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Research paper

Impact of fast and conservative freeze-drying on product quality of protein-mannitol-sucrose-glycerol lyophilizates

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ABSTRACT

Purpose: Mannitol/sucrose formulations are employed to generate lyophilizates for biopharmaceuticals with an elegant cake appearance. The aim of this study was to dry protein/mannitol/sucrose formulations as fast as possible without loss of cake appearance and protein stability. Glycerol was included as potential additional protein stabilizer. Three proteins (lysozyme and two monoclonal antibodies) at low and high concentration were analyzed comparing fast with conservative freeze-drying.

Methods: Freeze-drying cycle development was carried out with mannitol/sucrose formulations. A product temperature (Tp) close to the Te of mannitol and clearly above the Tg' of sucrose was targeted. Protein formulations were exposed to the final fast lyophilisation process and to a conservative freeze-drying cycle. Lyophilizates were characterized by differential scanning calorimetry, Karl-Fischer titration and X-ray diffractometry. Additionally, macroscopic cake appearance and reconstitution times were evaluated. Protein stability was characterized by UV/Vis spectroscopy, light obscuration and size exclusion chromatography.

Results: The fast freeze-drying cycle resulted in a primary drying time of 7 h (Tp: −10 °C) and a secondary drying time of 2 h in contrast to 47 h (Tp: −39 °C) and 12 h for the conservative cycle. Lyophilizates showed Tg values above 60 °C, a residual moisture level of 1%, reconstitution times of less than 35 s, δ-mannitol and elegant cake appearance. Mannitol/sucrose ratios below 4/1 did not lead to complete mannitol crystallization and were therefore not suitable for the selected process conditions. Characterisation of protein stability rendered low aggregation and particle levels for both, fast and conservative freeze-drying conditions.

Conclusions: It was shown that fast freeze-drying of mannitol/sucrose formulations above Tg' at a Tp of −10 °C resulted in good protein process stability and appropriate cake characteristics at maximum time reduction.

1. Introduction

Freeze-drying is frequently used to achieve long-term storage stability of sensitive biopharmaceuticals. More than 40% of the marketed biologicals are currently freeze-dried, a number which is expected to even increase in the future [1,2]. The manufacturing process is time- and cost-intensive with regular process times of several days or even weeks [3]. Especially for drugs in high demand and full capacity utilization, faster drying would enable higher throughput at preferably reduced production costs. Several approaches were already investigated with respect to process optimization at high sublimation rates and product temperatures. Amorphous formulations were dried above the glass transition temperature of the freeze concentrate (Tg') but below the collapse temperature (Tc) [2,4]. Highly concentrated protein formulations benefitted more since Tc was raised more than Tg' with increasing protein concentration. That enabled drying at higher product temperature (Tp) of up to −10 °C at 100 mg mL^{-1} mAb with a Tc of −8 °C. A 5 mg mL^{-1} protein formulation could only be dried at a Tp below −30 °C [2]. Another approach was to combine mannitol (Man) with sucrose (Suc) and dry at high Tp so that the amorphous matrix of Suc and protein collapsed while Man formed a crystalline scaffold without macroscopic visible defects such as e.g. eutectic melting [5]. Man and Suc are regularly combined due to their complementary characteristics. Suc probably the most frequently utilized protein stabilizer replaces water binding sites of proteins and decreases protein mobility in the dried state by vitrification [6–8]. Upon freeze-drying

Abbreviations: A, annealing step; aFD, after freeze-drying; API, active pharmaceutical ingredient; AUC, area under the curve; bFD, before freeze-drying; CN, controlled nucleation; F, freezing step; FD, freeze-drying; Gly, glycerol; hh, hemihydrate; HP-SEC, high performance size exclusion chromatography; HPW, highly purified water; LO, light obscuration; Lyso, lysozyme; mAb, monoclonal antibody; Man, mannitol; mDSC, modulated differential scanning calorimetry; PD, primary drying step; RM, residual moisture; SD, standard deviation/secondary drying step; Suc, sucrose; Tc, collapse temperature; Te, eutectic temperature; Tg, glass transition temperature of the freeze-dried cake; Tg', glass transition temperature of the freeze-concentrated solution; Tp, product temperature; XRPD, X-Ray powder diffraction

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Suc remains amorphous which is a prerequisite for protein stabilization [8]. In contrast, the bulking agent mannitol crystallizes during the process while losing its stabilization properties [9]. The crystalline scaffold leads to an elegant cake without shrinkage or cracks [10] and helps to increase the mass of the cake or to achieve the desired tonicity of the rehydrated formulation [11,12]. Typically, Man and Suc have to be combined at a Man/Suc ratio of at least 4/1 due to inhibition of Man crystallization by Suc at lower Man/Suc ratios [5,13]. Moreover, proteins also suppress bulking agent crystallization [14].

At high ratios of Man to Suc, the crystallization of mannitol minimizes the potential collapse of amorphous sucrose, eliminates the need to conduct primary drying below the Tg' or Tc, and thereby, provides the option to perform drying at higher temperatures and enables shorter processing times.. Drying of crystalline excipients to products of appropriate macroscopic appearance is limited by their eutectic temperature (Te), in case of Man (−1.5 °C [5]). It has already been demonstrated that collapse of the stabilizers is not necessarily coupled with protein destabilization [15–21]. To which extent macroscopic appearance should be accepted given that the protein stability is not affected is an ongoing discussion but complete collapse is not acceptable if not induced intentionally [22]. Thus, freeze-drying cycle development should aim at adequate cake appearance, unless protein stability is superior in collapsed cake structure.

The small molecular weight plasticizing agents glycerol and sorbitol were found to be beneficial for protein stability in the dried state [23–25]. Their potential lies in the prolongation of ß-relaxation times or local motions [23,25–27]. Due to their small size they might be able to reduce free volumes and increase the packing density better than larger stabilizers [26,28]. Because of their low Tg values (glycerol: −93 °C and sorbitol: 0 °C [29,30]) they would lead to macroscopic collapse if used as single component in a formulation. They would also lower Tg' values of Suc formulations to a considerable extent. As a consequence, fast drying at high product temperatures would be difficult in Suc/Glycerol formulations without macroscopic collapse. The appearance of crystalline mannitol reduces the potential for gross collapse, and provides the additional advantage of faster drying. In this study, glycerol was included as an additional stabilizer in a matrix comprising of amorphous sucrose and crystalline mannitol. Glycerol replaced 5%/10% of the amorphous Suc as these low concentrations showed antiplasticizing effects in previous studies [31].

All process steps were accelerated (that is, faster cooling or heating rates) to minimize the cycle duration. The freezing step plays an important role for the whole process because it determines ice crystal growth, size and distribution, as well as the crystallization behavior of the excipients [13,32,33]. Annealing above Tg' is commonly performed to ensure complete mannitol crystallization or to force the formation of a certain Man polymorph [13,34,35]. Moreover, it can be effectively utilized to minimize the effects of the inter-vial variability in the ice nucleation temperature, which causes differences in the ice structures (size of the crystals) across a batch of vials [33]. Annealing results in a more homogeneous (or uniform) ice structure and reduces the inter-vial heterogeneity. Hence the freezing step already determines characteristics of the dried matrix. Primary drying (PD) is the step with the highest optimization potential due to its long duration [2]. The last step, secondary drying, takes only a few hours [3]. Nevertheless, the final residual moisture (RM) content and the conversion of potentially formed Man hemihydrate into the anhydrous form depends on selection of sufficiently high secondary drying temperature and duration [5]. Thus, the characteristics of freeze-dried products are mainly influenced by the FD cycle conditions and the formulation composition, but are also affected by further parameters such as container, stoppers or filling volume which are not discussed here [36].

This study aimed at a cycle development of a fast freeze-drying cycle with Man/Suc formulations that were indistinguishable from conservative dried formulations regarding their physical characteristics. Protein formulations were investigated to show that a high Tp during PD, likely coupled to higher protein mobility, does not negatively impact protein process stability. For a comprehensive analysis, freeze-dried protein formulations were characterized by DSC, Karl-Fischer titration and XRD, checked for macroscopic appearance and reconstitution time, and protein stability was tested by LO, turbidity and HP-SEC. After development of the fast cycle with Man/Suc/Glycerol formulations, the results were compared to conservative freeze-drying conditions that provided non-collapsed amorphous matrices. Lysozyme and two monoclonal antibodies in different concentrations enabled a detailed study on impact of formulation and process. Lower Man/Suc ratios were tested with focus on complete Man crystallization and the impact of different freezing protocols.

2. Materials and Methods

2.1. Materials

Stock solutions of mannitol (Man) (VWR International, Ismaning, Germany), sucrose (Suc) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and glycerol (AppliChem GmbH, Darmstadt, Germany) were prepared in 10 mM L-histidine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) pH 7.0 formulation buffer. Different mannitol/sucrose/glycerol (Man/Suc/Gly) weight ratios were investigated: 40/10/0, 40/9.5/0.5, 40/9/1, and 40/0/0 [mg/mL]. Protein formulations contained protein, mannitol, sucrose and glycerol. For the second study, different Man/Suc ratios dissolved in highly purified water (HPW) were examined without protein: 20/30, 25/25, 30/20, 40/10 [mg/mL]. All formulations were filtered with 0.2 µm polyethersulfone (PES) membrane syringe filters (VWR International GmbH, Ismaning, Germany) prior to use.

Hen egg white lysozyme (MW 14.3 kDa, ε = 38.94 mL mg^{-1} cm^{-1}, Dalian Greensnow Egg Products Development Co., Ltd., Dalian, China) and two different monoclonal IgG_1 antibodies (MW ~ 150 kDa, ε = 1.49 mL mg^{-1} cm^{-1}, referred to as mAb1 and mAb2) served as model proteins within the Man/Suc/Glycerol formulations. mAb 1 was used at lower concentration as it results in viscous formulations at high concentration. Lysozyme was dissolved in formulation buffer whereas the stock solutions of the mAbs were prepared by tangential flow filtration in Minimate™ capsules (30 kDa Omega membrane, PALL Life Science, Port Washington, NY, USA) out of lower concentrated bulk solutions. Each protein was formulated at 2 mg mL^{-1} and, additionally, lysozyme at 100 mg mL^{-1}, mAb1 at 7.5 mg mL^{-1} and mAb2 at 80 mg mL^{-1}.

2.2. Methods

2.2.1. Lyophilization

Lyophilization stoppers (B2-TR coating, West Pharmaceutical Services Deutschland GmbH & Co. KG, Eschweiler, Germany) and DIN 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with highly purified water and dried for 8 h at 100 °C and 60 °C, respectively. A filling volume of 1.2 mL was dispensed into the vials which were semi-stoppered subsequently. The two outer rows of vials were not used for subsequent analysis. Thermocouples in different vial positions on the shelf recorded temperature values during freeze-drying.

The protein formulations were freeze-dried according to the given freeze-drying protocols (Table 1, Process 1 and Process 8). Controlled nucleation (CN) according to Geidobler et al. was conducted [37]. The vials were equilibrated at −5 °C for 1.5 h. At a Tp of −4 °C to −4.5 °C, the chamber pressure was reduced to 4 mbar. CN was induced by ventilation via the condenser chamber. The method was selected to enable potential scale up to larger freeze-dryers avoiding potential adjustments to the individual formulations. The different Man/Suc ratios were investigated with the fast freeze-drying cycle conditions (Process 8) and two variations of the freezing step: (1) equilibration at −5 °C, but without CN or (2) including an annealing step at −20 °C for

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