Investigation of lyophilized antibody formulations to enable short freeze-drying cycles and storage at room temperature

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FÜR MEINE ELTERN

The important thing is not to stop questioning.
~Albert Einstein~

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SUMMARY

Antibodies are dominating the biopharmaceutical market and are expected to grow further. Aiming to improve existing treatments, new antibody derivatives of improved efficacy and safety are being developed in a competitive market. Antibody derivatives include but are not limited to antibody-drug conjugates and Fc-fusion proteins. Due to their higher complexity, they are often less stable as liquids, increasing the demand for lyophilized formulations to ensure protein storage stability over the desired shelf life. In many cases, these antibody formats require lower doses, posing challenges to formulation and freeze-drying process development. Commercialized lyophilized antibodies typically contain disaccharides, most frequently sucrose, as a stabilizer and bulking agent. The low glass transition temperature of sucrose requires time- and energy-intensive, thus expensive freeze-drying cycles. At lower protein concentrations, this becomes even more relevant, raising the risk of product collapse during freeze-drying. Collapse occurs when primary drying is performed above the glass transition temperature or collapse temperature of the formulation. It is current dogma to design freeze-drying cycles that provide pharmaceutically elegant lyophilisates as collapse leads to batch inhomogeneity causing rejects, higher complaint rates, and most importantly may potentially be detrimental to protein storage stability. Thus, there is a need to look into alternative excipients for future freeze-dried antibody formulations.

The presented work investigated amorphous excipients to be used as alternative excipients to sucrose for freeze-dried antibody formulations, increasing the formulation's glass transition temperature. The main objectives were to investigate their ability to render pharmaceutically elegant lyophilisates upon short freeze-drying cycles, and to stabilize antibodies during freeze-drying and subsequent storage. Special focus was given to storage stability at elevated temperatures with the aim to study the potential for room temperature stable formulations.

At first, an imaging technique was established to evaluate the impact of excipients and freeze-drying cycles on cake appearance and structure. Different imaging techniques were compared regarding qualitative and quantitative characterization of the entire lyophilisate, and their potential for non-invasive evaluation of structure and morphology in the glass vial (Chapter 1). The comparative analysis revealed limitations of scanning electron microscopy, the current state of the art technique to characterize cake morphology. Micro-computed tomography was introduced as a technique allowing for comprehensive and reproducible imaging of cake structure and morphology.

Having established a method for evaluation of cake appearance, the next step of this work was focused on formulation development. Dextrans of different molecular weight from 1 to 500 kDa (Chapter 2) followed by HPBCD-based formulations in combination with other amorphous compounds (Chapter 3) were investigated. Their impact on thermal properties, cake appearance, other physico-chemical product quality attributes, and protein stability of two model antibodies was characterized. In particular, HPBCD was found to be a promising excipient, while dextran showed several limitations. Large dextrans of 40 kDa or higher were shown to increase the viscosity of the formulations leading to long reconstitution times, and did not sufficiently stabilize the antibodies during freeze-drying compared to smaller dextrans and HPBCD. The work highlighted limitations of dextrans with regards to protein stability, due to antibody glycation during storage at elevated temperatures. HPBCD

rendered lyophilisates with good product quality attributes and ensured antibody stability during freeze-drying and even at elevated storage temperatures. Best antibody stability was obtained in combination with sucrose, highlighting the fact that disaccharides will remain a mandatory part of freeze-dried antibody formulations. To further maximize protein stability, a thorough characterization of the optimal ratio of HPBCD and sucrose will be essential.

These formulations which provided good stability and product quality attributes were subsequently used for freeze-drying process optimization (Chapter 4). Primary drying parameters were optimized for a short freeze-drying cycle that renders pharmaceutically elegant lyophilisates. The presented work demonstrated that amorphous excipients with higher glass transition temperatures allow for shorter freeze-drying cycles while providing lyophilisates with improved cake appearance. Ultimately, the HPBCD-based formulation with addition of sucrose enabled the development of a short, single-step freeze-drying cycle while maintaining pharmaceutically elegant lyophilisates eventually reducing cycle time by 50%.

Overall, the current work demonstrated the potential of alternative amorphous excipients, which in contrast to crystalline bulking agents contribute to protein stability while avoiding additional complexity in the freeze-drying cycle. The combined use of HPBCD with sucrose may provide a formulation for low concentrated protein formulations that enables the development of short freeze-drying cycles while maintaining pharmaceutically elegant lyophilisates. The presented work may encourage considerations to store freeze-dried formulations at (controlled) room temperature rather than refrigerated conditions in the future.

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LIST OF ABBREVIATONS

ADC	Antibody-drug conjugate
API	Active pharmaceutical ingredient
BET	Brunauer-Emmett-Teller
BSA	Bovine serum albumin
cIEF	Capillary isoelectric focusing
Dex	Dextran
DSC	Differential scanning calorimetry
Fab	Antigen-binding fragment
FDA	Food and Drug Administration
FDM	Freeze-drying microscopy
FTIR	Fourier-transform infrared spectroscopy
His/His-HCl	Histidine/histidine-hydrochloride
HMWs	High molecular weight species
HPBCD / CD	Hydroxypropyl-betacyclodextrin
IgG	Immunoglobulin
LC-ESI-MS	Liquid chromatography electrospray ionization mass spectrometry
mAb	Monoclonal antibody
M_W	Molecular weight
µ-CT	Micro-computed tomography
NMR	Nuclear magnetic resonance
p_c	Chamber pressure
PDMS	Polydimethylsiloxane
Ph. Eur.	European Pharmacopeia
pI	Isoelectric point
PVDF	Polyvinylidene difluoride
PVP	Polyvinylpyrrolidone
rHA	Recombinant human albumin
SD	Standard deviation
SEC-MS	Size exclusion chromatography - mass spectrometry
SE-HPLC	Size exclusion high-performance liquid chromatography
SEM	Scanning electron microscopy
SSA	Specific surface area
Suc	Sucrose
T_c	Collapse temperature
T_{eu}	Eutectic temperature
T_g' / T_g	Glass transition temperature
T_p	Product temperature
T_s	Shelf temperature
$t_{1/2}$	Half-life
US	United States
USP	United States Pharmacopeia
w/v	Weight per volume
w/w	Weight per weight
XRPD	X-ray powder diffraction

INTRODUCTION

Biopharmaceuticals and the prevalence of antibodies

The era of biopharmaceuticals started in 1982 with the approval of Humulin®, a recombinant human insulin, which was created using recombinant DNA technology. The growing acceptance of biopharmaceuticals due to their potential to cure diseases while having less side effects at the same time, led to their fast dispersion. By 2018, 374 biopharmaceuticals had been approved in the United States or the European Union, containing 285 distinct active pharmaceutical ingredients (API) [1]. The global biopharmaceutical market has reached $237.3 billion in 2018 and is expected to grow to $389 billion by 2024, witnessing an annual growth of 8.6% [2]. Biopharmaceuticals comprise different product types such as monoclonal antibodies (mAb), hormones, vaccines, and coagulation factors. Recently, new modalities such as nucleic acids (genes) and cell therapies entered the biopharmaceutical market. mAbs are the largest class of biopharmaceuticals, accounting for more than 36% of all biopharmaceuticals approved by 2018 [3-5]. Figure 1 shows that the number of mAb approvals increased strongly over the last four years and there is a huge pipeline of mAbs still to be commercialized, thus driving further substantial market growth [6].

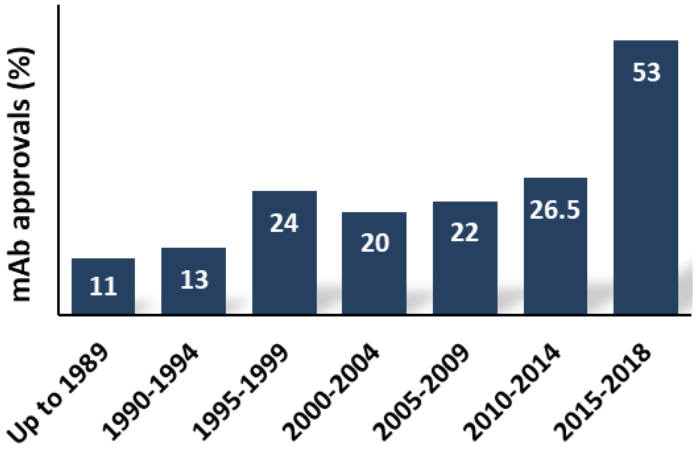

Figure 1. First time mAb approvals as a percentage of total biopharmaceutical approvals in the indicated time periods. Adapted figure from [1].

The origin of mAbs can be either murine, chimeric, humanized or human, with a subsequent decreased immunogenicity. The group of mAbs includes full-size mAbs and derivatives, such as bispecific antibodies, antigen-binding fragments (Fab), antibody-drug conjugates (ADC), and Fc-fusion proteins. Therapeutic mAbs have various indications but the vast majority is used for cancer treatment, followed by anti-inflammatory applications [5].

Biopharmaceuticals need to be administered parenterally, as they have very limited bioavailability when administered orally. Typical routes of administrations are either intravenous by infusion or injection, which shows the highest bioavailability, or subcutaneous injection, which is more convenient

compared to intravenous administration. Other less common routes of administration are intramuscular (e.g. Synagis) and intravitreal (Lucentis®). Parenterally administered biopharmaceuticals are formulated as liquid solutions, filled into vials, syringes, or cartridges, or as lyophilized (freeze-dried) powders, typically packaged in vials, that need to be reconstituted prior to injection. In general, liquid formulations are preferred to lyophilisates due to a less complex and more economical manufacturing process as well as higher convenience for administration. However, proteins in liquid formulations are susceptible to chemical and physical degradation during storage and transportation. Therefore, in some cases, it might not be possible to ensure stability over the desired shelf life. In such cases, lyophilized formulations are chosen due to slowed down degradation mechanisms in the dried state. Looking at the entire market, roughly 40% of all biopharmaceuticals are freeze-dried [3, 7]. Among these, mAbs cover the largest group with ~34% of all lyophilisates, followed by ~27% for coagulation factors, which are all manufactured as lyophilized formulations (Figure 2).

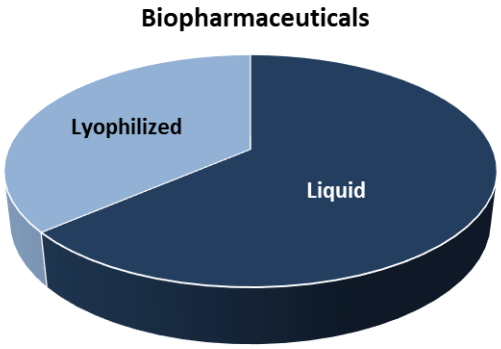

Figure 2. Share of lyophilized and liquid products that have been approved by the EMA from 1995 until 2018 shown for Biopharmaceuticals. [3].

In particular, new mAb derivates such as bispecific mAbs (e.g. Blincyto®), ADCs (e.g. Mylotarg®, Cablivi®, Adcetris®, Bespona®, Kadcyla®), and Fc-fusion proteins (e.g. Elocarte®, Alprolix®, Orencia®) are often marketed as lyophilisates. This suggests that these new, more complex mAbs require lyophilized formulations, potentially leading to a growing demand for lyophilized formulations in the future [8]. To date, liquid as well as lyophilized mAbs (with exception of Nucala® which can be stored at <25°C) require refrigerated storage conditions at 2-8°C. This necessitates complex and expensive supply chains, and raises the risk of recalls in case of temperature excursions. Moreover, supply to developing countries is challenging as there is often limited cold chain capacity.

The principles of freeze-drying

Freeze-drying, or lyophilization, is used during drug product manufacturing of parenterals to remove water from the product. It is a unit operation, which is applied after compounding, sterile filtration, and filling. Vials are stoppered partially, transported, and loaded into the lyophilization unit. After freeze-drying, the vials are stoppered within the lyophilizer, unloaded, and crimp-capped. In general, the lyophilization process itself comprises of three major steps, as depicted in Figure 3.

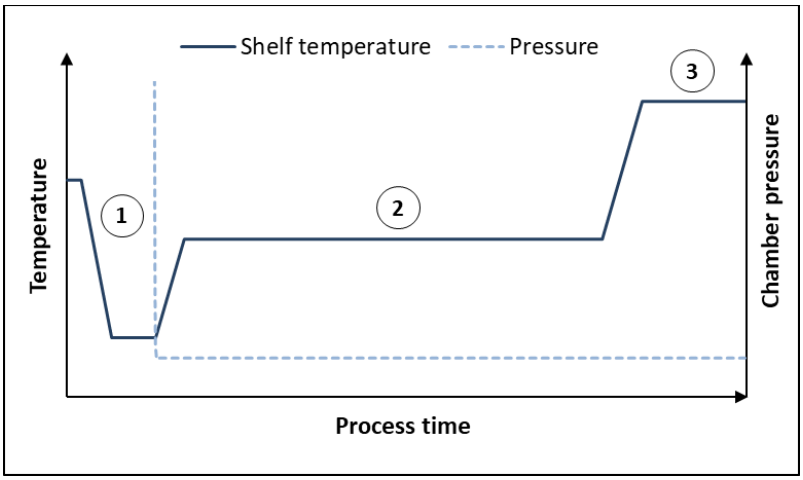

Figure 3. Schematic illustration of a lyophilization cycle. 1) Freezing, 2) primary drying, and 3) secondary drying.

1) Freezing

To achieve more homogenous freezing, a short equilibration phase at 5°C is often applied prior to freezing [9]. During freezing, the shelf temperature is decreased, typically at a linear rate [10], to the desired temperature and is held there long enough to ensure the entire solution has solidified. During cooling, the solution first nucleates, followed by ice crystal growth leading to a mixture of solid ice and solute concentrate [11]. In order to ensure complete solidification of the drug product solution, the final shelf temperature (T_s) needs to be below the eutectic temperature (T_{eu}) of crystalline compounds or the glass transition temperature of the maximally freeze-concentrated solution (T_g') for amorphous formulations [12]. T_{eu} and T_g' are also important formulation characteristics during the subsequent primary drying phase. The freezing step is of importance as the freezing rate and thus the nucleation temperature influences the morphology of formed ice crystals, which in turn directly influences the primary drying rate and potentially other product quality attributes like residual moisture or protein stability [12, 13]. After freezing, an optional annealing step can be applied (not shown in Figure 3) to either allow crystallization for formulations containing crystalline excipients or to increase ice crystal size [14].

2) Primary drying

Primary drying is initiated by reducing the chamber pressure below the water vapor pressure of ice at the given product temperature (T_p). This process step is defined by sublimation of frozen water directly into the gas phase and removes the majority of the water. During primary drying, T_s is increased to supply the energy consumed by ice sublimation. The drying rate *dm/dt* can be described by the following equation [15]:

$$\frac{dm}{dt} = \frac{(P_0 - P_c)}{(R_s + R_p)}$$

where P_0 is the vapor pressure of ice at T_p, P_c is the camber pressure, and R_p and R_s are the product and stopper resistance, respectively. Considering the fact that T_s and chamber pressure are the only controllable process parameters during primary drying, the sublimation rate *dm/dt* is thus maximized

by using a high T_p and a low chamber pressure in order to maximize the term $(P_0 - P_c)$. While T_p is influenced by T_s, the consumption of energy by the endothermic sublimation results in T_p being lower than T_s. Moreover, it is important to ensure that T_p stays below the collapse temperature (T_c) of the formulation during primary drying. If T_p exceeds T_c, which depends on T_g' of the amorphous formulation, the lyophilized product collapses. In brief, collapse during primary drying refers to a viscous flow of the glassy matrix leading to the loss of macroscopic pore structure [16]. It is thus essential to know T_g' and T_c of the drug product solution prior to freeze-drying cycle development. They are typically determined by differential scanning calorimetry [17] and freeze-drying microscopy [18, 19], respectively. Overall, primary drying is ideally performed at a target T_p slightly below the formulation's T_c. Typically, this requires low T_s leading to long primary drying times. This can be achieved by multiple chamber pressure and T_s combinations with typical chamber pressures in the range of 50 to 200 mTorr [9, 14, 20]. Primary drying is finished when all water has sublimated, i.e. additional energy supply results in an increase of T_p, reaching T_s. This can be monitored by using temperature probes, pressure rise test, or other techniques such as comparative pressure measurement using a Pirani gauge versus capacitance manometer, and tunable diode laser absorption spectroscopy [21, 22].

3) Secondary drying

During secondary drying T_s is raised to remove the unfrozen, bound water by desorption. It is well-established that efficiency of secondary drying is independent of chamber pressure, therefore it is typically kept at the level of primary drying [14]. Secondary drying is performed until the residual moisture reaches the desired level, typically ≤ 1% [23, 24].

Overall, freeze-drying is a time-consuming and energy-intensive, thus expensive drug product batch manufacturing process. The time-determining process step is primary drying. While both freezing and secondary drying typically last for a few hours, primary drying often requires several days [9]. Therefore, efforts to achieve shorter and more efficient lyophilization cycles mostly center around a solution that shortens the primary drying phase.

Protein stability in the solid state

Instabilities of proteins

While freeze-drying generally increases the marginal stability of proteins in particular upon transportation and long-term storage, degradation reactions may also occur in the solid state. Protein degradation can be divided into chemical and physical instabilities. Chemical instabilities involve a covalent modification of the protein via bond cleavage or formation, including but not limited to deamidation, oxidation, as well as glycation reactions. Deamidation is the hydrolysis of preferably asparagine residues and has been reported also for freeze-dried products. However, amorphous matrices were shown to stabilize proteins against deamidation in the solid state [25, 26]. Glycation or Mailllard reaction is the non-enzymatic reaction between reducing sugars and primary or secondary amino residuals of the protein occuring preferably at higher temperatures. Physical instabilities include

denaturation, which refers to the unfolding of the globular structure, and aggregation. Aggregation is the most common instability of antibodies and needs to be characterized and quantified for product release [27]. Aggregates form via different mechanisms, which have been discussed extensively in several reviews [28-30]. They can form during the manufacturing processes or upon storage and be either soluble or insoluble with the latter being present in the form of sub-visible or visible particles.

Mechanisms of protein stabilization

During the freeze-drying process, the protein is exposed to freezing stresses such as the ice-liquid interface and cryoconcentration, as well as drying stresses when water is removed, which can be drivers for protein denaturation or aggregation [31-33]. In order to minimize cryoconcentration, and to moreover avoid unnecessarily low T_g' values, the buffer and salt concentration is reduced to the minimum amount needed to control the pH [34]. For freeze-dried formulations amino acid buffers such as histidine buffers are preferred as they show smaller pH shifts during freezing compared to phosphate buffers for example [3]. To protect the protein from interfacial stresses such as the ice-liquid interface, surfactants such as polysorbate 20 or 80 or poloxamer 188 are added at low concentrations. While both buffer and surfactant are also part of liquid formulations, lyophilized formulations require additional excipients, namely cryo- and lyoprotectants. Cryoprotectants stabilize the protein during freezing and in the frozen state, whereas lyoprotectants are responsible for stabilization upon water removal and in the dried state. The mechanism of cryoprotection has in the past often been described by the preferential exclusion/hydration hypothesis [35-37], a thermodynamic mechanism that was initially proposed to account for stabilization by e.g. sugars in aqueous solutions [38, 39]. However, more recent literature suggests that stabilization by cryoprotectants during the freezing step of lyophilization might rather be explained by a kinetic mechanism, which is referred to as vitrification and also relevant for protein stabilization in the dried state [32, 40]. Vitrification (or glass dynamics) and water replacement theory are the two main hypotheses discussed to describe protein stabilization during drying and subsequent storage in the dried state [24, 41]. Water replacement theory describes a thermodynamic stabilization of the protein's native structure. It states that lyoprotectants can form hydrogen bonds with the protein, acting as a substitute for water molecules, which are removed during drying [42]. Vitrification explains the stabilization in the solid state through immobilization of the protein in an amorphous glassy matrix, thereby drastically slowing down degradation mechanisms [43]. It thus describes protein stabilization by a kinetic mechanism that is linked to the T_g of the amorphous matrix. Both theories are generally accepted to be of paramount importance for interpreting protein stability. However, recent literature suggests further refinement of these theories is required in relation to the detailed mechanisms of protein stabilization [44].

The relevance of the glass transition temperature

The glass transition temperature is generally defined as the temperature above which a glassy matrix transitions into a rubbery state, thus lowering viscosity and increasing mobility. A glass transition temperature exists in the frozen state and is referred to as T_g' and is an important characteristic for freeze-drying cycle development. The glass transition temperature of the freeze-dried formulation is referred to as T_g. The glass transition temperature of a formulation is characterized by the components

and their ratio to each other, but not their absolute concentration. T_g is of particular importance for protein stability described by the vitrification theory. It is generally accepted that storage above the T_g of a formulation increases protein degradation. Moreover, studies by Hancock *et al.* and Grasmeijer *et al.* have shown that depending on the molecular size and accordingly the molecular mobility of the sugar, T_g should be up to 50°C above the intended storage temperature to ensure complete vitrification [41, 45]. What is more, T_g is negatively correlated to residual moisture of a lyophilisate [46]. A small increase in residual moisture can strongly depress T_g, which is an important consideration regarding the intended storage temperature. On the other hand, it has been shown that the relationship between protein stability and T_g is not universal. While storage above T_g is a strong indicator for accelerated protein degradation, storage below T_g does not conclusively guarantee good protein stability [40, 41].

Excipients used as cryo-/lyoprotectants or bulking agents

Excipients used as cryo-/lyoprotectants or bulking agents typically belong to the classes of sugars, polyols, amino acids, or polymers. Bulking agents are excipients that are added to the formulation, in particular for low concentrated protein formulations, to render an intact lyophilisate. Some excipients may combine two or more features. Looking at lyophilized mAbs, besides amino acids only a very limited number of excipients is used in marketed drug products (Figure 4).

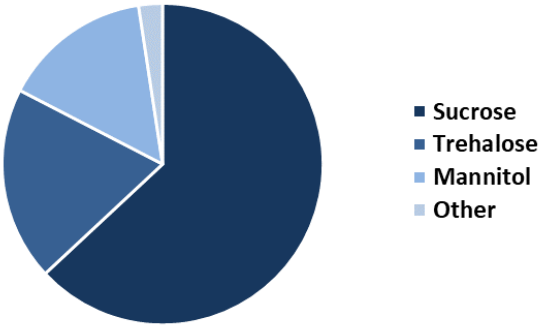

Figure 4. Percentage of non-amino acid stabilizers (cryo-/lyoprotectants) and/or bulking agents used in individual marketed mAb lyophilisates from 1995 to 2018. Other includes maltose and dextran [3].

The non-reducing disaccharides sucrose and trehalose are by far the most commonly used stabilizers, well-known to combine both cryo- and lyoprotective characteristics. As small, amorphous sugars they stabilize the protein via both vitrification and water replacement. With regards to the T_g considerations discussed above, trehalose (T_g of dry trehalose: ~115°C) would be expected to be preferred over sucrose (T_g of dry sucrose: ~70°C) [47]. However, trehalose is known to be more prone to crystallization during freeze-concentration, which could impair its lyoprotective properties [48]. This, in addition to the higher cost of the raw material [49], could be reasons why trehalose is used far less frequently in marketed formulations. In fact, more than 70% of all approved mAb lyophilisates contain sucrose (Figure 4). Mannitol is a polyol, which is added as a bulking agent in order to improve cake appearance. Its use in marketed freeze-dried biopharmaceuticals other than mAbs is relatively frequent, in

particular for low concentrated protein formulations [3]. Mannitol crystallizes during freeze-drying, thus is not able to stabilize the protein and is therefore most commonly used in combination with sucrose. Moreover, it is important that all mannitol crystallizes during freezing as crystallization during storage can be detrimental to protein stability. Although the addition of the polyols glycerol and sorbitol was shown to be beneficial for protein stability in the dried state [50, 51], they are typically not included in freeze-dried formulations. Due to their plasticizing characteristics, thus their very low T_g values, they are not attractive for economic freeze-drying cycles. Addition of amino acids such as arginine hydrochloride to sucrose was shown to improve protein stability, however chloride as counter ion strongly reduced the formulations T_g' [52]. Horn *et al.* recently reported the use of phenylalanine, leucine, and isoleucine as promising alternative bulking agents to mannitol [53]. Polymers, such as dextran and polyvinylpyrrolidone (PVP), for example, have been reported as excipients for freeze-dried protein formulations in the past [54]. To date, Mylotarg® is the only marketed lyophilisate containing a polymer (dextran) as an excipient. Polymers may be used as bulking agents but also stabilizers via the vitrification theory due to their high glass transition temperatures. Literature indicates that the use of polymers necessitates a case by case evaluation. For instance, Allison *et al*. found that the addition of a disaccharide is needed for dextran to ensure stability of freeze-dried actin during storage [55], while Larsen *et al.* reported superior stability of freeze-dried lactate dehydrogenase formulated with dextran compared to sucrose [56]. Both studies reported good cryo- and lyoprotective properties for dextran.

Moreover, the use of other classes of excipients has been described in literature and a non-exhaustive overview is provided here: A review on cyclodextrin describes the use of hydroxypropyl-betacyclodextrin (HPBCD) as a protein stabilizer in the dried state [57]. Human albumin can frequently be found in marketed freeze-dried cytokines [3].

The classes of excipients with high glass transition temperatures have the potential to serve as promising excipients for future mAb formulations with regards to designing short lyophilization cycles and storage at ambient temperatures.

Quality aspects of freeze-dried products

For release testing, important quality attributes specific to freeze-dried products include but are not limited to protein stability and potency, residual moisture, reconstitution time, and cake appearance [58, 59]. Freeze-drying aims to remove water in order to increase protein stability, consequently residual moisture levels should be low and specified. Although not necessarily assessed during commercial production, residual moisture levels need to be determined during development and protein stability has to be investigated for the worst case residual moisture level of the designed freeze-drying process. Higher residual moisture levels than specified can evoke or accelerate protein instabilities. For lyophilisates, stability of the API is assessed after reconstitution. It is generally accepted that reconstitution of a lyophilisate should be fast, although some marketed highly concentrated products have reconstitution times of more than 30 min [60]. The endpoint of reconstitution of a product is described by the US Pharmacopeia as the point in time, when all solids are completely dissolved and no visible particulates should be present [61]. Although recently under

diverse discussion, pharmaceutical elegance is generally strived for, i.e. pharmaceutically elegant lyophilisates (Figure 5A) are desirable to ensure intra- and inter-batch homogeneity.

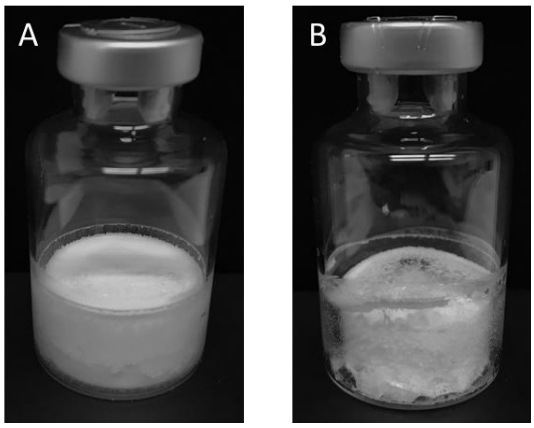

Figure 5. Pictures of a pharmaceutically elegant A) and collapsed B) lyophilisate.

After lyophilization, 100% visual inspection is performed to detect container-closure defects, extraneous particulates, or defects in cake appearance [62]. Defects in cake appearance can either be cosmetic or critical, with no established standard in characterizing the degree of a defect. Patel *et al.* recently summarized the various types of defects and encouraged for a science and risk-based rationale for evaluating whether a defect should be considered acceptable or not [59]. Examples for common cosmetic defects are fogging, which describes the phenomenon of solution creeping up the inner vials surface [63], or cracking, a result of released drying tension during water desorption [64, 65]. It has been shown that both fogging and cracking can be mitigated by the use of hydrophobic coated vials [66, 67]. On the other hand, there are cake defects which might influence product quality. An inspection guide for lyophilized parenterals by the US Food and Drug Administration describes that "Critical aspects would include the presence of correct volume of the cake and the cake appearance. With regard to cake appearance, one of the major concerns is meltback. Meltback is a form of collapse (…)" [68]. As described above, collapse is a loss of pore structure as a result of T_p higher than T_c (Figure 5B). This loss of pore structure can lead to increased reconstitution times of the lyophilisate [69]. More importantly, collapse can restrict water sublimation leading to higher residual moisture levels of the lyophilisate, which may increase protein instability [70, 71]. Although the negative impact of collapse on protein stability was shown to be not universal [72, 73], collapse in any case gives rise to complaints as it renders inhomogeneous batches [74]. Thus, to date, collapse is generally considered unacceptable leading to rejects [59].

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SCOPE

The growing market of therapeutic antibodies and particularly their next generation derivatives, which are often less stable as liquids, increase the need for lyophilized formulations. In addition, these new antibody formats often require lower doses than conventional antibodies, posing challenges with regards to formulation and lyophilization process development. Lower protein concentrations may require a higher amount of bulking agent to render mechanically stable and elegant lyophilisates. For typical sucrose formulations, this results in low glass transition and collapse temperatures due to the amended sugar/protein ratio, increasing the risk of collapse during short and aggressive freeze-drying cycles.

The focus of this work was to study alternative amorphous excipients for freeze-dried antibody formulations, which have considerably higher glass transition temperatures compared to commonly used disaccharides. The aim was to investigate their ability to provide pharmaceutically elegant lyophilisates while developing short freeze-drying cycles. Additionally, their ability to stabilize antibodies during freeze-drying and subsequent storage with an emphasis on the potential for room temperature stable formulations was investigated.

The first objective was to identify a method that allows for a comprehensive evaluation and differentiation of cake appearance and structure of different lyophilisates. In **Chapter 1**, µ-CT was introduced as an imaging technique and compared to different novel and established techniques with regards to their ability to provide a comprehensive, qualitative/quantitative, and non-invasive evaluation of cake appearance as well as structure and morphology.

Having established a method that allows for differentiation of cake appearances, the focus of the next two chapters (**Chapter 2** and **Chapter 3**) was on formulation development. Alternative excipients in low concentrated freeze-dried antibody formulations were characterized with regards to their impact on glass transition temperature, cake appearance upon aggressive freeze-drying, and protein stability upon freeze-drying and during storage. Particularly, their potential and limitations for storage at room temperature were analyzed and compared to a reference sucrose formulation. **Chapter 2** described the impact of dextrans of different molecular weights from 1 to 500 kDa. In **Chapter 3**, HPBCD-based formulations and the influence of adding other excipients such as recombinant human albumin, PVP, dextran, and sucrose were studied.

Developed formulations, which provided good stability and product quality attributes, were used for freeze-drying cycle optimization. The objective of **Chapter 4** was to develop a short lyophilization cycle using aggressive primary drying conditions resulting in high product temperatures, while maintaining pharmaceutically elegant lyophilisates with good physical-chemical product quality attributes and protein stability.

CHAPTER 1

Imaging techniques to characterize cake appearance of freeze-dried products

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Research Paper

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Abstract

Pharmaceutically elegant lyophilisates are highly desirable implying a stable and robust freeze-drying process. In order to ensure homogenous and intact cake appearance after process scale-up and transfer, characterization of lyophilisates during formulation and cycle development is required. The present study investigates different imaging techniques to characterize lyophilisates on different levels. Cake appearance of freeze-dried BSA formulations with different dextran/sucrose ratios was studied by visual inspection, 3D laser scanning, polydimethylsiloxane (PDMS) embedding, scanning electron microscopy (SEM), and micro-computed tomography (µ-CT). The set of techniques allowed a holistic evaluation of external cake appearance and internal structure providing complementary information at macroscopic and microscopic scale. In comparison to state of the art technologies like visual inspection or SEM, 3D laser scanning and µ-CT provided quantitative information allowing comparison of visual cake appearance. In particular, µ-CT enables a global, qualitative, and quantitative characterization of external and internal cake structure with a single measurement detecting heterogeneities of lyophilisates. We even demonstrated the use of non-invasive µ-CT for qualitative imaging of internal cake structure through the glass vial. Providing meaningful characterization of the entire lyophilisate, µ-CT can serve as a powerful tool during development of freeze-drying cycles, process scale-up, and transfer.

INTRODRUCTION

Lyophilization is a commonly used technique to enhance storage stability of biopharmaceuticals [1, 2]. Major constraints of freeze-drying are the additional time-consuming and costly manufacturing process after drug product filling as well as the complex development and scale-up. With biosimilars just having entered the pharmaceutical market [3], development of stable but cost conscious drug manufacturing is even more important. In terms of lyophilization, this drives the need to develop appropriate formulations which endure aggressive (i.e. shorter) lyophilization cycles with the prerequisite to deliver consistent product quality by a robust freeze-drying process. This also means in most cases to develop pharmaceutically elegant cakes with reproducible cake appearance. A homogeneous cake structure with minimal intra- and inter-vial variability is thus desired although inadequate cake appearance may not negatively impact product quality as recently reported by Schersch *et al*. [4]. Furthermore, a pharmaceutically elegant cake is not only implying a stable freeze-drying process, but correct cake volume and cake appearance are considered critical aspects for inspection of parenterals by health authorities [5].

During formulation and freeze-drying cycle development, the freeze-dried cakes are thoroughly characterized [6]. Besides cake appearance, this comprises characterization of the freeze-dried product in terms of microstructure, reconstitution time, and residual moisture. Microstructure and pore structure of the freeze-dried product are typically studied by scanning electron microscopy (SEM) [7] and specific surface area (SSA) measurements according to the Brunauer-Emmett-Teller (BET) [8] method. Rambathla *et al.* also showed that an increase in SSA as a result of a higher degree of supercooling during the freezing step may subsequently lead to a longer water vapor transition time [9]. Crystallinity and the type of polymorph formed in formulations can be assessed using X-ray powder diffraction [10]. Finally, residual moisture levels are determined using Karl Fischer titration or near-infrared spectroscopy (NIR) [11] for optimization of secondary drying [12, 13] and to characterize and control this factor which is critical for product stability.

Visual appearance of the final drug product is typically inspected by the naked eye. With regard to aggressive freeze-drying cycles, macroscopic collapse of the lyophilisate can often be observed when the product temperature exceeded the glass transition temperature (T_g') or collapse temperature (T_c) of the formulation during primary drying. A review of the different visual defects providing best-practice guidance for acceptance criteria was recently published by Patel *et al.* [14]. Besides external visible cake defects, partial collapse inside the lyophilisate or intra-vial heterogeneity have also been reported [15, 16], which may not be detected by conventional characterization techniques. However, partial collapse inside the lyophilisate can potentially be critical for the drug product, e.g. by leading to increased residual moisture. Furthermore, if such defects inside the lyophilisate exist during process development in a laboratory freeze-dryer, they can be indicative for an increased risk to process transfer to a pilot plant or commercial scale. It is well known that the thermal product history in various freeze-dryers is not necessarily the same [17, 18]. Thus, small changes in product temperature can then result in visually detectable cake defects after transfer to another freeze-dryer unit. In addition, characterization of cake morphology, which is defined during the freezing step by the formation of ice crystals, was recently correlated to the cake resistance to water vapor flow by Pisano *et al*. [19].

We therefore believe that holistic imaging of the lyophilisate structure beyond the information provided by visual inspection and SEM is advantageous during formulation and process development,

scale-up, and transfer. This includes characterization of the lyophilisate in terms of external cake appearance and inner macroscopic as well as microscopic cake structure. In particular, methods are needed, which provide qualitative information on a global level as well as quantitative data. Ideally, such methods are non-destructive, fast, and do not require removal of the cake from the glass vial.
The scope of the present study was to evaluate different imaging techniques and their applicability to study cake appearance of freeze-dried products allowing for a sound evaluation of cake defects on different scales. The methods were applied on lyophilisates of bovine serum albumin (BSA) as model protein. Different T_c were obtained using blends of sucrose and dextran. This composition enabled to obtain lyophilisates with different cake appearances within the same freeze-drying cycle. Freeze-dried cakes were visually inspected and classified from collapsed to pharmaceutically elegant cakes. The set of characterization methods included the well-established SEM, recently developed methods like Polydimethylsiloxane (PDMS) embedding [15] and micro-computed tomography (µ-CT) [20, 21]. In addition, a method allowing for non-invasive µ-CT imaging through the vial and three-dimensional (3D) laser scanning as a new technique was introduced. The techniques were compared with regard to their ability to provide qualitative and quantitative data, the local or global level of information on cake structure, as well as time and effort required for sample preparation, measuring time, and data processing. The results are discussed with respect to the added value for formulation and process development.

MATERIALS AND METHODS

Materials

Preparation of lyophilisates

Formulation. BSA Fraction V (Roche Diagnostics, Penzberg, Germany) was used as model protein. It was formulated at 10 mg/mL in a 20 mM histidine/histidine-HCl buffer pH 6.0 (Ajinomoto, Tokyo, Japan) with addition of 0.02% (w/v) polysorbate 20 (Croda International, Snaith, UK) and a total solid content of additional excipients of 8%. Six formulations with mixtures of dextran 1 kDa (Pharmacosmos, Holbaek, Denmark) and sucrose (Ferro Pfanstiehl Company, Mayfield Heights, Ohio) at different ratios (w/w) were prepared: Dex0/Suc100, Dex20/Suc80, Dex40/Suc60, Dex60/Suc40, Dex80/Suc20, Dex100/Suc0. All formulations were sterile filtered through 0.22 µm PVDF filter units (Millipore, Bedford, MA, USA). 10.6 mL of each formulation (n=20) were filled into 20R TopLyo® vials (Schott, Müllheim, Germany).

Freeze-drying process. Freeze drying was performed on a FTS LyoStar II freeze-dryer (FTS Systems Inc, New York, NY). To minimize radiation effects on cake appearance, edge vials were filled with sucrose solution and not further evaluated. Formulations were randomly distributed over three shelves. Vials were equilibrated at 5°C for 1 h and frozen to -35°C with shelf cooling rate of 0.3°C/min. The shelves were kept at -35°C for 3 h before applying vacuum (100 mTorr). For primary drying, shelves were heated to 10°C at 0.2°C/min and held for 56 h. Secondary drying was performed at 25°C (ramp rate 0.2°C/min) and 100 mTorr vacuum for 8 h. The vials were stoppered under nitrogen at 760 mbar.

Methods

Visual inspection

Lyophilisates were visually inspected in front of black background and classified for cake defects like collapse, dents, and cracks. Cake appearance was recorded with a camera.

3D laser scanning

3D laser scanning was used to visualize cake appearance and to obtain quantitative information on the cake volume. Vials were cut horizontally below the neck with a diamond grinding wheel to allow removal of the cake without damage (Fig. 1A). The cake was placed onto a plate, equipped with optical reflectors, which served as a reference system for the 3D scanner. The cake was scanned with a HandyScan 700™ 3D scanner (Creaform Inc., Leinfelden-Echterdingen, Germany). 50 images per second were recorded at a resolution of 0.2 mm until a closed mesh was obtained. In total, three scan sessions of 30-40 s were performed per cake rotated by 120° each time. Raw data of the three scan sessions were merged with the VXelements software version 6 (Creaform Inc.) into one image. Netfabb® software (Autodesk Inc., San Rafael, CA) was used to close the open mesh and to calculate the cake volume. Each formulation was scanned in triplicates. Cake volumes are given as mean and standard deviation (SD).

Polydimethylsiloxane (PDMS) embedding

To macroscopically visualize the internal cake structure, PDMS embedding of the lyophilisates was performed based on a method recently described by Lam and Patapoff [15]. Embedding was performed in duplicates per formulation. PDMS was prepared by mixing Sylgrad 184 Base and Curing agent (Dow Corning, Michigan, MI) at a 1:10 ratio. The mixture was degassed under vacuum in a desiccator and filled into 50 mL disposable syringes (BD, Drogheda, Ireland), which tips were covered with parafilm. Holes that were stitched into the syringe headspace allowed for degassing during the embedding process. The syringes were placed into a syringe holder with the tips directing into the open vials. A wire was fixed around the syringe opening and placed on top of the lyophilisate to avoid floating of the cakes during embedding. The setup was placed into a FTS LyoStar II (FTS Systems Inc, New York, NY) and the pressure was reduced to approximately 38 Torr for degassing. After a hold time of 30 min, the shelves were gently lowered to push down the syringe plunger and to release the PDMS. The setup was left in the lyophilizer under reduced pressure for two hours to let the PDMS fully penetrate into the cakes. The samples were subsequently placed into a heating oven (Heraeus Instruments GmbH, Hanau, Germany) at 40°C to cure the PDMS. The glass was removed and the embedded cake was cut into vertical and horizontal slices (thickness: approx. 2 mm) using a toggle press (HK 800, Berg & Schmid, Remseck, Germany). The slices were visualized at a 0.65-fold magnification under a Stemi 200 OC Stereomicroscope (Carl Zeiss AG, Feldbach, Switzerland).

Scanning electron microscopy (SEM)

Local morphology and pore structure of the freeze-dried cake were analyzed by SEM using a Sigma VP system (ZEISS, Oberkochen, Germany) under high vacuum and acceleration voltage of 3 keV. SEM was performed in duplicates per formulation. The vials were opened in a glove box under nitrogen and fragments of the top and bottom layer of the lyophilisate were extracted using a spatula. The samples were sputtered with gold (120 s, 30 mA, 0.1 bar Ar) using a Cressington 108 Auto sputter (Elektronen-Optik-Service GmbH, Dortmund, Germany) to increase their conductivity. Images at 50-, 100-, 500-, and 1000-fold magnification were recorded.

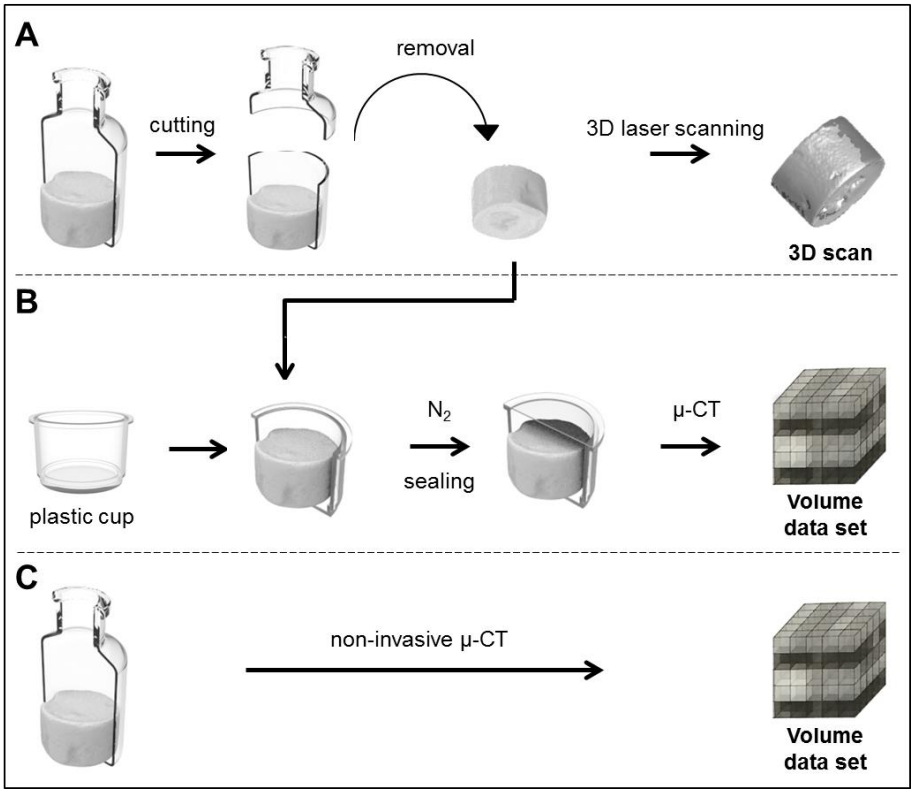

Figure 1. Sample preparation for innovative imaging techniques: A) 3D laser scanning or B) micro-computed tomography (µ-CT) after transfer to sealed plastic cups, and C) non-invasive µ-CT through the glass vial.

Micro-computed tomography (µ-CT)

Global 3D information on cake structure of the lyophilisate was obtained using µ-CT measurements.

Non-invasive µ-CT (within glass vial)

A nanotomOR instrument (phoenix|x-ray, GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany) was used to study the ability of µ-CT to visualize the cake structure through the glass vial (Fig. 1C). The lyophilisate was measured without any sample preparation using an acceleration voltage of 60 kV and a beam current of 310 µA. To allow for direct comparison with µ-CT measurements performed outside of the vial, pixel length was set to 18 µm. Total measurement time was 1.5 h.

Invasive µ-CT (removal of lyophilisate)

Analysis was performed in triplicates using a SkyScan 1275 X-Ray microtomograph (Bruker MicroCT, Kontich, Belgium). The freeze-dried cake was removed from the vial, as described above (Fig. 1B). The lyophilisate was immediately transferred into a custom made sealable plastic container, overlaid with nitrogen, and sealed (Fig. 1B). Thus the cake was protected from atmospheric humidity over the time frame of analysis. An acceleration voltage of 20 kV and a beam current of 175 µA were applied and pixel length was set to 18 µm. An exposure time of 650 ms with 6 averages per projection was applied. The lyophilisate was rotated over 360° with a step size of 0.25°. Total measurement time was 1.5 h. Reconstruction of the projections using the NRecon software (Bruker, Kontich, Belgium) resulted in an image stack of tomograms. Quantitative results are given as mean and SD.
High-resolution µ-CT images were obtained on a SkyScan 1272 X-Ray microtomograph (Bruker MicroCT, Kontich, Belgium). A piece (approx. 1/3) of the lyophilisate was transferred into a plastic cup as described above. Scans were acquired using an acceleration voltage of 30 kV and a beam current of 212 µA. An exposure time of 500 ms with 10 averages per projection was applied. The lyophilisate was rotated over 360°with a step size of 0.05°. With a pixel length of 4 µm, total measurement time was 18 h.

Data analysis

To perform reproducible and accurate data analysis, a central image stack of 600 slices per lyophilisate was used. Edge slices of top and bottom showing common µ-CT artifacts (cone beam error, beam hardening [22]) were not included.
Quantitative analysis required binary images, which were obtained by applying a threshold value to each image stack. In brief, threshold values were determined in OriginLab (OriginLab, Northampton, MA) by a multiple Gaussian fit using the histogram of a 600-slice image stack (see supporting information Fig. S1 for details).
Quantitative evaluation of cake integrity was performed on binary images using the analyze particles function in the open-source software Fiji [23], which calculates the number of white pixels per binary image. Using the pixel count per slice, the cake volume was calculated according to the following equation:

$$V = \sum_{i=1}^{n} pix_i \times \frac{V_{pix}}{10^{12}}\ cm^3 \quad (1)$$

with V = cake volume [cm^3], n = number of slices, V_{pix} = volume per pixel (18 μm^3), and pix = pixel count per slice. Surface to volume ratios of the binary image stack were calculated by CTAn 1.17 (Bruker, Kontich Begium), which uses the marching cube model to calculate surface and volume [24].

RESULTS

A panel of imaging techniques was investigated for their ability to characterize lyophilisates qualitatively and quantitatively with regards to cake appearance and macroscopic as well as microscopic cake structure. For a holistic testing, covering a broad range of cake defects, BSA based

formulations with Dex/Suc mixtures were lyophilized using an aggressive primary drying temperature of +10°C in order to obtain a variety of different cake appearances.

External cake appearance by visual inspection

External cake appearance was visually inspected and evaluated by taking pictures through the glass vial. In general, a more elegant visual appearance was observed with higher dextran concentrations (Fig. 2A). Dex0/Suc100 lyophilisates were found to be collapsed for the applied freeze-drying cycle. Dex20/Suc80 formulations were slightly improved but showed partial collapse and severe dents at the bottom of the cake. Severe to major dents were found for Dex40/Suc60 formulations and Dex60/Suc40 showed minor dents. The dents were reduced for higher Dex/Suc ratios, resulting in pharmaceutical elegant cakes for Dex80/Suc20 and Dex100/Suc0.

External cake appearance and cake volume by 3D laser scanning

3D laser scanning was used to investigate visual appearance qualitatively and to obtain quantitative information: For this purpose, the intact lyophilisate was removed from the vial (s. Fig. 1A). Cake structure integrity was visually confirmed over the scanning processing. The 3D scans of the lyophilisates reflected the defects observed by visual inspection for the different formulations (Fig. 2B). The qualitatively observed trend to more elegant lyophilisates with increasing Dex/Suc ratio was supported with quantitative data on cake volume, which was found to increase from 7.74 cm^3 for Dex0/Suc100 to 8.43 cm^3 for Dex100/Suc0 (Table 1). Variability of the cake volume was higher for collapsed formulations with standard deviations of 2.7% and 1.6% for Dex0/Suc100 and Dex20/Suc80 compared to 0.2% and 0.7% for Dex80/Suc20 and Dex100/Suc0, respectively.

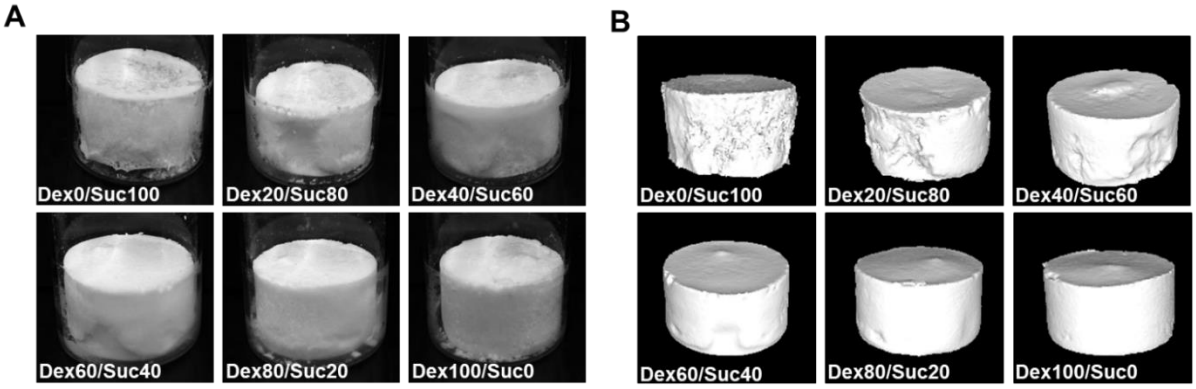

Figure 2. Visual appearance of lyophilisates. A) Pictures of lyophilisates in the intact glass vial. B) Images of 3D scans of lyophilisates removed from the vial. Approx. diameter of lyophilisates: 28 mm.

Table 1. Cake characterization data for different formulations using different imaging technologies. Quantitative data is given as mean of triplicates and standard deviation. The positions of cake defects are indicated in brackets with b=bottom, m=middle, t=top, v=vertical, e=edge.

Formulation	Visual inspection	3D scanning	PDMS embedding	SEM	µ-CT	
		cake volume (cm^3) ± std.			cake volume* (cm^3) ± std.	surface/volume ratio (1/mm) ± std.
Dex0/Suc100	collapse	7.74 ± 0.21	total collapse (m-t)	collapsed pores (b) complete loss of pore structure (t)	5.25 ± 0.14	7.25 ± 0.67
Dex20/Suc80	partial collapse, severe dents	7.94 ± 0.13	dents (b) cracks (b, v) collapse (e, t)	collapsed pores (t)	5.81 ± 0.25	9.47 ± 1.61
Dex40/Suc60	severe -major dents	8.02 ± 0.07	dents (b) cracks (b, v) collapse (e, t)	sponge-like melted pores (b, t)	6.78 ± 0.18	5.89 ± 1.00
Dex60/Suc40	minor dents	8.25 ± 0.03	cracks (b, v) onset collapse (m-t)	sponge-like melted pores (b)	7.16 ± 0.14	6.95 ± 1.26
Dex80/Suc20	elegant	8.27 ± 0.02	cracks (b, v)	honeycomb-like intact pores	7.67 ± 0.08	4.23 ± 0.96
Dex100/Suc0	elegant	8.43 ± 0.06	cracks (b, v)	honeycomb-like intact pores	7.78 ± 0.08	3.58 ± 0.46

* extrapolated to total cake height

Internal macroscopic structure by PDMS embedding

The internal macroscopic structure of the lyophilisates was investigated by embedding the lyophilisate in PDMS, cutting and microscopically analyzing a vertical middle cross-section and two horizontal slices taken from bottom and top regions. Figure 3 shows representative images for formulations which were classified by visual inspection as collapsed (Dex0/Suc100), with dents (Dex60/Suc40), and pharmaceutically elegant (Dex100/Suc0). The vertical cross-section of Dex0/Suc100 demonstrated the intra-vial heterogeneity of the lyophilisate. The region from vial bottom to half height of the cake showed an intact cake structure and the vial edges as well as the top region showed collapsed lyophilisate structure. This observation became even more prominent with horizontal cross-sections of top and bottom regions. Holes and dents were observed at the bottom close to the vial wall surrounding the inner intact lyophilisate. In contrast, an overall collapsed cake structure was observed for the top region. A more homogenous cake structure was observed for the Dex60/Suc40 lyophilisate. Dex60/Suc40 still showed dents for the cross-sections of the bottom region but a homogenous intact cake structure for the top region. However, within the vertical cross-section a collapsed part in the upper third of the lyophilisate reaching from one vial side to the other was found. This information of the internal macroscopic structure was not noticeable upon visual inspection or 3D laser scanning. A rather dense and uniform cake structure was observed for formulations Dex100/Suc0, with bottom and top regions both showing intact cake structure all over the cross-section. Cracks could be identified within the bottom and vertical images of Dex60/Suc40 and Dex100/Suc0. The macroscopic structure observed for the different formulations was similar for the duplicates investigated.

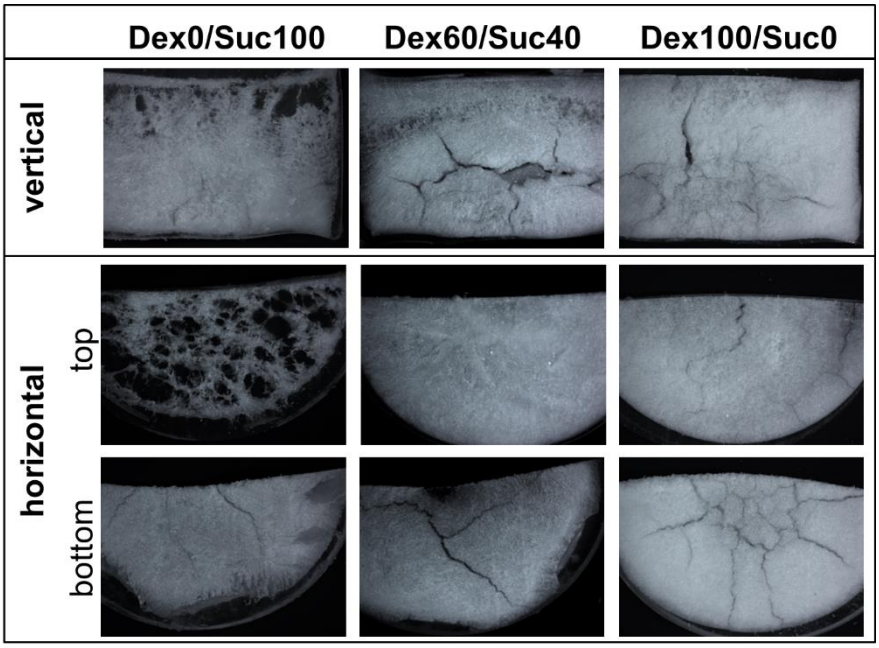

Figure 3. Analysis of PDMS embedded lyophilisates. Representative images of selected vertical and horizontal slices of lyophilisates. Approx. 20 mm sections of the entire lyophilisate are shown.

Internal microscopic structure and characterization of pore structure by SEM

To investigate the microscopic structure and in particular the pore structure, small parts of the vial bottom and top regions were extracted using a spatula and investigated by SEM. At 100-fold magnification, Dex0/Suc100 shows a highly porous microscopic sponge like structure for the bottom layer (Fig. 4A), matching the expectations based on the results of the previously described methods. For the top region, no intact morphology could be identified on a microscopic scale indicating that the formulation was completely collapsed. An intact and less porous microscopic structure was observed for top and bottom layers for Dex60/Suc40. At 500-fold magnification of the bottom region merging of the matrix in between two pores could be revealed. These fused regions within the material indicate onset of physical collapse of the cake. However, the second Dex60/Suc40 lyophilisate analyzed even showed fusion of pores in the top layer (see supporting information Fig. S2). With regard to the internal partial collapse of Dex60/Suc40 observed by PDMS embedding, this leads to the assumption that the samples were extracted from the intact top layer (vial 1) and the collapsed region in the upper third (vial 2) indicating criticality of sample preparation for SEM. Dex100/Suc0 formulations showed a different microscopic morphology compared to Dex0/Suc100 and Dex60/Suc40, which resembled a sponge like structure. In contrast, the arrangement of Dex100/Suc0 in the 500-fold SEM image in Figure 4B has a more lamellar, stapled appearance. This morphology for dextran based formulations has been observed before in a study performed by Padilla *et al.* [25].

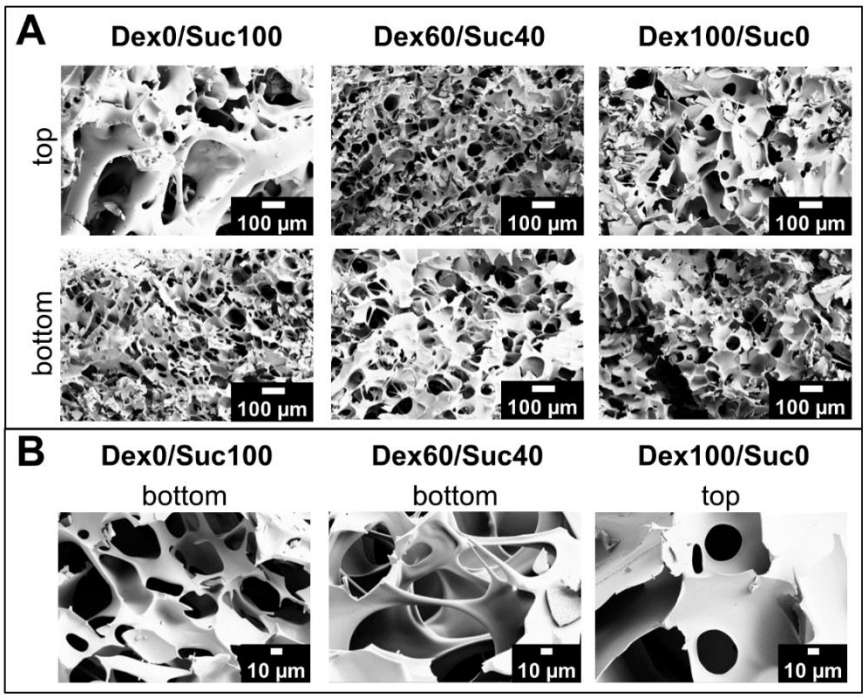

Figure 4. Scanning electron microscope (SEM) analysis of lyophilisates. Representative images from top or bottom layer of freeze-dried cakes at A) 100-fold magnification or B) 500-fold magnification.

Global cake structure by µ-CT in plastic cups

Qualitative evaluation of 3D reconstruction

µ-CT was performed to study external and internal cake structure globally on different levels. Greyscale images of the entire lyophilisate were obtained by 3D computer reconstruction. In general, material of higher density results in higher contrast and consequently, grey to white parts represent solid matrix, and black regions represent air. The 3D images of the lyophilisates as shown in Figure 5A support the observations made by visual inspection and 3D laser scanning. Formulations with Dex0/Suc100, which showed collapse, exhibited voids i.e. collapsed regions. In addition, the majority of the solid part had a white appearance compared to the rather grey character in case of Dex60/Suc40 and Dex100/Suc0 indicating densification of the solid material due to collapse. For Dex60/Suc40 formulations, dents close to the vial bottom were observed and in addition some voids (black areas) all over the lyophilisate which could represent either regions of partial collapse or cracks reaching to the glass vial. Finally, the formulation Dex100/Suc0, which was found to provide a visually elegant cake, also provided a rather homogenous appearance in the 3D µ-CT reconstruction.

Representative horizontal cross-sections for bottom, middle, and top regions of the lyophilisates are shown in Figure 5B. At the bottom region of Dex0/Suc100 large black regions were staggered around the inner cake. The voids increased for slices in the middle of the lyophilisate, completely surrounding the cross-section. At the top, almost the total area was black, interspersed only by some light-grey to white regions. The same intense contrast was observed for the boundary layer between air and inner cake structure for both bottom and middle slices. The light-grey to white parts again revealed matrix of higher density where the collapsed and shrunken material concentrates. Replacing 60% of the sucrose content with dextran (Dex60/Suc40), less black area was observed. The bottom layer of

Dex60/Suc40 showed more grey part compared to Dex0/Suc100, i.e. less dents and less collapse. However, partial collapse was observed for the middle slice, reflecting the observations made by the PDMS embedding technique. A significant improvement in cake structure for higher dextran concentration was observed for the top slice. Dex0/Suc100 showed total collapse, whereas Dex60/Suc40 exhibited a rather intact homogenous cake structure. Intact cake structure for all three slices was found for Dex100/Suc0. Collapse or dents were not observed for any slice. The cross-section from the middle of the lyophilisate showed larger lamellar-like structures particularly around the crack in the middle.

Figure 5C shows vertical cross-sections of the middle of the lyophilisates. Intra-vial heterogeneity was observed for all three formulations. For Dex0/Suc100, growth of collapsed regions was observed from vial bottom to top. White parts on the edges indicate that collapse and dents close to the vial wall occurred. The cross-section of Dex60/Suc40 visualized the collapsed region in the upper third, which has also been observed by PDMS embedding. In total, the overall cake structure was more uniform compared to Dex0/Suc100. For Dex100/Suc0, collapsed parts were not observed and regions with large lamellar-like structures, mainly in the middle of the lyophilisate, were found. For all formulations, cracks inside the cakes were observed.

Figure 5D shows a close-up for selected regions of Dex60/Suc40, obtained by a high resolution scan (4 µm) of a selected piece of lyophilisate. The upper image (1) reveals the fusion of microscopic pore structure as also observed by SEM for Dex60/Suc40 lyophilisates. In contrary, the lower image (2) shows intact sponge-like pore structure as observed by SEM for the very top layer. In addition, the top left of the image shows a rather lamellar structure. Based on the SEM observations for Dex100/Suc0 lyophilisates, this might indicate a region where preferably dextran is present. The distribution of protein and excipients within the lyophilisate is subject of ongoing research.

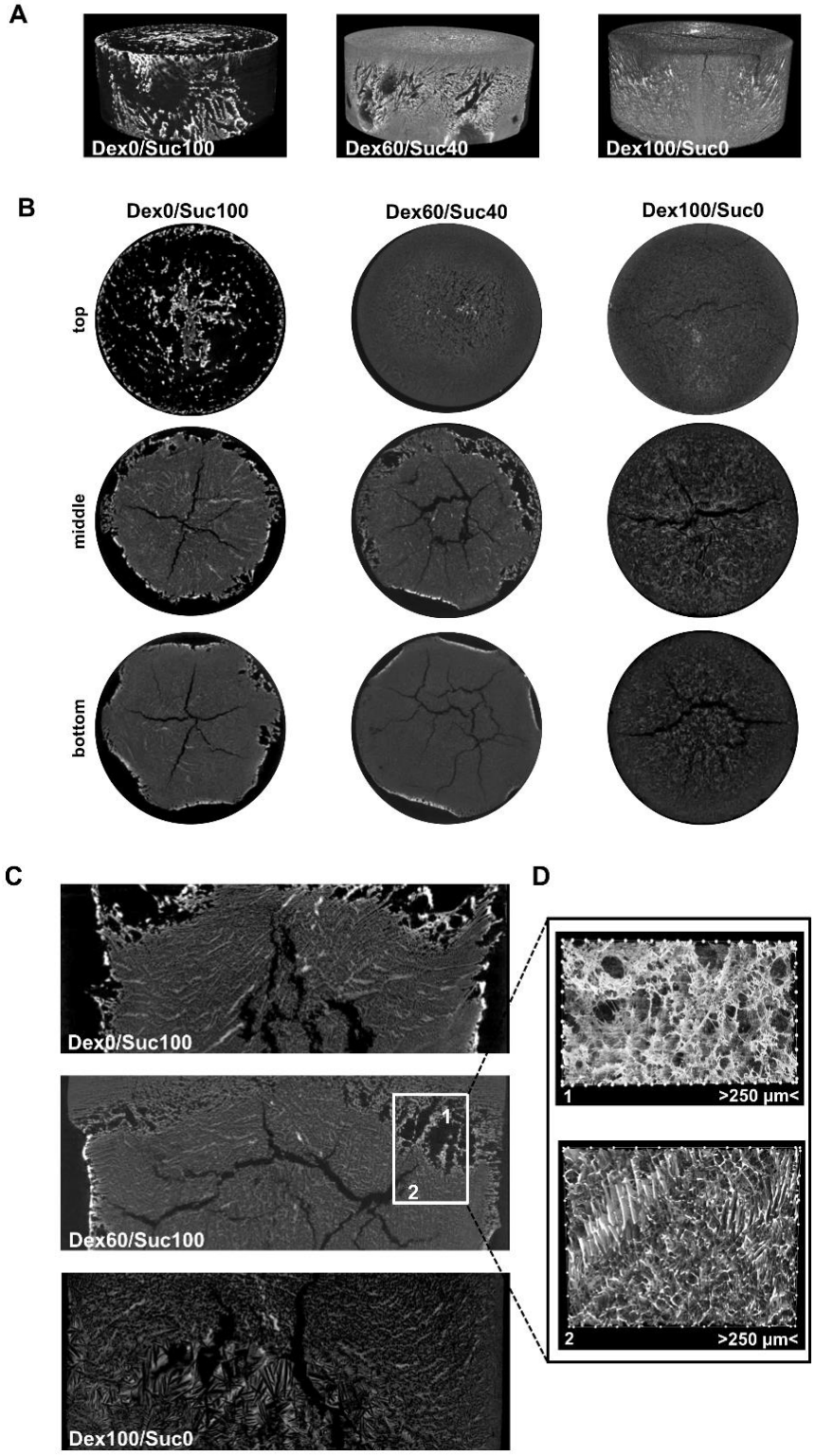

Figure 5. Representative µ-CT images of lyophilisates after transfer to plastic cups. A) Representative 3D-images of freeze-dried cakes. B) Representative images of horizontal cake structure at different cake heights from bottom to top layer. C) Representative images of vertical cake structure (middle of the freeze-dried cake). The white box indicates the region that has been used for high-resolution µ-CT. D) High-resolution µ-CT (4 µm) of partial cake: (1) collapsed microstructure and (2) intact sponge-like structure. Images are based on 3D computer reconstructions. Approx. cake diameter: 28 mm.

Quantitative evaluation of binary images

Quantitative evaluation was based on analysis of 600 slices throughout each lyophilized cake. Binary images were obtained for each slice by applying a threshold. In order to perform consistent and reproducible analysis, a threshold was chosen that was to the detriment of fine pore structures (see supporting information Fig. S3). By summing up the white pixels per slice plotted against the slice number, a quantitative measure for intra-vial integrity was obtained as shown in Figure 6. A steady decrease in pixel count was observed for Dex0/Suc100 from slice 300 to 600 indicating a loss of structure and reflecting the qualitative results obtained by PDMS embedding (Fig. 3) as well as µ-CT slices of middle and top regions (Fig. 5B). With higher Dex/Suc ratios the total pixel count per slice was increased and found more consistent throughout the cake. The collapsed region within the upper third of Dex60/Suc40 was detected by a lower pixel count between slices 300 - 450 which increased again for slices 450 - 600. An almost constant pixel count was found for Dex80/Suc20 and Dex100/Suc0. In a next step, the cake volume was calculated for each formulation. An increase in volume was found with higher Dex/Suc ratios as shown in Table 1. In general, volumes obtained by µ-CT were found to be lower compared to calculated volumes for 3D laser scanning (Table 1). In addition, surface to volume ratio were obtained, which were in general lower for higher dextran concentrations. This suggests that addition of dextran led to more dense and less porous lyophilisates. This quantitative evaluation corresponds to the porous morphology of Dex0/Suc100 samples observed in SEM.

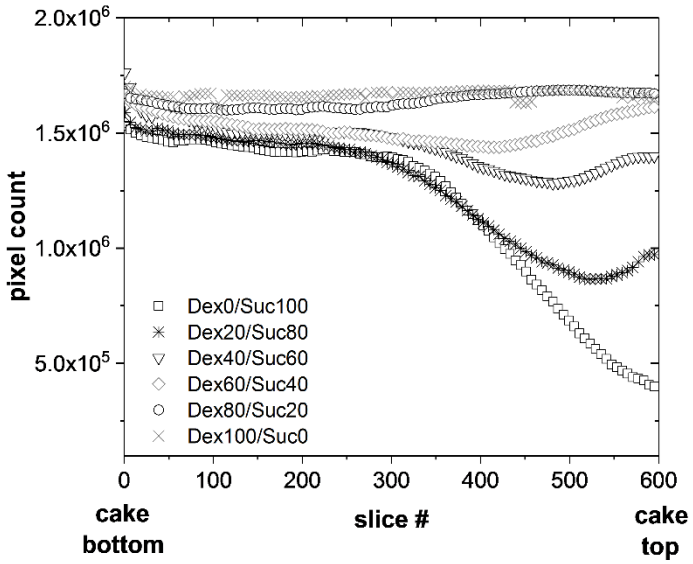

Figure 6. Quantitative evaluation of cake integrity by µ-CT. Integrity of vertical cake shown by pixel count per horizontal slices dependent on cake height. A higher pixel count represents higher integrity of the cake. Cake height is divided into 600 slices of equal thickness (18 µm). Pixel count of binary µ-CT images is plotted as mean value. For better visualization every sixth point is plotted.

Non-invasive µ-CT imaging through the vial

Additional µ-CT measurements were performed to evaluate the potential of µ-CT to study cake structure non-invasively through the glass vial. In order to confirm the validity of images obtained by scanning through the glass vial, the results were compared to a µ-CT scan of the identical lyophilisate after transfer into a plastic cup. Both images reveal details of the lyophilisate inner structure to the same extent (Fig. 7). Overall, a higher contrast between cake matrix and air was achieved by analysis after removal from the vial. In addition, a bright region surrounding the lyophilisate, referred to as beam hardening [22], is observed when analysis is performed in vials.

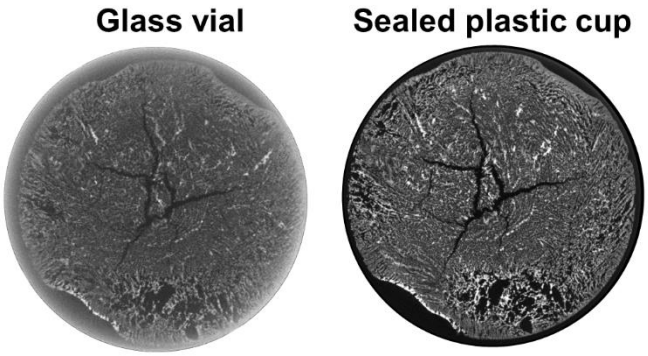

Figure 7. Non-invasive µ-CT for imaging of cake structure. Horizontal greyscale µ-CT slice of the same Dex60/Suc40 lyophilisate. µ-CT was performed through the glass vial at 60 kV (left image) or after transfer intro a plastic cup at 20 kV (right image).

DISCUSSION

The aim of the present study was to investigate and compare different imaging techniques for their ability to provide information on cake appearance as well as internal cake structure of lyophilisates on a local and global level. Special emphasis was hereby given to innovative imaging techniques: 3D laser scanning and non-destructive µ-CT methods. In order to evaluate the tested methods with respect to their ability to differentiate between cakes of different appearance, formulations with the same solid content, but different Dex/Suc ratios were examined. Dextrans, which have previously been used as excipients for protein formulations [26-28], were chosen as the T_g' is higher than that of sucrose. The T_g' and the T_c onset for 80 mg/mL sucrose or dextran 1 placebo solutions were -31.8°C/-30.3°C and -19.8°C/-19.5°C, respectively. Product temperature during primary drying was approximately -24°C. As a result, different cake appearances were intentionally obtained while using the same freeze-drying process parameters. It needs to be mentioned that optimization of freeze-drying conditions was not within the scope of the present study. Cracks depend on the water desorption behavior and were expected under the aggressive freeze-drying cycle conditions [29].
Visual inspection was performed representing state of the art for 100% control after lyophilization [30]. It allows for a fast and non-invasive evaluation of the visual appearance of a lyophilisate through the glass vial. However, this method faces some significant challenges. First, a defect might clearly be visible for the operator by eye, but recording the same with a camera in case of fully automated optical

inspection can be challenging. For instance, Patel *et al.* pointed out that in case of fogging camera based visual inspection may lead to false rejects [14]. Furthermore, cake appearance and its evaluation is one of the most subjective product quality attributes depending on the training of the inspector and therefore inter-individual variability in the reporting is common. Last but not least, a lyophilisate might appear pharmaceutically elegant upon visual inspection, but partial collapse may occur inside of a lyophilisate, hence cannot be seen by pure external inspection [14, 15].

We introduced 3D laser scanning as a novel technique for characterization of lyophilized cakes to drive the evaluation of visual appearance towards an objective and hence comparable analysis. In order to mitigate artifacts due to the curved surface of the glass container, the cake needs to be removed from the vial and exposed to the atmosphere during the measurement making it destructive. With this technique, a 3D image of the pure lyophilisate was recorded similar to visual inspection providing 360° information of the external cake appearance. In addition, quantitative data of the cake volume was provided. Higher cake volumes were obtained for formulations of higher Dex/Suc ratios correlating to an improved visual appearance (see Table 1). Thus, we could quantitatively demonstrate the improvement of visual appearance. This numerical information might be of advantage during formulation or process development allowing an objective comparison of the impact of different formulation or process parameters. 3D laser scanning was found to be an easy to use method to quantify visual appearance but it also revealed some constraints. The technique required removal of the lyophilisate from the vial, leading to exposure of the cake to the atmosphere for the timeframe of the measurement. Risks associated with this procedure are false cake volume either because of deformation or shrinkage of the lyophilisate during measurement or due to cake residuals remaining in the vial. Data for imaging techniques in this manuscript were obtained by lyophilization in TopLyo® vials and in 20R Fiolax® (data not shown). The remaining cake residues in the glass vial when removing the lyophilized cake becomes prominent for freeze-drying performed in uncoated vials. The lyophilisates tended to stick to the vial bottom. Thus, measurement of triplicates resulted in generally higher standard deviations for uncoated vials compared to hydrophobic coated vials (supporting information Fig. S4).

As mentioned above, some cake defects may not be noticeable by external inspection, making imaging of the internal cake necessary. An adapted protocol (e.g. imaging without coloration of the embedded slices) of the PDMS embedding technique, originally proposed by Lam and Patapoff [15], was used allowing for visualization of internal cracks and collapsed regions. It provides a valuable tool for imaging selected regions of the cake via vertical or horizontal cross-sections. One drawback of this technique is the time consuming sample preparation, with embedding plus curing taking several days. Furthermore, within other research studies, we found the applicability of the embedding technique to be dependent on the formulation composition. The phenomenon of incomplete encapsulation and challenges related to high fill height have previously been described by Lam and Patapoff [15]. Attempts to convert pictures into binary images for quantitative assessment were not successful as differentiation between embedded cake matrix and pores was insufficient due to little contrast.

SEM is a very common technique used to study the microscopic structure and the pores of lyophilisates during both formulation [7, 31] and process development [32-34]. It provided valuable information on the microscopic structure of different Dex/Suc formulations in our study. For instance, a 500-fold magnification of Dex60/Suc40 (Fig. 4B) showed onset of pore fusion, which suggests that the product was freeze-dried close to or above its T_c. It also revealed that high dextran concentrations seem to shift

the microscopic structure from a sponge-like arrangement towards a dense rather lamellar, honeycomb-like appearance. We hypothesize that this difference in microstructure is of importance as it might impact other product quality attributes, e.g. reconstitution time due to a different wettability. SEM is further considered as a powerful technique to visualize “micro-collapse”, which has been named by Milton *et al.* and refers to small holes which appear in an otherwise intact pore structure [33]. However, SEM has significant drawbacks. In general, very small pieces are extracted of the lyophilisate’s top and bottom regions by using a spatula. This bears the risk that the native structure of the lyophilisate is damaged during sample preparation or due to exposure to atmospheric humidity. Furthermore, for very porous lyophilisates fixation of the material on the sample holder was found to be challenging, resulting in a sample loss in the microscope or potentially severe damage of the instrument. Overall, SEM analysis requires considerable effort and immediate analysis of the prepared sample. Most importantly, the sample parts investigated by SEM represent only local pore structure which might not be representative for the entire lyophilisate, which can be critical particularly for lyophilisates that show strong intra-vial heterogeneity. This bears the risk of misleading interpretation of inter-vial heterogeneity and makes reproducible SEM analysis difficult.

The imaging techniques discussed above allowed for qualitative and in case of 3D laser scanning to some part quantitative characterization of lyophilisates of either the external or the internal cake structure. A comparative overview of the applicability of the methods is summarized in Table 2. In order to obtain a comprehensive understanding of both external cake appearance and internal cake structure, three different techniques would be required: Visual inspection or 3D laser scanning for evaluation of cake appearance and classification of cosmetic defects, PDMS embedding to visualize internal macroscopic cake structure and potential intra-vial heterogeneity, and last but not least SEM to obtain relevant microscopic information on pore structure. As both SEM and PDMS embedding methods are destructive at least a sample size of three (or four, if including 3D laser scanning) is required in order to obtain a sound cake characterization. This number of samples may however not be available in early development phases. Moreover, due to the invasiveness, macroscopic and microscopic evaluation of cake appearance cannot be performed using the same lyophilisate. Furthermore, images obtained by PDMS embedding and SEM represent only structural information of selected regions of the lyophilisate. Consequently, this drives the need to look into techniques which cover analysis of the global internal cake structure and the external cake appearance within the same sample. Ideally, these techniques provide qualitative and quantitative data at the same time and allow analysis without destroying the sample.

Table 2. Comparison of imaging techniques to characterize cake appearance of freeze-dried formulations. The overall effort is indicated as low=minutes, medium=hours, high=days.

Method	External cake appearance	Internal macroscopic structure	Internal microscopic structure/ pore morphology	Quantitative data	Global analysis	Measuring time/ Overall effort
Visual inspection [a)]	X	–	–	–	–	n.a./ low
3D scanning	X	–	–	X	–	~3min/ low
PDMS embedding [b)]	–	X	–	–	–	n.a./ high
SEM[b)]	–	–	X	–	–	n.a./ medium
µ-CT	(X)	X [a)]	X	X	X [a)]	1.5 h (18 µm), 18 h (4 µm)/ low-medium[c)]

[a)] non-invasive
[b)] destructive
[c)] µ-CT scanning time and effort vary depending on sample size, resolution, and µ-CT detector as well as desired read-out

µ-CT provides detailed cross-sectional and three-dimensional images allowing for a qualitative evaluation of cake structure of the entire lyophilisate at much higher resolution (18 µm) compared to the PDMS embedding technique and in addition visualizes regions where collapsed material concentrates by a higher contrast. We were able to relatively compare vertical cake homogeneity as pixel count per slice. In addition, with cake volume as well as surface to volume ratios, µ-CT provided quantitative data on cake structure. It is important to mention, that cake volumes obtained from 3D laser scanning and µ-CT differed. In general, volumes for total cake height obtained from µ-CT were found to be lower compared to those obtained with 3D laser scanning (see Table 1). This discrepancy is based on the way the volumes are calculated. 3D laser scanning provides a mesh of the lyophilisate outer surface and only dents, shrinkage and other defects exposed to the surface contribute to a reduction of the resulting calculated volume. In contrast, cake volume of µ-CT was calculated according to equation (1) in the material and methods section. The calculation was performed based on 3D reconstructed µ-CT greyscale images converted into binary images. With the applied threshold, internal cake structure attributes such as cracks or voids due to collapsed regions contributed to the calculated volume compared to volume obtained by 3D laser scanning using information from external cake appearance only. Hence, more detailed information is provided by the cake volume derived from µ-CT as it considers not only external visual appearance but also internal cake structure.

Previous studies from Parker *et al.* and Izutsu *et al.* compared SEM images with micro-structural attributes obtained by µ-CT measurements of lyophilisates extracted from the glass vial. Izutsu *et al.* used SEM and high-resolution µ-CT (pixel resolution 0.5 – 8 µm) to investigate the effect of post-freezing treatment and controlled nucleation on lyophilisate morphology [35]. They found both techniques to provide comparable information of microstructure suggesting µ-CT as alternative to SEM. Parker *et al.* characterized the effect of shelf temperature during primary drying on microstructural attributes using µ-CT (pixel size 4.5 µm). They correlated quantitative attributes like the pore size and the fragmentation index, which characterizes the connectivity of the cake matrix, to

the microstructure visualized by SEM [20]. The focus of both studies was to investigate microscale morphology of lyophilisates, in particular of small sample volumes using formulations of 1 µL and 1.2 mL, respectively. Therefore scans could be performed at very high resolutions with relatively short measurement times (e.g. 20 minutes [35]).

In contrary, the aim of the present study was to probe the ability of µ-CT to characterize external and internal global cake structure of the entire lyophilisate in dimensions of common marketed therapeutic antibodies. With typical dosage regimens for antibodies between 2 – 10 mg/kg [36], this means vial sizes of 10 – 50 mL for the marketed drug products [37-39]. Therefore, a 20 mL vial with a fill volume of 10.6 mL was used within our study. The objective was further to characterize the cake appearance and internal structure of the entire lyophilisate of approximately 28 mm diameter with a single and thus fast scan. For this purpose, we used a SkyScan 1275 with a 1944x1536 pixel detector, which allowed for a maximum resolution of 18 µm including a safety margin of approximately 3 µm to compensate for deviations of sample position. A single scan of the entire lyophilisate at 18 µm resolution clearly visualizes the differences in internal cake structure of the formulations. In order to further evaluate regions of intra-vial inhomogeneity on a microstructural level, high-resolution images at 4 µm resolution were obtained of a selected part of the Dex60/Suc40 sample using the SkyScan 1272 instrument. It needs to be mentioned, that high-resolution measurements are generally very time consuming. Hence, we propose to first perform a scan of the entire lyophilisate at lower resolution for global characterization of the macroscopic cake structure, optionally followed by a high-resolution measurement of smaller cake parts to investigate microstructure for selected regions of interest. A major advantage of high-resolution µ-CT compared to SEM is that larger parts of the lyophilisate can be evaluated on a microstructural level, thus allowing investigation of the “native” microstructure inside the partial cake and locating its position within the entire lyophilisate. Therefore, the use of µ-CT has the potential to overcome shortcomings of SEM, like potentially altered microstructure due to cutting or exposure of the sample to atmospheric humidity, as well as the low reproducibility. In addition, high-resolution µ-CT allows to correlate the pore size calculated from 3D reconstructed images to the cake resistance, which is linked to the sublimation rate during the drying step. This was recently shown by Pisano *et al.* exemplified for mannitol formulations [19]. Overall, µ-CT analysis requires less effort by the researcher than SEM, despite its long scanning time. The time determining factor of µ-CT analysis is the long scanning time, which however does not require any human monitoring.

One aim of the present study was to provide quantitative information on cake appearance and internal structure. To perform quantitative analysis, binary images are needed, which requires a consistent contrast throughout the 3D reconstructed greyscale µ-CT images. Thus, removal of the freeze-dried cake was necessary, in order to prevent artifacts due to the different densities of the glass vial and the lyophilized cake. The proposed procedure requires little sample preparation only (transfer of the lyophilisate from the glass vial into a plastic cup). In addition, overlay of nitrogen followed by sealing of the plastic cups assured cake integrity for several days, making it non-destructive. Hence, this allows for using the same lyophilisate after µ-CT for further physico-chemical analysis of other quality attributes e.g. SSA. Nevertheless, a completely non-invasive approach to qualitatively evaluate the internal cake structure is desirable. A first attempt was proposed by Ullrich *et al*., who performed µ-CT measurements to analyze cracks within the 2R glass vial [21]. However, they opened the vial to insert a styrofoam between the cake and the stopper for sample preparation. We aimed for a fully non-

invasive, non-destructive approach. To this end, we performed additional µ-CT experiments using a high-power X-ray tube at higher voltage. Higher voltages are needed in order allow penetration of the photons through the glass vial. In return, increased voltages are to the detriment of the contrast between the porous cake matrix and air in the greyscale images. The obtained greyscale images provided the same level of qualitative information as for measurements that were performed after removal of the lyophilisate from the vial. It allowed detecting macroscopic cracks and dents, but also fine structural changes like partially collapsed regions and even visualized structures of concentrated material. Thus, we demonstrated a completely non-invasive µ-CT application to visualize the inner cake structure and detect cake heterogeneities. Non-invasive µ-CT provides a powerful alternative tool to PDMS embedding in order to qualitatively evaluate the macroscopic structure on a global level and at much higher resolution through the vial. This non-invasive approach, however, may not be used for quantitative analysis as the resulting artifacts from the glass vial do not allow for accurate thresholding. The primary objective of the present study was to examine the applicability of µ-CT for characterization and differentiation between lyophilisates and its potential in comparison to other imaging technologies, rather than the ultimate optimization of µ-CT measuring and data processing parameters. Yet, in an ongoing study, we have further optimized the X-ray parameters (e.g. voltage) and reconstruction settings allowing for non-invasive µ-CT using not only the nanotomOR instrument but also the SkyScan instrument. In general, µ-CT instruments have different limitations. With regard to the SkyScan instruments used in our study, the two models differ in the physical size of the detectors, i.e. the field of view, and the number of pixels, which defines the maximum resolution for a sample of a given size. The resolution relates to the accuracy of the quantitative read-out during data processing when converting into binary images. Hence, for investigation of fine pore structures and quantitative analysis, an instrument model with a high pixel number of the detector would be superior, like the SkyScan 1272 in our study (16 Megapixel) compared to the SkyScan 1275 (3 Megapixel). However, it needs to be mentioned that high resolution scans can create massive data sets, which make data analysis challenging. In contrary, when aiming to identify intra-vial heterogeneities during process optimization, an instrument allowing to scan various vials formats with a single scan at acceptable resolution and thus considerable shorter measurement time would be preferred. The size of the sample that can be measured within in a single scan relates to the field of view, meaning the maximal distance between the detector and the X-ray source, as well as the physical dimensions of the detector. Hence, for this intention an instrument with a larger field of view like the SkyScan 1275 would be preferred. Choosing the adequate instrument model is thus a case by case approach depending on the desired application and result.

In summary, while most studies focus on one parameter of cake appearance only, e.g. either cracks [21], microscopic structure [20, 40], or visual appearance [14], our study provided a panel of imaging techniques giving complementary information on external cake appearance and internal cake structure at different scales. An overview of the results obtained by the different methods is given in Table 1 showing consistency between the results of cake appearance for the same level obtained by different methods. We compared the methods with regard to their ability to characterize cake appearance on different levels and in providing qualitative or quantitative data. Table 2 summarizes the applicability of visual inspection, PDMS embedding, SEM, 3D scanning, and µ-CT, highlighting that only the last two are able to provide a quantitative measure. We identified challenges associated with each method and evaluated the effort for sample preparation and measurement time.

CONCLUSION

Reproducible and homogenous cake appearance of lyophilized product is an important quality attribute indicating a stable and robust freeze-drying process. Detailed information on cake appearance during process and formulation development is thus essential. The combination of imaging techniques investigated in our study allowed a sound evaluation and differentiation of cake appearance at macroscopic and microscopic scale evaluated for different formulations to detect levels of collapse and cake defects. The techniques investigated provided consistent and complementary information being able to provide not only qualitative but also quantitative information allowing comparison of visual cake appearance.

In particular, we found µ-CT as a promising and valuable technique for formulation and process development as it allows global characterization of cake structure with one single measurement and little sample preparation. With our study, we could demonstrate that µ-CT is able to both qualitatively and quantitatively detect between changes in cake structure and to provide characterization on different levels. Overall, this technique allows detecting cake defects which may have been missed by current state of the art techniques like visual inspection and SEM. Information and data quality obtained from a µ-CT measurement will depend on the formulation composition, with higher total solid content resulting in higher contrast. In addition, the dimensions of the lyophilisate are important as the smaller the cake the higher is the resolution achievable when analyzing the entire lyophilisate. Finally, in particular non-invasive µ-CT provides a powerful tool during formulation development and especially freeze-drying cycle development, scale-up, and transfer to provide meaningful cake characterization throughout the lyophilized product.

X-ray based imaging technologies such as µ-CT are at present rapidly evolving. Future generations will potentially allow for a faster and automated measurement of samples. Such novel technologies could be used during the manufacturing process for non-invasive at-line monitoring of freeze-dried drug products.

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SUPPORTING INFORMATION

Figure S1 shows the procedure of transforming a greyscale image stack into binary images for quantitative analysis of µ-CT images. Figure S1 (A) shows a typical histogram calculated for an image stack of 600 slices. Thresholding was done performing a multiple gauss fit to the histogram using OriginLab as shown in (B). The inlection point of the cumulated fit was chosen as threshold value and verified experimentally by comparing a greyscale image with the identic binary one. While other studies performed thresholding by empirically only (Parker *et al.,* 2010,J Pharm Sci. 4616-4629), we aimed for a mathematic based and repeatable thresholding procedure. The applied thresholding procedure was found to be the most suitable and for the images obtained by µ-CT using a SkyScan 1275.

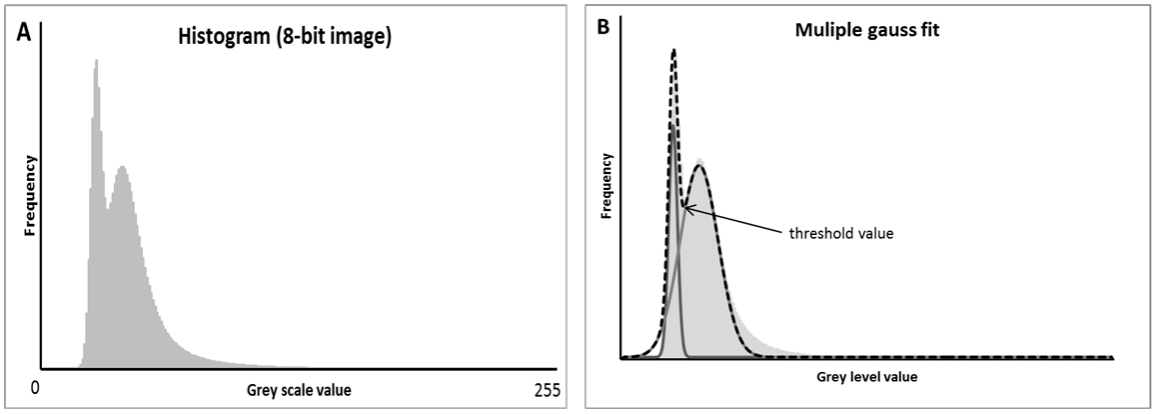

Figure S1. Schematic graphs for thresholding procedure. A) Histogram calculated for 8-bit image stack of 600 slices. B) Histogram overlaid with multiple gauss fitted curves (2 peak). Threshold value was chosen as inflection point of the cumulated fit curve (- - -).

Figure S2 shows the pore structures for the top layer observed by SEM at 100-fold magnification for samples of Dex60/Suc40 lyophilisates from two different vials. While vial 1 showed an intact porous pore structure of the top layer, fusion of pores was observed for the top layer of vial 2, indicating freeze-drying close to the collapse temperature. This reveals the low reproducibility of SEM.

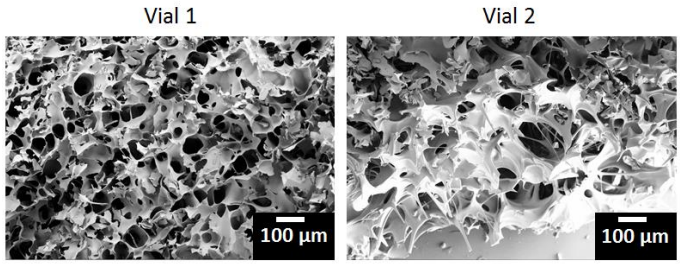

Figure S2. SEM images of Dex60/Suc40 lyophilisates from top layer at 100-fold magnification.

Figure S3 shows level of detail of the applied threshold highlighting that in order to keep a constant and reproducible thresholding procedure, fine pore structures have not been considered.

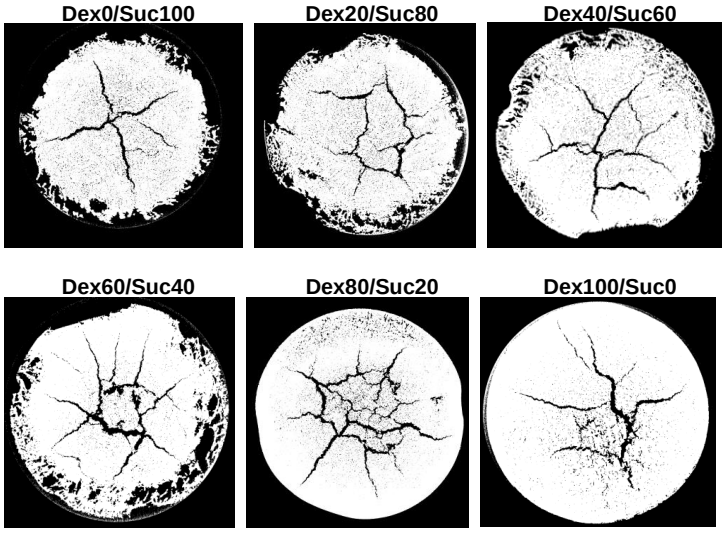

Figure S3. Representative binary images from the middle of the image stack (slice 280).

Figure S4 shows the effect of vial type used for freeze-drying on cake volume accuracy derived from 3D scanning. The figure shows that for hydrophobic coated vials (TopLyo), better reproducibility (i.e. lower standard deviation) and higher accuracy (continuos increase of cake volume with higher dextran concentration) were obtained.

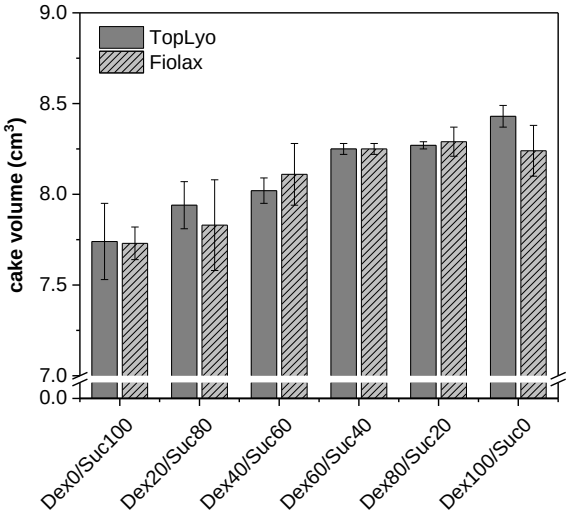

Figure S4. Cake volume calculated from meshes obtained by 3D scanning. Freeze-drying was performed in either Fiolax vials (hatched bars) or TopLyo vials (grey bars). Cake volume is presented as mean value (n=3) with standard deviation.

CHAPTER 2

Impact of dextran on thermal properties, product quality attributes, and monoclonal antibody stability in freeze-dried formulations

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Research Paper

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Abstract

Freeze-drying is commonly used to improve stability of liquid formulations of labile biopharmaceuticals. Lyo- and cryoprotectants such as sucrose are traditionally utilized as excipients, but have low glass transition (T_g') and collapse temperatures (T_c). Consequently, these formulations require low primary drying temperatures making the lyophilization cycle time-consuming and costly. We investigated different dextrans (1, 40, 150, and 500 kDa) and mixtures of dextran with sucrose as alternative excipients. The influence of dextran on thermal properties, cake appearance, and other quality attributes in the solid state was studied using bovine serum albumin as model protein. Especially at higher weight ratios of dextran to sucrose, dextrans of medium to high molecular weight (M_W) of 40-500 kDa showed up to 20°C higher T_c compared to sucrose, which was reflected in elegant lyophilisates. However, this resulted in longer reconstitution times. Addition of dextran led to lower residual moisture levels and higher T_g values compared to sucrose. We confirmed the thermal properties for two monoclonal antibodies (mAb) at two weight ratios of sucrose and dextran with different M_W and tested for stability at 40°C for 14 days. While no loss in relative potency of the antibodies was observed after storage, size exclusion chromatography and isoelectric focusing revealed a strong increase in high molecular weight species (HMWs) and acidic species, which were dependent on the M_W of the dextrans. With further characterization of selected formulations (dextran 1 kDa) by boronate affinity chromatography and mass spectrometry analysis, we demonstrated that HMWs were a result of glycation by free terminal glucose of the dextran. This chemical modification was strongly reduced when adding sucrose, which protects the protein possibly by shielding its surface. Our results demonstrate that formulation scientists need to use dextrans as excipients in freeze-dried mAb formulations with caution. A binary mixture of sucrose and dextran in adequate ratio however might potentially be superior to pure sucrose formulations allowing for faster freeze-drying cycles resulting in elegant lyophilisates and good protein stability.

INTRODUCTION

Biopharmaceuticals such as monoclonal antibodies are often lyophilized in a final drug product manufacturing step in order to improve their shelf life, if liquid formulations show limited stability. Instability of the proteins can occur via physical (e.g. unfolding, aggregation [1]) or chemical (e.g. oxidation [2], deamidation [3], condensation reactions [4]) degradation pathways. Freeze-drying immobilizes the protein in a dry glassy matrix, which retards degradation mechanisms. During the freeze-drying process, the protein needs to be protected against freezing and drying stresses, i.e. at the ice-liquid interface due to cryoconcentration and water removal, which can lead to protein denaturation or aggregation [5]. The added stabilizers can be differentiated into cryoprotectants, which stabilize the protein during freezing and in the frozen state, and lyoprotectants, which are responsible for protein stabilization upon water removal and in the dry state. Excipients may provide both, cryo- and lyoprotection, like e.g. sucrose and trehalose, which are common excipients in biopharmaceutical formulations. In addition, particularly for low concentration protein formulations, crystallizing excipients such as sugar alcohols or amino acids can be added to the formulation. The addition of mannitol for example improves the macroscopic cake structure and decreases the likelihood of cake collapse. However, these compounds serve as bulking agent only, as they are inferior to amorphous ones in terms of protein stabilization [6].

Different mechanisms of protein stabilization by sugars have been described. During freezing proteins are stabilized according to the so called “preferential exclusion” theory [7]. According to the theory, sugar molecules are preferentially excluded from the protein surface, which results in a loss in entropy. Stabilization in the dried state is mainly ascribed to two phenomena, “water replacement” [8] and “vitrification” [9]. Water replacement describes a thermodynamic stabilization suggesting that the hydroxyl groups of sugars form hydrogen bonds with the protein as water molecules are removed. Vitrification describes the kinetic stabilization by immobilizing the protein in a glassy matrix. More recent literature refined the vitrification theory hypothesizing that rather local mobility than global mobility of the sugar matrix is important for protein stability [10].

In particular sucrose was found to be a potent stabilizer and is used in most freeze-dried biopharmaceutical formulations approved by the FDA within the last years [11]. However, sucrose has constraints due to its low glass transition temperature of the maximally freeze-concentrated solution (T_g') as well as in the dry state (T_g) [12, 13].

In order to obtain pharmaceutically elegant lyophilisates, collapse during freeze-drying needs to be avoided. Collapse is mostly considered an unacceptable defect and may lead to rejects [14]. Therefore, the product temperature (T_p) during primary drying should not exceed the collapse temperature (T_c) of the formulation. T_c in turn, is generally 2-3°C above T_g'. Collapse is defined as the loss of pore structure induced by a viscous flow of the glassy matrix at temperatures above T_g'/T_c. Collapse does not only indicate a freeze-drying cycle not under control, but it may also lead to increased reconstitution times and residual moisture in the freeze-dried drug product. Higher residual moisture results in a lower T_g [15]. The vitrification theory suggests that in the rubbery state above T_g, immobilization and thus sufficient stabilization of the protein can get lost. Grasmeijer *et al.* showed that T_g should be at least 10-20°C above the storage temperature in order to ensure protein stability [16]. Presumably for this reason, many approved freeze-dried drug products containing sucrose need to be stored at 2-8°C [11]. In addition, formulations containing sucrose require conservative, thus time-consuming and costly freeze-drying cycles at low T_p.

In the view of these challenges, alternatives to sucrose are needed. Polysaccharides such as inulin [17, 18], hydroxyethyl starch [19], or dextran [20] exhibit markedly higher T_g' and T_g than sucrose. Hence, they could allow for drying at higher T_p and thus faster freeze-drying without collapse. This would have multiple benefits: increased production turnover at lower costs per freeze-drying cycle, less rejects during 100% visual inspection, and finally easier and thus faster technical transfer due to increased cycle robustness. Furthermore, a higher T_g of the freeze-dried product can potentially allow for storage at room temperature.

Dextran is an interesting polysaccharide to include in the freeze-dried products since dextran 40 kDa and 70 kDa are approved for parenteral use as plasma expander. Moreover, dextran 40 kDa has already been used as excipient in a marketed freeze-dried antibody-drug conjugate formulation [21]. The protein stabilizing effect of dextran has been reported diversely for different molecular weight dextrans, different proteins, and formulation compositions. Some literature suggests that dextran may not be able to sufficiently stabilize proteins upon freeze-drying and subsequent storage, due to limited interaction of the rigid polysaccharide with the protein. More precisely, Tonnis *et al.* reported that rigid dextran provided inferior protein stability (hepatitis B surface antigen, lactate dehydrogenase, β-galactosidase, insulin) compared to flexible inulin molecules of approximately the same size (6 and 4 kDa) [17]. Allison *et al.* demonstrated that in order to achieve good protein stability of actin, a globular multifunctional 42 kDa protein, sucrose needed to be added to dextran 200 kDa [22]. In contrast, Santana *et al.* and Larsen *et al.* found improved stability of rhEGF and LDH lyophilisates using dextran 40 kDa compared to sucrose upon storage at 40°C [20, 23]. In another study by Gloger *et al.*, low molecular weight dextran of 1 kDa was more effective to stabilize a 57 kDa bispecific protein during freeze-drying compared to higher molecular weight dextrans [24]. Overall, to the best of our knowledge, no systematic research has been presented studying the impact of different molecular weight dextrans on the solid state characteristics of proteinaceous lyophilisates. In addition, the effect of dextran on the stability of freeze-dried monoclonal antibodies is unclear.

The aim of this study was therefore to investigate the application of dextran of different molecular weight for freeze-dried monoclonal antibody (mAb) formulations. This would allow for aggressive freeze-drying cycles resulting in pharmaceutically elegant lyophilisates, which could be stored at elevated temperatures. Dextran 1, 10, 40, 150, and 500 kDa alone and mixtures of these dextrans with sucrose were investigated in terms of thermal properties, cake appearance, reconstitution time, residual moisture as well as specific surface area using bovine serum albumin as model protein. In a second step, two mAb formulations containing either dextran alone or 1:1 mixtures (w/w) of dextran and sucrose were characterized including protein stability at elevated storage temperatures.

MATERIALS AND METHODS

Manufacturing

Materials and formulations

BSA formulations. BSA Fraction V (Roche Diagnostics, Penzberg, Germany) was used as model protein. It was formulated at 10 mg/mL in a 20 mM histidine/histidine-HCl buffer (Ajinomoto, Tokyo, Japan) at

pH 6.0 containing 0.02% polysorbate 20 (Croda International, Snaith, UK) and 80 mg/mL additional excipients. The total excipient content consisted of mixtures of different dextrans (1, 40, 150, and 500 kDa, Pharmacosmos, Holbaek, Denmark) with sucrose (Ferro Pfanstiehl Company, Mayfield Heights, Ohio) as shown in Table 1. The formulations were sterile filtered through a 0.22 µm hydrophilic PVDF filter unit (Millipore, Bedford, MA, USA). 20 mL Fiolax® vials (Schott, Müllheim, Germany) were filled with 10.6 mL and partially stoppered with 20 mm Lyo-stoppers D777-1 (DAIKYO Seiko Ltd., Tokyo, Japan) before freeze-drying.

mAb formulations. Two different proprietary monoclonal antibodies mAb1 (IgG_1, pI ~9.4, 148 kDa) and mAb2 (IgG_1 pI ~8.2, 149 kDa) were prepared in formulations with 10 mg/mL mAb in 20 mM histidine/histidine-HCl buffer pH 6.0 containing 0.02% polysorbate 20. mAb1 was formulated with either 80 mg/mL sucrose as reference, with dextran of different molecular weight (1, 10, 40, 150, and 500 kDa), or with 1:1 dextran/sucrose mixtures according to Table 1. 2.7 mL were filled into 6 mL Fiolax® vials (Schott) stoppered with 20 mm Lyo-stoppers D777-1 (DAIKYO). Formulations were freeze-dried and subsequently stored at 40°C/75% relative humidity for 14 days.

Freeze-drying

Freeze-drying was performed on a FTS LyoStar II freeze-dryer (FTS Systems Inc, Stone Ridge, NY). To reduce edge effects, edge vials were filled with sucrose solution and not further evaluated. The vials filled with formulations were randomly distributed over the three shelves. After an equilibration step at 5°C for 1 h, the shelves were cooled to -35°C with a shelf cooling rate of 0.3°C/min and equilibrated at -35°C for 3 h. Vacuum (133 µbar) was subsequently applied and primary drying was performed at +10°C (ramp rate 0.2°C/min). For secondary drying, the shelves were heated to +25°C (ramp rate 0.2°C/min, 133 µbar) and kept for 8 h. The vials were stoppered under nitrogen at 760 mbar. After unloading, the vials were sealed with aluminum crimp-caps.

Table 1. Composition of protein formulations. All formulations were prepared in 20 mM His/His-HCl buffer pH 6.0 containing 0.02% polysorbate 20. Pure sucrose (80 mg/mL, Suc) was used as a reference formulation.

		Excipient[1]			BSA (mg/mL)	mAb (mg/mL)
		Sucrose (mg/mL)	Dextran (mg/mL)	% of dextran (w/w)		
BSA formulations						
Dex[2]/Suc Ratio	100/0	-	80	100	10	-
	80/20	16	64	80	10	-
	60/40	32	48	60	10	-
	40/60	48	32	40	10	-
	20/90	64	16	20	10	-
	0/100	80	-	0	10	-
mAb formulations						
Dex1 kDa		-	80	100	-	10
Dex10 kDa		-	80	100	-	10
Dex40 kDa		-	80	100	-	10
Dex150 kDa		-	80	100	-	10
Dex500 kDa		-	80	100	-	10
Dex1 kDa /Suc		40	40	50	-	10
Dex10 kDa /Suc		40	40	50	-	10
Dex40 kDa /Suc		40	40	50	-	10
Dex150 kDa /Suc		40	40	50	-	10
Dex500 kDa /Suc		40	40	50	-	10
Suc		80	-	0	-	10

[1] total excipient content for all formulations is 80 mg/mL
[2] Dextran (Dex) types (MW): 1, 40, 150, or 500 kDa

Thermal properties

Differential scanning calorimetry

T_g' and T_g were determined by differential scanning calorimetry (DSC) using a Tzero™ DSC Q2000 (TA Instruments Inc., New Castle, Delaware) instrument.

For determination of T_g', 20 µL solution were pipetted into a T_{zero} aluminum pan and hermetically sealed. The sample was frozen at 3°C/min to -50°C. After an isothermal equilibration phase of 10 min the temperature was increased with 1°C/min to 20°C. T_g' was reported as midpoint at half height of the glass transition step. Measurements were performed in triplicates and T_g' was reported as mean (standard deviation < 0.2).

For analysis of T_g, 5-10 mg of the lyophilisate were transferred into a T_{zero} aluminum pan, compacted, and hermetically sealed under nitrogen. The sample was equilibrated at -20°C and in a first scan heated at 20°C/min to either 100 or 150°C (depending on the formulation). After cooling down to -20°C again, a second heating step at the same rate to 150°C or 250°C (depending on the formulation) was performed. T_g of the second heating step was reported as midpoint at half height of the glass transition step. Each formulation was analyzed in triplicates and was reported as mean with standard deviation.

Freeze-drying microscopy

The temperature of onset of collapse (T_c) of BSA-based formulations was determined by freeze-drying microscopy (FDM) using a Linkam FDC196 freeze-drying stage (Linkam Scientific Instruments, Surrey, UK) and a Zeiss Axio Imager.A1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany) with a 20-fold magnification. Pictures were captured in a 5 s interval during the freezing step and a 2 s interval during heating, using a digital camera. A sample volume of 2 µL was cooled with 3°C/min until the respective formulation was frozen, followed by a fast freezing rate of 5°C/min to -45°C and a 10 min isothermal equilibration. Full vacuum was applied after 8 min. The samples were heated under full vacuum at 3°C/min to 10°C above the expected T_c and then at 1°C/min until the formulation collapsed. T_c was determined in single measurements as onset of collapse, where first gaps were visible.

Product quality attributes

Cake appearance

Cake appearance was evaluated by visual inspection and lyophilisates were classified as either pharmaceutical elegant or classified by the extent of defects such as cracks, dents, and collapse. Type and extent of defects are reported as percentage of in total 20 vials evaluated per formulation. The term ‘defect’ is used throughout the manuscript to indicate deviations from pharmaceutically elegant cake appearance in order to compare formulations that were lyophilized with the same freeze-drying cycle. Defects were not formally classified into critical or non-critical differentiating, which means into cosmetic defects and defects relevant to protein stability, but are discussed with regard to this point.

Residual moisture

Residual moisture was determined using a C30 Coulometric Karl Fischer titrator (Mettler Toledo, Greifensee, Switzerland). The lyophilized vial was weighed and the sample was reconstituted in dry methanol. The vial was vortexed, ultrasonicated, and allowed to dissolve in total 1 h before Karl Fischer titration. An aliquot was extracted with a 10 mL BD syringe, transferred into the measuring cell, and titrated with standardized KF reagent. The vial was washed with water and ethanol and heat-dried at 40°C for 10 min in order to determine the weight of the cake by reweighing the empty vial. Karl Fischer titration was performed for three vials per formulation and the residual moisture was reported as mean value in percent per weight of the total lyophilized cake with standard deviation.

Specific surface area

Specific surface area (SSA) was determined according to Brunauer-Emmett-Teller (BET) using the Quadrasorb evo surface area and pore size analyzer (Quantachrome, Odelzhausen, Germany) with Krypton as adsorbate. At least 300 mg lyophilisate were transferred into a 9 mm bulb sample cell. Samples were degassed overnight under vacuum at 40°C followed by nitrogen overlay prior to analysis. Krypton adsorption at 77 K was measured for nine measuring points over a pressure range of 0.05-0.25 mbar. SSA was determined by fitting the data points using the BET equation. Each formulation was analyzed in triplicates and SSA was reported as mean with standard deviation.

Reconstitution time

Reconstitution time was determined by a stopwatch and reported in minutes and seconds. 10 mL water for injection were injected from a 20 mL BD syringe through the stopper onto the inner glass wall of the vial, which was held slightly titled. Timing started when injection was completed. The vial was gently shaken manually. Timing was stopped when no solid residues were visible. Reconstitution was performed in triplicates and was reported as mean with standard deviation.

Viscosity

Dynamic viscosity was determined using a MCR 301 plate and cone rheometer (Anton Paar AG, Zofingen, Switzerland) at 20°C. The rheometer was equipped with a 25 mm, 0.5° measuring cone (Anton Paar AG). A sample volume of 70 µL was used and the shear rate was increased from 0 to 1000 s^{-1} within 2 min. Viscosity was reported as mean of 24 measurement points at a shear rate of 1000 s^{-1}.

Protein stability

Size exclusion high-performance liquid chromatography

To assess monomer loss and soluble aggregates of the mAb during storage, size exclusion high-performance liquid chromatography (SE-HPLC) was performed on an Alliance 2695 HPLC instrument (Waters Corporation, Milford, MA) equipped with a 2475 fluorescence detector (Waters Corporation). The reconstituted samples were diluted to a mAb concentration of 1.0 mg/mL with mobile phase. 5 µL of diluted sample were injected into a TSKG3000SWxl, 7.8 × 300 mm column (Tosoh Bioscience, Stuttgart, Germany) and eluted with mobile phase containing 0.2 M K_2HPO_4/KH_2PO4 and 0.25 M KCl of either pH 7 (mAb1) or pH 6.2 (mAb2) at a flow rate of 0.5 mL/min. The auto sampler was set to 5°C and the column temperature was 25°C. Fluorescence was measured at λ_{ex}278/λ_{em}350 nm. High molecular weight species (HMWs) were reported as percentage of total peak area using the Empower 3 Chromatography Data System software (Waters Corporation).

Capillary isoelectric focusing

Charge heterogeneity of mAb1 was studied by imaged capillary isoelectric focusing (cIEF) using an iCE280 Analyzer (ProtenSimple, San Jose, CA) with a FC-coated capillary cartridge (ProteinSimple). Anode solution was 0.08 M phosphoric acid and cathode solution was 0.1 M NaOH, both with 0.1% methylcellulose (MC). mAb1 formulations were diluted with purified water to a concentration of 2.5 mg/mL. A reagent mix of diluted mAb1 samples with 1% MC, Pharmalyte 8-10.5 (GE Healthcare, Chicago, IL), Biolyte 7-9 (BioRad, Cressier, Switzerland), lower (8.18) and upper (9.77) pI marker (ProteinSimple), and 8 M urea was prepared. Samples were focused for a time period of 11 min and detection was performed at 280 nm. Acidic species were reported as percentage of total peak area.

Boronate affinity chromatography

Separation of glycated and non-glycated mAb1 was performed on a TSKgel Boronate 5PW, 7.5 cm x 7.5 mm column (Tosoh Bioscience) using the same HPLC system as described above equipped with a 2487 UV detector (Waters Corporation). The column was equilibrated at 40°C with mobile phase A (100 mM Hepes, 200 mM NaCl, 70 mMTris, pH 8.6) at a flow rate of 1 mL/min and conditions were maintained after injection for 10 min. Within 5 min, a linear gradient of mobile phase B (mobile phase A + 500 mM sorbitol) from 0 to 100% was applied. Conditions were kept for 3 min followed by a change of mobile phase to initial conditions within 1 min. These washing conditions were kept until a total runtime of 30 min. Signal was detected at 280 nm and percentage of glycated mAb1 was reported as area percent.

Mass spectrometry

Native size exclusion chromatography with on-line mass spectrometry (SEC-MS) was performed to further characterize the HMWs of mAb1. Sample separation was performed using a Vanquish UHPLC+ focused chromatography system (Thermo Scientific). 20 µg mAb1 were injected onto an ACQUITY UPLC Protein BEH SEC Column, 200A, 1.7 µm, 4.6mm x 300 mm (Waters Corporation) and eluted with 100 mM ammonium acetate at a constant flow rate of 0.25 mL/min. A column temperature of 25°C was used and the autosampler was set to 4°C. On-line mass spectrometry was performed using an Exactive Plus EMR Orbitrap mass spectrometer (Thermo Scientific) with electron-spray ionization source. Data analysis was performed manually. 25-fold charged spectra were reported.

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was used to confirm covalent glycation of mAb1. Prior to analysis, samples were derivatized. Reduction was performed by addition of 0.4 M Tris, 8 M guanidine hydrochloride and 650 mM DTT, and incubation for 1 h at 50°C. The samples were subsequently diluted with a solution of iodoacetic acid in purified water and incubated for 30 min under light protection for decarboxymethylation. Finally, the samples were desalted via Protein Desalting Spin Columns (Thermo Scientific).

Sample separation was performed using a UPLC system (Thermo Scientific) on an ACQUITY UPLC Protein BEH300 C4 reversed-phase column, 2.1 x 50 mm, pore size 1.7 µm (Waters Corporation). The column temperature was set to 80°C and the column was equilibrated with 80% Eluent A (0.1% formic acid in purified water) and 20% Eluent B (0.1% formic acid in acetonitrile). Initial conditions were maintained for 4 min after injection, then a gradient was applied, increasing Eluent B to 50% within 5.6 min and up to 100% within further 1.4 min. Conditions were kept for 2 mins followed by a change to initial conditions within 5 min. A constant flowrate of 0.4 mL/min was used throughout the gradient.

Mass spectrometry was performed on an Impact II Mass Spectrometer (Bruker, Billerica, MA) with electrospray ionization source. Data analysis was performed with the DataAnalysis software (Ver. 4.3, Bruker). The deconvoluted singly charged spectra were generated by using the MaxEnt V. 3.4.1 software [25].

RESULTS

Thermal properties (T_g' and T_c)

Characterization of the thermal properties of the liquid formulation (T_g' and T_c) is essential for the design of the primary drying process parameters in freeze-drying. The impact of weight fraction and M_W of dextran on T_g' is shown in Table 2. In general, a stepwise increase of T_g' with higher dextran fraction was observed. Addition of 20% dextran increased T_g' by 0.9 to 1.7°C compared to pure sucrose formulation. A remarkable increase in T_g' by approximately 8°C compared to sucrose was observed when a dextran/sucrose ratio of at least 60/40 was used, resulting in T_g' values of approximately -21°C for dextran 40, 150, and 500 kDa. For dextran 1 kDa a smaller impact on T_g' with a T_g' of -24°C at a 60/40 ratio was observed. Complete substitution of sucrose resulted in T_g' values of approximately -10°C for dextran 40, 150, and 500 kDa, which is almost 20°C higher compared to the pure sucrose formulation. Identical results were obtained for mAb1 and mAb2 formulated in 100% dextran (s. Table S1 in the supporting information) and formulations with 1:1 dextran/sucrose mixtures showed a T_g' of approximately -24°C, which also was in line with the trend observed for BSA formulations. There was no considerable difference in T_g' between dextran 40, 150, and 500 kDa. In contrast, 100% dextran 1 kDa showed a lower T_g' of -20.3°C. T_g' values for placebo formulations compared to 10 mg/ml BSA formulations differed most for sucrose as excipient. The sucrose formulation with BSA showed a T_g' of -28.7°C which is 3°C above the placebo formulation, while no significant difference was observed for 100% dextran formulations with or without BSA. T_g' is indicative for the maximal T_p during primary drying in order to avoid collapse of the freeze-dried matrix. In order to determine the actual collapse temperature, FDM was performed. The T_c values were in good agreement with the T_g' results obtained by DSC. Onset of collapse was generally observed at temperatures slightly above T_g', as it is often described in literature [26-28]. T_c for dextran 1 kDa formulations was 1°C above T_g', whereas dextran 40, 150, and 500 kDa showed higher differences (up to 5°C) between T_g' and T_c when mixed with sucrose, which became lower again for formulations with 100% dextran.

Table 2. Thermal properties for BSA formulations. (P) = T_g' for formulations without protein. (T_g'/T_g: n=3, T_c: n=1).

Type of Dextran	Dex/Suc Ratio	Thermal properties		
		T_g'	T_c	T_g
1 kDa	Dex100/Suc0	-20.3 -19.8 (P)	-19.0	120.6 ± 1.4
	Dex80/Suc20	-22.9	-20.9	95.4 ± 4.5
	Dex60/Suc40	-24.8	-23.5	98.8 ± 2.2
	Dex40/Suc60	-26.5	-25.5	74.3 ± 1.2
	Dex20/Suc80	-27.6	-26.0	62.1 ± 2.4
40 kDa	Dex100/Suc0	-11.0 -10.9 (P)	-9.4	195.9 ± 0.9
	Dex80/Suc20	-16.4	-12.1	164.8 ± 0.8
	Dex60/Suc40	-20.9	-15.1	128.9 ± 1.9
	Dex40/Suc60	-24.7	-19.7	92.9 ± 4.8
	Dex20/Suc80	-27.4	-23.2	72.0 ± 0.1
150 kDa	Dex100/Suc0	-10.7 -10.2 (P)	-9.5	197.8 ± 0.6
	Dex80/Suc20	-15.9	-11.2	164.2 ± 0.6
	Dex60/Suc40	-20.8	-16.0	127.2 ± 1.2
	Dex40/Suc60	-24.7	-20.5	93.6 ± 1.4
	Dex20/Suc80	-27.4	-26.6	74.2 ± 4.1
500 kDa	Dex100/Suc0	-10.7 -10.4 (P)	-7.9	201.9 ± 0.4
	Dex80/Suc20	-16.1	-15.6	163.6 ± 0.8
	Dex60/Suc40	-21.1	-16.1	128.6 ± 1.2
	Dex40/Suc60	-25.1	-19.8	91.8 ± 0.6
	Dex20/Suc80	-27.0	-23.4	74.2 ± 3.5
none	Dex0/100	-28.7 -31.8 (P)	-30.3	42.7 ± 0.5

Product quality attributes

T_g values of the freeze-dried product are an important parameter for the upper limit of storage temperatures but are also dependent on residual moisture acting as a plasticizer [29]. Residual moisture was highest for the sucrose formulation (2.8% ± 0.7) and residual moisture levels decreased with higher dextran content and M_W (Figure 1a). In general, when using the same freeze-drying cycle, residual moisture levels of dextran 1 kDa formulations were higher (2.2% to 0.8%) compared to dextran 40, 150, and 500 kDa formulations. Dextran 500 kDa had a moisture level of 1.8% for 20% dextran and 0.7% for 100% dextran. Lower residual moisture levels correlated with higher T_g values and increasing SSA at higher dextran/sucrose ratio formulations. Freeze-dried BSA with 80 mg/mL sucrose showed the lowest T_g with a temperature of 42°C. For all other formulations, T_g was above 60°C and even above 190°C for 100% dextran 40, 150, and 500 kDa (Table 2). As observed already for T_g', stepwise addition of dextran resulted in higher T_g values following the Gordon-Taylor equation [30], which predicts the T_g of amorphous mixtures. This was independent on the M_W of dextran above 40 kDa ($R^2 > 0.997$).

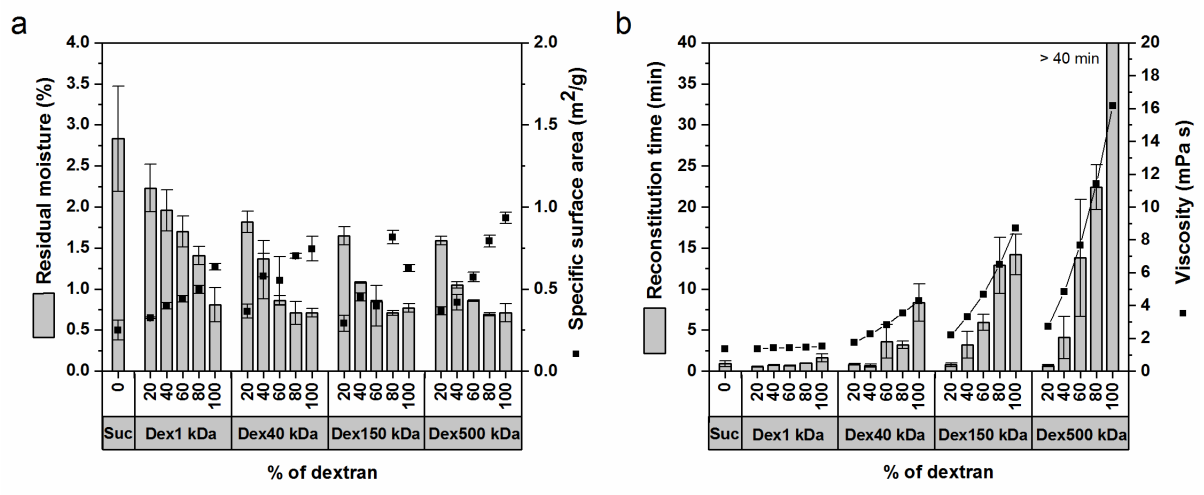
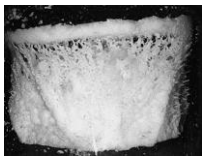
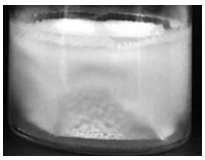
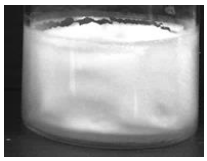
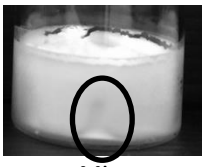
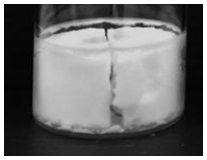


Figure 1. Quality attributes of BSA formulations in the solid state. (a) Residual moisture and specific surface area (n = 3). (b) Reconstitution time (n=3) and viscosity.

Additional important solid state characteristics are cake appearance and reconstitution time. 100% visual inspection was performed to evaluate cake appearance. Deviations from elegant cake appearance were classified in three categories: collapse, dents, and cracks. It was further differentiated between minor/partial and major/total defect as shown in Table 3. Overall, T_g'/T_c were reflected in cake appearance. All vials of the sucrose formulation were collapsed as T_p during freeze-drying was 6°C above the collapse temperature. Collapse was slightly reduced for dextran 1 kDa at 20/80 or 40/60 ratio, but still partially existing. For 100% dextran 1 kDa, neither collapse nor dents were observed. While no collapse was observed for dextrans of higher M_W at any ratio independent of M_W, dents were still present particularly at lower dextran fractions. Interestingly, despite the same T_g' and T_c, dextran 40 and 150 kDa at 20/80 ratio with sucrose showed major dents, while dextran 500 kDa containing products were pharmaceutically elegant at any ratio with only minor but consistent dents at a dextran/sucrose ratio of 20/80. At dextran/sucrose ratios of 60/40 or higher, practically no dents (minor dents in two vials of dextran 150 kDa) were observed for dextran 40, 150, and 500 kDa. In general, at high dextran weight fractions, cakes showed a higher degree of cracking which was more pronounced with increasing M_W of dextran.

Reconstitution times increased drastically at increasing weight fraction of dextran and with increasing M_W (Figure 1b). Reconstitution of a formulation with dextran 150 kDa at a dextran/sucrose ratio of 60/40 took more than 10 min and more than 40 min were necessary for complete re-dissolution of the product based on 100% dextran 500 kDa. The long reconstitution times were mostly due to a very small solid residue which remained in an otherwise clear solution and dissolved only slowly within the solution. Longer reconstitution went along with increasing viscosities for higher dextran/sucrose ratio formulations (Figure 1b).

Table 3. Categories and evaluation of cake defects with exemplary images. The percentage of 20 inspected vials with defects is reported. Defects were classified as t=total, p=partial, m=minor, and M=major.

Defect category	Type of Dextran	Dex/Suc Ratio	Percentage and extent of defects		
			collapse	dents	cracks
Collapse  Total  Partial	1 kDa	100/0	-	-	15% (m)
		80/20	-	100% (m)	-
		60/40	-	100% (m)	-
		40/60	30% (p)	-	-
		20/80	100% (p)	-	-
	40 kDa	100/0	-	-	-
		80/20	-	-	40% (m)
		60/40	-	-	-
		40/60	-	100% (m)	-
		20/80	-	100% (M)	-
Dents  Major  Minor	150 kDa	100/0	-	-	-
		80/20	-	-	70% (M)
		60/40	-	10% (m)	-
		40/60	-	75% (m)	-
		20/80	-	100% (M)	-
Cracks  Major  Minor	500 kDa	100/0	-	-	65% (M)
		80/20	-	-	85% (M)
		60/40	-	-	-
		40/60	-	-	-
		20/80	-	85% (m)	-
	none	0/100	100% (t)	-	-

Protein stability

Protein stability of two different mAbs was investigated after freeze-drying and after storage at 40°C for 14 days for formulations in either pure dextran 1, 10, 40, 150 or 500 kDa or 1:1 mixtures of dextran with sucrose. A formulation with 80 mg/mL sucrose was used as reference. Protein stability was analyzed with respect to soluble aggregate formation by SE-HPLC and changes in the distribution of charge variants by cIEF. The reference formulation with sucrose did not change in HMWs level after freeze-drying or storage (shown for mAb1 in Figure 2a). In contrast, formulations with 100% dextran 40, 150, and 500 kDa showed slightly higher HMWs (~1-3%) directly after freeze-drying. After storage at 40°C, the amount of HMWs strongly increased for all formulations. Dextran 500 kDa showed 12.4% HMWs which became even higher for lower M_W dextrans with up to 60.7% HMWs (dextran 10 kDa). While the HMW peak of dextran 1 kDa increased only marginally by 0.35%, the monomer peak in the chromatogram was shifted to a higher M_W (Figure 4a). Hence, less HMWs were formed in higher M_W dextrans formulations during storage at elevated temperatures. Furthermore, the HMWs formed in formulations of higher dextran M_W eluted earlier, i.e. were larger compared to the HMWs formed in formulations with lower M_W dextrans. The same HMWs pattern was obtained for a second mAb (mAb2) in the same dextran formulations with increasing M_W (s. Figure S1a in the supporting information).

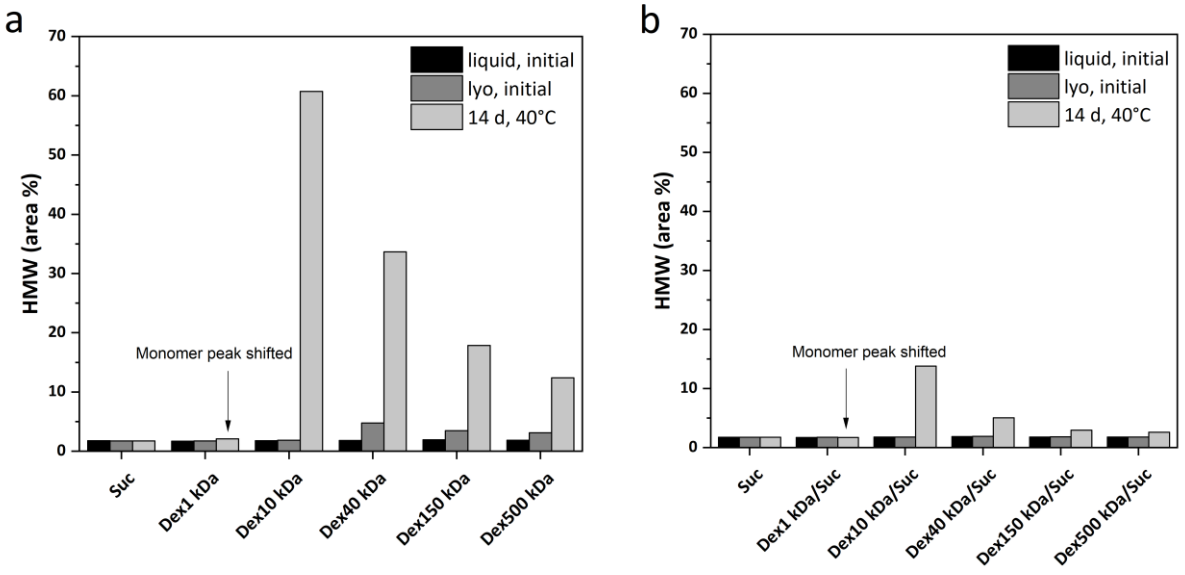


Figure 2. Amount of HMWs by SE-HPLC. 10 mg/mL mAb1 formulated with 80 mg/mL of either (a) pure dextran or sucrose or (b) 1:1 dextran/sucrose mixtures.

mAb1 formulated in equal mixtures of dextran and sucrose led to overall less HMWs (Figure 2b) compared to pure dextran formulations. No change in HMWs was observed directly after freeze-drying for any dextran/sucrose formulation. Upon storage at 40°C, the monomer peak of mAb1 in dextran 1 kDa/sucrose showed a slight shift to a higher M_W similar to the pure dextran 1 kDa formulation. All other mixtures of sucrose and dextran (10-500 kDa) showed approximately 5 times less HMWs compared to pure dextran formulations. mAb1 formulated with 1:1 dextran 10 kDa/sucrose mixtures showed the highest HMWs amount (13.8%), and HMWs of dextran 150 kDa/sucrose and dextran 500 kDa/sucrose increased by only 1.1% and 0.8%, respectively. Similar results were observed for mAb2 (s. Figure S1b in the supporting information).

Charge variants were characterized by cIEF. mAb1 formulation with pure dextran 1 kDa revealed an increase in acidic species of 13% directly after freeze-drying (Figure 3a) and a pronounced acidic shift was observed after storage at 40°C (Figure 4b). For mAb1 formulated with dextran 10 kDa or higher, no change in charge variants was observed upon freeze-drying (Figure 3a), but acidic peaks were higher after storage at 40°C. Similar to the change seen in SE-HPLC, acidic species decreased with increasing M_W of the dextrans: whereas acidic species for mAb1 formulated with dextran 10 kDa increased by 29.3% after storage, only 4-5% acidic species were formed for formulations with dextrans of higher M_W. No change in acidic species was observed for mAb1 formulated in pure sucrose as well as in 1:1 mixtures of dextran ($\geq$ 40 kDa) and sucrose upon both freeze-drying and storage. Only mAb1 formulated in 1:1 dextran/sucrose mixtures with dextran 1 and 10 kDa revealed increased acidic species during storage (Figure 3b). The instability of both formulations was less pronounced than for pure dextran formulations.

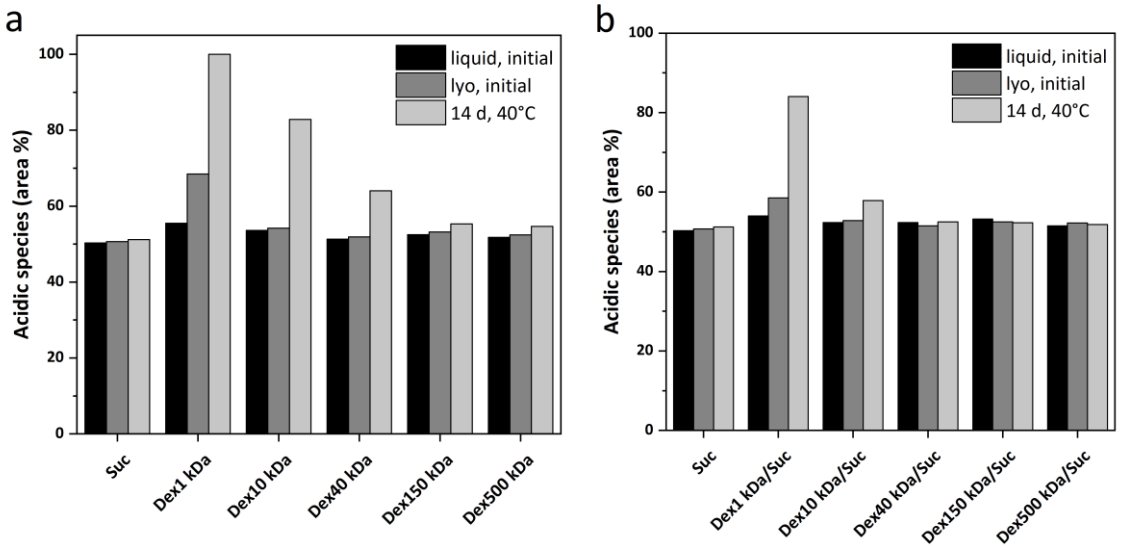


Figure 3. Amount of acidic species by cIEF. 10 mg/mL mAb1 formulated with 80 mg/mL of either (a) pure dextran or sucrose or (b) 1:1 dextran/sucrose mixtures.

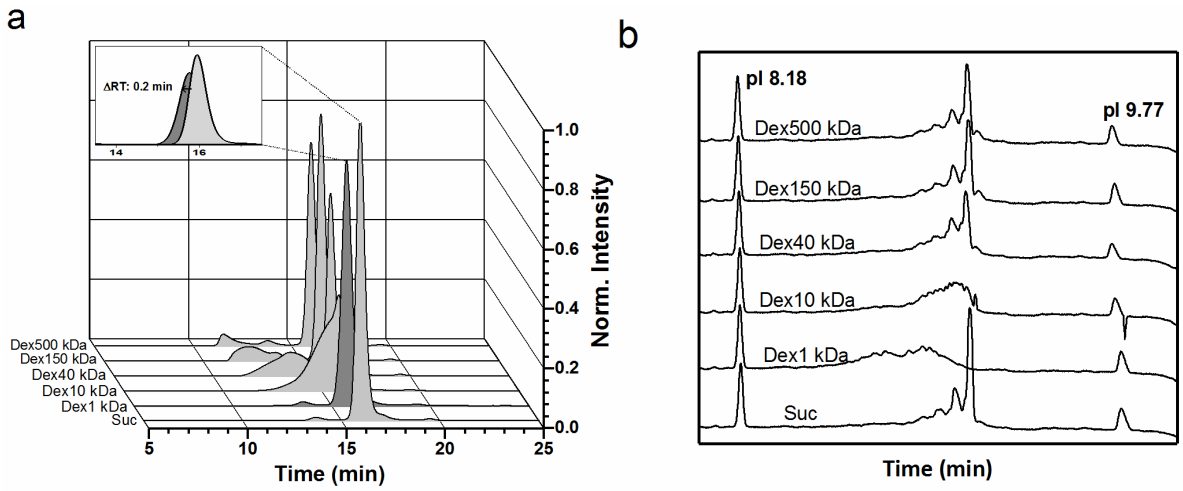


Figure 4. (a) Representative SE-HPLC chromatogram and (b) cIEF electropherogram. 10mg/mL mAb1 formulated with 80 mg/mL dextran or sucrose after storage at 40°C for 14 days.

To elucidate the root cause of HMWs formation upon storage and the drastic changes seen in cIEF, HMWs were further characterized for mAb1. We hypothesized that the increase in HMW species might be a result of glycation due to covalent binding of dextran to the mAb. Emphasis was therefore placed on boronate affinity chromatography. This analysis was accompanied by SEC-MS and LC-ESI-MS to investigate modifications of mAb1. Storage of mAb1 formulated with dextran 1 kDa at different temperatures (5, 25, and 40°C) for 14 days resulted in an increase of the retained fraction in boronate affinity chromatography. The retained fraction increased from 6.3% initially after freeze-drying to 7.4% after 14 days at 5°C and up to 80.7% after 14 days at 40°C being indicative for glycated species (Table 4).

The reference formulation (sucrose) showed a retained fraction of 3.9% only, both after freeze-drying and after storage at 40°C.

Table 4. Amount of glycated mAb by boronate affinity chromatography. 10mg/mL mAb1 formulated with 80 mg/mL of dextran or sucrose or 1:1 dextran/sucrose mixtures.

Formulation	Storage	Glycated mAb (area %)
mAb/Suc	initial lyo	3.9
mAb/Suc	14 days / 40°C	3.9
mAb/Dex1 kDa	initial lyo	6.3
mAb/Dex1 kDa	14 days / 5°C	7.4
mAb/Dex1 kDa	14 days / 25°C	22.8
mAb/Dex1 kDa	14 days / 40°C	80.7
mAb/Dex1 kDa/Suc	14 days / 40°C	73.2

Native SEC-MS was performed in order to analyze the mass of the shifted monomer peak for the dextran 1 kDa formulation. Figure 5a shows no change in mass during storage at 40°C for mAb1 formulated in sucrose. Figure 5b shows successive addition of multiple 162 Da units upon storage of mAb1 lyophilisates formulated with dextran 1 kDa. The M_W increase was negligible directly after freeze-drying, but became more pronounced upon storage at 25 and 40°C. After storage at 40°C for 14 days, mAb1 was fully converted to adducts. Maximal intensity was obtained at m/z of 6010-6040, which corresponds to an increase in M_W of approximately 2.25-3 kDa as compared to the 148 kDa of the pure mAb. This suggests that most mAb1 molecules were conjugated with approximately 2-3 dextran 1 kDa molecules. It is important to note that the M_W of 1 kDa is a normative value. Given the polydispersity of the dextran M_W, an absolute number of conjugated dextran molecules cannot be given. Finally, LC-ESI-MS, with prior reduction and alkylation of cysteines in order to break disulfide bonds, was utilized to confirm covalent conjugation of dextran 1 kDa to the mAb. Figure 6 shows mAb1 glycation in both light (Figure 6a) and heavy (Figure 6b) chain by dextran 1 kDa by several mass changes of +162 Da. This is in contrast to mAb1 formulations with sucrose, where no mass changes were observed.

For higher M_W dextrans, distinct resolution of glycated species was not possible as shown exemplary in Figure S2 in the supporting information by SEC-MS for the dextran 40 kDa formulation. The weight distribution of HMWs of mAb1 were overlaid by a broad distribution of multiple charge states, which did not allow for precise assignment of charge states and thus calculation of protein mass. Nevertheless, the obtained spectrum markedly differed from a typical native MS spectrum of pure protein aggregates (i.e. dimers, tetramers) [31], suggesting that also the HMWs of higher M_W dextran formulations are adducts between antibody and dextran.

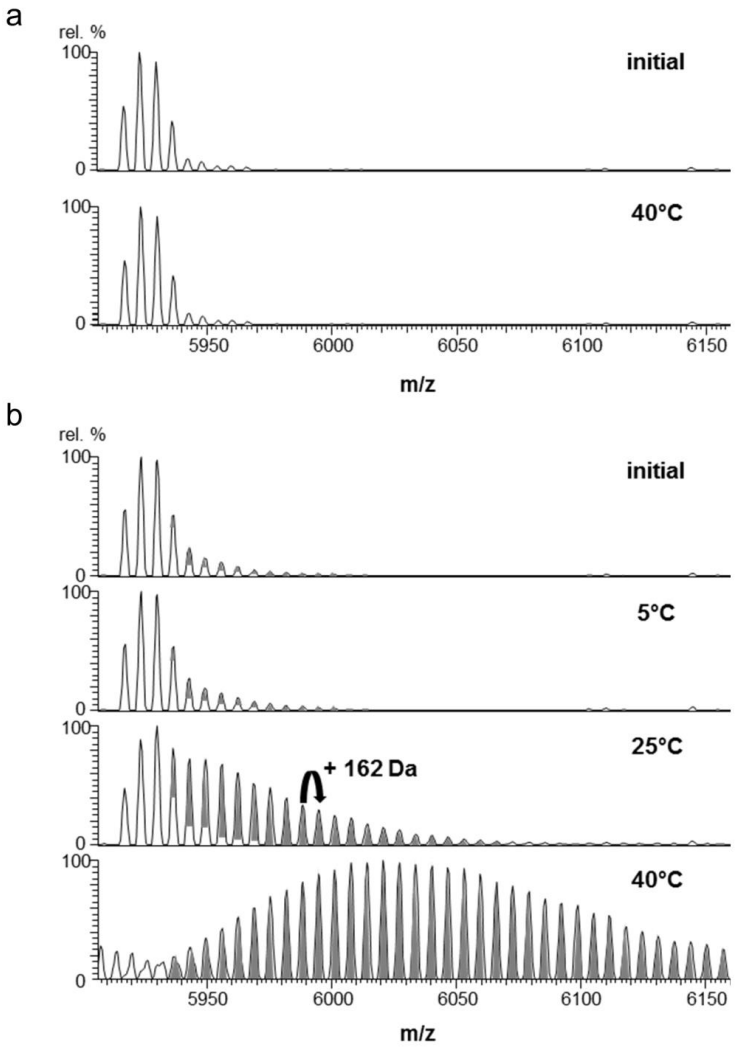


Figure 5. Native SEC-MS spectra. mAb1 formulated with (a) 80 mg/mL sucrose or (b) 80 mg/mL dextran 1 kDa initially after freeze-drying or after storage for 14 days at different temperatures. Mass changes by glycation (+162 Da) are highlighted in dark grey. z = 25.

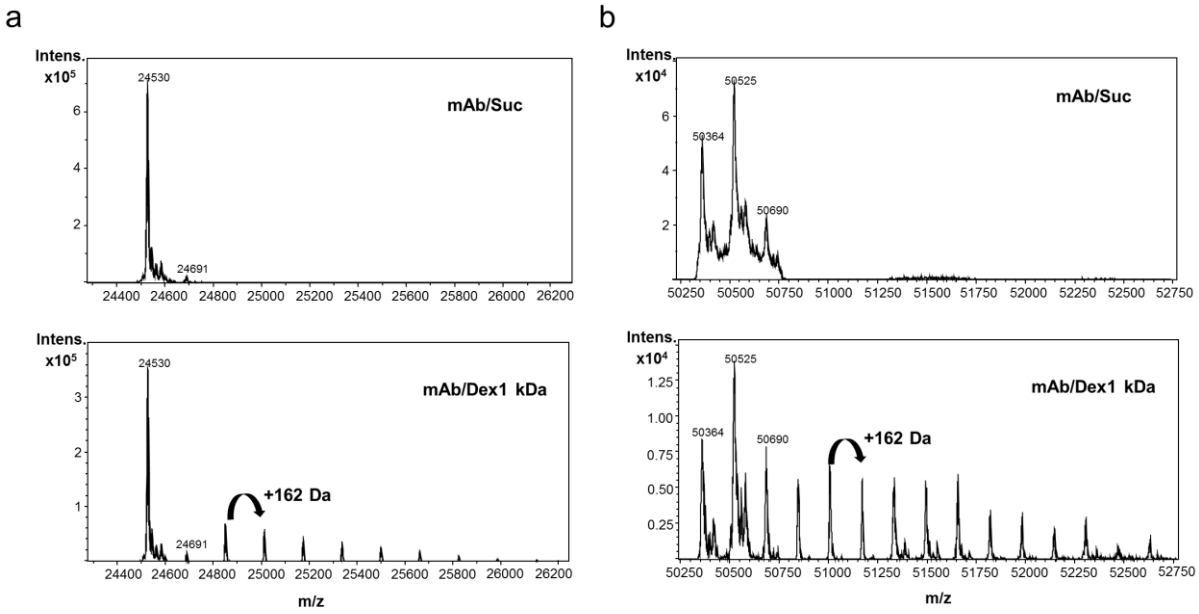


Figure 6. LC-ESI-MS spectra. (a) Light and (b) heavy chain of mAb1 formulated with 80 mg/mL sucrose or dextran 1 kDa after storage at 40°C for 14 days.

DISCUSSION

Major constraints in freeze-drying of parenteral drug products are costs and duration of the lyophilization process. The formulations often contain sucrose (T_g': -30°C) as protein stabilizer or bulking agent, requiring low primary drying temperatures for formulations without additional crystalizing bulking agent in order to avoid collapse. This results in low sublimation rates and time-consuming freeze-drying cycles. Within this study, we investigated dextrans of different M_W as potential excipients in freeze-dried formulations of mAbs, which would allow for more aggressive freeze-drying at higher T_p.

Initially, T_g' and T_c of the formulations were analyzed. Both BSA and mAb1/2 formulations with pure dextran 40, 150, and 500 kDa showed similarly high T_g' values of approximately -11°C, leading to the assumption that all of them allow for similar primary drying conditions (T_p < -11°C). This finding for protein formulations containing only dextran as stabilizer was in good agreement with a previous study by Larsen *et al.*, who characterized the T_g' of 5% (w/V) solutions in water for 10 different dextrans between 1-500 kDa and also observed a plateau in T_g' beginning at a M_W of 40 kDa [20]. Dextran 1 kDa, in contrast, would require a more gentle freeze-drying cycle at T_p < -19°C, which is still approximately 9°C higher than for pure sucrose formulations. In dextran/sucrose mixtures with dextrans of higher M_W, this increase was already achieved by adding 60% of dextran 40, 150 and 500 kDa. Furthermore, we observed an increasing difference between T_g' and T_c with increasing M_W. A T_c significantly above T_g', as frequently seen at higher protein concentrations, may be caused by higher viscosity of the solutions and their freeze concentrates [27, 32, 33]. This is supported by the increasing viscosity of the dextran formulations with higher M_W.

Consequently, the impact of dextran on drug product quality attributes like cake appearance, residual moisture, and reconstitution time as well as mAb stability at elevated temperatures was investigated. BSA and mAb1/2 formulations with different dextran/sucrose ratios were freeze-dried using a single freeze-drying cycle with an aggressive shelf temperature of +10°C during primary drying challenging cake appearance. Process optimization to maximize efficiency was beyond the scope of the study. The resulting T_p in the steady state of sublimation was approximately -24°C and consequently the sucrose formulation collapsed. The dextran containing formulations resulted in elegant cakes, sometimes with cracks or dents. Cracks have been described previously to be a result of relieved stress when unfrozen water is removed during drying [34]. Patel *et al.* suggested in a commentary on acceptable cake appearance that cracks should not be considered as critical defects being only a result of the mechanical properties of a freeze-dried formulation and in most cases without impact on protein stability [14]. While shrinkage or dents may be caused by similar physical principles as cracks, dents at the bottom of the vial can also be attributed to early onset of collapse without necessarily being detrimental to protein stability. In the present study, the occurrence of dents correlated with lower T_c/ T_g' of the formulation, i.e. indicated a T_p during primary drying close to the formulation's T_c. Interestingly, the dextran/sucrose ratio required to reduce dents increased in the order of dextran 40 < 150 < 500 kDa, although T_g' and T_c for identical ratios were similar. This indicates that T_c may not be the only relevant factor impacting local mobility during drying. Colandene *et al.* reported comparable observations for highly concentrated protein formulations which showed no collapse when freeze-dried above T_g' and even above the onset temperature for collapse, but below the temperature of full collapse. They hypothesized that higher viscosity may prevent macroscopic collapse due to limited freedom of movement [27]. The viscosity of our formulations increased for formulations with increasing M_W as well as content of dextran. The same trend was observed for reconstitution times, with up to 15 min (100% dextran 150 kDa) and even up to more than 40 min (100% dextran 500 kDa). Long reconstitution times have been described in previous publications on highly concentrated protein formulations [35-37]. Viscosity levels of such highly concentrated protein formulations are e.g. ~16 mPa s for a 151 mg/mL antibody solution [33]. This corresponds to the viscosity of our 10 mg/mL BSA formulation with 100% dextran 500 kDa. Thus, we hypothesize that both improved cake appearance and longer reconstitution times can be attributed to the increased viscosity for formulations with dextrans of high M_W. However, in regard to reconstitution time, viscosity is also not the only responsible factor. Wettability [38], cake density, and liquid penetration into the interior of the cake [36] are important, which are related to the pore structure of the cake. It is of general assumption that higher SSA leads to faster reconstitution times. However, it is important to consider that it is the combination of SSA and porosity that influences reconstitution time. For instance, recent literature reported shorter reconstitution times for lyophilisates with lower SSA, but large spherical pores [38, 39]. More precisely, Beech *et al.* and Shire *et al.* found longer reconstitution times for lyophilisates that showed dense, lamellar pores by SEM, although SSA for these samples was high [39, 40]. In a previous study related to this work, we characterized cake structure of dextran/sucrose formulations and found that dextran used in lyophilized formulations resulted in cake structures with dense lamellar pores compared to sponge like spherical pores formed in sucrose formulations [41]. Correspondingly, our formulations showed both increased SSA and slow reconstitution with higher fraction and M_W of dextran. Although, there are FDA approved products on the market that have reconstitution times up to 40 min [42-44], faster reconstitution is desirable, due to a higher patient's

convenience and a reduced risk that patients may not receive the desired dose in case of incomplete reconstitution.

Improved cake appearance and increased SSA for higher dextran proportion and M_W correlated with lower residual moisture levels, which were below 1% for pharmaceutically elegant cakes. In particular, the higher SSA and thus smaller pores allow for enhanced desorption during secondary drying. For lower residual moisture levels, molecular mobility of proteins is reduced and stability increases when the freeze-dried product is stored in the glassy state below T_g. For our dextran formulations, T_g is a function of the molecular weight fraction of the dextran but also influenced by residual moisture. T_g decreases with higher residual moisture levels [45]. For formulations with sucrose, which generally have a lower T_g, this can be detrimental for protein stability if the lyophilisates are stored at elevated temperatures. Therefore, it is generally desired to have freeze-drying processes that result in low residual moisture (~1%) [46]. However, Breen *et al.* found that intermediate residual moisture levels of 2-3% provided optimal physical stability, while lower residual moisture was beneficial in terms of chemical stability [47]. Schersch *et al.* reported less soluble aggregates if a monoclonal antibody was stored with residual moisture higher than 1% and good stability for a 70 kDa enzyme even at more than 4% residual moisture [13]. Thus, whether the low residual moisture levels obtained by addition of dextran are advantageous requires further investigation. Previous studies have reported that storage temperature should be 10-20°C below T_g to ensure complete vitrification [16]. All types of dextran investigated, already at low fractions, rendered lyophilisates with a T_g higher than 60°C up to 200°C which is far beyond intended storage temperatures. It is generally believed that there is a correlation between high T_g and improved storage stability of proteins at elevated temperature [16, 48].Thus, the significantly higher T_g compared to sucrose based formulations might potentially allow for increasing storage temperature, which is currently under investigation. Considering both, the thermal properties as well as the solid state properties of the drug product, our findings suggest that high concentrations of >80% dextran 1 kDa may be used as T_g' modifier to markedly increase T_g', to accelerate primary drying at higher shelf temperature and provide elegant cakes. For dextran 40, 150, and 500 kDa ideal dextran/sucrose ratios might be between 40/60 and 80/20, as higher dextran fractions increased reconstitution times drastically up to more than 10 min.

Dextran has been studied as excipient in lyophilisates for different types of molecules as described in the introduction section. However, its stabilizing properties have been discussed controversially [20, 22, 24]. Within these studies protein stability was investigated only in terms of protein activity or change in secondary structure. However, further characterization is necessary to understand protein stability in detail. Within our study, we assessed antibody stability in terms of relative potency by ELISA. Furthermore, protein purity was analyzed as loss of monomer and formation of soluble aggregates as well as charge heterogeneity. Finally, we performed a detailed characterization of protein modifications by boronate affinity chromatography, native SEC-MS and LC-ESI-MS.

No change in relative potency was found for mAb1 for dextran 1 kDa formulations after storage at elevated temperatures (see Table S2 in the supporting information). Similar findings were reported by Larsen *et al.* demonstrating improved retained activity of LDH in dextran 1 kDa formulations compared to sucrose, which was further increased for higher M_W of dextran [20]. However, SE-HPLC results revealed slightly higher levels of HMWs already after freeze-drying for formulations with higher M_W of dextran. A strong increase in HMW levels (or shift in monomer peak for formulations with dextran 1 kDa) was obtained after storage at elevated temperatures, which correlated with the M_W of dextrans. Typically, HMWs that are related to formation of protein aggregates result in increased levels of the

respective HMWs rather than in a change of the HMWs pattern or elution time. Thus, our observations rather suggest a chemical interaction between mAb1 and dextran than protein aggregation. As no monomer degradation was observed upon freeze/thaw with pure dextran formulations (data not shown), we hypothesize that the increase after freeze-drying could be attributed to protein aggregates as a result of insufficient protein stabilization by the large and rigid polysaccharide molecules. However, the increase in HMWs after storage in contrast, might be attributed to a chemical interaction between protein and dextrans. In addition, the fact that mAb1 revealed no change in acidic species directly after freeze-drying but after storage further supports this hypothesis.

A shift in acidic species during storage was observed by cIEF, which again was more pronounced for dextrans of lower M_W. An acidic shift in cIEF has been reported previously in a study by Quan *et al.* characterizing glycated forms of a rhumAb derived from cell culture [49]. This acidic shift was attributed to the formation of a Schiff base, which is the first reaction product of multiple steps of a Maillard reaction. Thus, we hypothesized that the terminal glucose units of dextran potentially led to glycation of mAb1 during storage at elevated temperatures, and manifests in the SE-HPLC chromatogram. This hypothesis was previously raised by Kanojia *et al.*, who also reported diffuse HMWs in freeze-dried TNF formulations with dextran [50]. However, no further characterization to confirm this hypothesis was done. Dextran is a polysaccharide consisting of multiple α-1,6-linked glucose units with 1,3-branching. Glucose itself is well-known to have reducing properties, which can cause glycation by the reducing carbonyl group of the sugar and amino groups of the protein (e.g. lysine residues) [51]. In order to confirm this hypothesis, HMWs formed during storage were further characterized by a panel of analytics including boronate affinity chromatography, native SEC-MS, and LC-ESI-MS. This was performed exemplarily for mAb1/dextran 1 kDa formulations, which showed a shift of the monomer to a higher M_W in SE-HPLC after storage. Boronate affinity chromatography is typically used to quantify the amount of sugar-bound (e.g. glycosylated [52] or glycated protein [51]) and unbound protein by interaction of the cis-diol groups of the sugar with the column. Indeed, boronate affinity chromatography demonstrated an increase of the retained (i.e. glycated) fraction with increasing storage temperature for dextran 1 kDa formulations. Eventually, covalent modification as a result of glycation was confirmed by LC-ESI-MS, which revealed mass changes by addition of multiple +162 Da units for both light and heavy chain of mAb1. Mass changes of +162 Da, as also suggested by SEC-MS, correspond to hexose units of the polydisperse dextran molecule. Thus, the monomer shift observed during storage was caused by glycation with oligo-glucose units, i.e. dextran molecules. The dependency of glycation on storage temperature is in line with previously published research investigating the time course of temperature dependent IgG glycation [53]. The observed trend of decreasing levels of HMWs formed in SE-HPLC, i.e. decreased glycation with increasing dextran M_W, can be explained by the lower abundance of reactive terminal groups of dextran. This lower glycation level combined with the polydispersity and the high molecular weight of dextrans made proof of glycation by MS for the dextran with a M_W of 40 kDa and above challenging: The SEC-MS spectrum of mAb1/dextran 1 kDa after storage at 40°C nicely revealed the polydispersity of the dextran. This became even more prominent with dextran 40 kDa and no spectral assignments and mass calculations could be performed (s. Figure S2 in the supporting information). Another analytical limitation for high M_W dextrans was that boronate affinity chromatography could not be applied, as the number of available cis-diols groups, which could interact with the column, is limited for large polysaccharides [54].

Although, mAb potency did not change upon storage of the dextran 1 kDa formulations, HMW species and chemical modification are generally of concern in terms of immunogenicity [55-57]. Therefore, in order to use dextran in freeze-dried mAb formulations mitigation strategies to reduce glycation are needed. By mixing dextran with sucrose in a 1:1 ratio no increase in HMWs was observed for higher M_W of dextrans directly after freeze-drying indicating enhanced lyoprotection by sucrose. This is in line with Allison *et al.*, who reported prevention of protein unfolding during freeze-drying for combinations of sucrose and high M_W dextran [22]. Furthermore, we could strongly reduce glycation for all dextrans as revealed by low HMW levels. For mAb1, glycation was for example reduced from 80.7% after storage at 40°C for 14 days in pure dextran 1 kDa formulations to 26.8% glycated fraction in formulations with 1:1 dextran 1 kDa/sucrose mixtures. We hypothesize that the improved protein stability by addition of sucrose might potentially be due to "shielding" the protein and thereby protecting its surface from glycation by dextran.

Finally, we postulate that the combination of dextran with disaccharides presents one possible mechanism to reduce glycation and thus such excipient combinations may potentially be of advantage in freeze-dried mAb formulations. Long-term stability studies of such formulations are currently ongoing.

CONCLUSION

The stepwise addition of dextran 1, 40, 150, and 500 kDa to sucrose as excipient in freeze-dried formulations of proteins increased the T_g'/T_c of protein formulations. A remarkable increase by 8°C was already achieved by 60% dextran fractions of dextran ≥40 kDa and was increased by even 18°C at 100% dextran as demonstrated for BSA and two mAb formulations. This would allow for more aggressive freeze-drying cycles at higher T_p during primary drying resulting in shorter cycles and thus lower costs. The cake appearance was markedly improved, which facilitates visual inspection and leads to overall less rejects. Care should be taken for formulations with dextran 150 and 500 kDa as fractions of more than 60% lead to unacceptable long reconstitution times. Moreover, formulations with pure dextrans showed insufficient lyoprotection of the antibody upon freeze-drying. Furthermore, the study demonstrated antibody glycation in pure dextran formulations stored at elevated temperatures. In 1:1 dextran/sucrose mixtures however, sucrose ensured protein stability upon freeze-drying and glycation during storage was strongly reduced.

Formulations scientists should use dextrans in freeze-dried mAb formulations with caution especially when used as single stabilizer and bulking agent. A mixture of medium M_W dextran with sucrose at an ideal sucrose to dextran ratio might provide the best compromise at all levels. Such excipient combinations might be used as alternative excipients for freeze-dried mAb formulations being superior compared to conventional formulations. They potentially allow for more aggressive freeze-drying cycles that result in elegant lyophilisates with good protein stability. Long-term stability studies of such formulations at room temperature and above are currently ongoing. During stability studies, it is recommended to use adequate analytical methods to detect possible glycation reactions.

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SUPPORTING INFORMATION

Table S1 shows the glass transition temperatures (T_g') of the maximally freeze-concentrated antibody formulations. For the different dextrans, similar T_g' values were observed independent of the type of protein (mAb1, mAb2, BSA). T_g' values for BSA are presented in Table 2 in the main manuscript.

Table S1. T_g' of mAb formulations. 10 mg/mL mAb formulated in 80 mg/mL of either pure dextran or sucrose or 1:1 dextran/sucrose mixtures. T_g' is based on single measurements.

Dextran Type	T_g' (°C) of mAb in pure dextran		T_g' (°C) mAb in 1:1 dextran/sucrose	
	mAb1	mAb2	mAb1	mAb2
None	-28.5	-28.9	-	-
Dextran 1 kDa	-20.5	-20.9	-25.6	-25.8
Dextran 10 kDa	-12.1	-12.1	-23.9	-23.6
Dextran 40 kDa	-10.6	-10.9	-24.2	-24.1
Dextran 150 kDa	-10.8	-10.7	-23.9	-23.9
Dextran 500 kDa	-10.5	-10.8	-23.5	-24.4

Figure S1 shows HMWs for mAb2 formed upon freeze-drying and storage. The increase in HMWs revealed the same pattern as shown for mAb1 in Figure 2 in the main manuscript, thus highlighting that protein stability effects were not specific for only one antibody.

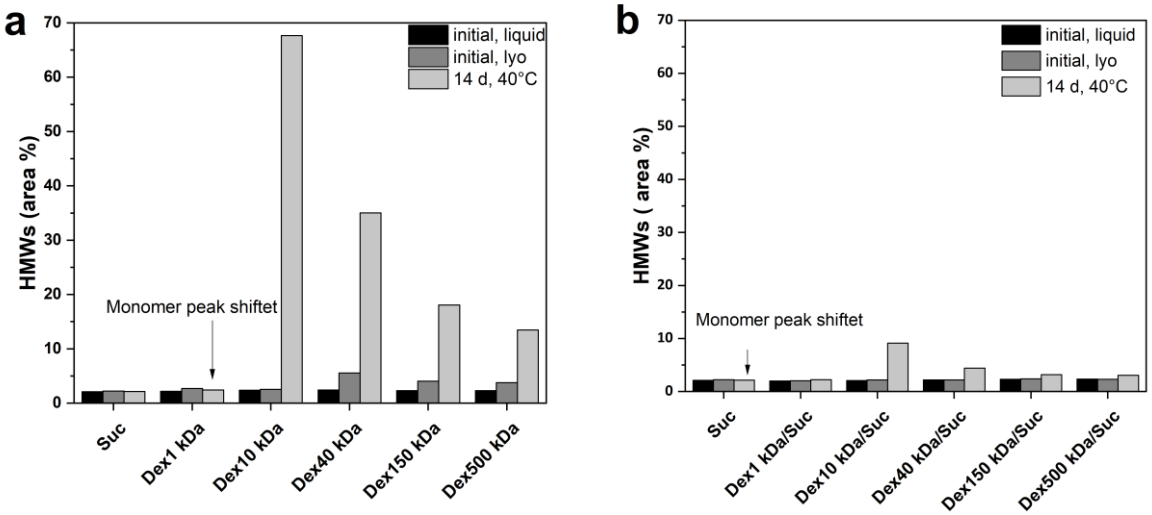

Figure S1. Amount of HMWs of mAb2 by SE-HPLC. 10 mg/mL mAb2 formulated with 80 mg/mL of either (a) pure dextran or sucrose or (b) 1:1 dextran/sucrose mixtures.

Table S2 shows results of relative potency of mAb1 determined by ELISA. Despite that glycation of the antibody was observed, CDR binding was not impaired.

Table S2. Potency by ELISA. Relative potency is given as mean % with standard deviation for mAb1 formulated in either 80 mg/mL sucrose or dextran 1 kDa. ELISA was performed in triplicates.

Formulation	Storage conditions	Relative potency (%)
mAb1/Suc	Initial	111 ± 3
mAb1/Dex1 kDa	2 weeks at 5°C	110 ± 5
	2 weeks at 25°C	111 ± 4
	2 weeks at 40°C	109 ± 2

Figure S2 shows a representative SEC-MS spectrum for mAb1 formulated in high M_W dextran (40 kDa). HMWs were not attributed to protein aggregates, but revealed a diffuse mass spectrum due to multiple charge ions. The diffuse pattern originates from the glycated mAb due to polydispersity of dextran.

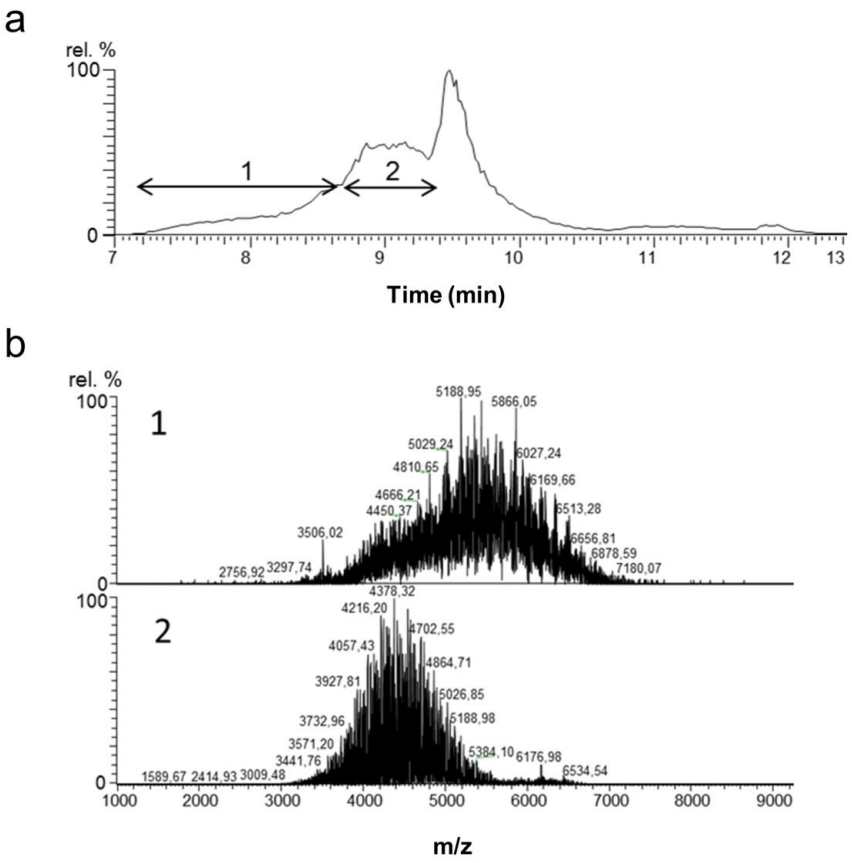

Figure S2. Exemplary SEC-MS data of mAb1 formulated with dextran 40 kDa. (a) Total ion count chromatogram. (b) Diffuse mass spectrum of two selected regions of the HMW fraction.

CHAPTER 3

Excipients for room temperature stable freeze-dried monoclonal antibody formulations

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Research Paper

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Abstract

Sucrose is a common cyro- and lyoprotectant to stabilize labile biopharmaceuticals during freeze-drying and storage. Sucrose based formulations require low primary drying temperatures to avoid collapse and monoclonal antibody (mAb) containing products need to be stored refrigerated. The objective of this study was to investigate different excipients enabling storage at room temperature and aggressive, shorter lyophilization cycles. We studied combinations of 2-hydroxypropyl-beta-cyclodextrin (CD), recombinant human albumin, polyvinylpyrroldione (PVP), dextran 40 kDa (Dex), and sucrose (Suc) using two mAbs. Samples were characterized for collapse temperature (T_c), glass transition temperature of the liquid (T_g') and freeze-dried formulation (T_g), cake appearance, residual moisture, and reconstitution time. Freeze-dried formulations were stored at 5°C, 25°C, and 40°C for up to nine months and mAb stability was analyzed for color, turbidity, visible and sub-visible particles, and monomer content. Formulations with CD/Suc or CD/PVP/Suc were superior to pure Suc formulations for long-term storage at 40°C. When using aggressive freeze-drying cycles, these formulations were characterized by pharmaceutically elegant cakes, short reconstitution times, higher T_g', T_c, and T_g. We conclude that the addition of CD allows for shorter freeze-drying cycles with improved cake appearance and enables storage at room temperature, which might reduce costs of goods substantially.

INTRODUCTION

Formulation development of monoclonal antibodies (mAbs) aims to identify excipients that maintain the protein in its intact native state. This means that the protein is stabilized throughout the drug product manufacturing process, shipment, storage, and administration to the patient. If stability cannot be assured in the liquid state, freeze-dried formulations are pursued. The absence of water in lyophilized preparations limits the molecular mobility and hereby retards degradation mechanisms, which ultimately improves storage stability. Although freeze-drying is a gentle drying method, the mAb is exposed to additional stress including cryoconcentration during the freezing step, exposure to the ice-liquid interface as well as the removal of hydrogen bonds with water molecules. Excipients are needed since they protect the mAbs during freezing (cryoprotection), drying (lyoprotection), as well as throughout storage in the dried state. To date, the majority of freeze-dried biopharmaceuticals on the market contain trehalose and more commonly sucrose as excipient, which are well known to be potent stabilizers, providing both lyo- and cryoprotection [1]. The excellent protein stabilization by sucrose is attributed to its ability i) to form hydrogen bonds with protein molecules, which is described by the water replacement theory [2] and ii) to form a glassy matrix in the dried state in which protein molecules are immobilized as described by the vitrification theory [3].

Recent literature demonstrates that stabilization via vitrification holds true as long as the storage temperature is at least 10 to 20°C below the glass transition temperature (T_g) of the freeze-dried product [4]. Dry sucrose itself has a T_g of ~60-70°C [5]. The T_g substantially decreases with increasing residual moisture of the lyophilisate [6]. For instance, a T_g of only ~42°C has been reported for a sucrose-based mAb lyophilisate with a residual moisture of ~2.5% [7]. Thus, marketed sucrose-based freeze-dried mAbs require refrigerated storage conditions at 2-8°C [8]. An exception is Mepolizumab (Nucala®) that can be stored at temperatures up to 25°C [9]. Overall, the need for refrigerated storage conditions is therefore not only related to the instability of the mAb, but is also attributed to the excipient sucrose. Being able to store freeze-dried biopharmaceuticals at room temperature would provide a considerable benefit by making cold chains redundant. Consequently, there is the strong need to look into novel excipients and excipient combinations, which combine water replacement with a high T_g enabling storage at room temperature. A higher T_g typically also comes along with a higher glass transition temperature of the maximally freeze-concentrated solution (T_g'). An increased T_g' would allow for a more aggressive lyophilization at higher product temperature (T_p) during primary drying, shortening the freeze-drying cycle. Thus, formulations with a higher T_g' and T_g will result in a strong reduction of the costs of goods of freeze-dried biopharmaceuticals by i) a shorter, more efficient manufacturing process and ii) avoiding cold chains throughout the supply chain of the drug product.

Traditionally, mannitol is often added to low concentration protein formulations as a bulking agent to improve cake appearance and to enable primary drying temperatures above the T_g' of sucrose. However, mannitol does not contribute to proteins stability due to its crystalline nature requiring a certain amount of amorphous stabilizer. On the other hand, a relatively high amount of mannitol, e.g. a 4:1 ratio of mannitol to sucrose, is needed to ensure complete crystallization of mannitol increasing the total excipient content [10, 11]. Incomplete crystallization of mannitol during freeze-drying can induce protein instability over storage. Moreover, mannitol can crystallize in different polymorphs depending on the freezing protocol and an annealing step is often used to improve crystallization and reduce primary and secondary drying times [12, 13]. Depending on the polymorph formed, protein

stability might be impaired. In the light of these challenges of mannitol containing formulations, amorphous excipients which increase the formulations T_g' and contribute to protein stability would be beneficial as substitute or additives to sucrose.

Regarding potential alternative amorphous excipients, high potential can be ascribed to 2-hydroxypropyl-beta-cyclodextrin (CD). CD is a cyclic oligosaccharide, consisting of seven glucopyranose units, and is one of the few cyclodextrins approved for parenteral use. CD remains fully amorphous after freeze-drying and can interact with the protein through its multiple hydroxyl groups [14]. It is a non-reducing sugar, which is of utmost importance to avoid glycation and covalent aggregation during storage at elevated temperatures [7, 15]. A mAb lyophilisate based on CD at high concentrations showed similar stability at accelerated conditions as a trehalose based formulation [16]. Branchu *et al.* and Iwai *et al.* investigated the amphiphilic, surfactant like properties using low concentrations (up to 1%) of CD to stabilize beta-galactosidase and lactate dehydrogenase upon spray- or freeze-drying. They found CD to be superior compared to sucrose or trehalose [17, 18]. To the best of our knowledge, no comprehensive study on the impact of CD on freeze-dried mAb formulations including long-term stability data has been reported. Furthermore, limited and controversial data is available on the T_g of CD formulations with 55°C reported for freeze-dried invertase with 10% (w/v) CD and 108°C for freeze-dried IL-2 formulated with 0.5% (w/v) CD, although both formulations had a similar residual moisture level of approximately ~2.5% [19, 20]. Another excipient repeatedly utilized in freeze-drying is polyvinylpyrrolidone (PVP) [21-23]. PVP alone will most likely not sufficiently stabilize the protein due to the sterically limited potential for hydrogen bonding between the large polymer and the large protein molecules. But, it has been hypothesized that the combination of polymers with saccharides could further improve protein stability by strengthening the glassy matrix [24]. In particular, PVP K17 is of interest as it is approved for parenteral use. Recently, human serum albumin has regained attention as excipient to stabilize therapeutic proteins with the availability of recombinant versions at larger scale and at reasonable costs. In particular, the high T_g of albumin makes it an interesting excipient for lyophilized formulations intended for storage at room temperature [25, 26]. In addition, for more than 20 years albumin is approved as excipient in parenteral protein formulations including lyophilisates like Avonex® [27]. Furthermore, dextran 40 kDa (Dex) has been shown to strongly increase T_g' and T_g in mAb formulations. However, Dex alone failed to ensure sufficient protein stability at elevated temperatures, but stability was strongly improved when used in combinations with sucrose [7].

Hence, the objective of this research was to systemically study the impact of CD, recombinant human albumin (rHA), PVP K17, and Dex, mixtures thereof, as well as combinations with sucrose on the stability of mAb lyophilisates with a focus on storage at elevated temperatures (i.e. 9 months at temperatures up to 40°C). We investigated the critical formulation temperatures in the liquid (T_c, T_g') as well as the freeze-dried state (T_g) of various formulations using two different proprietary model mAbs. Additionally, product quality attributes like cake appearance, residual moisture, and reconstitution time were analyzed after freeze-drying. Since therapeutic mAbs have a propensity to form aggregates, special emphasis was put on the analysis of loss of mAb monomer, the formation of visible and sub-visible particles, as well as changes in turbidity and color upon storage.

MATERIALS AND METHODS

Materials

Formulations

Two proprietary model monoclonal antibodies, mAb1 (IgG_1 pI ~9.4, 148 kDa) and mAb2 (IgG_1 pI ~8.2, 149 kDa), from F. Hoffmann-La Roche were formulated at a protein concentration of 10 mg/mL in 20 mM His/His-HCl buffer (Ajinomoto, Tokyo, Japan) at pH 6.0 with 0.02% polysorbate 20 (Croda International, Snaith, UK). Additional excipients with a total solid content of 80 mg/mL were added as described and abbreviated in Table 1. These excipients included 2-hydroxypropyl-betacyclodextrin (CD; Roquette, Beinheim, France), recombinant human albumin (rHA; Sigma Aldrich, Steinheim, Germany), Kollidon® 17 PF (PVP; BASF, Ludwigshafen, Germany), dextran 40 kDa (Dex, Pharmacosmos, Holbaek, Denmark) as well as sucrose (Suc; Ferro Pfanstiehl Company, Mayfield Heights, Ohio). The formulations were sterile filtered through a 0.22 µm hydrophilic PVDF filter membrane (Millipore, Bedford, MA). A volume of 3.2 mL per formulation was filled into 6 mL Fiolax® vials (Schott, Müllheim, Germany) and partially stoppered with 20 mm Lyo-stoppers D777-1 (DAIKYO Seiko Ltd., Tokyo, Japan) before freeze-drying.

Table 1. Formulation composition. All formulations contained 10 mg/mL of either mAb1 or mAb2 in 20 mM His/HCl buffer pH 6.0 with 0.02% polysorbate 20. Additional excipients used were sucrose (Suc), 2-hydroxypropyl-betacyclodextirn (CD), polyvinylpyrrolidone 17 (PVP), recombinant human albumin (rHA), and dextran 40 kDa (Dex).

Formulation	Sucrose (mg/mL)	CD (mg/mL)	rHA (mg/mL)	PVP (mg/mL)	Dex (mg/mL)
Suc	80	-	-	-	-
CD	-	80	-	-	-
CD/Suc_{low}	16	64	-	-	-
CD/Suc_{high}	24	56	-	-	-
CD/rHA_{low}	-	72	8	-	-
CD/rHA_{mid}	-	56	24	-	-
CD/rHA_{high}	-	40	40	-	-
CD/rHA/Suc	24	39.2	16.8		-
CD/PVP_{low}		72	-	8	-
CD/PVP_{mid}	-	56	-	24	-
CD/PVP_{high}	-	40	-	40	-
CD/PVP/Suc	24	39.2	-	16.8	-
CD/Dex	40	-	-	-	40
CD/Dex/Suc	24	39.2	-	-	16.8
Suc/Dex	48	-	-	-	32

Freeze-drying

A FTS LyoStar II (FTS Systems Inc, Stone Ridge, NY) was used. Samples were frozen at 0.3°C/min to -35°C. For primary drying, a vacuum of 133 µbar was applied and shelf temperature was increased by 0.2°C/min to +10°C. End of primary drying was determined when the chamber pressure monitored by a Pirani probe reached the same value as the capacitance probe. For secondary drying, vacuum remained unchanged and shelf temperature was increased by 0.2°C/min to +25°C and kept for 6 h. At the end of freeze-drying, vials were stoppered under nitrogen at 760 mbar and sealed with aluminum crimp-caps after unloading.

Thermal properties

Differential scanning calorimetry

Differential scanning calorimetry was performed on a T_{zero} DSC Q2000 instrument (TA instruments Inc., New Castle, Delaware) to determine T_g' and T_g. For determination of T_g', 20 µL were transferred into T_{zero} aluminum pans and hermetically sealed. Samples were frozen to -50°C with a freezing rate of 5°C/min followed by a 10 min isothermal phase and a heating step at the same rate up to 25°C. For determination of T_g of the lyophilized cake, 5-10 mg were weighed into T_{zero} aluminum pans under dry nitrogen and hermetically sealed. Samples were heated to either 100°C (Suc) or 150°C (all other formulations) at 20°C/min, cooled down to -20°C followed by a second heating step to 200°C or 250°C. T_g' and T_g were determined at half height of the glass transition step. Measurements were performed in triplicates and data was reported as mean ± standard deviation.

Freeze-drying microscopy

T_c was determined by freeze-drying microscopy (FDM) according to Haeuser *et al.* [7]. A Linkam FDC196 freeze-drying stage (Linkam Scientific Instruments, Surrey, UK) and a Zeiss Axio Imager A1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany) with 20-fold magnification were used. T_c was reported as the onset of collapse.

Viscosity

Dynamic viscosity was determined using a MCR 301 plate and cone rheometer (Anton Paar AG, Zofingen, Switzerland) equipped with a 25 mm, 0.5° cone (Anton Paar AG). 70 µL were analyzed at 20°C. The shear rate was increased from 0 to 1000 s^{-1} within 2 min and viscosity was reported as the mean of 24 measurement points at 1000 s^{-1}.

Product quality attributes

Cake appearance

Lyophilized cakes were visually inspected and representative pictures of the lyophilisates were taken in front of a black background. Cake appearance was evaluated based on the presence of visible cracks, dents, and collapse.

Reconstitution time

For reconstitution, 3.0 mL water for injection (Fresenius Kabi, Oberdorf, Switzerland) were injected into the vial through the stopper using a disposable syringe with the syringe tip connected to a 0.22 µm Millex® GV sterile syringe filter (Millipore, Bedford, MA) and a 21G x 1.5" needle (BD, Franklin Lakes, New Jersey). The reconstitution time was determined for three lyophilisates per formulation using a stop watch and was reported as mean ± standard deviation.

Residual moisture

Residual moisture was analyzed in triplicates by Karl Fischer titration according to Haeuser *et al.* [7]. A C30 Coulometric Karl Fischer titrator (Mettler Toledo, Greifensee, Switzerland) was used.

Protein stability

Samples were analyzed directly after freeze-drying and after 1, 3, 6, and 9 months of storage at 5°C, 25°C / 60% r.H., and 40°C / 75% r.H.. Prior to analysis, the lyophilisates were reconstituted as described above. For determination of color, turbidity, and sub-visible particles, ~2 mL of the reconstituted formulation were transferred into particle free glass tubes under laminar air flow conditions.

Visible particles

Visible particles of the reconstituted formulations were analyzed by visual inspection according to Ph. Eur. 2.9.20. in front of a black and white background [28]. Presence of visible particles was reported as I. "practically free from particles", II. "with few particles", and III. "with many particles".

Turbidity

Turbidity of reconstituted formulations was determined using a 2100AN Turbidimeter (HACH, Dusseldorf, Germany) according to Ph. Eur. 2.2.1. [29]. Data were reported as Nephelometric Turbidity Unit (NTU).

Color

Color of reconstituted formulations was determined using a LICO 690 Colorimeter (HACH). Color was classified according to the color scale described in Ph. Eur. 2.2.2. [30].

Size-exclusion chromatography

Loss of monomer was determined by size exclusion high-performance liquid chromatography (SE-HPLC) using an Alliance 2695 or an Alliance e2695 HPLC instrument (Waters Corporation, Milford, MA) equipped with a 2487 or 2489 UV/visible-detector (Waters Corporation), respectively. The autosampler was set to 5°C and the column oven to 25°C. 100 µg of mAb were injected on a TSKG3000SWxl, 7.8 × 300 mm column (Tosoh Bioscience, Stuttgart, Germany). The sample was eluted over 30 min with 0.2 M K_2HPO_4/ KH_2PO_4 and 0.25 M KCl of either pH 7.0 (mAb1) or pH 6.2 (mAb2) at a flow rate of 0.5 mL/min. Signal was detected as UV absorbance at 280 nm. Empower 3 Chromatography Data System software (Waters Corporation) was used for data processing and the loss of monomer was reported as percentage of total peak area.

Methods specific to data shown in the supporting information only are described in the supporting information. These methods comprise of sub-visible particle analysis according to USP <787> [31] and Ph. Eur. 2.9.19 [32], X-ray powder diffraction, and determination of water sorption isotherms.

RESULTS

Thermal properties and product quality attributes

T_g' and T_c were determined to assess the maximal product temperature during primary drying to avoid collapse. The data for mAb1 is summarized in Table 2 and reveal that all excipient combinations investigated within this study showed markedly higher T_g' and T_c values compared to the pure Suc formulation. Data for mAb2 were not considerably different and are provided in Table S1 in the supplementary information. Complete substitution of Suc with CD resulted in a strong increase of T_g' by 18.5°C. Addition of rHA to CD-based formulations resulted in even higher T_g' values. CD/rHA_{high} showed the highest T_g' of -9.3°C and T_c of -7.5°C amongst all tested formulations. On the contrary, adding PVP to CD caused a decrease in T_g'. However, even at the highest PVP concentration of 40 mg/mL, T_g' was 13.3°C above T_g' of the pure Suc formulation. The T_g' values of the mAb formulations with CD only or a 1:1 mixture of Dex and CD were similar. For all excipient combinations, T_g' was strongly reduced when Suc was added to the formulation with a negative linear correlation between sucrose content and T_g'. Consequently, the 48/32 mg/mL Suc/Dex formulations showed the smallest increase in T_g' of ~4°C compared to the pure Suc formulations. The T_c values were determined for mAb1 formulations and reflected the T_g' results. T_c was generally slightly (1 - 4°C) above T_g'. The greatest difference of 4°C was found for Suc/Dex.

Correspondingly to the different T_g' and T_c values, freeze-drying of the formulation with the same aggressive cycle (+10°C shelf temperature during primary drying) resulted in different cake appearances: all pure Suc-based mAb formulations were collapsed. Cake appearance improved with lowering the Suc fraction. No collapse but major dents were observed for Suc/Dex (T_c: -21.6°C), which became less pronounced for CD/PVP/Suc (T_c: -18.7°C), and completely disappeared for formulations that had a T_c of -17.8°C or higher. Formulations with high amounts of CD demonstrated a “brittle” cake appearance (s. Figure 1a) and showed prominent cracks, when ≥24 mg/mL PVP or rHA were present. Lyophilisates without any visual defects were obtained for CD/Dex/Suc and CD/rHA/Suc formulations.

Table 2. Formulation attributes. T_g', T_c, and viscosity of the liquid formulation, and reconstitution time, T_g, and residual moisture after freeze-drying. Data are shown for mAb1 formulations.

Formulation	Liquid			Lyophilisate			
	T_g' [1] (°C)	T_c (°C)	Viscosity (mPa s)	Reconstitution time (min:ss)	T_g (°C)	Residual moisture (%)	Cake appearance
Suc	-29.3	-30.3	1.3	00:51 ± 00:04	43.2 ± 5.2	4.2 ± 1.1	collapse
CD	-10.8	-10	1.4	00:45 ± 00:11	200.9 ± 1.2	0.2 ± 0.05	brittle
CD/Suc_{low}	-16.8	-14.8	1.5	00:42 ± 00:03	164.9 ± 0.1	0.11 ± 0.01	elegant – brittle
CD/Suc_{high}	-18.6	-17.8	1.5	00:39 ± 00:01	153.3 ± 0.2	0.13 ± 0.01	elegant – brittle
CD/rHA_{low}	-10.6	-9.1	1.4	01:41 ± 00:35	200.6 ± 2	0.2 ± 0.03	brittle
CD/rHA_{mid}	-10.2	-8.1	1.5	02:09 ± 00:38	199.9 ± 0.1	0.2 ± 0.02	cracks
CD/rHA_{high}	-9.3	-7.5	1.5	04:23 ± 01:33	196.3 ± 1.5	0.3 ± 0.04	cracks
CD/rHA/Suc	-18.8	-16.4	1.4	02:15 ± 00:41	153.7 ± 1.1	0.3 ± 0.05	elegant
CD/PVP_{low}	-11.7	-10.6	1.5	01:23 ± 00:10	192.2 ± 0.9	0.2 ± 0.02	cracks
CD/PVP_{mid}	-13.3	-12.2	1.6	01:30 ± 00:16	179.0 ± 3.4	0.2 ± 0.01	cracks
CD/PVP_{high}	-16.0	-14	1.7	02:22 ± 00:02	162.6 ± 2.5	0.2 ± 0.03	brittle
CD/PVP/Suc	-19.4	-18.7	1.5	00:44 ± 00:09	150.3 ± 1.6	0.2 ± 0.04	minor dents
CD/Dex	-10.6	-9.2	2.0	02:51 ± 01:11	199.3 ± 1.9	0.3 ± 0.06	brittle
CD/Dex/Suc	-19.0	-17.6	1.8	01:49 ± 00:35	157.8 ± 2.0	0.3 ± 0.04	elegant
Suc/Dex	-25.6	-21.6	2.2	03:35 ± 00:09	96.8 ± 0.6	1.1 ± 0.19	major dents

[1] standard deviation ≤ 0.2

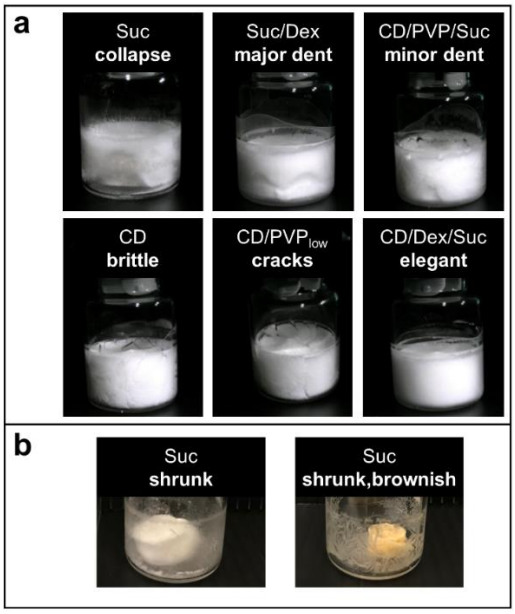

Figure 1. Representative images of different cake appearances. a) Lyophilisates after freeze-drying and b) Suc lyophilisates after storage at 40°C for 9 months.

Overall, viscosities for all formulations were similar and in the range of 1.3 to 2.2 mPa s (s. Table 2). Addition of Dex showed the strongest impact on viscosity and Suc/Dex was the formulation with the highest viscosity. A correlation between viscosity and T_c was not observed for the other formulations. Thus, there was also no clear correlation of viscosity and reconstitution time: all mAb1 lyophilisates were reconstituted within 5 min. Pure CD and Suc formulations had similar reconstitution times of 45 and 51 s, respectively. Formulations with higher polymer content (PVP, Dex) showed increased

reconstitution times and had viscosities of 1.6 to 2.2 mPa s. Interestingly, reconstitution time for mAb1 formulated with CD/rHA$_{high}$ was more than 4 min, although viscosity was slightly lower than that of polymer containing formulations. In general, reconstitution of mAb2 formulations took slightly longer compared to mAb1 formulations. This was most pronounced for Suc/Dex, where reconstitution of mAb1 lyophilisates took less than 4 min whereas it took more than 6 min for mAb2 cakes (s. Table S1 in the supporting information). In contrast, mAb2 lyophilisates showed a trend to reconstitute slightly faster throughout all CD/rHA formulations compared to mAb1 lyophilisates.

Another important product quality attribute of the freeze-dried formulation is residual moisture as it can be detrimental to protein stability, e.g. by acting as plasticizer increasing molecular mobility or as potential reaction partner. Formulations which showed major dents (Suc/Dex) and collapse (Suc) had increased residual moisture levels of 1.1 and 4.2 %, respectively. Residual moisture for all other lyophilisates was low between 0.1-0.3 %.

The T_g of the freeze-dried product was one major product criteria investigated in this study as it is believed to be indicative for the allowable storage temperature. Except for the pure Suc formulation (T_g: ~43°C), all other excipient combinations resulted in T_g values between 96 and 200°C, which is far beyond intended storage temperatures of proteinaceous formulations. A strong increase in T_g was obtained when the mAb was lyophilized with pure CD, resulting in a T_g of ~200°C. Mixtures of CD with rHA or Dex did not change the T_g compared to pure CD formulations. When CD was used in combination with PVP, T_g decreased with higher PVP content to 162.5°C for the highest PVP fraction. As expected, when Suc was added to polymer formulations, T_g of the formulation decreased with increased Suc content. Overall T_g values followed the trend observed for T_g'.

Protein stability

Protein stability was assessed at 5°C, 25°C, and 40°C for up to 9 months in terms of formation of visible and sub-visible particles, changes in color and turbidity, as well as loss of monomer content. Pure Suc formulations were analyzed as a reference. While all lyophilisates stored at 40°C were collapsed, some cakes turned brownish (Figure 1b). As a result, inconsistent stability behavior was observed. Hence, for time points of 6 or 9 months at 40°C, a brownish and a white lyophilisate were analyzed for Suc formulations. The visible change in color of the lyophilized cake was confirmed by color measurements of the reconstituted solution (Table 3). In addition, many visible particles were seen in the brownish vial for both mAbs, while no visible particles were formed in the white lyophilisates. Correspondingly, a markedly higher turbidity of 13.7 NTU (mAb1) and 11.6 NTU (mAb2) as well as a higher amount of sub-visible particles (s. Figure S1 in the supporting information) was obtained for brownish Suc formulations.

The majority of the other formulations did not show signs of instability based on visible particles, color, and turbidity after 9 month storage at 5°C, 25°C, and 40°C (Table 3 and Table S2 in the supporting information). Pure CD and combinations with Suc, as well as PVP and Dex containing formulations did not show any visible particles (Table 3) and levels for sub-visible particles were low in general (s. Figure S1 in the supporting information). In addition, those formulations showed no considerable change in color and increase in turbidity after storage at 40°C for 9 months was less than 1 NTU.

Table 3. Storage stability of lyophilisates. Visible particles, turbidity, and color after storage at 40°C for 9 months.

Formulation	Storage condition	Visible Particles[1]		Turbidity (NTU)		Color	
		mAb1	mAb2	mAb1	mAb2	mAb1	mAb2
Suc	initial	I	I	2.77	3.15	B7	B8
	9mo, 40°C #1	I	I	2.87	3.41	B7	B9
	9mo, 40°C #2[2]	III	III	13.7	11.6	B5	B4
CD	Initial	I	I	2.78	3.29	B7	B8
	9mo, 40°C	I	I	2.95	3.58	B7	B8
CD/Suc_{low}	initial	I	I	3.13	3.72	B7	B8
	9mo, 40°C	I	I	3.06	3.59	B7	B9
CD/Suc_{high}	initial	I	I	3.20	3.65	B7	B8
	9mo, 40°C	I	I	2.98	3.62	B7	B9
CD/rHA_{low}	initial	I	I	8.94	7.38	B6	B7
	9mo, 40°C	III	III	10.4	7.91	B6	B6
CD/rHA_{mid}	initial	I	I	17.7	14.9	B5	B5
	9mo, 40°C	III	III	22.3	19.0	B5	B5
CD/rHA_{high}	initial	I	I	22.4	19.6	B5	B5
	9mo, 40°C	III	III	36.1	29.5	B4	B4
CD/rHA/Suc	initial	I	I	14.3	10.9	B5	B6
	9mo, 40°C	I	I	14.5	11.8	BG5	B6
CD/PVP_{low}	initial	I	I	2.98	3.31	B7	B8
	9mo, 40°C	I	I	3.10	3.76	B7	BG7
CD/PVP_{mid}	initial	I	I	2.91	3.34	B7	B8
	9mo, 40°C	I	I	3.18	3.84	BG6	BG7
CD/PVP_{high}	initial	I	I	2.82	3.36	BG6	B8
	9mo, 40°C	I	I	3.36	3.65	BG6	BG7
CD/PVP/Suc	initial	I	I	2.83	3.27	B7	B8
	9mo, 40°C	I	I	3.14	3.85	B7	B8
CD/Dex	initial	I	I	3.08	3.52	B7	B8
	9mo, 40°C	I	I	3.08	4.42	B7	BG7
CD/Dex/Suc	initial	I	I	2.88	3.32	B7	B8
	9mo, 40°C	I	I	3.36	4.23	B7	B8
Suc/Dex	initial	I	I	2.82	3.16	B7	B9
	9mo, 40°C	I	I	3.11	3.80	B7	B8

[1] I = practically free of particles, II = with few particles, III = with many particles
[2] #1 white lyophilisate, #2 brownish lyophilisate (see Figure 1)

Formulations with rHA were less stable. After 9 months, visible particles were formed for all CD/rHA formulations independent of storage temperature (Table 3 and Table S2 in the supporting information) and an increase in turbidity of 13.7 NTU (mAb1) and 9.9 NTU (mAb2) was observed at 40°C. Even when stored at 5°C, CD/rHA formulations showed visible particles for both mAb (s. Table S2 in the supporting information). In general, CD/rHA formulations with mAb1 showed an earlier onset of particle formation. Already after 4 weeks at 40°C, all CD/rHA formulations of mAb1 showed many particles, whereas for mAb2 particles were only seen in CD/rHA_{high}. After 3 months many visible particles were seen in all CD/rHA formulations for both mAbs stored at 40°C (data not shown). In addition, a high

amount of sub-visible particles was formed for all CD/rHA formulations for both mAbs. The total count per mL was higher for mAb1 as shown in Figure S1 in the supporting information compared to mAb2. Interestingly, CD/rHA formulations with mAb2 showed no considerable increase in number of particles ≥ 10 µm. In general, formulations with increasing rHA content were less stable. However, when 30% sucrose was added to a mixture of CD/rHA, no particles and no increase in turbidity was observed after storage, although the rHA content in CD/rHA/Suc (16.8 mg/mL) was twice the amount of CD/rHA$_{low}$ (8 mg/mL).

Special emphasis to assess protein integrity was given to the loss of monomer content. Regarding the reference formulations with pure Suc, both mAbs were stable if stored at 5°C and 25°C (Figure 2a and Figure S3 in the supporting information). At 40°C, lyophilisates collapsed and those that remained white showed good protein stability with a monomer content of more than 96%, whereas a strong loss in monomer content occurred for brownish lyophilisates (Figure 2b). The decrease in monomer content correlated with a high amount of aggregates of around 20% for mAb1 and 30% for mAb2 after storage at 40°C for 9 months. Additional characterization by X-ray powder diffraction demonstrated that sucrose showed almost no tendency to crystallize in white lyophilisates, whereas in brownish lyophilisates sucrose had almost completely crystallized (s. Figure S2 in the supporting information).

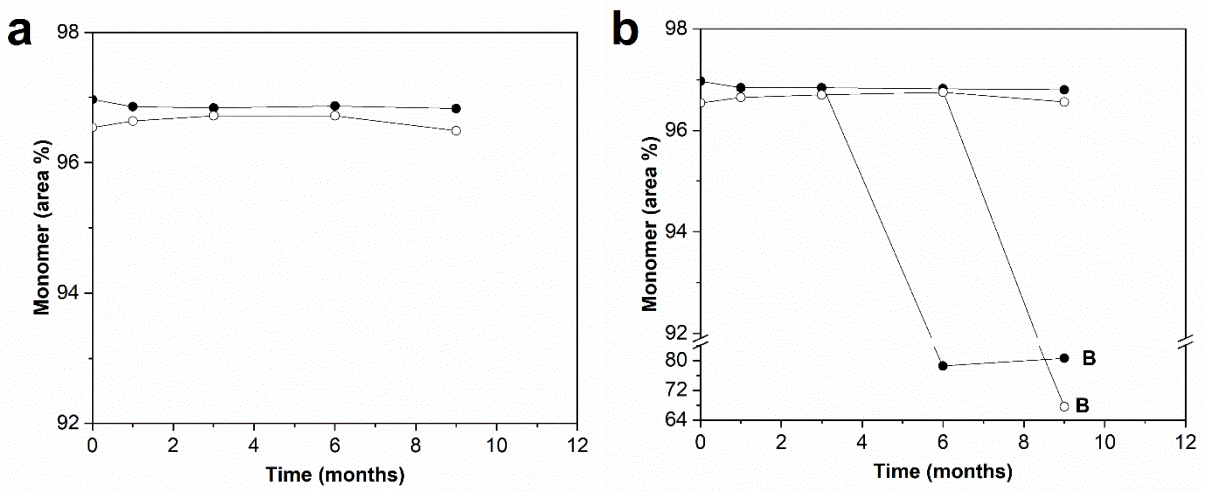

Figure 2. mAb stability in lyophilisates by SE-HPLC. Monomer loss of mAb1 (filled symbols) and mAb2 (open symbols) formulated with 80 mg/mL sucrose upon storage for 9 months at a) 25°C or b) 40°C. "B" indicates lyophilisates that had turned brownish upon storage.

Figure 3 and 4 show the loss of monomer content for mAb1 and mAb2 upon storage at 25°C and 40°C, respectively, for formulations with CD and CD/Suc, rHA, PVP as well as Dex, which corresponded to an increase in high molecular weight species (Stability at 5°C shown in Figure S3 in the supporting information). Overall, loss of monomer was higher after storage at 40°C compared to 25°C. Similar stability behavior was observed for mAb1 and mAb2 with in general a slightly higher loss of monomer for mAb2. At 25°C, both mAbs remained stable in formulations with pure CD or in combination with Suc. At 40°C, the mAbs were better stabilized when a mixture of CD/Suc was used with a loss of monomer of less than 1% compared to pure CD, which showed a loss of monomer of ~3%.

Corresponding to the substantial particle formation, addition of rHA to CD-based formulations resulted in a pronounced loss of monomer, depending on the rHA concentration. While formation of visible and sub-visible particles was more pronounced for mAb1, the formation of higher molecular weight species was higher for mAb2 formulations. The monomer content decreased by 3% after 9 months at 25°C for CD/rHA$_{high.}$ Protein degradation was much faster at 40°C with 80% (mAb2) and 83% (mAb1) monomer content for CD/rHA$_{high}$ after 9 months. If 24 mg/mL Suc was added to a CD/rHA mixture, protein stability was strongly improved, resulting in a remaining monomer content of 94% after 9 months at 40°C. Interestingly, although rHA content in this formulation (16.8 mg/mL) was twice the amount of CD/rHA$_{low}$ (8 mg/mL), CD/rHA/Suc exhibited only a minor monomer loss of ~2% compared to ~4% for CD/rHA$_{low}$ (Figure 4b).

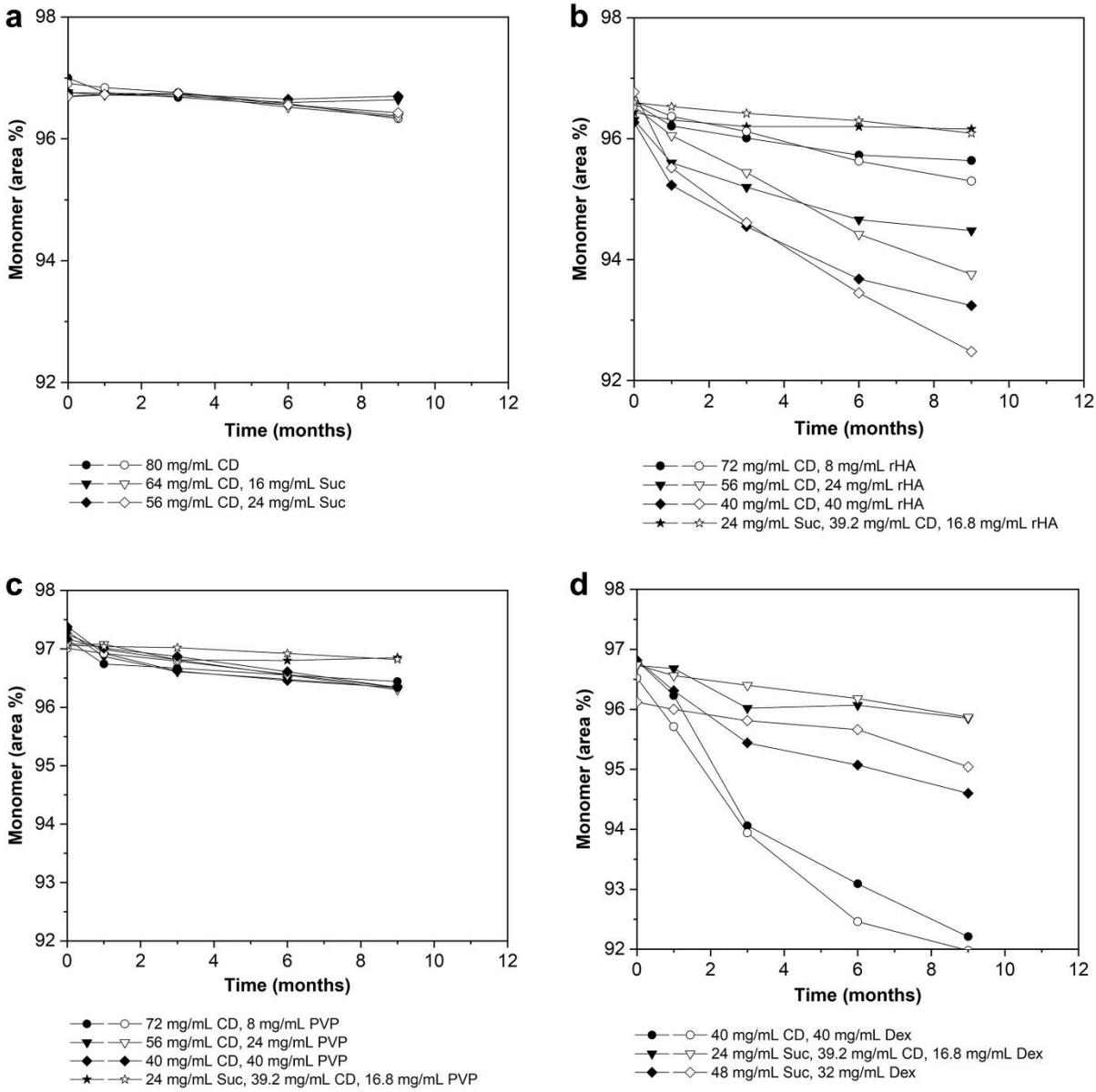

Figure 3. mAb stability in lyophilisates by SE-HPLC. Monomer loss of mAb1 (filled symbols) and mAb2 (open symbols) after storage at 25°C for formulations with a) CD, b) rHA, c) PVP, and d) Dex.

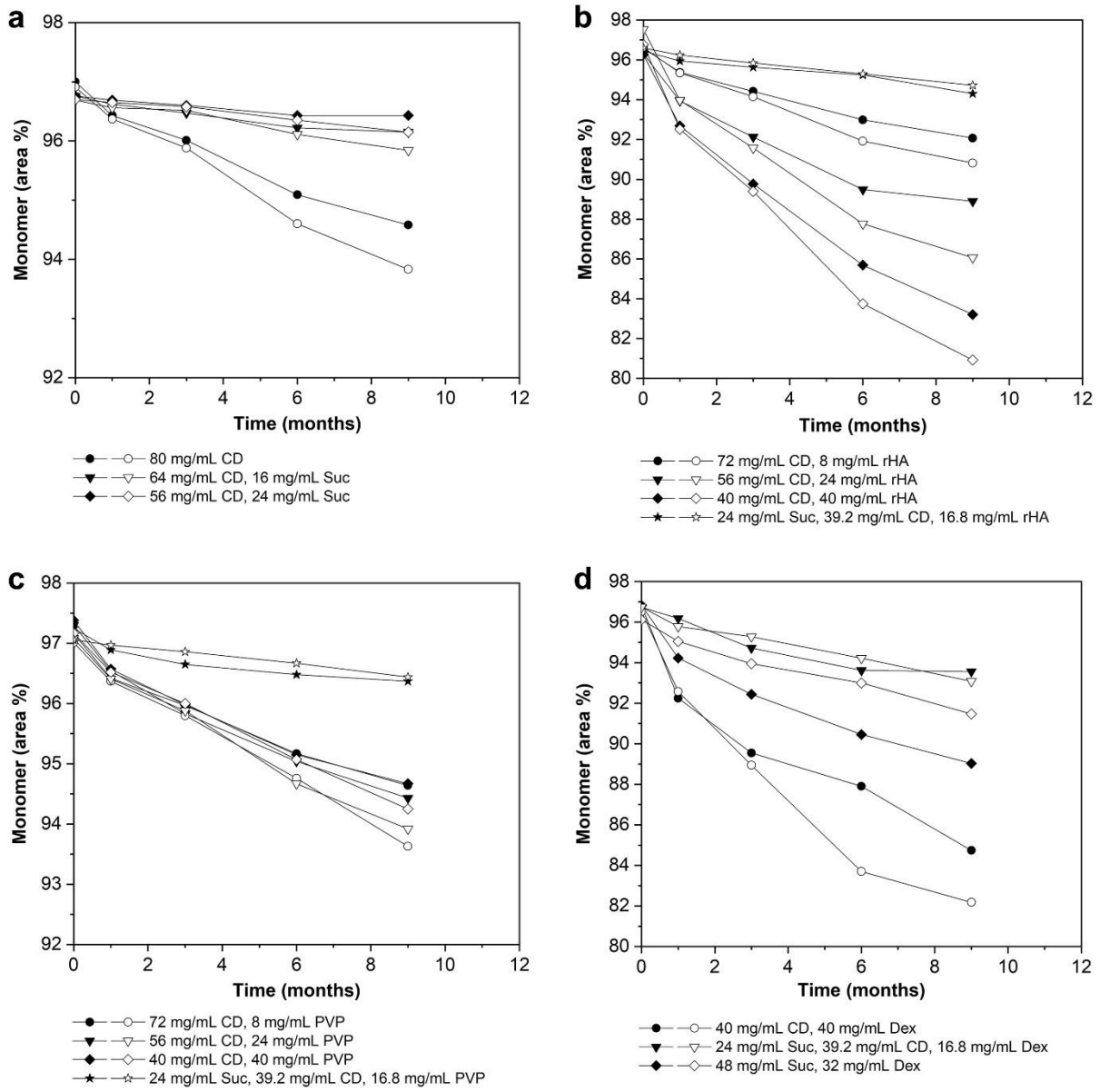

Figure 4. mAb stability in lyophilisates by SE-HPLC. Monomer loss of mAb1 (filled symbols) and mAb2 (open symbols) after storage at 40°C for formulations with a) CD, b) rHA, c) PVP, and d) Dex.

All formulations with PVP demonstrated good protein stability at 25°C storage. Best mAb stability was achieved in CD/PVP/Suc without changes at 25°C and a minor decrease in monomer content of 1% at 40°C after 9 months. Formulations with CD/PVP revealed similar stability as pure CD formulations with no change at 25°C and only a reduction of 2-3% in monomer content at 40°C after 9 months. For mAb2 but not for mAb1, the decrease in monomer content after 9 months at 40°C appears to be less pronounced with higher PVP content.

The Dex formulations were of inferior stability compared to the other excipient combination. Best mAb stability was achieved for the lowest dextran concentration of 16.8 mg/mL with a loss of monomer content of 3.1% for mAb1 and 3.6% for mAb2.

Overall, the stability differences between the mAb1 and the mAb2 formulations are marginal. They may be attributed to differences in the molecular properties like hydrophobicity and distribution of hydrophobic patch on the protein surface. This subject is beyond the scope of this study.

DISCUSSION

Review of existing literature on freeze-dried protein formulations reveals that the excipients included in the formulations have a major impact on the ideal freeze-drying cycle conditions, the resulting product quality attributes, as well as protein stability [4, 24, 33]. During formulation and process development, typically T_g' of the liquid and T_g of the freeze-dried product are analyzed as important formulation characteristics. T_g' is assessed to determine the maximum primary drying temperature to prevent collapse and T_g is believed to correlate with protein stability at a specific storage temperature. Most marketed freeze-dried mAb formulations contain trehalose or more commonly sucrose, potent lyo- and cryoprotectants but with moderate to low glass transition temperatures (T_g' and T_g). We believe that this standard use of disaccharides as protein stabilizer is, at least partially, responsible for higher costs of goods due to time-consuming freeze-drying cycles and refrigerated storage conditions. In this light, the present study systemically investigated novel excipient combinations compared to pure sucrose with regards to their potential to i) run more aggressive freeze-drying cycles while obtaining pharmaceutically elegant lyophilisates and ii) enable long-term storage at room temperature. It is important to mention that the goal of the present study was not the development of an ideal freeze-drying cycle for each formulation. Within this study we employed an aggressive lyophilization cycle which results in a T_p of ~-21°C during primary drying, significantly above the T_c of Suc formulations. The freeze-drying cycle employed within the present study (T_s: +10°C) shortened primary drying time by 17% compared to a typical freeze-drying cycle with a T_s of -10°C during primary drying for sucrose-based formulations. By this, we evaluated the potential for alternative excipient combinations to perform faster freeze-drying processes while maintaining elegant cake appearance.
In general, the T_g' values were indicative for the resulting cake appearances. Formulations with a T_g' markedly lower than T_p were fully collapsed (Suc), formulations with a T_g' slightly below T_p showed major dents (Suc/Dex), and a T_g' slightly above T_p resulted in minor dents (CD/PVP/Suc). While it has been shown that collapse does not necessarily harm protein stability [33], elegant cake appearance is often considered an important quality attribute included for lot release [34, 35]. The results also highlight the importance of determining T_c by FDM in addition to T_g' for precise prediction of resulting cake appearance. Although CD/PVP/Suc and CD/Dex/Suc showed similar T_g' values, the first showed minor dents while CD/Dex/Suc resulted in an elegant cake due to the small but considerable difference in T_c values (ΔTc = 1.1°C). The presence of major dents when T_p equals T_c has also been reported previously by Depaz *et al.* [34]. Formulations which contained a high fraction of CD resulted in brittle lyophilisates. This was also reported by Geidobler when a mAb was formulated with a 1% CD-based formulation [36]. Additionally, we observed formation of a film on the inner surface of the glass vial for formulations that contained mainly CD. We hypothesize that this phenomenon might be a combination of creeping, shrinkage and detachment and related to the amphiphilic nature of CD and investigations are ongoing whether cake appearance of formulations containing CD can be improved by using alternative vial types.

Besides enabling aggressive freeze-drying conditions, we targeted to enable storage at room temperature. Consequently, according to ICH guidance for drug product storage at 25°C, stability data for storage at 40°C for up to 6 months without significant changes is required [37]. T_g is associated with the transition from a glassy matrix to a viscous flow. Hence, at temperatures below T_g, immobilization of the protein is ensured and thus degradation mechanisms are expected to slow down. Several studies demonstrated strong correlation between increasing protein stability and higher glass transition temperatures [19, 38, 39]. Grasmeijer *et al.* reported that the storage temperature of freeze-dried formulations should be 10-20°C below T_g to ensure optimal protein stability [4]. However in some instances, it has been observed that protein stability does not necessarily or at least not only depend on T_g. Moorthy *et al.* found that the rank order of T_g was poorly correlated to the aggregation propensities of the formulations [40]. Pikal *et al.* found that T_g was predictive for the stability of lyophilized human growth hormone in disaccharide systems for storage temperatures near the formulations' T_g, whereas for storage at temperatures far below T_g, stability behavior seemed to be unrelated to T_g [41]. Our results support the observations by Pikal *et al.*.

All vials of the Suc formulation showed collapse. This collapse during lyophilization resulted in high residual moisture levels (4.2%) leading to a rather low T_g of 43.2°C as compared to typically reported values for sucrose [5], which was close to the storage temperature of 40°C. Consequently, inconsistency between vials with respect to stability was observed for Suc samples when stored at 40°C. Some Suc samples showed remarkable loss in monomer content, formation of visible particles, and changes in color, starting already after 6 months of storage. Other vials remained stable over a time period of 9 months at 40°C. Performing a conventional freeze-drying cycle with Suc formulations, which did not introduce collapse but major dents, resulted in residual moisture levels of 1-2% and correspondingly higher T_g values of 65°C (data not shown). These conservatively freeze-dried lyophilisates remained stable during storage and remaining monomer content of mAb2 was comparable to white lyophilisates of the aggressive cycle (s. Figure S4 in the supporting information). However, for sucrose formulations this would require time-consuming freeze-drying cycles to avoid collapse and render lyophilisates of low residual moisture. As mentioned above, the scope of the present study was to enable shorter freeze-drying cycles and therefore a Suc formulation freeze-dried with the same freeze-drying cycle served as a reference. Characterization of the morphology of brownish Suc lyophilisates revealed crystallization of sucrose and thus loss of the stabilizing amorphous matrix. A model developed by Bhugra *et al.* to predict the onset of crystallization of amorphous sucrose clearly showed that only small differences between storage temperature and T_g have a major impact on the onset of crystallization [42]. This might explain why some Suc vials did crystallize while others remained amorphous. The additional brownish appearance is likely to be ascribed to chemical modifications facilitated by the high residual moisture. Upon heat-stress, sucrose can hydrolyze into fructose and the reducing sugar glucose. Glucose can eventually lead to glycation of the protein [43, 44]. We confirmed mAb glycation by mass spectrometry showing an increased peak with a mass shift of +162 Da (s. Figure S5 in the supporting information). Slightly increased glycation levels were even found for white lyophilisates after 9 months at 40°C. These findings highlight the need for alternative excipients, enduring aggressive freeze-drying cycles without collapse and providing drug products with higher T_g. All tested excipient combinations within this study had much higher T_g values compared to Suc, suggesting sufficient immobilization of the mAb at storage temperatures of 25°C and 40°C. Additionally, we determined the residual moisture after storage at 25°C for select CD-based

formulations (CD, CD/PVP/Suc, CD/Suc_{high}). The levels were marginally increased by approximately 0.2-0.3%, and no increase was observed for pure sucrose formulations (s. Table S3 in the supporting information). Dynamic vapor sorption (DVS) measurements showed that residual moisture levels upon storage are far below moisture levels, where changes like crystallization become an issue. Changes in sorption behavior for CD-based formulations containing sucrose occurred at much higher moisture levels (approximately 15%) whereas onset of crystallization of pure sucrose occurred already at approximately 6-7% (s. Figure S6 in the supporting information). Thus, the low residual water content after storage and the low tendency of CD-based formulations to crystallize further support the possibility of room temperature storage.

CD is a promising alternative excipient, as in contrast to the unmodified parent cyclodextrins, CD has been shown to be toxicologically safe without adverse effects for intravenous administration of 250 mg/kg/day for 21 days [45]. However, care should be taken if drugs are intended for patients with kidney impairment due to renal toxicity concerns related to the unmodified parent cyclodextrin [46]. Moreover, CD does not only function as a bulking agent but also as amorphous stabilizer. Ressing *et al.* reported that CD preserves antigen-binding capacity of a freeze-dried mAb during storage at 56°C for up to 32 days better compared to sucrose [47]. These findings are partially in line with the results of our study. Storage at 25°C resulted in equally good mAb stability for both CD and Suc formulations. When lyophilisates were stored at 40°C, remaining mAb monomer content was much higher in CD formulations (94.6% mAb1, 94.0% mAb2) compared to brownish Suc lyophilisates (80.6% mAb1 67.6% mAb2), and slightly lower than in white Suc samples (96.5% mAb1, mAb2). However, no increase in mAb glycation levels was observed in CD samples compared to white Suc samples. mAb aggregation in pure CD was faster when stored at 40°C compared to storage at 25°C. The remaining monomer content after 3 months at 40°C was already lower than after 9 months at 25°C, although both storage conditions are far below the T_g of 200 °C. This is consistent with the results of a study by Faghihi *et al.*, who found similar mAb stability in terms of aggregate formation for pure trehalose or CD formulations after storage at 40°C for 3 months, but with slightly higher aggregation rates for CD [16]. The fact that different stability behavior was observed when stored at 25°C or 40°C, although both temperatures are far below the T_g, suggests that protein stabilization by CD at storage temperatures far below T_g is unrelated to T_g. This is consistent with previous observations [41]. Addition of 30% Suc to CD improved stability, resulting in mAb formulations that were stable upon long-term storage at 40°C. This leads to the hypothesis that CD may be inferior in suppressing local motions (β-relaxations) compared to sucrose. β-relaxations were shown to account for protein stability at storage temperatures far below the T_g [48]. Using tetrahertz spectroscopy, Smoohl *et al.* could demonstrate a distinct transition temperature for mAb formulations, which was associated with the onset of local motions [49]. We speculate that storage at 40°C might be above this transition temperature for the pure CD formulation, causing local motions which lead to increased protein instability.

Addition of PVP to CD formulations did not impact the mAb stability behavior compared to pure CD formulations with a monomer loss <1% when stored at 25°C and more pronounced aggregation at 40°C. PVP acts as a bulking agent for low concentration protein formulations. Correspondingly, stability of a freeze-dried protein stored at 40°C for 12 days was shown to be inferior when formulated with pure PVP compared to sucrose [50]. In combination with sucrose, PVP enhances protein stability at elevated temperatures compared to pure sucrose due to formation of hydrogen bonds between sucrose and PVP resulting in a more stable, amorphous glass [22, 51]. Previous studies used PVP (K40)

or larger, which have a higher T_g compared to the PVP (K17) used in our study [22, 23, 39, 52]. However, these higher molecular weight PVPs are not approved for parenteral use, hence were not included in our study. In a preliminary experiment, we did not find a considerable impact of PVP (K17) addition to pure sucrose on T_g hence we investigated the use of PVP in combination with CD. This novel combination of PVP as a bulking agent with CD, which stabilizes the mAb upon storage at 25°C, or CD/Suc provide overall good protein stability at 40°C. CD/PVP/Suc resulted in similar, very good properties in terms of mAb stability, T_g', reconstitution times, and residual moisture compared to CD/Suc_{high}, but with slightly reduced cake appearance due to minor dents.

In contrast, addition of rHA to CD destabilized both mAbs, regardless of the storage temperature. Formation of visible particles was already observed after 4 weeks at 40°C. This is of concern since protein aggregates can reduce therapeutic efficacy and have a greater immunogenic potential [53, 54]. After 9 months, particles were present in all rHA formulations independent of the storage temperature. If stored at 40°C, mAb1 formulated with CD/rHA_{high} even exceeded the compendial requirements for sub-visible particles of less than 6000 particles ≥10 µm per container [28]. Interestingly, particles were also present in placebo formulations, but no particle formation was observed if sucrose was present in the formulation. These observations point out that rHA itself is unstable in the presence of CD, but stable if combined with sucrose. These findings are consistent with a study by Costantino *et al.,* who reported formation of insoluble aggregates for various freeze-dried excipient-rHA combinations. rHA stability was found to be strongly dependent on the chosen excipient, although a relationship between molecular structure of the excipient (e.g. presence of hydroxyl groups) and rHA stability was not shown [55]. Finally, although formulation stability was improved by the addition of Suc, CD/rHA/Suc was not superior to CD/Suc_{high} or CD/PVP/Suc. Hence, while human albumin has been successfully used as a stabilizer for freeze-dried cytokines [27], our results suggest that the addition of rHA does not add value to the stability of freeze-dried mAb formulations.

Similarly, addition of Dex to CD decreased mAb stability. While dextran containing formulations did not show any significant changes in visible particles, SE-HPLC data revealed similar levels of loss of monomer for CD/Dex as for CD/rHA_{high} formulations. This is consistent with our previous study. We reported impaired mAb stability due to glycation when formulated with dextrans only. Stability was improved when mixtures of Dex and Suc were used [7]. There is a marketed lyophilized antibody-drug conjugate available, formulated with a mixture of Dex and Suc, which is intended for refrigerated storage [56]. Hence, we included a formulation with a similar Dex to Suc ratio in the present study to test whether this combination would also allow for elevated storage temperatures. While we confirmed good mAb stability after 9 months at 2-8°C (s. Figure S3 in the supporting information), we observed loss in monomer content of 5% (mAb2) and 7% (mAb1) when stored at 40°C. Both Suc/Dex and CD/Dex formulations showed considerable loss of monomer content, while mAb stability in Suc/Dex appeared slightly superior to CD/Dex. Potentially, this could be explained by a hypothesis previously described suggesting a better protection of the mAb by sucrose (342 Da) molecules compared to the sterically larger CD (~1kda) [7]. This hypothesis requires further confirmation as the Dex fraction varies across the Suc/Dex and CD/Dex formulations.

In summary, the present data set demonstrates that CD is a promising alternative excipient for freeze-dried mAb formulations as it does not only increase the glass transition temperatures but in contrast to crystalline bulking agents such as mannitol also functions as amorphous stabilizer. In particular, combinations of CD/Suc or CD/PVP/Suc are highly attractive, enabling aggressive freeze-drying cycles

without adding additional complexity to the process leading to shorter cycle time. Further process optimization is subject of ongoing research. Moreover, they ensure protein storage stability at 25°C and higher, thereby overcoming constraints of pure sucrose formulations. These formulations with high T_g' may be particularly interesting for potent, low concentrated novel therapeutic protein modalities such as antibody-drug conjugates or Fc-fusion proteins. Due to their increased complexity, they are often less stable in liquid dosage forms requiring freeze-dried formulations. However, the applicability and stabilizing properties of CD for such new formats needs to be investigated.

CONCLUSION

Biopharmaceutical formulations, which can be stored at room temperature, are highly attractive. Especially the possibility to freeze-dry under aggressive conditions while maintaining elegant cake appearance at the same time will further reduce costs of goods. Low concentration mAb formulations with pure sucrose are not adequate for aggressive freeze-drying cycles and product storage at room temperature. We investigated alternative excipients and demonstrated that low concentration mAb formulations with CD/Suc or CD/PVP/Suc endure aggressive freeze-drying cycles without collapse resulting in good product quality attributes such as cake appearance, residual moisture, and reconstitution time. Most importantly, these excipient combinations enable storage at room temperature or above for at least 9 months at the same time. While PVP acts as bulking agent only contributing to a stable amorphous matrix, CD is a particularly interesting excipient as it does not only increase the formulation's T_g but is also able to stabilize the protein. Whether these formulations are able to stabilize new mAb derivatives is yet to be verified. We demonstrated that mAbs are well stabilized by CD for storage temperatures up to 25°C, whereas at higher temperatures the addition of 30% sucrose to pure CD or mixtures of CD with PVP is beneficial for protein stability. We propose that the addition of CD in mAb formulations might reduce costs of goods of freeze-dried biopharmaceuticals by allowing for shorter freeze-drying cycles and making cold chain for drug product storage eventually redundant.

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SUPPORTING INFORMATION

Methods

This paragraph lists methods specific for data shown in the supporting information. Other methods are described in the main manuscript.

Sub-visible particles

Number and size distribution of sub-visible particles was analyzed by light obscuration in analogy to USP <787> [31] and Ph. Eur. 2.9.19. [32] requirements using a HIAC 9703+ liquid particle counter (Skan, Allschwil, Switzerland). After rinsing the system with 0.5 mL sample solution, 4 aliquots of 0.2 mL were analyzed. Analysis and classification of particles into $\geq$ 2 µm, $\geq$ 5 µm, $\geq$ 10 µm, and $\geq$ 25 µm was performed by the Pharmspec 3 software. The first measurement was discarded, and the mean ± standard deviation of run 2 to 4 was reported as cumulative particles per mL.

X-ray powder diffraction

X-ray powder diffraction (XRPD) was performed using a STOE STADI (Stoe & Cie, Darmstadt, Germany) powder diffraction system. Pure $K_{\alpha 1}$ radiation was produced using a copper generator (40 kV, 40 mA). Measurements were performed from 3° to 42° $2-\vartheta$ at a resolution of 0.5° $2-\vartheta$ and duration of 20 s per step.

Water sorption isotherms

Water sorption behavior was determined using a DVS Intrinsic Plus (SMS Surface Measurements Systems, Middlesex, UK) moisture balance system. 10-20 mg sample were weighed into the sample holder. After equilibration at 0% r.H. for 300 min, the sorption isotherms were measured in a range from 0% r.H to 90%-r.H. at 25°C with a dynamic increase of 5% r.H. per minute.

Table S1 shows formulation attributes of the liquid and freeze-dried mAb2 formulation. The values for T_g', viscositiy, reconstitution time, T_g, and residual moisture were not considerable different from mAb1 formulations. Correspondingly, cake appearance for the different formulations was similar for mAb1 or mAb2 containing formulations.

Table S1. Characterization of liquid formulation (T_g', $T_{c,}$ and viscosity), as well as quality attributes of the freeze-dried product (reconstitution time, T_g, residual moisture) directly after freeze-drying. For abbreviations see Table 1.

Formulation	Liquid		Lyophilisate			
	T_g' [1] (°C)	Viscosity (mPa s)	Reconstitution time (min:ss)	T_g (°C)	Residual moisture (%)	Cake appearance
Suc	-29.5	1.3	01:02 ± 00:27	43.0 ± 0.6	4.0 ± 0.4	collapse
CD	-10.9	1.4	00:53 ± 00:13	201.6 ± 0.2	0.2 ± 0.06	brittle
CD/Suc_{low}	-16.5	1.4	00:49 ± 00:02	163.7 ± 2.2	0.1 ± 0.00	elegant – brittle
CD/Suc_{high}	-18.6	1.4	00:50 ± 00:01	154.5 ± 0.5	0.1 ±0.01	elegant – brittle
CD/rHA_{low}	-10.9	1.4	01:17 ± 00:14	201.5 ± 0.0	0.2 ± 0.03	brittle
CD/rHA_{mid}	-10.6	1.5	01:43 ± 00:31	202.4 ± 0.5	0.2 ± 0.04	cracks
CD/rHA_{high}	-9.7	1.5	04:07 ± 01:07	198.1 ± 0.3	0.3 ± 0.05	cracks
CD/rHA/Suc	-18.8	1.4	01:43 ± 00:13	154.1 ± 1.4	0.2 ± 0.01	elegant
CD/PVP_{low}	-10.7	1.5	02:14 ± 00:32	199.3 ± 1.3	0.2 ± 0.06	cracks
CD/PVP_{mid}	-13.7	1.6	02:51 ± 01:26	191.8 ± 0.8	0.2 ± 0.04	cracks
CD/PVP_{high}	-16.1	1.7	03:32 ± 01:05	183.5 ± 0.9	0.2 ± 0.05	brittle
CD/PVP/Suc	-19.2	1.5	01:24 ± 00:15	155.7 ± 1.0	0.2 ± 0.01	minor dents
CD/Dex	-10.8	2.0	03:58 ± 00:48	203.0 ± 1.2	0.3 ± 0.05	brittle
CD/Dex/Suc	-18.8	1.7	02:35 ± 07:23	160.2 ± 0.0	0.3 ± 0.01	elegant
Suc/Dex	-26.3	2.2	06:18 ± 00:56	96.6 ± 1.3	0.9 ± 0.15	major dents

Table S2 shows formulation stability in terms of visible particle formation, changes in turbidity or color for both mAbs stored at 5°C or 25°C for 9 months. In CD/rHA formulations visible particles were also formed when stored at 5 or 25°C for 9 months. All other formulations remained stable in terms of visible particles or changes in color and turbidity.

Table S2. Storage stability of lyophilisates. Visible particles, turbidity, and color after storage at 5 or 25°C for 9 months.

Formulation	Storage condition	Visible Particles[1]		Turbidity (NTU)		Color	
		mAb1	mAb2	mAb1	mAb2	mAb1	mAb2
Suc	9mo, 5°C	I	I	3.13	3.50	BG7	B9
	9mo, 25°C	I	I	2.98	3.72	BG7	B9
CD	9mo, 5°C	I	I	3.27	3.77	BG7	B9
	9mo, 25°C	I	I	2.99	3.97	BG7	B9
CD/Suc_{low}	9mo, 5°C	I	I	3.02	3.48	B7	B8
	9mo, 25°C	I	I	3.06	3.67	B7	B8
CD/Suc_{high}	9mo, 5°C	I	I	2.96	3.54	B7	B9
	9mo, 25°C	I	I	3.00	3.51	B7	B9
CD/rHA_{low}	9mo, 5°C	III	III	9.24	7.91	B6	B7
	9mo, 25°C	III	III	9.64	8.70	B6	B7
CD/rHA_{mid}	9mo, 5°C	III	III	17.4	15.8	B5	B5
	9mo, 25°C	III	III	18.6	16.7	B5	B5
CD/rHA_{high}	9mo, 5°C	III	III	21.9	20.9	B5	B5
	9mo, 25°C	III	III	25.7	24.5	B5	B5
CD/rHA/Suc	9mo, 5°C	I	I	14.5	11.4	B6	B6
	9mo, 25°C	I	I	14.5	11.7	BG5	B6
CD/PVP_{low}	9mo, 5°C	I	I	3.17	3.57	B7	B8
	9mo, 25°C	I	I	3.19	3.85	B7	B8
CD/PVP_{mid}	9mo, 5°C	I	I	3.15	3.64	B7	B8
	9mo, 25°C	I	I	3.16	3.75	B7	B8
CD/PVP_{high}	9mo, 5°C	I	I	3.12	3.69	B7	BG7
	9mo, 25°C	I	I	3.54	3.95	BG6	BG7
CD/PVP/Suc	9mo, 5°C	I	I	3.09	3.5	B7	B8
	9mo, 25°C	I	I	14.5	3.99	B7	B8
CD/Dex	9mo, 5°C	I	I	3.17	3.88	B7	B8
	9mo, 25°C	I	I	3.26	3.79	B7	B9
CD/Dex/Suc	9mo, 5°C	I	I	3.03	3.65	BG7	B8
	9mo, 25°C	I	I	3.14	4.17	BG7	B8
Suc/Dex	9mo, 5°C	I	I	3.38	3.63	B7	B9
	9mo, 25°C	I	I	3.04	3.83	BG7	B8

Table S3 shows residual moisture levels of mAb2 formulations after storage at 25°C for 15 months revealing a small but consistent increase in residual moisture for CD-based formulations upon storage, whereas no increase in residual moisture was observed for pure sucrose formulations.

Table S3. Residual moisture of mAb2 lyophilisates after storage at 25°C for 15 months given as mean (n=3) with standard deviation.

Formulation	**Residual moisture (%)**	
	after freeze-drying	**after storage at 25°C**
Suc	4.0 ± 0.4	3.8 ± 0.45
CD	0.2 ± 0.06	0.5 ± 0.01
CD/PVP/Suc	0.2 ± 0.01	0.5 ± 0.01
CD/Suc_{high}	0.1 ±0.01	0.4 ± 0.11

Figure S1 shows sub-visible particles formed in mAb1 and mAb2 lyophilisates during storage at 40°C. Considerable and consistent increase in sub-visible particles is shown for rHA containing formulations for both mAbs as well as brownish Suc formulations. Increased particle counts (≥ 2 µm) for mAb1 formulations after 1 month and for mAb2 formulations after 3 and 6 months can be ascribed to the high measurement variability of light obscuration.

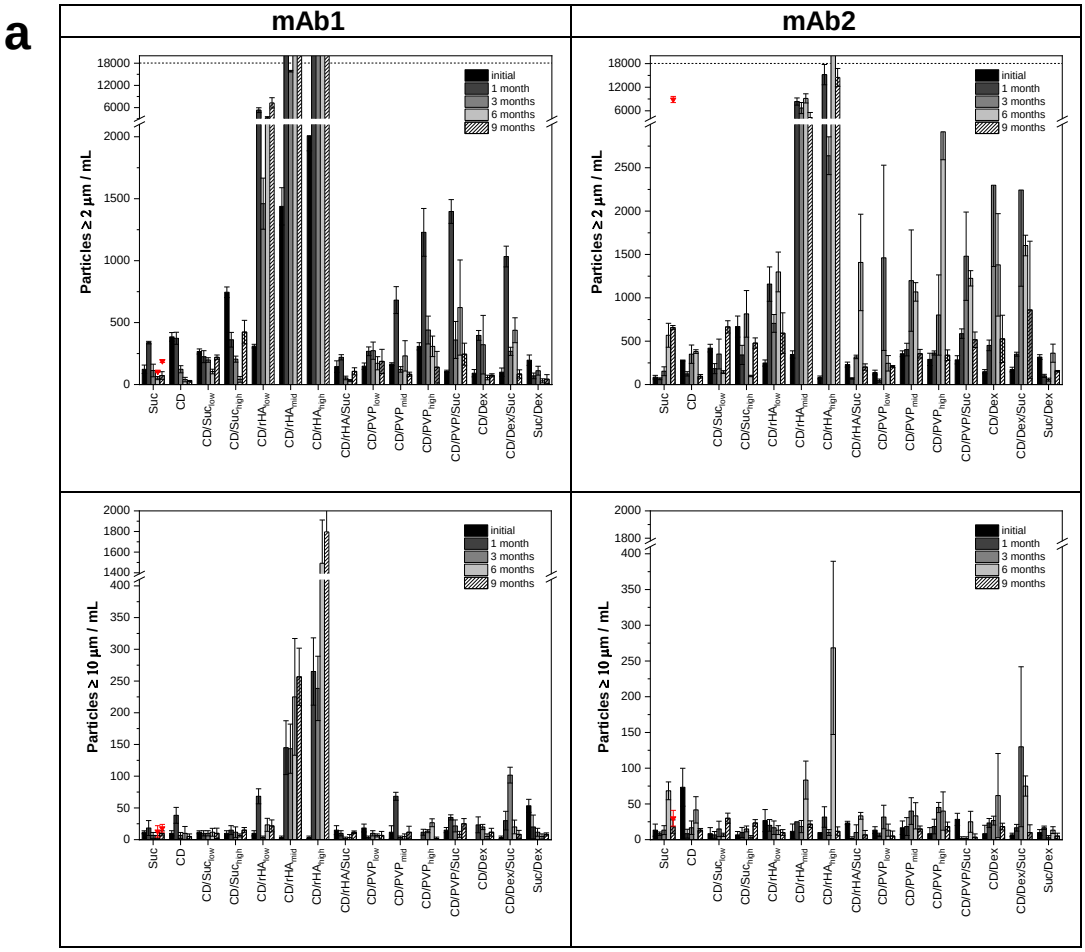

Figure S1. Analysis of particulate matter by light obscuration. Cumulative sub-visible particle count ≥ 2 µm (top row) and ≥ 10 µm (bottom row) are shown for mAb1 (left panel) and mAb2 (right panel) after storage at a) 40°C, b) 25°C and c) 5°C. Red triangle shows sub-visible particles of brownish Suc samples. Dotted line indicates upper limit of detector.

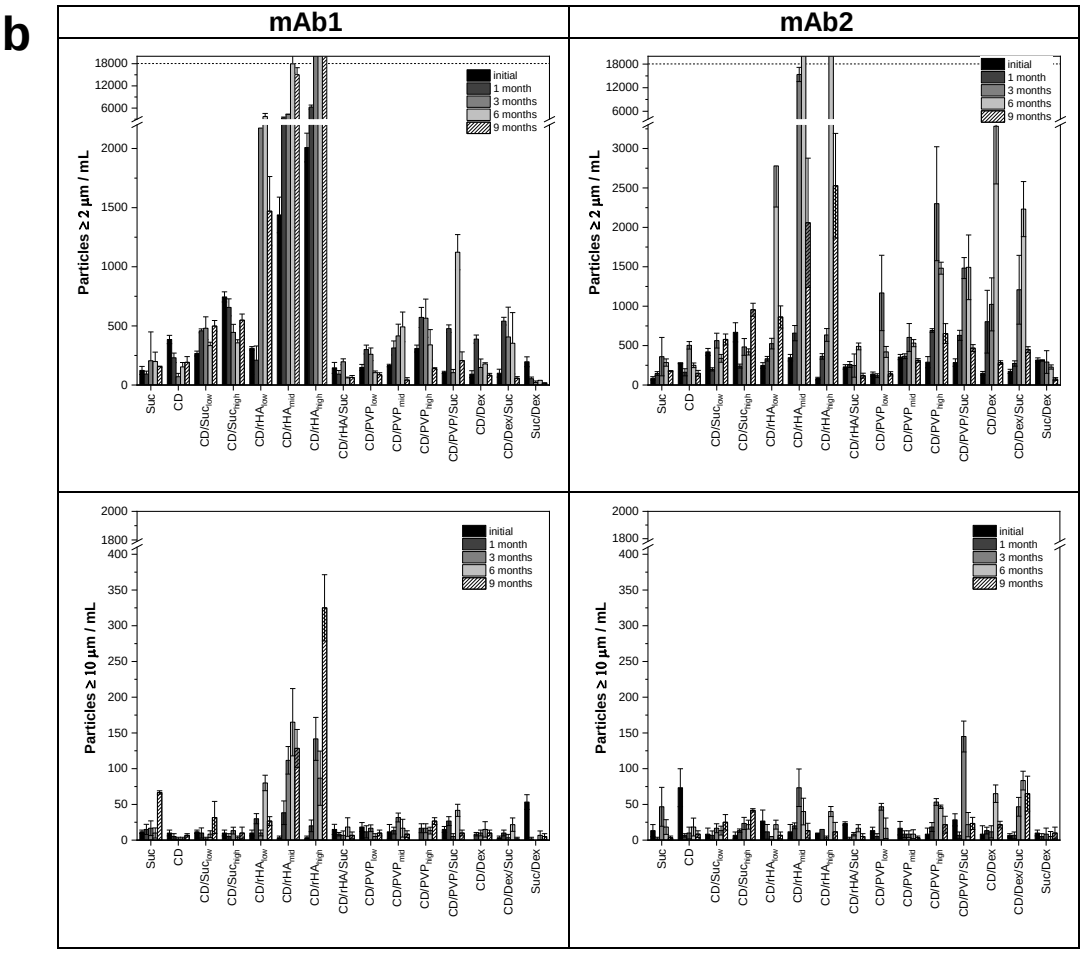

Figure S1 (continued). Analysis of particulate matter by light obscuration. Cumulative sub-visible particle count ≥ 2 µm (top row) and ≥ 10 µm (bottom row) are shown for mAb1 (left panel) and mAb2 (right panel) after storage at a) 40°C, b) 25°C and c) 5°C. Red triangle shows sub-visible particles of brownish Suc samples. Dotted line indicates upper limit of detector.

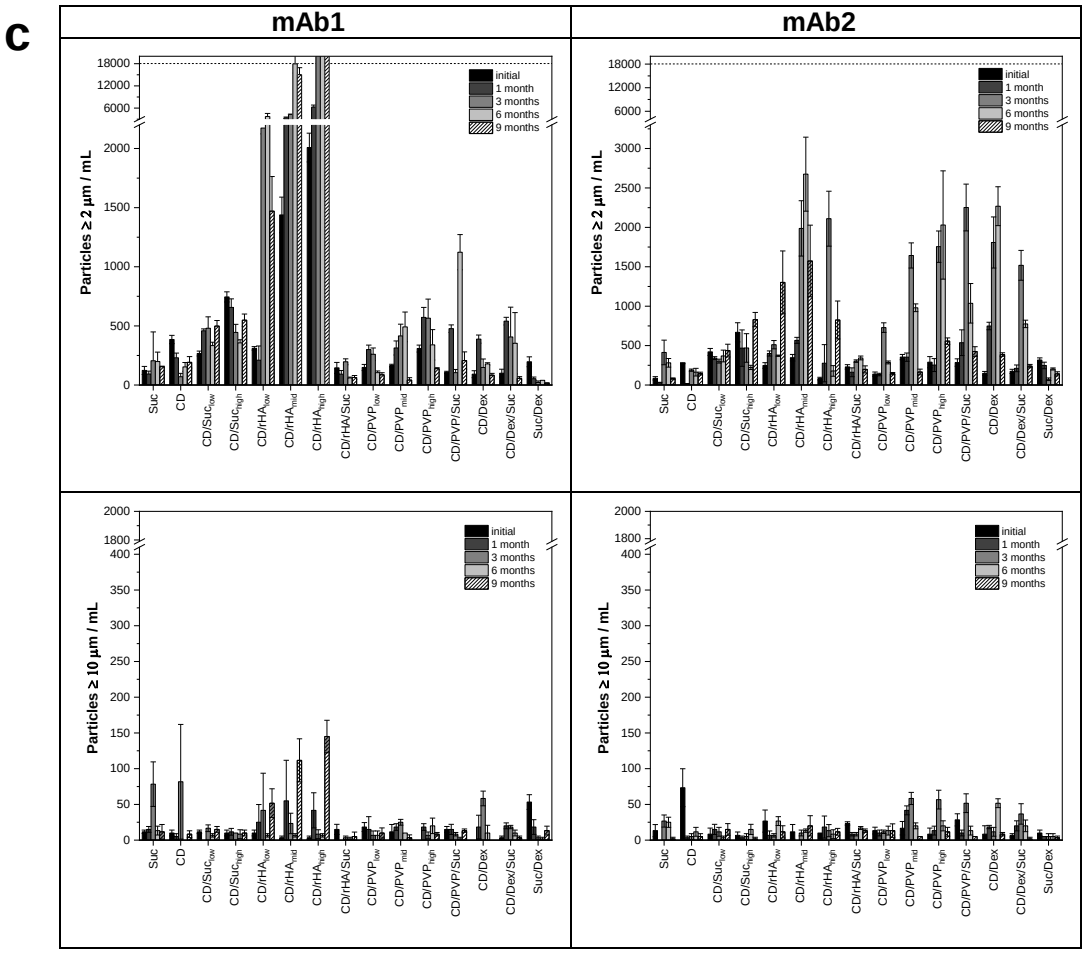

Figure S1 (continued). Analysis of particulate matter by light obscuration. Cumulative sub-visible particle count ≥ 2 µm (top row) and ≥ 10 µm (bottom row) are shown for mAb1 (left panel) and mAb2 (right panel) after storage at a) 40°C, b) 25°C and c) 5°C. Red triangle shows sub-visible particles of brownish Suc samples. Dotted line indicates upper limit of detector.

Figure S2 shows structural characterization by X-ray powder diffraction of Suc formulations for mAb1 after storage at 40°C for 9 months revealing crystallization of sucrose in lyophilisates that turned brownish upon storage.

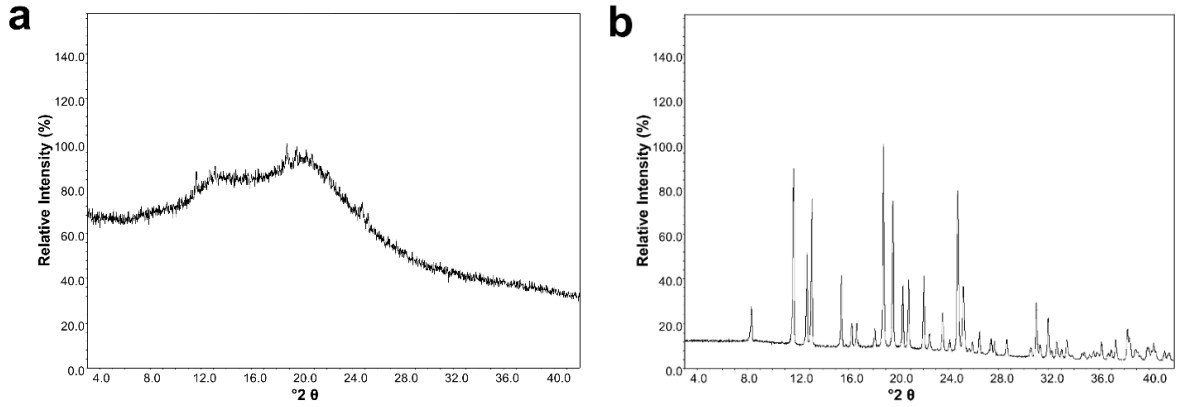

Figure S2. Representative X-ray diffractograms. X-ray diffraction signals of a) white and b) brownish mAb1 lyophilisates formulated with 80 mg/mL sucrose after storage at 40°C for 9 months.

Figure S3 shows loss of monomer at different time points for mAb1 and mAb2 formulations after storage at 5°C. Formulations with Suc, CD, or PVP show no loss of monomer after 9 months at 5°C. In general, rHA and Dex containing formulations are less stable. Formulations with 40 mg/mL rHA or 40 mg/mL Dex in a 1:1 ratio with CD show a decrease in monomer content by approximately 1% after 9 months.

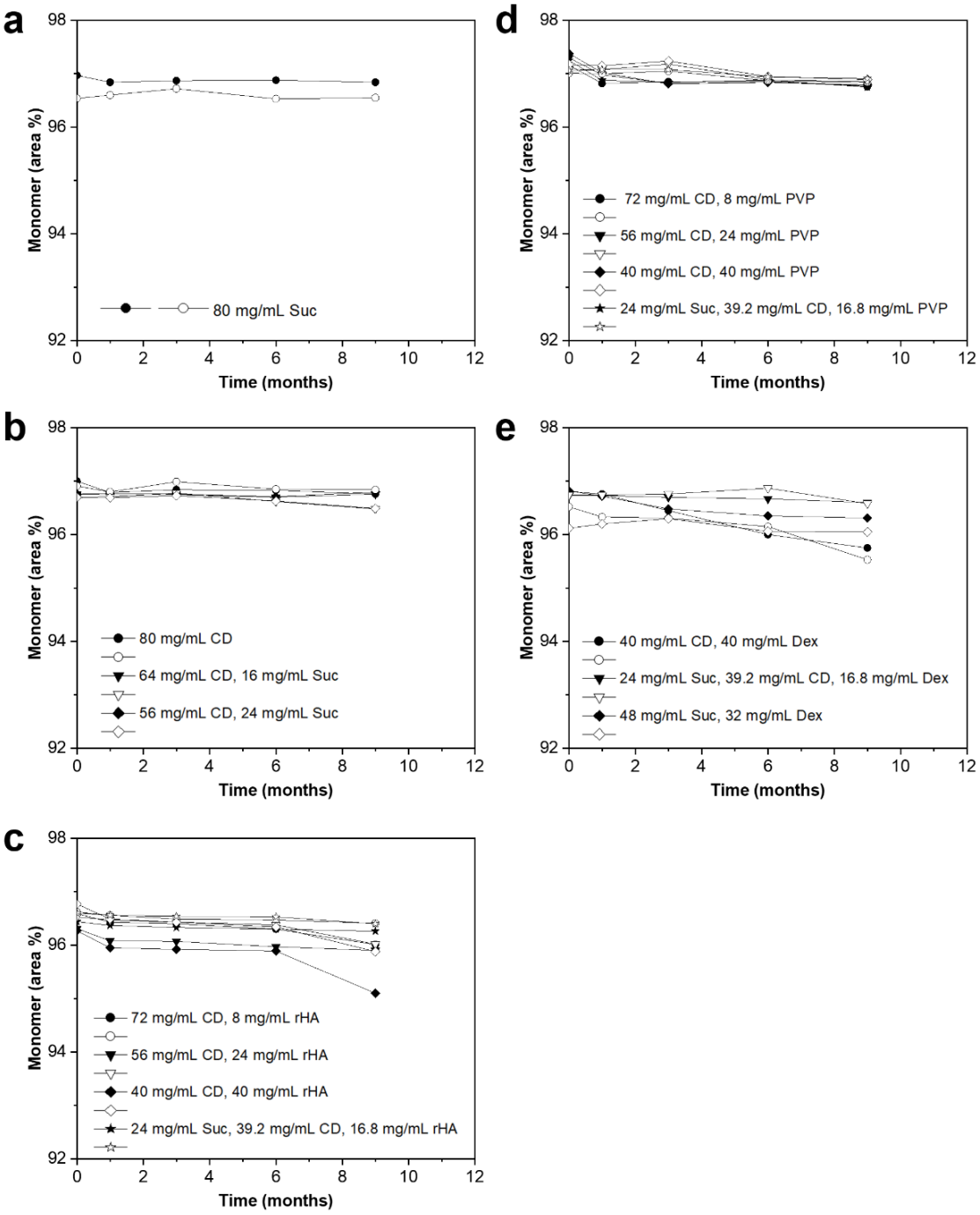

Figure S3. mAb stability in lyophilisates by SE-HPLC. Loss of monomer of mAb1 (filled symbols) and mAb2 (open symbols) after storage at 5°C for formulations with a) Suc, b) CD, c) rHA, d) PVP, and e) Dex.

Figure S4 shows monomer loss of mAb2 in pure Suc for a conservatively freeze-dried formulation, i.e. non-collapsed lyophilisate. Samples collapsed less compared to the aggressive freeze-dried lyophilisates upon storage and revealed comparable monomer levels to “white” aggressively freeze-dried lyophilisates.

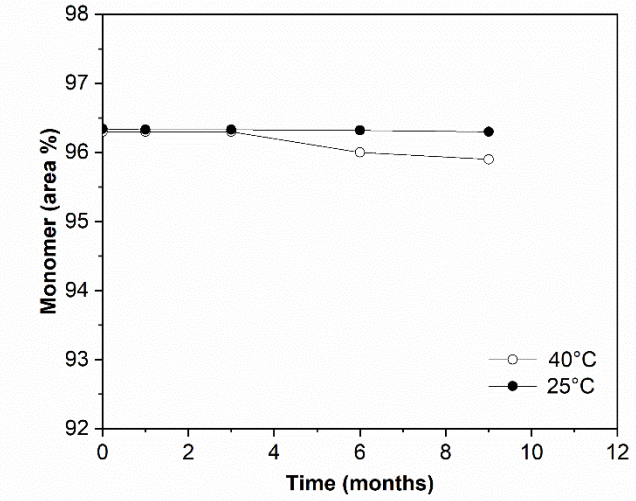

Figure S4. mAb stability in non-collapsed sucrose lyophilisates. Loss of mAb2 monomer in pure Suc formulations after storage at 25 and 40°C.

Figure S5 reveals mAb glycation in pure Suc formulations when stored at 40°C for 9 months. Glycation in brownish lyophilisates is twice that of white lyophilisates.

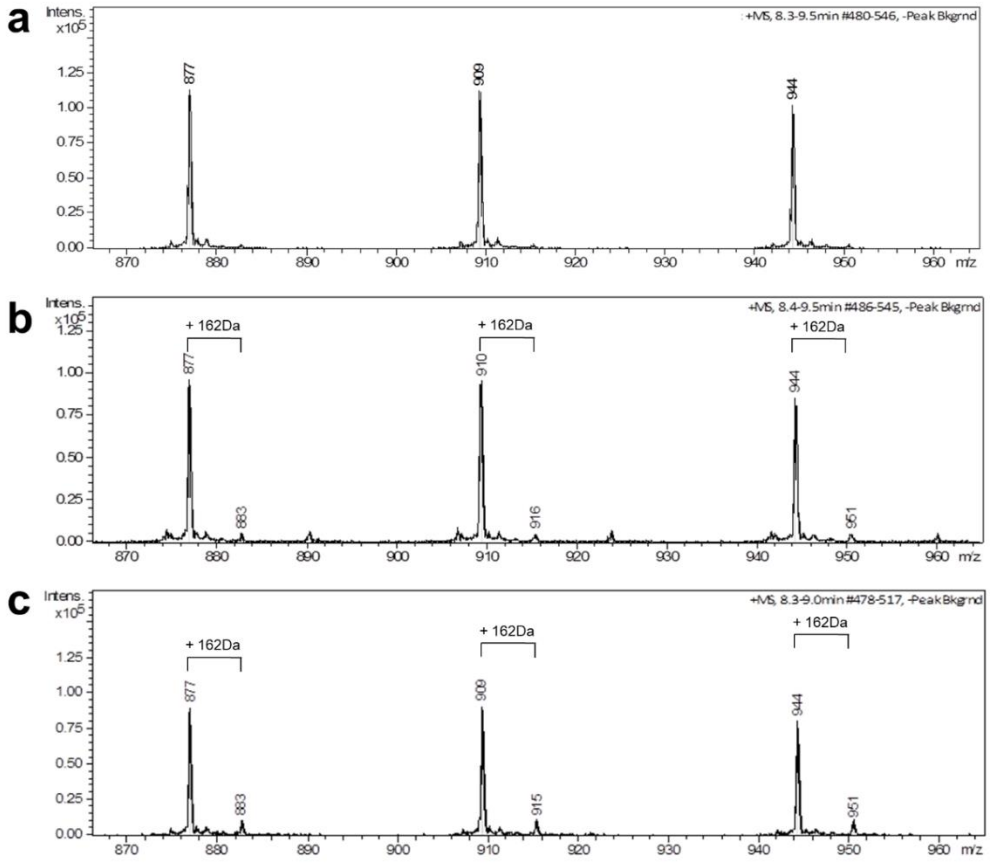

Figure S5. Representative mass spectrogram of mAb1 formulated with sucrose. a) directly after freeze-drying and b) white lyophilisate or c) brownish lyophilisate after 9 months at 40°C. z = 27.

Figure S6 shows dynamic vapor sorption behavior for mAb2 lyophilisates. Changes in sorption behavior for CD-based formulations containing sucrose occurred at much higher moisture levels (approximately 15%) compared to pure sucrose (approximately 6-7%), thus ware far above moisture levels relevant during long-term storage at 25°C.

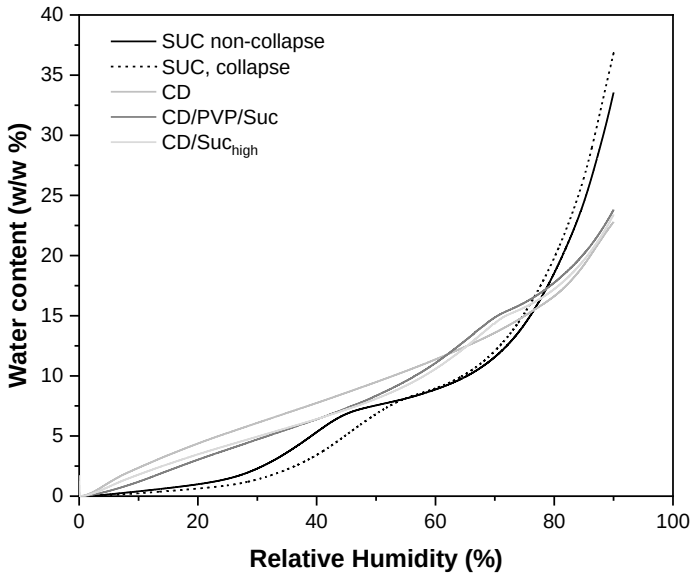

Figure S6. Water sorption isotherms for mAb2 lyophilisates.

CHAPTER 4

Be aggressive! Amorphous excipients enabling single-step freeze-drying of monoclonal antibody formulations

Pharmaceutics, 11 (**2019**) 616

Research Paper

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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-betacyclodextrin (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.

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 (T_g') of the maximally freeze concentrated solution 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°C 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 Pansare *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-hydroxyproyl-betacyclodextrin (HPBCD), which has a T_g' similar to dextan 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 protein to sugar ratios 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.

MATERIALS AND METHODS

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 (s. Table S1): F_S contained 80 mg/mL sucrose (Ferro Pfanstiehl Company, Mayfield Heights, Ohio), 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 µm PVDF sterile filter unit (Millipore, Bedford, MA, USA). 3.2 mL per formulation were filled into 6 mL TopLyo® vials (Schott, Müllheim, Germany) and partially stoppered with 20 mm Lyo-stoppers D777-1 (DAIKYO Seiko Ltd., Tokyo, Japan).

Freeze-drying

Freeze-drying was performed using a FTS Lyostar II (FTS Systems Inc., Stone Ridge, NY). 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.

Table 1. Overview of process parameters of the different freeze-drying cycles employed. All cycles had a loading step of 60 min at 5°C prior to freezing. End of primary drying time was determined as $\Delta p_c \leq 1$ mTorr between Pirani and capacitance probe.

		Primary drying			Secondary Drying			
Cycle #	Freezing	Ramp (°C/min)	T_s (°C)	p_c (mTorr)	Ramp (°C/min)	T_s (°C)	Hold time (min)	Pressure (mTorr)
0 (Reference)	Ramp 0.3°C/min Temp. -35°C Hold 180 min	0.2	-10	100	0.2	+25	360	100
1		0.2	+10	100	0.2	+25	360	100
2		0.2	+30	100	0.2	+40	360	100
3		1.0	+30	100	0.2	+40	360	100
4		0.2	+30	155	0.2	+40	360	155
5		0.2	+30	100			-	

T_s: shelf temperature, p_c: chamber pressure

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 T_{zero} DSC Q2000 instrument (TA instruments Inc., New Castle, Delaware). 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).

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, Göttingn, Germany) at 20-fold magnification [19].

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.

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 AI 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.

Reconstitution time

For reconstitution, 3.0 mL or 2.9 mL of water for injection were added to the 10 mg/mL 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].

Specific surface area

Specific surface area was determined in triplicates according to Brunauer-Emmett-Teller (BET) using the Quadrasorb evo surface are 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.

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].

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) equipped with a 2487 UV/visible detector (Waters Corporation). Samples were held at 5°C and the column temperature was set to 25°C. 50 mg/mL mAb formulations were diluted to 10 mg/mL with formulation buffer,

10 mg/mL mAb formulations were analyzed without further preparation. 10 µL of the sample were injected on a TSKG3000SWxl, 7.8×300 mm column (Tosoh Bioscience, Stuttgart, Germany) and eluted over 30 min with a 0.2 M K_2HPO_4/KH_2O_4 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 (Waters Corporation) and monomer content was reported as percentage of total peak area.

RESULTS

Thermal properties of the liquid formulations

To assess the critical T_p, T_g' and T_c were determined for 10 mg/mL 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 $F_{CD/P/S}$ (-19.2°C) and $F_{CD/S}$ (-18.6°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°C and -8.3°C for 10 mg/mL 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 mg/mL to 50 mg/mL compared to an increase of only 2.6°C for F_{CD} for example. In addition, for F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$ 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 $F_{CD/P/S}$ 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
$F_{CD/P/S}$	10	-19.2	-18.5
	50	-16.5	-14.6
$F_{CD/S}$	10	-18.6	-16.0
	50	-15.2	-10.9

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_c = 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}, $F_{CD/P/S}$, and $F_{CD/S}$ 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°C 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 did only slightly increase 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).

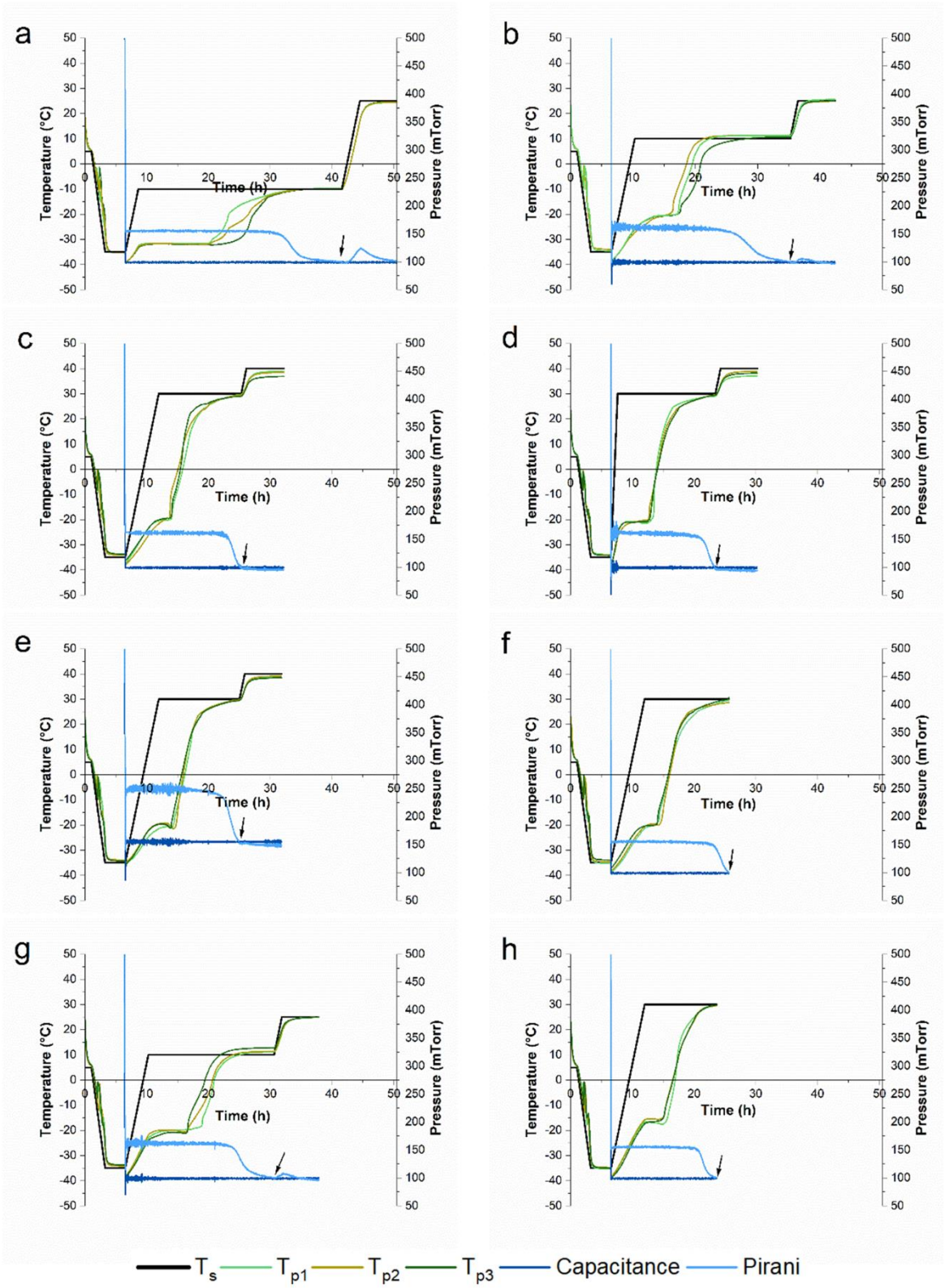

Figure 1. Freeze-drying cycles. Process monitoring data of a-f) 10 mg/mL mAb formulations and of g-h) 50 mg/mL mAb formulations. a) shows the conservative cycle (C0), b) and g) C1, c) C2, d) C3, e) C4 and f) and h) C5. The arrow indicates end of primary drying. T_s = shelf temperature; T_p = product temperature determined by thermocouples.

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) (Figure 2a and Figure 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}, $F_{CD/P/S}$, 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 $F_{CD/P/S}$ 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 $F_{CD/S}$ and $F_{CD/P/S}$ 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}, $F_{CD/P/S}$, 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.

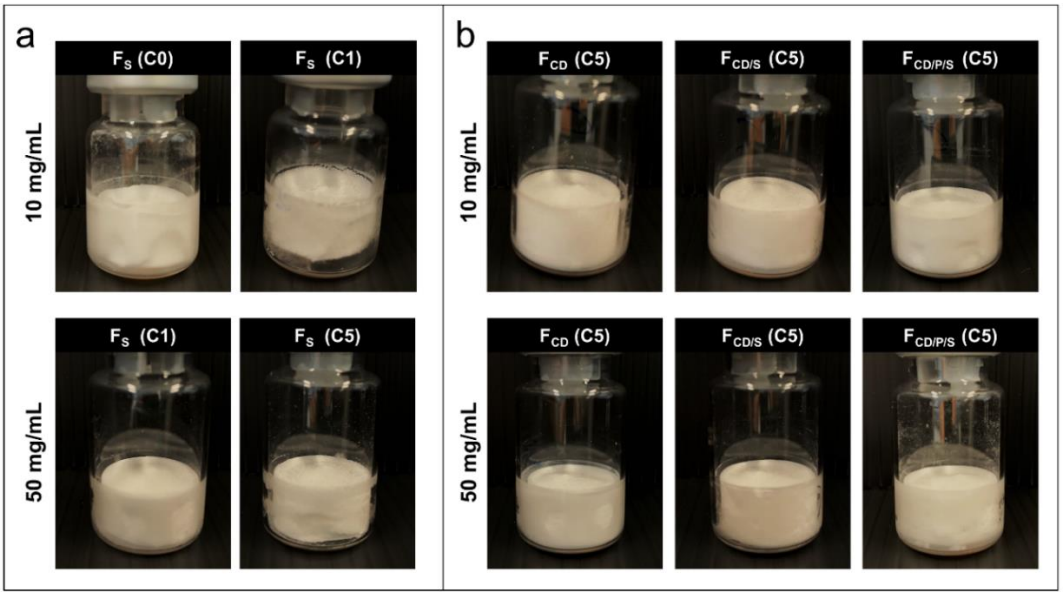

Figure 2. Cake appearance. Representative lyophilisates for 10 mg/mL formulations (upper row) and 50 mg/mL formulations (lower row). a) different cake appearances for F_S depending on freeze-drying cycle and b) cake appearance obtained for F_{CD}, $F_{CD/S}$, and $F_{CD/P/S}$ exemplarily shown for C5.

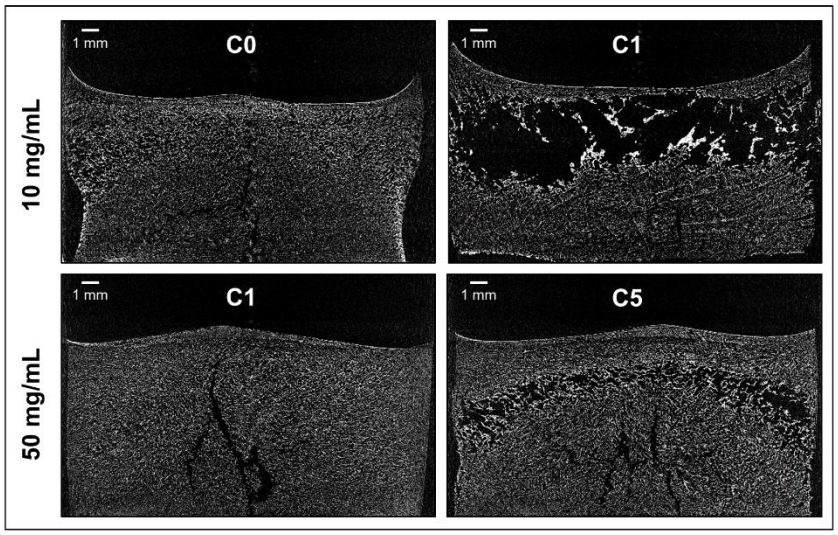

Figure 3. Internal cake structure of F_S. Representative µ-CT images for 10 mg/mL and 50 mg/mL formulations freeze-dried with different lyophilization cycles.

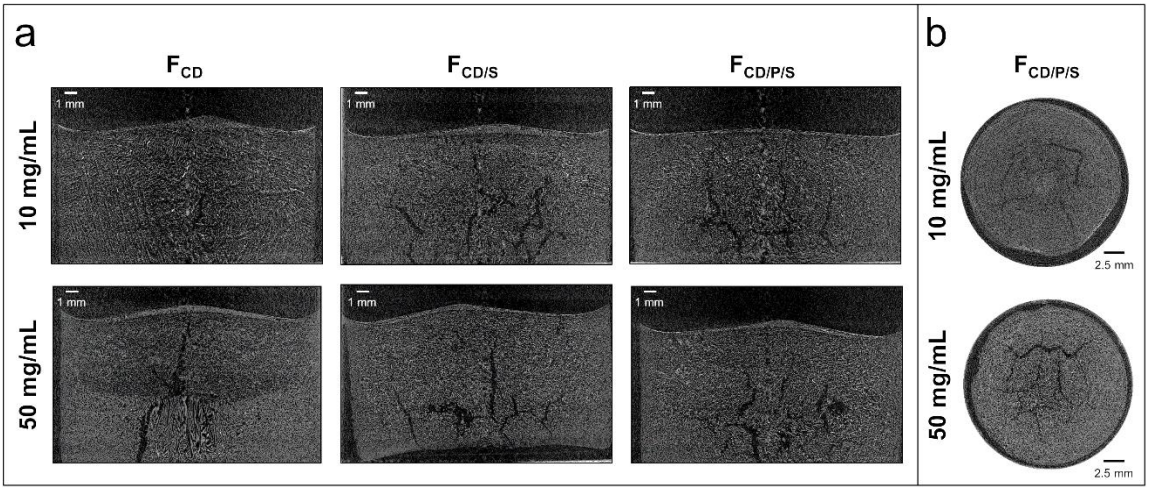

Figure 4. Internal cake structure. Representative µ-CT images for 10 mg/mL and 50 mg/mL formulations freeze-dried with C5 showing a) internal cake structure through a vertical cross section of F_{CD}, $F_{CD/S}$, and $F_{CD/P/S}$ and b) cake structure at the bottom of the vial through a horizontal cross section of $F_{CD/P/S}$.

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 sec) 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 F_S at 50 mg/mL mAb took the longest with ~80 sec compared to F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$, 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 mg/mL and 50 mg/mL formulations. (Figure S2). Only F_S 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 F_{CD}, $F_{CD/P/S}$, $F_{CD/S}$ was less than 0.5%, with marginal differences amongst the different formulations. In contrast, F_S had a residual moisture of 1.2% when freeze-dried with the conservative cycle C0. When freeze-dried with the more aggressive cycle C1, residual moisture levels increased to 4.1%. For C2 to C4 (or C1 for 50 mg/mL mAb formulations), some vials were stoppered at the end of primary drying to evaluate the remaining moisture content at the end of primary drying. Residual moisture of these stoppered samples varied more between the different formulations but without a clear trend. Products from C3 showed the least variation in residual moisture at the end of primary drying. Overall, already after primary drying residual moisture was below 0.5%, supporting the observations for the Pirani signal (Figure 1c-e).
Residual moisture of F_S with 50 mg/mL mAb was generally higher compared to F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$ (Figure 5b). C1 resulted in a residual moisture for F_S of 0.64%, while freeze-drying with C5 led to a residual moisture of 1.1%. F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$ 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.

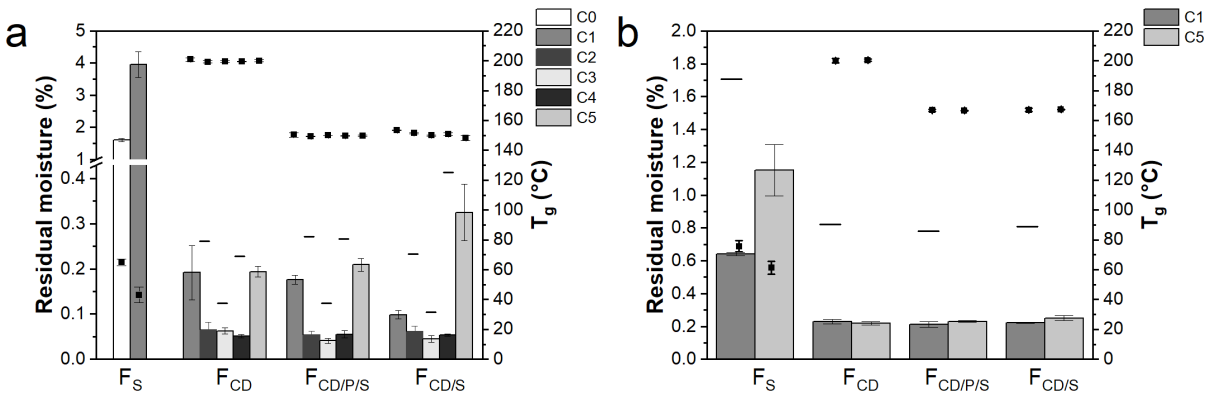

Figure 5. Residual moisture and T_g. Data is given for a) 10 mg/mL mAb formulations and b) 50 mg/mL mAb formulations for different formulations and freeze-drying cycle conditions. Residual moisture levels are shown as bars and lines. Lines show residual moisture level of vials stoppered after primary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

The varying residual moisture levels of 10 mg/mL F_S 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 F_S was considerably lower with 43.2°C. T_g values of F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$ were much higher than compared to F_S and were comparable for all cycles employed. T_g of $F_{CD/P/S}$ and $F_{CD/S}$ showed a similar T_g at ~150°C, while F_{CD} 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 F_S when freeze-dried with C1 or C5.

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 F_S 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 F_{CD}, $F_{CD/P/S}$, and $F_{CD/S}$ at 10 mg/mL mAb was comparable to F_S. F_S, $F_{CD/S}$, and $F_{CD/P/S}$ 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). F_{CD} 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 F_s after storage for both freeze-drying cycles. The remaining monomer content after storage was much lower for F_{CD} with levels of 80.7% and 84.3% for C1 and C5, respectively. Better and similar mAb stability was obtained for $F_{CD/S}$ and $F_{CD/P/S,}$ with a loss of monomer content of 4.1% for C1 and 2.7% for C5. Interestingly, for F_{CD}, $F_{CD/S,}$ and $F_{CD/P/S}$ with 50 mg/mL mAb stability was generally slightly improved when lyophilized with C5 compared to C1.

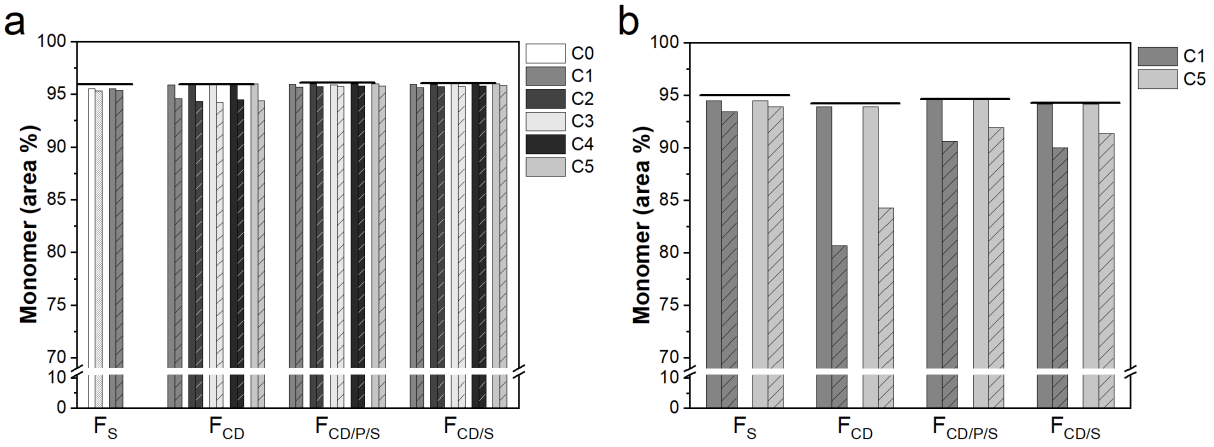

Figure 6. Protein stability by SE-HPLC. Percentage of remaining monomer for a) 10 mg/mL mAb formulations and b) 50 mg/mL mAb for different formulations and freeze-drying cycle conditions. Bars without pattern show monomer content directly after freeze-drying and bars with pattern show monomer content 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.

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.

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_c may be used interchangeable. 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 (ΔT) increases with higher protein concentration. For a 50 mg/mL mAb formulation with 7-8% disaccharide, a ΔT 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 ΔT 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 ΔT is less pronounced. For $F_{CD/S}$ and $F_{CD/P/S}$ 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 addition, our data suggests that T_p should ideally be at least 4°C below T_c in order to result in a throughout elegant lyophilisate. If the difference between T_c and T_p was less than 3°C, minor dents were introduced. In terms of internal cake structure, µ-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 µ-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.

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°C to +10°C (C1) and from +10°C 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°C 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_c is a balance between batch homogeneity and prevention of collapse or meltback [3, 41]. Previous studies demonstrated an increase of p_c 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 time 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, $F_{CD/P/S}$, and $F_{CD/S}$ 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].

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_p above T_g' during primary drying [4]. The results of the present study showed good and comparable storage stability for $F_{CD/P/S}$ and $F_{CD/S}$. Collapse did not impact protein stability of F_S when stored at 40° 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 $F_{CD/S}$, $F_{CDP//S,}$ and F_{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/w) are close to our 7:1 and 1.6:1 ratio for 10 mg/mL and 50 mg/mL mAb. 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 $F_{CD/S}$, $F_{CDP//S,}$ and F_{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/w) 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.

CONCLUSION

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.

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SUPPORTING INFORMATION

Table S1 shows the composition of the different formulations. F_S served as reference formulation throughout this study.

Table S1. Formulation composition. All formulations were prepared in 20 mM histidine/histidine-HCl buffer pH 6.0 containing 0.02% polysorbate 20.

Formulation	mAb (mg/mL)	Sucrose (mg/mL)	HPBCD (mg/mL)	PVP (mg/mL)
F_S	10 50	80	-	-
F_{CD}	10 50	80	-	-
$F_{CD/P/S}$	10 50	24	39.2	16.8
$F_{CD/S}$	10 50	24	56	-

HPBCD: 2-hydroxypropylbetacyclodextrin
PVP: Polyvinylpyrrolidone

Figure S1 shows the reconstitution times of 10 mg/mL and 50 mg/mL mAb formulations freeze-dried with different cycle parameters. For 10 mg/mL mAb formulations reconstitution times were comparable for all formulations and cycle conditions. Reconstitution of 50 mg/mL mAb formulations took generally longer and reconstitution of F_S took around 80 seconds (sec) longer compared to F_{CD}, $F_{CD/P/S}$, and $F_{CD/S,}$ independent of the cycle employed.

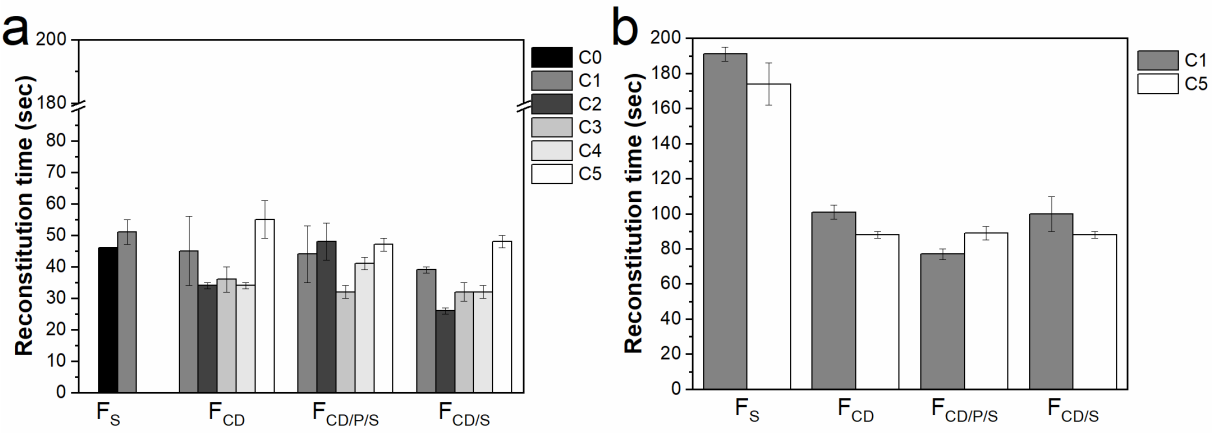

Figure S1. Reconstitution time of lyophilisates. a) 10 mg/mL mAb formulations and b) 50 mg/mL mAb formulations freeze-dried with different cycles.

Figure S2 shows the specific surface areas of 10 mg/mL and 50 mg/mL mAb formulations freeze-dried with different cycle parameters. In general, F_S showed a slightly lower specific surface area compared to F_{CD}, $F_{CD/P/S}$, and $F_{CD/S.}$ In addition, $F_{S\ at}$ 10 mg/mL mAb, when freeze-dried with C1 had a lower specfic surface area, which is indicative for the collapsed lyophilisate.

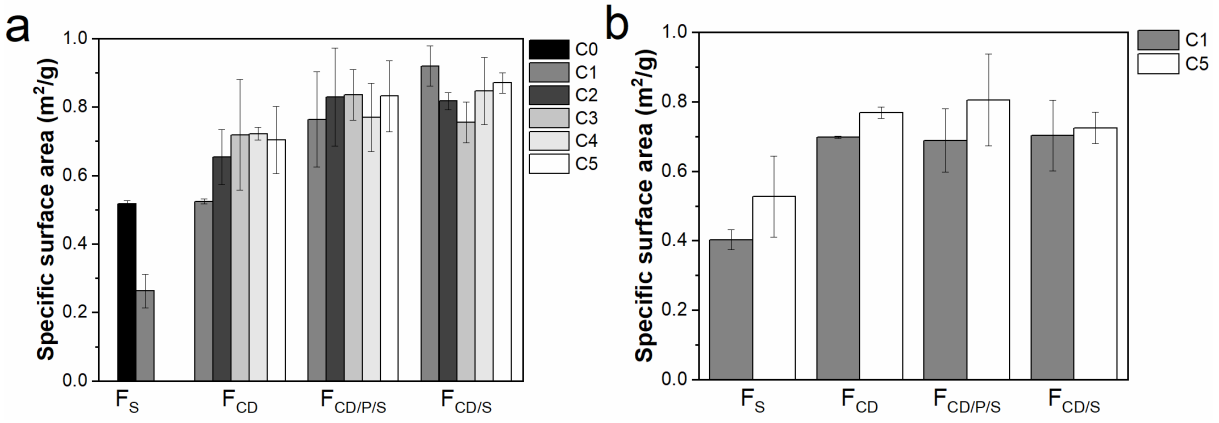

Figure S2. Specific surface area of lyophilisates. a) 10 mg/mL mAb formulations and b) 50 mg/mL mAb formulations freeze-dried with different cycles.

Figure S3 shows mAb stability after 3 months 40°C of 10 mg/mL lyophilisates that were spiked to different residual moisture levels to investigate whether very low residual moisture levels might be detrimental to mAb stability during storage. Stability was not affected in a moisture level range between 0.2% and 2%.

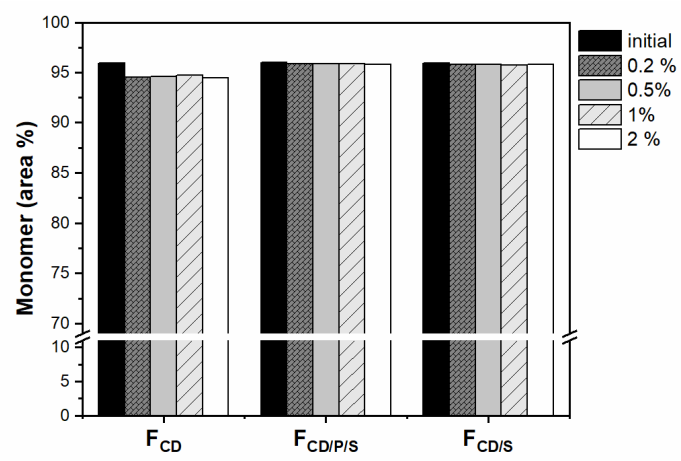

Figure S3. Protein stability dependent on residual moisture (in %). Monomer content by SE-HPLC is given for 10 mg/mL formulations after freeze-drying and after storage for 3 months at 40°C.

DISCUSSION AND OUTLOOK

Freeze-drying is a unit operation during drug product manufacturing used to increase stability, if proteins are unstable as liquids. However, typical disaccharide-based formulations have low glass transition temperatures. Particularly for low concentrated antibody formulations, this poses challenges for freeze-drying cycle development due to an increased risk of collapse. Collapse increases the risk of rejects and complaints, and may be detrimental to protein stability.
The aim of this work was to investigate amorphous excipients as alternatives to sucrose for low concentrated freeze-dried antibody formulations. The main objectives were to investigate the excipients with regards to their ability to stabilize antibodies during freeze-drying and particularly during storage at elevated temperatures, while enabling short freeze-drying cycles that provide pharmaceutically elegant lyophilisates. To address the impact of different excipients and freeze-drying cycles on the lyophilisate, µ-CT was established to visualize differences in cake appearance and structure.

Cake appearance by µ-CT: Opportunities and limitations

Objective evaluation of cake appearance can be difficult and is dependent on the training of the operator. In commercial manufacturing, automated visual inspection machines are often used, which help to bring the evaluation of cake appearance to a more objective and reproducible process. However, lyophilized products can provide challenges for camera-based inspection systems, e.g. for fogging, necessitating re-inspections [1, 2]. Moreover, during freeze-drying cycle development, not only cake appearance, but also internal cake structure and morphology are investigated to detect characteristics that may influence drug product quality attributes such as reconstitution time or protein stability. This is typically done through PDMS embedding and scanning electron microscopy. Because of their destructive nature only select regions of the lyophilisate can be investigated. Moreover, this approach limits the possibility to directly link the individual cake structure of a lyophilisate to its impact on product quality attributes. In light of these challenges, µ-CT was introduced as an alternative method in Chapter 1. It enabled imaging of the cake structure and morphology of the entire lyophilisate with one single measurement, thus being advantageous compared to PDMS embedding and scanning electron microscopy. Moreover, this method was applied to evaluate the impact of different excipients on the lyophilisates, investigated in Chapters 2 and 3, and to detect collapsed regions in the lyophilisates upon development of an aggressive freeze-drying cycle (Chapter 4).

Quantitative characterization

In addition to qualitative imaging, the applicability of µ-CT to quantitatively differentiate between cake appearances resulting from different formulations freeze-dried with the same lyophilization cycle was demonstrated in Chapter 1. Performing the X-ray scan with a resolution of 10 µm, *surface/volume* ratio

and *pixel count/slice* were calculated to achieve a quantitative measure for collapse. This additional quantitative characterization of lyophilisates can be valuable during freeze-drying cycle development and process transfers. For instance, Parker *et al.* used a resolution of 4.5 µm to compare porosity of lyophilisates, which were freeze-dried with aggressive or conservative conditions [3]. Izutsu *et al.* performed µ-CT with a high resolution of 0.5 µm to study the impact of freezing protocols on the morphology of freeze-dried solids [4]. Recent studies by Pisano *et al.* and Foerst *et al.* used µ-CT images with a resolution of 6 µm and 1 µm, respectively, to calculate the pore size of lyophilisates for estimation of the mass transfer rate during primary drying [5, 6]. On the one hand, these studies highlight the potential of µ-CT for a quantitative characterization, while on the other hand the different acquisition parameters, e.g. resolution, simultaneously reveal its limitations. Any quantification is based on binary images, meaning prior processing of the tomographs is needed. The outcome of this is dependent on image quality, i.e. the contrast of the µ-CT image, resolution, and thresholding algorithms. As the glass vial, which needs to be penetrated by the X-rays, has a much higher density than the lyophilisate, obtaining images of high contrast is challenging. For this reason, the above mentioned studies removed the lyophilisate from the glass vial for analysis. In the study presented in Chapter 1, we used a custom-made plastic container, which was overlaid with nitrogen and sealed to avoid structural changes due to water uptake. Besides contrast, the resolution of the X-ray scan has considerable impact on the quantification of pore size, as a resolution that is lower than the size of individual pores will make any quantification of pores inaccurate. Additionally, the different available thresholding algorithms further influence the accuracy of the quantitative information, turning the idea of objective quantification into subjective evaluation. Therefore, caution should be taken when comparing data from different studies using different acquisition parameters. Nevertheless, the relative comparison of quantitative characteristics of lyophilisates within one study can be of great advantage during development, for example to compare different formulations or process parameters. However, the use of µ-CT to characterize morphology is not a high-throughput method. Measurement time increases strongly with higher resolutions and larger vial sizes. Dependent on the µ-CT equipment, i.e. detector and field of view, analysis of one lyophilisate can easily take more than a day. The lyophilization process is known to result in inter-vial heterogeneities [7], requiring a representative sample size of the batch for statistical significant analysis. This leads to time-consuming analysis and immense data sets, limiting the applicability for routine quantitative process characterization.

Non-invasive imaging

As mentioned above, µ-CT of the lyophilisate through the glass vial is more challenging due to the different X-ray intensities needed for the glass vial and lyophilisate. In Chapter 1, the feasibility of µ-CT for a non-invasive analysis of the internal cake structure to qualitatively identify cake defects, or intra- and inter-vial heterogeneities was demonstrated. The method presented in Chapter 1 resulted in considerable loss of contrast and marked appearance of so called beam hardening artefacts [8]. Over the course of this work, using another µ-CT instrument and adapting the measurement as well as reconstruction parameters, non-invasive imaging with much higher quality and without strong artefacts was achieved, as shown by µ-CT images in Chapter 4. Nevertheless, reductions in obtained contrast are expected and would be considered acceptable for a purely qualitative approach. It is possible that further method development, or tools such as phase contrast enhancement would

enable quantitative analysis of µ-CT images that were acquired through the glass vial. While most research focuses on a quantitative readout, qualitative evaluation is already beneficial, as in contrast to scanning electron microscopy it comprehensively visualizes the entire cake structure. Most importantly, the non-invasive imaging allows to directly link the structure or morphology to other product quality attributes by using the same lyophilisate for further characterization, being highly beneficial during formulation and process development, and transfers. In addition, for the qualitative evaluation of macroscopic defects in cake appearance, such as collapsed regions, a considerably lower resolution than used in the presented studies might be sufficient and thus increase throughput. Such qualitative evaluation could also provide a promising in-process control for commercial production complementary to visual inspection, revealing internal critical defects such as meltback or partial collapse. To implement µ-CT as a routine analysis, further studies would be needed to first identify an ideal µ-CT instrument suitable to cover all typical vial dimensions and second to establish the optimal acquisition parameters with minimal measurement to achieve the resolution needed to identify macroscopic cake defects. The full potential of µ-CT goes beyond imaging of cake structures. For example, µ-CT has been reported to detect container closure integrity issues [9, 10] and an X-ray based system that detects external particles, such as glass or fibers within lyophilisates, was presented [11]. Future µ-CT instruments may potentially enable a combined analysis of these different aspects.

Potential and challenges of alternative amorphous excipients

Sucrose is the most frequently used excipient in lyophilized protein formulations. Despite its excellent protein stabilizing properties, its low glass transition temperature provides limitations, leading to time-consuming freeze-drying cycles and potentially requiring refrigerated storage conditions. As proteins increase the formulation's glass transition temperature, these challenges are less pronounced for high concentrated antibody formulations. However, as new mAb derivatives such as ADCs, bispecific mAbs, and Fc-fusion proteins often require lower doses, development of a short freeze-drying cycle providing elegant lyophilisates becomes challenging. Chapter 2 and 3 investigated alternative amorphous excipients, which are known to have high glass transition temperatures to a) render elegant lyophilisates upon a freeze-drying cycle that demonstrated collapse in Chapter 1 for a pure sucrose formulation and b) stabilize the two model mAbs during freeze-drying and in particular during storage at elevated temperatures. Chapters 2 and 3 studied the impact of dextrans of different molecular weight (1 to 500 kDa) and HPBCD-based formulations on freeze-dried mAb formulations.

Impact on properties of freeze-dried formulations

The excipients (and combinations thereof) investigated in the presented work rendered formulations that exhibited markedly higher glass transition temperatures of both the liquid and the freeze-dried formulation compared to the pure sucrose formulation, which served as the reference throughout this work. Comparing the glass transition temperatures obtained for dextrans and HPBCD illustrates that besides molecular weight, structural aspects like cross-linking and branching strongly influence the glass transition temperature. The glass transition temperature of HPBCD (T_g': -10.8°C), a cyclic oligosaccharide of approximately 1 kDa with hydroxypropyl side chains, was similar to that of high

molecular weight dextrans (dextran 40-500 kDa, Tg': -10.5°C). Within this work it was shown that a smaller molecule with a similar higher glass transition temperature might be preferable with regards to product quality attributes and antibody stability. A drawback of higher molecular weight dextrans was their increased viscosity, which correlated with much longer reconstitution times. In contrast, HPBCD rendered formulations with the same glass transition temperature, but did not contribute to viscosity, providing lyophilisates with short reconstitution times. Both, dextran and HPBCD-based lyophilisates significantly improved cake appearance and provided lower residual moisture levels (Chapters 2, 3) compared to a non-collapsed sucrose formulation (Chapter 4).

Antibody stability: glass dynamics and molecular interactions

During freeze-drying HPBCD (Chapter 3) and smaller dextrans (Chapter 2) better stabilized the two model mAbs compared to high molecular weight dextrans (≥40 kDa), which showed a loss of monomer content after freeze-drying. In general, two mechanisms for protein stabilization are discussed, namely vitrification and water replacement, which have been described in the 'Introduction' section in detail. Stabilization through water replacement has been reported to depend on the molecular ratio of excipient to protein, which at a constant excipient content of 80 mg/mL used within this work, was lower for large dextrans in comparison to smaller dextrans. However, this raises the question whether the molecular ratio of the dextran or of the single glucose unit might account for the interaction. The molar ratio of glucose units to antibody would be the same for all dextran formulations. To fully elucidate this question, further studies with varying antibody/dextran ratios would be needed. Presumably, a better explanation for the observed lower stability might be that high molecular weight dextrans are only able to stabilize via vitrification. Their rigid nature, which increases with higher molecular weight, might prevent dextrans from interacting via hydrogen bonds. Vitrification of the protein is important for stability in the dried state during storage, as for storage temperatures below the glass transition temperature molecular mobility (α-relaxation) is reduced, slowing down degradation mechanisms. Stability studies at elevated temperatures within this work showed that protein stability during storage is not exclusively linked to the glass transition temperature of the formulations. Storage of the collapsed sucrose formulation close to its glass transition temperature resulted in sudden, strong degradation of both mAbs. For dextran and HPBCD formulations, the storage temperature of 40°C was far below their glass transition temperature of approximately 200°C. According to the vitrification theory, good and comparable mAb stability would have been expected for both formulations. However, dextrans could not stabilize the mAbs during storage at elevated temperatures (which will be outlined in more detail below). On the other hand, HPBCD stabilized the mAb during freeze-drying and provided significantly better stability during storage, while best stability was obtained with the addition of 30% sucrose. Addition of PVP did not further contribute to mAb stability. It has been described previously that at storage temperatures far below the glass transition temperature, water replacement rather than vitrification is the dominating stabilizing mechanism [12]. These findings highlight that sucrose is superior to HPBCD in stabilizing the antibody by molecular interactions. Recently, Tonnis *et al.* reported that not only the size, but rather the flexibility of a sugar accounts for its ability to stabilize proteins via hydrogen bonds [13].

To address the ability of molecular interactions, Mensink *et al.* proposed solid-state nuclear magnetic resonance spectroscopy (NMR) to characterize the $^{1}HT_{1\rho}$ and $^{1}HT_{1}$ relaxation times as an indirect

measure for the miscibility of protein and sugars in the lyophilisate [14]. Solid-state NMR studies to assess the antibody-sugar interactions in dextran and HPBCD formulations are subject of ongoing research. A direct measure for hydrogen bonds can be given by Fourier-transform infrared spectroscopy (FTIR) to investigate the ability of a sugar and/or the mass fraction needed to maintain the secondary structure of a protein [15]. Good protection of the native confirmation of a freeze-dried antibody by HPBCD was reported by Faghihi *et al*. [16]. *A*dditional studies with the formulations established within this work would help to get a profound understanding whether HPBCD shows less interaction via hydrogen bonds compared to sucrose. If close interactions were given, fast molecular motions have been described to contribute to protein stability at storage temperatures far below the glass transition temperature. Using neutron scattering, Pikal *et al.* demonstrated that sucrose, despite its high molecular mobility (i.e. α-relaxation), strongly depresses fast β-relaxations, which might explain its outstanding stabilizing properties [17]. Thus, additional studies using neutron scattering would be useful to get a deeper understanding of fast dynamics in HPBCD formulations. Depending on these outcomes, the ratio of HPBCD and sucrose with regards to optimal mAb stability could potentially be derived.

Risk of antibody glycation at elevated storage temperatures

Besides physical instability of the antibody if stored at temperatures close to the glass transition temperature, some chemical modifications are particularly accelerated at elevated temperatures. Chapter 2 highlighted the risk of antibody glycation at elevated storage temperatures for dextrans. Although our results showed no impact on potency, glycation and particularly the formation of accelerated glycation end-products is of concern with regards to immunogenicity [18]. Moreover, Mensink *et al.* reported impaired affinity for an antibody due to glycation [14]. Thus the impact on protein affinity, immunogenicity, and safety has to be evaluated on a case-by-case basis. Most importantly, Chapter 3 showed that glycation can also occur in lyophilisates with sucrose at elevated storage temperatures. Particularly at higher residual moisture levels, sucrose can hydrolyze, eventually leading to antibody glycation. This finding is of key importance regarding the possibility of storage at room temperature and demonstrates the limitations of dextrans and sucrose as excipients. On the contrary, HPBCD is a non-reducing sugar that does not hydrolyze unless exposed to extreme acidic conditions, thus being beneficial for room temperature stable formulations. Whether glycation by sucrose would be an issue in binary formulations of HPBCD and sucrose and therefore limit the amount of sucrose, needs to be investigated.

Applicability for antibody formulations

The findings of the studies in Chapter 2 and 3 showed that sucrose is and will be an important excipient for stabilization of the two model mAbs. However, for short freeze-drying cycles of low concentrated mAb formulations and potential storage at room temperature, sucrose alone will likely result in impaired cake appearance and stability. Based on that, the addition of alternative excipients becomes relevant. Dextrans were found to be not suitable due to glycation of the antibody. On the other hand, HPBCD with addition of sucrose was found to provide good stability at elevated temperatures. Having a high fraction of HPBCD, the advantage of the high glass transition temperature was maintained, resulting in overall good product quality attributes. This can be beneficial with regards to development

of short lyophilization cycles, leading to more elegant lyophilisates, which would cause less rejects. The potential of storage at room temperature would allow to make costly cold chains redundant and in particular enable a better supply of developing countries, where maintaining controlled storage temperatures can be challenging.

Due to the possible different modes of interaction with the protein, it is essential to determine the influence of HPBCD on *in vitro* affinity and *in vivo* pharmacokinetic in toxicological studies. In general, HPBCD is parenterally approved and its use can be considered save up to a daily dose of 250 mg/kg/day for more than two weeks [19]. Due to the short $t_{1/2}$ of HPBCD, a longer administration period with less frequent administrations, as is often the case for biopharmaceuticals is likely to be without adverse effects. HPBCD is eliminated through the kidney without being metabolized, thus literature emphasizes that caution might be needed for application in patient groups with impaired renal activity, due to accumulation of HPBCD [19]. Beyond toxicology, HPBCD-based formulations are expected to be hypotonic, which needs to be taken into account during administration. However, this can be easily counteracted by reconstitution with 0.9% sodium chloride solution.

Single-step freeze-drying

Freeze-drying is a time-consuming batch process requiring a lot of energy, resulting in an expensive drug product manufacturing process. The lengthy process is mainly ascribed to the primary drying step, which can last several days. Increasing the shelf temperature or chamber pressure during primary drying would shorten the primary drying phase. However, the increase of shelf temperature is considerably limited by the glass transition/collapse temperature of common sucrose-based formulations. To this end, Chapter 2 and 3 investigated alternative excipients that render freeze-dried antibody formulations with much higher glass transition temperatures than sucrose formulations. The best performing formulations across all aspects, developed in Chapter 3, were used for freeze-drying process development.

Shortening the freeze-drying cycle

Chapter 4 focused on freeze-drying cycle optimization by shortening primary drying time through optimization of the two adjustable process parameters: shelf temperature and chamber pressure. It is well-established that increasing the shelf temperature rather than the chamber pressure renders higher sublimation rates at lower product temperatures [20]. Therefore, the main parameter varied was shelf temperature. The HPBCD-based formulations enabled the usage of much higher shelf temperatures during primary drying, shortening the required time by 48% compared to a reference cycle without leading to collapse. An additional increase in chamber pressure was tested, but did not further shorten primary drying time. In addition, the use of high shelf temperatures enables the initiation of the desorption phase of unfrozen water already during the primary drying step, making a separate secondary drying step redundant. Thus, the applicability of a single-step freeze-drying cycle for HPBCD-based formulations could ultimately be demonstrated. Single-step freeze-drying is a rarely studied concept transforming the conventional freeze-drying cycle outlined in the ‘Introduction’

section. So far, this principle has been demonstrated successfully twice in literature for sucrose-based formulations at protein concentrations above 50 mg/mL [21, 22]. At lower protein concentrations however, it was detrimental to cake appearance and necessitated the addition of a crystalline bulking agent. The presented work developed alternative amorphous formulations that enabled single-step freeze-drying for low concentrated (10 mg/mL) protein formulations.

This single-step freeze-drying process ultimately shortened the total cycle time by 50% compared to the conservative reference cycle. However, it is important to understand that the 50% cycle time reduction may not be considered universal. For instance, batch size and fill volume per vial impact the primary drying time. A higher fill height will increase product resistance and thereby reduce the mass transfer. Moreover, Pansare *et al.* observed partial collapse when increasing the fill height, concluding that the fill height has a considerable impact on cake appearance for a single-step freeze-drying cycle [22]. This might as well apply for the studied formulations in Chapter 4 and would require further investigation.

Besides focusing on optimization of the primary drying step itself, recent literature highlights the impact of the freezing step and proposes strategies to manipulate the freezing behavior, improving the efficacy of primary drying [23]. In conventional shelf cooling, nucleation occurs randomly, leading to inter-vial heterogeneity and typically small ice crystals, both contributing to a longer primary drying time. Recently, techniques have been introduced that enable controlled nucleation during the freezing step. It has been shown that controlled nucleation reduces inter-vial heterogeneity [7], leads to formation of larger ice crystals that allow for faster sublimation during primary drying and thus shorten the primary drying step [24-26]. Whether and how controlled nucleation would impact the primary drying of a single-step freeze-drying cycle cannot be fully answered at this point in time, as in contrast to conventional lyophilization cycles, there is no steady sublimation period during primary drying. In addition, large ice crystals reduce efficiency of water desorption due to a lower specific surface area available for desorption [25]. Consequently, higher residual moisture levels have been reported for lyophilisates that were controlled nucleated [24]. Dependent on the magnitude of the increase in residual moisture, this could potentially impact protein stability during storage. Therefore, further studies would be needed to elucidate the applicability and potential of controlled nucleation for single-step freeze-drying to further shorten the cycle time.

Scale-up considerations

As much as short freeze-drying cycles are desired, it is important to be aware that the lyophilizer itself has limitations as well. In Chapter 4, only one third of the lyophilizer's capacity was loaded. Aiming for efficient freeze-drying, lyophilization should be carried out at full load, which in turn results in overall higher sublimation. This becomes particularly important in scale-up to pilot plant and eventually production freeze-dryers. Depending on the lyophilizer's design and capacity, freeze-dryer overload can become an issue for high sublimation rates. Freeze-dryer overload can occur either via choked flow [27] or condenser overload. The cross-sectional area and aerodynamic properties of the gas flow path between the vials and the condenser impose an upper limit to the maximum sublimation rate that a lyophilizer can handle. At sublimation rates exceeding that upper limit, the water vapor flow is choked, meaning the required mass transfer through the duct cannot be maintained at the set pressure, leading to an increase in chamber pressure above the set point and eventually an out-of-control process.

Condenser overload refers to the situation where water vapor in the chamber is generated at a faster rate than the condenser can trap it, leading to an increase in condenser temperature. Hence, the applicability of the developed single-step freeze-drying cycle for production scale has to be investigated carefully and process parameters potentially need to be adapted. If a short single-strep freeze-drying process can be implemented, this will strongly increase the throughput, which is generally limited by the capacity of the lyophilizer due to the batch process. Consequently, further increases of throughput would trigger intensive research into continuous freeze-drying concepts, which are already evaluated by some researchers [28, 29].

Process robustness and transfer aspects

In general, transfer of a freeze-drying process either during scale-up or from one site to another is challenging. Often, modeling is used to facilitate process transfer. However, modeling approaches might be difficult for single-step freeze-drying cycles, as there is no steady sublimation phase and secondary drying already takes place during the primary drying phase. Besides changes to batch size and capacity, differences in radiation, pressure variation within the chamber, as well as differences in heating due to variances in the course of the fluid flow are examples of typical parameters that might change during a transfer. Here, the single-step freeze-drying cycle (Chapter 4) and the formulations proposed (Chapter 3) might be beneficial. We demonstrated, that drying with the single-step freeze-drying process is more homogenous compared to a conservative cycle. Additionally, there is a smaller potential for premature initiation of secondary drying before primary drying was finished for all vials, as secondary drying is triggered by completed primary drying for each vial individually. Moreover, Pansare *et al.* have shown that single-step freeze-drying is less influenced by radiation compared to a conventional freeze-drying cycle [22]. Furthermore, the high collapse temperatures of the formulations developed in the presented work allow to design a freeze-drying process with a strong safety margin for considerable chamber pressure or shelf temperature variations, while still rendering a short freeze-drying cycle.

Outlook: Next steps to implement future freeze-dried antibody formulations

For implementation of HPBCD-based freeze-dried antibody formulations in the future, there are still open questions that need to be answered with regards to protein stability and single-step freeze-drying. Important next steps include the investigation of the molecular interactions between the protein and HPBCD. Investigation of the miscibility of HPBCD and the antibody using solid-state NMR is currently being initiated. Further investigation by FTIR and neutron scattering would be valuable to understand the protein stabilizing mechanisms for HPBCD. A thorough characterization using these analytical techniques will help to optimize the ratio of HPBCD and sucrose, which is essential to maximize protein stability. Studying the deuterium uptake of lyophilisates might provide a valuable tool to predict long-term antibody stability in formulations with different HPBCD to sucrose ratios [30]. Subsequently, real life stability studies for up to 24 months at 25°C and higher are essential to address whether such formulations would enable storage at controlled room temperature (20-25°C), or maybe even higher ambient temperatures. In addition to the standard analytics used to investigate physical

and chemical stability during storage, characterization by boronate affinity chromatography and mass spectrometry would be important to evaluate the risk of antibody glycation by sucrose at elevated temperatures for HPBCD-based formulations. Potentially, strategies to mitigate the risk of antibody glycation might become relevant, e.g. by using a scavenger which is the subject of current investigations. Ultimately, due to the possible different modes of interaction with the protein, the influence of HPBCD on *in vitro* affinity using e.g. ELISA or cell-based assays and *in vivo* pharmacokinetic in toxicological studies need to be addressed. Most importantly, to thoroughly elucidate the potential of HPBCD for future freeze-dried mAb formats, its impact on cake appearance and particularly protein stability for formulations with mAb derivatives such as ADCs and Fc-fusion proteins needs to be investigated. For implementation of the developed single-step freeze-drying cycle, the impact of different vial sizes and fill heights and most importantly aspects relevant for process scale up such as choked flow and condenser overload have to be evaluated.

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CONCLUSIONS

The presented work investigated alternative amorphous excipients to sucrose-based formulations to achieve high glass transition temperatures. The aim was to enable short freeze-drying cycles while providing pharmaceutically elegant lyophilisates. We demonstrated that the combined use of HPBCD with sucrose was particularly suitable, reducing cycle time by 50% and enabling improved storage stability at elevated temperature.

As a first step, µ-CT was introduced as an imaging technique that allows for visualization of cake appearance, such as partial collapse, cake structure, and morphology. Most importantly, it enabled a non-invasive imaging of the entire lyophilisate. It was demonstrated that µ-CT can also be used for quantitative characterization of cake appearance. However, the long measurement times at high resolution limit its applicability for the high throughput measurements needed for statistically significant quantitative information. Nevertheless, qualitative imaging will be beneficial for future process development and transfers. In addition, it may be useful in commercial production as a selective in-process control measurement complementary to visual inspection.

Investigation of alternative excipients initially focused on dextrans of 1 to 500 kDa. The results showed that while dextran formulations improved cake appearance, the increasing viscosity at higher molecular weights strongly impacted reconstitution time. Furthermore, dextrans of 40 kDa or higher did not sufficiently stabilize the two model mAbs upon freeze-drying. Most importantly, dextrans provided overall inferior protein stability compared to a pure sucrose reference formulation during storage due to antibody glycation. Using a mixture of equal fractions of dextrans and sucrose did improve protein stability of the formulation, however the data indicated that high amounts of sucrose may be needed for sufficient protein stability, which will reduce the formulation's glass transition temperature and therefore reduce the positive effect on cake appearance.

Therefore, in a next step, HPBCD-based formulations were investigated and the impact of the addition of other excipients was studied. It was shown that HPBCD provided elegant lyophilisates with good physico-chemical product quality attributes that stabilized the model mAbs. However, best protein stability during long-term storage was achieved when sucrose was added. These findings highlight that the excipients investigated within this work alone can not fully surpass the excellent stabilizing properties of sucrose, and the addition of sucrose will remain mandatory in antibody formulations. The combined use of HPBCD and sucrose may enable storage at elevated temperature or room temperature.

Based on the most promising HPBCD-based formulations, the last part of this work focused on the development of a short freeze-drying cycle, using aggressive primary drying conditions. The study showed, that the HPBCD-based formulation with addition of sucrose allowed for a shelf temperature during primary drying of +30°C. This enabled the development of a single-step freeze-drying cycle reducing cycle time by 50%. The obtained lyophilisates were characterized using µ-CT and demonstrated homogenous cake structures without defects. In contrast, a formulation that additionally contained PVP showed dents while having similar protein stability.

Overall, the presented work demonstrated the relevance of looking into alternative excipients, which in contrast to crystalline bulking agents add to protein stability while avoiding additional complexity in

the freeze-drying cycle. HPBCD with the addition of sucrose was found to be a promising formulation for future freeze-dried antibodies. This formulation with high collapse temperature facilitates development of short freeze-drying processes and may be beneficial during transfers. Providing elegant lyophilisates with short freeze-drying cycles, it avoids the uncertainties regarding the acceptance of products showing collapse. In addition, short single-step freeze-drying cycles would reduce costs of drug product manufacturing and increase throughput. The potential of room temperature storage would be a considerable improvement for pharmaceutical antibodies overall. In particular, it would make market access to developing countries easier by making complex cold chains redundant.