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RESEARCH PAPER

Optimization of the Fine Particle Fraction of a Lyophilized Lysozyme Formulation for Dry Powder Inhalation

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ABSTRACT

Purpose A new dry powder inhalation technology creates inhalable particles from a coherent lyophilized bulk at the time of inhalation. The aim of this study was to evaluate several approaches to improve the fine particle fraction (FPF) and to understand underlying mechanisms.

Methods Lysozyme was chosen as model drug. Phenylalanine and valine were added, and the freezing process was varied. Lyophilisate characteristics as well as aerosolization behavior was analyzed.

Results The addition of the crystalline amino acids rendered a dose independent three-fold increase of the FPF. This is possibly due to enhanced fracture properties of the lyophilisates upon impact of the air stream and reduced particle agglomeration/cohesion caused by a rougher surface. This positive effect was well preserved over 3 months of storage. The structure of the lyophilisate was influenced by the freezing process which in turn affected the aerosolization behavior. Liquid nitrogen and vacuum-induced freezing performed best, doubling the FPF. The special cake morphology with elongated channels enabled easy disintegration. The resulting large porous particles comprise a low density being advantageous for a high FPF.

Conclusion The variation of the lyophilization process and formulation utilizing excipients enabled an optimization of the FPF of the novel lyophilisate based DPI system.

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KEY WORDS dry powder inhalation · fine particle fraction · freeze-drying · freezing process · lyophilisate

ABBREVIATIONS

ACI	Andersen Cascade Impactor
API	active pharmaceutical ingredient
DPI	dry powder inhaler
ED	emitted dose
FPF	fine particle fraction
Lys	lysozyme
MD	metered dose
Phe	L-phenylalanine
RH	relative humidity
SEM	scanning electron microscopy
Tg′	glass transition temperature of the max. freeze concentrated solution
Val	L-valine
XRD	x-ray diffractometry

INTRODUCTION

Since first marketed in 1970, dry powder inhalers (DPIs) have been subject to continuous improvement. Device technology developed continuously to overcome the relatively low efficiency in fine particle fraction (FPF) and inconsistencies in the emitted dose (ED) of the first generation DPIs (1). Most DPIs are breath-activated passive systems where powder aerosolization is achieved by the patient's own inspiration. These devices mostly have the disadvantage of inspiratory flow-dependent de-agglomeration of the powder (2). Some newer devices like the Clickhaler® and the Taifun® show an *in vivo* deposition which is relatively independent from the inspiratory effort (3,4). Furthermore, active devices were developed which enable respiratory force independent dosing precision and reproducible aerosolization (5). These devices are primarily

designed for systemic pulmonary delivery and for conditions where the inspiratory power of the patient cannot be relied upon (6). Examples for active devices are the Nektar Pulmonary Inhaler for Exubera® or the Aspirair™ device, which both use compressed air for the powder de-agglomeration and aerosolization process (6,7).

New powder formulation methods are equally important to sophisticated devices. The two main formulation methods to enhance powder flowability and dispersibility are carrier systems mainly utilizing lactose and controlled agglomeration of pure drug particles, called soft pelletization (8). The growing interest in pulmonary delivery of therapeutic proteins, nucleic acid nanocarriers, and vaccines as dry powder aerosols demands for alternative formulation methods to replace the commonly used but problematic micronization of the active pharmaceutical ingredient (API). Micronization may generate local hot spots in the processed materials and reduces their stability which is particularly a problem for thermolabile biopharmaceuticals (9). A variety of new methods like controlled crystallization, supercritical fluid precipitation, spray drying, and spray freeze-drying were recently summarized by Chow *et al.* (9) with regards to the production of more uniform particles in terms of morphological state (e.g. crystallinity), particle size distribution, and shape. All these particle engineering methods directly produce a powder, which can reduce some challenges of micron-sized powder particles such as poor flow behavior and a high tendency to aggregate, but handling and precise metering remain difficult.

Freeze-drying is a common method for the stabilization of labile bioproducts. The lyophilized products are porous cakes in the dimensions of the former fill volume. For the production of inhalable particles the lyophilisate can be milled, which adds another manufacturing step and additional stress for the API. Alternatively, impacting air can be used for the disintegration of a freeze-dried preparation into inhalable particles (10). Thus, DPI formulation is stored in a non-powdered form and the formation of fine particles occurs at the time of inhalation, thereby avoiding formulation problems like inadequate flowability and dispersibility of the powder. In addition, the manufacturing process is suitable for chemical entities as well as labile biopharmaceuticals. Because the formulation is metered as a liquid, extremely high accuracy at dose metering and a high preparation yield are further advantages of this technology. Nevertheless, freeze-drying is a time consuming and energy intensive manufacturing process (11). A previous study revealed that placebo lyophilisates, disintegrated by compressed air in a custom designed test device, show material dependent differences in the fine particle output (12). This led to the question whether the FPF of lyophilisates which exhibit poor dispersing behavior can be enhanced by the addition of excipients which demonstrate good aerosolization performance. The freeze-drying process or, more specifically, the freezing step is considered as another possible approach to increase fine particle output. The freezing process is a key step in lyophilization because it determines the ice crystal structure (shape and dimensions) and therefore governs the resulting lyophilisate morphology (13,14). The morphology of the lyophilisate should impact the dispersibility and aerosolization of the freeze-dried cake. Overall, the ice nucleation temperature, the freezing rate, as well as the freezing mechanism influence the ice crystal formation (15). Shelf-ramped freezing renders a low ice nucleation temperature and fast ice crystal growth, resulting in a high number of small ice crystals. The addition of an annealing step enables a rearrangement and secondary ice crystal growth (16). Freezing on a precooled shelf causes higher nucleation temperatures and slower freezing rates from vial bottom to top compared to shelf-ramped freezing and leads to a large heterogeneity between vials (17). Vacuum-induced freezing allows controlled ice nucleation at a defined temperature. The ice nucleation starts at the top surface, followed by a top-down freezing which results in vertical ice crystals (18). A very fast cooling method is immersion in liquid nitrogen, where freezing occurs by directional solidification resulting in small lamellar-oriented pores (19).

The aim of this study was to optimize the fine particle output of the model API lysozyme which demonstrated a poor emitted dose and fine particle fraction when lyophilized solitarily. High molecular weight species such as proteins form soft and elastic sponges after freeze-drying, which is utilized, for example, in flexible gelatin-containing wound dresses (20). For the disintegration into small particles by an air impact, these properties could be impedimental. Lysozyme was used as a model substance and the intention was to develop a pulmonary delivery system for lyophilisted formulations in general. Therefore protein stability and activity was not tested specifically. Instead, in a first approach, it was intended to achieve an optimized FPF by the addition of excipients. The two amino acids phenylalanine and valine performed best as single excipient lyophilisates in a previous study, showing FPF related to metered dose (MD) of nearly 50% (12). They were therefore chosen as potential excipients to enhance the fine particle output of lysozyme. To gain a better understanding of the underlying mechanisms of the improvement, lyophilisate characteristics as well as aerosolization properties were investigated in addition to fine particle measurements. Furthermore, storage stability for 3 months at 25°C/60% RH and 40°C/75% RH was evaluated for a selected formulation. In a second approach to increase the fine particle output, various freezing methods as part of the lyophilization process of two selected formulations were applied. The obtained lyophilisates were investigated with respect to their morphology, mechanical properties, and aerosolization performance.

MATERIALS AND METHODS

Materials

The model protein lysozyme from chicken egg white (Lys) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany) as a crystalline powder in hydrochloride form. Excipients used were L-phenylalanine (Phe) (Merck KGaA, Darmstadt, Germany) and L-valine (Val) (Fagron GmbH&Co KG, Barsbüttel, Germany). Solutions were made with highly purified water (Purelab Plus, Elga LabWater, Celle, Germany).

Formulation Preparation

0.5 ml aqueous solutions of 12 mg/ml lysozyme with 2 to 8 mg/ml phenylalanine or valine were filled into 2R glass vials (Fiolax® clear, Schott AG, Müllheim, Germany) and vials were equipped with rubber stoppers (1079-PH 701/40/ow/winered, West Pharmaceutical Services, Eschweiler, Germany). Freeze-drying was carried out in a laboratory scale freeze-drier (Lyostar II, FTS Systems, Stone Ridge, NY, USA). The samples were frozen at −1°C/min to −45°C for 1 h. Primary drying was performed at a shelf temperature of −15°C (shelfs were ramped at +0.2°C/min) and a pressure of 100 mtorr for 20 h. For secondary drying the shelf temperature was increased to +30°C at a ramp rate of +0.1°C/min for 6 h. For comparison purposes also 4 mg/ml phenylalanine and 12 mg/ml valine solutions were freeze-dried similarly.

Additional four different freezing methods were tested besides the normal shelf ramped freezing at −1°C/min:

- Annealing at −10°C for 10 h: The samples were frozen at −1°C/min to −45°C with a hold at −45°C for 1 h, followed by a shelf temperature increase to −10°C at +1°C/min and annealing for 10 h at −10°C before the samples were frozen again at −1°C/min to −45°C with another hold at −45°C for 1 h.
- Precooled shelf at −70°C: The samples were placed on a precooled shelf at −70°C for 1.5 h.
- In liquid nitrogen: Samples were immersed in liquid nitrogen for 1 min and were subsequently placed on a precooled shelf.
- Vacuum-induced at −3°C: The samples were equilibrated on the shelfs at −3°C for 1 h. According to (18) the chamber was evacuated as fast as possible to 600 mtorr to induce freezing. Afterwards the shelf temperature was quickly decreased to −45°C for 1 h.

X-Ray Diffractometry (Xrd)

The content of three vials was pooled for investigation with a Seifert X-ray diffractometer XRD 3000 TT (Seifert, Ahrensburg, Germany) equipped with a copper anode (40 kV, 30 mA, wavelength 154.17 pm). The samples were measured from 5 to 40° 2θ at a step rate of 0.05° 2θ with 2 s measuring time per step.

Device

The lyophilized formulations were dispersed into aerosols using a custom-designed device (12). Briefly, it consists of two capillaries with a diameter of 0.75 mm pierced through the stopper of the vial housing the lyophilisate. One capillary is the air inlet, the other capillary the air outlet, which is connected to the mouthpiece. Compressed air is stored in a 20 ml pressure reservoir at a pressure of 3 bar. Pressing a lever releases the compressed air through the inlet capillary, thereby starting dispersion of the powder.

Fine Particle Fraction (FPF) Analysis

The FPF was measured using a short stack version of the Andersen Cascade Impactor (ACI) (8-Stage Non-Viable Sampler Series 20–800, Thermo Andersen, Smyrna, GA, USA) at a flow rate of 39 l/min (corresponds to a pressure drop of 4 kPa with the HandiHaler®, which is recommended by USP <601> (21)). Baffle plates were coated with a solution of 83% glycerin (AppliChem GmbH, Darmstadt, Germany), 14% ethanol (central supply LMU, Munich, Germany) and 3% Brij 35 (Serva Electrophoresis GmbH, Heidelberg, Germany). Filters used were type A/E glass fiber filters 76 mm (Pall Corporation, Ann Arbor, MI, USA). By removing stages 2 to 7, the whole FPF (particle fraction <4.94 μm) is collected on the filter directly below stage 1 and can easily be quantified by weighing the filter before and after powder deposition. The herein contained total lysozyme content was calculated by taking the varying amount of excipients into account. The FPF was calculated as the percentage of the total lysozyme content in the vial. The emitted dose (ED) was measured by weighing the vial before and after aerosolization and also calculated as the percentage of the total lysozyme content in the vial. The FPF analyses were performed in triplicate and the mean value including the standard deviation is presented in the figures.

Mechanical Testing

The mechanical properties of the lyophilisates were investigated using a Texture Analyzer (TA.XT.plus, Stable micro Systems, Godalming, United Kingdom) equipped with a 5 kg load cell and a cylindrical stainless steel probe with a diameter of 5 mm at a test speed of 1 mm/s and a maximal immersion into the lyophilisate of 2 mm. The cylindrical probe acts on the lyophilisate by proceeding at constant speed and the force needed for the immersion is recorded.

The resulting force represents the force necessary for breakage of the lyophilisate. At the end of the immersion, the probe touches the bottom of the vial which results in a steep increase of the immersion force curve and can therefore be disregarded. Measurements were performed in triplicate and averaged.

High Speed Camera Recording

The aerosolization behavior of the lyophilisates in the vial was recorded using a Fastcam 1024 PCI (Photron, San Diego, CA, USA) with a sample rate of 1000 fps. Dispersion of the lyophilisates was performed using the device at standard settings (20 ml compressed air at 3 bar).

Microscopy and Scanning Electron Microscopy (SEM)

The morphology of the lyophilisates was analyzed by digital light microscopy (VHX-500F, Keyence Corporation, Osaka, Japan) equipped with a VH-Z20R objective with variable lighting.

The particle morphology of the dispersed fine particles was analyzed with a Jeol Scanning Electron Microscope (JSM-6500F, Jeol, Ebersberg, Germany) at 5 kV. Sample preparation was performed using a second short stack version of the ACI where stages 3 to 7 have been removed. Conductive self-adhesive tape (Ø 12 mm Leit-Tabs, Plano GmbH, Wetzlar, Germany) was placed on the coated baffle plate after stage 2. The particles collected on the tape therefore have an aerodynamic cut off diameter between 4 and 4.94 μm at a flow rate of 39 l/min (12). The samples were sputtered with carbon under vacuum.

Stability Testing

For stability testing the sealed lyophilized samples were stored at 25°C and 60% relative humidity (RH) above a saturated ammonium nitrate (VWR International, Leuven, Belgium) solution or at 40°C and 75% RH above a saturated sodium chloride (Merck KGaA, Darmstadt, Germany) solution for 3 months. Stoppers used were dried before lyophilization at 105°C for 4 h.

The lyophilisates were analyzed for morphological state, moisture content, mechanical properties, microscopic morphology, as well as ED and FPF.

Residual Moisture Content Analysis

The residual moisture content of lyophilisates was determined by Karl-Fischer titration (Metrohm 756 KF Coulometer, Herisau, Switzerland) using Hydranal Coulomat AG (Riedel-deHaën, Seelze, Germany) as titration reagent. The lyophilisates were dissolved in the vials in 1.0 ml of anhydrous methanol (Hydranal-Methanol dry, Riedel-deHaën, Seelze, Germany), additionally dried with molecular sieve 3Å (VWR International GmbH, Darmstadt, Germany). As blanks, empty freeze-dried vials, treated similarly, were used. The injection volume was 500 μl each. The measurements were performed in triplicate in order to calculate mean values.

RESULTS

Optimization of the FPF by Addition of Excipients

A custom-designed test device was used for disintegration of the lyophilisates and delivery of the fine powder. The resulting FPF of the lysozyme formulations were measured with a short-stack version of the ACI. Lyophilized lysozyme from 12 mg/ml lysozyme solution showed an ED of 37.4% and a small FPF of only 4.8% related to the MD of 6 mg. In order to improve the FPF, 2 to 8 mg/ml phenylalanine and valine, which demonstrated good performance in a previous study (12), were added.

XRD demonstrated that freeze-dried lysozyme without excipients was completely amorphous. Mixtures with the amino acids demonstrated an increase in crystallinity of the formulations for concentrations >4 mg/ml of phenylalanine or >2 mg/ml of valine (Fig. 1). The XRD patterns of these

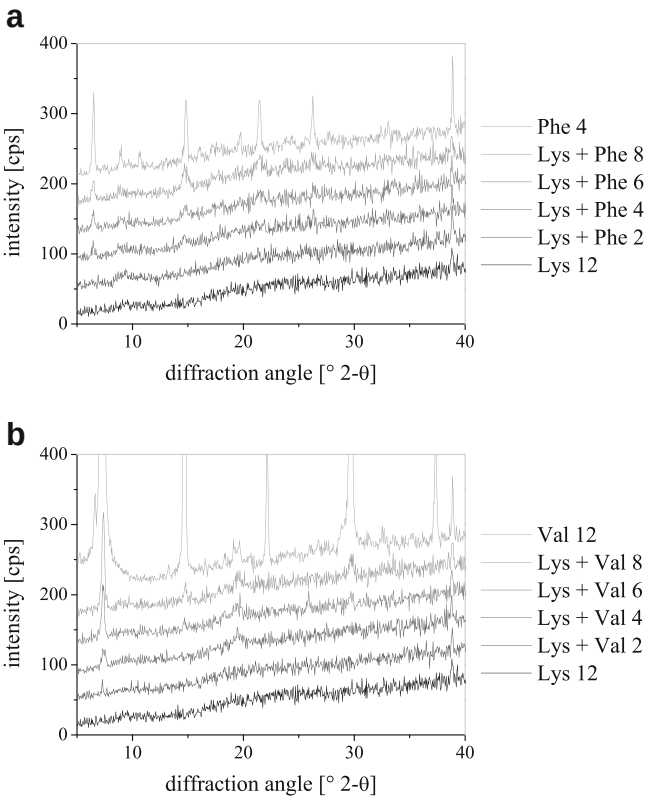

Fig. 1 XRD patterns of 12 mg/ml lysozyme lyophilisates and the mixtures with phenylalanine (**a**) or valine (**b**) in the concentration of 2 to 8 mg/ml and pure freeze-dried phenylalanine at 4 mg/ml (**a**) or valine at 12 mg/ml (**b**).

lyophilisates showed crystalline reflections of phenylalanine-monohydrate and valine, respectively.

The different lysozyme formulations demonstrated that the addition of phenylalanine or valine resulted in a dose-independent increase of the FPF of lysozyme. The ED is plotted against the FPF related to ED (left y-axis) in Fig. 2. From the contour lines and the right y-axis, it is also possible to determine the FPF related to MD from the same plot. The ED was almost doubled to around 70% by addition of the excipients. The FPF related to MD was increased up to 17.0% by adding 4 mg/ml phenylalanine or elevated up to 14.1% by adding 6 mg/ml valine, mainly a consequence of the doubled ED.

To understand underlying mechanisms for the successful FPF optimization, the lyophilisate characteristics as well as the aerosolization properties were further investigated. The force necessary for breakage of the lyophilisate was tested by immersion of a cylindrical probe. Steady fracture at a constant force, resulting in a plateau, was found for pure phenylalanine and valine lyophilisates in the concentration of 4 mg/ml and 12 mg/ml (Fig. 3) with force values of 0.014 N and 0.006 N, respectively. For lysozyme, a continuous increase of the immersion-force-curve at a slope of 0.07 N/mm was observed which represents a more elastic structure where mainly compression takes place. The immersion-force-curves of the mixtures, in contrast, revealed a plateau, indicating steady fracture at 0.08 N in the case of the phenylalanine mixture or 0.09 N for the valine mixture, exemplarily shown for the two formulations with the best performance.

High speed camera recordings were performed in order to gain insights into the aerosolization behavior of the different lyophilisates. For all formulations the disintegration of the lyophilisate started by fragmentation into larger subunits (Fig. 4). These pieces disintegrated further into fine particles which swirled around the endings of the capillaries until they left the vial. After aerosolization of pure lysozyme, a substantial amount of particles adhered to the capillaries (Fig. 4a). If lysozyme was formulated with one of the crystalline amino acids, in contrast, adhesion of the dispersed particles to the capillaries and other surfaces in the vial was lower, as can be seen from Fig. 4b, c. For the lysozyme lyophilisate almost all particles, which did not stick to the surfaces, left the vial after about 100 ms. Fine particles of those formulations that included an amino acid in contrast swirled in the vial for a longer period of time until about 500 ms. This could be a result of the larger total content in the vial.

The microscopic morphology of the different freeze-dried formulations was inspected with the reflected light microscope. The lyophilisates of both pure lysozyme and the mixtures showed a porous comb-like structure. Fig. 5 demonstrates the pore size for the upper surface of the lyophilisates of around 100 µm for pure freeze-dried lysozyme and slightly bigger pores of around 150 µm for the formulation with phenylalanine. The pore walls of pure lyophilized lysozyme were very thin and translucent.

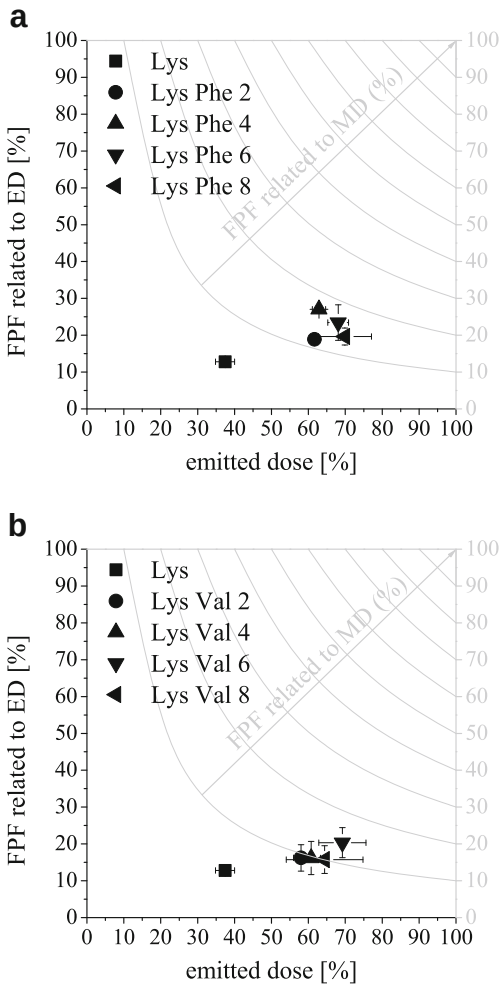

Fig. 2 ED *versus* its related FPF as well as the FPF related to metered dose of lyophilisates of pure lysozyme and lysozyme formulated with 2–8 mg/ml phenylalanine (**a**) or valine (**b**).

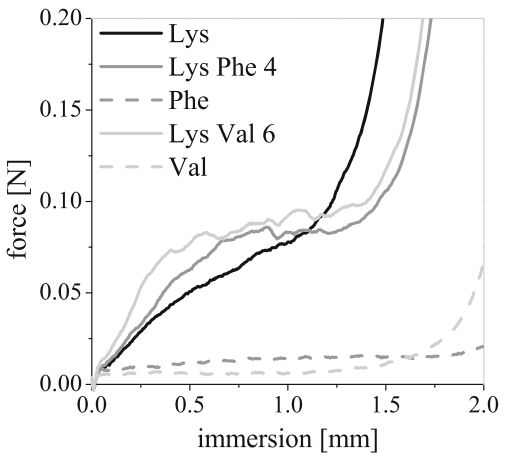

Fig. 3 The texture characterization of lysozyme 12 mg/ml lyophilisates in comparison to lysozyme 12 mg/ml + phenylalanine 4 mg/ml, lysozyme 12 mg/ml + valine 6 mg/ml, phenylalanine 4 mg/ml and valine 12 mg/ml.

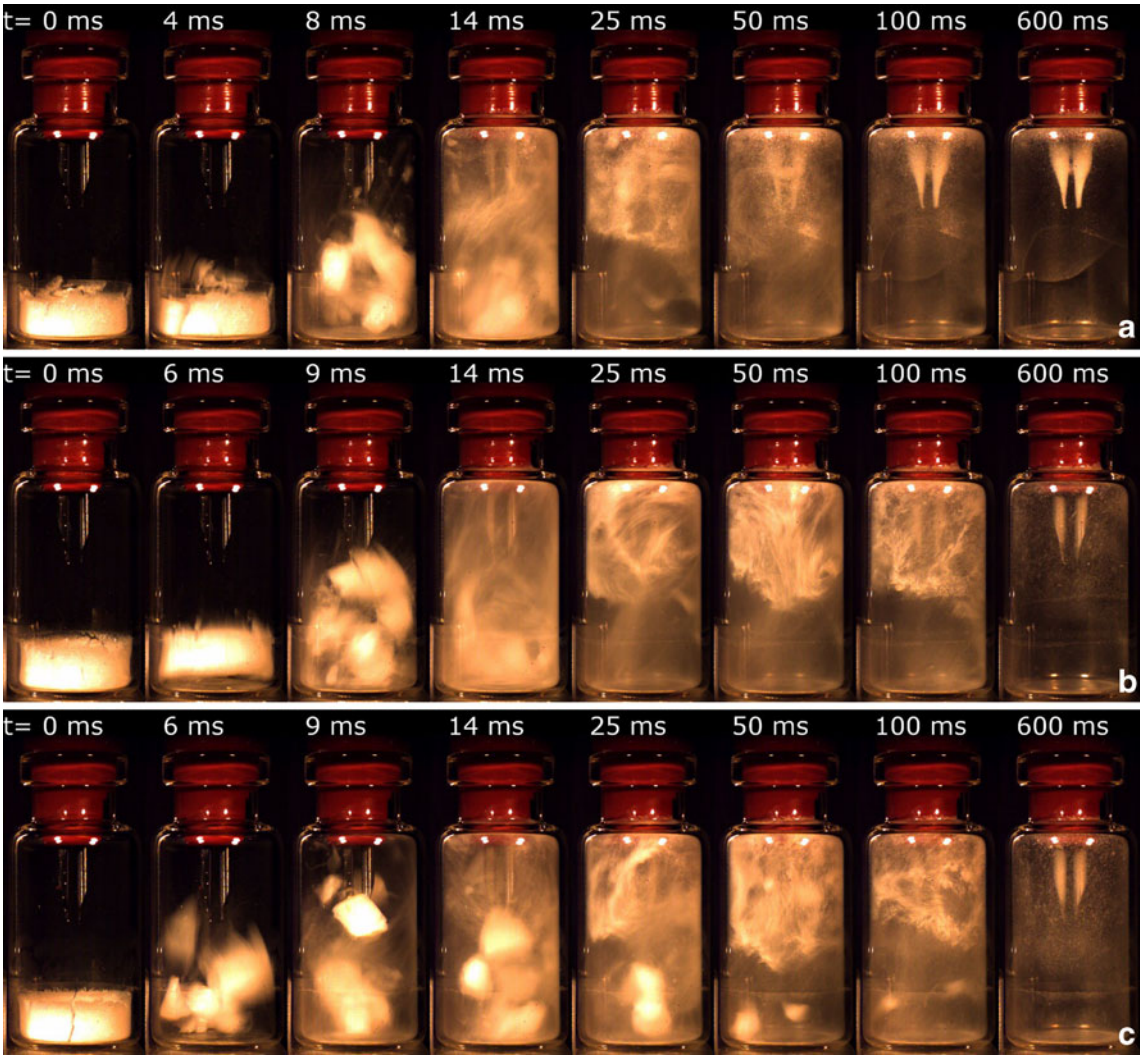

Fig. 4 High speed camera recordings of the disintegration process of lysozyme lyophilisate (**a**), lysozyme in the combination with 4 mg/ml phenylalanine (**b**) or with 6 mg/ml valine (**c**).

Whereas the pore walls of the mixtures appeared whitish and clearly visible. This is exemplarily shown for the phenylalanine mixture in Fig. 5b.

In order to determine the geometric size and shape of the dispersed particles released from the test device, the particles were investigated with SEM. For comparison of particles with identical aerodynamic size, the specimens were collected on the baffle plate after stage 2 of the ACI, having an aerodynamic cut off diameter of 4–4.94 μm. At the border of the impacted particle spots (Fig. 6a, c, e), it was possible to observe particles which were clearly separated from each other. These geometric particle sizes of approximately 10–30 μm were comparable for the different lysozyme formulations. The fragments of the pure lysozyme lyophilisates showed a very smooth surface (Fig. 6b), whereas the fragments of the lysozyme phenylalanine formulation revealed a rough surface with needle-shaped crystal structures embedded (Fig. 6d). The fragments of the lysozyme valine formulation demonstrated also a rougher surface (Fig. 6f).

Storage Stability of 12 mg/mL Lysozyme-4 mg/mL Phenylalanine-Formulation

Freeze-drying is known to improve the stability of labile drugs (22). If a freeze-dried formulation contains amorphous or partially crystalline modifications which are thermodynamically unstable, one always has to consider the possibility of recrystallization during storage (14). Furthermore, the moisture content of a freeze-dried formulation during storage may increase due to moisture coming from the vial stopper (23) or insufficient integrity of the container adversely influencing physicochemical stability and aerosol performance (9). Consequently, freeze-dried 12 mg/ml lysozyme with 4 mg/ml phenylalanine formulation was stored at 25°C/60% RH and 40°C/75% RH up to 3 months.

The XRD pattern of the lyophilized lysozyme phenylalanine formulation showed no difference between freshly freeze-dried or stored lyophilisates. The crystalline reflections of phenylalanine-monohydrate remained at the same

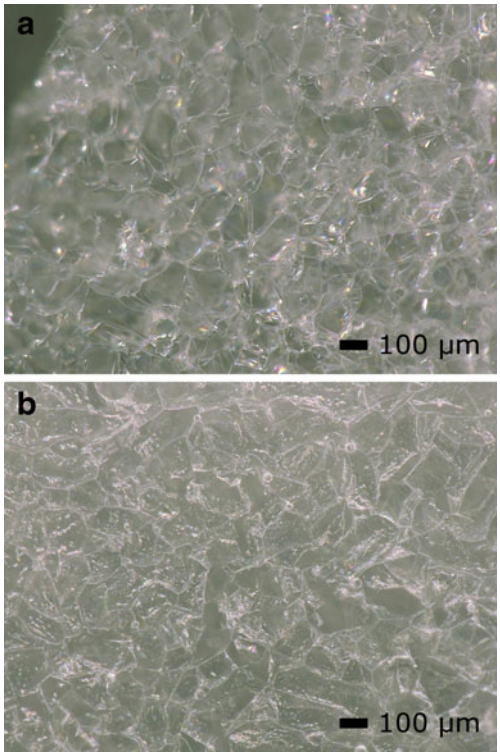

Fig. 5 Microscopic morphology of lysozyme lyophilisates: upper surface of pure lysozyme (**a**) in comparison to lysozyme with 4 mg/ml phenylalanine (**b**).

peak position and height. During storage the moisture increased from 0.3% to 0.8% and 1.9% after 1 month and to 1.5% and 2.7% after 3 month at 25°C/60% RH and 40°C/75% RH, respectively. Samples stored at the higher temperature and relative humidity absorbed significantly larger amounts of water. The mechanical behavior of the lyophilisates remained stable during storage. The position and shape of the immersion-force-curve of the mechanical testing did not change. Furthermore, no change in the microscopic morphology was detectable. ACI measurements revealed slight but not significant differences in ED as well as FPF of the lyophilisates. At t_0 samples demonstrated an ED of 57% which slightly increased to 64% during storage. The FPF related to ED amounted to 34% at t_0 and varied between 30% and 34% after 1 and 3 months of storage. Thus, stability of the formulation with respect to physicochemical properties and aerosol performance over 3 months at 40°C/75% RH could be demonstrated.

Variation of the FPF by Variation of the Freezing Method

The morphology of the solid matrix of a lyophilisate is determined by the freezing process, comprising ice nucleation and ice crystal growth (18). Different freezing methods were applied to alter the lyophilisate properties in order to

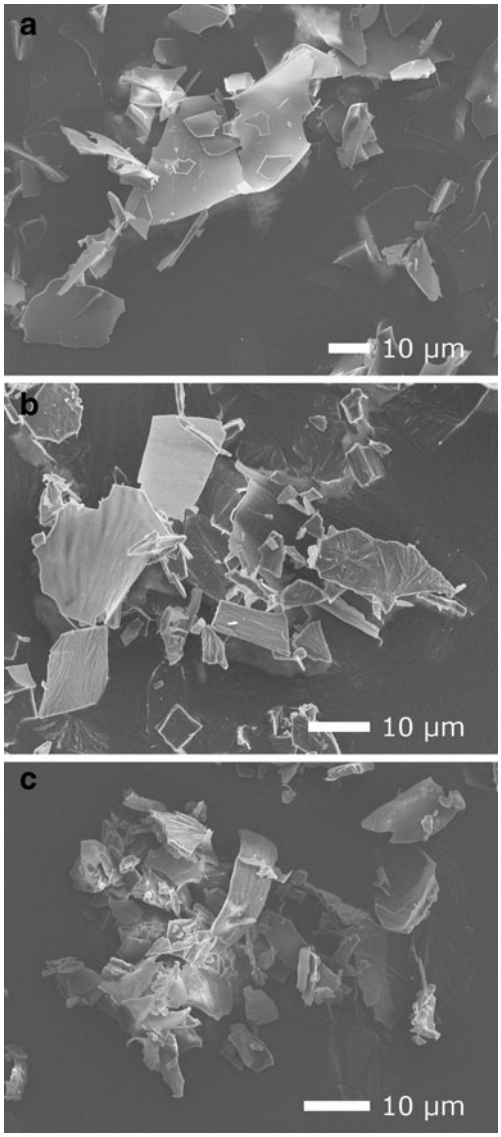

Fig. 6 SEM of the particles with an aerodynamic cut off diameter between 4 and 4.94 μm of lysozyme 12 mg/ml (**a**) and lysozyme 12 mg/ml + phenylalanine 4 mg/ml (**b**) as well as lysozyme 12 mg/ml + valine 6 mg/ml (**c**).

improve ED and FPF. 12 mg/ml lysozyme solutions containing 4 mg/ml phenylalanine or 6 mg/ml valine were freeze-dried. Besides the normal shelf-ramped freezing at −1°C/min, an additional annealing step at −10°C for 10 h, freezing on a precooled shelf at −70°C, freezing by immersion into liquid nitrogen and vacuum-induced freezing at −3°C were performed. The crystallinity of the lyophilisates was analyzed by XRD. Lysozyme was always present in the amorphous state. All lyophilisates in the combination lysozyme and valine showed the characteristic peak pattern of crystalline valine with the main peaks at 7.4° and 19.5° 2θ (Fig. 7a). Phenylalanine exhibited very small peaks, indicating a partial crystallinity in the form of the monohydrate for

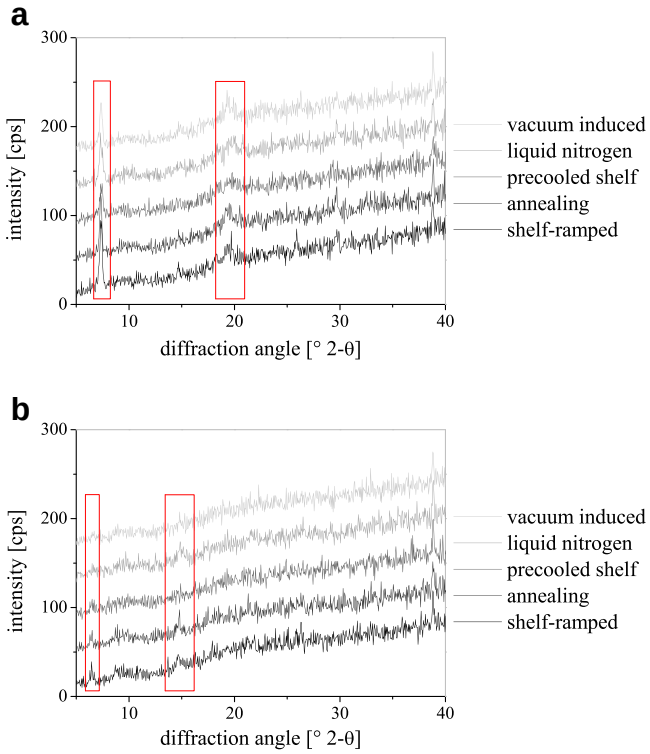

Fig. 7 XRD pattern of 12 mg/ml lysozyme/6 mg/ml valine (**a**) and 12 mg/ml lysozyme/4 mg/ml phenylalanine (**b**) lyophilisates prepared with different freezing procedures.

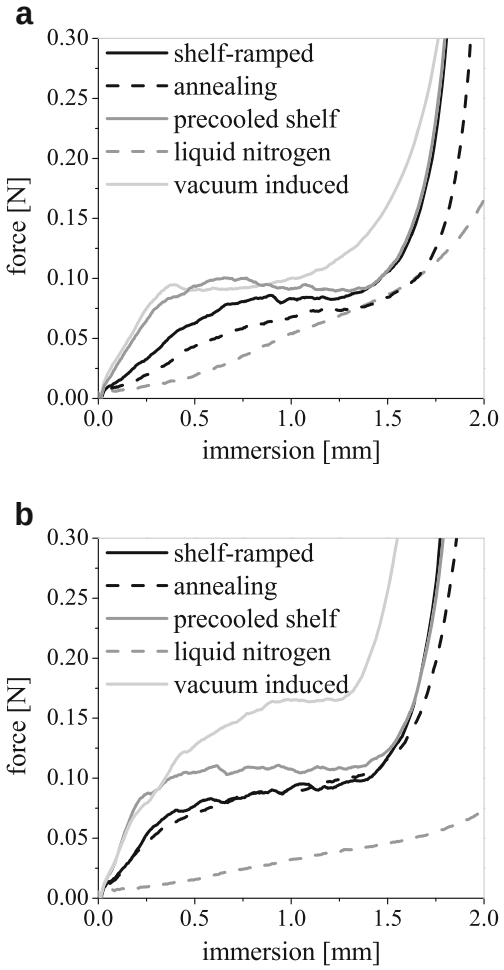

Fig. 8 The mechanical testing of 12 mg/ml lysozyme/4 mg/ml phenylalanine (**a**) or 12 mg/ml lysozyme/6 mg/ml valine (**b**) prepared with different freezing procedures.

all different frozen lyophilisates (Fig. 7b). The partial crystallinity of phenylalanine was analyzed in a previous study (12).

Mechanical testing revealed marked differences of the mechanical properties of lyophilisates prepared with different freezing processes, as can be seen in Fig. 8. For samples frozen in liquid nitrogen, a constant positive slope of the immersion-force-curve was observed. Vacuum-induced freezing resulted in a force peak with a maximum at 0.09 N at the beginning of the immersion for the lysozyme/phenylalanine lyophilisates, whereas the lysozyme/valine lyophilisates required a higher overall force of 0.16 N for fracture. The lysozyme/valine lyophilisates frozen on a precooled shelf required also a higher force for breakage of 0.11 N compared to the lyophilisates frozen in the conventional shelf-ramped process or additionally with annealing, which both demonstrated a force of 0.09 N for breakage. Lysozyme/phenylalanine lyophilisates frozen on a precooled shelf as well showed a plateau of constant fracture at 0.09 N which was higher than the lyophilisate frozen by shelf-ramping (0.08 N) or including annealing (0.07 N).

Microscopy of lyophilisates frozen via shelf-ramping, with an annealing step, or on a precooled shelf showed a similar pore structure. Differences in the pore sizes, particularly at the bottom side, were apparent. For the lysozyme/phenylalanine lyophilisates, for example, the pore size at the bottom was around 100 µm for samples frozen via shelf-ramping. Annealing caused an increase to about 160 µm, whereas precooled shelf freezing resulted in smaller pores of approximately 30 µm. Both samples frozen in liquid nitrogen demonstrated a special morphology with lamellar pores oriented in the direction from outside to the center, which was clearly visible for the inner and bottom structure of the lyophilisate (Fig. 9a). The samples obtained by vacuum-induced freezing had a very dense upper surface structure with tiny little pores of approximately 30 µm for the formulation with phenylalanine (Fig. 9b) and 20 µm for the formulation with valine. The bottom of these lyophilisates showed small pores of 50–60 µm as well.

Analysis of the dispersed particles by SEM demonstrated that the fragments of the conventionally frozen (at −1°C/min) lysozyme/phenylalanine lyophilisates had a platelet-like structure, whereas the samples frozen in liquid nitrogen or by vacuum-induced freezing were fluffy and porous. The fine particles with an aerodynamic size between 4 and 4.94 µm

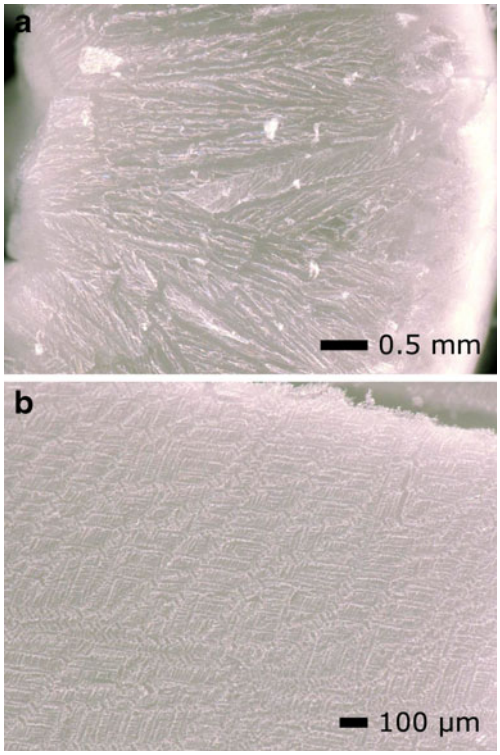

Fig. 9 The bottom surface morphology of lyophilisates frozen in liquid nitrogen (**a**) and the upper surface morphology of the vacuum induced frozen lyophilisates (**b**) of 12 mg/ml lysozyme/4 mg/ml phenylalanine.

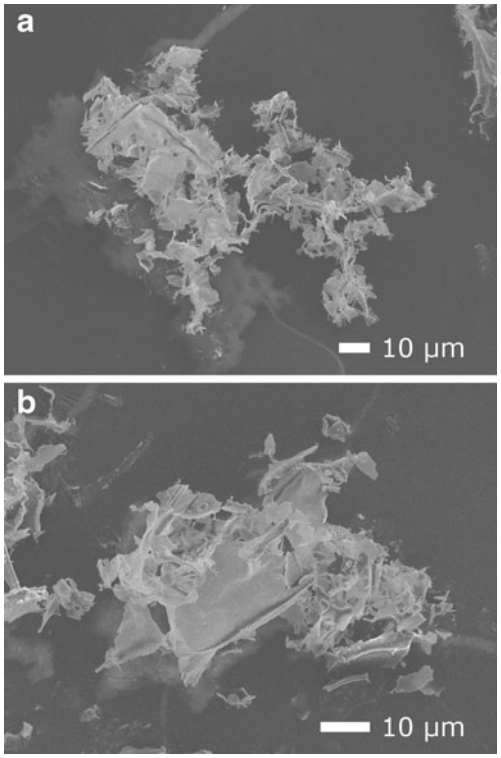

Fig. 10 SEM of the particles with an aerodynamic cut off diameter between 4 and 4.94 μm. Aerosolized 12 mg/ml lysozyme/4 mg/ml phenylalanine lyophilisates frozen in liquid nitrogen (**a**) and frozen vacuum induced (**b**).

consisted of agglomerates of smaller fragments and demonstrated different geometric sizes, depending on the freezing process: particles from normal freezing were 10–30 µm, the particles from vacuum-induced freezing of approx. 80 µm, and the ones frozen in liquid nitrogen of approx. 110 µm in size (Fig. 10). The aerodynamic diameter (d_A) is defined by the relationship

$$d_A \cong d_V \sqrt{\frac{\rho}{\chi \rho_0}} \quad (1)$$

and is dependent on the size (d_V), shape (χ) and mass density of the particle (ρ) for $\rho_0 = 1$ g/cm^3. By disregarding the shape assuming round particles, a particle density can be estimated based on the aerodynamic and geometric size. The particle density decreased from around 0.05 g/cm^3 for the small particles generated from lyophilisates prepared by normal freezing to a density of around 0.002 g/cm^3 for the large particles generated from lyophilisates prepared by liquid nitrogen freezing.

Additional high speed camera recordings revealed a different behavior of the various frozen lyophilisates during the disintegration process in the vial. For the lysozyme/phenylalanine formulation, for example, the disintegration into particles of the lyophilisates frozen at −1°C/min (Fig. 4b) and with the annealing step (Fig. 11a) was already finished after about 25 ms. The disintegration into small particles of samples prepared by precooled shelf freezing lasted about 50 ms (Fig. 11b). Lyophilisates from vacuum-induced freezing (Fig. 11d), in contrast, disintegrated into smaller subunits from which particles were scaled off over a longer period of time (more than 100 ms). The lyophilisates frozen in liquid nitrogen disintegrated quickly into voluminous particles, resulting in a complete fill of the vial (Fig. 11c). The valine formulations showed similar aerosolization behavior.

The described variations of lyophilisate morphology and properties partly resulted in differences in the fine particle output, as can be seen from Fig. 12. For both, lysozyme/phenylalanine and lysozyme/valine formulations annealing did not result in a significant difference in ED and FPF compared to regular shelf freezing. The precooled shelf method in comparison to shelf-ramped freezing led to a significant increase of the ED from 63% to 79% and therefore also to an increase of the FPF related to MD from 17% to 30% for the phenylalanine formulation. The valine formulation, in contrast, showed no significant differences. By freezing in liquid nitrogen, it was possible to significantly enhance the ED and the FPF for both formulations. The ED increased to 88% in the case of the phenylalanine formulation with an increase in the FPF related to ED from 27% to 44% while the valine formulation showed an enhanced ED from 69% to 86% and FPF related to ED from 20% to 30%. The samples

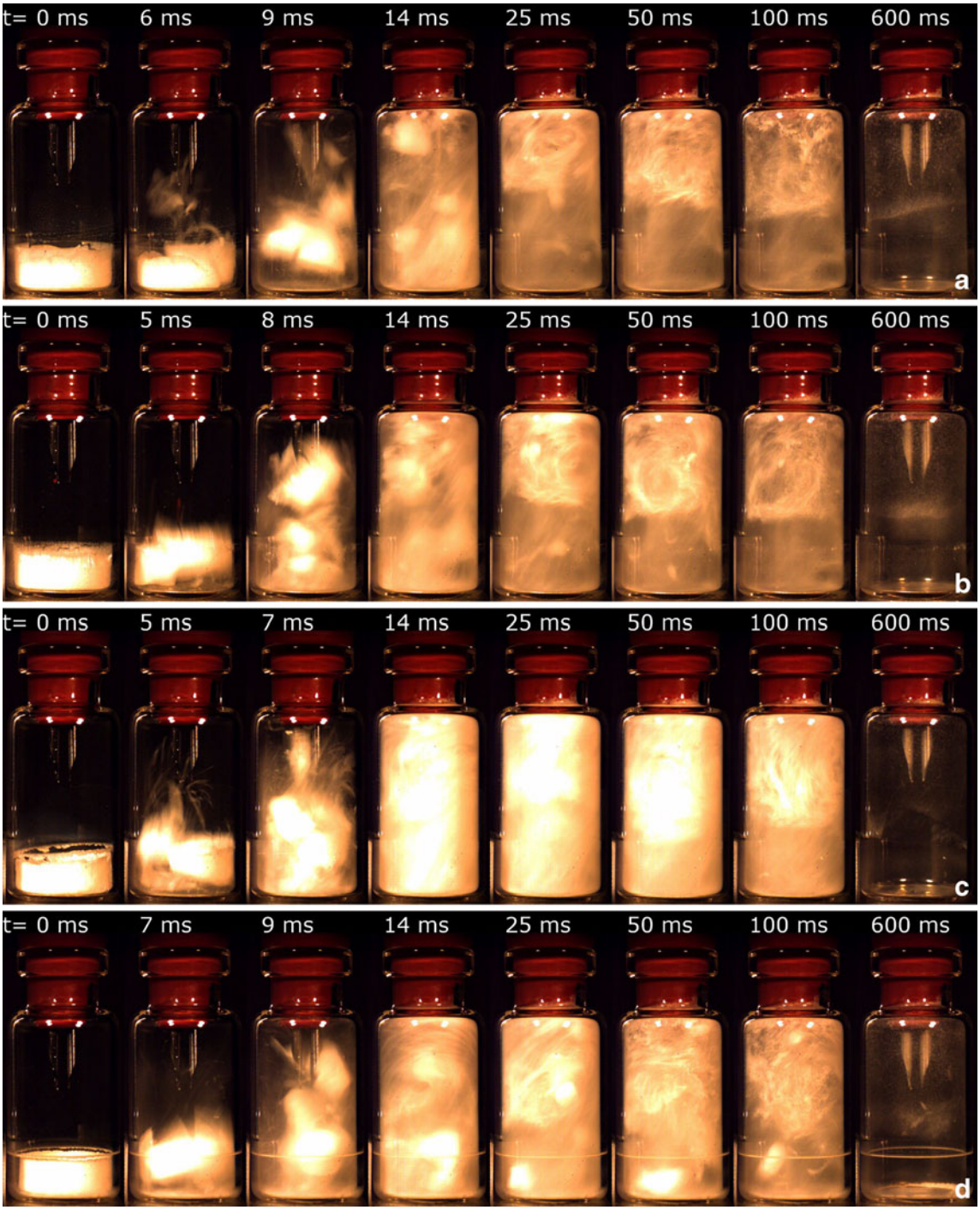

Fig. 11 High speed camera recordings of the disintegration process of 12 mg/ml lysozyme/4 mg/ml phenylalanine lyophilisates frozen with annealing (**a**), on precooled shelf (**b**), in liquid nitrogen (**c**) and vacuum induced (**d**).

from vacuum-induced freezing also exhibited a significant escalation of the FPF for both formulations to 49% and 32% as well as a significant increase of the ED to 80% for the phenylalanine formulation. Freezing in liquid nitrogen and vacuum-induced freezing resulted for the phenylalanine formulation in a more than twofold elevation of the FPF related to MD from 17% to 39%.

DISCUSSION

Optimization of the FPF by Addition of Excipients

It was possible to significantly increase the FPF of the lyophilized model API lysozyme by addition of the amino acids phenylalanine or valine. These two excipients are not

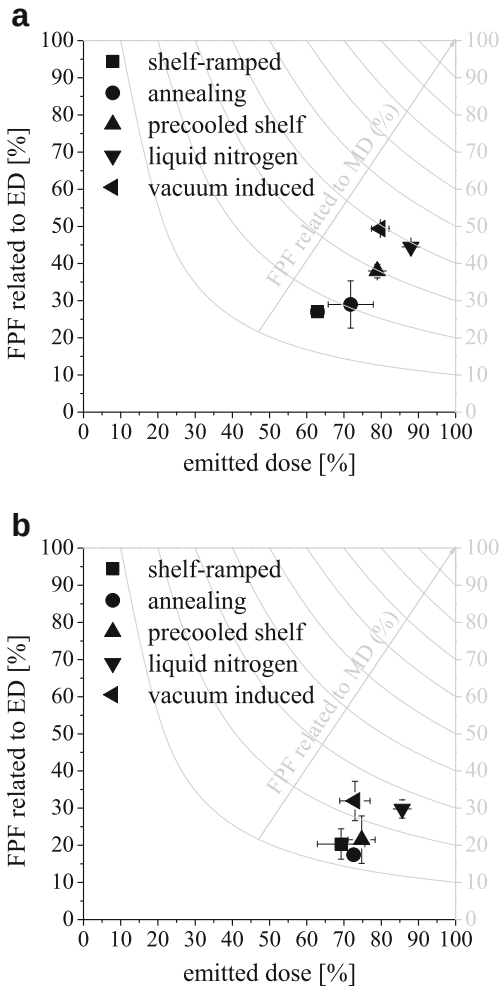

Fig. 12 The impact of the different freezing processes of lyophilized 12 mg/ml lysozyme/4 mg/ml phenylalanine (**a**) or 12 mg/ml lysozyme/6 mg/ml valine (**b**) on the ED and the FPF related to ED as well as the FPF related to metered dose.

commonly used in freeze-drying or dry powder formulation for inhalation but were chosen because of their good performance in a previous study exhibiting high FPF related to MD of nearly 50% (12). In principle, most amino acids crystallize during freeze-drying and are therefore suitable as bulking agents (24). Glycine, for example, is a commonly used bulking agent in freeze-dried formulations (25). Valine crystallizes as well during freeze-drying whereas phenylalanine forms a monohydrate and is only present in a partially crystalline state (12).

The magnitude of the FPF elevation of 12 mg/ml lyophilized lysozyme was independent of the amount of excipient added. A small amount of 2 mg/ml phenylalanine or valine was sufficient to double the FPF related to MD from 4.8% to 11.7% and 9.4%, respectively. Consequently, the amino acids had a great effect on the lyophilisate properties and aerosolization behavior of freeze-dried lysozyme. These lyophilisate properties and characteristics are discussed in the following to understand underlying mechanisms.

As expected, lysozyme was present in the amorphous state in all formulations. Proteins, in general, do not crystallize during freezing but form an amorphous solid at the glass transition temperature of the freeze concentrate (Tg′) (14). This purely amorphous glass appeared translucent in light microscopy, resulting in a glassy sheen on the lyophilisate surface (26), whereas the pore walls appeared whitish in the formulations with an amino acid. As intended, valine crystallized during freeze-drying. In contrast, phenylalanine was partly present in the amorphous state. Because crystallization is dependent on the presence of other solutes (27), the amorphous protein prevented phenylalanine from crystallization at the highest protein to amino acid ratio of 6:1. At higher phenylalanine concentrations, the amino acid was only partially crystalline as indicated by small crystalline reflections appearing in combination with an amorphous halo. This was not only caused by the presence of the amorphous protein since pure freeze-dried phenylalanine also exists in a partly amorphous state (12). During freeze-drying phenylalanine crystallizes in the form of the needle-shaped monohydrate (12) which is the stable pseudo-polymorph when crystallization occurs at temperatures below 37°C (28). The crystalline nature of formulations containing an amino acid corresponded to a rough surface of the pore wall fragments in SEM. The distinct striped appearance of the needle-shaped phenylalanine-monohydrate crystals coated with a thin layer of amorphous material was also reported previously (29). This surface roughness could explain the lower adhesiveness of the particles to the capillaries and the vial surface, as well as to each other, because of a smaller effective contact area compared to the planar surface of lyophilized lysozyme fragments. The enhanced dispersibility of particles with irregular surfaces was also observed for spray-dried particles (8,30,31). Less adhesion to the capillaries and vial surface enhanced the ED and consequently the FPF related to MD. A longer time period during which particles swirled in the vial until their escape through the outlet capillary was noticeable, which could be an effect of a larger total content in the vial or of less adhesion resulting in a larger amount of free swirling particles in the vial.

The rough surface is not the only parameter responsible for the successful increase of the FPF by addition of phenylalanine or valine. Mechanical properties of the lyophilisates can be examined by texture analysis. The cylindrical probe acts on the lyophilisate by proceeding at a constant speed. The resulting force needed for the immersion is constantly measured and represents the force necessary for breakage of the lyophilisate. The texture analysis revealed that lyophilisates containing phenylalanine or valine, which show steady fracture at a constant force, have good aerosolization properties with a high ED and FPF. It is important to note, that the force necessary to fracture the lyophilisate was much higher in the lysozyme/amino acid combinations (0.08–0.09 N) compared

to the pure amino acid (0.006–0.014 N) but did not hinder the disintegration of the lyophilisates. Lysozyme, on the other hand, showed a continuous positive slope of the immersion-force curve, which points to an elastic structure for which mainly compression takes place instead of breakage. Since the lyophilisate needs to fracture into fine particles during the dispersion process, one can easily conclude that brittleness and fragility of lyophilisates shown by a plateau in the immersion-force curve of the mechanical testing and therefore steady fracture at a constant force are beneficial for a good dispersibility and a high fine particle output. By the addition of crystalline excipients it was possible to improve the fracture properties of lyophilized lysozyme and consequently the FPF.

Storage Stability of 12 mg/mL Lysozyme-4 mg/mL Phenylalanine-Formulation

During storage the aerosolization performance of the lyophilisates may become reduced and amorphous material may recrystallize due to moisture uptake. The formulation was stored at 25°C and 60% RH or 40°C and 75% RH, as recommended by the International Conference on Harmonization (32). The morphological state of the lyophilisate did not change during storage of 3 months. Even during storage at 40°C, which is above the transition point of phenylalanine in water where the anhydrous form is more stable (28), phenylalanine remained a monohydrate. Furthermore, the partly amorphous phenylalanine state was preserved over the storage period. The residual moisture of lyophilisates immediately after freeze-drying was less than 1%, which is considered optimal for storage stability (22). The moisture content of the freeze-dried formulation increased during storage. The higher the storage temperature, the greater was the increase in moisture content, which agrees with other storage stability studies (24,33). Residual moisture in the stopper is often responsible for the moisture uptake of the formulation up to an equilibrium value which is characteristic for the product, amount of product, and stopper treatment method, whereby the time to the equilibrium is dependent on the temperature (23). Lyophilisates stored at 25°C did not reach the equilibrium of greater or equal to 2.7% within 3 months. Since water has a plasticizing effect on amorphous materials (34), variations in the mechanical characteristics were expected. Nevertheless, the mechanical property of the formulation did not change for all storage conditions and time periods. Potentially the crystalline component had a stabilizing effect on the mechanical behavior of the lyophilisate. The cascade impactor measurements demonstrated that an increased moisture content was not detrimental for the aerosolization performance of the formulation.

Variation of the FPF by Variation of the Freezing Method

As the manner of ice crystal formation and growth during the freezing process fixes the texture and porosity of the final dried product, the degree of supercooling, the freezing rate, and the time required for complete solidification directly impact ice crystal morphology (15). In general, freezing methods for which supercooling exceeds 5°C result in a freezing of samples by global supercooling, which causes a spherulitic morphology. This was the case for lyophilisates prepared with standard shelf-ramped freezing, annealing, and precooled shelf freezing. Directional solidification, in contrast, is often seen at very high cooling rates, or when ice nucleation is induced close to the equilibrium freezing point (15). Lyophilisates frozen by directional solidification show lamellar plate morphology (35), which was in this study only observed for freezing by immersion in liquid nitrogen. It causes extreme temperature gradients along the vial bottom and sides, resulting in directional solidification from those surfaces inward (17). The microscopic morphology of both liquid nitrogen frozen formulations showed the characteristic thin pores of lamellar-orientation in the direction from outside toward the middle. A supercooling of less than 5°C occurred also for the controlled nucleation at −3°C by vacuum-induced freezing. Nevertheless, these formulations were frozen by global supercooling, which resulted in spherulitic pore morphology visible on the bottom of the lyophilisates (Fig. 7a). In addition, observation of the formulations during the freezing process showed a sudden change in the appearance of the vial content from a clear liquid to an opaque/translucent slush, which is indicative of global supercooling (36).

As Table I summarizes, the different freezing processes applied resulted not only in a varied morphology but also in a variation of cake characteristics and aerosolization performance. For both lysozyme formulations (with 4 mg/ml phenylalanine or 6 mg/ml valine), freezing in liquid nitrogen and vacuum induced freezing demonstrated the best results for ED and FPF by about doubling again the FPF related to metered dose. In the following, differences and commonalities of the samples prepared by the different freezing procedures are discussed in order to understand underlying mechanisms of the aerosolization performance improvement.

The physical state of the compounds in a lyophilisate can be influenced by the freezing procedure. In addition to enabling rearrangement and growth of ice crystals, annealing or thermal treatment of frozen systems is a process which facilitates crystallization of a bulking agent such as glycine or mannitol (14). On the other hand, fast freezing, for example by immersion into liquid nitrogen, can prevent solutes from crystallizing. XRD, however, showed no differences in the peak height of the different frozen lyophilisates.

Table I Results Overview of the Cake Characteristics and Aerosolization Performance of Lysozyme/Phenylalanine or Lysozyme/Valine Lyophilisates Prepared with Different Freezing Procedures

	Standard shelf-ramped	Annealing	Precooled shelf	Liquid nitrogen	Vacuum induced
Lyozyme 12 mg/ml + phenylalanine 4 mg/ml					
pore structure	spherulitic	spherulitic	spherulitic	lamellar	spherulitic
mechanical testing	force plateau at 0.08 N	force plateau at 0.07 N	force plateau at 0.09 N	constant slope of 0.06 N/mm	force peak max at 0.09 N
disintegration characteristics	fast, within 25 ms	fast, within 25 ms	within 50 ms	fast into voluminous particles	into subunits and scaling off
ED	63%	72%	79%	88%	80%
FPF (ED)	27%	29%	38%	44%	49%
FPF (MD)	17%	21%	30%	39%	39%
particle morphology	platelet-like	–	–	fluffy, porous	fluffy, porous
particle size	10–30 μm	–	–	80 μm	110 μm
Lysozyme 12 mg/ml + valine 6 mg/ml					
pore structure	spherulitic	spherulitic	spherulitic	lamellar	spherulitic
mechanical testing	force plateau at 0.09 N	force plateau at 0.09 N	force plateau at 0.11 N	constant slope of 0.03 N/mm	force plateau at 0.16 N
disintegration characteristics	into subunits and scaling off	fast, within 25 ms	into subunits and scaling off	fast into voluminous particles	into subunits and scaling off
ED	69%	73%	75%	86%	73%
FPF (ED)	20%	17%	22%	30%	32%
FPF (MD)	14%	13%	16%	25%	23%
particle morphology	platelet-like	–	–	–	–
particle size	10–30 μm	–	–	–	–

Thus, no further crystallization of phenylalanine occurred during annealing and fast freezing by immersion into liquid nitrogen did not prevent valine or phenylalanine from crystallization.

For the differently frozen lyophilisates, texture analysis revealed a different mechanical behavior. Because the physical state remained the same, the differences must be a result of the structural properties. Conventional shelf-ramped freezing and annealing resulted, for all samples, in an immersion-force curve with a plateau that indicates brittle cake fracture. Both samples prepared by precooled shelf freezing demonstrated a steeper slope of the immersion-force-curve until the plateau was reached, suggesting that a higher force was needed to break at least the upper half of the cake. Freezing on a precooled shelf can cause an enormous temperature gradient throughout the fill volume, which leads to smaller pores at the bottom and large pores near the top (15) as observed in this study. Phenylalanine samples from vacuum-induced freezing demonstrated a peak at the beginning of the immersion-force-curve, indicating that a higher force was needed to break the top part. Compared to the other freezing methods, the microscopic picture revealed a very dense upper surface of this sample, which therefore can explain the harder and more destruction resistant structure. The reduction of the pressure to 600 mtorr for vacuum-induced freezing results in an evaporation of water, which lowers the temperature on the surface of the solution and induces ice nucleation (18). During this vaporization process, the solutes concentrate at the surface (13) and thus form a dense upper surface layer. Both formulations frozen in liquid nitrogen showed a constant slope of the immersion-force-curve without a plateau and thus demonstrated an elastic behavior. These sample characteristics conflict with the above conclusion that brittleness of the lyophilisate instead of an elastic behavior is beneficial for a good disintegration and a high fine particle output. This demonstrates, however, that there must be several factors influencing the aerosolization behavior which more or less can compensate for other characteristics. While a brittle structure can improve the disintegration behavior for lyophilisates with a spherulitic morphology, it is not essential for good disintegration of samples with a special morphology such as the narrow lamellar oriented pores resulting from directional solidification. Possibly, the narrow pore structure does not fracture in the same way as the spherulitic pores but particles form due to separation and rupture of the lamellae.

High speed camera recordings demonstrated quick and complete disintegration of lyophilisates frozen in liquid nitrogen into voluminous particles. Consequently, the directional lamellar pore structure of the cake can easily disaggregate into particles without the need of superfine breakup. SEM showed highly porous particle agglomerates with a huge geometric size of about 110 μm but an aerodynamic size in the respirable size range. Therefore, these porous particles must have a very small density. The harder samples of vacuum-induced or precooled shelf freezing disintegrated first into smaller pieces, from which fine particles were scaled off continuously. SEM revealed that cakes from vacuum-induced freezing disintegrated also into porous particles which had a bigger geometric size of about 80 μm compared to the conventionally frozen samples with a geometric particle size of approx. 20 μm. Porous particles with a big geometric size and a low density are therefore advantageous for a high ED and FPF, which can be most effectively achieved by freezing by immersion in liquid nitrogen prior to lyophilization. It has been already demonstrated in literature that porous particles can be efficiently aerosolized from standard DPIs, exhibiting elevated respirable fractions. These porous particles were produced by spray drying (37).

Despite good performance of the sample frozen in liquid nitrogen, one should consider that freezing sensitive proteins by immersing the vial in liquid nitrogen can result in increased protein aggregation or decreased enzyme activity compared to a slow cooling rate (38). The reason for this is the formation of higher surface areas by quench freezing (36), resulting in increased surface denaturation at the ice-water interface. A protection can be achieved by the addition of surfactants as cryoprotectants (38). Furthermore, freezing in liquid nitrogen is not appropriate for full-scale GMP production of sterile pharmaceuticals (35). Vacuum-induced freezing, in contrast, can be considered more suitable for production scale.

CONCLUSION

This study demonstrates two methods for a successful improvement of the FPF of a lyophilization-based novel formulation for inhalation. This includes the addition of excipients, as well as the variation of the lyophilisate structure by varying the freezing method. It was also possible to demonstrate some underlying mechanisms responsible for the improved disintegration and aerosolization behavior of the lyophilized formulations. Furthermore, storage stability and unchanged aerosolization property of a selected formulation was demonstrated.

In summary, the FPF of lyophilized lysozyme formulations could be enhanced by addition of the crystalline excipients phenylalanine or valine. The amount of excipient added was less important. The crystalline excipients improved the fracture properties of the lyophilisate and enhanced its dispersibility and fine particle output. Additionally, crystals possibly coated with a thin layer of amorphous material resulted in a rough surface of the

fragments which reduced particle aggregation and adhesion to vial and capillary surfaces and therefore enhanced the ED.

Storage at 25°C/60% RH and 40°C/75% RH for 3 months demonstrated physical stability and unchanged aerosolization performance, despite a slight increase in the moisture content. Possibly, the crystalline excipients served as mechanical stabilizers.

Different freezing methods resulted not only in different pore structures of the lyophilisates but also in a variation of the mechanical properties as well as in the aerosolization behavior. A further improvement of the FPF of lyophilized lysozyme formulated with phenylalanine or valine could be achieved by freezing in liquid nitrogen or by vacuum-induced freezing at −3°C. Considering the mechanical behavior of these different frozen lyophilisates, it was obvious that not only improved fracture properties of the lyophilisates can have an enhancing effect on the fine particle output. In addition, a special structure, such as the fine directional lamellar pore structure resulting from freezing in liquid nitrogen, could positively affect the ED and FPF. This special structure disaggregated quickly and completely into particles having good aerodynamic properties without the need of superfine breakup. The resulting fine particles had a geometric size of about 110 μm but an aerodynamic size in the respirable size range because of a low density and high porosity.

In conclusion, the novel formulation for inhalation based on dispersion of lyophilisates enabled an improvement of the FPF by different strategies. This included the utilization of excipients showing good disintegration properties as well as the variation of the lyophilization process. The freezing method, in particular, resulted in a variation of the lyophilisate structure and thereby affected its aerosolization properties.

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