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Research paper

Preparation and physicochemical characterization of supercritically dried insulin-loaded microparticles for pulmonary delivery

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Abstract

In the search for non-invasive delivery options for the increasing number of therapeutic proteins, pulmonary administration is an attractive route. Supercritical fluid (SCF) drying processes offer the possibility to produce dry protein formulations suitable for inhalation. In this study, insulin-loaded microparticles suitable for pulmonary administration were prepared and characterized. *N*-Trimethyl chitosan (TMC), a polymeric mucoadhesive absorption enhancer and dextran, a non-permeation enhancer, were used as carriers for insulin. The particles were prepared by spraying an acidic water/DMSO solution of insulin and polymer into supercritical carbon dioxide. The mean size of the particles was 6–10 μm (laser diffraction analysis) and their volume median aerodynamic diameter ca. 4 μm (time-of-flight analysis). The particles had a water content of ca. 4% (w/w) (Karl–Fischer), and neither collapsed nor aggregated after preparation and storage. In the freshly prepared dried insulin powders, no insulin degradation products were detected by HPLC and GPC. Moreover, the secondary and tertiary structures of insulin as determined by circular dichroism and fluorescence spectroscopy were preserved in all formulations. After one-year storage at 4 °C, the particle characteristics were maintained and the insulin structure was largely preserved in the TMC powders. In conclusion, SCF drying is a promising, protein-friendly technique for the preparation of inhalable insulin-loaded particles.

Keywords: *N*-Trimethyl chitosan microparticles; Supercritical carbon dioxide; Pulmonary delivery; Insulin; Physicochemical characterization

1. Introduction

The advances in biotechnology have resulted in the availability of a large number of protein-based drugs. Up until now, most therapeutic proteins are administered by injection because of their low stability and bioavailability after oral administration and poor absorption at other mucosal sites. Recently, pulmonary delivery has attracted much attention as a non-invasive administration route for macromolecules such as proteins and peptides, because the respiratory tree has a large surface area (~75 m^2), extensive vasculature, a thin membrane and low enzymatic activity [1–3]. The transport of macromolecules across the absorptive area, the alveolar wall, occurs by transcytosis for proteins >22 kDa and through tight-junctional paracellular processes for smaller molecules [1–3]. However, the clearance rate of transcytosis is so slow that it is of little importance for pulmonary delivery of protein drugs.

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Protein-containing particles in the aerodynamic size range 1.5–3 μm (in combination with a short breathhold period) may yield a more effective alveolar deposition [1,3,4]. The major factors limiting pulmonary absorption of the proteins are the poor deposition of the protein formulations at the alveolar region, low absorption from the respiratory epithelial barriers and the mucociliary escalator, which rapidly removes protein solution or particles from the central respiratory tract and prevents the access of the protein to the alveoli. Therefore, a suitable delivery system is required for efficient administration of protein deeply into the lungs. Moreover, the biological activity and structural stability of proteins are of crucial importance during pharmaceutical manufacturing of protein formulations. Proteins can undergo different chemical and physical degradation reactions particularly when formulated as a solution. It has been shown that the long-term stability of proteins can be greatly enhanced when they are stored in a dried state [5,6]. Importantly, proteins from dried formulations are more efficiently absorbed in the lung compared to proteins in solutions [7].

Supercritical fluid (SCF) drying is an attractive technique to prepare dried protein formulations [8–11]. SCF drying is a fast and mild process, is cost effective and offers the possibility to produce small microparticles suitable for inhalation [8,10–12]. A fluid is defined as supercritical when its pressure and temperature exceed their critical values. Above the critical points, the SCF has a liquid-like viscosity and density and gas-like diffusivity properties and can therefore penetrate into substances like a gas and dissolve materials like a liquid [13]. Supercritical carbon dioxide (SC-CO_2) has been commonly used as a SCF for drying pharmaceutical proteins, because proteins have a very low solubility in SC-CO_2. Consequently, it can act as an anti-solvent which results in the precipitation of the protein from its solution [14]. Moreover, CO_2 is inexpensive, non-toxic and has a moderate critical temperature (31 °C) [9,10,15].

Dried proteins and peptides are often formulated with sugars as carriers for pulmonary administration and as stabilizers to protect proteins from degradation during processing and storage [10,15]. However, the pulmonary bioavailability of proteins is still low as compared to the intravenous and subcutaneous bioavailability [3]. Therefore, polymeric particles with absorption-enhancing properties may be suitable alternative carriers for pulmonary protein delivery. Particles consisting of peptides and proteins associated with polymeric chitosan and chitosan derivatives have been shown to enhance the absorption of these macromolecules across mucosal epithelia [16–21]. Chitosan-based polymers are mucoadhesive and are capable of opening the tight junctions. Both properties may help to stimulate the uptake of the encapsulated protein [16–21]. In contrast to chitosan, which is soluble at low pH and insoluble at neutral pH, *N*-trimethyl chitosan chloride (TMC), a partially quaternized chitosan derivative, shows good water solubility over a wide pH range. Hence, TMC has mucoadhesive properties and excellent absorption-enhancing effects even at neutral pH [22,23]. Because of these properties, TMC could be an attractive alternative to chitosan for the design of protein-loaded particles.

The aim of the present work was to prepare insulin-loaded microparticles for pulmonary delivery using a SCF drying process. TMC with a degree of quaternization (DQ) of 20% (TMC20, as a mucoadhesive) and 60% (TMC60, as a mucoadhesive and an absorption enhancer) [22,24] and dextran (as a non-mucoadhesive and non-permeation enhancer) were selected as polymeric carriers. After preparation of TMC- and dextran–insulin microparticles, their physical characteristics such as shape, geometric and aerodynamic size distributions, water content, and insulin content were studied. Moreover, the structural integrity of insulin as well as the long-term stability of the dry powders was evaluated.

2. Materials and methods

2.1. Materials

Chitosan ($M_n = 40$ kDa, $M_w = 177$ kDa, determined by gel permeation chromatography (GPC) using poly [ethylene glycol] (PEG) standards [25], degree of deacetylation 93%) was a generous gift from Primex (Avaldsnes, Norway). *N*-Trimethyl chitosan with two DQ, 20% (TMC20) and 60% (TMC60), was synthesized by methylation of chitosan by using CH_3I in the presence of a strong base (NaOH) and analyzed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy as previously described [26]. Dextran (M_w: 64–76 kDa), recombinant human insulin ($M_w = 5.807$ kDa, 29 IU/mg) and FITC-labeled human insulin were purchased from Sigma–Aldrich (Schnelldorf, Germany). DMSO was purchased from Across Organics. All other chemicals used were obtained from commercial suppliers and were of analytical grade.

2.2. Preparation of insulin-loaded microparticles

Insulin-loaded dextran and TMC microparticles were prepared by spraying a HCl/DMSO solution of insulin/polymer into SC-CO_2. First, 30 mg insulin was dissolved in 2.7 ml of HCl 0.01 M. Then dextran or TMC (270 mg) was dissolved in this solution. The percentage of insulin/TMC or insulin/dextran was 10% (w/w). After TMC or dextran was fully dissolved, 2.7 ml polymer-insulin solution was mixed with 93.7 g of DMSO.

A scheme of the experimental setup is presented in Fig. 1 and details about the apparatus and the method were reported before [27]. At the selected operating temperature (40 °C), pressure (110 bar) and mixing ratios CO_2, water and DMSO are fully miscible resulting in a single phase in the supercritical region. The following experimental conditions gave spherical microparticles. First, at 40 °C SC-CO_2 was introduced with a constant flow rate of 333 g/min using a diaphragm pump (Lewa) into a 1-L precipitation

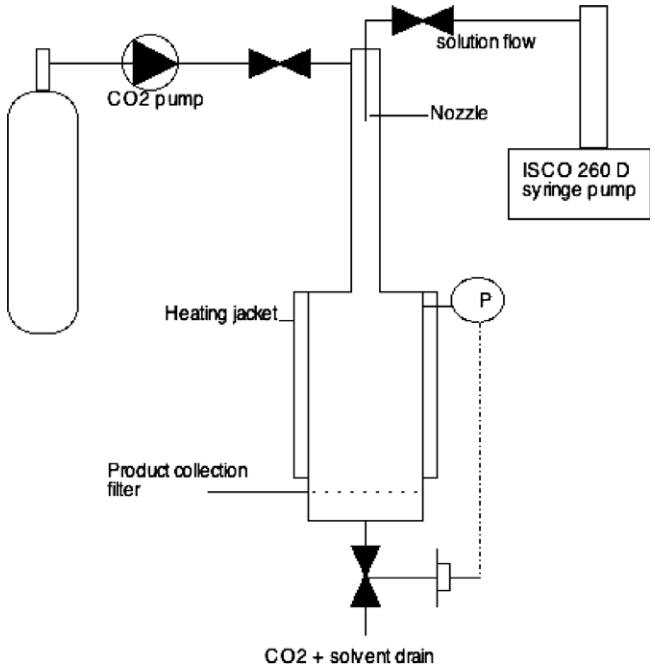

Fig. 1. Scheme of experimental setup of the SCF drying system.

vessel until the pressure reached 110 bar. Next, the polymer-insulin solution (total volume around 100 ml; flow rate 4.5 ml/min) and SC-CO_2 (333 g/min) were directly fed into an atomization device (a nozzle, diameter 0.08 mm) using two ISCO 260 syringe pumps. The ongoing co-current flow of supercritical CO_2 (333 g/min) dispersed the sprayed polymer-insulin solution. The mixture was passed through a residence-time tube (internal diameter 2 cm; length 70 cm) into the precipitation vessel. The use of a residence-time tube was crucial, because the supersaturation level was very high and phase split took place at the same time as mixing of the solution and SC-CO_2 [28]. A water jacket was used to maintain the pumps and vessel at a temperature of 40 °C. Electrical heating was used to keep the temperature of the nozzle and residence tube at 40 °C. The pressure in the vessel (110 bar) and CO_2flow (333 g/min) were controlled by exit valves and maintained at 40 °C and 110 bar during the whole experiment. Once the solution of polymer-insulin in HCl/DMSO was entirely sprayed into the precipitation vessel, the solution line was closed. Subsequently, SC-CO_2 at a flow rate of 200 g/min and ethanol at a flow rate of 4 ml/min were mixed and sprayed into the precipitation vessel for 12 min in order to remove the residual solvents (H_2O and DMSO). Finally, the vessel was flushed for 30 min with SC-CO_2 (200 g/min) to further extract residual solvents including ethanol. The dry powder was collected from the filter at the bottom of the vessel, once the pressure was released and stored in closed containers at 4 °C. To test the real-time stability, a part of each batch was stored in air-tight containers, which were closed under nitrogen to prevent water absorption during storage. The bottles were placed in a tightly closed box containing silica and stored for one year at 4 °C in refrigerator.

2.3. Characterization of the insulin-loaded microparticles

2.3.1. Particle morphology

The morphology of the TMC microparticles was determined using a JEOL JSM-5400 scanning electron microscope (SEM) (Peabody, USA). Samples of the particles were fixed onto aluminum SEM stubs using self-adhesive carbon disks, and were subsequently sputter-coated with a conducting gold layer.

2.3.2. Particle size analysis

The volume median diameter (VMD) of particles and size distribution were determined with a laser diffraction apparatus (HELOS BF MAGIC, Clausthal-Zellerfeld, Germany) equipped with a dry powder dispersion system RODOS (Sympatec GmbH, Germany). Approximately 2 mg of bulk powder was placed into the RODOS ring and dispersed through a laser beam (628 nm) with 3 bar of air pressure. Measurements were performed with a 100 mm lens and calculations were based on the Fraunhofer theory. In another measurement the particles were introduced via a DP-4 insufflator (PENN CENTURY Inc., Philadelphia, USA) designed for *in vivo* studies in rats, into the LD particle analyzer.

2.3.3. Aerodynamic particle size analysis

The volume median aerodynamic diameter (VMAD) of the microparticles was assessed by aerolization of the powders in an Aerosizer™ (TIS Inc., Minneapolis, USA) based on direct time-of-flight measurements. Half a milligram of each powder was introduced into the Aerosizer using a DP-4 insufflator (see above). The volume median aerodynamic diameters (VMADs) and the volume fraction smaller than 5 μm of the particles were calculated from the particle number distributions presented by the Aerosizer. The mass mean aerodynamic diameter (MMAD) of particles is equal to the VMAD when particles of different sizes all have the same density.

2.3.4. Confocal laser scanning microscopy (CLSM)

FITC-insulin-loaded TMC microparticles were prepared by drying in SC-CO_2, as described in Section 2.2. The particles were suspended in dichloromethane (DCM) and mounted on a slide. After evaporation of the DCM, the particles were visualized with a confocal laser scanning microscope (Bio-Rad, Alphen a/d Rijn, The Netherlands). The distribution of the FITC-insulin within the particles was investigated by scanning the particles in the x, y plane with a z-step of 0.2 μm.

2.3.5. Water content analysis

The water content of the different TMC- and dextran–insulin particles was determined by Karl–Fischer titration. In brief, 2–3 mg powder, accurately weighed, was dissolved (TMC particles) or suspended (dextran particles) in 500 μl methanol. Fifty microliters of sample was injected into the titration cell and the amount of water of the powders was

calculated after subtraction of the background (methanol only) signal.

2.4. Quantification and characterization of insulin

2.4.1. Reversed-phase HPLC

The insulin content of the TMC microparticles was determined by reversed-phase HPLC. This technique also allows the detection of insulin degradation products. A Prosphere C_{18} (300 Å; 5 μm, 250 × 4.6 mm) column (Alltech Breda, The Netherlands) in combination with an All-guard C18 pre-column (Alltech, Breda, The Netherlands) was used. The column was equilibrated with a solvent mixture consisting of 25% acetonitrile/75% H_2O/0.1% trifluoroacetic acid (TFA) for one hour at a flow rate of 1 ml/min. The microparticles were dissolved in 0.01 M HCl (1 mg/ml containing 0.1 mg insulin) and 50 μl of the solutions was injected onto the column. A calibration curve was made by injecting volumes of 0.5–50 μl of a freshly prepared insulin (1 mg/ml) solution in 0.01 M HCl onto the column.

A gradient was run from the starting composition, acetonitrile/H_2O (25/75%)/TFA 0.1%, to acetonitrile/H_2O (38/62%)/TFA 0.1% in 25 min. The mobile phase was delivered to the column at a flow rate of 1 ml/min by a Waters 600 gradient pump equipped with a Waters 717 plus autosampler (Waters Corporation, Milford, MA, USA). Chromatograms were recorded with a Waters 600 absorbance detector set at 280 nm.

2.4.2. Gel permeation chromatography (GPC)

GPC analyses were performed with a method adapted from [29] and [30] on a GPC max, VE 2001 instrument (Viscotek, Oss, The Netherlands) equipped with a Superdex 75 10/300 GL column, exclusion limit 1×10^5 Da (GE Healthcare Europe GmbH, Amsterdam, The Netherlands). The insulin-loaded particles were dissolved in acetic acid 20% (w/w) in water at a concentration of 1 mg/ml of insulin. Then the samples were centrifuged at high speed for 1 min to remove insoluble particles, if any. The mobile phase was 100 mM phosphate-buffered saline (pH 7.4). Two hundred microliters of each sample was injected onto the column; elution was done at a flow rate of 0.5 ml/min. Chromatograms were recorded with a TDA 302 tetra detector (Viscotek, Oss, The Netherlands). UV (at 280 nm) and right angle light scattering (RALS) signals were used to analyze the data. A dn/dc value of 0.189 ml/g [31] was assumed for calculation of the molecular weight of insulin from the light scattering data.

2.4.3. Circular dichroism (CD) spectroscopy

The secondary and tertiary structures of insulin were monitored by far-UV and near-UV circular dichroism (CD) spectroscopy, respectively. Insulin, a physical mixture of insulin and dextran/TMC 1/10 w/w and insulin-loaded TMC/dextran microparticles were dissolved in 0.01 M HCl. The final concentration of insulin was ca. 0.5 mg/ml. Prior to CD spectroscopy, all samples were centrifuged for 1 min at 10,000*g* to remove particulate matter, if any. Far-UV (200–260 nm) CD and near-UV (250–320 nm) CD spectroscopy were performed at room temperature, in a 0.02-cm quartz cuvette and a 0.5-cm quartz cuvette, respectively, using a dual beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA, USA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic grating (2400 lines/nm, blaze wavelength 230 nm) and 1.24 mm slits. Each spectrum is the average of 5 scans. Spectra of 0.01 M HCl, empty TMC20, TMC60 and dextran particles dissolved in 0.01 M HCl were recorded and subtracted from the corresponding sample spectrum. The measured CD signals were corrected for concentration differences and converted into delta molar extinction ($\Delta\varepsilon$), based on a mean residual weight of 113.86 (M_w/51 amino acids) [32].

2.4.4. Fluorescence spectroscopy

Insulin, a physical mixture of insulin and dextran or TMC (1/10 w/w) and insulin-loaded microparticles were dissolved in 0.01 M HCl. Fluorescence emission spectra (290–450 nm, 1-nm step) of the different samples were measured in 1-cm quartz cuvettes in a Fluorolog III Fluorimeter (Jobin Yvon-Horriba, Edison, NJ, USA) at 25 °C while stirring. Excitation was at 280 nm and the slits (excitation and emission) were set at 3 nm. The integration time per data point was 0.1 s and the average of the 5 scans was taken. Spectra of 0.01 M HCl, empty TMC20, TMC60 and dextran particles dissolved in 0.01 M HCl and plain polymer solutions were subtracted from the corresponding sample spectrum. Before analysis, the solutions were diluted with 0.01 M HCl to obtain an absorbance <0.1 at 280 nm and the spectra were normalized for concentration differences.

3. Results and discussion

3.1. Preparation of insulin-loaded dextran and TMC microparticles

In this study we have investigated the preparation and characterization of polymeric particles suitable for the pulmonary delivery of insulin. Particles based on TMC, a mucoadhesive absorption enhancer, and dextran, an inert polymer, were prepared using a SCF process with SC-CO_2 as an anti-solvent. The experimental conditions were based on a previous study in which the drying of a solution of dextran in DMSO resulted in small dextran microparticles, suitable for inhalation [33]. The yield of particle production was about 60%. This relatively low yield can be explained by the relatively small scale at which the powders were produced, resulting in relatively large losses of the solution in the tubing of the SCF apparatus and some loss of material due to agglomeration around the spraying device. The optimum polymer concentration was found to be 0.3% (w/w) in HCl/DMSO. At lower polymer con-

centrations agglomerated particles were collected, whereas at higher concentrations TMC20 and TMC60 were not fully soluble in the HCl/DMSO mixture. Because of the poor solubility of the TMCs and insulin in DMSO, insulin and the polymers were first dissolved in an acidic aqueous solution (0.01 M HCl). Then, the protein/polymer solution was mixed with DMSO. The ratio of 0.01 M HCl/DMSO of 2.7/97.3 (w/w) was chosen based on the phase behavior of the water/DMSO/CO_2mixture reported before [27]. Lower or higher ratios resulted in agglomerated particles or wet materials, respectively.

3.2. Particle characterization

Fig. 2 shows representative SEM photographs of insulin-loaded TMC and dextran microparticles. This figure shows that the size distributions of the three particle formulations were comparable and in the micrometer range (ca. 1–30 μm). The TMC20 and TMC60 microparticles were spherical with a smooth surface (Fig. 2a and b), whereas the shape of the dextran microparticles was somewhat irregular (Fig. 2c). The cumulative volume distributions of the freshly prepared TMCs and dextran particles directly after preparation and as measured by the laser diffraction technique are shown in Fig. 3a and b. The cumulative volume distributions of the particles as function of aerodynamic diameter are shown in Fig. 3c. All three different insulin powders showed a fairly narrow, unimodal size distribution, indicating the absence of agglomerates. From Fig. 3a it appears that 90% of the particles have a size between 1 and 30 μm, which is in excellent agreement with SEM analysis. The size distribution of the particles introduced into the analyzer with a DP-4 insufflator was similar to that of the particles introduced with the dry-powder air dispersion system using an air pressure of 3 bar (cf. Fig. 3a and b). This comparison is important to evaluate the potential of the insufflator in order to properly disperse and introduce the powders into the Aerosizer for further aerodynamic size measurements.

The VMADs of the microparticles, as determined by the time-of-flight technique and the VMDs, as determined by laser diffraction technique, are given in Table 1. The volume fractions in particles <5 μm obtained from the cumulative volume distribution graphs as function of aerodynamic diameter are presented in Table 1. This indicates that more than 60% of the particles delivered from the insufflator have a volume aerodynamic diameter less than 5 μm. The VMADs of the TMC and dextran particles were about 4 μm, which is suitable for reaching the peripheral respiratory tract at a relatively low flow rate [1,4,34]. The VMADs of the TMC20–insulin and TMC60–insulin particles were significantly smaller than their VMDs (Table 1), which indicates that the TMC–insulin particles have a density below 1. Since the true density of saccharides is around 1.6 g/cm^3, this implies that the insulin microparticles are porous or hollow. CLSM images of the FITC-insulin TMC microparticles (Fig. 4) show that both small and large particles were loaded with FITC-insulin. Z-scan images of the FITC-insulin particles (Fig. 4) indicated that the FITC-insulin was homogeneously distributed over the individual particles. From the CLSM analysis and aerodynamic size characterization it can be concluded that the particles are porous but not hollow, because, FITC-insulin was distributed over the whole particles and not solely present in the shell. The VMAD of the dextran–insulin powders was also smaller than the VMD. However, the difference between VMAD and VMD is substantially smaller for the dextran–insulin particles as compared to the TMC–insulin particles, which indicates that the dextran particles are less porous than the TMC particles.

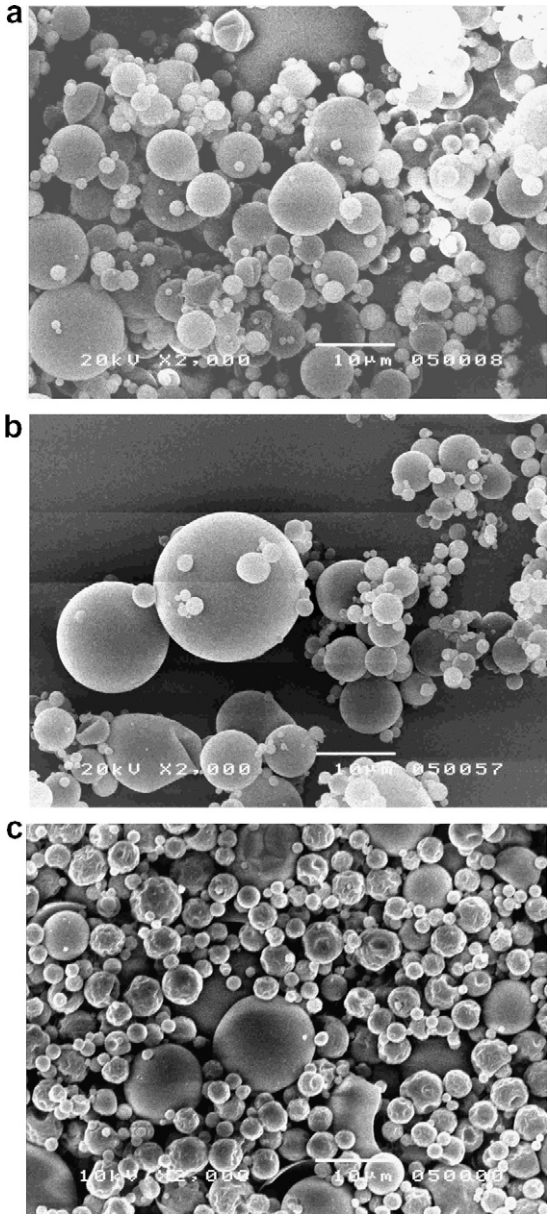

Fig. 2. Scanning electron microscopy (SEM) images of: (a) TMC60–insulin; (b) TMC20–insulin; and (c) dextran–insulin microparticles.

Table 1 shows the water content of the freshly prepared particles. The water content of the powders was around 4%

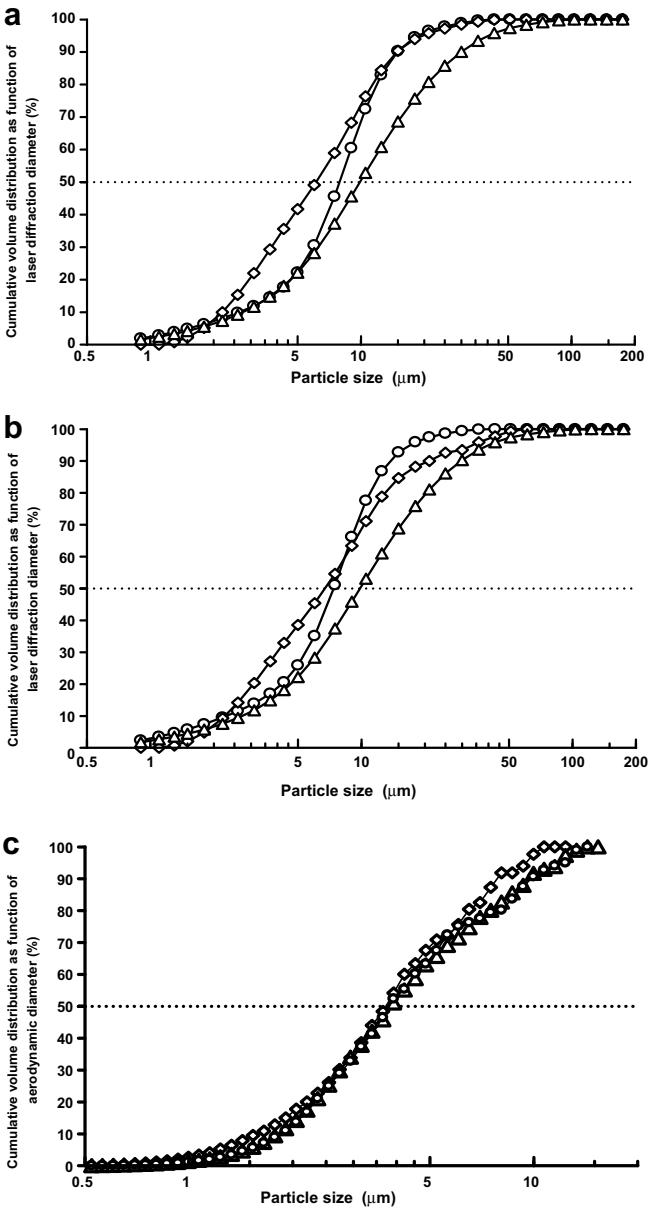

Fig. 3. Cumulative volume distribution curves as function of laser diffraction diameter (a and b) and as function of aerodynamic diameter (c) for dried insulin formulations. For laser diffraction analysis, the particles were either introduced with a DP-4 insufflator (a) or with a dry-powder air dispersion system using an air pressure of 3 bar (b). (○) TMC60–insulin; (△) TMC20–insulin; and (◇) dextran–insulin particles.

(w/w). Although relatively high, the water content resulted neither in particle agglomeration nor collapse (see Section 3.4.1.).

3.3. Insulin loading and structural integrity

HPLC analyses of insulin after dissolution of the powders showed that the loading was 9.0–9.7% which is close to the feed ratio (10%). Moreover, no peaks indicative for chemical degradation products were detected.

Possible formation of covalent insulin aggregates (e.g. due to disulfide reshuffling) as a result of the SCF process was studied with GPC analysis. For GPC analysis, the particles were dissolved in 20% (v/v) acetic acid in water, a solvent in which non-covalent dimers dissociate [29]. The recovery of insulin was 100% for the TMC60 and dextran formulations, which means that all insulin present in these formulations was dissolved and eluted afterwards. It was not possible to analyze the TMC20–insulin formulation with GPC because of the low solubility of TMC20 in the elution buffer (PBS, pH 7.4). Fig. 5 shows the GPC chromatograms of the insulin formulations. Both UV and RALS detection revealed a peak with an elution volume of ca. 15.5 ml for native insulin (untreated insulin dissolved in acetic acid 20% (w/w)), and insulin formulated in dextran and TMC60 microparticles. This elution volume corresponds to monomeric insulin (6000 g/mol). No additional peaks that would indicate the presence of covalent dimers were detected. TMC60–insulin particles showed a broad peak at a retention volume of 8.5 ml. This peak was also seen for TMC60 polymer alone, which showed tailing and was partly overlapped with the insulin peak (Fig. 5).

Table 1
Particle characteristics of the dried insulin formulations

Dried formulations	Characteristic laser diffraction diameters (μm)[a]			VMAD (μm)[b]	FPF < 5 μm (percentage of the emitted dose)[b]	Water content[c] (%)
	X10	X50 (VMD)	X90			
TMC60–insulin particles	2.6	7.9	14.9	4.0	64.1	4.1
TMC20–insulin particles	2.7	9.9	30.0	4.1	62.1	4.1
Dextran–insulin particles	2.2	6.1	14.8	3.9	67.5	4.2

[a] Determined by laser diffraction (LD) analyses ($n = 2$).
[b] Determined by time-of-flight measurements ($n = 2$).
[c] According to Karl–Fischer titration ($n = 2$).

Possible changes in the three-dimensional structure of insulin due to the drying process were monitored by circular dichroism (CD) spectroscopy. Near-UV CD was used to gain information about the tertiary structure, whereas far-UV CD gives information about the secondary structure [32]. Insulin-loaded TMC/dextran particles and native insulin (untreated insulin dissolved in HCl 0.01 M) were dissolved in 0.01 M HCl. At pH 2, insulin (pI = 5.3) is positively charged and consequently electrostatic interactions with TMC are minimized. Furthermore, at this pH equilibrium exists between insulin in its monomeric and dimeric form [30,35]. The CD spectra obtained after dissolution of freshly prepared insulin formulations are shown in Figs. 6a and b. Fig. 6a shows the near-UV CD spectra of the different insulin samples. Insulin exhibits one peak at 255 nm characteristic for phenylalanines (3 per insulin molecule) and a broad peak around 276 nm, characteristic for tyrosines (4 per insulin molecule) [35,36]. These signals are mainly attributed to the interactions in the monomer–

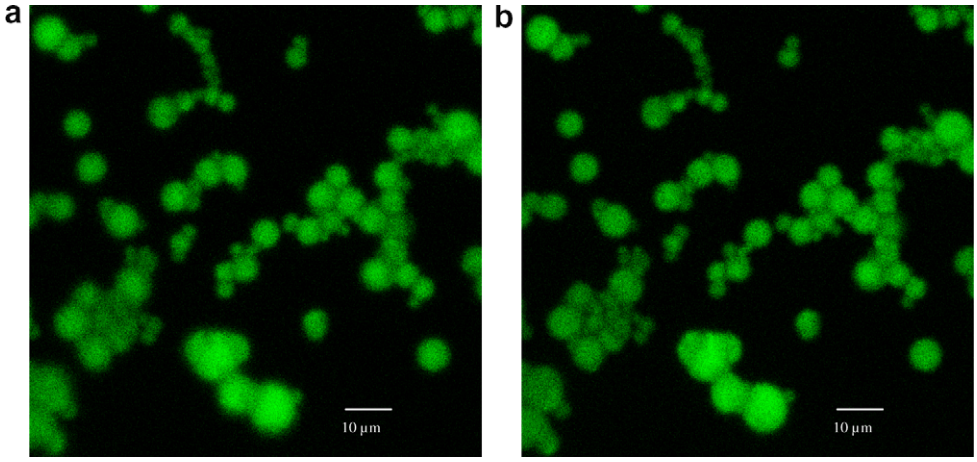

Fig. 4. CLSM images of FITC-labeled insulin–TMC microparticles (image b is 1 μm deeper inside the microparticles than image a).

monomer interface of the dimers [35]. The CD spectrum of insulin in the presence of TMC20 or TMC60 was the same as that of native insulin (data not shown). In comparison with native insulin, the CD spectrum of the dissolved insulin-loaded TMC and dextran particles shows an increase of the signal at a vicinity of 276 nm, indicating subtle changes in the tyrosine environments [35–37]. There were no significant spectral changes at 255 nm characteristic of phenylalanine in insulin (Fig. 6a). The overall spectral shape was essentially retained, which indicates that the tertiary structure was largely preserved.

Far-UV CD spectra are shown in Fig. 6b. Native insulin exhibits a spectrum with minima at 210 and 222 nm, which is typical for a protein with an α-helical structure. No significant spectral changes in shape and intensity were observed after dissolution of TMC60–insulin particles compared to native insulin (Fig. 6b). Small increases were observed in the vicinity of 224 and 210 nm in the spectra of the insulin–dextran and TMC20–insulin particles (Fig. 6b), which indicates minor increases in the α-helical structure. The results of near-UV and far-UV CD indicate that the secondary and the tertiary structures of the insulin were mainly preserved during the drying process.

The tertiary structure of the insulin was also studied by fluorescence spectroscopy. Fig. 7 shows the fluorescence spectra of the samples, dissolved in 0.01 M HCl. Dextran- and TMC–insulin formulations showed similar fluorescence intensities as native insulin (untreated insulin dissolved in HCl 0.01 M) with an emission maximum at 305 nm, indicating that the tertiary structure of insulin was preserved. These results are in agreement with the near-UV CD data.

3.4. Characterization of the aged insulin microparticle formulations

3.4.1. Particle size

After one-year storage at 4 °C in closed bottles, the powders were still free flowing. Moreover, the particle size distributions, as measured by laser diffraction analysis, were comparable to those of the freshly prepared particles (shown in Table 1), indicating that the dried particles were stable in time and that neither aggregation nor particle collapse had occurred upon storage.

3.4.2. Structural integrity of insulin

The insulin integrity in the formulations stored for one year at 4 °C was compared with the insulin structure in the freshly prepared batches. The HPLC chromatograms of the aged TMC–insulin particles showed a single peak, representative of insulin. In contrast, the dextran–insulin formulation showed an extra peak at higher retention time of insulin (data not shown), which might be due to degraded (e.g., deamidation of asparagine residues) insulin [38]. The percentage of the degradation product was less than 10% (w/w) of the total insulin content. Fluorescence spectroscopic analysis of the aged insulin powders indicates some extent of tyrosine oxidation (see the fluorescence spectroscopy results, below). These oxidized products are more hydrophilic than native insulin and consequently are expected to have a shorter retention time in HPLC. However, such peaks were not detected and likely these oxidized products co-elute with native insulin as previously demonstrated for methionine oxidized interleukin 2 [39].

Possible formation of covalent insulin aggregates during the storage of the dried formulations was studied with GPC analysis. The recovery of insulin was 100% which means that all insulin present in the formulation was dissolved and eluted afterwards. The GPC chromatograms of the aged insulin formulations are shown in Figs. 5b and c. The chromatograms of the aged dextran- and TMC60–insulin powders largely overlapped with those of the freshly prepared formulations. No other peaks were detected that correspond to higher or lower molecular weight components, indicating that neither chain cleavage occurred nor covalent aggregates were formed. Both the TMC60 and dextran peak showed tailing and partly overlapped with the insulin peak (Fig. 5).

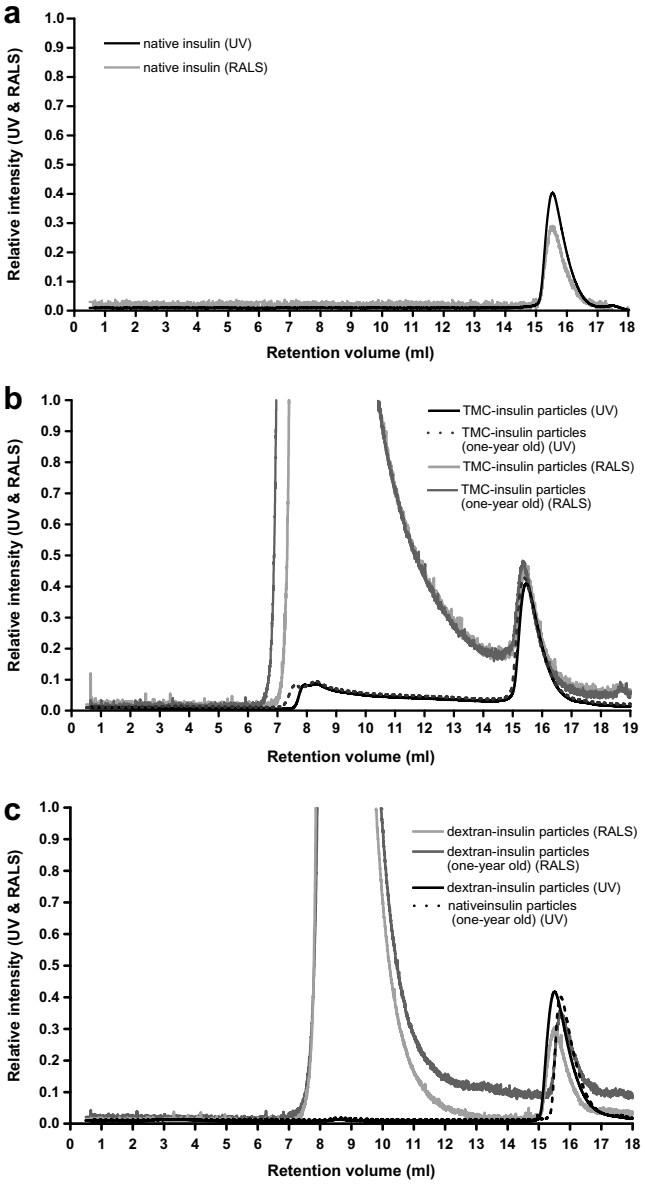

Fig. 5. GPC chromatograms of the dried insulin formulations. (a) Native insulin; (b) TMC60–insulin particles; and (c) dextran–insulin particles. Particles were dissolved in 0.01 M HCl prior to analysis.

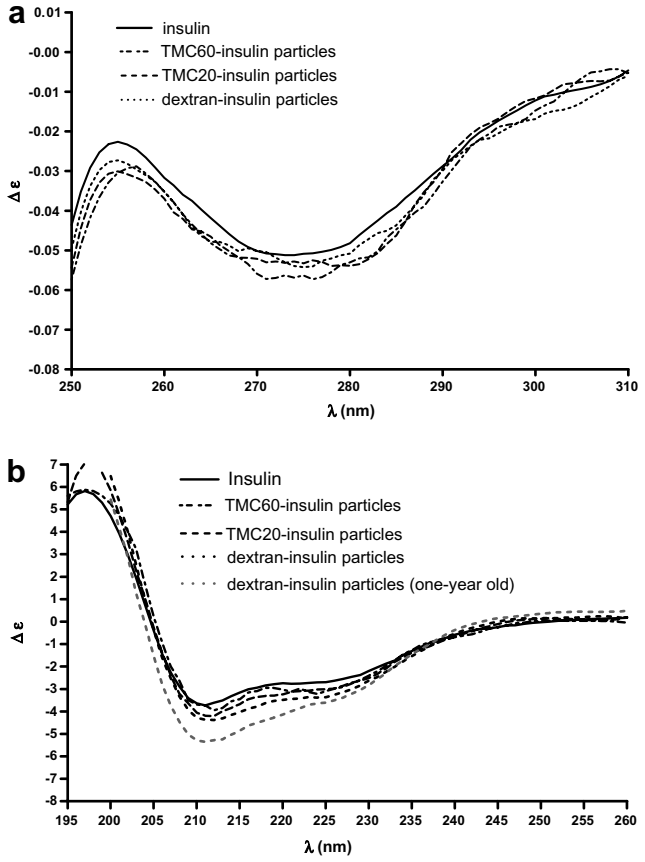

Fig. 6. Conformational analysis of insulin dry powders by CD spectroscopy. (a) Near-UV CD spectra; (b) far-UV CD spectra. Particles were dissolved in 0.01 M HCl prior to analysis.

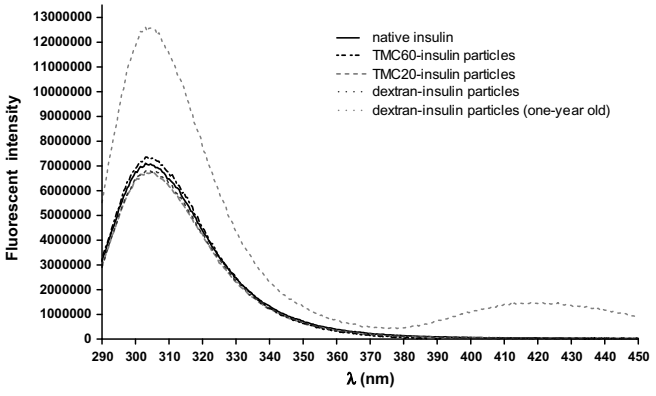

Fig. 7. Fluorescence emission spectra of the insulin dried formulations. Particles were dissolved in 0.01 M HCl prior to analysis.

The secondary and tertiary structures of insulin after one-year storage of the dried insulin formulations were investigated by far- and near-UV CD. The CD spectra of TMC60- and TMC20–insulin and the near-UV CD spectrum of dextran–insulin did not substantially change, except that there was a slight increase in intensity, which may either be due to slight conformational changes but more likely reflect a calibration error in the protein assay because different insulin standards were used. In comparison with native insulin, the far-UV CD spectrum of the dissolved insulin-loaded dextran particles (Fig. 6b) showed an increase of the signals particularly at a vicinity of 210 nm, showing increase of α-helical structure and reduced accessibility of tyrosines [29,36] and consequently, subtle changes in the secondary structure of insulin.

The tertiary structure of insulin after storage for one year at 4 °C was also studied by fluorescence spectroscopy. Both TMC–insulin powders showed after dissolution a slight increase in fluorescence intensity compared to that of native insulin. The fluorescence peaks (emission maximum 305 nm) did not shift to a higher or lower wavelength,

which indicates that the tertiary structure of insulin was preserved (data not shown). These results are in agreement with the near-UV CD data. Dextran–insulin particles showed a significant increase in fluorescence intensity at 305 nm plus an extra peak around 410 nm (Fig. 7). The increase in the intensity of the tyrosine emission peak suggests a conformational change [40], whereas the newly formed emission peak at higher wavelengths (ca. 410 nm) is indicative of tyrosine oxidation [41].

Altogether, the aging study revealed that the insulin structure of the TMC–insulin formulations was essentially preserved after storage for one year at 4 °C, whereas the aged dextran–insulin formulations showed both chemical and physical degradation of the protein. The stabilizing action of TMC, as compared to dextran, is unknown, but may be related to the cationic character of TMC: electrostatic interactions between TMC and insulin might protect insulin better during storage in the dried state.

4. Conclusions

This study demonstrates that SCF drying is a suitable method to produce inhalable insulin powders with defined particle characteristics and preserved structure of insulin. Porous spherical TMC and dextran particles had similar VMAD of ≈4 μm, which should yield a comparable pulmonary deposition and consequently allow a direct comparison of the particles in *in vivo* studies under comparable inspiratory conditions. Further *in vivo* studies are needed to evaluate the applicability and potential of these polymeric-insulin powders as pulmonary delivery systems.

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References

[1] J.S. Patton, Mechanisms of macromolecules absorption by the lungs, Adv. Drug. Deliv. Rev. 19 (1996) 3–36.
[2] D.R. Owens, B. Zinman, G. Bollit, Alternative routes of insulin delivery, Diabet. Med. 20 (2003) 886–898.
[3] J.S. Patton, C.S. Fishburn, J.G. Weers, The lungs as a portal of entry for systemic drug delivery, Proc. Am. Thorac. Soc. 1 (2004) 338–344.
[4] S.P. Newman, A. Hollingworth, A.R. Clark, Effect of different modes of inhalation on drug delivery from a dry powder inhaler, Int. J. Pharm. 102 (1994) 127–132.
[5] M.C. Manning, K. Patel, R.T. Borchardt, Stability of protein pharmaceuticals, Pharm. Res. 6 (1989) 903–918.
[6] J.F. Carpenter, M.C. Manning, Rational Design of Stable Protein Formulations, Theory and Practice, Kluwer Academic/Plenum Publishers, New York, 2002.
[7] A. Hussain, Q.H. Majumder, F. Ahsan, Inhaled insulin is better absorbed when administered as a dry powder compared to solution in the presence or absence of alkylglycosides, Pharm. Res. 23 (2006) 138–147.
[8] S.D. Yeo, G.B. Lim, P.G. Debenedetti, H. Bernstein, Formation of microparticulate protein powders using a supercritical fluid antisolvent, Biotechnol. Bioeng. 41 (1993) 341–346.
[9] N. Elvassore, A. Bertucco, P. Caliceti, Production of insulin-loaded poly(ethylene glycol)/poly(l-lactide) (PEG/PLA) nano-particles by gas antisolvent techniques, J. Pharm. Sci. 90 (2001) 1628–1636.
[10] N. Jovanovic, A. Bouchard, G.W. Hofland, G.J. Witkamp, D.J.A. Crommelin, W. Jiskoot, Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying, Eur. J. Pharm. Sci. 27 (2006) 336–345.
[11] N. Jovanovic, A. Bouchard, G.W. Hofland, G.J. Witkamp, D.J.A. Crommelin, W. Jiskoot, Stabilization of proteins in dry powder formulations using supercritical fluid technology, Pharm. Res. 21 (2004) 1955–1969.
[12] M. Tservistas, M.S. Levy, M.Y. Lo-Yim, R.D. O'Kennedy, P. York, G.O. Humphrey, M. Hoare, The formation of plasmid DNA loaded pharmaceutical powders using supercritical fluid technology, Biotechnol. Bioeng. 72 (2001) 12–18.
[13] E. Reverchon, Supercritical antisolvent precipitation of micro-and nano-particles, J. Supercrit. Fluids 15 (1999) 1–21.
[14] P.M. Gallagher, M.P. Coffey, V.J. Krukonis, N. Klasutis, Gas antisolvent recrystallization: new process to recrystallize compounds insoluble in supercritical fluids, ACS Symp. Ser. 406 (1989) 334–354.
[15] H. Todo, H. Okamoto, K. Iida, K. Danjo, Improvement of stability and absorbability of dry insulin powder for inhalation by powder-combination technique, Int. J. Pharm. 271 (2004) 41–52.
[16] I.M. van der Lubben, G. Kersten, M.M. Fretz, C. Beuvery, J.C. Verhoef, H.E. Junginger, Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice, Vaccine 28 (2003) 1400–1408.
[17] I.M. van der Lubben, F.A. Konings, G. Borchard, J.C. Verhoef, H.E. Junginger, In vivo uptake of chitosan microparticles by murine Peyer's patches: visualization studies using confocal laser scanning microscopy and immunohistochemistry, J. Drug Target. 9 (2001) 39–47.
[18] P. Calvo, C. Remunan-Lopez, J.L. Vila-Jato, M.J. Alonso, Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines, Pharm. Res. 14 (1997) 1431–1436.
[19] R. Fernandez-Urrusuno, P. Calvo, C. Remunan-Lopez, J.L. Vila-Jato, J.A. Alonso, Enhancement of nasal absorption of Insulin using chitosan nanopartocles, Pharm. Res. 16 (1999) 1576–1581.
[20] M. Amidi, S.G. Romeijn, G. Borchard, H.E. Junginger, W.E. Hennink, W. Jiskoot, Preparation and characterization of protein-loaded *N*-trimethyl chitosan nanoparticles as nasal delivery system, J. Control. Release 111 (2006) 107–116.
[21] A. Vila, A. Sanchez, K. Janes, I. Behrens, T. Kissel, J.L. Vila Jato, M.J. Alonso, Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice, Eur. J. Pharm. Biopharm. 57 (2004) 123–131.
[22] J.H. Hamman, M. Stander, A.F. Kotzé, Effect of the degree of quaternization of *N*-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia, Int. J. Pharm. 232 (2002) 235–242.
[23] M. Thanou, J.C. Verhoef, J.H.M. Verheijden, H.E. Junginger, Intestinal absorption of octeriotide: *N*-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue in vitro and in vivo, J. Pharm. Sci. 89 (2000) 951–957.
[24] D. Snyman, J.H. Hamman, A.F. Kotze, Evaluation of the mucoadhesive properties of *N*-trimethyl chitosan chloride, Drug Dev. Ind. Pharm. 29 (2003) 61–69.

[25] X. Jiang, A. van der Horst, M.J. van Steenbergen, N. Akeroyd, C.F. van Nostrum, P.J. Schoenmakers, W.E. Hennink, Molar-mass characterization of cationic polymers for gene delivery by aqueous size-exclusion chromatography, Pharm. Res. 23 (2006) 595–603.
[26] A.B. Sieval, M. Thanou, A.F. Kotzé, J.C. Verhoef, J. Brussee, H.E. Junginger, Preparation and NMR-characterisation of highly substituted *N*-trimethyl chitosan chloride, Carbohydr. Polym. 36 (1998) 157–165.
[27] Y. Pérez de Diego, H.C. Pellikaan, F.E. Wubbolts, G. Borchard, G.J. Witkamp, P.J. Jansens, Opening new operating windows for polymer and protein micronisation using the PCA process, J. Supercrit. Fluids 36 (2006) 216–224.
[28] Y. Pérez de Diego, F.E. Wubbolts, G.J. Witkamp, P.J. Jansens, T.W. de Loos, Improved PCA process for the production of nano- and microparticles of polymers, AIChE Journal 50 (2004) 2408–2417.
[29] A. Ahmad, I.S. Millett, S. Doniach, V.N. Uversky, A.L. Fink, Stimulation of insulin fibrillation by urea-induced intermediates, J. Biol. Chem. 279 (2004) 14999–15013.
[30] B. Metz, G.F. Kersten, G.J. Baart, A. de Jong, H. Meiring, J. ten Hove, M.J. van Steenbergen, W.E. Hennink, D.J.A. Crommelin, W. Jiskoot, Identification of formaldehyde-induced modifications in proteins: reactions with insulin, Bioconjug. Chem. 17 (2006) 815–822.
[31] J. Demeester, S.S. de Smedt, N.N. Sanders, J. Haustraete, in: D.J.A. Crommelin (Ed.), Methods for Structural Analysis of Protein Pharmaceuticals, AAPS Press, Arlington, 2005, pp. 245–276.
[32] M. Bloemendal, W. Jiskoot, in: D.J.A. Crommelin (Ed.), Methods for Structural Analysis of Protein Pharmaceuticals, AAPS Press, Arlington, 2005, pp. 83–130.
[33] Y. Pérez de Diego, F.E. Wubbolts, G.J. Witkamp, T.W. de Loos, P.J. Jansens, Measurements of the phase behaviour of the system dextran/DMSO/CO2 at high pressures, J. Supercrit Fluids 35 (2005) 1–9.
[34] M.P. Timsina, G.P. Martin, C. Marriott, D. Ganderton, M. Yianneskis, Drug delivery to the respiratory tract using dry powder inhalers, Int. J. Pharm. 101 (1994) 1–13.
[35] K. Huus, S. Havelund, H.B. Olsen, M. van de Weert, S. Frokjaer, Thermal dissociation and unfolding of insulin, Biochemistry 44 (2005) 11171–11177.
[36] A. Ahmad, I.S. Millett, S. Doniach, V.N. Uversky, A.L. Fink, Partially folded intermediates in insulin fibrillation, Biochemistry 42 (2003) 11404–11416.
[37] E.H. Strickland, D. Mercola, Near-ultraviolet tyrosyl circular dichroism of pig insulin monomers, dimers, and hexamers. Dipole–dipole coupling calculations in the monopole approximation, Biochemistry 15 (1976) 3875–3884.
[38] J. Brange, Chemical stability of insulin. 4. Mechanisms and kinetics of chemical transformations in pharmaceutical formulation, Acta Pharm. Nord. 4 (1992) 209–222.
[39] J.A. Cadee, M.J. van Steenbergen, C. Versluis, A.J. Heck, W.J. Underberg, W. den Otter, W. Jiskoot, W.E. Hennink, Oxidation of recombinant human interleukin-2 by potassium peroxodisulfate, Pharm. Res. 18 (2001) 1461–1467.
[40] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer/Plenum Press, New York, 1999.
[41] C. Arigita, L. Bevaart, L.A. Everse, G.A. Koning, W.E. Hennink, D.J.A. Crommelin, J.G. van de Winkel, M.J. van Vugt, G.F. Kersten, W. Jiskoot, Stability of mono- and trivalent meningococcal outer membrane vesicle vaccines, Infect. Immun. 71 (2003) 5210–5218.