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Characterization of a new inhalable thymopentin formulation

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ABSTRACT

The present work describes a new dry powder containing thymopentin (TP5) suitable for inhalation. A total of 21 dry powders were produced by co-spray drying TP5 with lactose or mannitol as a bulking agent, leucine as a dispersibility enhancer and poloxamer 188 as a drug stabilizer. Analyses by scanning electron microscopy, laser diffractometry, thermogravimetry, Twin Stage Impactor and HPLC were performed to characterize the manufactured powders. The results revealed that formulation compositions greatly influenced the physical characteristics of the powders, such as the angle of repose, tapped density, particle size and aerodynamic diameter which, in turn, affected their aerodynamic behavior. A higher loading of leucine in the formulations (>63% by dry weight) improved the aerosolization properties of the powders by producing aerodynamically lighter particles. The optimum formulation, which had a tapped density of 0.31 g/cm^3, an aerodynamic diameter of 1.9 μm and an in vitro deposition of 45%, was obtained by combining TP5/mannitol/leucine in the ratio of 10/18/72. In addition, it was interesting to find that poloxamer 188 had a significant impact on improving the powder flowability rather than stabilizing TP5. In conclusion, the chosen composition promises an enhanced aerosol performance for the new TP5 inhalation formulation, suitable for deep lung deposition.

1. Introduction

Recent advances in biotechnology have made it possible to use peptides and proteins as active pharmaceutical ingredients (APIs). Recent practical routes for administering such biopharmaceuticals include intravenous, intramuscular and subcutaneous injection. However, drug injection in a hospital or a doctor's office can be expensive and inconvenient for patients. In addition, many patients find self-injectable therapies unpleasant. As a result, the injection regimens for many chronic or subchronic diseases are associated with poor patient compliance. Ideally, a suitable drug delivery system should be safe, reproducible, non-invasive, convenient and has a high bioavailability (Johnson, 1997).

In the search for needle free delivery, pulmonary drug delivery shows great potential for the delivery of proteins and peptides to allow them to exhibit systemic activity. The efficiency of the lung as a systemic absorption site is due to the fact that the lung has a very large absorptive surface area, a very thin diffusion path to the blood stream, an elevated blood flow, a relatively low metabolic activity and first-pass hepatic metabolism is avoided (Adjei and Gaupta, 1997). In terms of the pulmonary administration, oral inhalation is well accepted by the general population in most societies (Byron, 1990). Also, dry powder inhalations (DPIs) have many advantages over nebulizers and metered dose inhalations (MDIs) because they are propellant-free, portable, easy to operate and low-cost devices with improved stability of the formulation because it is in a dry state (Prime et al., 1997; Arakawa et al., 1993).

Thymopentin (TP5), a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr), consists of the residues 32–36 of the 49-amino acid human hormone thymopoietin. This pentapeptide exhibits a similar biological activity to thymopoietin and is, therefore, considered to be the active sequence (Goldstein et al., 1979). TP5, acting as an immunomodulator, can bring the immune dysequilibrium, which may be either hyperresponsiveness or hyporesponsiveness, towards normal state. A multitude of in vivo studies have shown the efficacy of TP5 treatment for the therapy of a variety of diseases, including primary and secondary immune deficiencies, autoimmunity, infections, cancer and AIDS (Clumeck et al., 1985; Singh et al., 1998). TP5 has been used clinically in the form of injections and a course of TP5 treatment usually lasts one to six months. However, the long-term and repeated injections are associated with problems of poor patient compliance. Therefore, the development of a TP5 inhaler would expand the range of delivery strategies available to the physician, and potentially overcome some of the drawbacks of the other alternative delivery routes.

To prepare inhalable biopharmaceutical powders, spray-drying is a commonly used method (Bosquillon et al., 2001; Steckel and Brandes, 2004; Rabbani and Seville, 2005). In such a one-step process the liquid is evaporated immediately and the droplets are then cooled. Thus, it is naturally preferable for drying the heat-sensitive

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macromolecules as no degradation takes place under optimal conditions. In addition, the characteristics of the final products can be controlled by adjusting the formulation and process variables (Ståhl et al., 2002).

In this study, a group of dry powders were prepared by co-spray drying TP5 with a bulking agent (mannitol or lactose) and a dispersibility enhancer (leucine) in different ratios. In addition, poloxamer 188 is supposed to act as a stabilizer for TP5. Based on the characterization of these powders, the dependence of the in vitro aerosolization performance of the particle component and their physical characteristics were evaluated with a view to produce an optimum formulation suitable for inhalation.

2. Materials and methods

2.1. Materials

TP5 (purity: 99.11%) was purchased from Soho-Yiming Pharmaceutical Co., Ltd. (Shanghai, China); mannitol and lactose were obtained from Bodi Chemicals (Tianjin, China); poloxamer 188 (Lutrol® F68) was kindly donated by BASF (Shanghai, China). Other reagents were of analytical or chromatographic grade. Double-distilled water was used throughout the study.

2.2. Formulation of the dry powders

TP5 was employed in all formulations with a constant dry weight loading (10%, w/w). The binary carrier systems consisted of leucine/mannitol or leucine/lactose in different ratios. For a comparison, mannitol, lactose and leucine were used separately in three formulations at a drug/carrier ratio of 1:9. Poloxamer 188 was added to each formulation at a concentration of 2% (w/w) of the dry powder mass.

2.3. Preparation of spray dried powders

Aqueous solutions containing drugs and excipients were prepared with a total powder mass of 1% (w/v). The prepared formulations were subsequently spray-dried using an Eyela® SD-1000 spray-dryer (Tokyo Rikakikai Co. Ltd., Japan), under the following standard operating conditions: inlet temperature, 110 °C; atomization pressure, 170 kPa; feed rate, 7 mL/min; aspirator setting, 0.6 m^3/min. These conditions resulted in an outlet temperature of 70–75 °C. The resultant powders were stored in a desiccator at ambient temperature until analysis.

2.4. Powder characterization

2.4.1. Spray-drying yield and drug content

The yields of spray-dried powders were quantified as a percentage mass of the anticipated total powder yields. The TP5 content of each prepared powder was measured in triplicate, with analysis by HPLC, and expressed as a percentage of the nominal load.

2.4.2. Scanning electron microscopy

Selected samples of dry powder formulations were sputter layered with gold. Representative scanning electron micrographs of the powders were taken using a scanning electron microscope (SSX-550, Shimadzu Co. Ltd., Tokyo, Japan).

2.4.3. Water content

Thermogravimetric analysis (TGA) was used to determine the water content of the spray-dried powders. TGA (TGA 50, Shimadzu Co. Ltd., Tokyo, Japan) was performed on accurately weighed samples which were run at a heating rate of 10 °C/min under a nitrogen purge.

2.4.4. Powder flowability

The most frequently employed method for characterizing the powder flowability is the angle of repose (Carstensen, 1993). Powders were poured through a funnel to form a cone-shaped pile which had an angle, α, to the horizontal. The value of α was calculated by measuring the height and radius of the pile. A large angle of repose is indicative of poor flow properties while a small angle of repose indicates a free-flowing powder. The angle of repose and the tapped density of powders were measured using a Powder Characteristics Tester PT-R (Hosokawa Micron Corp., Japan).

2.4.5. Particle size and powder tapped density

The volume particle size was measured with a laser diffractometer (LS 230, Backman Coulter, USA) in dry powder form after dispersing with compressed air. Values presented are the average of three determinations.

The powder density (ρ) was determined by tap density measurements, i.e. following 1000 taps which allowed the density to reach a plateau (Bosquillon et al., 2004). Assuming a perfect packing, the tapped density of monodisperse spheres is approximately 21% underestimate of the particle density due to the void spaces between particles. In the case of polydispersed particles, the void spaces are reduced but this is probably counterbalanced by incomplete packing (Vanbever et al., 1999).

Theoretical estimates of the particle primary aerodynamic diameter (d_{ae}) can be derived from the particle sizing and tapped density data, according to Eq. (1) (Hinds, 1999):

$$d_{ae} = d(\rho/\rho_1)^{1/2} \quad (1)$$

where $\rho_1 = 1\ \mathrm{g/cm^3}$.

2.4.6. In vitro powder deposition

The pulmonary deposition of the dry powders was investigated in vitro using a Twin Stage Impinger (TSI, custom made). Water was introduced to the upper (7 mL) and lower (30 mL) stages of the TSI. Powders (30 mg) were loaded into size 3 HPMC capsules and placed in an Aerolizer® inhaler (Schering, Co.). The capsules were pierced and the liberated powder was drawn through the TSI at a flow rate of 60 L/min for 10 s. In all cases ten capsules were discharged into the apparatus per determination and each experiment was repeated in triplicate. After each determination, the lower stage was rinsed with water and made up to a final volume of 50 mL. The amount of TP5 in the washings was determined by HPLC.

The emitted dose (ED), defined as the percentage of the total loaded powder mass exiting the capsule, was determined gravimetrically. The respirable dose (RD) was defined as the mass of drug recovered from the lower stage of the TSI (effective cut-off diameter 6.4 μm). The respirable fraction (RF), defined as the ratio of the RD to the total loaded dose, was expressed as a percentage and corrected for the actual TP5 content in each powder (Rabbani and Seville, 2005).

2.5. HPLC analysis of TP5

The TP5 concentration was determined with a Hitachi D-7000 HPLC system. A reversed-phase ODS-C18 HPLC column 250 mm × 4.6 mm was operated at room temperature. The mobile phase was methanol (10%, v/v) and 0.02 mol/L phosphate buffer (pH 7.0, 90%, v/v) at a flow rate of 1 mL/min and the detection was performed at 244 nm.

2.6. Statistical analysis

Significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by a Newman–Keul post hoc test and

the tests were carried out using SPSS 12.11 software. Differences were considered significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Spray-dried powder characteristics

A total of 21 spray-dried powders were investigated. All these powders showed similar drug contents with a range of 93.3–97.6%, indicating that TP5 did not suffer denaturation during the spray-drying process. The residual moisture levels in the powders were quite low, about 0.5% of the dry weight. As the water content of powders can significantly affect particle size and excipient crystallization during long-term storage, thereby adversely affecting the dispersion performance of the power (Masters, 1985), a relatively low moisture content is of great importance. In addition, particular attention was paid to the four physical properties of dry powders, i.e. flowability, tapped density, particle size and aerodynamic diameter of the particles, which are affected by the formulation components and, in turn, influence the aerosolization performance of the powders.

As shown in Fig. 1a, the incorporation of leucine into mannitol or lactose contributed to the better flowability compared with the individual use of these three carriers. The best flowability was observed when the carrier consisted of mannitol/leucine with a ratio of 2:8 (angle of repose = 37.7°). The formulation exhibiting good flow properties displays better aerosolization characteristics, as has been demonstrated previously (Rabbani and Seville, 2005). In contrast to mannitol, lactose rendered the powder more cohesive and, hence, reduced the flowability and the yield. It was observed that as the lactose loading increased from 36% to 45%, a mass of particles adhered to the inside wall of the cyclone during spray-drying. Therefore, the yield dropped sharply from about 50% down to 32%. However, in terms of the TP5/mannitol/leucine powders, altering the leucine proportion from 81% to 9% by weight had a negligible effect on the yield, which ranged from 47.9% to 53.7%.

The tapped density is an important physical property of dry powders. Previous investigations have indicated that a lower tapped density is associated with better aerosolization properties (Bosquillon et al., 2001). Fig. 1b shows that the proportion of leucine in carriers greatly affects the tapped density of the powders. The more leucine incorporated in the formulation, the lighter the particles produced. So, the lightest powder (0.231 g/cm^3) was the one which employed leucine as an individual carrier. By contrast, the powder only containing lactose as a carrier had the greatest tapped density of 0.713 g/cm^3. In the cases of formulations with binary carriers, the tapped density of the TP5/mannitol/leucine powders ranged from 0.270 g/cm^3 to 0.520 g/cm^3, whereas the TP5/lactose/leucine powders presented higher densities ranging from 0.279 g/cm^3 to 0.689 g/cm^3. As demonstrated previously, some lighter powders could be reasonably expected to perform better in terms of aerosolization than others.

Interestingly, the geometric particle size (Fig. 1c) showed a very similar pattern to Fig. 1a with an increase of the fraction of leucine in the formulations. It decreased upon addition of leucine, remained steady and then increased slightly on addition of more leucine. This was also observed with the aerodynamic diameter (Fig. 1d), which was affected by both the particle size and density. Given that all the powders contained identical amounts of TP5 and poloxamer 188, the differences in physical parameters must be attributable to the compositions of the carriers. Previous research has shown that leucine is a particularly hydrophobic amino acid and its surfactant-like properties may result in the ability of leucine to migrate to the droplet surface during the rapid drying phase of the spray-drying and, hence, influence the surface characteristics of the resultant particles (Seville et al., 2007; Gliniski et al., 2000). Furthermore, leucine is able to act as an anti-adherent in the case of dry powders. It is assumed that leucine interferes with the weak bonding forces, such as Van der Waal's and Coulomb forces, between the small particles which helps to keep the particles separated and may be thought of as weak links or "chain breakers" between the particles (Chougule et al., 2007). These particular properties of leucine

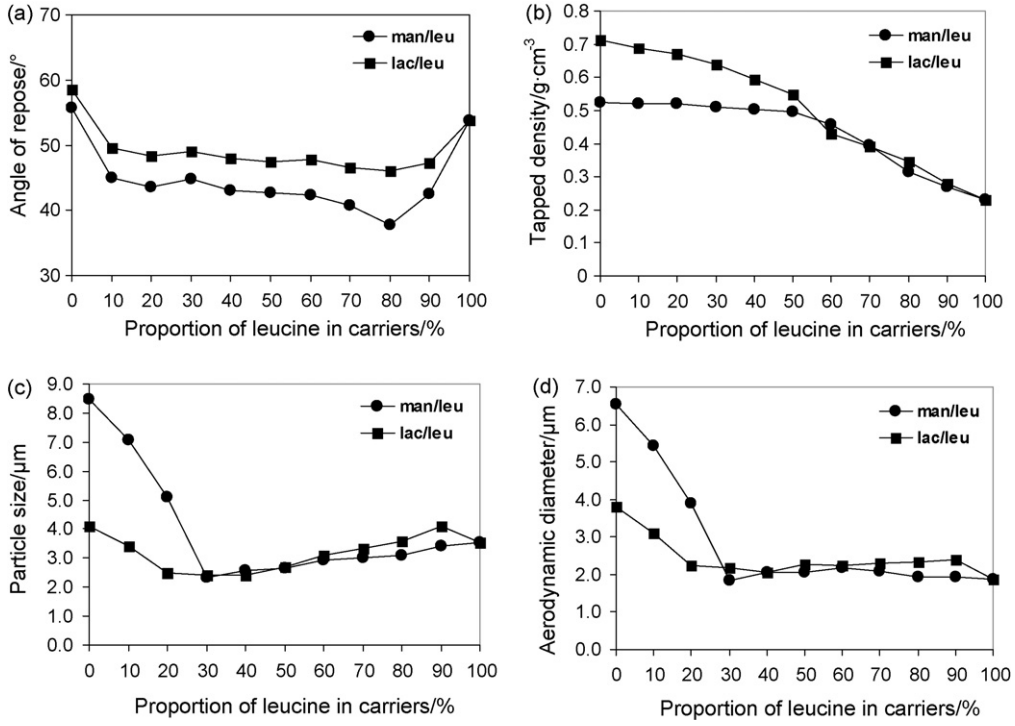

Fig. 1. Influence of carrier compositions on the physical properties of TP5 DPI. (a) Angle of repose vs. leucine proportion; (b) tapped density vs. leucine proportion; (c) particle size vs. leucine proportion; (d) aerodynamic diameter vs. leucine proportion. Man, Lac and Leu represented mannitol, lactose and leucine, respectively. TP5 and poloxamer 188 were employed in all formulations with a dry weight loading of 10% and 2% (w/w), respectively.

give the powders a better flowability and produce smaller particles. Nevertheless, higher concentrations of leucine might lead to highly charged particles, thereby adversely affecting the powder flow properties.

As shown in Fig. 1d, most powders exhibited an aerodynamic diameter below 5 μm, indicating that they were of a suitable particle size to avoid deposition by inertial impaction in the oropharyngeal cavity (Larhrib et al., 1995). The two exceptions were TP5/mannitol/leucine 10:81:9 and TP5/mannitol 1:9, with aerodynamic diameters of 5.62 μm and 6.54 μm, respectively.

3.2. Particle shape and morphology

Overall, the SEM images indicated that the powders were amorphous in nature as there were no obvious crystalline particles visible (Fig. 2). This was as expected since powders produced by spray-drying are known to be predominately amorphous in nature (Corrigan, 1995). SEM also showed that the carrier components significantly influenced the particle shape and morphology. The Man and Lac powders consisted of smooth spherical particles (Fig. 1a and b) presumably due to the addition of poloxamer 188 to the formulations. Poloxamer, as a low-molecular surfactant without surface visco-elasticity, is very mobile and tends to accumulate at the air/liquid interface to form a layer, and to render the surface of the particles smoother (Elversson and Millqvist-Fureby, 2006). However, the increasing surface smoothness usually increases adhesion forces between particles as a result of an increased contact area between the interacting species (Zeng et al., 2001). Thus, a smooth surface of powders may not be necessary for inhalation. In addition, what is more notable for the formulation TP5/mannitol 1:9 is that the SEM showed that it consisted of particles of diameter below 5 μm, but the measured mean particle size of the powder was 8.47 μm. Similar results have also been reported by Rabbani and Seville, who suggested that the larger size obtained during particle sizing reflects the cohesion of individual particles to form larger aggregates that fail to disperse during the sizing procedure.

Although poloxamer was employed in all the powders at the same concentration, the particles containing leucine exhibited a very different shape and morphology as hollow semispherical particles with a wrinkled surface (Fig. 2c–e). This can be explained by the surfactant-like properties of leucine, which made it likely to accumulate at an air–water interface. Moreover, the molecular weight of leucine is lower than that of poloxamer so that leucine could competitively concentrate on the surface of droplets. Leucine molecules

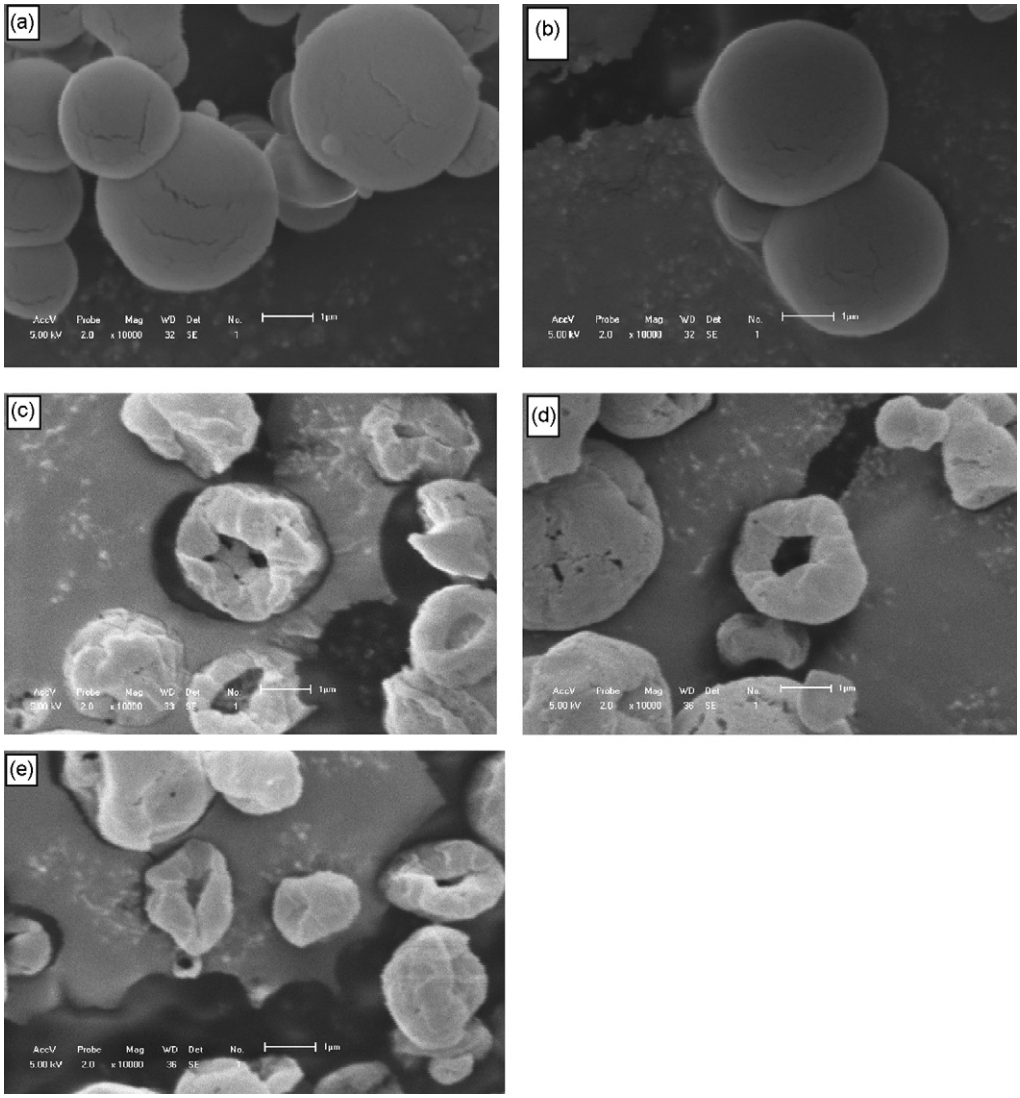

Fig. 2. Scanning electron micrographs of selected spray-dried powders. (a) The powders prepared with TP5/mannitol (1:9); (b) the powders prepared with TP5/lactose (1:9); (c) the powders prepared with TP5/leucine (1:9); (d) the powders prepared with TP5/mannitol/leucine (10/45/45); (e) the powders prepared with TP5/lactose/leucine (10/45/45). Poloxamer 188 was contained in each powder at a concentration of 2% by dry weight. Scale bars = 1 μm.

probably form a layer (but might not be integrated) on the surface of droplets that inhibits the passage of water vapour and, as a consequence, the surface layer expands like a balloon. When the water fully evaporates, the leucine surface layer collapses resulting in the observed wrinkled structure (Raula et al., 2007). The advantage of wrinkled, hollow particles is that they have a reduced tapped density and there is less contact between particles, thereby resulting in a better in vitro deposition (Ranade, 1987).

3.3. Powder aerosolization properties

3.3.1. Emitted dose

The emitted dose (ED) of the resultant powders is shown in Fig. 3. The majority of the powders with binary carriers (M1–M8, L1–L8) had an ED of over 90% of the total capsule contents, which is clinically acceptable. The more leucine included in the formulation, the higher the ED obtained. A relatively high ED of about 98% was observed with M1 and M2. Interestingly, some powders had a higher ED in spite of their poorer flowability, which could be due to the fact that turbulence was able to deagglomerate the particles to some extent. The two leucine-free powders, Man and Lac, exhibited a lower ED, 87% and 83%, respectively. In the case of formulations with a low ED, it was found that the residual particles adhered to the wall of inhaler and/or capsule shells due to electrostatic attractions.

3.3.2. In vitro deposition

The dry powders showed great variations in terms of the respirable fraction (RF, Fig. 4). Obviously, the difference in RF observed for the powders must be solely related to the differences in the formulation components prior to spray-drying, as the same spray-drying operating conditions were employed for each powder. Among all the powders, Leu, M1, M2 and M3 had higher RF values (44.8%, 45.6%, 44.9% and 43.8%, respectively) than M4 ($P < 0.05$).

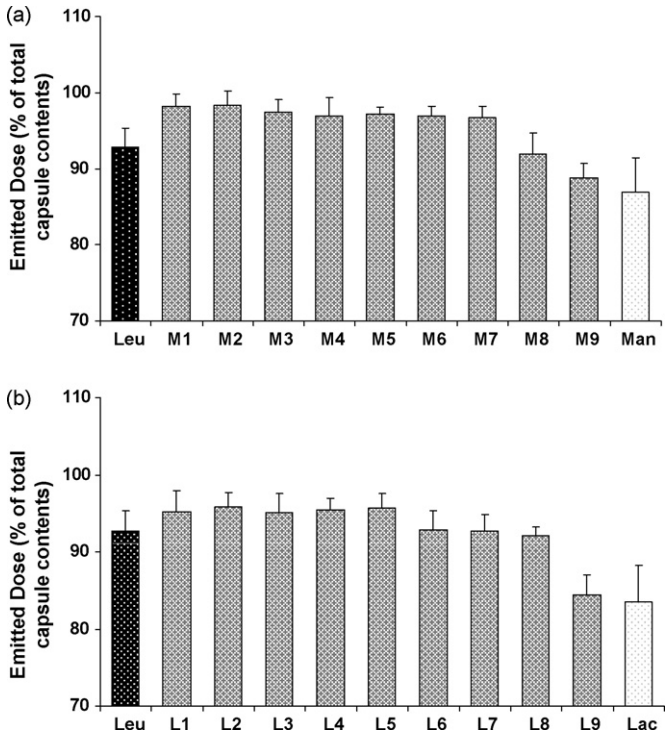

Fig. 3. Emitted dose of spray-dried powders. (a) From Leu to Man, powders were generated from TP5/mannitol/leucine with the ratio of 10:0:90, 10:9:81, 10:18:72, 10:27:63, 10:36:54, 10:45:45, 10:54:36, 10:63:27, 10:72:18, 10:81:9 and 10:90:0, respectively. (b) From Leu to Lac, powders consisted of TP5/lactose/leucine at the same ratios described in (a). Poloxamer 188 was added in each powder at a concentration of 2%, w/w.

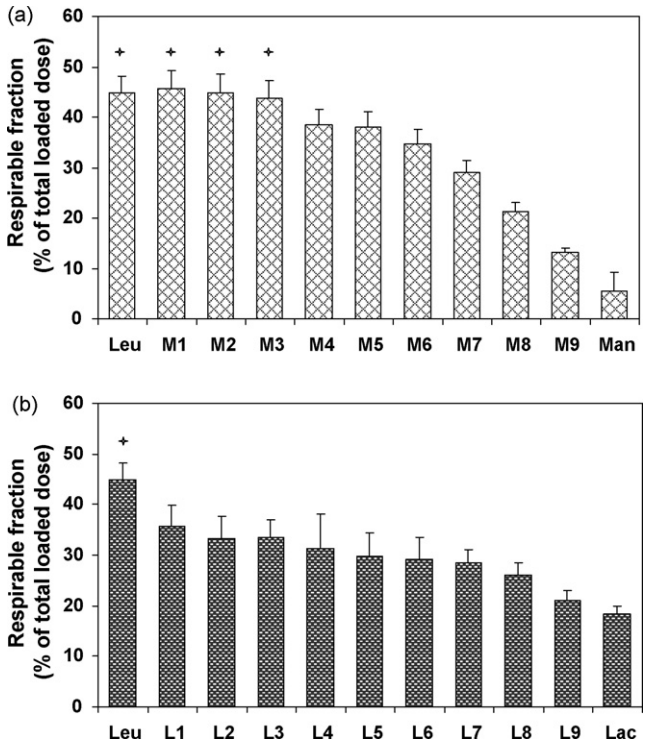

Fig. 4. Respirable fraction of spray-dried powders. The powder compositions were described in Fig. 3. Values are mean ± SD, $n = 3$. (a) $^{*}P < 0.05$, compared to M4; (b) $^{*}P < 0.05$, compared to L1.

In particular, powder M2 exhibited the best flowability and, so, could be considered as a suitable formulation. Fig. 4 demonstrated that a higher loading of leucine in the formulation contributed to a better aerosolization performance, which was more apparent with TP5/mannitol/leucine powders than with TP5/lactose/leucine powders. Decreasing the leucine concentration was reflected in a reduction in the RF. The Man powder exhibited the poorest RF of 5.43%.

The influence of leucine on powder aerosolization has been evaluated previously (Najafabadi et al., 2004; Chew et al., 2005). These researchers reported that the incorporation of leucine into sodium cromoglycate resulted in a more dispersible powder, and attributed this to the surface activity of leucine leading to its accumulation at the particle surface during the spray-drying process. However, it is necessary to examine whether the results obtained by these two groups could be more widely applied to other drugs, given that the powders they produced consisted of over 90% sodium cromoglycate. Recently, Seville et al. (2007) have demonstrated that incorporating a range of amounts of leucine (5–20%) in formulations, with a relatively low drug loading (4% salbutamol), can significantly reduce the interactions between the resulting particles, leading to an enhanced dispersibility and functional in vitro pulmonary deposition.

Compared with the previous investigations mentioned earlier, in this study, the amount of leucine employed in the formulations covered a wider range (9–81% by dry weight). The results of RF revealed that leucine improved the aerosolization performance of the TP5-contained dry powder and was more effective at higher concentrations (>63% of total weight). Combining more leucine with less mannitol (e.g. powders M1, M2 and M3) resulted in a satisfactory RF exceeding 40%. The contribution of leucine to the improved aerosolization could be due to its surfactant-like and anti-adherent properties. When agglomerates of particles are formed, the addition of leucine reduces the stability of those agglomerates so that they are more likely to break up in the turbulent air stream

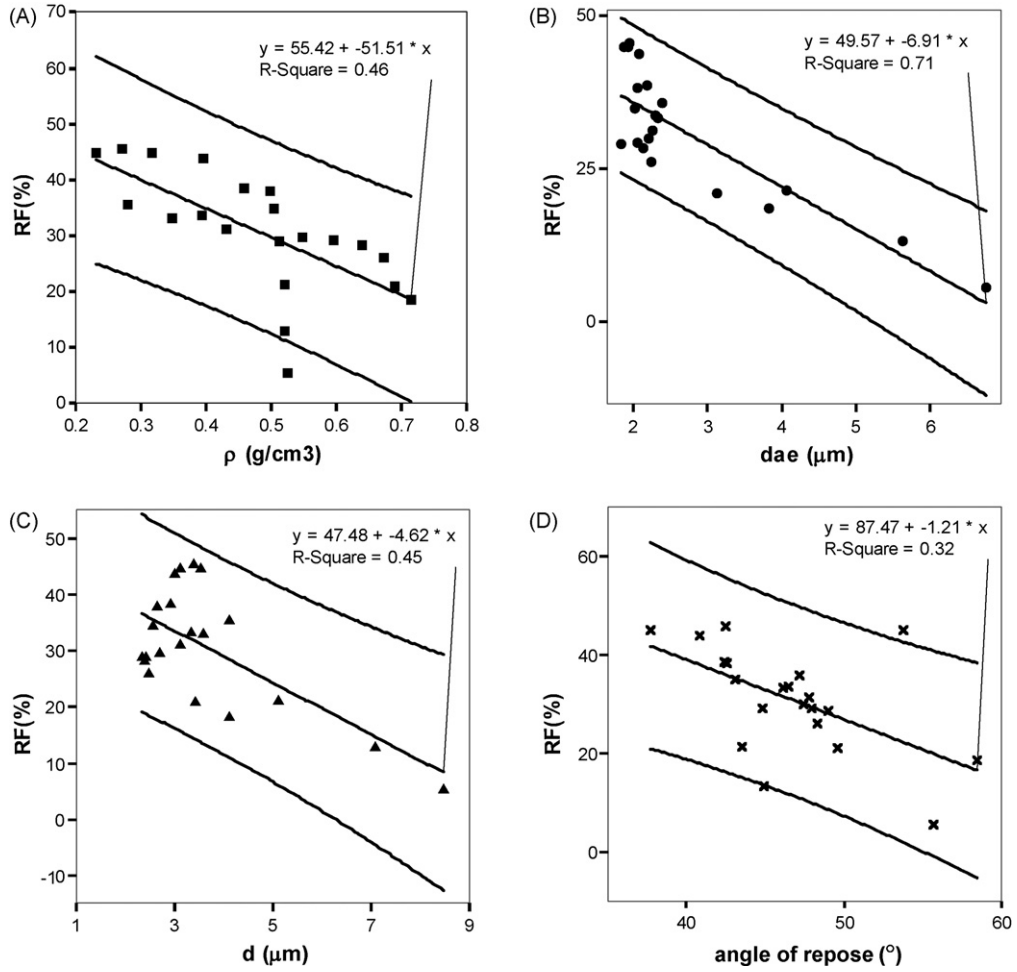

Fig. 5. Influence of the physical properties on the respirable fraction (RF) of dry powders. (A): Tapped density (ρ) vs. RF, standard error of the estimate (SE) = 8.06; (B): aerodynamic diameters (d_{ae}) vs. RF, SE = 5.85; (C): geometric size (d) vs. RF, SE = 8.09; (D): angle of repose vs. RF, SE = 9.05. Linear regression with 95% individual prediction interval was performed. The analysis of variance showed the correlations between RF and the different parameters were significant ($P < 0.01$).

created on inhalation to form small individual particles which are likely to reach the lower lung (Chougule et al., 2007).

3.4. Influence of the physical characteristics on the aerosolization properties

It has been reported that the aerodynamic behavior of a powder depends on multiple interrelated factors, such as primary particle size, particle density, powder composition, powder crystallinity, surface properties and powder cohesiveness (Bosquillon et al., 2001). Hence, the influence of the physical characteristics of the powders on their RF was investigated in this study. As shown in Fig. 5, the tapped density, geometric size, aerodynamic diameter as well as the repose angle of the dry powders could all affect the respirable fraction in the same way, i.e. a lower level of these factors resulting in a higher RF. Although lower R-square values were obtained from the test, the correlations between these physical properties and the RF could be fitted to linear models and the results exhibited very statistically significant differences ($P < 0.01$).According to the observations described above, investigations should focus on the development of porous/low density particles having a smaller particle size, a density below 0.4 g/cm^3, and an aerodynamic diameter between 1 μm and 3 μm to achieve a higher respirable fraction and avoid the natural clearance mechanism operating in the lungs (alveolar macrophage uptake) due to the higher geometric diameter of the particles. Such aerodynamically light particles also provide a solution to particle aggregation and preserve the flowability of the powder by reducing particle interactions (Edwards et al., 2005).

3.5. Roles of poloxamer 188 in the formulations

Inhaled biopharmaceuticals need to have their size reduced by suitable techniques that not only produce particles within the target range of 1–5 μm but also with optimal surface properties to aid pulmonary delivery without loss of biological activity (Shoyele and Cawthorne, 2006). During spray-drying, proteins and peptides can be absorbed at the air–liquid interface of droplets, unfold and aggregate at the droplet surface to cause surface denaturation. Surfactants have been shown to reduce this surface denaturation by excluding the protein or peptides from the interface (Adler et al., 2000; Maa et al., 1998a,b). The surfactants used for stabilizing macromolecule drug formulations include serum albumin, amino acids, polysorbate 80, and poloxamer 188 (Johnson, 1997). Another function of the surfactants is to control the surface properties of micronized particles and, thus, prevent the particles from agglomerating and becoming highly charged. Steckel and Brandes (2004) have reported that poloxamer 188 plays a special role in determining the spray-dried particle morphology. They found that increasing the amount of poloxamer 188 affected the flowability and dispersibility of the powders as a higher number of fine particle fractions was obtained. Nevertheless, higher concentrations of

poloxamer 188 (>2%) lead to highly charged particles limiting the use of poloxamer 188 to a concentration of 2%.

In our study, poloxamer 188 was employed in each formulation at a concentration of 2%. The determination of drug contents exhibited no difference between the powders with and without poloxamer (data not shown), indicating that poloxamer is not necessary to protect TP5. This can be presumably explained by the simple structure of TP5, which has only five amino acid residues. As an oligopeptide, TP5 is less amphipathic for absorption at the air–liquid interface and denaturation. However, the addition of poloxamer 188 had a positive effect on the flowability of powders. For example, powder M2 containing poloxamer exhibited a repose angle of 37.7° in contrast to 48.9° with the removal of the polymer from the system.

4. Conclusion

This paper describes a new concept involving the development of DPI containing TP5, which has received little attention to date. In this study, lactose, mannitol and leucine were selected as candidate carriers for TP5 inhalation. The results revealed that the formulation composition had significant effects on the physical characteristics of the dry powders, such as tapped density, geometric and aerodynamic size as well as flowability and, thus, influenced their aerosolization performance. It was found that the aerodynamically light particles provided a solution to particle aggregation and, accordingly, resulted in higher in vitro deposition. Among the developed powders, the formulation TP5/mannitol/leucine 10/18/72 was preferred; this was free-flowing and the respirable fraction was as high as 45% and, so, it would be anticipated to deposit in the lower regions of the respiratory tract, thereby facilitating systemic delivery of TP5.

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