Leucine Coated Inhalable Insulin and Thymopentin Peptide Powders Produced by Aerosol Flow Reactor Method

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Summary

Pulmonary route of drug delivery has been the focus of intense research in order to improve the efficacy of therapeutic peptides both for local and systemic treatment. However, formulation of such inhalable powders is challenging since the peptide may be in inactive conformation due to the thermodynamic effects of particle preparation process. In this research, inhalable insulin and thymopentin peptide powders were prepared by the aerosol flow reactor method from precursor solutions of peptide, trehalose, sodium citrate and leucine for the protection of the labile peptides. Scanning electron microscopy images of inhalable peptide powders showed intact and separated particles with spherical and partly-buckled shapes. Particle size distribution of insulin particles was 1.13 μm and thymopentin particles was 0.63 μm. Aerosolization of inhalable powders were measured with an inhalation testing device showed that emitted dose (ED) of the insulin powders were 1.9 mg/dose and fine particle fractions (FPF) of the insulin powders were 55-59%. Thymopentin powders exhibited 1.5-1.7 mg/dose of ED and 27-36% of FPF. Additionally, inhalable insulin powders were analysed by circular dichroism after the particle preparation process and results indicated similar α-helical conformation which assures the conformational stability of insulin after the aerosol process. Furthermore, high performance liquid chromatography analysis of both thymopentin and insulin powders indicated a similar corresponding solute peak as the pristine samples at the same retention time. Protective encapsulation of peptides was shown to be successful and temperature pulse did not affect the peptide stability. In the near future, cellular interaction, cytocompatibility and drug permeation properties of the peptide powder particles will be investigated.

Introduction

The discovery of new therapeutic peptides and the improvement of conventional therapeutic treatments demand new strategies for an efficient drug delivery. Innovative biopharmaceutical molecules are proposed to treat the rising number of diseases such as for cancer treatments with monoclonal antibodies [1], vaccines [2] and insulin for the treatment of diabetes [3]. However, the efficient delivery of the therapeutic peptides has remained a challenge in current pharmaceutical research due their large molecular size, hydrophilicity and chemical and enzymatic lability [4]. Peptides are rapidly degraded by enzymes or by low pH conditions in gastrointestinal tract and as injected the peptides face rapid hepatic degradation and low patient compliance. These challenges stimulated much of the research towards the pulmonary delivery of the peptides as a promising non-invasive alternative route for the delivery of labile peptides.

An ultimate demand in the particle engineering of peptides is that the peptides are still active in their resulting inhalable formulation. Therefore, we have developed an aerosol-based method [5] where peptide particles are gently dried followed by rapid consecutive decrease and increase in the saturation condition of a coating material L-leucine. This renders particles flowable and dispersible [6] and also provides protective encapsulation [7] for peptides while the activity of peptides in sustained.

Materials and Methods

Human insulin in powder form was purchased from Benjamin Pharmaceutical Chemical Co. Ltd., China. Insulin solution was prepared by lowering the pH to 2.8 with 0.1 M acetic acid solution. After dissolution of the insulin, the pH was adjusted to 7.5 with 1 M NaOH solution. D-Trehalose (Sigma Aldrich, USA), sodium citrate tribasic dihydrate (Sigma Aldrich, Japan) and L-Leucine (Alfa Aesar, Germany) were weighed and dissolved in the insulin solution. Drug-to-excipient ratio was 1:9. Final solution was used in the aerosol process. Resulting insulin particles is referred as *Ins-Tr-L particles* in this paper. Peptide-free excipient (trehalose, leucine and sodium citrate) particles are referred as *Tr-L particles*.

Thymopentin (TP5) in powder form was ordered from Huajin Pharma, China. Precursor thymopentin (2.5 g/l) solution contained trehalose (12.9 g/l), L-leucine (6.4 g/l) and sodium citrate (3.2 g/l) dissolved in ion-exchanged water. Final solution was used in the aerosol process. The resulting thymopentin particles is referred as *TP5-Tr-L particles* in this paper.

Droplets were generated with an ultrasonic nebulizer and transferred with nitrogen gas into a laminar flow reactor that was set to 50°C. Liquid volume consumption was 7×10^{-4} with a gas flow rate 20 l/min. Thereafter the temperature was pulsed up to 230 °C for 0.25 s of residence time. At the downstream of the reactor, the dry particles were cooled and diluted in a porous tube. Insulin microparticles were collected and stored at 20°C at 0% relative humidity for further analysis.

Particle size distributions in the gas phase were determined with an electrical low-pressure impactor, ELPI (Dekati LTD., Finland). Morphology of the Ins-Tr-L particles, TP5-Tr-L particles and Tr-L particles were imaged with a scanning electron microscope (Zeiss Sigma VP) at 1.5 kV.

High Performance Liquid Chromatography (HPLC) elution profiles of pristine insulin, pristine thymopentin, Ins-Tr-L particles, TP5-Tr-L particles and Tr-L particles were analysed using Agilent-Hewlett Packard 1050 Series HPLC System with UV detector at ambient temperature. Chromatography was performed with a SunFire RP-C18 column (4.6x150 mm, 5µm). Sample concentrations of 3 to 5 mg/ml were run with the injection volume of 40 µl and the peptide was detected by UV at wavelength 220 nm. The mobile phase consisted of acetonitrile and 0.1% trifluoroacetic acid. A linear gradient from 0 to 60% acetonitrile was applied in 30 min at 1 ml/min.

Circular dichroism (CD) data were collected using a JASCO J-720 CD spectropolarimeter. Pristine insulin and insulin aerosol samples were diluted in 0.1 M acetic acid solution to get final concentration of protein 0.1 mg/ml. CD spectra were collected in the wavelength range of 190-260 nm using 1mm path length cuvette. Temperature controller was used and measurements were carried out at 20°C. Each final spectrum was averaged from three consecutive scans. Spectra of the acetic acid solution and the excipients were recorded and subtracted from Ins-Tr-L particle sample spectrum. The resulting spectra were smoothed by the Savitzky–Golay function using a convolution width of 20 points.

Fine powder aerosolization of powders was studied using an inhalation testing device developed in-house. The detailed operating principles has been discussed before [6][8]. Briefly, Easyhaler® reservoir was filled with 1 g of the peptide powder and inhalation was performed 10 times. Inspiration flow rates of 40 L/min and 55 L/min were adjusted by pressure drops to 2 kPa and 4 kPa, respectively. Particles were collected on the stages of a Berner-type low pressure impactor (BLPI) wherefrom mass distributions were measured gravimetrically. Mass median aerodynamic diameters (MMAD), geometric standard deviation (GSD), of each deposited powders were calculated and fine particle fractions (FPF) were calculated according to the emitted dose (ED).

Moisture sensitivity analysis of peptide particles (Ins-Tr-L and TP5-Tr-L particles) and excipients particles (Tr-L particles) were conducted in desiccators, where 44%, 65%, and 75% relative humidity (RH) were achieved with saturated solutions of the salts $NaNO_2$, K_2CO_3, and NaCl at 25^oC, respectively. Solid state properties of the samples were then characterized with scanning electron microscopy (SEM), differential thermal analysis (DTA) and x-ray diffraction (XRD) at 2nd and 7th days. XRD and DTA results are still underway.

Results and Discussion

The SEM images (Figure 1) of inhalable powders show intact and separated particles with spherical and partly-folded shape. The incorporation of insulin or thymopentin did not affect the surface morphology of the particles since no significant morphological differences are observed between Figure 1A, 1B and 1C. Number average size of Tr-L (only excipients: trehalose, sodium citrate and leucine) particles was 0.93 µm, Ins-Tr-L particles was 1.13 µm and TP5-Tr-L particles was 0.63 µm in gas phase.

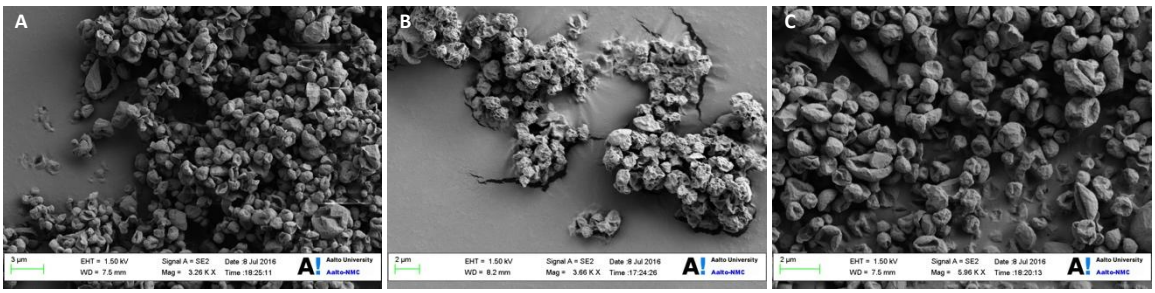

Figure 1 - SEM images of (A) Tr-L particles (excipients particles) (B) Ins-Tr-L particles (insulin particles) (C) TP5-Tr-L particles (thymopentin particles).

Powder aerosolization of excipient powders and peptide powders were compared in Table 1. Excipient (Tr-L) powders emitted dose (ED) was higher than insulin particles (Ins-Tr-L) and thymopentin (TP5-Tr-L) particles. On the other hand, fine particle fraction (FPF) of insulin (Ins-Tr-L) powders was highest compared to excipient (Tr-L) particles and thymopentin (TP5-Tr-L) particles.

The pristine peptides and peptide powders were further characterized and compared by reversed-phase high performance liquid chromatography. Both pristine insulin and insulin (Ins-Tr-L) particles indicated a similar corresponding solute peak and elution profile with retention time at 24.9 min and similarly, retention time of both pristine thymopentin and thymopentin (TP5-Tr-L) particles were at 13.9 min.

Table 1 - Aerosolization results of the L-leucine coated powders from Easyhaler® at two pressures and inhalation flows.

SAMPLE	ED (mg/dose)		CV_{ED}		FPF (≤ 5μm,%)		MMAD (μm)		GSD	
	2 kPa	4 kPa	2 kPa	4 kPa	2 kPa	4 kPa	2 kPa	4 kPa	2 kPa	4 kPa
Ins-Tr-L	1.9	1.9	0.3	0.4	55	59	1.6	1.3	1.9	1.7
TP5-Tr-L	1.6	1.7	0.6	0.5	36	27	1.2	1.1	2.1	2.0
Tr-L	2.3	2.6	0.2	0.1	46	53	2.0	2.0	1.8	1.9

Circular dichroism spectra (Figure 2) of pristine insulin (green line) and insulin (Ins-Tr-L) particles (red line) were almost the same indicating similar α-helical conformation which assures the conformational stability of insulin after the aerosol process. Therefore, it can be stated that the new way of engineering the leucine coating can be successfully applied for the preparation of inhalable peptide particles. Stability of insulin particles were further studied with particles that were stored at room temperature (RT) at 0% relative humidity for 87 days. The CD spectra of stored particles (blue line) did not exhibit irregular shaped peptide structure and exhibited similar spectra as the pristine insulin, indicating an α-helical conformation. Therefore, the insulin particles are stable at room temperature at 0% relative humidity.

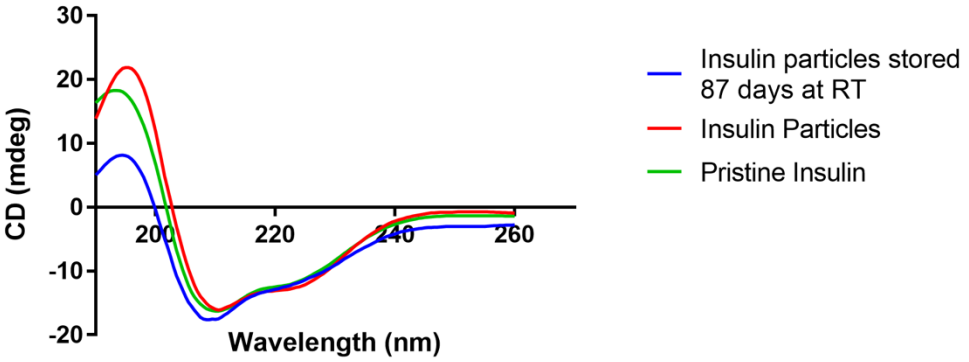

Figure 2 – Circular dichroism spectra of pristine insulin (grey line), insulin particles (orange line) and insulin particles stored at room temperature at 0% relative humidity for 87 days.

Stability of the inhalation powders were further investigated under humid conditions and morphology analysis of each particle is shown in Figure 3. In general, all the particles under humid conditions did not strongly coagulated as clumps and remained as individual particles. However, formation of necks with adjacent particles was observed on insulin particles (Ins-Tr-L) at 75% RH on Day 7. The aerosolization and agglomerate formation of particles under humid conditions will be further analysed by inhalation test device. Moreover, solid state characteristics of particles under humid conditions will be investigated by XRD analysis and DTA analysis.

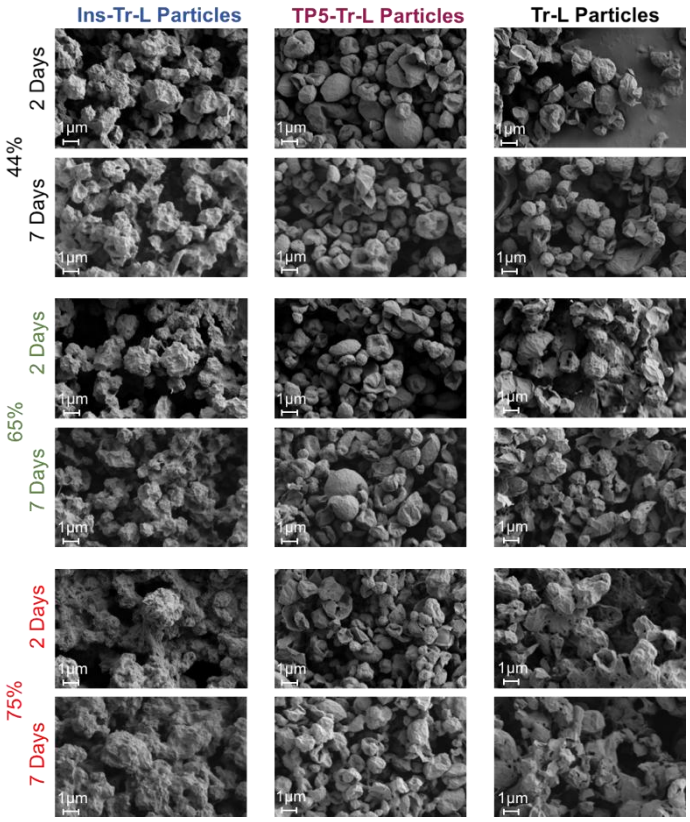

Figure 3 – The SEM images of the particles under humid conditions (44%, 65%, 75% RH) at room temperature. The samples were collected and imaged on Day 2 and Day 7 after conditioned.

Conclusions

We have demonstrated the production of conformationally stable insulin and thymopentin inhalable dry powders. A rapid change in temperature needed for the leucine coating did not affect the structure and the conformation of the peptides. Protective encapsulation of peptides was shown to be successful and temperature pulse did not affect the peptide stability. We will further analyse the solid state properties of the peptide powders with other methods such as XRD, DTA and FTIR spectroscopy to confirm the peptide stability. Furthermore, particle permeation through cell monolayer experiments will be studied in the near future for analysing the efficacy of peptide particles.

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