides a major boost for research on DPIs.

However, the systemic delivery of proteins and peptides by inhalation is technically challenging. First, the dry powders tend to agglomerate into larger aggregates because of the Van der Waals and electrostatic forces between particles, which thus decreases the airflow properties of the powders and their subsequent deposition into the deep lung (9,10). Second, the powders are rapidly cleared from the lungs due to mucociliary clearance, enzymatic degradation and phagocytosis by alveolar macrophages (11). To circumvent the problems, a carrier system, the microparticles containing nanoparticles (size within the nanometer range, including solid lipid nanoparticles), has been developed by co-spray-drying nanoparticles with bulking agents and dispersibility enhancers (12,13). The microparticles presented hollow structures, the shells of which were composed of the nanoparticle aggregations (14).The hybrid microparticles have combined the drug release and delivery potential of the nanoparticles with the ease of flow, processing and aerosolization potential of the large porous particles (12). Jeffrey prepared the microparticles with the fine particle fraction (FPF%) as high as 40% (15). Importantly, the microparticles have been shown to disassociate into the primary nanoparticles once they were exposed to an aqueous environment, such as in the alveolar lung region. It is reported that the phagocytic activity is maximum for particles of 1–2 μm, decreasing for both smaller and larger particles out of this range (16). Therefore, the nanoparticles could remain in the lung lining fluid until absorption, while avoiding unwanted phagocytic mechanism. Meanwhile, the nanoparticles could protect proteins and peptides from the proteolytic degradation, and the carrier itself may exhibit certain absorption enhancer effects (17).

To be therapeutically effective, the microparticles must readily disassociate into the primary nanoparticles in an aqueous medium without significant destruction on the delivery advantages associated with the nanoparticles systems (18). In this regard, the presence of a water-soluble excipient (i.e. mannitol) that forms "excipient bridges" interconnecting the nanoparticles has been found to be necessary in enabling the microparticles redispersion. The mannitol forms a layer around the nanoparticles, which could prevent the particle's coalescence (19). As reported, the microspheres enabled an immediate release of the nanoparticles due to the high solubility of the mannitol, which therefore only acts as an inert carrier of the nanoparticles (20). More importantly, the physicochemical properties of nanoparticles and the release profile of the therapeutic agents were shown to not be negatively affected by the spray-drying (21,22).

Research in this area has proposed the hybrid microparticles as valuable vehicles for efficient drug delivery to the lungs (12–15). However, this research mainly focused on the characterization, aerodynamic properties, as well as formulations of the microparticles *in vitro* (18–22). No further studies *in vivo* have been reported to estimate the potential of the microparticles as carriers for the pulmonary delivery of the nanoparticles. In our present work, we prepared the inhalable microparticles using the spray-drying technique and systematically estimated their feasibility for the pulmonary delivery of the solid lipid nanoparticles (SLNs) by careful investigations of their characteristics, aerosolization properties, as well as pharmacokinetics and pharmacodynamics.

MATERIALS AND METHODS

Materials and Animals

TP5 was bought from Chengdu Kaijie Biotechnologies Co. Ltd., China. Fluorescein isothiocyanate-labeled TP5 was synthesized by our lab. Glyceryl monostearate and soybean phosphatidylcholine were from Shanghai Taiwei Pharmaceutical Co. Ltd., China. Mannitol and bromophenol blue were purchased from Tianjin Bodi Chemical Plant, China. Leucine, sodium cholate and sodium pentobarbital were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were used without further purification.

Superoxide dismutase (SOD) kit was obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China); CD3-FITC, CD4-PE, CD8-PerCP, isotype control antibody IgM-FITC, IgG2a-PE, IgG1-PerCP and hemolysin were supplied by Biolegend (San Diego, CA, USA).

Wistar rats (280±20 g) were supplied by Experimental Animal Center of Sichuan University. Animal experiments were performed according to the requirements of the National Act on the use of experimental animals (P.R. China) and were approved by Animal Ethics Committee of Sichuan University.

Preparation of the TP5-Loaded SLNs

TP5-loaded SLNs (TP5-SLNs) were prepared by double emulsion technique (23). Typically, 0.25 ml of TP5 solution (40 mg/ml) containing 20 mg of sodium cholate was added to 2.5 ml of chloroform and ether solution (1:1, *v/v*) containing 200 mg of glyceryl monostearate and soybean phosphatidylcholine (1:1, *w/w*, oily phase). This mixture was dispersed with an ultrasonic probe (Ningbo Scientz Biotechnology Co. Ltd., China) for 2 min at 100 W, leading to a primary water in oil emulsion (W/O). A water-in-oil-in-water emulsion (W/O/W) was formed after addition of 10 ml of 0.5% poloxamer 188 solution (outer aqueous phase) to the previous W/O emulsion, followed by sonication for 30 s at 80 W. The organic solvent was removed by evaporation under reduced pressure using a rotary evaporator (BÜchi, Switzerland).

Preparation of the Inhalable Microparticles

The aqueous suspension containing a total powder mass of 2.5% (*w/v*) was prepared with mannitol/leucine/TP5-SLNs at a ratio of 1/3/1 (*w/w*). Bromophenol blue was incorporated into the suspension to quantify the powder aerodynamic properties *in vitro*.

The prepared suspension was subsequently spray-dried using a laboratory scale spray-dryer (Büchi® Mini Spray Dryer, B-290, Switzerland) under the following conditions: inlet temperature, 110°C; press airflow, 600 L/h; aspirator setting, 100%; feed rate, 4.0 ml/min. The resulting outlet temperature was about 52°C. Five batches of the microparticles were prepared and stored in a desiccator until analysis.

Characterization of the Microparticles

The morphology of the microparticles was viewed using a conventional scanning electron microscope (SEM, JSM-5900LV, JEOL, Japan) at an accelerating voltage of 20 kV. The microparticles were spread on a graphite surface, and then the sample was coated with gold using an Ion Sputter.

Confocal Laser Scanning Microscope (CLSM, Leica TCS SP5, Mannheim, Germany) was also used to investigate the structure of the microparticles and the distribution of the SLNs in the microparticles. To facilitate the CLSM investigation, TP5 was labeled with fluorescein isothiocyanate (FITC-TP5). The dry powder was spread on a glass slide and then was examined with CLSM. Green fluorescence was observed with a 520 nm emission filter under 495 nm laser illumination. The pinhole diameter was set at 104 μm. Stacks of images were collected every 0.8 μm along the z-axis. A videocapture (AxioVision Release 4.1, Carl Zeiss, Oberkochem, Germany) was used to acquire digital images.

The size of the microparticles was measured by light diffraction using a Mastersizer 2000 equipped with a Scirocco dry disperser (Malvern Instruments Ltd., UK). Each sample was measured in triplicate. The data were expressed as the volume median geometric diameter. The tap density of the inhalable microparticles was determined by tap density measurement, i.e. following 1000 taps, which allowed the density to reach a plateau (24).

Aerodynamic Properties Evaluation

The aerodynamic properties of the microparticles were investigated using a Twin Stage Impinger (TSI) according to the Pharmacopoeia of P.R. China (2005). Double-distilled water was introduced to the upper (7 ml) and lower (30 ml) stages of the TSI. The microparticles were loaded into size 3 gelatin capsules. The capsules were pierced, and the liberated powders were drawn through the TSI at a flow rate of 60 L/min for 10 s aspiration. Each deposition experiment involved the aerosolization of ten capsules and was repeated in triplicate.

As described above, bromophenol blue was incorporated into the formulation to evaluate the aerodynamic properties of the microparticles. The fractions of the microparticles deposited in the upper and lower stages of the TSI were quantified by UV spectrophotometer (Varian, USA).

The emitted dose (ED), defined as the percent of total loaded powder mass exiting the capsule, was determined gravimetrically. The respirable dose (RD) was defined as the mass of powder deposited in the lower stage. The respirable fraction (RF) was the ratio of RD to total loaded does.

Stability of the SLNs During Spray-Drying

In order to recover the TP5-SLNs, the microparticles were dispersed into pH 7.4 phosphate buffer (PBS) under mild magnetic stirring. After that, the characteristics of the TP5-SLNs before and after spray-drying were analyzed ($n=5$). The encapsulation efficacy (EE%) of the TP5-SLNs was measured using established HPLC method. The particle size and polydispersity index were determined using a Malvern zetasizer Nano ZS90 (Malvern Instruments Ltd., UK) based

on quasi-elastic light scattering. Size measurements were performed following a 1/10 (*v*/*v*) dilution of the SLN suspension at 25°C. The zeta potential of the SLNs was measured using the same instrument at 25°C following a 1/10 (*v*/*v*) dilution. Each sample was analyzed in triplicate.

The effect of spray-drying on the release behavior of the SLNs was also investigated. The release experiments were performed by incubating the SLNs or the microparticles in the pH 7.4 PBS with horizontal shaking at 37°C. At predetermined time intervals, the cumulative release amount of the individual samples were measured using established HPLC method after separating free drugs from the SLNs by ultracentrifugation (23).

Pharmacokinetic Studies

Drug Administration

The animals were randomly divided into four groups of 5 animals each to receive different TP5 formulations at a dose of 290 μg/kg. The rats in group 1 were given FITC-TP5 solution by tail vein injection. The rats in group 2 through group 4 received an intrapulmonary administration of FITC-TP5 solution, FITC-TP5-SLNs suspension and FITC-TP5 microparticles, respectively. For intrapulmonary drug administration, a non-invasive pulmonary delivery method was used as described later.

Blood samples were collected at scheduled time. Plasma concentrations of FITC-TP5 were measured using an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) (25).

Validation of the Non-invasive Pulmonary Delivery Method

In order to investigate the validation of the non-invasive pulmonary delivery method, the microparticles containing FITC-TP5-SLNs were delivered to the lungs using the non-invasive pulmonary delivery method. Briefly, holding the rat tightly, a small blunted forceps was used to displace the tongue for maximal oropharyngeal exposure. After a clear view of the trachea was obtained, the tip of the dry powder insufflator (Penn-Century Inc., USA) was endotracheally inserted, and the microparticles were sprayed to the lungs.

The rat was sacrificed 10 min after the administration; afterwards, the lungs and the stomach were dissected and visualized using a Whole Body Fluorescence Imaging System (lightools Research, Encinitas, CA, USA).

Pharmacodynamics

The rats were equally divided into seven groups (five each), and for three consecutive days, the rats in group 2 through group 7 were given cyclophosphamide solution intraperitoneally at a dose of 35 mg/kg once daily to build immunodepression rat models. The rats in group 1 and 2 without further treatment were used as the normal control and the immunodepression control, respectively. In the following seven days, the rats in group 3 were given TP5 solution (120 μg/kg) by tail vein injection, and the rats in group 4 through group 7 received an intrapulmonary administration of TP5 solution (120 μg/kg), TP5-SLNs suspension (120 μg/kg), TP5 microparticles (120 μg/kg) and blank microparticles, respectively, using the non-invasive pulmonary delivery method.

On the eleventh day, blood samples were collected from the rats and were put into the anticoagulant tubes for the superoxide dismutase (SOD) activity assay and the T-lymphocyte subsets analysis.

SOD Activity

The superoxide dismutase (SOD) activity assay was based on the SOD kit instruction, using a UV spectrophotometer (Varian, USA). For each sample assay, 20 μl of plasma was used, and all the assay validation met the requirements of the SOD kit instruction.

Measurement of the Lymphocyte Subsets

The lymphocyte subsets were determined by multiparameter flow cytometry with three-color analyses. The immunofluorescent staining of the whole blood was performed as follows: briefly, 100 μl of the anticoagulant whole blood was added to a test tube containing preadded fluorescent antibodies (FITC: CD3/PE:CD4/PerCP:CD8) or isotype control (FITC: IgM/PE:IgG2a/PerCP:IgG1). After incubation for 20 min at 25°C in dark, red blood cells were lysed using the hemolysin following the manufacturer's instruction. The samples were washed thoroughly in PBS by centrifugation (350×*g*, 5 min), then the cell sediments were resuspended in 0.5 ml of PBS. The samples were kept on ice, and the T-lymphocyte subsets were analyzed within 4 h using a BD FACS Canto™ II flow cytometer (USA). Twenty-thousand total events were collected for each sample.

Statistical Analysis

Statistical analysis was carried out using the Student's *t*-test; in all cases, $P < 0.05$ was considered significant, and the data were expressed as mean ± standard deviation (S.D.).

RESULTS

Effect of the Spray-Drying on the SLNs

In the current study, we recovered the SLNs by incubating the microparticles in PBS. It was observed that after the

incubation in the aqueous medium, the aerosol excipients of the microparticles were immediately dissolved, forming an SLN suspension. Therefore, the SLNs could be easily recovered from the microparticles.

The influences of the spray-drying on the SLNs were investigated for five batches of the microparticles, and each sample was measured in triplicate. As shown in Table I, after spray-drying, there was a slight change in the EE% of the SLNs, which dropped from 61.2% to 55.0%, while no significant differences were observed for the zeta potential. However, the mean particle size increased from 147 nm to 218 nm after the spray-drying, and the PDI also increased correspondingly. This might be attributed to the aggregation of the SLNs during the spray-drying.

Drug release study was done to investigate whether the SLNs could maintain sustained drug release character after spray-drying. Fig. 1 illustrates the release profiles of TP5 from the SLNs and the microparticles. The results showed that both the SLNs and the microparticles displayed a sustained drug release pattern, while no burst effect was observed. The result showed that there was discrepancy between the SLNs and the microparticles. The drug release rate of microparticles was more rapid than that of the SLNs. However, the microparticles maintained sustained drug release character, which met our goal.

Characteristics and Aerodynamic Properties of the Microparticles

As depicted in Fig. 2a, the resulted microparticles were almost spherical-shaped and had a high dispersibility rather than being aggregated. Fig. 2b shows a close-up view of the microparticle surface, and it was obvious that the particles containing SLNs were porous and hollow, suggesting a low tap density. However, the surface of the microparticle without SLNs (Fig. 2c) had an irregular surface and a dense spherical shape. Additionally, the size of the microparticles containing SLNs was larger than that of the particles without SLNs. The confocal microscopy image (Fig. 3) confirmed that the microparticles containing SLNs were hollow and the SLNs were homogeneously distributed within the shell.

According to the Mastersizer 2000 particle size analysis, the volume median geometric diameter of the microparticles was 4.1 ± 0.1 μm. By virtue of the hollow and porous structure, the microparticles had a low tap density of 0.175 ± 0.02 g/cm^3. In our research, the prepared microparticles presented a high emitted dose (ED) of $98.0\% \pm 1.23\%$ and a respirable fraction (RF) of $51.07\% \pm 1.21\%$.

Validation of the Non-invasive Pulmonary Delivery Method

After the pulmonary administration of the FITC-TP5-SLNs microparticles, strong fluorescence was visualized in the lungs. As depicted in Fig. 4b, green fluorescence can be visualized from the trachea, main bronchi, peripheral and central part of the lungs, which presented a spot distribution, indicating dry powders spread in the pulmonary alveolus. However, no fluorescence was observed in the esophagus and the stomach (Fig. 4c).

The results confirmed that the non-invasive pulmonary delivery method could effectively spray powders to the lungs through the trachea, avoiding drug loss by intragastric application via the esophagus.

Pharmacokinetic Studies

The pharmacokinetic parameters of the TP5 could not be characterized due to the rapid degradation of the TP5 *in vivo* ($t_{1/2} < 30$ s). Alternatively, the FITC-labeled TP5 was used for pharmacokinetic analysis in the present work. The time courses of FITC-TP5 concentrations in blood after the administrations of different FITC-TP5 formulations are shown in Fig. 5, and the pharmacokinetic parameters are listed in Table II.

Plasma FITC-TP5 concentration reached a value close to 180 ng/ml after i.v. administration of the FITC-TP5 solution, which was significantly higher than those of any other groups. However, FITC-TP5 eliminated quickly from the plasma with a short half-life of 43 min; thus, its plasma concentration was not detectable after 10 h. By contrast, after the intrapulmonary administration of the FITC-TP5 solution, a rapid increase in the plasma FITC-TP5 level was observed, and the T_{max} was 18 min. However, the absorption effect was transient followed by a stiff drop to 3 ng/ml within 1 h. The FITC-TP5-SLNs suspension group exhibited an evident increase in serum FITC-TP5 concentration and reached a maximal value up to 82 ng/ml

Table I Effect of Spray-Drying on the SLNs (Mean ± S.D., $n = 5$)

SLNs	Size (nm)	Zeta potential (mV)	PDI	EE%
Pre-spray-drying	147.4 ± 4.0	-53.0 ± 1.9	0.18 ± 0.01	61.2 ± 4.1
After spray-drying	217.9 ± 8.3^b	-48.9 ± 2.0	0.25 ± 0.03^a	55.0 ± 3.4^a

[a] $P < 0.05$, [b] $P < 0.005$ *vs.* SLNs prespray drying

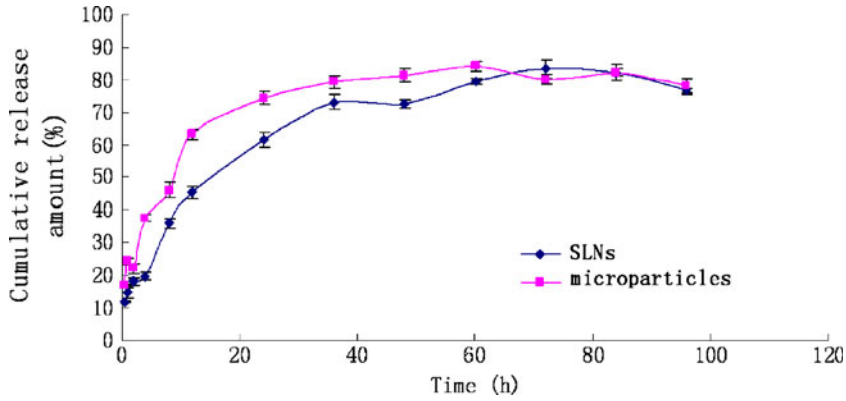

Fig. 1 Release profiles of TP5 from the SLNs and the microparticles (mean ± S.D., $n = 5$).

within 1 h after the administration. Furthermore, prolonged FITC-TP5 release was found, and the serum concentration maintained at a relatively high level for 48 h. Compared with the FITC-TP5-SLNs group, the microparticle group performed slower absorption, in which the serum FITC-TP5 concentration increased gradually to 52 ng/ml over 2 h. However, sustained drug release and similar elimination phase were also detected after the intrapulmonary administration of the microparticles. Moreover, the C_{max}, AUC and MRT were significantly increased after the intrapulmonary delivery of the SLNs and the microparticles compared with the i.v. TP5 group.

SOD Activity

The changes of the SOD activity in peripheral blood could illuminate the immune abnormality of the organism, which was chosen to reflect the pharmacodynamic actions of different TP5 formulations (26).

As described in Table III, the SOD values of the immunodepression rats were significantly reduced compared with those of the normal control rats, indicating that the immunodepression models have been established. Also as seen in Table III, the SOD levels of the immunodepression rats could be raised after the administration of different TP5 formulations. Moreover, the SOD values of the SLN group and the TP5 microparticles group were significantly increased when compared with those of the others, while there were no significant differences between the two groups. The results demonstrated that the TP5 microparticles had a strong pharmacodynamic action after pulmonary administration. Furthermore, the bioequiavailability of the TP5-SLNs and the TP5 microparticles illuminated that the microparticles are suitable for pulmonary delivery of the SLNs without significant compromise on the SLNs characteristics.

Peripheral Blood T-lymphocyte Subsets

The peripheral blood $CD4^+/CD8^+$ ratio for normal human is confirmed to be stable, while in the immunological suppression or deficient patients, the ratio is irregularly changed. As an immunomodulator, TP5 can bring the $CD4^+/CD8^+$ ratio towards the normal state (27). Therefore, this ratio was chosen as the index of pharmacodynamic detection.

As shown in Table IV, the $CD4^+$% of the immunodepression rats had a remarkable increase, while a significant decline was observed for $CD8^+$%; thus, the $CD4^+/CD8^+$ ratio in the immunodepression rats was markedly increased. The results indicated that the T-lymphocyte subsets were significantly affected by the immunodepression. After the

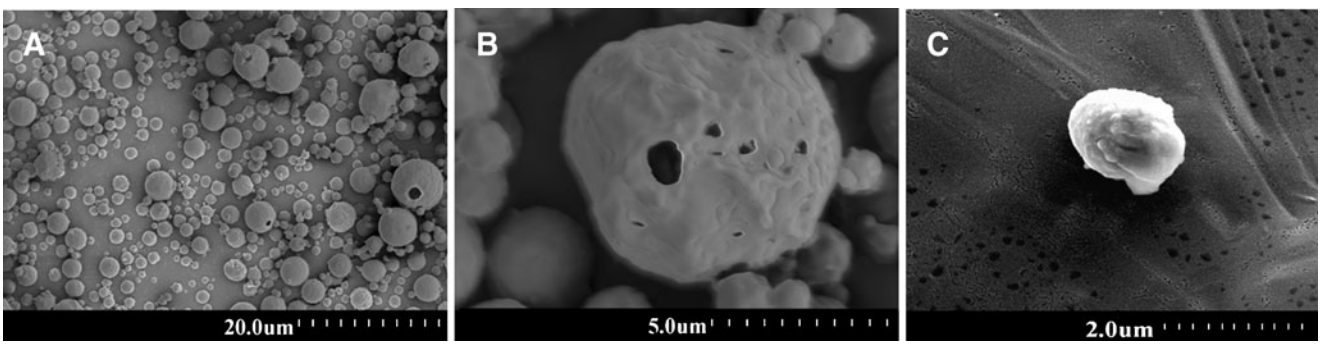

Fig. 2 SEM microphotographs of microparticles **a**, **b** containing SLNs and **c** without SLNs.

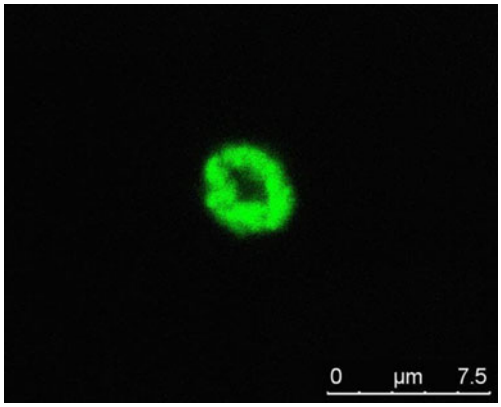

Fig. 3 Confocal microscopy image of microparticles containing SLNs.

administration of different TP5 formulations, the $CD4^+$% was decreased and the $CD8^+$% was increased. Correspondingly, the increased $CD4^+/CD8^+$ ratio in the immunodepression rats was reduced. There was no significant difference between the blank powder group and the immunodepression control, indicating that the excipients did not have therapeutic activity. On the other hand, the $CD4^+$%, $CD8^+$% as well as the $CD4^+/CD8^+$ ratio were significantly reversed to the normal values, respectively, after the pulmonary administration of the TP5-SLNs and the TP5 microparticles compared with the other TP5 formulations. Those results suggested that the TP5-SLNs and the TP5 microparticles had remarkably immunomodulating effects on the immunodepression rats.

As a commonly used immunosuppressive agent, cyclophosphamide could inhibit the proliferation and the differentiation of T-lymphocyte, causing irregular changes in the numbers of the T-lymphocyte subsets. However, the T-lymphocyte subsets show different sensitivity towards cyclophosphamide. For example, the $CD8^+$ cells are more sensitive than the $CD4^+$ cells, which can explain the increase in the $CD4^+/CD8^+$ ratio in the immunodepression rats.

DISCUSSION

In this research, we prepared the inhalable microparticles and systematically estimated the feasibility of the microparticles for the pulmonary delivery of the SLNs. The microparticles containing SLNs (nanoparticles) have been proposed as valuable vehicles for efficient drug delivery to the lungs. The hybrid microparticles presented much better flow and aerosolization properties than the SLNs. Moreover, the microparticles have been expected to disassociate into the primary SLNs once deposited in the lungs, with the drug release and delivery advantages associated with the SLNs delivery systems.

The particle size of the DPIs is a crucial factor in DPIs' formulation. DPIs should be small enough to pass through the mouth, throat and conducting airways and reach the deep lung, but not so small that they fail to deposit and are breathed out again. There is no consensus concerning the ideal size of DPIs. However, it has been reported that DPIs ranging from 1 to 5 μm are the optimum size to maximise the deposition in the lower respiratory tract (24). In our research, the size of the microparticles was 4.1 ± 0.1 μm with a low tap density of 0.175 g/cm^3, which was suitable for inhalation. As depicted in Fig. 2b and Fig. 3, the microparticles were spherical and hollow, and the shells were composed of the SLN aggregations. More importantly, owing to their physical characteristics, the microparticles presented appropriate aerodynamic characteristics for lung delivery (emitted dose of $98.0\% \pm 1.23\%$ and respirable fraction of $51.07\% \pm 1.21\%$). These kinds of large hollow microparticles possess a high aerosolization efficiency for the following reasons: the large geometric size reduces cohesion between microparticles, which consequently improves the flowability of the particles off the inhaler and promotes the disaggregation into fine particles, once emitting out of the inhaler (28). Furthermore, by virtue of the low-density, an effective lung delivery was achieved, while the oropharyngeal deposition of the particles was minimized (12).

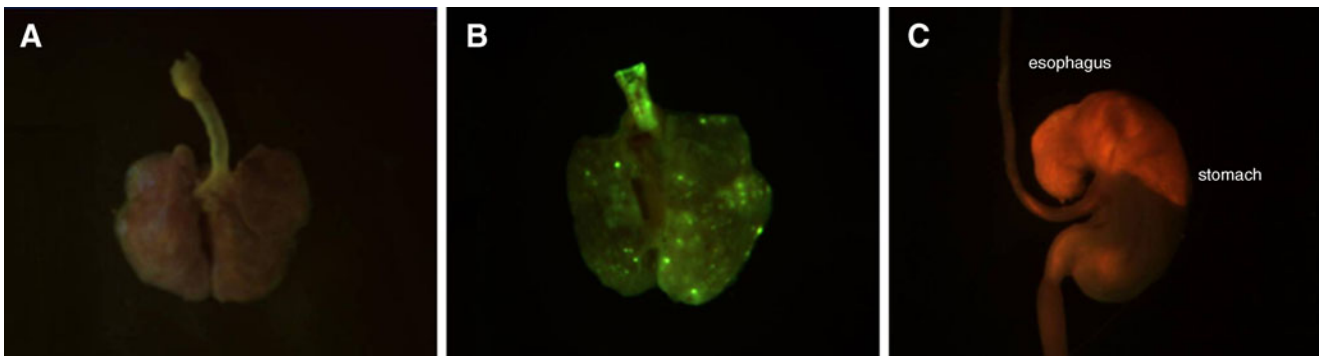

Fig. 4 Whole body fluorescence images of **a** blank lungs; **b** lungs of rat received intrapulmonary delivery of microparticles; **c** esophagus and stomach of rat received intrapulmonary delivery of microparticles.

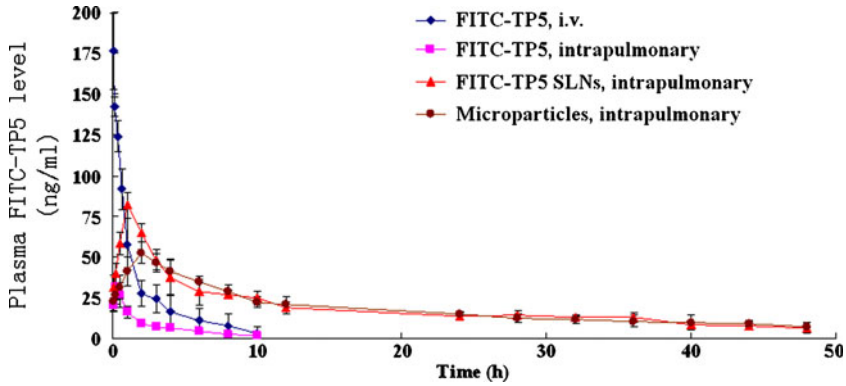

Fig. 5 Mean plasma FITC-TP5 concentration-time curve of each experiment group (mean ± S.D., $n = 5$).

The microparticles suitable for inhalation were prepared by spray-drying the SLN suspension. The spray-drying technique has been successfully developed to produce dry powders containing labile pharmaceutical proteins. By optimizing formulations and process conditions, this one-step process could produce dry powders suitable for inhalation (29). In the formulation, mannitol, which is known for being nontoxic and nonhygroscopic (30,31), was chosen as a drying auxiliary agent, and leucine as a dispersibility enhancer. The presence of mannitol provided increased stability to the SLNs, as the formation of excipient layer around the SLNs could prevent the lipids' coalescence (19). In addition, the mannitol could protect the proteins against thermal stresses and denaturation during the spray-drying (32). The mechanism for its stabilizing action is most likely water replacement (33). The incorporation of leucine into the formulation was shown to improve the aerosol behavior of the spray-dried powders significantly by reducing moisture sorption and surface tension (10).

The effect of spray-drying on the SLNs was investigated. As depicted in Table I, after spray-drying, there was a slight change in the EE% of the SLNs, while a significant difference was observed for the particle size. In the formulation process, the irreversible aggregation of the SLNs could be prevented by the aerosol excipient. However, as a result of the thermal conditions during the drying process, some SLNs may aggregate together, which accounted for the increase of the particle size. However, the release profiles of TP5 from the SLNs and the microparticles showed that, for both formulations, sustained drug release patterns were observed, which demonstrated that the spray-drying had a slight effect on the drug release behavior of the SLNs. Therefore, taking all these results into account, we believe that after inhaling the microparticles, the SLNs could be easily released from the microparticles without significant changes in the drug release and delivery advantages associated with the SLNs delivery systems.

Investigations *in vitro* demonstrated that the microparticles containing SLNs had ample potential for the pulmonary delivery of the SLNs, which was confirmed by the pharmacokinetic and pharmacodynamics studies *in vivo*. As demonstrated in Fig. 5 and Table II, the C_{max}, AUC and MRT were significantly increased after the pulmonary delivery of the microparticles, as compared with the i.v. TP5 group. These results clearly showed that the TP5 absorption was markedly enhanced after the entrapment in the SLNs, thus improving its bioavailability. Also, the long MRT demonstrated the sustained drug release of the SLNs. Furthermore, the results of the pharmacodynamics greatly supported the feasibility of the inhalable microparticles for the lung delivery of the SLNs. In pharmacodynamic study, SOD activity and lymphocyte subsets were chosen as the biomarkers to reflect the therapeutic efficacies of different TP5 formulations. The results demonstrated that the TP5 microparticles had remarkable immunomodulating effect on the immunodepression rats compared with i.v. TP5

Table II The Pharmacokinetic Parameters of Each Experiment Group (Mean±S.D., $n = 5$)

Parameters	FITC-TP5 solution, i.v.	FITC-TP5 solution, intrapulmonary	FITC-TP5-SLNs, intrapulmonary	Microparticles, intrapulmonary
$t_{1/2}$ (h)	0.72 ± 0.21	1.47 ± 0.31[a]	6.22 ± 3.66[a, b]	13.62 ± 2.74[a, b, c]
C_{max} (ng/ml)	176.26 ± 23.16	34.32 ± 6.72[a]	81.69 ± 8.36[a, b]	52.47 ± 6.38[a, b, c]
Tmax (h)		0.3 ± 0.11	1.0[b]	2.0[b, c]
AUC (ng/ml h)	260.04 ± 72.42	73.33 ± 9.01[a]	907.81 ± 98.05[a, b]	882.76 ± 34.97[a, b]
MRT (h)	2.19 ± 0.64	2.91 ± 0.29	16.04 ± 0.79[a, b]	16.85 ± 1.43[a, b]

[a] $P < 0.05$ *vs.* FITC-TP5 solution, i.v.; [b] $P < 0.05$ *vs.* FITC-TP5 solution, intrapulmonary; [c] $P < 0.05$ *vs.* FITC-TP5-SLNs, intrapulmonary

Table III The SOD Activity of Each Experiment Group (Mean±S.D., $n=5$)

Groups	SOD (U/ml)
Normal control	$201.7\pm10.6^{a, b, c}$
Immunodepression control	$100.8\pm14.8^{b, c}$
TP5 solution, i.v.	$147.2\pm9.6^{a, c}$
TP5 solution, intrapulmonary	$130.4\pm10.2^{a, b, c}$
TP5-SLNs, intrapulmonary	$178\pm9.4^{a, b}$
TP5 microparticles, intrapulmonary	$166.1\pm11.7^{a, b}$
Blank powder, intrapulmonary	$101.7\pm18.2^{b, c}$

[a] $P<0.05$ *vs.* immunodepression control; [b] $P<0.05$ *vs.* TP5 solution, i.v.; [c] $P<0.05$ *vs.* TP5 microparticles, intrapulmonary

solution. Additionally, in the SOD activity study, the bioequiavailability of the TP5-SLNs and the TP5 microparticles illuminated that the microparticles were suitable for the pulmonary delivery of the SLNs without significant compromise on the SLNs' characteristics.

The enhanced bioavailability and therapeutic efficacy of TP5 by the inhalation of the TP5 microparticles may be attributed to the advantages of the microparticles and the SLNs' systems. In our research, the inhalable microparticles had combined the drug release and delivery potential of the SLNs with the ease of flow, processing and aerosolization potential of large porous particles. Investigations *in vitro* demonstrated that the prepared microparticles presented suitable aerodynamic characteristics for the lung delivery (emitted dose of 98.0%±1.23% and high respirable fraction of 51.07%±1.21%). Once emitting out of the inhaler, the microparticles with a high respirable fraction could be effectively spread in the lung alveolus, with a slight oropharyngeal deposition. After inhalation, the microparticles would have to overcome at least two obstacles before exerting the therapeutic efficacy: the alveolar macrophages and the enzymatic activity. It is reported that the phagocytic activity is maximum for particles of 1–2 μm, decreasing for both smaller and larger particles out of this range (16). Therefore, SLNs could remain in the lung lining fluid until absorption, while avoiding unwanted phagocytic mechanism. Furthermore, the SLNs could protect TP5 from the proteolytic degradation, and the carrier itself may exhibit certain absorption enhancer effects (17).

When making a comparison between the SLNs and the microparticles, we found that, the bioavailability and therapeutic efficacy of the SLNs were slightly higher than those of the microparticles. It seemed that the SLNs were more suitable for the pulmonary delivery of TP5. However, long-term stability of drugs can be achieved when stored as a dry product. This may be important for the SLNs containing proteins or peptides, which are susceptible to hydrolysis. Therefore, the microparticles provide a more stable environment for the peptide than the SLNs. Furthermore, the dry powder inhalers (DPIs) have many advantages over nebulizers and metered dose inhalations, such as robust, portable, propellant-free and breath-actuated. Taking those into account, we believe that the microparticles are the suitable candidates for the pulmonary drug delivery. Additionally, the bioavailability and therapeutic efficacy of the microparticles were close to those of the SLNs, which just confirmed the feasibility of the microparticles as carriers for the pulmonary delivery of the SLNs.

CONCLUSIONS

In our present study, the inhalable microparticles with a high aerosolization efficiency were prepared by spray-drying the SLN suspension. With the protective effect of mannitol and leucine, the SLNs had no essential changes in their properties or the drug release behavior after incorporation into the microparticles. Therefore, the microparticles would disassociate into the SLNs once deposited in the lungs, without significant changes in the drug release and delivery advantages associated with the SLNs delivery systems. The pulmonary administration of the microparticles showed an enhanced drug absorption and a sustained drug release. Moreover, the pharmacodynamic study showed that the TP5 microparticles had a remarkably strengthened therapeutic efficacy compared with i.v. TP5 solution. In conclusion, both *in vitro* and *in vivo* results illustrated that the inhalable microparticles possessed an

Table IV Change of $CD4^+$/$CD8^+$ Between Groups (Mean±S.D., $n=5$)

Groups	$CD4^+$%	$CD8^+$%	$CD4^+/CD8^+$
Normal control	66.9±2.5	34.8±3.0	$1.91\pm0.25^{a, b, c}$
Immunodepression control	82.5±7.7	15.5±1.8	$5.33\pm0.22^{b, c}$
TP5 solution, i.v.	78.1±1.0	22.4±1.0	$3.49\pm0.19^{a, c}$
TP5 solution, intrapulmonary	81.1±1.5	19.9±1.5	$4.1\pm0.38^{a, b, c}$
TP5-SLNs, intrapulmonary	71.2±2.7	29.3±2.8	$2.45\pm0.32^{a, b, c}$
TP5 microparticles, intrapulmonary	76.3±1.4	25.6±1.5	$2.99\pm0.22^{a, b}$
blank powder, intrapulmonary	84.7±1.8	16.2±1.1	$5.25\pm0.44^{a, b, c}$

[a] $P<0.05$ *vs.* Immunodepression control; [b] $P<0.05$ *vs.* TP5 solution, i.v.; [c] $P<0.05$ *vs.* TP5 microparticles, intrapulmonary

ample potential for the pulmonary delivery of the SLNs. Our work provided some positive exploration for researche on pulmonary drug delivery. However, further studies need to be done for the clinical application of the inhalable microparticles, such as the dose delivery accuracy and the chronic effects of the carrier on the lungs.

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