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The influence of lysozyme on mannitol polymorphism in freeze-dried and spray-dried formulations depends on the selection of the drying process

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ABSTRACT

Freeze-drying and spray-drying are often applied drying techniques for biopharmaceutical formulations. The formation of different solid forms upon drying is often dependent on the complex interplay between excipient selection and process parameters. The purpose of this study was to investigate the influence of the chosen drying method on the solid state form. Mannitol–lysozyme solutions of 20 mg/mL, with the amount of lysozyme varying between 2.5% and 50% (w/w) of total solid content, were freeze-dried and spray-dried, respectively. The resulting solid state of mannitol was analysed by near-infrared spectroscopy in combination with multivariate analysis and further, results were verified with X-ray powder diffraction. It was seen that the prevalence of the mannitol polymorphic form shifted from β-mannitol to δ-mannitol with increasing protein concentration in freeze-dried formulations. In spray-dried formulations an increase in protein concentration resulted in a shift from β-mannitol to α-mannitol. An increase in final drying temperature of the freeze-drying process towards the temperature of the spray-drying process did not lead to significant changes. It can thus be concluded that it is the drying process in itself, rather than the temperature, that leads to the observed solid state changes.

1. Introduction

In the manufacturing of biopharmaceutical products, freeze-drying and spray-drying are commonly applied unit operations transforming the formulation from the liquid state to the solid state and thus increasing the stability of the protein or peptide. Owing to the complex conformational structure, proteins have a tendency to unfold, aggregate and denature during drying (Wang, 1999). One strategy to stabilise proteins against heating, freezing or shearing stresses involved in the drying process is the inclusion of the disaccharides sucrose or trehalose (Back et al., 1979; Bakaltcheva et al., 2007) or of polyols such as sorbitol (Wimmer et al., 1997) and mannitol (Costantino et al., 1998) in the formulation.

Mannitol, classified as a non-hygroscopic additive, is often added in freeze-dried protein formulations to provide a stable matrix structure. It can also be used as a stabiliser to prevent proteins from stress involved in the dehydration process. Besides the amorphous form of mannitol, there exist at least four crystalline solid forms of mannitol, namely anhydrous α-, β-, and δ-mannitol as well mannitol hemihydrate (Burger et al., 2000; Nunes et al., 2004). Several studies have shown that the variation in the solid state form of mannitol can affect the performance of a formulation (Costantino et al., 1998) and the stability (Izutsu et al., 1994; Yoshinari et al., 2002) of the dried protein system. In general, crystalline mannitol is considered to be ineffective in preserving protein activity. This finding has so far not been further specified whether the three anhydrous polymorphs might behave differently in this regard. In addition, the phase separation due to recrystallization of mannitol upon storage may cause degradation of protein.

Mannitol polymorphism is described to depend upon both processing and formulation factors. It has been reported that different drying conditions and the co-solute added in protein formulations may result in various solid state forms of mannitol in the final product. For example in freeze-drying, changes in the freezing cycle may result in the formation of amorphous mannitol or the various crystalline polymorphs of mannitol, alone or in mixtures (Kim et al., 1998). Different spray-drying conditions can also result in a mixture of polymorphic forms of mannitol (Hulse et al., 2008; Lee et al., 2011). It was recently reported though that the processing conditions might not have an influence on the polymorphic state of mannitol, when highly concentrated solutions between 15% and 20% (w/w) of mannitol are spray-dried, as β-mannitol was obtained in all cases (Littringer et al., 2012). For

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freeze-drying it has been shown that the concentration of mannitol can influence the polymorphic outcome. While concentrations of 5% (w/w) or lower result predominantly in the formation of the β-polymorph, mannitol concentration of 10% (w/w) usually result in the formation of δ-mannitol (Grohganz et al., 2011; Kim et al., 1998; Pyne et al., 2003). A design of experiment approach on the influence of formulation parameters such as the mannitol concentration on mannitol polymorphism has been reported (De Beer et al., 2007).

Compared with the research on the interplay between mannitol crystallinity and protein activity, little work has been done to elucidate how co-solutes (e.g. protein drug, buffer salt, amino acid or other components) direct the solid state form of mannitol upon dehydration. As an example, the presence of protein in a freeze-drying process resulted in δ-mannitol, whereas β-mannitol and mannitol hemihydrate were observed in the absence of protein (Grohganz et al., 2011). Furthermore, the addition of protein was found to be able to stabilise the metastable hemihydrate form of mannitol (Cao et al., 2013). It was reported that, when co-spray-dried with mannitol, lysozyme induced β- and δ-mannitol whereas trypsin induced amorphous mannitol at 1:1, 1:9 and 9:1 (w/w) protein–mannitol ratios (Hulse et al., 2009). Our study on co-spray-drying of lysozyme and mannitol showed that a mixture of α- and β-mannitol was produced at 1:1 and 1:9 (w/w) protein–mannitol ratios (Lee et al., 2011). However it remains unclear as to the exact molecular mechanism causing this difference. A mechanistic understanding of mannitol polymorphism in the presence of co-solute will be desired in order to improve the quality of the dehydrated protein product, which is also underlined by the growing interest in the Quality by Design (QbD) concept (Yu, 2008).

Freeze-drying involves freezing and slow sublimation under vacuum, while a different heat/mass transfer and higher temperatures are involved in spray-drying. For the freeze-drying process, it has been shown that that freezing rate and annealing temperature have an impact on the polymorphic form of mannitol (Liao et al., 2007). As spray-drying is based on evaporation of solvent upon heating, a different drying mechanism is applied. This may result in a different polymorphic outcome and this outcome can be influenced by the particle size (Lee et al., 2011). The two processes were applied in this study to investigate mannitol polymorphism in identical lysozyme formulations under distinct drying processes. Due to the dependency of mannitol polymorphism on the mannitol concentration itself, a rather low concentration of the solution was investigated as a stronger impact of the formulation component could be expected. X-ray powder diffraction (XRPD) and near-infrared spectroscopy (NIR) in combination with multivariate analysis were applied to identify and estimate mannitol polymorphic forms in the dehydrated protein product. This choice is based on the fact, that XRPD is still the gold standard in the identification of polymorphic forms, while NIR is widely regarded as the standard process analysis tool. As spectroscopic techniques both Raman and NIR spectroscopies have been applied for in-line measurements during freeze-drying (Bruells et al., 2003; Romero-Torres et al., 2007), but also for more traditional pharmaceutical processes such as tableting (Karande et al., 2010). Furthermore, it was shown in several studies that NIR is well suited to differentiate between the polymorphic forms of dried mannitol formulations (De Beer et al., 2007; Grohganz et al., 2010). The wider application possibilities of NIR and Raman spectroscopy for in-process monitoring of pharmaceutical production processes have recently been reviewed (De Beer et al., 2011).

The aim of this study is to investigate how two different drying operations, namely freeze-drying and spray-drying in combination with different protein concentrations influence mannitol polymorphism in the final product. Lysozyme was used as the model protein.

2. Materials and methods

2.1. Materials and sample preparation

D-Mannitol was purchased from VWR (Poole, UK). Lysozyme from chicken egg white (Fluka Analytical) was obtained from Sigma–Aldrich (Steinheim, Germany). For the drying operations, solutions of mannitol and lysozyme were produced at a concentration of 20 mg/mL total solid content. The investigated concentration of lysozyme was 2.5%, 10%, 20%, 30%, 40% and 50% (w/w) of the total solid content for both processes.

2.2. Spray-drying

Feed solutions containing lysozyme at five different concentrations, ranging from 2.5% to 50%, were prepared and spray-drying was performed using a Büchi B-290 mini spray-dryer (Flawil, Switzerland). The operating conditions were kept constant at the following parameters: inlet temperature 130 °C, feed flow rate 4 g/mL, atomisation 11.1 g/min, drying air flow rate 0.47 m^3/min and outlet temperature 55 °C. The resulting outlet temperature was found to vary between 54 °C and 58 °C. The spray-dried powders were collected into Parafilm-sealed glass scintillation vials, and the closed vials were stored at room temperature prior to analysis.

2.3. Freeze-drying

The freeze-drying was performed in an Epsilon 2-4 LSC freeze-dryer (Martin Christ, Osterode, Germany). The freeze-drying cycle consisted of freezing to −50 °C, followed by a 2 h annealing step at −10 °C and reduction of the temperature to −50 °C again.

Two different drying schemes were applied in order to investigate the influence of the final process temperature on polymorphism. In the standard cycle, primary drying was performed over 35 h at −30 °C and secondary drying was performed over 8 h at +20 °C. In the second cycle at elevated temperature, primary drying was performed over 30 h at −30 °C and secondary drying was performed over 13 h at +40 °C, resulting in the same cycle length for both processes. The pressure during primary and secondary drying was kept constant at 0.05 mbar (5 Pa). Samples were closed under vacuum in the freeze-dryer at the end of the cycle, and stored at room temperature prior to analysis.

2.4. Morphology

The morphology of the dry powders was visually examined using a JSM-5200 scanning electron microscope (SEM; JEOL Ltd., Tokyo, Japan). Samples were transferred onto carbon sticky tape and mounted in metal stubs, followed by sputter coating with a thin layer of gold-palladium for 120 s with a E5200 Auto Sputter Coater (BIO-RAD, Polaron Equipment Ltd., Watford, England) under an Argon gas purge (Air Liquide, Taastrup, Denmark). The specimens were then imaged at an accelerating voltage of 10 kV energy at magnifications between 350× and 10,000×.

2.5. X-ray powder diffraction

X-ray powder diffraction (XRPD) was performed on a PANalytical X'Pert Pro equipped with a PIXcel detector (PANalytical BV, Almelo, The Netherlands) using Cu Kα radiation (45 kV, 40 mA, λ = 1.54187 Å). The samples were placed on rotating aluminium sample holders and measured with a step size of 0.026°2θ and a scan speed of 0.082°2θ/s.