Be Aggressive! Amorphous Excipients Enabling Single-Step Freeze-Drying of Monoclonal Antibody Formulations

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Abstract: Short freeze-drying cycles for biopharmaceuticals are desirable. Formulations containing an amorphous disaccharide, such as sucrose, are prone to collapse upon aggressive primary drying at higher shelf temperature. We used 2-hydroxypropyl-beta-cyclodextrin (HPBCD) in combination with sucrose and polyvinylpyrrolidone (PVP) to develop an aggressive lyophilization cycle for low concentration monoclonal antibody (mAb) formulations. Glass transition temperature and collapse temperature of the formulations were determined, and increasingly aggressive cycle parameters were applied. Using a shelf temperature of +30 °C during primary drying, the concept of combining sublimation and desorption of water in a single drying step was investigated. Cake appearance was evaluated visually and by micro-computed tomography. Lyophilisates were further analyzed for reconstitution time, specific surface area, residual moisture, and glass transition temperature. We demonstrated the applicability of single-step freeze-drying, shortening the total cycle time by 50% and providing elegant lyophilisates for pure HPBCD and HPBCD/sucrose formulations. HPBCD/PVP/sucrose showed minor dents, while good mAb stability at 10 mg/mL was obtained for HPBCD/sucrose and HPBCD/PVP/sucrose when stored at 40 °C for 3 months. We conclude that HPBCD-based formulations in combination with sucrose are highly attractive, enabling aggressive, single-step freeze-drying of low concentration mAb formulations, while maintaining elegant lyophilisates and ensuring protein stability at the same time.

Keywords: glass transition; collapse; freeze-drying; cyclodextrin; antibody; cycle optimization; single-step freeze-drying

1. Introduction

Freeze-drying is frequently used to manufacture drug products of proteins which are unstable as liquids. Proteins are generally more stable in the dried immobilized state as physical (e.g., aggregation) and chemical (e.g., hydrolysis) degradation mechanisms are slowed down. Although around 40% of all biopharmaceuticals are freeze-dried, liquid formulations are often preferred due to a significantly less complex manufacturing process [1,2]. Freeze-drying is a time-consuming low throughput batch process which usually takes several days up to weeks [3], requires much energy, and is ultimately costly. Freeze-drying consists of three process steps, (i) freezing, (ii) primary drying, where crystallized
water is removed under vacuum by sublimation, and (iii) secondary drying, where desorption of the bound water takes place. Primary drying is the most time consuming of the three steps. Hence, efforts to optimize the lyophilization cycle time often focus on the primary drying step.

During primary drying it is important that the product temperature ($T_p$) stays below the critical formulation temperature to avoid collapse. Collapse may [4,5] or may not [6,7] be detrimental to the storage stability of monoclonal antibodies, but in any case is often considered as a defect of the drug product, which might lead to rejects during 100% visual inspection [8]. The collapse temperature ($T_c$) is typically 1–3 °C above the glass transition temperature of the maximally freeze concentrated solution ($T_g'$) of an amorphous formulation. Disaccharides such as sucrose and trehalose, which are commonly used in freeze-dried antibody formulations [1], have low $T_g'$ and $T_c$ values of around $-28$ to $-32$ °C [9]. This makes low pressure and shelf temperature ($T_s$) during primary drying necessary, resulting in long primary drying time.

Efforts to shorten freeze-drying cycle time have re-gained attraction and different approaches are described in recent literature. For example, microwave assisted freeze-drying enables much faster primary drying compared to conventional freeze-drying [10]. With regards to conventional freeze-drying, it is well-known that increasing $T_p$ significantly impacts cycle time, e.g., an increase of $T_p$ by 1 °C may shorten primary drying by 10% [3]. In this light, Colandene et al., Bjelosevic et al., and Depaz et al. successfully optimized primary drying by freeze-drying above $T_g'$ without introducing collapse [11–13]. However, this approach is only applicable for formulations with high protein concentrations or high protein to sugar ratios, as they show a $T_c$ which is markedly higher compared to $T_g'$. Another approach is to change the formulation composition in order to obtain a higher critical formulation temperature. To this end, crystalline excipients can be included as bulking agents in combination with amorphous sucrose as stabilizer. Recent publications by Horn et al. and Lansare et al. showed that combinations of crystalline and amorphous excipients allow for aggressive freeze-drying of low concentration protein formulations, while maintaining elegant lyophilisates [14,15]. The ideal ratio of crystalline and amorphous excipients has to be chosen carefully. Crystallization has to be assured which asks for less amorphous excipient content. Yet, the crystalline excipients insufficiently stabilizes the protein [16], which makes a higher amorphous stabilizer necessary. Additional technical problems can arise, e.g., an excessive mannitol content can lead to glass breakage during freeze-drying [17].

Little literature is available on the use of alternative amorphous excipients, such as polysaccharides or polymers, for aggressive cycle development. With their high $T_g'$ and $T_c$ as well as potentially protein stabilizing properties due to their amorphous state, such excipients could enable aggressive, short freeze-drying cycles. Larsen et al. recently investigated dextrans, polysaccharides with a $T_g'$ of $-21$ °C up to $-9$ °C depending on their molecular weight, for freeze-drying of lactate dehydrogenase. The dextrans enabled much faster primary drying and provided elegant lyophilisates with good protein stability [18]. In contrast, we found that dextrans were inferior stabilizers for mAbs compared to sucrose [19]. Another excipient of interest is 2-hydroxypropyl-betacyclodextrin (HPBCD), which has a $T_g'$ similar to dextran 40 kDa [20], and a $T_c$ of $-9$ to $-6.5$ °C [21]. It can be found in approved small molecule parenterals. Furthermore, the use of HPBCD to stabilize proteins in the dried state is described in literature [22]. HPBCD was able to stabilize an antibody during freeze-drying and supercritical fluid drying comparable to trehalose at a protein to sugar ratio of 1:4 (w/w) [23,24]. Additionally, HPBCD could provide protein stability during storage at elevated temperatures superior to sucrose [25,26]. The high $T_g'$ and the protein stabilizing properties of HPBCD might allow for a high $T_p$ during primary drying while resulting in elegant lyophilisates. To the best of our knowledge, so far, no studies on freeze-drying cycle optimization for HPBD-based mAb formulations are available.

This study aimed to shorten the lyophilization cycle time by using aggressive primary drying conditions for HPBCD-based low concentration mAb formulations (10 mg/mL) containing either pure HPBCD, or combinations with sucrose and polyvinylpyrrolidone (PVP) as excipients, which have all been shown to render amorphous lyophilisates [27–30]. For a comprehensive study, 50 mg/mL mAb formulations were included as well. The critical formulation temperatures, $T_g'$ and $T_c$, were determined
and the formulations were freeze-dried with increasingly more aggressive process conditions. Shelf temperatures typically applied for secondary drying were used during primary drying and the hypothesis whether this might enable combining sublimation and desorption of water in one single drying step was tested. The lyophilisates were characterized with regards to cake appearance and structure, reconstitution time, specific surface area, residual moisture, and glass transition temperature of the freeze-dried formulation \( T_g \), as well as protein stability upon freeze-drying and after storage and compared to a conservatively freeze-dried reference formulation with pure sucrose.

2. Materials and Methods

2.1. Materials

A F. Hoffmann-La Roche proprietary monoclonal antibody (IgG\(_1\), pI ~8.2, 149 kDa) was used at 10 mg/mL (low concentration) and 50 mg/mL (high concentration) in 20 mM histidine/histidine-HCl buffer pH 6.0 (Ajinomoto, Tokyo, Japan) with 0.02% polysorbate 20 (Croda International, Snaith, UK). Formulations (F) were prepared with different excipient concentrations as shown in Table S1. \( F_S \) contained 80 mg/mL sucrose (Ferro Pfanstiehl Company, Mayfield Heights, OH, USA), \( F_{CD} \) 80 mg/mL 2-hydroxypropyl-betacyclodextrin (HPBCD, Roquette, Beinheim, France), \( F_{CD/S} \) 56 mg/mL HPBCD and 24 mg/mL sucrose, and \( F_{CD/P/S} \) 39.2 mg/mL HPBCD, 16.8 mg/mL polyvinylpyrrolidone K17 (PVP, BASF, Ludwigshafen, Germany), and 24 mg/mL sucrose. Prior to filling, the formulations were filtered through a 0.22 \( \mu \)m PVDF sterile filter unit (Millipore, Bedford, MA, USA). Then, 3.2 mL per formulation were filled into 6 mL TopLyo\(^{\circledR} \) vials (Schott, Müllheim, Germany) and partially stoppered with 20 mm Lyo-stoppers D777-1 (DAIKYO Seiko Ltd., Tokyo, Japan).

2.2. Freeze-Drying

Freeze-drying was performed using an FTS Lyostar II (FTS Systems Inc., Stone Ridge, NY, USA). Each freeze-drying cycle was performed with one shelf fully loaded. The vials containing the different formulations were distributed randomly over the shelf. To reduce the impact of edge effects on the results, edge vials were excluded from further evaluation. Overall, six different freeze-drying cycles were employed with varying primary and secondary drying conditions as shown in Table 1. Product temperature was determined by three thermocouples in center vials. Chamber pressure \( (p_c) \) was monitored using a Pirani and a capacitance probe. Cycle 0 (C0) represented a typical conservative freeze-drying cycle and was only performed for \( F_S \). Formulations \( F_{CD} \), \( F_{CD/S} \), and \( F_{CD/P/S} \) at 10 mg/mL mAb were freeze-dried with cycles C1 to C5. \( F_S \) was freeze-dried with C0 and with C1. C1 and C5 were employed for the 50 mg/mL mAb formulations. At the end of freeze-drying, vials were stoppered at 760 mbar under nitrogen and sealed with aluminum crimp-caps after unloading.

| Cycle | Freezing | Primary Drying | Secondary Drying |
|-------|----------|---------------|------------------|
|       |          | Ramp \(^{\circ}C/\text{min}\) | \( T_s \) \(^{\circ}C\) | \( p_c \) (mTorr) | Ramp \(^{\circ}C/\text{min}\) | \( T_s \) \(^{\circ}C\) | Hold Time (min) | Pressure (mTorr) |
| 0     | Ramp     | 0.2           | -10              | 100               | 0.2           | +25              | 360              | 100               |
| 1     | 0.3 \(^{\circ}C/\text{min}\) | 0.2           | +10              | 100               | 0.2           | +25              | 360              | 100               |
| 2     | Temp.    | 0.2           | +30              | 100               | 0.2           | +40              | 360              | 100               |
| 3     | -35 \(^{\circ}C\) | 1.0           | +30              | 100               | 0.2           | +40              | 360              | 100               |
| 4     | Hold     | 0.2           | +30              | 155               | 0.2           | +40              | 360              | 155               |
| 5     | 180 min  | 0.2           | +30              | 100               |              |                 |                  |                   |

\( T_s \): shelf temperature, \( p_c \): chamber pressure.
2.3. Differential Scanning Calorimetry

$T_g'$ and $T_g$ were determined by differential scanning calorimetry according to Haeuser et al. [26]. Measurements were performed using a Tzero DSC Q2000 instrument (TA instruments Inc., New Castle, DE, USA). $T_g'$ and $T_g$ were determined in triplicates and reported as mean ($T_g'$, standard deviation < 0.2 °C) and as mean with standard deviation ($T_g$).

2.4. Freeze-Drying Microscopy

Freeze-drying microscopy (FDM) was used to determine $T_c$ as the temperature of the onset of collapse. Measurements were performed according to Haeuser et al. using a Linkam FDC196 freeze-drying stage (Linkam Scientific, Instruments, Surrey, UK) and a Zeiss Axio Imager.A1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany) at 20-fold magnification [19].

2.5. Visual Cake Appearance

Cake appearance of lyophilisates was evaluated by visual inspection. Representative pictures were taken using a camera in front of a black background.

2.6. Micro-Computed Tomography

Micro-computed tomography (µ-CT) was performed using an evolved version of the methodology introduced previously [31]. The lyophilisates were analyzed without any further sample preparation though the glass vial with a SkyScan 1272 X-Ray microtomograph (Bruker MicroCT, Kontich, Belgium). Scans were acquired using an acceleration voltage of 40 kV and a beam current of 250 µA. To ensure monochromatic X-rays with enough energy to pass through the glass vial, a 0.5 mm Al filter was applied. The vial was rotated over 360° with a step size of 0.1°. An exposure time of 2388 ms with 10 averages per projection was applied. Projections were reconstructed using the NRecon software (Bruker, Kontich, Belgium) to obtain an image stack of tomographs.

2.7. Reconstitution Time

For reconstitution, 3.0 or 2.9 mL of water for injection were added to the 10 and 50 mg/mL mAb formulations, respectively, using a 5 mL disposable syringe equipped with a 21 G needle. Reconstitution time was determined in triplicates as described previously and reported as mean with standard deviation [19].

2.8. Specific Surface Area

Specific surface area was determined in triplicates according to Brunauer–Emmett–Teller (BET) using the Quadrasorb evo surface area and pore size analyzer (Quantachrome, Odelzhausen, Germany) with Krypton as adsorbate. Analysis was performed in a 9 mm bulb sample cell filled with at least 100 mg lyophilisate. Prior to analysis, the samples were degassed overnight under vacuum at 40 °C and overlaid with Nitrogen. Krypton adsorption was determined for nine measuring points at 77 K over a pressure range of 0.05 to 0.25 mbar. Specific surface area was determined by fitting the data points using the BET equation and reported as mean with standard deviation.

2.9. Residual Moisture

Residual moisture was determined in triplicates according to Haeuser et al. using a C30 Coulometric Karl Fischer titrator (Mettler Toledo, Greifensee, Switzerland). Residual moisture was reported as mean with standard deviation [19].

2.10. Size-Exclusion Chromatography

Stability of the mAb was analyzed by size-exclusion high-performance liquid chromatography (SE-HPLC) using an Alliance e2695 HPLC instrument (Waters Corporation, Milford, MA, USA).
equipped with a 2487 UV/visible detector (Waters Corporation, Milford, MA, USA). Samples were held at 5 °C and the column temperature was set to 25 °C. Then, 50 mg/mL mAb formulations were diluted to 10 mg/mL with formulation buffer, 10 mg/mL mAb formulations were analyzed without further preparation. A total of 10 μL of the sample was injected on a TSKG3000SWxl, 7.8 × 300 mm column (Tosoh Bioscience, Stuttgart, Germany) and eluted over 30 min with a 0.2 M K2HPO4/KH2PO4 and 0.25 M KCl of pH 6.2. Signal was detected as UV absorbance at 280 nm. Data processing was done using the Empower 3 Chromatography Data System software v. 4 (Waters Corporation, Milford, MA, USA) and monomer content was reported as percentage of total peak area.

3. Results

3.1. Thermal Properties of the Liquid Formulations

To assess the critical \( T_p, T_g', \) and \( T_c \) were determined for 10 and 50 mg/mL mAb formulations (Table 2). The low concentration reference formulation (\( F_S \)) had a much lower \( T_g' \) of \(-29.5 \) °C compared to the other formulations. Similar \( T_g' \) values were obtained for \( F_{CDPS} \) (\(-19.2 \) °C) and \( F_{CDS} \) (\(-18.5 \) °C), which were both more than 10 °C above the \( T_g' \) of \( F_S \). The highest \( T_g' \) values among all formulations were obtained for pure HPBCD formulations with \(-10.9 \) and \(-8.3 \) °C for 10 and 50 mg/mL mAb, respectively. In contrast to the other formulations where \( T_c \) was slightly above \( T_g' \), \( F_S \) had a \( T_c \) of \(-31.0 \) °C, which was slightly lower than its \( T_g' \). Generally, formulations with 50 mg/mL mAb showed markedly higher \( T_g' \) and \( T_c \) values compared to 10 mg/mL mAb formulations. The impact of protein concentration on \( T_g' \) was more pronounced the lower the \( T_g' \) of the formulations compared to each other. This means that the \( T_g' \) of \( F_S \) increased by \( 4.1 \) °C from \(-29.5 \) to \(-25.4 \) °C when increasing the mAb concentration from 10 to 50 mg/mL compared to an increase of only \( 2.6 \) °C for \( F_{CD} \) for example. In addition, for \( F_{CD}, F_{CDPS}, \) and \( F_{CDS} \) with 50 mg/mL mAb, the difference between \( T_g' \) and \( T_c \) was at least twice that of 10 mg/mL mAb formulations. For instance, at low mAb concentration, \( T_c \) of \( F_{CDPS} \) was \( 0.7 \) °C and for high mAb concentrations \( 1.9 \) °C above the \( T_g' \).

| Table 2. Critical formulation temperatures. Glass transition temperature (\( T_g' \)) and onset of collapse temperature (\( T_c \)) of the liquid formulations. |
|-----------------|-------|-------|
| Formulation     | mAb Concentration (mg/mL) | \( T_g' \) (°C) | \( T_c \) (°C) |
| \( F_S \)       | 10    | -29.5 | -31.0 |
|                 | 50    | -25.4 | -20.4 |
| \( F_{CD} \)    | 10    | -10.9 | -10.1 |
|                 | 50    | -8.3  | -6.3  |
| \( F_{CDPS} \)  | 10    | -19.2 | -18.5 |
|                 | 50    | -16.5 | -14.6 |
| \( F_{CDS} \)   | 10    | -18.6 | -16.0 |
|                 | 50    | -15.2 | -10.9 |

3.2. Impact of Freeze-Drying Parameters on \( T_p \) and Primary Drying Time

As reference, a conservative freeze-drying cycle (C0), typically used for sucrose-based formulations, with \( p_r = 100 \) mTorr and \( T_S = -10 \) °C during primary drying was performed for \( F_S \). Freeze-drying with C0 resulted in a \( T_p \) in the steady state of primary drying of \(-32 \) °C and a primary drying time of \(~35 h \) with 50.5 h total cycle time (Figure 1a). As a start, \( T_S \) during primary drying was increased to \(+10 \) °C (C1), showing a much shorter steady state during primary drying with a mean \( T_p \) of \(-21 \) °C, as shown in Figure 1b. Primary drying time, i.e., total cycle time, was shortened by 6 h, corresponding to a reduction of 12%. To further shorten the freeze-drying cycle time, \( F_{CD}, F_{CDPS}, \) and \( F_{CDS} \) were lyophilized with a \( T_S \) during primary drying of \(+30 \) °C and a \( T_S \) of \(+40 \) °C during secondary drying (C2). Figure 1c shows that increasing \( T_S \) from \(+10 \) to \(+30 \) °C resulted in a freeze-drying cycle with a very short steady state phase at a \( T_p \) of \(-20 \) °C. Ultimately, primary drying time was reduced to \(~19 h \). Using a low ramp rate (0.2 °C/min) from freezing to primary drying and the high temperature
difference of 65 °C to overcome, the ramping step contributed with ~17% markedly to the total cycle time of 32.2 h. Hence, C3 was performed using a ramp rate from freezing to primary drying of 1 °C/min. Consequently, primary drying time was reduced by another 2 h and showed a slightly more pronounced steady sublimation phase compared to C2 (Figure 1d). Another approach to accelerate primary drying is to increase the pressure. To this end, primary and secondary drying in C4 were performed at a p_c of 155 mTorr (Figure 1e). Increasing p_c only slightly increased T_p to ~−19.0 °C, but did not result in a shorter primary drying phase compared to C2. The aggressive lyophilization cycles C2–C4 showed no increase in the Pirani signal (Figure 1c–e) during secondary drying, indicating that the desorption phase was already finished at the end of primary drying. Hence, a final single-step freeze-drying cycle was used without a secondary drying step, leading to a total cycle time of ~25 h (Figure 1f). This resulted in a 50% reduction of total cycle time compared to the conservative cycle. Highly concentrated mAb formulations were freeze-dried using only two cycles, the initial C1 and the final C5. In general, T_p in the steady state during primary drying was slightly higher compared to 10 mg/mL mAb formulations, with a T_p of ~−20 °C compared to ~−21 °C during C1 and a T_p of ~−17 °C compared to ~−20 °C during C5. Consequently, primary drying was completed earlier, i.e., after 24 h for C1 and after 17 h for C5 (Figure 1g–h).

3.3. Cake Appearance and Structure

Cake appearance was investigated visually as well as by µ-CT to obtain a comprehensive evaluation of the structure. In preliminary experiments, better, less brittle or cracked cake appearance was obtained for all formulations when freeze-dried in TopLyo® vials compared to Fiolax® vials. Therefore, only TopLyo® vials were used for all experiments. F_S with 10 mg/mL mAb showed major dents when freeze-dried with the reference cycle (C0) (Figures 2a and 3). The increase of T_s during primary drying to +10 °C resulted in collapse (Figure 2a). µ-CT images of the internal structure revealed a total loss of cake structure in the upper half of the lyophilisate (Figure 3). F_CD, F_CD/PS, and F_CD/S at 10 mg/mL mAb resulted in elegant lyophilisates throughout all lyophilization cycles C1–C5, with only minor dents at the bottom of the vial for F_CD/PS as shown in Figure 2b. These dents were slightly more pronounced in C4. Differences in the internal cake structure between the formulations were detected by µ-CT. Pure HPBCD formulations rendered homogenous cakes. Some cracks formed in F_CD/S and F_CD/PS formulations (Figure 4).

At 50 mg/mL mAb F_S resulted in pharmaceutically elegant lyophilisates (Figure 2) with internal cracks, as shown in Figure 3, when freeze-dried with C1. At more aggressive conditions with a T_s of +30 °C (C5), major dents were observed by visual inspection (Figure 2) and µ-CT revealed a collapsed internal cake structure in the upper half of the lyophilisate. F_CD, F_CD/PS, and F_CD/S at high mAb concentration resulted in visually elegant lyophilisates for both cycles C1 and C5. Interestingly, the internal cake structure of F_CD at 50 mg/mL mAb was different compared to the 10 mg/mL mAb formulation. At higher mAb concentration, the cake showed a large lamellar like structure in the middle-bottom region and a smaller spherical structure, similar to 10 mg/mL formulations, in the upper half of the lyophilisate (Figure 4). To elucidate on the root cause of this observation was beyond the scope of this study.
Figure 1. Freeze-drying cycles. Process monitoring data of (a–f) 10 mg/mL mAb formulations and of (g–h) 50 mg/mL monoclonal antibody (mAb) formulations. (a) The conservative cycle (C0), (b,g) C1, (c) C2, (d) C3, (e) C4, and (f,h) C5. The arrow indicates end of primary drying. $T_s =$ shelf temperature; $T_p =$ product temperature determined by thermocouples.
FCD/S at 10 mg/mL mAb resulted in elegant lyophilisates throughout all lyophilization cycles C1–C5, with only minor dents at the bottom of the vial for FCD/P/S as shown in Figure 2b. These dents were slightly more pronounced in C4. Differences in the internal cake structure between the formulations were detected by µ-CT. Pure HPBCD formulations rendered homogenous cakes. Some cracks formed in FCD/S and FCD/P/S formulations (Figure 4).

At 50 mg/mL mAb FS resulted in pharmaceutically elegant lyophilisates (Figure 2) with internal cracks, as shown in Figure 3, when freeze-dried with C1. At more aggressive conditions with a $T_s$ of $+30 \, ^\circ\text{C}$ (C5), major dents were observed by visual inspection (Figure 2) and µ-CT revealed a collapsed internal cake structure in the upper half of the lyophilisate. FCD, FCD/P/S, and FCD/S at high mAb concentration resulted in visually elegant lyophilisates for both cycles C1 and C5. Interestingly, the internal cake structure of FCD at 50 mg/mL mAb was different compared to the 10 mg/mL mAb formulation. At higher mAb concentration, the cake showed a large lamellar like structure in the middle-bottom region and a smaller spherical structure, similar to 10 mg/mL formulations, in the upper half of the lyophilisate (Figure 4). To elucidate on the root cause of this observation was beyond the scope of this study.

**Figure 2.** Cake appearance. Representative lyophilisates for 10 mg/mL formulations (upper row) and 50 mg/mL formulations (lower row). (a) Different cake appearances for reference formulation (FS) depending on freeze-drying cycle and (b) cake appearance obtained for FCD, FCD/S, and FCD/P/S exemplarily shown for C5.

**Figure 3.** Internal cake structure of FS. Representative micro-computed tomography (µ-CT) images for 10 and 50 mg/mL formulations freeze-dried with different lyophilization cycles.

3.4. Other Product Quality Attributes

Other important product quality attributes to study when optimizing lyophilization processes are reconstitution time, specific surface area, residual moisture, and $T_g$. All 10 mg/mL mAb formulations reconstituted fast (<60 s) without an effect of the lyophilization cycle employed (Figure S1a). For 50 mg/mL mAb formulations, reconstitution was generally slower and took at least twice as long. Reconstitution of FS at 50 mg/mL mAb took the longest with ~80 s compared to FCD, FCD/P/S, and FCD/S, independent of the cycle (Figure S1b). In general, no major differences were found in specific surface area for the different excipient combinations and freeze-drying cycles employed for both 10 and 50 mg/mL formulations (Figure S2). Only FS with 10 mg/mL mAb showed a markedly lower specific surface area when freeze-dried with C1 compared to C0, indicating collapse. More pronounced differences were observed for the residual moisture as shown in Figure 5. For 10 mg/mL mAb formulations (Figure 5a), residual moisture of FCD, FCD/P/S, FCD/S was less than 0.5%, with marginal differences amongst the different formulations. In contrast, FS had a residual moisture of 1.2% when freeze-dried with C5.
Figure 3. Internal cake structure of FS. Representative micro-computed tomography (µ-CT) images for 10 and 50 mg/mL formulations freeze-dried with C5 showing (a) internal cake structure through a vertical cross section of FCD, FCD/S, and FCD/P/S, and (b) cake structure at the bottom of the vial through a horizontal cross section of FCD/P/S.

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Residual moisture of FS with 50 mg/mL mAb was generally higher compared to FCD, FCD/P/S, and FCD/S (Figure 5b). C1 resulted in a residual moisture for FS of 0.64%, while freeze-drying with C5 led to a residual moisture of 1.1%. FCD, FCD/P/S, and FCD/S with 50 mg/mL mAb showed low residual moisture levels of ~0.2%, identical for C1 and C5 and similar to the values for the 10 mg/mL mAb formulations.

The varying residual moisture levels of 10 mg/mL FS when freeze-dried with different cycles were reflected in different $T_g$ values. While C0 resulted in lyophilisates with a $T_g$ of 65 °C, the $T_g$ of the collapsed FS was considerably lower with 43.2 °C. $T_g$ values of FCD, FCD/P/S, and FCD/S were much higher than compared to FS and were comparable for all cycles employed. $T_g$ of FCD/P/S and FCD/S showed a similar $T_g$ at ~150 °C, while FCD had a $T_g$ of ~200 °C. Corresponding to the $T_g$ of the liquid formulations, $T_g$ was generally higher for 50 mg/mL mAb formulations with differences in $T_g$ for FS when freeze-dried with C1 or C5.
3.5. Protein Stability

Protein stability was investigated as remaining monomer content by SE-HPLC directly after freeze-drying as well as after storage at 40 °C for 3 months (Figure 6). All formulations demonstrated sufficient cryo- and lyoprotection for both mAb concentrations, independent of the freeze-drying cycle employed. Good storage stability was obtained for the 10 mg/mL mAb FS reference formulation after storage at 40 °C for 3 months when freeze-dried with the conservative as well as the more aggressive cycle C1. Stability of FCD, FCD/P/S, and FCD/S at 10 mg/mL mAb was comparable to FS. FS, FCD/S, and FCD/P/S showed a marginally but consistent decrease in monomer content of 0.1%-0.2% after storage (Figure 4a), which went along with an increase in high molecular weight species (data not shown). FCD lyophilisates were less stable during storage (~1.5% loss of monomer) throughout all freeze-drying cycles. While 50 mg/mL mAb formulations also demonstrated good protein stability during freeze-drying, they generally showed a higher loss of monomer during storage. A 1% decrease in monomer content was observed for FS after storage for both freeze-drying cycles. The remaining monomer content after storage was much lower for FCD with levels of 80.7% and 84.3% for C1 and C5, respectively. Better and similar mAb stability was obtained for FCD/S and FCD/P/S with a loss of monomer content of 4.1% for C1 and 2.7% for C5. Interestingly, for FCD, FCD/S, and FCD/P/S with 50 mg/mL mAb stability was generally slightly improved when lyophilized with C5 compared to C1.

Figure 5. Residual moisture and $T_g$. Data is given for (a) 10 mg/mL mAb formulations and (b) 50 mg/mL mAb formulations for different formulations and freeze-drying cycle conditions. Residual moisture levels are shown as bars and lines. Lines show residual moisture level of vials stoppered after primary drying. Squares show $T_g$ values of the lyophilisates. Values are means ($n = 3$) ± standard deviation.

Figure 6. Protein stability by size-exclusion high-performance liquid chromatography (SE-HPLC). Percentage of remaining monomer for (a) 10 mg/mL mAb formulations and (b) 50 mg/mL mAb for different formulations and freeze-drying cycle conditions. Bars without pattern show monomer content directly after freeze-drying and bars with pattern show monomer content after storage at 40 °C for 3 months. Lines show the monomer content of the liquid formulation prior to freeze-drying.
In an additional experiment, we investigated whether the aggressive freeze-drying cycle that we used might result in so-called overdrying of the mAb (i.e., ~0.2% residual moisture). Therefore, defined residual moisture levels were prepared for 10 mg/mL formulations by spiking of the lyophilisates with water droplets. Residual moisture dependent protein stability was subsequently investigated. Our data in Figure S3 shows similar protein stability for lyophilisates with a residual moisture of 0.2%, 0.5%, 1%, or 2% after 3 months at 40 °C.

4. Discussion

The aim of this study was to develop an aggressive, thus short freeze-drying cycle for amorphous formulations with a higher $T_g'$ and $T_c$ compared to pure sucrose that results in elegant cakes, low residual moisture levels, fast reconstitution time, and has no negative impact on protein stability. A sucrose-based formulation was included for reference purpose to (i) compare cycle time and product quality attributes with traditional sucrose-based formulations and (ii) demonstrate the limits of pure sucrose with regards to shortening freeze-drying.

4.1. Correlation Between $T_g'$, $T_c$, $T_p$, and Cake Appearance

In a first step during formulation and freeze-drying cycle development, it is essential to determine $T_g'$ and $T_c$ of the liquid formulation, as they are indicative for the $T_p$ which should not be exceeded during primary drying to avoid collapse. $T_c$ is commonly considered to be the more accurate predictor and is typically few degrees above $T_g'$ [32,33]. For formulations with low protein concentrations, $T_g'$ and $T_p$ may be used interchangeably. In fact, in our study $T_c$ of F$_S$ was even slightly lower than the $T_g'$. This is consistent with data previously reported by Colandene et al. for sucrose-based 10 mg/mL protein formulations [11]. At higher protein concentrations, $T_c$ is markedly higher than $T_g'$ and the difference ($\Delta T$) increases with higher protein concentration. For a 50 mg/mL mAb formulation with 7–8% disaccharide, a $\Delta T$ of 5 °C was reported and Depaz et al. found a $T_c$ 14 °C above the $T_g'$ for a 100 mg/mL mAb formulation [13,15]. This is in line with our results of a $\Delta T$ of 5°C for the 50 mg/mL pure sucrose formulation. For formulations containing excipients which have a high $T_g'$ themselves, the effect of protein concentration on $\Delta T$ is less pronounced. For F$_{CDSS}$ and F$_{CDPSS}$ both 10 mg/mL formulations showed very similar $T_g'$ values, but their $T_c$ differed by 2.6 °C. This directly translated into different cake appearance when freeze-dried with the same cycles with $T_p$ close to $T_g'$ (C1–C5). $T_c$ depends on several factors such as the solid concentration as well as sublimation rate. Thus, collapse in the vial might occur at slightly higher temperatures during freeze-drying than the $T_c$ determined by FDM [33]. Greco et al. used optical coherence tomography to determine $T_c$ of a 5% sucrose solution in the vial during freeze-drying and found it to be 3 °C above that temperature determined by FDM [34]. This is in line with the cake appearance observed in our study. F$_S$ at low protein concentration showed major dents when freeze-dried with the conservative lyophilization cycle, where $T_p$ was close to $T_c$. Collapse occurred only when $T_p$ was much higher than $T_c$, as it was the case for C1. In terms of internal cake structure, $\mu$-CT analysis showed minor cracks in most lyophilisates independent of the cycle employed. Patel et al. suggested that cracks should not be considered cake defects as they are only process artefacts which are not detrimental to product quality [8]. In fact, internal cracks have been found to be a result of relieved stress during secondary drying, when unfrozen water is removed [35,36]. Lam et al. suggested that the formation of splitted cakes might be linked to a complex interplay of events occurring during the freezing step. They also reported that the occurrence of cracks is highly variable, and observed that cracks can be present in lyophilisates that look pharmaceutically elegant from the outside [37], in line with our observations. More important are internal defects such as partial collapse, which was revealed by $\mu$-CT for e.g., 50 mg/mL F$_S$ when freeze-dried with C5. This highlighted in addition that the dents observed by visual inspection were truly correlated to the onset of collapse.
4.2. Impact of Process Parameters

It is well established that an increase of \( T_s \) during primary drying reduces cycle time [3]. Correspondingly, as we increased \( T_s \) during primary drying from \(-10 \) °C to ultimately \(+30 \) °C, we substantially shortened primary drying by 48%. Additional elimination of secondary drying shortened the overall cycle time by in total 50%. Although both increases in \( T_s \) from \(-10 \) to \(+10 \) °C (C1) and from \(+10 \) to \(+30 \) °C (C2), strongly impacted process time, the latter only marginally increased \( T_p \). Similar to our results, Depaz et al. reported that increasing \( T_s \) from \(-30 \) to \( 0 \) °C resulted in a marked increase of \( T_p \) for a 25 mg/mL mAb formulation, and led to a brief steady sublimation phase, whereas a further increase of \( T_s \) to \(+15 \) °C further shortened the steady sublimation phase without impacting \( T_p \) [13]. In addition, aggressive primary drying temperatures resulted in a steeper drop of the Pirani signal. A fast drop of the Pirani signal implies a homogenously dried batch [38], which is desirable, in particular if aiming to omit secondary drying. Greater batch homogeneity was additionally demonstrated by smaller variations within the temperature probes for aggressive lyophilization conditions. When aiming for aggressive freeze-drying cycles at high \( T_s \), the ramping step contributes markedly to the total cycle time. Typically, ramp rates of less than \( 1.0 \) °C/min are applied, but faster ramp rates of e.g., \( 1 \) °C/min are also suitable. Horn et al. found no negative impact when increasing the ramp rate into primary drying from \( 0.5 \) °C to \( 1 \) °C/min [14]. Ohio et al. demonstrated that faster ramp rates might result in even better cake appearance for high \( T_s \) conditions during primary drying compared to very slow ramp rates [39,40]. In contrast, Pansare et al. reported more pronounced shrinkage for lyophilisates that were freeze-dried using a ramp rate of \( 0.5 \) °C/min compared to \( 0.1 \) °C/min [15]. In the present study, although product quality attributes like residual moisture, specific surface area, reconstitution time, and qualitative internal cake structure did not show any differences for C3 compared to C2, we visually observed some lifted cakes after freeze-drying. However, the time point when lifting occurred remained unknown. In the light of these observations and a potential gain of only 2 h in overall cycle time, we decided to stick with the low ramp rates for the following cycles C4 and C5. In general, selection of the optimal \( p_r \) is a balance between batch homogeneity and prevention of collapse or meltback [3,41]. Previous studies demonstrated an increase of \( p_r \) to be advantageous for shorter freeze-drying cycles, leading to higher \( T_p \) and thus shorter cycle times [14]. However, in the present study, although \( T_p \) was slightly higher in C4 compared to C2, no impact on primary drying was observed when using a pressure of 155 mTorr compared to 100 mTorr. At 155 mTorr, \( T_p \) showed a slight drop after a short steady state (C4) prior to its increase. It has been speculated that this might be indicative for micro-collapse [13,15]. For all aggressive cycles (C2–C4), the Pirani signal indicated end of overall drying, i.e., sublimation and desorption, already at the end of primary drying. Finally, \( F_s \), \( F_{CDS} \), and \( F_{CDDS} \) allowed for a single-step freeze-drying, resulting in comparable product quality attributes to a conservative cycle. Pansare et al. also applied a single step freeze-drying for amorphous formulations, but their disaccharide-based formulations resulted in product shrinkage and partial collapse for formulations with 25 mg/mL or less protein and minor shrinkage at 50 mg/mL. Addition of a crystalline excipient was necessary to obtain elegant lyophilisates, which however led to slightly higher aggregation rates compared to purely amorphous formulations during storage [15].

4.3. Protein Stability

When optimizing freeze-drying processes, it is of utmost importance to ensure protein stability. In our study, no negative impact of the process parameters on protein stability, including \( T_s \) of \(+30 \) °C during primary drying and \(+40 \) °C during secondary drying was observed for any formulation, which is in line with previous studies [13–15]. Tang and Pikal reported that dried protein formulations will not undergo denaturation at temperatures up to \( 100 \) °C for short periods [3]. In terms of storage stability, collapse is often of concern as it may result in higher residual moisture and lower \( T_g \). Various studies report that collapse itself did not reduce protein stability during storage [6,7]. On the contrary, Lueckel et al. demonstrated that collapse resulted in increased aggregation of an IL-6 lyophilisate in a sucrose/glycine formulation after storage [5]. Correspondingly, Passot et al. reported 25% loss
of activity for lyophilized toxins in a PVP/sucrose or PVP/mannitol matrix after 6 months of storage, when freeze-dried with a $T_g$ above $T_g$ during primary drying [4]. The results of the present study showed good and comparable storage stability for $F_{\text{CDP}}$ and $F_{\text{CD}}$. Collapse did not impact protein stability of $F_s$ when stored at 40 °C for 3 months. However, we previously found substantial protein degradation for a collapsed 10 mg/mL sucrose-based formulation when stored at 40 °C for 6 months or longer [26].

At 50 mg/mL mAb, all formulations showed a higher loss of monomer compared to the 10 mg/mL formulation after 3 months at 40 °C. The increase in aggregates was more pronounced for $F_{\text{CD}}$, $F_{\text{CDP}}$, and $F_{\text{CD}}$ compared to $F_s$. Within this study the excipient solid content was kept constant at 80 mg/mL for both low and high mAb concentrations. Thus, for the 50 mg/mL mAb formulations the excipient to protein ratio was too low to adequately protect the protein. Similarly, Lewis et al. reported good mAb stability when formulated at 5 mg/mL but observed protein aggregation at 20 mg/mL in 25 mg/mL sucrose lyophilisates [42]. Their excipient to protein ratios of 4:1 and 1.2:1 (w/v) are close to our 7:1 and 1.6:1 ratio for 10 and 50 mg/mL mAb, respectively. Cleland et al. reported that a molar excipient to protein ratio of at least 360:1 is necessary in order to ensure good protein stability at 40 °C for 3 months [43]. This ratio was easily exceeded for the 10 mg/mL mAb formulations in our study. For the 50 mg/mL mAb formulations, only $F_s$ (695:1) was at this level, whereas $F_{\text{CD}}$, $F_{\text{CDP}}$, and $F_{\text{CD}}$ reflected ratios of only 290:1, 321:1, and 163:1, respectively. Interestingly, we achieved a better storage stability for 50 mg/mL mAb formulations freeze-dried with C5 compared to C1, although no differences were observed in the physico-chemical product quality attributes. To elucidate the influence of excipient to protein ratio and process parameters on formulations with a mAb concentration above 50 mg/mL was beyond the scope of the present study.

In summary, a binary combination of HPBCD and sucrose at a 7:3 ratio (w/v) provides a highly attractive amorphous formulation. This formulation allows for a 50% cycle time reduction compared to the conventional reference cycle through single-step freeze-drying of low concentration biopharmaceuticals, resulting in elegant lyophilisates with short reconstitution times, low residual moisture, high $T_g$, and good protein stability during storage. Moreover, the actual effect on cycle time might be even more pronounced as the reference cycle with sucrose formulations at a mAb concentration of 10 mg/mL would require even lower primary drying in order to eliminate dents.

5. Conclusions

Within the present study we demonstrated that scientists can be very aggressive during freeze-drying, using HPBCD-based formulations in combination with sucrose or PVP/sucrose. We were able to reduce cycle time by 50%, obtaining pharmaceutical elegant lyophilisates for pure HPBCD and HPBCD/sucrose, while HPBCD/PVP/sucrose showed minor dents. All other product quality attributes were similar, acceptable, and comparable to the conservatively freeze-dried sucrose formulation. Protein stability was ensured for all formulations upon freeze-drying and combinations of HPBCD/sucrose and HPBCD/PVP/sucrose at 10 mg/mL mAb provided good stability during storage at 40 °C for 3 months. We believe that the proposed excipient combinations can be applied for higher concentrated protein formulations as well by adjustment of excipient to protein ratio. We conclude that the proposed single-step freeze-drying cycle using a binary mixture of HPBCD/sucrose has the potential to significantly reduce costs of goods due to more efficient freeze-drying, while maintaining elegant lyophilisates and ensuring protein stability.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4923/11/11/616/s1, Table S1: Formulation composition. Figure S1: Reconstitution time of lyophilisates. Figure S2: Specific surface area of lyophilisates.

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