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Research paper

Formulation and in vitro evaluation of highly dispersive insulin dry powder formulations for lung administration

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ABSTRACT

The aim of this work was to develop highly dispersible and dry formulations of insulin for use in dry powder inhalers (DPIs) using high-pressure homogenisation (HPH) and spray-drying. Several formulations were evaluated, including formulations spray-dried without excipients and formulations coated with lipids. A physiological lipid composition based on a mixture of cholesterol and phospholipids was used to form the coating film around micronised drug particles. The production technique and excipients were chosen in order to limit the degradation of the active ingredient.

The resulting powders exhibited a size and shape suitable for the deep lung deposition of drugs, and good aerodynamic features were obtained for the different formulations tested, with fine particle fractions between 46% and 63% vs. 11% for raw insulin powder. The presence of a lipid coating of up to 30% (w/w) did not significantly affect the aerodynamic behaviour, and the coated formulations also exhibited a decreased residual moisture content of between 2.3% and 3.7% vs. 4.8% for raw insulin, which should improve the long-term stability of the protein formulations. No degradation of the insulin molecule occurred during the HPH/spray-drying process, as it was shown using an HPLC method (insulin content between 98.4% and 100.5%), and the content in high molecular weight proteins, assessed using a gel filtration method, stayed below 0.4%.

1. Introduction

Diabetes mellitus (DM) currently affects about 285 million adults worldwide, and this number is expected to increase to 439 million adults by the year 2030. It is the fifth leading cause of death in the United States [1,2]. Inadequate levels of insulin lead to blood glucose levels that are above normal. If this persists for long periods of time, the complications of diabetes, nephropathy, retinopathy, neuropathy and other serious microvascular and macrovascular complications begin to develop [3].

The benefits of tight glycemic control through the administration of exogenous insulin were shown in the landmark Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) in patients with type 1 and type 2 DM, respectively. However, many patients still do not achieve their glycemic goals [1].

For example, patients with type 2 DM, which account for about 95% of all diabetic patients, might benefit from improved glycemic control offered by exogenous insulin but are either not prescribed insulin or have a fear of injecting it. Therefore, alternative modes of administration may increase the number of patients that receive the benefits of insulin shown in the DCCT and UKPDS studies [1].

For this reason, research on non-invasive methods of insulin delivery such as oral, dermal, nasal and inhalative administration has been extensive for more than 30 years [4,5].

Several factors make the lungs an excellent site for drug absorption, including their vast surface area, their rich blood perfusion and the very low thickness of the alveolar epithelium (0.1–0.5 μm). Metabolic activity of the lung is also limited in comparison with that of the gastro-intestinal system, which should confer increased stability to the proteins administered by inhalation [6]. However, in comparison with subcutaneous (sc) insulin injection, inhalation exhibits a lower reproducibility and dosage precision [7]. It is also not possible to administer sufficient drug doses to the lungs because of an inadequate particle size spectrum that allows aerosol deposition in the bronchial system and not in the alveoli [8]. Liquid aerosols and dry powder formulations that are currently under development were designed in order to overcome these problems and show particular promise. The pharmacokinetic profiles of currently developed inhaled insulin formulations also exhibit interesting features. To control post-prandial blood glucose increase, the insulin regimen should mimic the physiological first-phase insulin response, with an early peak followed by a tail of activity correlating with the second phase of insulin release by β-cells [9]. In a number of single-dose clinical studies performed

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with different inhaled insulin formulations, inhaled insulin showed a faster onset of action than sc regular insulin. Maximum metabolic action and maximum insulin concentrations were also faster [8,10,11]. Inhaled insulin has, in fact, an onset of action comparable to sc short-acting insulin lispro ($t_{early\ 50\%}$ of 32 min and 41 min, respectively) and a duration of action comparable to sc regular insulin ($t_{late\ 50\%}$ of 387 min and 415 min, respectively) [12]. These features allow inhaled insulin to control post-prandial glucose more efficiently, thanks to the similarity of its pharmacokinetics and pharmacodynamics to the profile of natural insulin secretion [10,12]. Moreover, the within-subject variability of inhaled insulin appears to be comparable to that of sc regular insulin [8,11] and, after adjustment of the dosage, its clinical efficacy is comparable to that of sc regular insulin [9].

Treatment with inhaled insulin was previously considered safe and well tolerated, with most data regarding long-term tolerability published for the Exubera® and AERx iDMS® systems over study periods of up to 2 years. The main adverse effects were slight, reversible changes in lung function parameters and a rise in insulin antibodies (not associated with any clinical or safety parameters) [13].

However, the first pulmonary delivered version of insulin (Exubera®) was taken off the market in October 2007 because of unexpectedly low sales. A few months later, the FDA also published a press release reporting a potentially increased risk of bronchial carcinoma in ex-smokers treated with Exubera®. Insulin does indeed act as a weak growth factor after binding to the receptor for IGF-1 (efficiency of only 1/100 of IGF-1) and could therefore be involved in the six newly diagnosed cases of primary lung malignancies in clinical trials among Exubera®-treated patients [13]. However, there is, to date, no evidence for a relevant competitive effect of inhaled insulin at the IGF-1 receptors in the lung. Moreover, all of these patients had a prior history of cigarette smoking, and the number of cases was too small for a final risk evaluation [13].

In this context, we believe that even if this potentially increased risk for lung cancer should be subjected to further investigation prior to the product relaunch of an inhaled insulin therapy, there is still a benefit in developing insulin formulations for inhalation. It is a non-invasive alternative therapy with treatment satisfaction that is higher than that for sc insulin, as observed in the open-label studies [11]. It might be particularly useful in patients with type 2 diabetes who fail on oral antidiabetic therapy and whose switch to insulin treatment is often delayed, and in patients with needle phobia, who represent at least 10% of the population [13].

It is estimated that the bio-availability and bio-efficacy of the current inhalation systems is approximately 8–15% compared to sc insulin [9,11,14]. This poor performance can be mainly attributed to losses within the device and in the patient's mouth and oropharynx. Indeed, it was shown that about 30–50% of insulin that reaches the lungs is absorbed systemically [9,14]. Therefore, improvements in the formulation and/or delivery device could lead to better performance.

The deposition site in the respiratory tract and the efficiency of inhaled aerosols are critically influenced by the aerodynamic particle size distribution. Inhaled particles should have an aerodynamic diameter between 1 μm and 5 μm in order to reach the lower airways [15]. Because micronised particles are generally very cohesive and characterised by poor flow properties, the most widespread formulation strategy for a dry powder inhaler (DPI) consists of blending the drug with coarse and fine carrier particles [16]. Carbohydrates, in particular lactose, are widely used for this purpose in formulations for DPI [17].

Another strategy for improving the dispersion properties of powders is to use solid lipid microparticles (SLmP) [18,19]. This promising formulation alternative in terms of particle dispersibility and potential tolerance in lung tissue was used in this work by producing a lipid coating around insulin particles in order to develop DPI insulin formulations. The solid lipid microparticles were made of cholesterol and phospholipids that are physiological lipids of the pulmonary surfactant. They are expected to be well tolerated within the tracheo-bronchial tree due to their composition, which is comparable to liposomes [20]. Unlike the hydrophilic excipients commonly used in DPIs, the hydrophobic nature of cholesterol should reduce the adsorption of water vapour during storage, improving the long-term stability of the encapsulated protein. Water is indeed involved in all of the major stability issues of proteins [21].

Moreover, the limitation of water adsorption could limit the agglomeration of particles by decreasing the capillary forces between them. This could improve the inspiration-driven aerosolisation properties of the powder and subsequent pulmonary deposition. In addition, the lipid coating is made up of a mixture of amphiphile (i.e., phospholipids) and hydrophobic compounds (i.e., cholesterol), which should allow a large capacity of encapsulation to be obtained for both water-soluble and fat-soluble active ingredients. Phospholipids, on the other hand, could promote the dispersion and dissolution of the active ingredient in physiological liquid and, perhaps, act as an absorption enhancer [22]. They could also reduce the generation of electrostatic charges at the surface of the particles.

The aim of this work was to develop SLmP formulations of insulin for use in DPIs using spray-drying. High dispersion properties were sought, as well as deep lung deposition of the powders. The production technique and excipients were chosen in order to limit the degradation of the active ingredient. Insulin was chosen as a model protein because of the huge therapeutic and economic potential generated by the use of inhaled formulations in patients with diabetes. This is a well-known and thoroughly studied substance that will permit evaluation of the application of SLmP technology to other bioactive substances, such as proteins and monoclonal antibodies. Several formulations were evaluated, including two "reference" formulations spray-dried without excipients and formulations coated with lipids (SLmP formulations).

2. Materials and methods

2.1. Materials

Raw recombinant human insulin was supplied as a micronised powder from Sigma–Aldrich (St. Louis, MO). Cholesterol was purchased from Bufa (Uitgeest, The Netherlands). Phospholipon® 90H (Ph90H), hydrogenated soy lecithin, with more than 90% hydrogenated phosphatidylcholine, consisting of approximately 85% distearol phosphatidylcholine and 15% dipalmitoyl phosphatidylcholine, was purchased from Nattermann Phospholipids GmbH (Koln, Germany). Isopropanol and acetonitrile were of HPLC grade and were purchased respectively from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO). Low-viscosity silicone oil (20 cSt) was purchased from Dow Corning (Midland, Michigan). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Preparation of the particles from a solution without excipients

A formulation of spray-dried insulin particles with no excipient was prepared by spray-drying using a Büchi mini spray-dryer B-191 (Büchi Laboratory-Techniques, Switzerland) equipped with a pneumatic spraying system and a high-performance centrifugation cyclone. The chosen operating conditions for spray-drying were optimised and were inspired by the literature [23]: nozzle size, 0.7 mm; inlet temperature, 100 °C; resulting outlet temperature, 40 °C; spraying air flow rate, 800 l/h; drying air flow rate, 35 m^3/h; and solution feed rate, 5 ml/min.

The solution was constituted by a 5 mg/ml insulin solution in water, prepared by alkalinisation with a few drops of 0.1 M NaOH

followed by acidification to pH 7.4 with 0.1 M HCl. Batches of 500 mg were produced each time. Following these conditions, the process yield was around 66%.

2.2.2. Preparation of particles from a suspension, with or without a lipid coating

The lipid coating was made up of a mix of cholesterol and saturated phospholipids (Phospholipon® 90H). Saturated phospholipids have the advantage of presenting high transition temperatures (T_c ± 54 °C for Phospholipon® 90H). However, a preponderant ratio of cholesterol was sought to limit the softening phenomenon during spray-drying, as it has a melting temperature around 140 °C. A cholesterol/Phospholipon ratio of 75:25 w/w was used, which has already been found to give appropriate size, density and aerodynamic features to the coating of other active substances for inhalation, such as tobramycin and budesonide, as previously described [18,19].

In order to get a final particle size distribution compatible with pulmonary administration, the particle size of the raw insulin powder was first reduced using high-pressure homogenisation (HPH) [24,25]. The efficiency of the particle size reduction by HPH is dependent on the homogenising pressure applied, the number of homogenising cycles, the drug hardness characteristics and, possibly, the processing temperature [25].

Insulin was first suspended in isopropanol (2% w/v), and dispersion of the powder was ensured by 10 min of ultrasonication in a 40 kHz Branson® 2510 bath. The particle size was then reduced by HPH using an EmulsiFlex-C5 high-pressure homogeniser (Avestin Inc., Ottawa, Canada). Pre-milling low-pressure homogenisation cycles were first conducted on the insulin suspension to further decrease the particle size (10 cycles at 7000 PSI and 10 cycles at 12,000 PSI). HPH was then finally applied for 30 cycles at 24,000 PSI. These cycles were conducted by recirculating the processed suspension directly into the sample tank (closed loop). Because HPH causes a sample temperature increase (increase of 30 °C following 20 cycles at 24,000 PSI), all operations were carried out using a heat exchanger placed ahead of the homogenising valve, with the sample temperature maintained at 5 ± 1 °C. This permits limitation of the degradation of labile materials such as proteins. This protocol was chosen after particle size analysis of the samples between the different size reduction steps.

Lipids (cholesterol/phospholipids 75:25 w/w) in proportions of 0%, 10%, 20%, 30% or 40% w/w in relation to the insulin load were then dissolved in a small volume (<3 ml) of hot isopropanol (55 °C) before addition to the homogenised insulin suspension. Whereas insulin is insoluble in isopropanol, lipids are soluble in it and will coat the micron-sized particles during atomisation. The suspension was then spray-dried using the same equipment as previously described.

Some modifications were made to the commercial mini spray-dryer in order to improve its drying efficiency and to avoid partial melting or softening of the lipid excipients incorporated into the SLmP formulations [18,19]. The following optimal conditions of spray-drying were used for the coated and uncoated formulations: inlet temperature, 70 °C; resulting outlet temperature, 22–25 °C; spraying air flow rate, 800 l/h heated to 55 °C; drying air flow rate, 35 m^3/h; solution feed rate, 2.5 g/min; nozzle size, 0.75 mm; cold air temperature, −5 °C, generated at 10 m^3/h; and cold water circulation in the jacketed cyclone, 5 °C. Following these conditions, the process yield was about 60%.

2.2.3. Particle size

Particle size and size distribution was measured by laser diffraction using a Malvern Mastersizer 2000 laser diffractometer with a liquid sampling system (Hydro S, Malvern, UK).

The particle size was characterised by the *Volume median diameter*, $d_{(0.5)}$, corresponding to the size in microns at which the distribution is divided into two samples of the same volume, and the *Volume mean diameter*, $D_{[4.3]}$, which is the average diameter, balanced by the total volume of particles contained in each histogram class. This is the more representative value because each variation in size distribution will result in a change in $D_{[4.3]}$. In addition, $V\% < 5\ \mu m$ was the total volume percentage of particles with a diameter below 5 μm.

The insulin particles without coating were measured using the following parameters: refractive index of insulin: 1.544; absorption index: 0.1; dispersing liquid: low-viscosity silicone oil (20 cSt) containing 0.1% (m/v) Span 20 and 8% (v/v) isopropanol; pumping rate: 1750 rpm; and obscuration: 4%. This low obscuration rate was used in order to avoid multiple scattering. A 3-min ultrasonication sequence (70% power) was applied, and the sizes were measured after stabilisation of the laser obscuration (2 or 3 min).

The insulin particles with a lipid coating (SLmP formulations) were measured using the following parameters: refractive index of lipids: 1.6; absorption index: 0; dispersing liquid: distilled water containing 0.1% Polysorbate 80; pumping rate: 1750 rpm; and obscuration: 4%. A 3-min ultrasonication sequence (70% power) was applied, and the sizes were immediately measured.

2.2.4. Scanning electron microscopy (SEM)

Evaluation of particle size and morphology was achieved by scanning electron microscopy (SEM). Samples were scattered on a carbon tape that was then dusted to remove the excess. A 30-min depressurisation was applied in the coater before coating with a platinum layer (pressure before coating: 3×10^{-2} mbar; pressure under Ar: 6×10^{-2} mbar; coating duration and power: 40 s and 40 mA). The acceleration voltage during observation was 8 kV.

2.2.5. Insulin content and degradation

2.2.5.1. HPLC. An initial evaluation of insulin stability during the spray-drying process was carried out following an HPLC method described in European Pharmacopoeia 6.0 using an HP 1100 series apparatus (Agilent Technologies, Belgium) equipped with a quaternary pump, an autosampler, an oven heated to 40 °C and a variable wavelength UV detector set at 214 nm. The separation system, as prescribed in the insulin monograph, was a 25 cm × 4.6 mm stainless steel (5 μm particle size) reversed-phase C18 column (Agilent Technologies, Belgium).

The mobile phase (acetonitrile–phosphate buffer solution adjusted to pH 2.3 with phosphoric acid, 26:74) was run at a flow rate of 1.0 ml/min. Samples were dissolved in 0.01 M HCl.

In the presence of lipids, the mobile phase composition had to be adapted to a 28:72 acetonitrile/phosphate buffer ratio in order to avoid a small lipid peak appearing at the same retention time as insulin.

A method validation was performed following the procedure from "Guide de validation analytique de la SFSTP" [26]. Linearity, accuracy and precision were assessed. The values presented are the average of at least three determinations.

2.2.5.2. High molecular weight proteins. Another important point to consider in order to assess conservation of the biological activity of insulin in the formulations is the possible presence of high molecular weight proteins (HMWP) arising from the covalent aggregation of insulin. These HMWP were searched for using size-exclusion chromatography (SEC), a method that could also help in detecting a loss of insulin tertiary structure, as it is a separation technique based on the hydrodynamic diameter of the molecules.

The SEC HPLC method used is described in European Pharmacopoeia 6.0. We used an HP 1100 series apparatus (Agilent Technol-

ogies, Belgium) equipped with a quaternary pump, an autosampler and a variable wavelength UV detector set at 276 nm. The separation system, prescribed in the insulin monograph, was a TSK-GEL® G2000SWXL column (Tosoh Bioscience), usable for globular protein separation between 5×10^3 and 1.5×10^5 Da. The insulin aggregates were identified based on the retention times mentioned in the method of reference.

System suitability was established using raw insulin (solution at 4 mg/ml in 0.01 M HCl) subjected to one week of stirring at 40 °C in order to obtain a sufficient percentage of high molecular weight proteins (0.4%) to be detected.

2.2.6. X-ray diffraction

X-ray powder diffraction (XRPD) is a powerful tool that is widely used for crystalline state evaluation. Diffraction patterns were determined using a Siemens diffractometer D5000 (Siemens, Munich, Germany) with a Cu line as the source of radiation (WL1 = 1.5406 A, WL2 = 1.54439 A) and standard runs using a 40 kV voltage, a 40-mA current and a scanning rate of 0.02°/min over a 2θ angle range of 2–70°. The degree of crystallinity was evaluated by modelling the amorphous and crystalline areas using a pseudo-Voigt function (Topas fitting software).

2.2.7. Residual solvents

The residual water content of the powder formulations was assessed using thermogravimetric analysis (TGA) with a Q500 apparatus (TA instruments, New Castle, USA). Runs in triplicate were set from 30 to 300 °C at 10 °C/min using samples of between 5 and 10 mg in a ceramic pan. The weight loss observed between 30 and 160 °C was attributed to water evaporation from the powder, and the percentage of weight change was defined as the moisture content of the powder. However, this weight change was also expected to include a small loss in isopropanol, which is the only other potential solvent present in the powders. A specific determination of isopropanol content was performed using gas chromatography and is described hereafter. Weight loss occurring after 160 °C was probably due to thermal decomposition of insulin, which was observed through browning of the powder.

As isopropanol was used as a dispersing solvent during the manufacture of the formulations obtained from a suspension, the residual content was determined in two of these formulations, namely the 0% and 20% lipid-coated formulations. This was done in anticipation of the fact that these formulations were selected to be administered to patients during a future phase I pharmacoscintigraphic study.

A gas chromatography (GC) technique was used for the determination of isopropanol with the following parameters: GC equipment: Carlos Erba Instrument "Auto/HRG/MS" MFC 500; capillary column: CP-Sil 5CB 25 m × 0.32 mm (Chromopack, Belgium); gas vehicle: He, Pressure: 50 kPa; injector temperature: 200 °C; detector temperature: 240 °C; column temperature: 40 °C for 10 min then gradient of 30 °C/min to 200 °C, maintained for 15 min; acquisition time: 25 min; and injected volume: 1 μl.

Samples were put into solution in dimethylformamide (DMF) containing ethyl acetate as an internal standard. A calibration curve was constructed with standards of increasing concentrations in isopropanol (250–10,000 ppm).

2.2.8. Aerodynamic features

2.2.8.1. Spraytec® laser diffraction. The Spraytec® (Malvern, UK) is a laser diffraction apparatus equipped with an inhalation cell specifically modified for measuring the particle size diameter (PSD) generated from medicinal aerosols, including MDI, DPI and nebulisers. This allows the particle size properties of DPIs to be measured under simulated breathing conditions in order to collect information on the real agglomeration state of the powder, unlike the classic laser diffraction technique, in which particles are in the individualised state.

The acquisition parameters were as follows: triggering mode: level 10%; data acquisition rate: 2500 Hz; acquisition duty cycle: 50%; test duration: 3000 ms; and refractive index: 1.50 (standard opaque particles). The volume median diameter ($d_{(0.5)}$), volume mean diameter ($D_{[4.3]}$) and total volume percentage of particles with a diameter below 5 μm ($V\% < 5$ μm) were determined.

2.2.8.2. Impaction measurements. Powder aerodynamic evaluation was performed using a multi-stage liquid impinger (MsLI) (Copley Scientific Ltd., Nottingham, UK). This device, described in European Pharmacopoeia 6.0, is able to simulate lung deposition of an aerosol and consists of four successive stages and a terminal filter (stage 5). The powder dose is released from the device because of the air flow generated, and particles gradually impact on the various stages depending on their aerodynamic features.

A dry powder inhalation device (Aerolizer®, Novartis, Switzerland) was first filled with a no. 3 HPMC capsule (Capsugel, Bornem, Belgium) loaded with 5 mg of insulin. Five capsules were taken for each test. HPMC capsules were used because gelatin capsules have a tendency to break during the test and to produce agglomerates during particle size measurements. The Aerolizer® is a unidose device of simple design with low internal resistance. The applied air flow rate and the duration of the experiment are based on the internal resistance of the inhalation device and are chosen to obtain a pressure drop of 4 kPa within the device and a total aspirated air volume of 4 L. For the Aerolizer® device, the airflow rate was set to 100 L/min for 2.4 s. Depending on the air flow applied in the impactor, "cut-off" diameters can be calculated for each floor. They reflect the minimum aerodynamic diameter of the particles impacted at a given stage. At this flow rate, the cut-off diameters for stages 2, 3, 4 and 5 were, respectively, 10.1, 5.3, 2.4 and 1.3 μm.

The MsLI was filled with 20 ml of filtered 0.01 M HCl in each stage. After particle deposition, fractions were quantitatively collected in volumetric flasks and the volume adjusted with 0.01 M HCl. Two flasks were also dedicated to the collection of the powder that remained in the inhalation device and in the bent tube or "throat". Drug deposition in the device, the throat, the four stages and the filter was determined by the HPLC method previously described. For accuracy, each test was repeated three times.

In the presence of lipids, a 15-min ultrasonication sequence in a 40 kHz Branson® 2510 bath was applied in order to dissolve the insulin, as well as a filtration step (ProFill 0.45 μm syringe filter, Alltech) before injection of the samples.

A fine particle dose (FPD), fine particle fraction (FPF) and mass median aerodynamic diameter (MMAD) were calculated using the CITDAS software (Copley Scientific Ltd., Nottingham, UK), by interpolation from the cumulative fraction curve of the active ingredient against the cut-off diameter of respective stages. The FPF expresses the percentage of particles with an aerodynamic diameter of less than 5 μm, which are assumed to reach the deep lung. It was expressed as a percentage of the metered dose, which is the total drug dose measured in all of the fractions (device, throat, stages 1–5) after the impaction experiment.

2.2.8.3. Uniformity of the delivered dose. The uniformity of the delivered dose (UDD) was measured using a UDD device (Copley Scientific Ltd., Nottingham, UK) and the procedure described in European Pharmacopoeia 6.0 for assessment of powders for inhalation. An Aerolizer® device (Novartis, Switzerland) was first filled with a no. 3 HPMC capsule (Capsugel, Bornem, Belgium) loaded with 5 mg of insulin. The airflow rate was set to 100 L/min for 2.4 s. After particle deposition, the UDD device was rinsed with 0.01 M HCl, and the liquid was quantitatively collected in a volumetric flask. This procedure was repeated for nine other capsules.

Drug deposition in the UDD device was then determined by the HPLC method previously described. In the presence of lipids, a 15-min ultrasonication sequence in a 40 kHz Branson® 2510 bath was applied in order to dissolve the insulin, as well as a filtration step (ProFill 0.45 μm syringe filter, Alltech) before injection of the samples. All of the formulations were tested in triplicate.

2.2.9. Stability study

A one-month stability study was performed on two of the formulations produced from a suspension, namely the 0% and 20% lipid-coated formulations. This was done in anticipation of the fact that these formulations were selected to be administered to patients during a future phase I pharmaco-scintigraphic study.

The two formulations were freshly produced and size 3 HPMC capsules (Capsugel, Bornem, Belgium) were filled with 5.00 mg (±1%) insulin. The capsules were shared out and stored in 50-ml high-density polyethylene containers. The containers were stored for 30 days in drying ovens with controlled relative humidity (RH). Three storage conditions were chosen: 25 °C/60% RH, 40 °C/75% RH and a fridge condition (4–8 °C).

3. Results and discussion

A major concern with protein and peptide absorption by inhalation is the crossing of the alveolar epithelium [6]. Many absorption enhancers, such as protease inhibitors, cyclodextrins, bile salts and other surfactants or citric acid [27–30], have been studied and seem to be efficient at increasing macromolecule absorption. However, the mechanism of action of these absorption enhancers could be due to an irreversible distortion of the alveolar epithelial cell layer, which could generate safety concerns regarding possible long-term effects [29].

In this study, we coated insulin particles with cholesterol and phospholipids, both of which are endogenous compounds present in the pulmonary surfactant. Phospholipids could act as an absorption enhancer by hastening the surfactant recycling process in the alveolar cells, leading to enhanced uptake of the protein molecule into systemic circulation [22].

The presence of cholesterol should also ensure a good long-term stability of the protein, as well as appropriate aerodynamic properties of the powders [18,19].

Different powder compositions were formulated with the aim of studying the influence of the physical state of insulin and the coating level (in percentage) on the physicochemical and aerodynamic characteristics of the powders. Table 1 gives an overview of all spray-dried (SD) powder formulations studied. As the choice of excipients used to enhance the aerosol behaviour of powders is very limited for pulmonary applications [29], we also tried to develop two "reference" formulations without excipients, which were SD from a solution or suspension of insulin with appropriate particle size distribution and storage stability.

3.1. Physicochemical characterisation

The laser diffraction results presented in Table 2 and Fig. 1 show that the starting material had a median diameter of 9.30 ± 0.03 μm and a bimodal distribution, which is not compatible with pulmonary administration. For SD formulations, the median diameter could be reduced to values between 2.05 ± 0.07 μm and 2.32 ± 0.09 μm, which is a well-suited size range for peripheral lung deposition and subsequent systemic absorption. The size distributions were monodisperse and exhibited quite a narrow range, with the percentage of particles between 1.0 and 5.0 μm from 83% to 90%. No major differences were observed in the size distributions between the different formulation types, even for the coated formulations with a high content of lipid coating, as observed with median particle sizes of 2.05 μm for the F1 formulation and 2.19 μm for the F6 formulation.

Particles obtained after spray-drying of a suspension of insulin were as small as those obtained from a solution. This could be achieved due to an efficient preliminary size reduction step using HPH. Moreover, for each formulation, the median particle size was not significantly different before or after spray-drying (Student *t*-test, $p > 0.05$, N.S.), indicating that a single insulin particle was contained in each droplet during spray-drying of the suspensions and that no aggregation of particles occurred.

Table 1
Composition of the formulations (before and after spray-drying) used for the preparation of the dry insulin powders for inhalation.

	Physical state of insulin (before spray-drying)	Liquid composition (before spray-drying)		Solid composition (after spray-drying)	Coating composition
		Insulin (%) (w/v)	Lipids (%) (w/v)	Lipids[a] (%) (w/w)	Cholesterol/Phospholipon (%) (w/w)
F1	Solution	0.5	0	0	–
F2	Suspension	2	0	0	–
F3		2	0.2	10	75/25
F4		2	0.4	20	75/25
F5		2	0.6	30	75/25
F6		2	0.8	40	75/25

[a] Data expressed in percentage of insulin weight.

Table 2
Particle size characteristics, insulin content and water content of all formulations tested (mean ± SD, $n = 3$).

	Particle size characteristics				HPLC insulin content (%)	Water content (%)
	Before spray-drying (suspension)	After spray-drying (powder)				
	$d_{(0.5)}$ (μm)	$d_{(0.5)}$ (μm)	$D_{[4.3]}$ (μm)	% < 5.0 μm (%)		
Raw insulin	–	9.30 ± 0.03	9.55 ± 0.05	21.8 ± 0.8	100.0[a]	4.8 ± 0.1
F1	–	2.05 ± 0.07	2.25 ± 0.08	97.5 ± 0.9	98.4 ± 1.1	6.1 ± 0.1
F2	2.10 ± 0.05	2.11 ± 0.03	2.29 ± 0.06	98.1 ± 0.5	99.2 ± 1.1	4.9 ± 0.1
F3	2.10 ± 0.04	2.09 ± 0.05	2.37 ± 0.06	95.9 ± 0.6	100.4 ± 0.4	3.7 ± 0.1
F4	2.25 ± 0.05	2.32 ± 0.09	2.61 ± 0.05	93.8 ± 0.6	100.2 ± 0.4	2.6 ± 0.1
F5	2.15 ± 0.07	2.18 ± 0.05	2.65 ± 0.06	92.4 ± 0.6	100.5 ± 0.6	2.4 ± 0.1
F6	2.16 ± 0.07	2.19 ± 0.03	2.58 ± 0.07	93.1 ± 0.8	99.1 ± 1.2	2.3 ± 0.1

[a] Raw insulin (untreated material) was used as a reference for HPLC measurements.

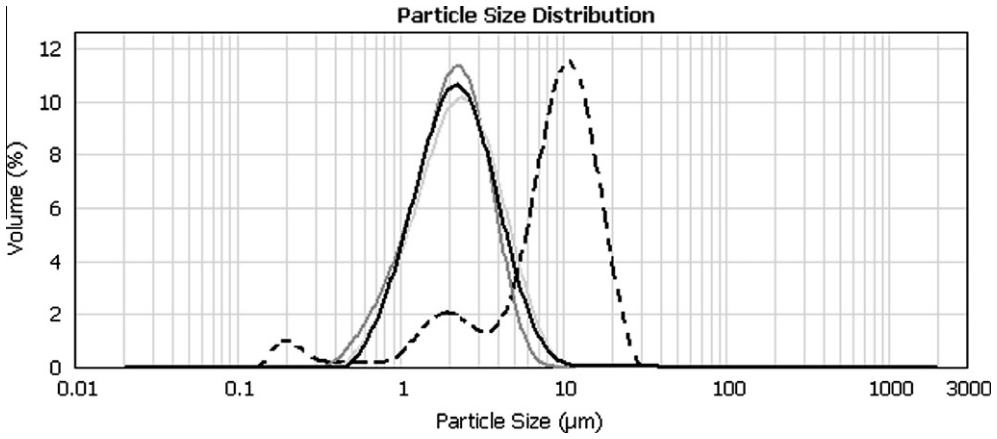

Fig. 1. Laser diffraction particle size distribution of raw insulin material (dashed line) and formulations F1 (dark-grey line), F2 (light-grey line) and F6 (black line) that were measured with the Mastersizer 2000®.

The absence of degradation of the insulin molecule during the HPH/spray-drying process was evaluated using an HPLC method. As shown in Table 2, the content of insulin lies within pharmacopoeia requirements for all tested formulations. In order to confirm the conservation of the biological activity of insulin in the formulations, we tested the presence of high molecular weight proteins (from the covalent aggregation of insulin) by size-exclusion chromatography (SEC). We first subjected raw insulin (solution at 4 mg/ml in 0.01 M HCl) to stirring at 40 °C for one week in order to obtain a certain percentage of high molecular weight proteins. Only insulin dimers were obtained, which were present at a concentration of 0.4%. This concentration corresponds to the limit of quantification. A test was also performed under more drastic storage conditions, with raw insulin powder stored at 40 °C for 6 months, which led to the formation of 13.4% of insulin dimers.

Chromatograms obtained for all insulin formulations (Fig. 2) did not exhibit detectable peaks, aside from the insulin monomer peak, so the content of HMWP was therefore considered to be below 0.4% in all cases.

The XRD spectra of the untreated insulin (raw material) and the different formulation types are shown in Fig. 3, with modelling of the amorphous and crystalline area using a pseudo-Voigt function. Some weak diffraction peaks appeared at 2θ angles of 10.4° and 19.5° for raw insulin, and the degree of crystallinity was determined to be 2.3 ± 0.1%. For the F1 formulation produced from an insulin solution, these peaks almost fully disappeared, suggesting that the insulin was in amorphous form. This is probably due to the very fast solvent evaporation during spray-drying that does not allow sufficient time for crystal nucleation, crystal growth and polymorph transitions when the solubility limit of insulin is reached at the surface of the droplet. However, other peaks at 31.6° and 45.4° were detected in the XRD pattern that were due to the presence of NaCl crystals (from neutralisation of the acidic solution of insulin with NaOH). These peaks were identified by comparison with a NaCl control sample. The pattern of the F2 formulation produced from an insulin suspension exhibited the same peaks as raw insulin, with a degree of crystallinity of 0.8 ± 0.1%. This decreased crystallinity could be due to the decreased particle size in comparison with raw insulin powder. It is known that in X-ray diffraction, the peak height is affected by the crystal size and crystallinity of the particles [31].

In order to detect any potential change in the crystal state of the lipids, blank particles were first prepared by spray-drying a mix of cholesterol and Phospholipon 90H (75:25) dissolved in isopropanol. The same spray-drying conditions as the preparation of the insulin formulations were used. As shown in Fig. 4, the pattern of

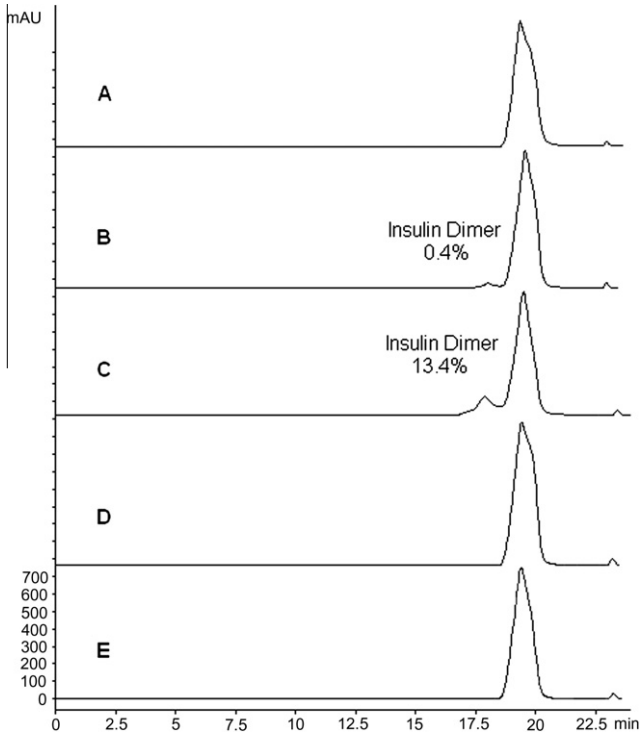

Fig. 2. Size-exclusion chromatograms of raw insulin material: (A) freshly prepared solution, (B) solution under stirring at 40 °C for one week, (C) powder stored at 40 °C for 6 months and of insulin formulations: (D) freshly prepared solution of F2, (E) freshly prepared solution of F4.

the blank particles exhibited features of the crystalline raw excipients. However, the percentage of amorphous phase in the blank particles was evaluated to be 30.1 ± 0.1%. Lipids were present in a partially amorphous state, as they are dissolved in isopropanol and solidified by rapid solvent elimination during spray-drying. The lipid-coated insulin formulations all exhibited patterns that corresponded to combining the pattern of the F2 formulation produced from an insulin suspension to that of the SD lipids, as can also be seen in Fig. 4. The degree of crystallinity in these powders gradually increased from 6.8 ± 0.1% for the F3 formulation to 19.9 ± 0.1% for the F6 formulation, in accordance with the increased proportion of lipids, showing that the lipids are also about 30% in the amorphous phase in the coated formulations. The pres-

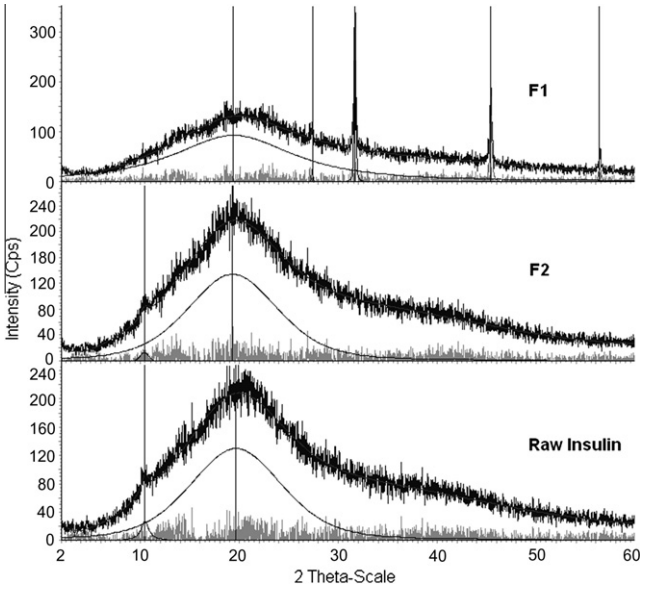

Fig. 3. X-ray powder diffraction patterns of raw insulin, the F1 and F2 formulations and modelling of the amorphous and crystalline areas using a pseudo-Voigt function.

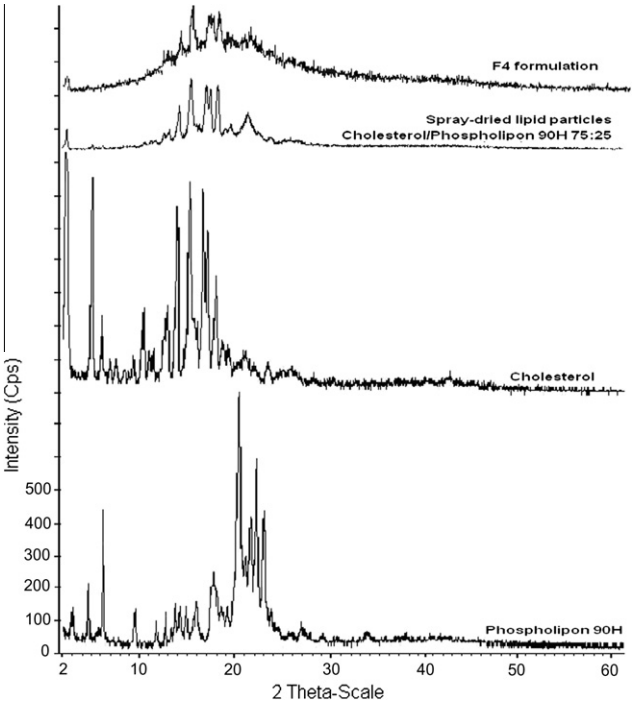

Fig. 4. X-ray powder diffraction patterns of raw lipid excipients, spray-dried blank lipid particles (cholesterol/Phospholipon® 90H 75:25 w/w) and the F4 formulation.

ence of amorphous insulin and lipids in the formulations could be an issue, as these may have a tendency to slowly crystallise during storage and lead to a change in the physical properties of the powders. Therefore, a long-term study should be set up in order to confirm the thermodynamic stability of the formulations. However, in a previous study carried out in our laboratory, it was shown that formulations made up of a physical blend of 98% SD lipid carrier particles (cholesterol/Phospholipon 90H in a ratio of 90:10 w/w) and 2% budesonide particles exhibited no change in their XRD patterns and degree of crystallinity after 12 months at 25 °C and 60% RH [18].

As the total water content of the DPI formulations and amount of water adsorbed around the particles influence the physical stability and control the magnitude of capillary forces that cause particle aggregation, the residual moisture content was measured using TGA. The water content was estimated to about 4.8% for raw insulin, 6.1% for the F1 formulation and 4.9% for the F2 formulation (Table 2). The percentage of water in the lipid-coated formulations gradually decreased (from about 3.7% to 2.3%) with an increase in the proportion of lipid coating. The use of isopropanol rather than water as a dispersing solvent and the presence of a lipid coating around the insulin particles decreased the re-adsorption of water by the particles, which should enhance the physical stability of the macromolecule in long-term storage and the deagglomeration of the powder during inhalation. More generally, the use of DPIs seems particularly favourable for the administration of peptides and proteins, thanks to the removal of water from the system, as well as providing longer storage stability and avoiding the need to store and distribute in a cold-chain.

As isopropanol is used during the manufacture of the formulations obtained from a suspension, the residual content was determined in the F2 and F4 formulations, which were selected in order to be administered to diabetic patients during a future phase I pharmaco-scintigraphic study. According to the ICH guidelines (CPMP/ICH/283/95), isopropanol is a class III solvent, a group that comprises solvents with low toxic potential in humans and for which the permitted daily exposure (PDE) is above 50 mg. No health-based exposure limit is required for class III solvents in final products but, as a precaution, we calculated the concentration limit in the same way as for class II solvents using the following equation:

$$\text{Concentration (ppm)} = 1.000 \times \text{PDE (mg)}/\text{Daily dose (g)}$$

The daily dose in this evaluation is generally fixed at 10.0 g, and the concentration limit would therefore be 5000 ppm. The two formulation types were under the limit of acceptable isopropanol residual content, with values of 2470 ± 30 ppm and 2810 ± 30 ppm for the uncoated and coated formulations, respectively.

Table 3
Particle size characteristics of the formulations $d_{(0.5)}$, $D_{[4.3]}$ and % < 5.0 μm (mean ± SD, n = 3) measured with the Spraytec® laser diffractometer.

	$d_{(0.5)}$	$D_{[4.3]}$	% < 5.0 μm
Raw insulin	7.8 ± 0.3	10.5 ± 0.7	22.9 ± 1.6
F1	4.3 ± 0.4	6.1 ± 0.7	61 ± 6
F2	2.9 ± 0.2	3.22 ± 0.12	93.1 ± 1.4
F3	2.6 ± 0.3	3.8 ± 0.3	80.6 ± 1.7
F4	2.5 ± 0.2	3.7 ± 0.2	80.3 ± 1.3
F5	2.6 ± 0.4	4.0 ± 0.6	78 ± 4
F6	3.8 ± 0.4	6.0 ± 0.5	65 ± 5

3.2. Aerodynamic evaluation

The Spraytec® laser diffraction results presented in Table 3 show that particle size values obtained for raw insulin were comparable to those obtained with the standard laser diffraction method. This indicated that the particles were easily dispersed in a fully individualised state, even under simulated breathing conditions, as shown in Fig. 5.

Spraytec® results for the formulation SD from an insulin solution without excipient (F1) exhibited a large increase in particle diameters in comparison with standard laser diffraction ($d_{(0.5)}$ of 4.3 ± 0.4 μm and 2.05 ± 0.07 μm, respectively) and a tremendous decrease in the percentage of particles with a diameter below

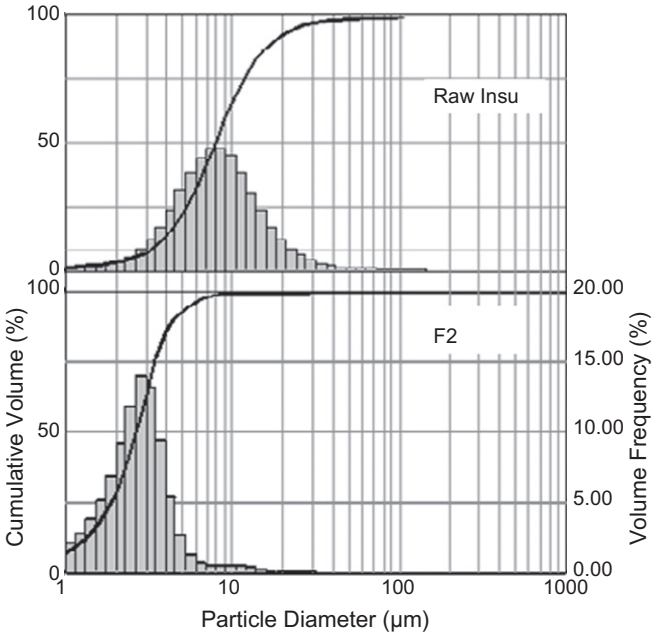

Fig. 5. Average particle size distribution and undersize curve of raw insulin material and the F2 formulation measured with the Spraytec®.

5 µm (61 ± 6% vs. 97.5 ± 0.9%), whereas the formulation SD from an insulin suspension without excipient (F2) had only slightly increased median diameters (2.9 ± 0.2 µm vs. 2.11 ± 0.03 µm) and a slightly decreased percentage of particles with a diameter below 5 µm (93.1 ± 1.4% vs. 98.1 ± 0.5%). The presence of powder aggregates in the formulations can be explained by the fact that inter-particle interactions are very high for micronised particles because of the increase in specific surface area. However, the different behaviours observed for the F1 and the F2 formulations could be explained by the differences in residual moisture content (6.1 ± 0.1% and 4.9 ± 0.1%, respectively), leading to the formation of liquid bonds in the inter-particle gaps in the F1 formulation. The surface properties of the particles could also be involved. As shown in Fig. 6, the F1 formulation had a fairly spherical shape with a smooth surface, whereas the F2 formulation, for which no insulin dissolution occurred in the solvent system used before spray-drying, retained a more corrugated surface, closer to the raw insulin powder features. This irregular surface could increase inter-particle distances and therefore reduce Van der Waals interactions, resulting in an increase in powder dispersibility [32].

The coated formulations (F3–F5) all showed a similar increase in particle aggregation in comparison with the uncoated F2 formulation and a decrease of about 10% in the percentage of particles with a diameter below 5 µm (Table 3). The F6 formulation, which possessed the highest content of lipid coating (40%), exhibited higher aggregation, with a particle size distribution comparable to the F1 formulation. A high lipid content seemed to induce some particle-sticking, probably because of the relatively low melting temperature of lipids and because of the phospholipids present in the formulations.

It is important to note that Spraytec® laser diffraction only gives geometric particle dimensions, whereas MsLI measures aerodynamic diameters. Even if the Spraytec® could give a good indication of the aerosolisation properties of DPI formulations and could be used as a quick preliminary test, we would still need

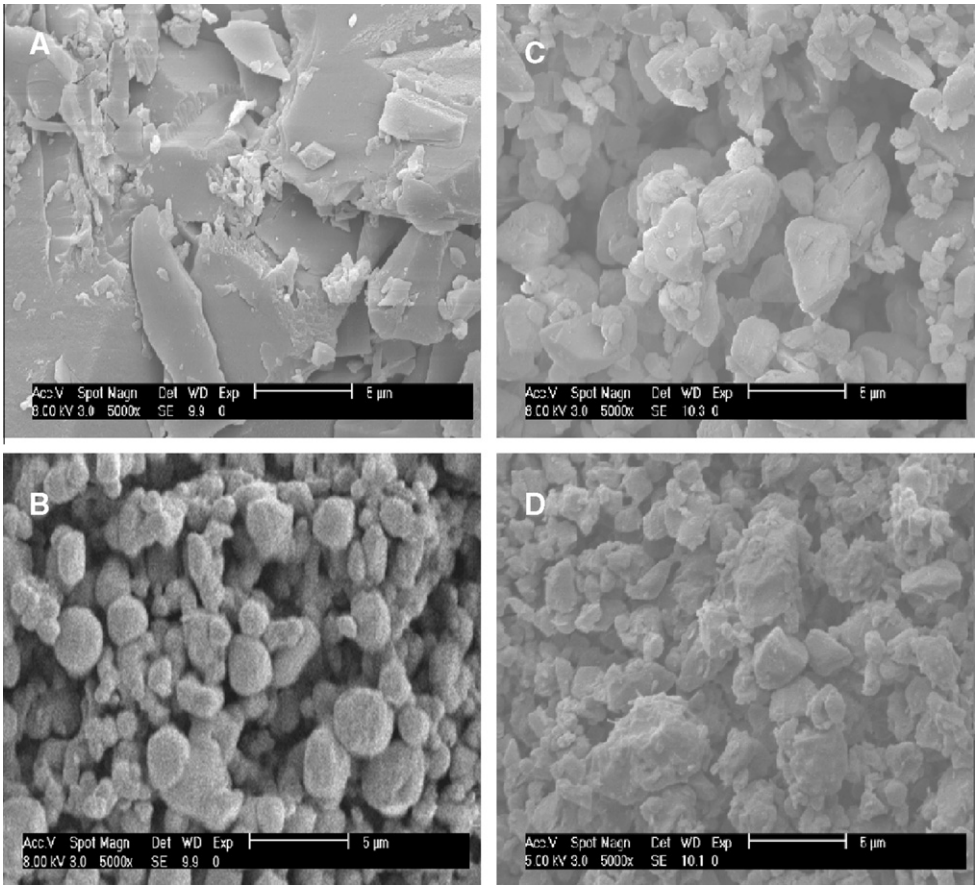

Fig. 6. SEM pictures obtained at a magnification of 5000 × from raw insulin (A) and from SD insulin formulations F1 (B), F2 (C) and F4 (D).

Table 4
In vitro deposition characteristics (mean ± SD, $n = 3$) of the different formulations measured by a MsLI, corresponding to a nominal insulin dose of 5.0 mg.

	Raw insulin	F1	F2	F3	F4	F5	F6
FPD (mg)	0.49 ± 0.16	2.17 ± 0.15	2.8 ± 0.2	2.8 ± 0.2	2.7 ± 0.3	2.6 ± 0.2	2.0 ± 0.2
MMAD (μm)	5.65 ± 0.06	3.53 ± 0.03	2.71 ± 0.05	2.68 ± 0.05	3.07 ± 0.06	3.02 ± 0.07	2.92 ± 0.07
Metered dose (mg)	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.4	4.60 ± 0.11	4.6 ± 0.3	4.4 ± 0.2	4.4 ± 0.3
FPF (%)	11 ± 3	48 ± 4	63 ± 4	61 ± 4	59 ± 4	59 ± 3	46 ± 3

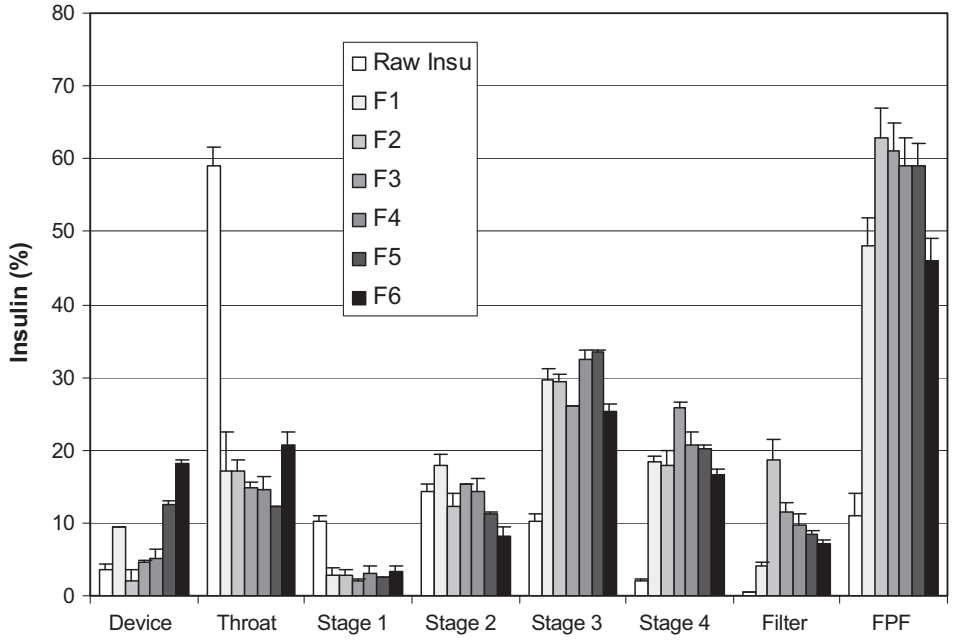

Fig. 7. In vitro deposition patterns (MsLI) of the different insulin formulations ($n = 3$).

impaction measurements to characterise the de-agglomeration behaviour of the powders in an air stream in order to understand how powder deposition in the lungs might occur. The aerodynamic behaviour of the different insulin formulations analysed in an MsLI is shown in Table 4 and Fig. 7.

The results indicated that the mass median aerodynamic diameters (MMAD) were between 5.65 μm for raw insulin and around 2.7 μm for the F2 and F3 formulations. On the other hand, FPF, which roughly corresponded to the drug deposition fraction at stages 3 and 4 and at the filter (cut-off diameters of 5.3 μm, 2.4 μm and 1.3 μm, respectively), varied within a range of 11% for raw insulin and 63% for the F2 uncoated formulation obtained from a suspension of insulin in isopropanol.

The metered dose of insulin, which is the dose that is recovered from the inhalator and the different parts of the MsLI, is in all cases comprised between 88% and 92% of the total drug loaded.

A comparison of the MMAD results with the Spraytec® median diameters, obtained by drawing a comparison plot (one method per axis), gave a good correlation between the two methods, as previously described in the literature [33]. A coefficient of determination R^2 of 0.92 was calculated, which does not indicate agreement between the two methods but measures the strength of the linear relationship between them. The most important difference between MMAD and $d_{(0.5)}$ results was obtained for the raw insulin powder (5.7 μm vs. 7.8 μm, respectively). This can be explained by the fact that the Copley® software used for determination of the MMAD only takes into account particles with an aerodynamic diameter below 10.1 μm (cut-off diameter of stage 2), whereas the determination of the $d_{(0.5)}$ with Spraytec® includes all particles exiting the device. This effect was mainly visible for the raw insulin powder, as it possesses an important fraction of particles with a diameter over 10 μm.

We also compared the FPF values obtained by impaction with the percentage of particles with a diameter below 5 μm obtained by the Spraytec®. Here again, there was a linear relation between the data generated from the two methods ($R^2 = 0.97$). However, the FPF values obtained for the different formulations using the impaction method were lower (46–63%) than for the percentage of particles with a diameter below 5 μm measured with the Spraytec® (61–93%).

No significant difference was observed for the fine particle fraction between the F2 uncoated formulation (63 ± 4%) and the coated formulations with a coating load of up to 30% (F3, F4 and F5 formulations), with FPF values of 61 ± 4%, 59 ± 4% and 59 ± 3%, respectively (1-way ANOVA test, $p > 0.05$, N.S.). However, the F1 and F6 formulations both exhibited lower FPF values that were below 50%. For the F1 formulation, this more agglomerated state of the powder was indicated by a higher deposition at stage 2 of the MsLI (cut-off: 10.1 μm) and by fewer particles deposited in the last filter stage of the impinger. As discussed above, this lower performance was probably due to the higher residual moisture content (6.1%) of this formulation.

An increase in the lipid content of the formulations to up to 40% (F6) also seemed to induce some particle-sticking because of the relatively low melting temperature of the lipids. This was shown by an increase in the particles impacted in the throat and by powder losses within the inhalation device (see Fig. 7). It is therefore important to be in the appropriate residual water content range in order to avoid the apparition of both electrostatic charges and liquid bonds leading to more inter-particle interactions.

The aerodynamic performance of the different formulations tested was very high in comparison with what could be obtained from other protein formulations for inhalation currently under development, which often have an in vitro deposition of around

30%. As an example, the insulin formulation Exubera® showed FPF values between 33% and 45% depending on the nominal dose (1 mg or 3 mg) [34]. Aside from insulin, other proteins such as growth hormone and parathyroid hormone have already been formulated for DPI administration using formulations presenting higher particle sizes and low powder densities. They are characterised by fine particle fractions of 38% and 61%, respectively [35,36].

Assessment of the uniformity of the delivered dose, performed on the F2 and F4 formulations, showed that all capsules delivered between 94.5% and 102.5% of the mean delivered dose for the F2 formulation and between 93.0% and 101.9% for the F4 formulation. The mean delivered doses were 74% and 87% of the loaded doses, respectively.

A one-month stability study was also performed on the F2 and F4 formulations. Results exhibited no significant difference between the initial features for the physicochemical characterisation (including HPLC insulin content and HMWP) and aerodynamic behaviour, for any of the three tested conditions (data not shown). However, a long-term stability study should be performed in the future in order to prove the stabilisation potential of these formulations.

4. Conclusion

In the present study, we evaluated insulin SLmP formulations and formulations without excipients, produced by high-pressure homogenisation and spray-drying. In the case of SLmP formulations, a physiological lipid composition based on a mixture of cholesterol and phospholipids was used to form a coating film around micronised drug particles, and the resulting powders exhibited a size and shape suitable for the deep lung deposition of drugs.

We were able to obtain good aerodynamic features for the different formulations tested, with fine particle fractions between 46% and 63% vs. 11% for raw insulin powder. These are high FPF values in comparison with those obtained for other protein formulations for inhalation currently under development, which often have an in vitro deposition of around 30%. The presence of a lipid coating of up to 30% (w/w) did not significantly affect the aerodynamic behaviour, and the coated formulations also exhibited a decreased residual moisture content, which should improve the long-term stability of the protein formulations. A preliminary one-month stability study showed no significant difference from the initial characteristics for the coated and uncoated formulation types. It still remains to be determined whether the formulations effectively give high deep lung deposition results *in vivo* and how insulin will be absorbed into the systemic blood stream. A pharmaco-scintigraphic clinical trial on type I diabetic patients will be done in order to evaluate the deep lung deposition, pharmacokinetics and bio-availability of these formulations.

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