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Aggressive Freeze-Drying – a fast and suitable method to stabilize biopharmaceuticals

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ERKLÄRUNG

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Für meine Eltern
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LIST OF ABBREVIATIONS

BET	Brunauer Emett Teller
BSA	Bovine serum albumine
CMC	Critical micelle concentration
DSC	Differential scanning calorimetry
DTA	Differential thermal analysis
ETA	Electrical thermal analysis
FDA	U.S. Food and Drug Administration
FDM	Freeze-dry microscopy
FNU	Formazine Nephelometric Units
FTIR	Fourier transformed infrared spectroscopy
G	Glycine
Gly	Glycine
HES	Hydroxyethylstarch
HMWS	High molecular weight species
HP-SEC	High performance size exclusion chromatography
Ig	Immunoglobulin
IgG	Immunoglobulin class G
ile, iso	isoleucine
LDH	L-lactic dehydrogenase
Leu	Leucine
M	Mannitol
mAb	Monoclonal antibody
Man	Mannitol
Met	Methionine
PBS	Phosphate-buffered saline
pc	Chamber pressure
PEG	Polyethylenglycol
Ph.Eur.	European Pharmacopoiea
Phe	Phenylalanine
PS	Polysorbate
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrrolidone
Rh-GCSF	Recombinant human granulocyte colony stimulating factor

RWC	Residual water content
S	Sucrose
SD	Standard deviation
SE-HPLC	Size exclusion high performance liquid chromatography
SEM	Scanning electron microscopy
SSA	Specific surtace area
Svp	Subvisible particle(s)
T	trehalose
T0	Temperature of zero mobility
Tc	Collapse temperature
Tcryst	Crystallization temperature
Tg	Glass transition temperature
Tg′	Glass transition temperature of the maximally freeze-concentrated solution
Tm	Melting temperature
TMA	Thermal mechanical analysis
Tp	Product temperature
Trp	Tryptophan
Ts	Shelf temperature
USP	United States Pharmacopoiea
XRD	X-Ray powder diffraction

CHAPTER 1 GENERAL INTRODUCTION

1. THE NEED FOR STABILIZATION

For more than 30 years biologicals like proteins, peptides, and hormones gain increasing importance for the treatment of human diseases. Due to the enormous potential of biologicals to act as very specific and high potency pharmaceutical substances, enormous efforts are put in developing such new molecules to function against severe illness. While those molecules persuade by their unique potency as pharmaceutical substances, they usually exhibit weak stability during processing into a final drug [1]. Minor thermodynamic stability as well as miscellaneous degradation pathways are characteristic of many biological (macro-) molecules. Hence, the need to protect these molecules during processing and over the entire shelf life against various stress vectors is most important.

For many small molecules, the way of administration is the oral route, which is a very comfortable way to administer a drug. However, the gastro-intestinal system in mammals was built to take large molecules like proteins to pieces. Moreover, molecules larger than 500-600 Da are not able to overcome the intestinal barriers of the biological membranes [2]. For this reason, biopharmaceutical drugs are usually administered via the parenteral route as a liquid formulation [3]. Consequently, those molecules need to be formulated as solutions or suspensions. Quite a number of biopharmaceutical drugs on the market are in fact available as liquid formulations [4]; however, many molecules cannot be stabilized in a solution throughout the whole shelf life of usually 24 months. Monoclonal antibodies, the most important class of biopharmaceutical molecules [5], are often susceptible to degradation in liquid environment. In order to be able to distribute the biopharmaceutical drugs to patients, the sensitive molecules need to be stabilized in a thermodynamically less active system. Although storage could theoretically be realized as a frozen solution, which would represent a thermodynamically less active system, for practical reasons storage and distribution at frozen condition is not feasible. Storing valuable biopharmaceuticals in the dried state therefore has become the method of choice already decades ago [6]. Most of the biological molecules to be stabilized are sensitive against temperatures above the body temperature of mammals. Thus, drying must be operated at low temperatures compared to other industrial drying processes. To reach reasonable drying rates at low temperatures (i.e. low vapor pressure of the solvent), lowering of the surrounding pressure is mandatory. However, pharmaceutical formulations that are only dried by applying vacuum, result in products that are often not easy to redissolve [7]. At that point “lyophilization” (or synonymously “freeze-drying”) bears crucial advantage. A solution is frozen to a system consisting of solvent crystals (most often ice) and a concentrated and therefore solidified interstitial matrix composed of the solutes of the initial solution. The crystallized solvent is subsequently removed by sublimation at low temperature. As soon as all of the solvent has been removed, the interstitial matrix forms a highly porous structure, which can easily be dissolved again. Embedded in those solid matrices sensitive biomolecules it can be stabilized successfully for the intended shelf lives.

In order to successfully stabilize a sensitive biomolecule within a freeze-dried product, a combination of necessary excipients and accurate control of the freeze-drying process is essential. Excipients are used to stabilize the molecule of interest against stress vectors arising during processing and subsequent storage. In the course of freeze-drying excipients generally need to provide protection against freezing-, drying-, and interfacial stresses [8]. Additional excipients are used to adjust the tonicity, maintain pH level, and provide a rigid structure to support the interstitial matrix after removing the solvent by sublimation.

The most important goal of a freeze-drying process is to conserve critical quality attributes of the product. In order to avoid deterioration of the quality of an embedded molecule of interest, during a freeze-drying process the temperature of the product needs to be kept below a critical temperature [9]. In order to control the product temperature, a controlled (low) pressure and energy (heat) input for a defined period is necessary. Most often, the so-called collapse temperature is considered as maximally allowable product temperature, as above the collapse temperature the interstitial matrix softens [10]. A collapsed product is regarded as failure of the process, particularly because of the corrupted macroscopic pharmaceutical elegance [11], but also as some data from literature report, an adverse effect of collapse on the stability of the embedded molecule of interest [12, 13]. Moreover, important physical parameters as for example residual water content [14] or dissolution time [15] are reported to be negatively affected in collapsed products, too.

On the contrary, having a closer look to stability data of collapsed lyophilizates, in several cases collapsed material did not perform worse than non-collapsed material regarding stabilization of embedded molecules. In a systematic study it was recently shown, that collapsed freeze-dried formulations are able to stabilize a number of sensitive proteins – in same cases even better than non-collapsed lyophilizates [16, 17]. This report is even more remarkably as the results apply to classical freeze-drying formulations (containing sugars and bulking agents) without further optimization. Collapsed products were achieved by increasing the product temperature above the collapse temperature by means of an aggressive freeze-drying cycle. This cycle, running at high shelf temperatures and high chamber pressures aimed at high energy input into the drying product. As already 1 °C increase in product temperature cause a decrease of 13% in primary drying time [11], high drying rates, and therefore short freeze-drying processes can be achieved. Shortening of freeze-drying cycles is of major interest in industry. Several patents and patent applications aim on shortening freeze-drying cycles by increasing product temperatures close to or above the collapse temperature [18-20].

The aggressive cycle, proposed by Schersch et al., seems to be promising in terms of short freeze-drying processes whilst the most critical product attributes (stability/activity, rehydration time) were at least comparable to conventional freeze-drying. However, in contrast to conventional freeze-drying essential knowledge is missing so far to establish aggressive freeze-drying as a reliable alternative to conventional freeze-drying. To fill that gap, this work presents systematic investigations of variations of process parameters and formulation designs and their influence on product quality. Moreover, robustness and reproducibility of aggressive collapse cycles as well as the applicability to different freeze-driers is studied.

2. FREEZE-DRYING PROCESS

Freeze-Drying, or interchangeably lyophilization, is a 3-step unit operation to remove a solvent from a material in order to improve stability of that material [21]. In contrast to many conventional drying techniques that use evaporation as drying mechanism, during freeze-drying the solvent (usually water [22]) is first solidified (step 1: freezing) and subsequently removed from the system via sublimation (step 2: primary drying). To reach the desired residual solvent levels a further step utilizing desorption is normally added (step 3: secondary drying). [6, 21, 23]

A major reason for this elaborate and time consuming drying operation is in most cases the heat sensitive nature of the material to be dried, often food or pharmaceutical products [24].

Since removing a solvent by sublimation is the major step in freeze-drying, the process needs to be controlled according to the phase transition behavior of the solvent, which is represented by a phase diagram. Figure 1.1 exemplarily shows a phase diagram of water schematically including the phase transitions during freeze-drying.

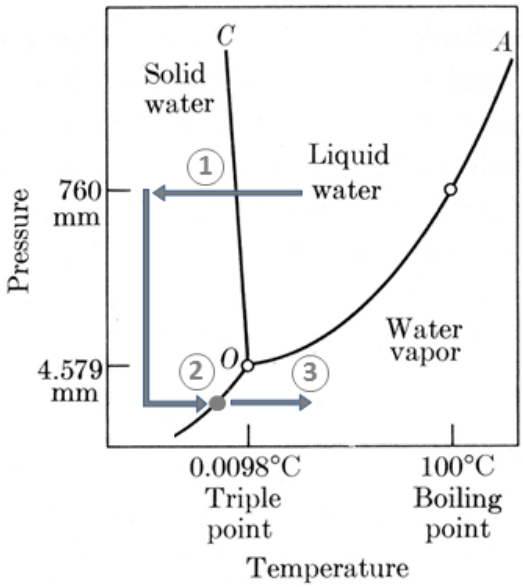

Figure 1.1: Schematic phase diagram of pure water (figure modified from [25]) including characteristic phase transitions during freeze-drying: 1:freezing, 2:primary drying, 3: secondary drying (pressure is displayed in mmHg=Torr, 760mmHg=1,013bar, 4,579mmHg =6,117mbar)

To realize the necessary steps given by the phase diagram, temperature as well as pressure needs to be controlled in the course of the drying process. A typical freeze-drying process is for that reason divided in the already mentioned three steps: Firstly, the solvent needs to be forced from the liquid to the solid state what is realized by cooling the system down. Secondly, the pressure needs to be reduced below the triple point followed by a distinct increase of temperature which forces the solid to pass the sublimation curve and be sublimated. Thirdly, to dry the material to the desired final solvent content, more energy is supplied via a further increase in temperature (while the pressure is main-

tained low). An example of a freeze-drying process is shown in Figure 1.2, also highlighting the three major steps during freeze-drying. In this case, an optional annealing step is included, which can become necessary for some excipients or sometimes bears advantages regarding drying rates. Annealing during the freezing step as well as a general description of the three major steps of a freeze-drying cycle are delineated in the next paragraph with focus on freeze-drying of aqueous pharmaceutical formulations.

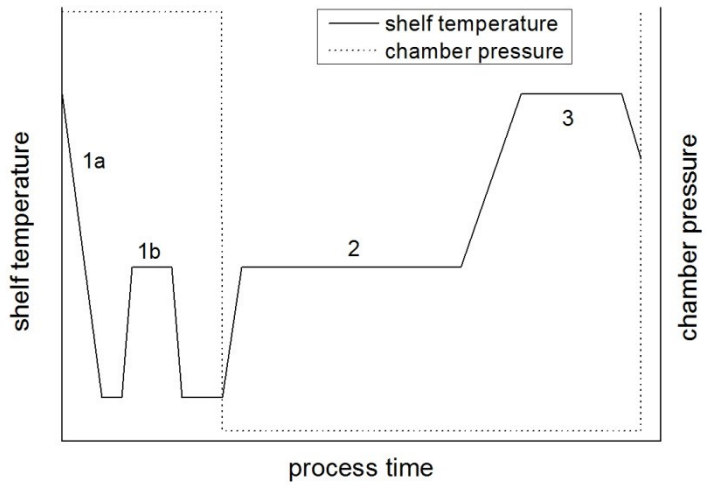

Figure 1.2: Schematic illustration of a typical freeze-drying cycle for pharmaceutical solutions. Numbers depict typical steps; 1: freezing (with optional annealing step 1b), 2: primary drying, 3: secondary drying.

2.1 FREEZING STEP

During the freezing step the temperature of a material is lowered to an extent that solvent(s) (hereafter "solvent" is replaced by water, as water is the most prominent solvent in freeze-drying [22]) as well as solute(s) are completely solidified to crystalline or amorphous solids [26]. Since the (micro-) structure formed by both, the ice crystals and the solidified solute determines characteristics like primary and secondary drying rate, product crystallinity, surface area, and protein stability, ice nucleation plays a major role in freeze-drying [27, 28]. The characteristics of ice nucleation depend directly on the degree of supercooling, which in turn depends on solution properties as well as process conditions [29]. A higher degree of supercooling results in faster ice nucleation and faster effective freezing and therefore smaller ice crystals, while a lower degree of supercooling result in slower freezing, allowing the ice crystals enough time to grow [14, 27]. Although the degree of supercooling plays a dominant role for the later morphology of the lyophilizates, that particular parameter is difficult to control, especially during scale-up of freeze-drying from laboratory (more impurities) to production scale in clean room areas [30]. However, also in laboratory freeze-drying intra-batch heterogeneity is reported [27, 28, 31], resulting from stochastic ice nucleation (meaning different degree of supercooling for each vial).

While freezing progresses, water is separated from the supercooled solution in the form of growing ice crystals, resulting in a continually concentration ("cryoconcentra-

tion" [32]) of the solutes within the remaining solution up to a 20-fold increase of concentration [33]. This removal of water from the solution is sometimes considered the main drying mechanism [33]. Depending on the nature of the solutes they either crystallize to an eutectic mixture, or remain in an amorphous state [23]. In the latter case, at a certain concentration c_g' or weight fraction w_g' and temperature T_g' the viscosity of the concentrated solution reaches a critical value (around 10^{12} Pa s [34]) and the now maximally freeze-concentrated solution undergoes a vitrification. Figure 1.3 shows both, a phase diagram of a sucrose water mixture as well as a phase diagram of a salt water mixture complemented by a typical behavior of a sucrose solution or a salt solution during freeze-drying (see description for details).

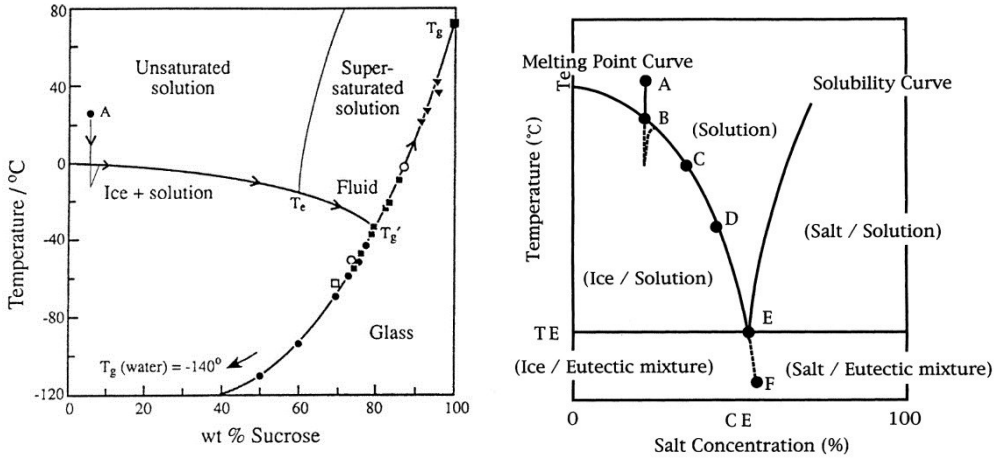

Figure 1.3: Left: Solid–liquid phase diagram of a sucrose–water mixture. As an example the route of a 5%wt solution of sucrose during freeze-drying is marked with arrows [33] (A → supercooling, ice nucleation and freezing of pure water → passing T_e without crystallization → forming a glass at T_g' → being dried towards T_g). Right: Solid–liquid phase diagram of a salt–water mixture, whereby the salt can be considered to represent also crystalline bulking agents. The route of a crystallizing solution is depicted in letters A to F [35] (A → cooling to B → supercooling and freezing of water to C and D → eutectic crystallization at E → eutectic mixture at F).

As already mentioned above, uniform ice nucleation is difficult to control within a batch of, for example, glass vials. In order to reduce inter-vial heterogeneity, a modified freezing step is suggested to change the micro-structure of the frozen product [36]. This method, called "annealing", recommends to increase the temperature of the frozen product after complete solidification (e.g. at -50°C) above the glass transition temperature of the interstitial matrix (e.g. to -20°C) for a certain amount of time [29]. This increase in temperature results in a re-softening of the interstitial matrix and allows (further) crystallization of solutes (e.g. crystalline bulking agents like mannitol)[37]. Moreover, due to the systems tendency to reduce the overall energy, Ostwald ripening increases larger ice crystals to the account of smaller ones. Consequently, the primary drying kinetics are improved (as larger ice crystals result in larger pores an therefore less resistance to water vapor) [32]. On the downside, the increases in pore size by annealing decreases the absolute number of pores and therefore decreases specific surface area [38] and thus slows down secondary drying kinetics. Moreover, during annealing the solutes are again in a

highly concentrated rubbery state, which is not a preferable situation especially for sensitive APIs and can lead to instabilities of those molecules.

Controlled ice nucleation seems to be an elegant way to optimize freeze-dried products regarding reduction of vial-to-vial heterogeneity (a particular problem in production-scale freeze-driers) and shortening freeze-drying cycles by increased primary drying rates [39]. Recently, different techniques were developed that aim for a better/complete control of ice nucleation during the freezing step [29, 40]. Controlled nucleation systems are already available for lab and pilot scale freeze-driers from different vendors (ControLyo™, SP Scientific/Praxair; FreezeBooster™, Millrock Technology; VERISEQ©Nucleation, Linde AG/Ima Life). For large-scale production freeze-driers this technology is under development.

2.2 PRIMARY DRYING STEP

The primary drying step during freeze-drying serves to remove the frozen solvent from the product by sublimation. In order to force a phase transition from solid to vapor state, conditions have to be set to a pressure and temperature below the triple point of the solvent (for the most common solvent water cf. Figure 1.1, p<6.11 mbar, T<0.0098 °C). Although, it would also be possible to achieve sublimation at atmospheric pressure (if the partial pressure of water is kept below the triple point) enormous technical effort like immense volume flows of cold and dry drying gas had to be realized [41].

To achieve sublimation, the necessary sublimation enthalpy needs to be provided by supplying energy in form of heat. Since freeze-drying products contain an interstitial matrix besides ice, and the mass rate to remove water vapor is technically restricted, the heat input has to be accurately controlled in order not to increase the product temperature above the critical temperature of the product [26]. As long as the heat input is controlled below that critical level at a certain product temperature, the provided heat is transduced into the sublimation of ice in a quasi-steady state until all ice is sublimated [42]. This situation can be expressed as coupled heat and mass transfer (cf. equation (1.1); dQ/dt represents the heat flow into the product, ΔH_S the sublimation enthalpy, dm/dt the mass flow of water vapor, $mc_p \partial T/\partial t$ the change of the product temperature). However, as the change in product temperature is small compared to the heat flow due to sublimation, the second part of equation (1.1) can usually be neglected. [43]

$$\frac{dQ}{dt} = \Delta H_S \frac{dm}{dt} + mc_p \frac{\partial T}{\partial t} \tag{1.1}$$

The necessary heat input into the product is generally realized by temperature controlled (i.e. heated) surfaces that are in direct contact with the product or, more common, a primary packing material containing the material to be freeze-dried is placed on those surfaces. The most common method for pharmaceuticals is to fill a solution into glass vials and place them onto shelves within a freeze-drier. In this case, the heat flow into the product can be expressed as equation (1.2) (dQ/dt heat flow into the product; A_V cross-section area of the vial; K_V heat transfer coefficient of the vial; T_S shelf temperature; T_P product temperature) [42].

$$\frac{dQ}{dt} = A_V K_V (T_S - T_P) \tag{1.2}$$

The overall heat flow is composed of three components: conduction between shelf and vial/product (K_c), thermal radiation (K_r), and heat transfer via gas convection (K_g) [44]. While heat transfer coefficients for conduction and radiation are independent from pressure, K_g is proportionally related to pressure [42]. Since at higher chamber pressures heat transfer is more efficient, shelf temperatures can be reduce to some extent, reducing the risk of melt back at the bottom (due to a temperature gradient within the sample) [26].

Driving force for sublimation of ice from an ice surface is the difference between water vapor pressure at the ice surface and partial water vapor pressure in the surrounding of the ice surface [45]. If there is no difference, no sublimation occurs. Usually, the partial water vapor pressure in the surrounding (i.e. product chamber) is decreased by a surface that is cooled down to lower temperatures than the actual product temperature (i.e. a so-called condenser). In common freeze-drying equipment and primary packaging material, there is a certain resistance to free water vapor transport [26]. More importantly, the freeze-drying product itself restricts the free transport of water molecules from the sublimation site to the condenser (so called product resistance). This so called "product resistance" is a function of dry layer thickness and increases with increasing layer thickness, which in turn increases with increasing drying time [46]. Summarizing, the sublimation rate dm/dt can be expressed as a combination of driving and hindering forces of sublimation (cf. equation (1.3), p_0: vapor pressure of ice at the sublimation front; p_{Cd}: condenser pressure; R_{total}: Resistance to water vapor flow, consisting of product resistance R_P, resistance of primary packaging material R_S, and resistance of the chamber R_C) [45].

$$\frac{dm}{dt} = \frac{p_0 - p_{Cd}}{R_P + R_S + R_C} = \frac{p_0 - p_{Cd}}{R_{total}} \tag{1.3}$$

$$ln(p_0) = 24.01849 - \frac{6144.96}{T_P} \tag{1.4}$$

As the vapor pressure of ice is dependent on the product temperature (equation (1.4)) [43], an increase in product temperature results in an increase of sublimation rate and therefore in shorter primary drying times. Experimental data underline the strong dependence of product temperature and primary drying time (cf. Figure 1.4 and Figure 1.5) [47]. For an increase of 1 K in product temperature a decrease of more than 10% in drying time is reported [11]. However, since product temperature needs to be maintained below the critical product temperature to avoid product collapse, an approach to increase drying rates due to higher product temperatures is limited, especially for amorphous products.

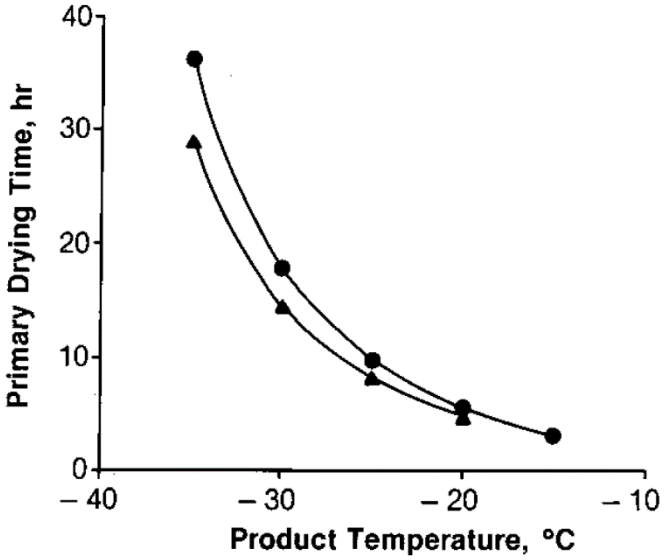

Figure 1.4: Effect of product temperature on primary drying time of 5% (v/v) solutions of KCL (dots) and PVP (triangles); filling height 1cm; from [47]

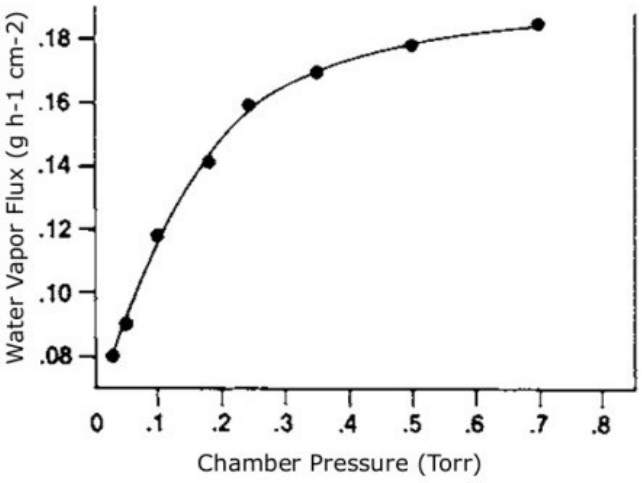

Figure 1.5: Effect of chamber pressure on sublimation rate (0.18; methylprednisolone sodium succinate, approx. 0.8 cm filling height, 45°C shelf temperature; modified from [47])

During progression of primary drying, the dry layer thickness increases, the more ice is removed by sublimation, resulting in increasing product resistance [43] and therefore decreased sublimation rate. If the reduced sublimation is not taken into account and the heat input is maintained at the same level (although less energy is required for sublimation), the product will warm up. Therefore, particularly freeze-drying processes that are optimized regarding product temperatures close to the critical product temperature, need to be carefully controlled and monitored. On the other hand, some degree of local collapse (also called microscopic or small scale collapse) can enhance primary drying rates, since due to increased pore size product resistance is remarkably reduced in micro-collapsed material [48].

2.3 SECONDARY DRYING STEP

After completion of primary drying, unfrozen water has to be removed via a secondary drying step by desorption [23] to reach residual moistures that are able to provide stabilization throughout an intended period of storage time [49] (most commonly 0.5 - 1%). During freezing, not the entire water is transformed into ice, but is distributed within the amorphous matrix formed by the solutes (most common situation for pharmaceuticals). Up to 30% of this interstitial matrix is composed of unfrozen water [36, 50]. In rare cases of pure crystalline products, unfrozen water is adsorbed to the surfaces of the crystalline matrix [51].

Since more energy is required to overcome the activation energy of desorption, more heat is provided to the product (via higher shelf temperatures, e.g. 25°C). Attention needs to be paid to the heating ramp rate of the shelf temperatures, as at the beginning of secondary drying the glass transition temperature of the product is low (due to high residual moisture) [50]. Because drying rates are not influenced by chamber pressure, no further adjustment needs to be realized compared to chamber pressure of the primary drying. Dependent on the temperature and specific surface area of the matrix, after a certain amount of time a plateau level of residual water is reached. [51]

Concerning the influence of secondary drying on stability, for heat sensitive biopharmaceuticals temperature and time of secondary drying need to be chosen carefully [52]. So called "over-drying", i.e. reducing the residual water content to very low amounts, can cause severe instabilities. [53, 54] Especially for proteins, a certain amount of remaining water in the matrix is of importance, as those molecules rely on the preservation of their native state, which is closely connected to water. Additionally, uneven distribution of residual water inside the product matrix, which is a highly probable scenario, can result in instability [55].

3. FREEZE-DRYING OF PROTEIN FORMULATIONS

The most convenient way to handle a protein pharmaceutical throughout production until administration is a liquid formulation. However, most proteins do not show sufficient stability in solution, as water serves as an excellent reaction partner or medium for hydrolysis, cross-linking, deamidation, oxidation, aggregation, disulfide rearrangements, etc. [33, 56]. Those degradation pathways in the majority of cases result in loss of activity. In addition, other severe consequences like immune response caused by aggregated protein species upon administration to patients are a serious concern. [57]. Hence, a cautious stabilization of protein pharmaceuticals is indispensable.

By reducing the amount of water (i.e. drying) degradation usually can be decelerated to an extend that sufficient stability is provided to store such products over extended periods of time (18-24 months) [49]. On the other hand, however, the proper activity of proteins is dependent on their native structure that is generally inevitable connected to the presence of surrounding water molecules [58]. Consequently, the preservation of the native structure at very low water presence plays a major role in stabilization of proteins. In order to achieve that, in freeze-drying of proteins excipients need to be employed. Most often a combination of cryo- and lyo-protectants, a surfactant and buffer agents is included in a freeze-drying formulation [49]. To account for further requirements also

crystalline bulking agents, tonicifiers, antioxidants, and/or preservatives can be part of the mixture [8, 59].

3.1 STABILIZATION OF PROTEINS DURING FREEZE-DRYING AND STORAGE

Although freeze-drying is the method of choice for long-term preservation of product quality, the process itself entails several risks for sensitive molecules like protein pharmaceuticals [33]. In which way proteins in aqueous solution can be affected by freezing- and drying-stress, and how they can be successfully stabilized against those stress vectors, will be discussed in the following.

3.1.1 FREEZING STEP

3.1.1.1 Stress vectors

As a first step in freeze-drying, by lowering the temperature of the solution, water has to be transferred into ice. Since the free energy of unfolding shows a temperature dependent parabolic profile, above and below certain temperatures (<10°C and >50-100°C, respectively) a denaturation is thermodynamically favored [60]. At low temperatures the solubility of hydrophobic parts of a protein molecule increases, resulting in a decrease of intra-molecular hydrophobic interactions that are responsible for the three-dimensional native structure of the molecule [61-63]. Hence, the native conformation is changed due to the low temperature, which is referred to cold denaturation.

As soon as the water is transferred to ice crystals, new interfaces are formed within the mixture. As proteins are surface active molecules they can adsorb to that solid-liquid interfaces, which can result in surface-induced denaturation [64-66].

Due to the removal of water in the form of ice, the remaining solution is concentrated. Thus, two effects can be observed that have an effect on protein stability: Firstly, every solute can reach levels of a more than 20 fold higher concentration compared to the initial concentration [67]. Regarding ionic strength, this dramatic increase can cause destabilization of the native conformation of a protein [60]. Moreover, the presence of reactive species (e.g. oxygen) in high concentrations can accelerate protein degradation [8]. Secondly, at high concentrations excipients can be separated from the protein to be stabilized [68, 69]. Due to selective crystallization of for example buffer salt, the pH value can change dramatically [70, 71]. Moreover, if cryo-/lyo-protectants crystallize during freezing, they are no longer able to ensure stabilization during freeze-drying [72]. But also non crystallizing excipients can exhibit phase separation from the protein to be stabilized [73].

3.1.1.2 Stabilization

Cryoprotectants are able to stabilize sensitive proteins during freezing successfully. It has been shown, that postulated mechanisms that account for stabilization of proteins in aqueous solution are also valid for cryoprotection [74]. In brief, the addition of a stabilizing excipient to an aqueous protein solution results in a preferential hydration of the surface of the protein due to thermodynamically favored interaction of the protein with the solvent. Consequently, the excipient is preferentially excluded from the protein domain, increasing the free energy of the system and therefore resulting in stabilization as the

unfolded state of the proteins is less favored. [75-78]. As preferential exclusion is not a direct interaction between excipient and protein, quite a number of substances can be used as cryoprotectans (sugars, polyols, amino acids, polymers, etc.) [79]. Most often sugars are employed as they do not show phase separation or crystallization (they are even able to suppress crystallization of other excipients [80]) as it is reported for other excipients that are used [72]. To protect proteins from denaturation at interfaces, non-ionic surfactants can be added to the formulation [65].

3.1.2 DRYING STEP

3.1.2.1 Stress vectors

During the drying step of freeze-drying, the environment of the protein is further altered compared to the situation during or after freezing. After freezing, the protein is distributed in an amorphous glassy matrix (if a suitable formulation has been chosen). This matrix contains a substantial amount of unfrozen water, which assists to keep the native conformation of the protein. However, as already during freezing, a major part of water is removed from the solution, "drying stress" already appears at that stage. In solution 1 g of fully hydrated protein contains 0.3-0.35 g water [81, 82], while during drying the residual water is reduced at least to one third of that [8]. Nevertheless, at which state of freeze-drying it occurs, dehydration of the protein environment usually results in denaturation [83, 84].

3.1.2.2 Stabilization

Stabilization of proteins against dehydration-induced stress is generally based on an amorphous matrix that encloses the protein as a sort of a solid dispersion. Good stabilization properties of the amorphous matrix are generally attributed to two major mechanisms, namely the vitrification in a rigid matrix and the replacement of water by solutes, respectively [85].

Vitrification Theory

The formation of an amorphous glass ("vitrification") is considered as the first major mechanism to stabilize a protein in a freeze-dried product [86-88]. This mechanism is termed "glass dynamics mechanism" by some researchers [89]. A precondition for a successful stabilization is a molecular dispersion of the protein within the glass. As amorphous glasses in terms of their structure are more comparable to liquids than to (crystalline) solids [90], they can also be considered as "solid solutions" with extremely increased viscosity (around 10^{12} Pa s [91]) [86]. To achieve the formation of an amorphous glass, which contains the dispersed protein, usually excipients like sugars or polymers are utilized. By cooling of such (aqueous) solutions during the freezing step of freeze-drying, the excipient/protein mixture undergoes a glass transition at the glass transition temperature of the maximally freeze-concentrated solution (T_g'). Due to the glassy state of the matrix, diffusion, which is the prerequisite for chemical and conformational degradation reactions, is kinetically slowed down dramatically [79]. The glassy structure that is formed during freezing is maintained throughout the entire freeze-drying process. Due

to the removal of water from the amorphous glass, the glass transition temperature is continuously increased during the freeze-drying process. For a successful stabilization of the protein in the final freeze-dried product, the glassy matrix needs to be stored below their glass transition temperature T_g [92] in order to maintain kinetic hindrance of degradation. Glasses formed by different substances can be characterized regarding their change in viscosity for a certain temperature change [91]. So-called fragile glasses exhibit a higher increase in viscosity for a particular temperature drop compared to so called strong glasses [91]. Therefore, excipients forming fragile glasses (like sucrose or trehalose) are preferable for protein stabilization [93]

Water Replacement Theory

A second major prerequisite for successful stabilization of a protein is to maintain the native conformation throughout processing and storage [83]. The native structure of proteins is adapted to aqueous environment including the formation of hydrogen bonds to surrounding water molecules. As water is removed during drying, the function of water needs to be replaced by an excipient (water replacement hypothesis) [94-97]. Hydrogen bonds between protein and specific excipients, like saccharides, preserve the native structure of the protein in the solid state and therefore act as water substituent [58, 98, 99].

Since the postulation of both theories, a number of studies have been evaluated to show the applicability to experimental results. Some findings show that their results fit better to the preservation of the native conformation by water replacement [85, 89, 99, 100], while other researchers rather confirmed kinetic stabilization [101-103]. Most probable a synergistic effect of both mechanism contribute to stability (as for example suggested by Yoshioka et al. [104, 105]).

3.1.3 Long-term Stabilization of dried proteins

Beyond stabilization of proteins just during a freeze-drying process, the ultimate goal of (freeze-) drying is still a preservation of product quality for an extended period of time (e.g. compared to stability in solution). Although, remaining water within a freeze-dried product is reduced to low amounts, causing stabilization in situ, during storage and shipping, degradation still can occur [100, 106, 107]. As pharmaceutical quality is diminished by both, chemical and physical degradation, in the following no distinction is made between instability arising from either of those mechanisms. Anyway, they are known to foster each other [108] resulting in deterioration of quality.

3.1.4 Factors affecting long-term stabilization

While the mechanisms to keep a protein stable in a freeze-dried product ("vitrification" and "water replacement") are known at least since the early 1990s, the connection between properties of the stabilizing matrix and stability of the proteins during storage is still somewhat ambiguous [109]. A tool that is able to predict precisely the long-term storage stability based on material characteristics is still lacking. However, in the last decade more and more studies point in the direction of the importance of molecular mobility or relaxation behavior respectively [101, 105, 110-116]. Since it is beyond the scope of this

work to review the mechanisms of molecular mobility in detail, in the following sub chapter an overview of factors affecting long-term stabilization in general is given.

3.1.4.1 Glassy dynamics

The dispersion within an amorphous matrix is a major requirement for most pharmaceutical proteins to achieve stabilization for long-term storage at reasonable temperatures [117]. Typical amorphous solids to protect proteins are composed of disaccharides which exist after freeze-drying in a glassy solid state, characterized by both, lacking long-range order compared to crystalline material and undergoing a glass transition (which is the general definition of a glass [118]). Inherently, glasses are thermodynamically not stable due to a non-equilibrium state, which is expressed as an excess in enthalpy [118] compared to an equilibrium solid state (=crystal). Figure 1.6 schematically depicts the difference between the enthalpy of an amorphous glass compared to a crystal of the same material.

In contrast to a crystal, which is spontaneously formed at the melting temperature T_m upon cooling, a glass is formed first by supercooling and then a glass transition at T_g. If a supercooled liquid is further cooled down, viscosity is increased and therefore a reduced mobility of the molecules is enforced. These molecules generally are prone to rearrange towards equilibrium like in the liquid state. However, below a certain temperature (glass transition temperature T_g) the molecules are not able to rearrange due to high viscosity, finally resulting in the formation of a non-equilibrium highly viscous state: a glass is formed, characterized by timescales (well) above 100 s for molecular rearrangements. [119]

Major prerequisite for glass transition is rapid cooling [120] and a comparable high viscosity around T_m, since otherwise the liquid will spontaneously crystallize at T_m. As the system is thermodynamically not in an equilibrium state, the molecules are driven by thermodynamic forces (reduction of enthalpy) to relax towards that equilibrium state [119]. In other words, there is still mobility inside a glass.

The enthalpy of a glass is dependent on the way of formation, for example rapid cooling results in higher enthalpy. However, the enthalpy of glass can be reduced by treatment of the glass at temperatures close to the glass transition temperature (e.g. 16 K below T_g) [121]. This intended or non-intended change in enthalpy (and therefore mobility/relaxation) is named “annealing” or “aging” [119].

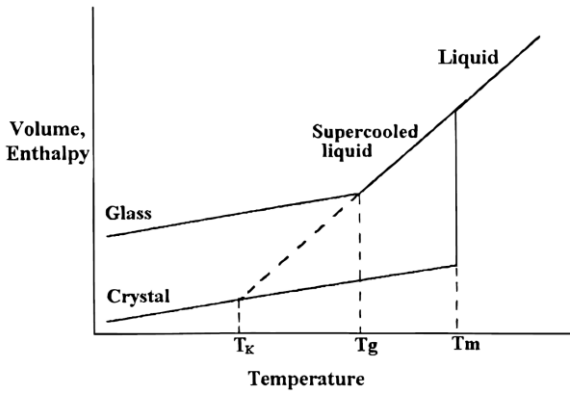

Figure 1.6: Schematic description of temperature dependent enthalpy of amorphous (glass) versus crystalline material (T_k=Kauzmanm temperature; T_g=glass transition temperature; T_m=melting temperature) from [120]

Molecular mobility and protein stability

Thinking back to the concept of stabilizing a sensitive protein inside the glassy matrix, mobility inside the glassy matrix seems to be unfavorable as every degradation reaction necessarily involves some degree of mobility [85]. The Kauzmann temperature T_K (theoretical intersection of enthalpy of a supercooled liquid and the crystalline state, for detailed description see e.g. [122]), lying about 50 K below T_g, is sometimes referred to as the temperature of zero configurational entropy [121]. Thus, some authors suggest best stability of a protein within a glass during storage at a storage temperature 50 K below T_g. In turn, they try to reach high glass transition temperatures by selecting appropriate excipients allowing storage at higher temperatures [49, 123, 124].

Having a closer look at the connection between stability on the one hand and characteristics of the amorphous glassy matrix on the other hand, there is more which needs to be considered than "just" a glass transition temperature [116]. As already outlined above, the amorphous matrix, which incorporates the protein, features a complex number of relaxation processes [125]. Depending on the scale of their occurrence they are classified in global and local dynamics, while global mobility (or α-relaxation) is connected to glass transition (and relaxation times of around 100s, local mobility (or β-relaxation) is connected to vibrational motions at temperatures below the glass transition (and relaxation times of picoseconds to microseconds [126]) [116, 127].

Several authors have shown, that stability against physical degradation [102, 128] and/or against chemical degradation [102, 129] can be explained by global mobility. In addition, a connection between higher glass transition temperatures and increased storage stability could be shown [130-132]. On the other hand, global mobility is not necessarily connected to storage stability in every case [92, 133].

Regarding the influence of local mobility on storage stability, some authors stated a more significant relevance of that phenomenon [89, 134-136]. A correlation between local mobility and stability against chemical and physical degradation was reported by some authors [137, 138]. Interestingly, adding a plasticizer to an excipient mixture and therefore increasing global mobility, resulted in a decrease in local mobility (expressed as increased relaxation time) and an increase in storage stability [126, 134]. Based on their findings, Cicerone and coworkers proposed to adapt the vitrification hypothesis regarding superior importance of β-relaxation compared to α-relaxation [126].

Beside the choice of appropriate anti-plasticizers, a modification in relaxation times can also be achieved via additional steps during or after freeze-drying. Treatment of (freeze-) dried amorphous glasses by high temperatures (below T_g) result in relaxation of the glass towards equilibrium [110]. Consequently, relaxation times are decreased. Since an increase in relaxation time is generally assigned to an increase in protein stabilization, additional annealing, or aging of amorphous glasses is advised to improve storage stability. [111, 139-142]

3.1.4.2 Residual water

The content of water remaining in the product after freeze-drying has a significant influence on the stability of a protein during storage. For freeze-drying of proteins without excipients, a certain level of water have to be maintained in order not to compromise the hydration shell of the protein resulting in instability [53]. However, as water can be

directly involved in degradation reaction as reactant, reaction medium and/or solvent [108, 143], sufficient low residual water content is mandatory.

Since in freeze-dried products protein pharmaceuticals are stabilized in glassy matrices, the amount of water in the amorphous phase plays a major role. Since water act as plasticizer of the amorphous matrix, molecular mobility is affected directly by water content, which is often reported as a decrease in glass transition temperatures as consequence of an increase in water content [54, 89]. The same situation can also be described by increasing water content, resulting in increasing mobility (α- and β-dynamics [144]) and therefore increasing reaction rates that are coupled to mobility [143].

3.1.4.3 Hydrogen ion and hydroxide ion activity

Although from a classical point of view there is no definition of pH in the solid state, hydrogen ions as well as hydroxide ions can influence degradation of proteins in the dried state (e.g. β-elimination) [107, 145-147]. This phenomenon can be explained as sort of a "pH memory" of proteins in the freeze-dried state. Depending on the pH in aqueous solution prior to lyophilization, the ionogenic groups preserve their ionization state in the dried state [148]. In order to access solid state "pH" recently new pH sensors were developed [149].

3.1.4.4 Temperature

As molecular mobility as well as chemical reactions in general are dependent on temperature (as described by the Arrhenius equation), this is an important factor for stabilization. Exposure of freeze-dried protein formulations to elevated temperatures therefore result in decreased stability, especially chemical degradation reactions are increased [107]. As some degradation reactions did not show a Arrhenius like temperature dependence [150], results from accelerated storage stability studies at elevated temperatures have to be judged carefully.

3.1.4.5 Crystallization of amorphous excipients

Crystallization of stabilizing amorphous excipients during storage causes a separation of the regarding excipient from the amorphous mixture. Consequently, the crystallized proportion of the excipient is no longer able to fulfill its task to stabilize the protein. [68, 151] Several examples have shown that crystallization of excipients deteriorates protein stabilization during storage and thus needs to be avoided [72, 152, 153]. In pharmaceutical freeze-drying, one widely used excipient that is prone to crystallization during storage, is mannitol [154, 155].

3.1.4.6 Specific surface area

As already outlined above, during freeze-drying new interfaces are generated during the freezing of solutions to be freeze-dried (cf. page 10). Once ice crystals are removed by sublimation, the character of these interfaces change from (interstitial amorphous) solid/ice to solid/void, however, they persist throughout the whole period of storage. Hence, a potential degradation of proteins at those interfaces can occur during an extended period. Consequently, the specific surface area of a freeze-dried product can play

an important role for storage stability. Higher specific surface area is equal to more protein molecules exposed at the surface of the dried solid (provided that the coverage of the surface by protein molecules is constant).

Generally, a uniform distribution of protein molecules inside the stabilizing amorphous matrix is thought to be essential for good protein stabilization [156]. However, due to their amphiphilic nature, proteins tend to accumulate at interfaces. Recently it was shown that in freeze-dried and spray-(freeze-)dried mixtures of proteins and sugars accumulation of protein at the surface of the dried matrix can be detected [157-159]. While spray-(freeze-)dried products show a significantly higher accumulation compared to freeze-dried products, the addition of a surfactants like polysorbate was able to depress the accumulation of protein at the surface [158-160]. As expected, also the storage stability of the incorporated protein was increased [159, 161].

Besides the observation that protein instability in dried products can generally be correlated to accumulation of protein at surfaces, a correlation between stability and the specific surface area of the product seems to be more feasible [139]. Abdul-Fattah et al. proposed that the "total protein surface accumulation", representing the total amount of protein that is exposed to the surface (in percentage of all protein present), is more precise to correlate possible instability to surface degradation [139]. For example, if the product exhibits a high protein accumulation but a low specific surface area, less protein could be present at the surface compared to low surface accumulation and high specific surface area.

Although they had no evidence for surface accumulation of the protein, Hsu and coworkers found that the formation of non-soluble aggregates of t-PA in freeze-dried and subsequently stored lyophilizates, linearly correlates with the specific surface area of a freeze-dried product. Since there was now instability in freeze-thawed samples, and all physical characteristics of the products except the SSA were identically, they conclude that instability necessarily arose from degradation at the solid/void interface [162].

3.2 SELECTION OF THE RIGHT EXCIPIENTS

There is a tremendous number of publications in literature that report effects of adding various substances to freeze-drying products in order to improve product quality. It is beyond the scope of this section to review all of them in detail. Therefore, the most important classes of excipients for freeze-drying of protein formulations are briefly introduced in the following. For further information the interested reader is referred to the detailed paper of Wang [8], where also a list of suitable formulations for different proteins can be found (see also [163] and for marketed products [164]).

As already noted above, a protein that needs to be dried, has to be stabilized against a number of stress vectors that arise during freeze-drying and storage (cf. pages 10 and 11). This is done mostly by adding one or more excipients of the following groups to the protein solution prior to freeze-drying: cryo- and lyo-protectant (carbohydrate, polymer, protein, or amino acid), pH buffer agent (salts, amino acids), bulking agent (sugar alcohols, amino acids, polymers), tonicity modifier (salts), surfactant (non-ionic), antimicrobial preservative.

Cryo- and lyo-protectants

The mechanism of cryo-protection is mainly based on the preferential exclusion of excipients from the protein surface, which in turn is preferentially hydrated [58]. As preferential exclusion is mediated by non-specific interactions, quite a number of excipients are able to act as cryo-protectants: sugars, polyols, certain amino acids (and derivatives), salts, polymers (e.g. proteins, PEG) [8, 76, 79]. However, most of those excipients fail as stabilizers during the drying step of freeze-drying as they do not provide either a glassy matrix or specific interaction or they lack both [79].

Amongst all stabilizers that were investigated for their use as stabilizers of proteins, non-reducing disaccharides (sucrose, trehalose) have shown a superior potential to keep proteins active during freeze-drying and subsequent storage [49, 79]. Besides their ability to act as cryo-protectants, major advantage of those sugars is their capability to stabilize a protein (1) kinetically in an amorphous glass (vitrification) [88] and (2) thermodynamically via direct interaction with the protein (water replacement) [94, 95]. Carpenter et al. recommend a minimal weigh-to-weight ratio of 5:1 between stabilizer and protein as a rule of thumb [49]. However, the most proper ratio needs to be reexamined from case to case.

Reducing sugars (like glucose, fructose, galactose, maltose, lactose) are also able to provide an amorphous matrix and interact with the protein; however, they are also likely to react with the protein via their reducing entity, especially during storage [8]. Also basic amino acids like arginine have been shown to form amorphous glasses and stabilize proteins during storage [165]. However, for sufficient residual water content and high glass transition temperatures they need to be combined with crystalline bulking agents [166].

Although they do not contribute to a direct stabilization due to lacking of specific interactions [49], sometimes the use of high molecular weight carbohydrates or polymers (PVP, dextran, HES) additionally to disaccharides as main stabilizers, is suggested in order to increase the glass transition of the dried mixture [8, 164].

Buffering agents

The first step to choose a proper buffer agent is to select the appropriate pH value for the best stabilization of the protein – in solution and in the dry state. Not necessarily the optimum pH in solution is the optimum pH for storage in the dried state [103]. In principle, most of the well-known buffer systems can be used (phosphate, acetate, citrate, glycine, histidine, TRIS, etc). However, sodium phosphates tend to selective crystallization of the basic salt during the freezing step resulting in enormous pH shifts of up to four pH values [167]. Instead of sodium phosphate potassium phosphate is recommended as suitable buffer system besides citrate, histidine, and Tris [49, 67]. Although the risk of selective buffer crystallization can be minimized by glass forming excipients [168], using the minimal buffer concentration, which is necessary to keep the pH stable, is recommended [49, 169]. 10mM to 20mM can be considered as a suitable starting point for optimization.

Bulking agents

In order to improve the formation of a mechanically strong cake, which also has an elegant appearance, an excipient that crystallizes during freezing can be utilized [8, 49]. Due to their crystalline structure these so called bulking agents do not contribute to the direct stabilization of proteins [72, 83]. However, even for proteins that are stable without

amorphous stabilizer, bulking agents can be necessary to hinder the (low concentrated) protein to be lost from the vial by being blown out by the water vapor stream [151]. Most frequently mannitol and glycine are used as crystalline bulking agents [164]. However, other amino acids also showed their potential to fulfill that role due to their crystallization propensity [7, 166]. Generally, bulking agents are used in excess to amorphous stabilizers regarding weight to weight ratios [164].

As the mixture of bulking agents with amorphous glass forming excipients significantly impacts (decreases) the crystallization behavior, accurate care needs to be taken to fully crystallize the bulking agent during the freezing step [170]. Remaining amorphous bulking agent is likely to crystallize during storage and therefore decrease stability of proteins [72]. Full crystallization can be achieved by annealing during freezing [154]. Moreover, the polymorphic form of the crystallized bulking agent needs to be considered as critical factor, as a later change during storage, for example from hydrate crystal to anhydrous crystal, can decrease protein stability [171].

Besides crystalline bulking agents like mannitol or glycine also the use of polymers like dextran (in amorphous state) can improve drying behavior by increasing the formulations glass transition temperature in the frozen state (T_g') and therefore allows to apply higher drying temperatures [49].

Surfactants

One of the major stress vectors for proteins during freeze-drying results from different interfaces present during the process: container-solution interface, air-solution interface, ice-solution interface, void-solid interface [124]. However, proteins can be stabilized against denaturation and aggregation at these interfaces by adding surfactants (usually non-ionic species) to the formulation [49]. These surfactants compete with the protein for adsorption at the interfaces [172, 173] or directly interact with the protein molecules preventing aggregation [174]. The addition of surfactants to the rehydration diluent can support stabilization, too [8, 65].

Quite a number of surfactants have been shown to be effective stabilizers against aggregation during freeze-thaw experiments (Tween 20, Tween 80, Brij 35, Brij 30, Triton X-10, Pluronic F127, SDS) [124]. Most often the polysorbates Tween 20 and 80 are used in freeze-dried products [164], due to their low critical micelle concentration (CMC) they are effective already at low concentrations [8](i.e. at concentrations not far above the CMC, e.g. 0.5mg/ml or 0.05%).

Although their overall beneficial properties for protein stabilization, especially polysorbates are prone to a number of chemical degradation reactions like auto-oxidation an therefore formation of peroxides, which can destabilize proteins [175].

Further formulation excipients

Parenteral protein formulations generally should be isotonic solutions. If a solution, after choosing appropriate stabilizers, still is hypotonic, a tonicity modifier has to be added. Carpenter and coworkers suggest glycine or mannitol instead of sodium chloride [49]. However, also sugars can be added [79], which are usually already present as stabilizers. In this case, just the amount of sugar needs to be increased (isotonic solution of sucrose approx. 8.7%, mannitol approx. 5.2%).

Although microbiological growth is per se inhibited in dried matter, one could think of a multi-dose freeze-dried biopharmaceutical, which for example is administered in a multiple way after reconstitution. In that case, an antimicrobial preservative would be necessary to be included into the formulation. However, from studies on liquid protein formulations it is known that antimicrobial preservatives often have a strong destabilizing effect on proteins [176].

4. COLLAPSE OF FREEZE-DRYING PRODUCTS

4.1 THE COLLAPSE PHENOMENON

Structural change of a frozen sample upon freeze-drying is a well-known phenomenon already for more than a hundred years, when Shackell described shrinkage of different foodstuffs if they are dried under vacuum without complete solidification by freezing [177] (which also is regarded as first documented use of sublimation as drying mechanism [178]). In general, collapse can be described as microscopic or macroscopic structural changes due to (intentionally or unintentionally) applied drying conditions [179]. Those changes occur, when the temperature of the product exceeds a critical value, which is usually termed "collapse temperature" [180]. Concerning aqueous solutions (e.g. protein formulations) collapse can be regarded as loss in the highly porous cake structure that is formed during freezing [181].

4.1.1 MACROSCOPIC COLLAPSE

As already the first descriptions of collapse lines out, shrinkage of the material during freeze-drying is an immediately visible sign of structural collapse [177]. This reduction of the volume of the dried product compared to the frozen product can also be observed in freeze-drying of aqueous solutions in glass vials [10, 23]. However, macroscopic collapse usually is rather described as bubbling, formation of cavitations, fenestration, foaming, spattering, etc. [10, 179]. Figure 1.7 exemplarily shows macroscopic appearance of collapsed vs. non-collapsed lyophilizates.

Figure 1.7: Macroscopic appearance of non-collapsed (left) as well as collapsed (right) lyophilizate (freeze-dried from 5% wt/wt aqueous sucrose solution)

4.1.2 MICROSCOPIC COLLAPSE

On a microscopic scale, collapse is characterized by a reduction of the average pore size (i.e. porosity) and an increase in bulk density, respectively [181, 182]. Since certain

changes on the microscopic scale show no or just minor changes on the macroscopic appearance of the product, this phenomenon is also regarded as "modified" or "limited" collapse [10]. Figure 1.8 shows microscopic pictures (SEM) exhibiting small holes in the amorphous sugar structure, which were formed by micro-collapse during freeze-drying [48]. Some authors also consider a slightly reduced cake volume (i.e. shrinkage) as collapse on the microscopic level and use "shrinkage" as a synonym for micro-collapse [183, 184].

Especially if mixtures of excipients are used, that form a partly crystalline and partly amorphous interstitial matrix, no signs of collapse can be observed macroscopically, even if micro-collapse occurs. In this case the crystalline fraction forms a supporting matrix for the collapsing amorphous fraction [10, 13, 183] (same mechanism also reported for vacuum drying without freezing [7]).

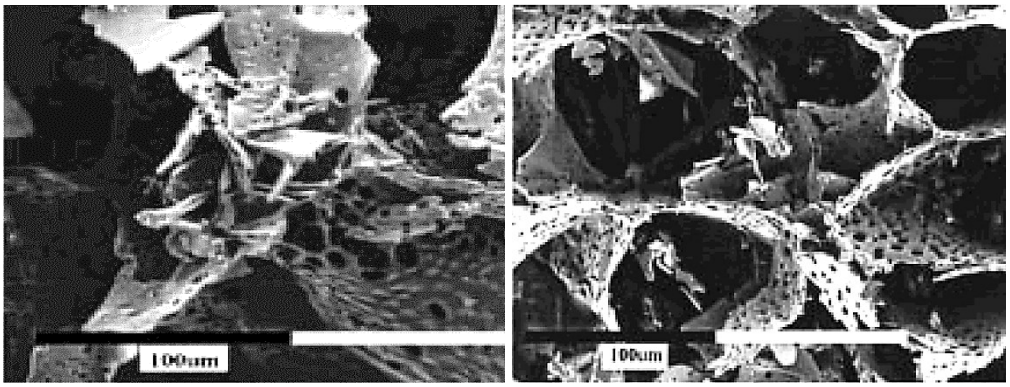

Figure 1.8: Scanning electron microscope pictures of sucrose based (left) as well as trehalose based (right) freeze-dried placebo formulation showing small pores (2-20μm) as visible sign of micro-collapse. (45mg/mL respective sugar, 0.1 mg/mL polysorbate 20, and 5 mM histidine, pH 6.0) (white scale bars represent 100μm) [48]

4.2 THE CAUSE OF COLLAPSE

Collapse during freeze-drying is caused by a reduction in viscosity due to rewarming of the previously solidified product above the critical product temperature (i.e. collapse temperature) followed by viscous flow caused by gravity and surface tension [10, 180, 183]. More precisely, on the microscopic scale collapse can be defined as viscous flow over the finite distance of a pore diameter during the timescale of freeze drying [185]. While first it was thought that viscous flow occurs due to the force to gravity [10], some authors outline the importance of surface tension as driving force for viscous flow [180, 185]. In case of a fully amorphous interstitial matrix, collapse is connected to the glass transition. For crystalline systems collapse can be observed at the eutectic temperature [186].

4.2.1 VISCOSITY AND VISCOUS FLOW

In order to find a way to describe collapse behavior in more quantitative terms, Bellows and King proposed a simple equation from the energy balance between surface energy decrease and viscous dissipation (equation (1.5), t represents the time necessary for collapse, γ the surface tension, η the viscosity of the amorphous phase, and R the pore

radius) [180]. Pikal and Shah formulated a similar description for a dynamic process dependent on temperature and measurement time (equation (1.6)), where x represents the distance of flow (e.g. pore radius for full collapse), t represents the time, γ the surface tension, η the viscosity of the amorphous phase, T the temperature [185].

$$t = \frac{\eta R}{2\gamma} \tag{1.5}$$

$$dx = 2\gamma \int_0^t \frac{dt}{\eta(T,t)} \tag{1.6}$$

Both equations highlight the importance of viscosity on the collapse. The authors reasonably assume a constant surface tension in the region of interest as well as a constant pore size and time of observation. Therefore, they were able to calculate critical viscosities necessary for collapse, which are in the order of $10^7 - 10^{10}$ cPoise [180] or around 10^8 Poise [185]. However, as these viscosities equal $10^4 - 10^7$ Pas, which is at least five orders of magnitude lower than the viscosity usually attributed to glass transition (10^{12} Pas [34]), an explanation of this gap is missing at the moment. Possible explanations include a deviation of glass transition viscosities of sugar based glasses from generally proposed viscosity of glass forming liquids [187] as well as deviations due to measurement setup (e.g. measurement time).

Nonetheless, viscosity is strongly dependent on temperature as illustrated in Figure 1.9 for aqueous solutions (25 wt %) of some low molecular sugars.

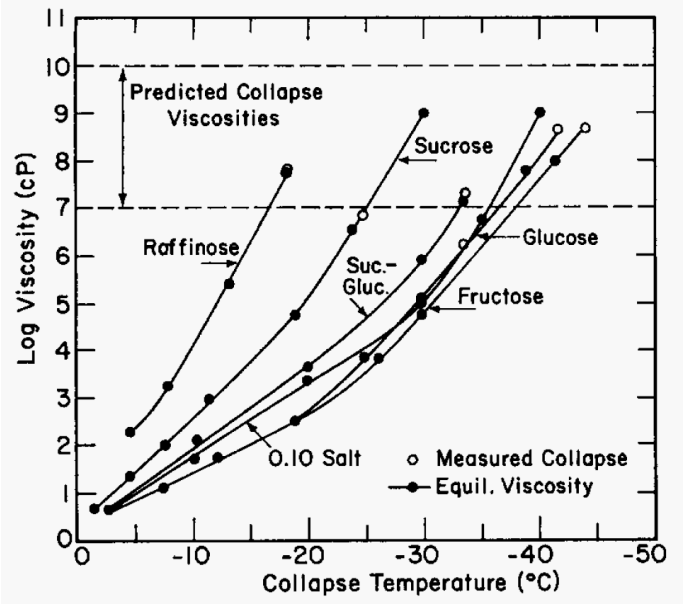

Figure 1.9: Relationship between equilibrium viscosity and (collapse) temperature of aqueous sugar soltuions (initial solute concentration 25 wt %). From [180]

The connection of viscosity η and temperature T in vicinity of the glass transition (characterized by the viscosity η_g and temperature T_g) can be expresses by the Williams-

Landel-Ferry equation (1.7). For many glass forming polymers the constants c_1 and c_2 for temperatures above T_g are similar and can by estimated as c_1=17.4 and c_2=51.6. [188].

$$log\left(\frac{\eta}{\eta_g}\right) = \frac{-c_1(T - T_g)}{c_2 + (T - T_g)} \tag{1.7}$$

If the temperature of the material is further increased, at a certain temperature ice melts and dilutes the (already viscous and collapsing) interstitial matrix resulting in further decrease of viscosity [179, 189].

4.2.2 Collapse Temperature

As outlined above viscosity is strongly coupled to temperature. Therefore, in order not to fall below the critical viscosity causing structural collapse, a certain temperature should not be exceeded during freeze-drying. This temperature is referred to as collapse temperature T_c [179, 180]. In general, the collapse temperature depends on the composition of the interstitial phase (molecular weight and structure, mixtures of solutes) between the ice crystals [10].

For low molecular sugars, collapse temperatures range about 1-5 K above glass transition temperatures detected by differential scanning calorimetry [8, 10, 190]. Collapse temperatures of sucrose and trehalose, the most prominent excipients for freeze-drying of pharmaceutical proteins, range around -30 °C. Adding higher molecular weight excipients (e.g. proteins) or cells to the mixture causes an increase in divergence between T'_g and T_c of up to 10 K [191, 192]. For fully amorphous mixtures, the divergence between T_g and T_c can be attributed to the fact, that either temperature is detected at slightly different states of the system. During measurement of T_g the interstitial matrix is in contact with ice and exhibits a certain non-frozen water amount, while during the measurement of T_c the ice has been removed as well as some of the non-frozen water has already been evaporated [185]. As water acts as plasticizer of the amorphous phase [193, 194], removing water increases the collapse temperature.

It is well known that higher product temperatures result in faster drying and therefore shorter processes. As the collapse temperature represents the upper limit of product temperatures allowing freeze-drying to a non-collapsed product, a considerable interest has been drawn to detect collapse temperatures [185]. On the other hand, detection of collapse temperatures is an analytical challenge. While the glass transition temperatures of the maximally freeze-concentrated solution T_g' can be determined via a number of methods (differential thermal analysis DTA, thermal mechanical analysis TMA, electrical thermal analysis ETA, or most commonly differential scanning calorimetry DSC)[168, 195, 196], the detection of T_c is limited by using a specialized microscope [197, 198]. These microscopes are equipped with a small temperature and pressure controlled chamber, where freeze-drying of a small amount of solution (2 – 10 µl) is operated between two cover slips and therefore can be optically monitored. Although there is a progress in well-engineered freeze-drying microscopes [199], every measurement inherently bears subjective judgment of the operator. Moreover, the collapse temperature determined by FDM might not represent the situation in a glass vial, since in the FDM just a thin layer of e.g. 25 µm [200] instead of a larger three-dimensional matrix is analyzed. Additionally, the detection of collapse at a certain temperature depends on the duration of the analytical

determination, where prolonged measurement time lead to observation of collapse at (slightly) lower temperatures [185]. Consequently, some caution needs to be exercised for designing freeze-drying cycles based on collapse temperature measurement. If observation times during T_c measurements are short, possibly artificially high collapse temperatures are detected, which in turn do not correspond to the actual temperature at which the sample collapses in a real freeze-drying cycle [181, 185].

Nevertheless, FDM is the most often applied technique to asses collapse temperatures of freeze-drying solutions [200]. Usually, two different temperatures can be read out from the microscopic determination, T_c^{micro} represents the temperatures, at which the very first visible signs of collapse (thin cracks or tiny holes, also referred to as T_c^{onset}) are apparent, while T_c^{macro} corresponds to the temperature at which a complete loss of structure is visible (also referred to as $T_c^{complete}$) [48, 191, 192].

Recently, optical coherence tomography-based freeze-drying microscopy was proposed as the more accurate technique to determine collapse temperatures directly in a vial during a real freeze-drying run. Comparing temperature measured by a thermocouple and optical collapse behavior according to the special microscope, collapse temperatures can be determined with a setting closer to the situation of the product during a freeze-drying run [201].

4.3 Manipulation of Collapse Behavior

As already outlined above, a higher product temperature during (freeze-) drying saves time. Hence, especially from a manufacturing perspective it is of high interest to increase the maximum allowable product temperatures of products to be freeze-dried as far as possible without risking a loss in product quality.

Having a look back to the determinants of collapse (cf. equations (1.5) and (1.6)), namely viscosity, temperature, pore radius, surface tension, and time, it becomes explicit that some of these parameters can be hardly influenced. Since the time that is necessary for collapse to occur lays in the magnitude of minutes and freeze-drying processes took at least hours, time cannot be influenced in a useful way. In addition, surface tension is difficult to be influenced directly. However, viscosity, temperature, and pore size can be influenced by adjusting process parameters and formulation composition, whereas changing viscosity by formulation composition plays a major role.

Following an experimental observation, that higher initial solute concentrations result in higher collapse temperatures, Tsourouflis et al concluded that during freezing of higher concentrated solutions less ice is formed, and therefore the system contains less void per volume after drying, which can be expressed as a lower inner surface. Further, lower inner surface reduces the surface tension, and therefore the system collapses at higher temperatures [179]. However, as the authors detected an inconsistent influence of freezing rate on collapse temperature for similar disaccharides, it is doubtful, if the modification of pore size by modification of freezing (e.g. by controlled nucleation) affects collapse temperatures considerably. In contrast to the observation of Tsourouflis et al, MacKenzie stated that the collapse temperature is independent from initial solute concentration as well as from freezing rate [10]. In a recent study, Meister and coworkers report a dependence of collapse temperature on both, initial solute concentration as well as nucleation temperature [202]. However, the authors also point out the difficulties to

determine reliable data, since the control of nucleation temperature is difficult in a freeze-drying microscope and the observation of collapse can be challenging for both, quite high as well as quite low solute concentrations.

Contrary to freeze-drying of food products, which exhibit collapse according to their structure and composition defined by nature, protein formulations can be compounded of numbers of different excipients. As described above, an amorphous matrix is necessary for successful stabilization of most proteins. Excellent stabilization can be achieved by low molecular weight sugars, especially non-reducing disaccharides (sucrose, trehalose). Unfortunately, these sugars exhibit low collapse temperatures (cf. Table 1.1).

Table 1.1: Collapse temperatures (Tc) and glass transition temperatures of the maximally freeze-concentrated solution (Tg') of a number of freeze-drying excipients.

excipient	Tc [°C]	reference	Tg' [°C]	reference
Arginine	-34	Ito [203]		
Dextran 2000	-9	Mackenzie [10]		
Dextran 70kDa	-11	Adams and Ramsay [204]	-10	Her et al [210]
Fructose	-48	Mackenzie [10]		
Glucose	-40	Mackenzie [10]		
Glycine			-37	Chang and Fischer [205]
Hp-beta-CD	-9.2a	Meister et al [202]		
Lactose	-31	Mackenzie [10]	-27	Her et al [210]
Mannitol	-2 (eutectic melt)	Johnson et al [206]	-27	Lueckel et al [154]
Phenylalanine			-10.5	own observations
PVP 40kDa	-24, -17.3	Adams and Ramsey [204], Meister et al [202]	-20	Her et al [210]
Sorbitol	-54	Adams and Ramsey [204]		
Sucrose	-32	Meister et al [202], Ito [203]	-32	Kasraian et al [198]
Trehalose	-29	Meister et al [202], Adams and Ramsey [204]	-30	Levine and Slade [207]

Viscosity of an amorphous mixture is strongly dependent on its composition, with higher molecular weight components, exhibiting higher viscosity and therefore higher collapse temperature [180, 208]. It was shown, that the addition of polysaccharides like starch [179], maltodextrins [179, 208], dextran [209], PVP, Ficoll [210], and proteins [211] distinctly increase glass transition temperatures. Since collapse temperatures are inherently linked to glass transition temperatures, above mentioned findings that excipients influence glass transition temperatures can be attributed to collapse temperatures, too. Moreover, the addition of protein in high concentrations (> 60 mg/ml) has elucidated an even more pronounced increase in collapse temperatures compared to glass transition

temperatures [212]. On the other hand, low molecular weight excipients like sodium chloride, glycerol, as well as glycine, decrease glass transition temperatures of amorphous mixtures [10, 15, 179, 198].

The correlation between the composition of an amorphous mixture and the resulting glass transition temperature can be characterized by the Gordon-Taylor equation or in a simplified form by the Fox equation [211, 213]. Based on their experimental data, To and Flink proposed the usability of the same correlation for collapse temperatures (cf. equation (1.8)) [208]. $T_{c,mix}$ represents the collapse temperature of the mixture, $T_{c,1}$ and $T_{c,2}$ the collapse temperatures of the solutes, w_1 and w_2 the weight fraction of the corresponding solutes. The factor B is difficult to detect and basically based on free volume theory. Assuming, according to Duddu and coworkers [211], the same simplification for B (namely to describe B as a constant), prediction of collapse temperature of a binary mixture from collapse temperatures of the pure components should hypothetically be possible according to equation (1.9) (modified Fox equation).

$$\frac{1}{T_{c,mix}} = \frac{\frac{w_1}{T_{c,1}} + \frac{w_2 B}{T_{c,2}}}{w_1 + w_2 B} \tag{1.8}$$

$$\frac{1}{T_{c,mix}} = \frac{w_1}{T_{c,1}} + \frac{w_2}{T_{c,2}} \tag{1.9}$$

Based on considerations about glass transition temperatures of frozen aqueous biological media (4 solutes plus water), Fonseca and coworkers proposed a simple linear equation to calculated T_g' as well as T_c based on properties of the single components in aqueous solution (determined experimentally or taken from literature sources) [192, 214]. Equation (1.10) shows the proposed correlation, $T_{c,mix}$ represents the collapse temperature of the mixture, $T_{c,i}$ the collapse temperature of solute i, and c_i denotes the concentration in % wt/wt of solute i in the multicomponent aqueous mixture.

$$T_{c,mix} = \frac{\sum_i T_{c,i}\, c_i}{\sum_i c_i} \tag{1.10}$$

In order to achieve mechanically stable and elegant product cakes, it is a common strategy during development of freeze-drying formulations, to include a solute into the mixture, which crystallizes during freezing and is therefore termed crystalline bulking agent [8]. By admixing sufficient amounts of a crystalline bulking agent to a formulation the macroscopic collapse temperature can be markedly increased [208], for example an 18 K increase for mannitol sucrose mixture is reported [192]. Hence, such formulations can be freeze-dried at high product temperatures without visible signs of collapse [206], since the crystalline bulking agent provides sufficient cake stability. On the microscopic scale, however, collapse of the amorphous phase towards the crystalline matrix occurs [10, 208].

4.4 Detection of Collapse

Collapse as macroscopic change of product structure has already been qualitatively described by Shakell in 1909 [177]. Although collapse was an often observed event during freeze-drying, the description of the structure of the freeze-dried product was based on macroscopic appearance and limited to non-collapsed, partially collapsed and completely collapsed for some decades [195].

Several microscopic techniques were applied to characterize the structure of freeze-dried products. While scanning electron microscopy has been used at least since the 1970s [10], fluorescence microscopy [48] and Micro-X-Ray Computed Tomography [215] are more recent technologies to qualitatively illustrate freeze-dried material. Although from the latter technique some quantitative facts can be calculated, microscopic methods are rather suitable for qualitatively illustrating cake structures.

On the other hand, several authors describe techniques to detect alterations in product structure associated with collapse in a more quantitative way. For example, a decrease in volume was assessed by simple determination of height and diameter of samples [216]. Levi and Karel used a method (previously described by Hwang and Harakawa), where specific volumes of freeze-dried samples were determined by a glass beads displacement method [181]. To assess changes in the true density due to collapse of freeze-dried samples also helium pycnometry can be utilized [182, 217]. Rambhatla et al made use of mercury intrusion porosimetry to detect different degree of shrinkage [183]. These authors also used Brunauer-Emmett-Teller (BET) gas adsorption to quantify specific surface area of shrunken cakes.

Despite the fact that collapse is a serious issue for freeze-dried pharmaceuticals, a sound investigation of the applicability of the different techniques for a reliable quantitative description of degree of collapse was lacking for a long time. However, recently Schersch investigated several techniques (helium pycnometry, mercury porosimetry, specific volume determination, density measurements, and BET SSA analysis) with the aid of lyophilizates exhibiting different degrees of collapse. Although "pore diameter and volume, the specific volume, the density and the apparent porosity all show some degree of correlation to the macroscopically determined degree of collapse", the determination of the specific surface area showed up as most sensitive tool to characterizes small differences in degree of collapse, also micro-collapse could clearly be detected [190].

4.5 Impact of Collapse

Based on a long history, most publications dealing with freeze-drying of sensitive goods point out that collapse is an unwanted event during freeze-drying. Also most reviews, which serve as sort of entry literature in the field of freeze-drying for young scientists, underline the importance to avoid product collapse [23, 36, 49, 169]. Therefore, there is a strong persuasion of scientists as well as responsible people from the industry that (at least macroscopic) collapse has to be avoided by all means, resulting for example in rejection of whole batches of freeze-dried products as far as collapse was observed [192]. In the following section, the documented impact of collapse on product characteristics shall be examined from relevant available literature emphasizing pharmaceutical freeze-drying.

4.5.1 Impact on physical characteristics

One of the most immediate signs of collapse is loss of structure, described in many variations, e.g. "bubbling, swelling, foaming, cavitation, fenestration, gross collapse, retraction and beading" [10]. Altogether, due to their loss in structure collapsed pharmaceutical products are described to lack a pharmaceutical elegance [14, 218].

On the other hand, a number of product properties are potentially affected by the structural changes during collapse occurs or in the final collapse material. MacKenzie as well as Tsourouflius et al already delineate the closure of pores as a result of viscous flow during collapse as dramatic limitation for water vapor transport from the proceeding sublimation front [10, 179]. They noticed that drying is reduced to ineffective evaporative mechanisms [179] or more generally that drying is drastically hampered [10]. The increased (product) resistance to water vapor flow is thought of leading to decreased sublimation rates and therefore a retarded primary drying [219]. In addition, due to reduced porosity (i.e. reduced specific surface area), desorptive drying during secondary drying becomes less effective resulting in high residual water contents [14, 15, 220].

Since the highly porous structure of freeze-dried products generally allows for a fast rehydration of such products, a reduced porosity is also considered to worsen the rehydration behavior of collapsed products [10, 15].

Darcy and Buckton report an increased tendency of spray-dried and subsequently collapsed lactose to undergo crystallization [221]. Obviously, the increased water content of the collapsed samples resulted in an increased tendency toward crystallization due to an increased mobility of the amorphous phase. Izutsu and coworkers showed that storage at elevated temperatures of inositol based lyophilizates caused collapse and crystallization [152], potentially resulting from increased mobility due to higher storage temperatures, too.

4.5.2 Impact on Protein Stability / Product Quality

Based on the serious influence of collapse on the morphology and physical characteristics of freeze-dried products, there are general concerns about adequate preservation of product qualities in collapsed material [10, 23, 218]. Especially for the production of freeze-dried pharmaceutical, the importance of a mechanically stable, non-collapsed caked is outlined [8, 185].

On the other hand, several approaches have shown that for a successful stabilization of pharmaceutical molecules a porous cake is not the only possible way. Particularly spray drying has been established as a beneficial tool to stabilize proteins [160, 222-224]. However, also vacuum drying, foam drying as well as supercritical fluid drying have shown their potential to stabilize embedded molecules without generation of porous cakes [7, 161, 166, 217, 225, 226].

Although there is the widespread opinion that product collapse needs to be avoided during freeze-drying, data confirming a clear negative effect of collapse on the stability of molecules to be stabilized are rather rare.

Lueckel et al observed a dramatic increase in IL-6 aggregation, if collapsed samples were stored at elevated temperatures, though right after freeze-drying there was no difference between collapsed and non-collapse samples. However, due to their high residual water content, collapsed samples exhibited low glass transition temperature, which laid below the respective storage temperatures. Therefore, degradation by a Maillard-ike

reaction was fostered due to high molecular mobility. Moreover, also non-collapse samples, if they exhibited high residual water content, showed similar increase in aggregation [227].

Passot and coworkers observed markedly decreased storage stability for toxins formulated in PVP-sucrose and mannitol-PVP mixtures, if those were freeze-dried above their glass transition temperature Tg'. These observations were independent from the macroscopic collapse, as PVP-sucrose showed macroscopic collapse while mannitol-PVP did not show a difference in macroscopic appearance compared to samples freeze-dried below Tg'. Therefore, micro-collapse seems to be the main cause for decrease of storage stability. In samples that were freeze-dried below their corresponding Tg', no loss of toxin activity could be detected [13].

Schneid et al operated a robustness testing for a model vaccine formulation (live bacteria, sucrose, buffer; quantities not specified), where they employed different freeze-drying protocols in order to achieve different product temperatures during primary drying, below and above the collapse temperature of the formulation. Although they did not identify a difference in stability right after freeze-drying, collapsed samples showed an increased degradation behavior compared to non-collapsed samples. However, also in this study, the collapsed and non-collapsed samples exhibit different residual water contents as well as different glass transition temperatures, i.e. collapsed samples show lower glass transition temperature and therefore higher mobility during storage at equal temperatures compared to non-collapsed samples [228].

Jiang and Nail directly assed the influence of process conditions on the protein stability during freeze-drying from solutions containing no excipients but buffers. By the variation of product temperature (due to variation of process parameters) they generated collapsed and non-collapsed samples. Comparing the recovery of protein activity, collapsed samples showed less activity recovery compared to non-collapsed samples, most pronounced for LDH samples. Also for non-collapsed samples, the authors observed a faster loss of activity during primary drying for higher product temperatures. However, since the authors outline a strong decrease in activity recovery at residual water contents below approx. 10%, a decrease in recovery during freeze-drying could also be attributed to the decrease in water content, as for higher product temperatures a faster decrease in water is expected. Moreover, the authors emphasized the importance of the freezing method, protein concentration as well as the use of stabilizers on the recovery of activity in general [12].

Adams and Iron reported no loss in enzyme activity (Erwinia L-Asparaginase) due to collapse, although their products exhibited complete structural collapse in lactose-sodium chloride mixtures [15]. In a further study, Adams and Ramsay also described that structural collapse of glucose based formulation did not hamper successful stabilization of the enzyme, whereas non-collapsed samples, based on mannitol, showed a poor enzyme activity [204].

Izutsu and coworkers freeze-dried β-galactosidase in increasing amounts of inositol and subjected these samples to storage at elevated temperatures. While right after freeze-drying all samples showed a non-collapsed appearance, by storing them at 60°C and 70°C for up to seven days they observed structural collapse during storage. Concurrently they noticed a decrease in recovery of enzyme activity. However, collapse was accompanied

by crystallization of the previously amorphous inositol. In turn, if crystallization was impeded by addition of polymers to the formulations, collapsed but not crystallized samples preserved activity equally well as non-collapsed samples, which were stored at lower temperatures [152].

Stimulated by the finding of Lueckel, Jiang, and Izutsu Wang et al designed a study to investigate the influence of structural collapse on the stability of α-amylase as well as recombinant Factor VIII (rFVIII) formulated in a glycine-sucrose mixture. By omitting the annealing step during freezing, they avoid crystallization of glycine resulting in low collapse temperature. In turn, annealing caused glycine crystallization followed by an increase in collapse temperature. Hence, both species dried by the same freeze-drying protocol resulted either in collapsed or non-collapsed samples. Though, the samples showed different residual water contents (collapsed as expected higher), the authors did not detect a difference in stability for both proteins between non-collapsed and collapsed samples, neither right after freeze-drying, nor after storage of 18 months. Even a slightly better preservation of activity was reported for 40°C storage of collapsed rFVIII samples [219].

Chatterjee et al subjected formulations composed of LDH and a mixture of raffinose or trehalose and glycine in different ratios to a freeze-drying protocol ensuring a product temperature 10 K above the glass transition temperature. Formulations at low glycine contents showed structural collapse, while increasing amounts of glycine result in macroscopically non-collapsed samples due to formation of a crystalline glycine matrix. For comparison, all formulations are freeze-dried at low product temperature according a conventional freeze-drying protocol, which ensures that samples did not exceed the collapse temperature during drying. Regarding preservation of LDH activity, no significant differences could be detected comparing low and high glycine amounts from the aggressive cycle, former collapsed latter not. Moreover, comparing collapsed samples from the aggressive cycle to non-collapsed samples from the conventional cycle, an equally well preservation of activity was detected [220].

Although the intention of their study was not an investigation of the influence of collapse on the activity preservation of incorporated bacteriophages, Puapermpoonsiri et al detected a better stabilization of bacteriophages in collapsed sucrose and PEG samples compared to non-collapsed samples after a storage period of 30 days. The authors attribute this surprising finding to higher residual water contents of the collapsed samples. Nevertheless, collapsed sucrose and PEG samples seem to be superior to non-collapsed samples [229].

During evaluation, whether primary or secondary drying step during freeze-drying is more critical for the stability of am model protein (LDH), Luthra and coworkers discovered that collapse during primary drying has no influence on recovery of LDH activity – even without lyo-protectant. Moreover, they found no difference between collapsed and non-collapsed samples after secondary drying (0.5% and 5% sucrose). In collapsed samples that contain 5% sucrose actually a full recovery of LDH activity was recorded [52].

In a two-section study Schersch et al investigated the direct influence of collapse on the stability of three model proteins (IgG1, LDH, and "PA01"), formulated in typical amorphous or partially crystalline formulations and freeze-dried to collapsed or non-collapsed lyophilizates by choosing a conventional or an aggressive freeze-drying cycle. To

act as amorphous stabilizers sucrose, trehalose (IgG1 LDH) or arginine (PA01) was employed, for partially crystalline mixtures PEG3350 was utilized (LDH). The authors applied quite a number of techniques to detect differences in protein aggregation, conformational stability, as well as activity recovery between collapsed and non-collapsed samples. Altogether, after freeze-drying they observed an excellent stability of IgG1 as well as PA01 independent from structural collapse of the samples. LDH samples showed increased levels of insoluble protein aggregates (detected by light obscuration), however, there were no differences between both species collapsed and non-collapsed. Regardless of the higher amounts of aggregates, the activity of LDH was preserved equally well (around 100%) in both collapsed and non-collapsed samples. The authors concluded that there is not necessarily a detrimental effect of structural collapse on protein stability [16].

In addition to investigations on the stability of proteins in collapsed lyophilizates right after freeze-drying Schersch and coworkers studied the influence of collapse on the storage stability of the lyophilizates that have been mentioned already above (IgG1, LDH, PA01). To accelerate potential degradation they stored samples at up to 50°C for up to 26 weeks. They found a comparable stability throughout the whole storage period for the potentially most sensitive model protein LDH, expressed in comparable aggregation, conformation, and activity recovery. For IgG1 as well as for PA01 collapsed samples exhibited even a slightly better stabilization, as especially at high storage temperatures collapsed samples showed less aggregation or less decrease in catalytic activity to some extent. Moreover, also partially collapsed samples performed worse than completely collapsed samples [17].

4.6 Intentional Use of Collapse

Growing evidence showed that collapse during freeze-drying does not necessarily damage the molecule to be stabilized. Thus, several companies/inventors filed patent applications dealing with freeze-drying using protocols that causes (micro-) collapse. Some applications explicitly claim freeze-drying processes (and formulations) resulting in product temperatures above the glass transition temperature Tg' but below the collapse temperature Tc consequently resulting in micro-collapsed products, but retaining the macroscopic structure [18, 230]. The reduction of porosity by micro-collapse decreases the dry layer resistance. Therefore, during primary drying at a certain product temperature, drying rates are increased, as water vapor can more easily pass the dry layer [48, 171, 186]. Applying those high product temperatures result in short freeze-drying processes as already outlined by several authors [36, 48, 206, 220]. Moreover, recently also Tchessalov and coworkers filed a patent application using high product temperatures during primary drying, too. In contrast to former mentioned applications, Tchessalov et al also apply product temperatures above the collapse temperature in order to decrease process time as much as possible [20].

Besides protein stability, also residual water content as well as reconstitution time belongs to the often mentioned concerns for collapsed products. However, for example Schersch et al as well as Tchessalov et al demonstrated that despite fully collapsed structure the samples can be dried to adequate residual water contents. Furthermore, reconstitution times are in the range of non-collapsed samples [16, 20].

5. SPECIFIC CONSIDERATIONS FOR AGGRESSIVE FREEZE-DRYING

Condensing the knowledge about collapse during freeze-drying and its effect on critical product qualities by now, a further development of "collapse-drying" [16] or aggressive freeze-drying towards a new freeze-drying technique promises to be valuable. The potential capability to manufacture products in considerably less time to at least the same quality, as already delineated by recent findings [16, 17, 20], renders a further investigation highly attractive. In order to develop aggressive freeze-drying towards a reliably technical option, some points needs to be considered.

Generally, a freeze-drying process has to produce high quality products in a reproducible way. Generating and maintaining of a precise low vacuum as well as precise control of the heat input into the product is a technical challenge. Therefore, a freeze-drying process also needs to be robust against small variations in process controlling factors (pressure, temperature).

In order to implement a new technique for (pharmaceutical) freeze-drying, a fast success would be more realistic if these techniques would be executable on already available technical equipment. Independent from structural collapse, freeze-drying at high product temperatures can unveil some new challenges for the technical equipment. For example especially during primary drying a larger amount of water vapor is generated per time compared to conventional freeze-drying cycles, that needs to be removed from the surrounding of the product. In laboratory as well as in pilot and production scale freeze-driers, this is usually realized by a combination of a condenser for water vapor and a vacuum system for non-condensable gases. Maintaining a certain precise chamber pressure is an inevitable prerequisite for successful freeze-drying. Hence, the condenser and vacuum machinery have to be capable to remove water vapor in the quantities that were generated by sublimation. Furthermore, if the connection between product chamber and condenser is constructed in a way that water vapor flow is restricted to a certain degree, the velocity of water vapor could potentially reach the speed of sound, resulting in choked flow and finally in loss of chamber vacuum [231]. Aggressive freeze-drying processes result in higher amounts of water vapor per time compared to conventional freeze-drying, therefore the condenser temperatures as well as the maintenance of chamber pressure has to be monitored carefully. Moreover, the system controlling the energy input into the product (usually in form of temperature controlled shelves) has to be able to supply more energy per time compared to conventional freeze-drying cycles.

For a better understanding – and a sound process design – of aggressive freeze-drying, the kinetic of primary and secondary drying is of high interest. It is widely accepted that freeze-drying at higher product temperatures result in a reduction of freeze-drying time as proposed by Pikal [11]. However, the drying kinetics of freeze-drying cycles at higher product temperatures are barely investigated so far. Generally, primary drying kinetics of aggressive freeze-drying cycles are expected to differ profoundly due to high energy input as well as due to micro- or structural collapse. It is known that secondary drying kinetics strongly depend on the microscopic structure [51], therefore, for collapsed products a distinction to conventional freeze-dried product has to be expected.

Contrary to the widespread opinion that reduced porosity, or better reduced specific surface area, solely bears corrupted product quality regarding achievable residual water

contents and rehydration behavior, Schersch and coworkers have shown a superior storage stability of collapsed samples with low specific surface area [17]. Observations of Hsu et al as well as of Abdul-Fattah et al (foam-dried material) also point in the direction of slightly better stability of products with lower specific surface area [162, 217]. Moreover, residual water content as well as reconstitution time of samples of a number of quite different formulations have not shown significant or stability influencing differences between collapsed and non-collapse material [16, 20]. However, bearing reports of deteriorated residual water content as well as reconstitution time in mind, it is important to keep an eye on those important product qualities.

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CHAPTER 2 OBJECTIVES

Many therapeutic proteins are not stable in liquid solutions. Freeze-drying is therefore the process of choice when it comes to stabilization. Over the last decades, pharmaceutical freeze-drying was established as a reliable technique to stabilize sensitive molecules. Already in the beginnings, the importance to avoid collapse of the product during drying and storage was highlighted [1]. Collapsed samples are regarded as irregular, unacceptable products, which exhibit higher water content, slower reconstitution and compromised protein stability.

In a recent, systematic investigation, however, it was shown that collapse during freeze-drying did not affect the stability of a sensitive model protein and two therapeutic proteins [2]. Moreover, compared to conventionally freeze-dried, non-collapsed lyophilizates the collapsed samples revealed superior stability during storage at elevated temperatures [3]. The collapsed lyophilizates were mainly produced by an aggressive freeze-drying cycle that increased the product temperature above the collapse temperature. Hence, the proven fact that aggressive freeze-drying does not affect the protein stability was taken as a starting point for the actual work.

The major aim of this thesis was to generate a foundation for the intended use of aggressive freeze-drying for stabilization of protein pharmaceuticals. Controllable and reproducible production of lyophilizates by aggressive freeze-drying was considered as fundamental requirement to facilitate aggressive freeze-drying as suitable alternative to conventional freeze-drying. Thus, the first part of the studies was focused on controllability of aggressive freeze-drying.

Aiming for definition of a design space for controllable and reproducible production of lyophilizates by aggressive freeze-drying, the influence of process parameters on product characteristics was evaluated. For a sound understanding of how to perform and potentially optimize aggressive freeze-drying, the drying kinetics of samples during aggressive freeze-drying were monitored. To gain understating how the formulation composition affects the drying behavior, the most common formulation strategies were taken into consideration. Disaccharide based formulations without bulking agent were compared to formulations of low and high bulking agent content. Beyond drying behavior, the final lyophilizate characteristics resulting from different process parameters were of utmost interest. Key lyophilizate qualities (e.g. water content, crystallinity) were determined and judged regarding the potential to ensure protein stabilization based on the concepts of water replacement and vitrification. Preserving the protein stability was regarded as a major determinant for successful use of aggressive freeze-drying within the studied design space.

The second major aim of the thesis was to shorten drying times of aggressive freeze-drying and maintain the superior protein stabilization. In previous work [4], collapsed samples have shown slow secondary drying due to the low specific surface area. As collapsed samples concurrently revealed preferable stabilization, it was aimed to develop a formulation strategy that result in collapsed samples, which can be dried within less than 24 h to < 1% residual water content.

Summarizing, the aims of the thesis were:

- Evaluation on how the variation of process parameters influences the drying kinetics, physico-chemical characteristics, and protein stabilization of aggressively freeze-dried lyophilizates in order to define a design space for aggressive freeze-drying (Chapter 4).

- Investigation of the influence of bulking agents, solid concentration, and protein concentration to identify formulation strategies that are able to achieve preferable physico-chemical characteristics of aggressively freeze-dried lyophilizates (Chapter 5).

- Characterization of the influence of bulking agents, solid concentration, and protein concentration on the ability to stabilize incorporated proteins during aggressive freeze-drying and subsequent storage in order to verify broad applicability or isolate the most promising formulation strategy (Chapter 6).

- Evaluation of the applicability of aggressive freeze-drying to further proteins in order to examine a potentially universal use of aggressive freeze-drying for the stabilization of proteins (Chapter 7).

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CHAPTER 3 MATERIALS AND METHODS

1. MATERIALS

1.1 MODEL PROTEINS

1.1.1 MONOCLONAL ANTIBODY MAB_T1

In order to represent the most important class of therapeutic antibodies [1] an IgG1 monoclonal antibody has been selected as model protein. Immunoglobulins like IgG (IgG = immunoglobulin subtype G) belong to the group of immunological active glycoproteins, which bind highly specific to an explicit epitope of a cell or the like. This makes them very effective therapeutics.

Like all antibodies, IgG molecules consist of each, two heavy and two light chains, forming a Y-shaped three-dimensional structure of around 150kDa. Enzymatic cleavage by papain results in two Fab-fragments (Fab = antibody binding fragment) as well as one Fc-fragment (Fc = crystallizable fragment). While the Fab-parts are associated with antigen binding, the Fc-part facilitates effector functions of the antibody [2].

Mab_T1 was available as bulk solution at a concentration of approx. 17 mg/ml in a phosphate buffer at 10.5 mM at pH of 6.4. Prior to use, the bulk was filtered using a syringe filter (PVDF membrane, 0.2µm pore diameter; PALL Lifesciences, Dreieich, Germany) in a laminar airflow bench (Herasafe, Heraeus Instruments GmbH, Hanau, Germany). After filtration, the exact concentration of mab_T1 was determined by UV absorption (UV 280nm, ε=1.499 ml mg^{-1} cm^{-1}) at a wavelength of 280 nm using a NanoDrop 2000 photometer (Thermo Fischer Scientific, Schwerte, Germany). For freeze-drying experiments, the protein concentration was adjusted to the desired value by mixing the filtered bulk solution with 10mM potassium phosphate buffer (pH=6.4) and various amounts of excipients.

1.1.2 MONOCLONAL ANTIBODY MAB_T2

In order to account for the prominent status of monoclonal antibodies in the therapeutic treatment, a second monoclonal antibody (mab_T2, IgG1) was utilized for freeze-drying investigations. Mab_T2 was available at a concentration of approx. 40 mg/ml as bulk solution containing histidine, trehalose, as well as polysorbate (pH=5.4).

Prior to use, the bulk solution was dialyzed overnight at 5°C (exchange of buffer medium after 2 and 4 hours) against a 10mM sodium citrate buffer (pH=5.4) using Pierce Slide-A-Lyzer® dialysis cassettes (cutoff 10 kDa, 12 ml; Thermo Fisher Scientific, Bonn, Germany). After dialysis, the solution was filtered using a syringe filter (PVDF membrane, 0.2µm pore diameter; PALL Lifesciences, Dreieich, Germany). The exact concentration of mab_T2 after filtration was determined by UV absorption (UV 280nm, ε=1.55 ml mg^{-1} cm^{-1}) at a wavelength of 280 nm using a NanoDrop 2000 photometer (Thermo Fischer Scientific, Schwerte, Germany). For freeze-drying experiments the protein concentration was adjusted to the desired value by mixing the mab_T2 solution with 10mM sodium citrate buffer (pH=5.4) and various amounts of excipients.

1.1.3 L-LACTATE DEHYDROGENASE (LDH)

Although L-lactate dehydrogenase is not employed as therapeutic protein, it is often used to reveal destabilizing stress vectors during freeze-drying [3-14], spray-drying [15, 16], vacuum drying [17, 18], or supercritical fluid drying [19]. Due to its sensitivity to freezing as well as drying stresses, if not proper stabilized by suitable cryo- and lyo-protectants, LDH is also reported to be a suitable model to investigate the effect of product collapse on the protein stability [20, 21].

LDH is an enzyme (oxidoreductase; EC 1.1.1.27) that catalyze the oxidation of lactic acid to pyruvate and concurrent the reduction of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) to the reduced form (NADH) and vice versa. LDH is present in a wide variety of animals and plants. For freeze-drying LDH from rabbit muscle frequently is utilized, which consists of four subunits forming a tetramer of 140 kDa molecular weight [22].

L-lactic dehydrogenase type II from rabbit muscle was acquired as aqueous suspension (approx. 12 mg/ml LDH, 800-1,200 units/mg protein, 3.2 M ammonium sulfate, pH=6.0; Sigma-Aldrich, Steinheim, Germany). Immediately before use, the suspension was dialyzed 24 hours at 5°C (exchange of buffer medium after 3 and 6 hours) against a 10mM sodium phosphate buffer (pH=7.5) using Pierce Slide-A-Lyzer® dialysis cassettes (cutoff 20 kDa, 0.3 – 5 ml; Thermo Fisher Scientific, Bonn, Germany). After dialysis the exact concentration of LDH was determined by UV absorption (UV 280nm, ε=1.49 ml mg^{-1} cm^{-1}) at a wavelength of 280 nm using a NanoDrop 2000 photometer (Thermo Fischer Scientific, Schwerte, Germany). For freeze-drying experiments the protein concentration was adjusted to the desired value by mixing the LDH solution with 10mM sodium phosphate buffer (pH=7.5) and various amounts of excipients.

1.1.4 RECOMBINANT PLASMINOGEN ACTIVATOR (RPA)

To broaden knowledge about aggressive freeze-drying and also to different classes of protein drugs, formulations of recombinant plasminogen activator (rPA) available at a concentration of 3.0 mg/ml formulated with 0.5M arginine and 0.25M phosphate (pH=7.2) were used. RPA is a modified form of human tissue plasminogen activator containing 355 amino acids (62.9 kDa) produced in *E.coli* and therefore present as non-glycosylated modification. After thawing rPA bulk was filtered using a syringe filter (PVDF membrane, 0.2µm pore diameter; PALL Lifesciences, Dreieich, Germany). Following filtration the exact concentration of rPA was determined by UV absorption (UV 280nm, ε=1.74 ml mg^{-1} cm^{-1}) at a wavelength of 280 nm using a NanoDrop 2000 photometer (Thermo Fischer Scientific, Schwerte, Germany). For freeze-drying experiments, the protein concentration was adjusted to the desired value by mixing the rPA solution with highly purified water and various amounts of excipients.

1.1.5 GRANULOCYTE COLONY-STIMULATING FACTOR (GCSF)

To complete the set of model proteins GCSF was chosen representing another therapeutic protein (treatment of neutropenia [23]). GCSF is a monomeric cytokine of 19kDa molecular weight, which is most stable at pH of 4.0 and exhibits poor stability upon freeze-drying without proper stabilization by excipients [24]. GCSF was available as frozen bulk solution containing 5%wt sorbitol and 0.004% polysorbate 80 in a 10mM sodium acetate buffer (pH=4.0).

Prior to use, the bulk solution was dialyzed overnight at 5°C (exchange of buffer medium after 2 and 4 hours) against a 10mM sodium citrate buffer (pH=4.0) using a Spectra/Por® dialysis membrane (cutoff 6-8 kDa, Spectrum Laboratories, Rancho Dominguez, CA, USA). After dialysis, the solution was filtered using a syringe filter (PVDF membrane, 0.2µm pore diameter; PALL Lifesciences, Dreieich, Germany). The exact concentration of GCSF after filtration was determined by UV absorption (UV 280nm, ε=0.815 ml mg^{-1} cm^{-1}) at a wavelength of 280 nm using a NanoDrop 2000 photometer (Thermo Fischer Scientific, Schwerte, Germany). For freeze-drying experiments the protein concentration was adjusted to the desired value by mixing the GCSF solution with 10mM sodium citrate buffer (pH=4.0) and various amounts of excipients.

1.2 FREEZE-DRYING EXCIPIENTS

Table 3.1 lists the excipients that were used in this work.

Table 3.1: List of excipients used in this thesis

excipient	specification	supplier
Citric acid monohydrate	Ph.Eur.	Jungbunzlauer (Ladenburg, Germany)
hp-β-cyclodextrin (Cavasol W 7 HP Pharma)		Wacker Chemie (Munich, Germany)
K2HPO4	p.A.	Applichem (Darmstadt, Germany)
KH2PO4	p.A.	Applichem (Darmstadt, Germany)
L-arginine	Emprove® Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
L-Glycine	>99%	Sigma Aldrich Chemie (Steinheim, Germany)
L-Glycine	Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
L-histidine	Emprove® Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
L-isoleucine	BioUltra, ≥99.5%	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
L-leucine	For biochemistry	Merck KGaA (Darmstadt, Germany)
L-methionine	Emprove® Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
L-Phenylalanine	>99%	Sigma Aldrich Chemie (Steinheim, Germany)
L-tryptophan	reagent grade, ≥98%	Sigma Aldrich Chemie (Steinheim, Germany)
Mannitol	Ph.Eur.	Cerestar (Cargill Europe BVBA, Mechelen, Belgium)
Na2HPO4 x 2 H2O	p.A.	Applichem (Darmstadt, Germany)
NaCl	p.A.	VWR (Darmstadt, Germany)
NaH2PO4 x 2 H2O	p.A.	Sigma Aldrich Chemie (Steinheim, Germany)
NaOH 1M		Applichem (Darmstadt, Germany)
Na-Succinate * 6 H2O	p.A.	Dr.Paul Lohmann (Emmerthal, Germany)
Polysorbate 20	Ph.Eur.	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
Polysorbate 80	J.T. Baker®, Ph Eur., BP, JP	VWR (Darmstadt, Germany)
Sucrose	p.A.	Suedzucker (Mannheim, Germany)
Trehalose		British Sugar (Peterborough, England)
α,α-Trehalose dihydrate	high purity, low endotoxin	Ferro Pfanstiehl Lab., Inc.(Waukegan, IL, USA)
α,α-Trehalose dihydrate	For Biochemistry	VWR (Darmstadt, Germany)

1.3 CHEMICALS AND REAGENTS

The chemicals and reagents used in this work are listed in Table 3.2.

Table 3.2: List of chemicals and reagents used in this thesis

substance	specification	supplier
Bovine Serum Albumine (BSA)	≥ 98%	Sigma Aldrich Chemie (Steinheim, Germany)
Hydranal® water standard 0.1	0.01% water	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
Hydranal® water standard 1.0	0.1% water	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
Hydranal® water standard 1.0	1.0% water	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
Hydranal®-Methanol dry	<0.01% water	Fluka Analytical (Sigma Aldrich Chemie, Steinheim, Germany)
Hydrochloric acid 1M		Applichem (Darmstadt, Germany)
L-arginine	Emprove® Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
Na2HPO4 x 2 H2O	p.A.	Applichem (Darmstadt, Germany)
NaCl	p.A.	VWR (Darmstadt, Germany)
NaH2PO4 x 2 H2O	p.A.	Sigma Aldrich Chemie (Steinheim, Germany)
Na-Pyruvat	99%	Sigma Aldrich Chemie (Steinheim, Germany)
β – NADH	98%	Sigma Aldrich Chemie (Steinheim, Germany)
Phohspohric acid 85%	BioReagent	Sigma Aldrich Chemie (Steinheim, Germany)
S-2288™ (H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroaniline dihydrochloride)		Chromogenix (Milano, Italy)
Trizma® base	BioUltra	Sigma Aldrich Chemie (Steinheim, Germany)

2. METHODS

2.1 FREEZE-DRYING

1 ml of the formulations to be freeze-dried was filled into pre-washed (HPW) DIN 2R glass vials (glass type I, "Fiolax" quality) from Schott (Schott, Mainz, Germany). This combination resulted in a filling height of approx. 1 cm. After filling, lyophilization stoppers (WESTAR® RS with FluroTec coating; West Pharmaceutical Services , Eschweiler, Germany) were placed in position 1 allowing water vapor to escape during freeze-drying. Especially for storage stability, investigation vials as well as stoppers were sterilized prior

to use. According to the Ph. Eur. vials were heat sterilized (160°C, 120 min), while stoppers were autoclaved (121°C, 15 min) and subsequently dried at 80°C over night assisted by vacuum.

Freeze-drying was performed utilizing one of the freeze-driers specified in Table 3.3. Vials were placed directly on the shelves surrounded by a metal frame. In order to minimize edge effects due to radiation from non-temperature controlled parts of the freeze-drier (chamber walls, door), in every experimental run at least two rows of vials filled with 5% wt. sucrose solution were placed at each side of the frame ("shielding" vials).

Table 3.3: Overview of freeze-driers used in this thesis

Type	FTS LyoStar II	FTS LyoStar III	Christ Epsilon 2-12D spezial	Christ Epsilon 2-6D
Manufacturer	SP Scientific, Stone Ridge, NY, USA	SP Scientific, Stone Ridge, NY, USA	Martin Christ, Osterode am Harz, Germany	Martin Christ, Osterode am Harz, Germany
Number of shelves	3	3	3	3
Shelf area	0.43 m²	0.43 m²	0.47 m²	0.21 m²
inner diameter connection chamber/condenser	9.9 cm	9.9 cm	30 cm	12 cm
Ice capacity	30 l	30 l	12 kg	6 kg
Number of temperature sensors	16	16	6	6
Pressure gauge 1	Capacitance	Capacitance	Pirani	Pirani
Pressure gauge 2	Pirani	Pirani	-	-

Freeze-drying was operated according to the freeze-drying cycles described below. After finishing the freeze-drying cycle, the freeze-drier was vented by nitrogen gas (quality 5.0) to 800 mbar and the vials were closed by lowering the shelves of the freeze-drier. Finally, the vials were crimped with aluminum flip-off caps (West Pharmaceutical Services, Eschweiler, Germany).

2.1.1 Compounding of Freeze-Drying Formulations

Formulations to be freeze-dried were prepared by mixing precise amounts of excipient stock solutions (excipient dissolved in formulation buffer), formulation buffer, and finally adding the protein bulk solution. For homogeneous mixture, the container was gently rotated. Finally, the formulations were filtered using a syringe filter (PVDF membrane, 0.2µm pore diameter; PALL Lifesciences, Dreieich, Germany). In order to assess the characteristics of the initial formulation, an aliquot of each sample was analyzed without being freeze-dried.

2.1.2 FREEZE-DRYING CYCLES

2.1.2.1 Aggressive cycles

Based on the work of Schersch [25] two basic aggressive cycles were chosen that were used throughout the work if not stated otherwise in the respective section. Basically, freezing of products was performed by lowering the shelf temperature from 20°C to -50°C at a rate of 0.8 K/min. To ensure complete solidification, -50°C was held for at least 120 minutes. In order to provide sufficient refrigeration power to the condenser, the shelf temperature was increased up to -40°C prior to applying vacuum.

For aggressive freeze-drying, a chamber pressure of 1 mbar is applied and the shelf temperature is increased to 45°C at a rate of 0.7 K/min. Primary and secondary drying is operated at 1 mbar and 45°C. See Table 3.4 for aggressive cycle 1, aggressive cycle 2 can be found in Table 3.5. Cycle 2 was derived from cycle 1 after it was recognized that for certain formulations much shorter drying cycles are suitable to generate lyophilizates of sufficiently low residual moistures, too. In order to evaluate a design space for aggressive freeze-drying shelf temperature as well as chamber pressure were varied in a dedicated study (cf. CHAPTER 4, section 2). Generally, the chamber pressure was controlled by nitrogen-injections via the gas injection valve of the freeze-driers.

Table 3.4: Aggressive freeze-drying cycle 1 (long cycle)

		shelf	chamber			summed up	
	ramp	temperature	pressure	hold time		process time	
	[K/min]	[°C]	[mbar]	[min]	[hh:mm]	[min]	[hh:mm]
loading		20	atm.				
freezing	0.78	-50	atm.			90	01:30
		-50	atm.	120	02:00	210	03:30
	0.33	-40	atm.			240	04:00
drying		-40	1	20	00:20	260	04:20
	0.71	45	1			380	06:20
		45	1	1440	24:00	1820	30:20

Table 3.5: Aggressive freeze-drying cycle 2 (short cycle)

	ramp	shelf temperature	chamber pressure	hold time		summed up process time	
	[K/min]	[°C]	[mbar]	[min]	[hh:mm]	[min]	[hh:mm]
loading		20	atm.				
freezing	0.78	-50	atm.			90	01:30
		-50	atm.	120	02:00	210	03:30
	0.33	-40	atm.			240	04:00
drying		-40	1	20	00:20	260	04:20
	0.71	45	1			380	06:20
		45	1	360	06:00	740	12:20

2.1.2.2 Conventional cycles

In order to benchmark the result of samples from aggressive freeze-drying cycles, conventional freeze-drying cycles were applied for selected formulations (cf. Table 3.6, Table 3.7, and Table 3.8, respectively). These gentle freeze-drying protocols ensured product temperatures below the collapse temperature of respective formulations.

Table 3.6: Conventional freeze-drying cycle 1, applied for placebo studies with 5% sucrose formulation

	ramp	shelf temperature	chamber pressure	hold time		summed up process time	
	[K/min]	[°C]	[mbar]	[min]	[hh:mm]	[min]	[hh:mm]
loading		20	atm.				
freezing	0.78	-50	atm.			90	01:30
		-50	atm.	120	02:00	210	03:30
	0.33	-40	atm.			240	04:00
primary drying		-40	0.09	20	00:20	260	04:20
	0.67	-20	0.09			290	04:50
		-20	0.09	1800	30:00	2090	34:50
secondary drying	0.2	5	0.09			2215	36:55
		5	0.09	420	07:00	2635	43:55

Table 3.7: Conventional freeze-drying cycle 2, applied for formulations containing LDH

	ramp	shelf temperature	chamber pressure	hold time		summed up process time	
	[K/min]	[°C]	[mbar]	[min]	[hh:mm]	[min]	[hh:mm]
loading		20	atm.				
freezing	0.78	-50	atm.			90	01:30
		-50	atm.	200	03:20	290	04:50
	0.33	-40	atm.			320	05:20
primary drying		-40	0.04	1830	30:30	2150	35:50
	0.1	-20	0.04			2350	39:10
		-20	0.04	1200	20:00	3550	59:10
secondary drying	0.2	10	0.04			3700	61:40
		10	0.04	600	10:00	4300	71:45

Table 3.8: Conventional freeze-drying cycle 3, applied for formulations containing mab_T1

	ramp	shelf temperature	chamber pressure	hold time		summed up process time	
	[K/min]	[°C]	[mbar]	[min]	[hh:mm]	[min]	[hh:mm]
loading		20	atm.				
freezing	0.9	2	atm.			20	00:20
		2	atm.	60	01:00	80	01:20
	0.8	-50	atm.			145	02:25
		-50	atm.	120	02:00	265	04:25
	0.33	-40	atm.			295	04:55
		-40	atm.	30	00:30	325	05:25
primary drying		-40	0.07	30	00:30	355	05:55
	0.33	-30	0.07			385	06:25
		-30	0.07	3600	60:00	3985	66:25
secondary drying	0.1	10	0.07			4385	73:05
		10	0.07	600	10:00	4985	83:05

2.1.3 PROCESS MONITORING

During freeze-drying especially the temperature of the drying product is of interest. To monitor product temperatures thermocouples (FTS LyoStar II) or resistance temperature, detectors (RTDs; Christ freeze-driers) were used. The measuring head was placed in a centered position slightly above the bottom of the glass vial. During early primary

drying, the product temperature is significantly below the shelf temperature due to sublimative self-cooling. The end of sublimation (or more specifically the end of primary drying according to the definition of primary drying as drying by sublimation) is indicated by an increase in product temperature, finally approaching the shelf temperature. In addition, the end of primary drying can be monitored by comparing pressure reading from a capacitance and a Pirani pressure gauge (end of primary drying indicated by approaching pressure readings). Since only the FTS LyoStar II freeze-drier was equipped by a capacitance as well as a Pirani pressure gauge (cf. Table 3.3) this method could just be applied to freeze-drying cycles in that machine.

In order to evaluate primary drying kinetics during aggressive freeze-drying by weight loss, a Christ CWS-40 freeze-drying balance (Martin Christ, Osterode am Harz, Germany) was employed in selected freeze-drying cycles. Additionally, a sample thief was used to draw samples from the running process during primary as well as secondary drying. Based on either determining weight loss or residual water content, primary and secondary drying kinetics were calculated.

2.2 CHARACTERIZATION OF PROTEIN STABILITY

2.2.1 HIGH PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY (HP-SEC)

Protein aggregation of samples prior and after freeze-drying was monitored via HP-SEC. Befor injection, samples were filtered or centrifuged in order to remove insoluble aggregates. From each species at least two samples were analyzed and each sample was injected twice. The chromatograms were integrated manually using the Chromeleon Software from Dionex (Dionex, Idstein, Germany). The integrated chromatograms were analyzed regarding percentage content of fragments, monomers, dimers, and high molecular weight species by dividing the partial area under the curve (AUC) of each species by the total AUC. Additionally, the recovery of monomer was calculated. To allow for comparison of results from different runs, standard calibration curves were collected from known concentrations of 0.25 to 1.0 mg/ml. Samples were analyzed undiluted.

mab_T1 and mab_T2

A Gynkotek HPLC system was used (Gynkotek, Germering, Germany) equipped with a TSKgel G3000SWxl column from Tosoh (Tosoh Bioscience, Stuttgart, Germany). An aqueous buffer containing 50 mM sodium phosphate and 300 mM sodium chloride (pH=7.0) was used as mobile phase at a flow rate of 0.5 ml/min. 50µl of sample were injected and absorbance was measured photometrically at λ=280 nm and recorded by the connected Chromeleon software.

LDH

A Gynkotek HPLC system was used (Gynkotek, Germering, Germany) equipped with a TSKgel G3000SWxl column from Tosoh (Tosoh Bioscience, Stuttgart, Germany). An aqueous buffer containing 10 mM sodium phosphate and 150 mM sodium chloride (pH=6.9) was used as mobile phase at a flow rate of 0.8 ml/min. 50µl of sample were injected and absorbance was measured photometrically at λ=280 nm and recorded by the connected Chromeleon software.

GCSF

A Dionex Ultimate 3000 HPLC system was used (Dionex, Idstein, Germany) equipped with a YMC-Pack Diol 120 DL 12S05 – 3008 WT column from YMC Europe (YMC Europe, Dinslaken, Germany). An aqueous buffer containing 50 mM sodium phosphate and 300 mM sodium chloride (pH=7.0) was used as mobile phase at a flow rate of 0.8 ml/min. 30µl of sample were injected and absorbance was measured photometrically at λ=215 nm and recorded by the connected Chromeleon software.

RPA

A Gynkotek HPLC system was used (Gynkotek, Germering, Germany) equipped with a TSKgel G3000SWxl column from Tosoh (Tosoh Bioscience, Stuttgart, Germany). An aqueous buffer containing 0.5 M arginine and 0.25 M phosphate (pH=7.5) was used as mobile phase at a flow rate of 0.5 ml/min. 50µl of sample were injected and the eluate was monitored by fluorescence (extinction λ=280 nm, emission λ=344nm) and recorded by the connected Chromeleon software.

2.2.2 Light Obscuration (LO)

Light obscuration was used in addition to HP-SEC to characterize samples prior to and after freeze-drying regarding protein aggregation. Via LO the concentration of sub-visible particles (svp) in the range of 1 to 200 µm was determined using a PAMAS SVSS-C40 device (PAMAS, Rutesheim, Germany). The system was calibrated annually. Ahead of use, the device was rinsed with HPW (0.2µm filtered) until control measurement showed a particle count of less than 100 particles > 1 µm per ml. To gain the necessary volume of 1.3 ml for one sample cycle (0.4 ml sample pre-rinse, 3 measurements each consuming 0.3 ml) two lyophilizates were reconstituted and pooled. At least two sample cycles were measured (i.e. four lyophilizates). To evaluate protein aggregation the mean svp count per ml of particles >1 µm, >10 µm, and >25µm were calculated.

2.2.3 Nephelometry (Turbidity)

To assess insoluble protein aggregates also in the range below 1 µm turbidity of samples prior and after freeze-drying was determined via nephelometry. The turbidity was measured by static light scattering at 90° (λ = 860 nm) using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany) and recorded in formazine nephelometric units (FNU). At least a duplicate of 2 ml of pre-lyophilization solutions or 2 pooled vials of reconstituted lyophilizate, respectively was analyzed and the mean values are calculated.

2.2.4 Fourier Transform Infrared (FTIR) Spectroscopy

The secondary structure of proteins prior to freeze-drying and after reconstitution of the freeze-dried products were investigated using Fourier Transform Infrared (FTIR) spectroscopy. A Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a temperature controlled (20°C) calcium fluoride flow through cell (Aqua Spec Flow Cell AS 1100 BM, path length 6.5 µm, Bruker Optics, Ettlingen, Germany) and

a nitrogen cooled mercury-cadmium-telluride (MCT) detector was utilized. Each spectrum was generated by 120 scans (LDH formulations: 240 scans) from 4000 to 850 cm^{-1} with a resolution of 4 cm^{-1}. In order to evaluate pure protein spectra, placebo excipient solutions were measured, which represented the exact composition of the respective formulation but without protein content. Either these placebo solutions were directly used as background spectra or they were subtracted by the OPUS software (OPUS 6.5, Bruker Optics, Ettlingen, Germany) prior to analyzing the measurement data. To evaluate secondary structure elements of proteins, the amide I region of the spectra (1720 – 1580 cm^{-1}) was vector normalized (i.e. area normalized) and the second derivative was calculated (with 17 smoothing points according to the Savitzky-Golay algorithm). At least two samples of each species were injected in duplicate. The mean spectra were calculated and samples before freeze-drying were visually compared to reconstituted freeze-dried samples. Beyond visual comparison for selected samples, a correlation coefficient of the spectra was calculated according to Prestrelski et al [26] (cf. Equation (3.1)), where r represents the correlation coefficient ($r = 1$ for identically spectra) whereas x_i and y_i represent the spectral absorbance values of the two spectra that should be compared. For calculation the range between 1720 and 1610 cm^{-1} was evaluated.

$$r = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}} \tag{3.1}$$

For heat stress spectra measurement of mab_T1, mab_T2, and GCSF a Bio ATR cell II (Bruker Optics, Ettlingen, Germany) was used. A temperature ramp was accomplished from 30°C to 90°C recording a spectrum every 10 minute. Initially, background spectra of the placebo solution were taken and subsequently the protein solution of about 10 mg/ml in the same buffer was measured. Second derivative spectra were calculated and standardized.

2.2.5 Catalytic Activity

LDH

The enzymatic activity of LDH was evaluated by monitoring the decrease of absorption at 340 nm using the enzyme assay provided by Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). 100 mM phosphate buffer pH 7.5 was prepared by dissolving 0.842 g monosodium phosphate dihydrate and 2.580 g disodium hydrogen phosphate dihydrate in 200 ml HPW. 23.05 mg β-NADH was dissolved in 5.0 ml 100 mM phosphate buffer and further diluted 1:50 with 100 mM phosphate buffer (final concentration 0.13 mM). 75.92 mg sodium pyruvate was dissolved in 1.0 ml 100 mM phosphate buffer and further diluted 1:10 with 100 mM phosphate buffer (final concentration 0.69 mM). For dilution of LDH samples (in order to inhibit adsorption of LDH to cuvettes etc.) a 1.0% (w/v) solution of bovine serum albumine (BSA) was prepared by dissolving 200.0 mg BSA in 20 mL 100 mM phosphate buffer. LDH samples were diluted to a final LDH concentration of 0.5 µg/mL (approx. 0.46 units/mL) with 1%(w/v) BSA solution (to minimize dilution errors a two-step dilution scheme was applied). Before measurement, the β-NADH and the pyruvate solution were equilibrated at 37°C. To start the measurement, a Poly (methyl

methacrylate) (PMMA) was placed in an Agilent 8453 UV-visible Spectrophotometer (Agilent Technologies, Waldbronn, Germany) temperature controlled at 37°C. 2.8 ml β-NADH solution was pipetted into the cuvette and 100 µl pyruvate solution was added. Finally, 100 µl sample solution was added and a 1000 µL Eppendorf pipette was used to mix the solution gently. Immediately after mixing the UV absorption (λ = 340 nm) it was measured for at least 120 seconds. The activity was calculated using equation (3.2) based on a linear slope ($\Delta A_{340nm}^{sample}$) obtained by regression. 3 (ml) represents the total volume of the assay, f is the dilution factor, 6.22 is the millimolar extinction co-efficient of β-NADH at 340 nm and 0.1 (ml) is the volume of the enzyme solution added to the assay. Finally, the activity in units per mg protein was calculated by equation (3.3).

$$activity\left[\frac{U}{ml_{enzyme}}\right] = \frac{\left(\Delta A_{340nm}^{sample} - \Delta A_{340nm}^{blank}\right)3f}{(6.22)(0.1)} \tag{3.2}$$

$$activity\left[\frac{U}{mg_{protein}}\right] = \frac{activity\left[\frac{U}{ml_{enzyme}}\right]}{\frac{mg_{protein}}{ml_{enzyme}}} \tag{3.3}$$

In order to correct day-to-day variations of the assay, a standard LDH solution was included in the daily measurements. This standard solution was prepared by directly diluting 83.3 µl of LDH suspension as purchased from Sigma-Aldrich with 916.6 µl 1% (w/v) BSA solution to a final concentration of 0.5 µg/ml.

RPA

The plasminogenolytic activity of rPA was analyzed by a method based on an assay described by Kohnert et al. [27]. Samples to be analyzed were diluted to a rPA concentration of 10 µg/ml with a 100 mM TRIS buffer (pH=7.5). The temperature of the diluted sample and of a 12 mM solution of S-2288™ was adjusted to 37 °C in a water bath. S-2288™ (H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroaniline dihydrochloride) is an artificial substrate of rPA. Cleavage of S-2288™ at the amide group results in the formation of p-nitroaniline.

To start the enzymatic reaction, 50 µl of diluted sample was mixed with 50 µl of the 12 mM S-2288™ solution in a pre-warmed (37 °C) quartz well plate. The concentration of p-nitroaniline was detected by UV absorption at 405 nm (extinction coefficient 9750 l mol^{-1} cm^{-1}) using a BMG Fluostar Omega at 37 °C (BMG Labtech, Offenburg, Germany) over 10 minutes. The activity of rPa was calculated from the linear fit (i.e. slope) of the first 120 data points (i.e. 120 s) of the measurement.

2.3 PHYSICAL CHARACTERISTICS OF LYOPHILIZATES

2.3.1 MACROSCOPIC APPEARANCE

Each species of lyophilizates was visually inspected regarding the macroscopic appearance. Characteristics like cracks, shrinkage, foaming, spattering, etc. were taken into consideration. In order to document the macroscopic appearance, pictures of at least two samples of each species were taken.

2.3.2 KARL-FISCHER (KF)

The residual water content of lyophilizates was analyzed by coulometric Karl-Fischer titration employing a 737 KF Coulometer (Metrohm, Filderstadt Germany). Prior to measurement, the calibration of the device was checked by water standards (Hydranal® water standard 1.0 and 0.1, Riedel-de-Haën, Sigma-Aldrich, Steinheim, Germany). For analysis of lyophilizates, 3 mL of dry methanol (Hydranal® -Methanol dry, Fluka, Sigma-Aldrich, Steinheim, Germany; kept dry by a molecular sieve, UOP Type 3A, Fluka, Sigma-Aldrich, Steinheim, Germany) was added to the sealed lyophilizate by injection. To allow for a complete extraction of water from the dispersed lyophilizate, the sample was placed in an ultrasonic bath for 10 minutes. After extraction, 1 ml of the methanol was injected into the reaction vessel and the amount of water was determined (maximum allowed start drift 10 µg/min). To correct for water content of the methanol, each time three blank measurements were performed at the beginning and at the end of each measurement series. Each lyophilizate species was measured at least in triplicate. The residual water content in percentage was calculated based on the weight of the lyophilizate (calculated from filling volume and solid content of the formulation).

2.3.3 DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential Scanning Calorimetry was used to characterize the formulations before freeze-drying and the freeze-dried (and stored) samples, respectively. A DSC 204 Phoenix (Netzsch, Selb, Germany) system was used, which was annually calibrated by an indium standard.

Glass transition of the maximally freeze-concentrated solution (T_g')

For analyzing T_g' 20 µl solution it was pipetted to aluminum crucibles that where subsequently closed by an aluminum lid and cold welded. As reference, an empty crucible was used (also sealed by a lid). To determine T_g' the samples were cooled down to -70°C at a cooling rate of 10 K/min and subsequently heated up to 20°C at a heating rate of 10 K/min. The midpoint of an endothermic shift of the baseline during the heating scan was regarded as T_g'. Samples were analyzed in triplicates.

Glass transition of the freeze-dried samples (T_g)

In order not to compromise the glass transition temperatures by exposure to air humidity, the preparation of samples were accomplished in an isolator flushed by dry nitrogen gas. About 5 to 15 mg of a freeze-dried sample was homogenized within the vial by a spatula and accurately weighted into aluminum crucibles and sealed by an aluminum lid. As reference, an empty crucible sealed under nitrogenwas used too. To determine T_g, samples were cooled to 0°C at a cooling rate of 10 K/min, then heated to 100 – 120°C (depending on the excipients used) at 10 K/min, again cooled to 0° at 10 K/min, and finally heated up to 160 - 200°C (depending on the excipients used) at a heating rate of 10 K/min. The first heating step was included in order to remove artifacts and not used for analysis. From the second heating scan the midpoint of endothermic shifts were regarded as glass transitions. Moreover, further endo- and exothermic events were assigned to melting or crystallization of sample compounds.

2.3.4 Freeze-Drying Microscopy (FDM)

The collapse temperatures of certain formulations were analyzed by Freeze-Drying Microscopy (FDM). A Linkham FDCS 196 freeze-drying stage (Linkham Scientific Instruments, Surrey, UK) with a liquid nitrogen cooling system, a programmable temperature controller, and a vacuum pump (TRIVAC, Oerlikon Leybold Vacuum, Köln, Germany) were used in combination with a Zeiss Axio Imager Zm1 microscope (Zeiss, Göttingen, Germany) and set up with a Pixelink digital camera (Linkham Scientific Instruments, Surrey, UK). Utilizing a Zeiss LD Epiplan 20x lens (Zeiss, Göttingen, Germany) a 200-fold magnification was used. Pressure was monitored with a Pirani sensor and controlled by a proportional bleeding valve. Temperature was determined via a thermocouple mounted in the oven, used to control the sample temperature. For analysis, a droplet of 2 µl was placed on a glass cover slide, which was positioned on top of the oven of the freeze-drying stage (for better thermal contact a thin film of silicon oil is applied between oven and cover slide). A precision cut spacer of 25µm thickness was used to ensure a constant thickness of the layer to be analyzed. Finally, a second glass cover slide was placed on top. Samples were frozen at a cooling rate of 1 K/min to -50°C. After reaching -50°C a vacuum of 0.02mbar was applied. In the next step, the sample was heated at a heating rate of 1 K/min and pictures were taken every second. Characteristic changes in the structure of the already dried material (behind the progressing sublimation front) were attributed to micro-collapse, macro-collapse, and meltback.

2.3.5 X-Ray Powder Diffraction (XRD)

To characterize the molecular structure of freeze-dried samples X-Ray Powder Diffraction (XRD) was utilized. Around 100 mg of sample was grinded to powder in the freeze-drying vial by a spatula and subsequently placed on a copper sample holder in a flat layer. Analyses were carried out using a Seifert XRD 3000 TT diffractometer (GE Sensing & Inspection Technologies, Ahrensburg, Germany) with a Bragg-Brentano theta-theta geometry and equipped with a copper anode (40 kV, 30 mA, CuKα at λ=0.15417nm). Samples were analyzed from 5° to 45° 2-θ with a step size of 0.05° 2-θ and a hold time of 2 seconds per angle. The resulting diffractograms were evaluated regarding diffraction peaks. Detected diffraction peaks were compared to reference diffraction patterns of the International Centre for Diffraction Data (ICDD) (formerly JCPDS) database. If no diffraction peaks were present in the diffractogram, the sample was regarded as fully amorphous.

2.3.6 Scanning Electron Microscopy (SEM)

To get an insight into the morphology of the lyophilizate scanning electron microscopy was applied. Small pieces of a sample were extracted carefully from the vial and put to a double-sided adhesive carbon tape (Bal-tec GmbH, Witten, Germany) on top of an aluminum sample holder. In order to enhance electrical conductivity, samples were prepared by low-vacuum sputter coating using carbon. For microscopy, a Jeol JSM-6500F (Jeol, Ebersberg, Germany) at magnifications of up to 10,000 was used.

2.3.7 Determination of specific surface area (SSA)

Krypton gas adsorption according to the Brunauer Emmett Teller (BET) method was used to determine the specific surface area (SSA) of lyophilizates. Measurements were performed by using an Autosorb-1 (Quantachrome Instruments, Odelzhausen, Germany) purged with Krypton 4.8. Two to five lyophilizates were pooled to reach a volume of about 1 cm^3 and weighted into a special glass tube. Subsequently, samples were degassed for at least two hours by vacuum at room temperature (by the Autosorb-1 degassing feature). For analysis, krypton adsorption at 77 K over a relative pressure range of 0.05 – 0.3 was accomplished and the Brunauer Emmett and Teller (BET) equation was used to fit the data employing at least 5 measurement points and calculate the SSA. According to the European Pharmacopeia the correlation coefficient of the fit was at least 0.9975 (r^2=0.995) [1].

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CHAPTER 4 INVESTIGATIONS ON THE AGGRESSIVE FREEZE-DRYING PROCESS

1. INTRODUCTION

A number of textbooks (e.g. [1-4]) and publications (e.g. [5-8]) are available concerning the proper performance of freeze-drying with respect to conventional strategy and resulting product qualities. More recently it was shown that also the application of comparably harsh conditions in terms of freeze-drying process parameters, resulted in freeze-dried products of admirable stability of the incorporated proteins – although they exhibited a collapsed appearance [9]. The proper stabilization which was reported not only for the stability of mentioned "collapse-dried" products right after drying, but also during accelerated storage stability studies [10], stimulates to reconsider the conventional approach of freeze-drying of pharmaceutical formulations. Most recently it was shown that, compared to conventionally freeze-dried (=elegant) lyophilizates, the collapsed lyophilizates are less susceptible to changes during storage at elevated temperatures [11].

Conventional freeze-drying ever since is known as a time-consuming production step. Throughout research and development the application of product temperatures as high as possible is strived for [12-15]. Bearing now the findings of Schersch et al [9, 10] in mind, the application of aggressive conditions for freeze-drying appears as an encouraging possibility to save time and therefore resources without compromising key product qualities, i.e. stability of the molecule to be stabilized. Therefore, an investigation of the robustness and potentially universal use of aggressive freeze-drying to different types of protein formulations is of high interest.

In contrast to conventional freeze-drying, for the quite new field of aggressive freeze-drying little is known concerning major topics like relation between variations of process or formulations parameters and later product characteristics by now. Regarding a potential evolution of aggressive freeze-drying from an unwanted aberration of conventional freeze-drying towards a competitive alternative, there is the utmost need to gain more knowledge about the relation of controllable process parameters and final product characteristics. In addition, the applicability of aggressive freeze-drying to different freeze-drier designs would be a prerequisite for successful establishment of aggressive freeze-drying as a reliable drying technology. As final product qualities inherently depend on the initial solution, and quite a number of different formulations are reported to be conventionally freeze-dried [6, 8], also the suitability of aggressive freeze-drying for a broad range of formulations is a topic of high interest, which is outlined in Chapter 5 of this thesis.

In addition to the relationship between process parameter variations and final product qualities, kinetics of primary and secondary drying mechanisms of aggressive freeze-drying were investigated. As a third topic, the use of different freeze-drier designs for aggressive freeze-drying were studied.

Important physicochemical characteristics of aggressive freeze-dried products like residual water content and glass transition temperature were determined. To gain further insight in the qualities of those products, a set of techniques (e.g. specific surface area analysis, X-Ray powder diffraction) were applied. Besides the importance of basic physicochemical characteristics, also the impact of different process conditions on the stability of incorporated proteins was evaluated. In order to cover a significant range of marketed biopharmaceutical proteins [16], an IgG_1 antibody (mab_T1) was used as main model protein. Due to its susceptibility to freezing and drying stresses, mab_T1 is not stored in a lyophilized form, but as refridgerated liquid bulk solution. Upon freeze-drying without addition of appropriate cryo- and lyo-protectants or by using improper freeze-drying protocols, mab_T1 undergoes remarkable instability expressed as pronounced aggregate formation. Therefore, this sensitive molecule is regarded as ideal model to reveal even slightly non-ideal conditions that possibly can occur during aggressive freeze-drying. In order to compare the results from aggressively freeze-dried samples to the state of the art technique, selected samples were also freeze-dried according to conventional freeze-drying protocols. As the main instability pathway of mab_T1 is of aggregative nature, the investigation of protein stability was focused on analytical tools, which account for soluble and non-soluble protein aggregates.

2. VARIATION OF PROCESS CONDITIONS

To dry a product, or more specific a pharmaceutical formulation at gentle conditions, the temperature of the material needs to be controlled at low values. In common freeze-driers, the product temperature during freeze-drying is controlled by temperature controlled shelves, where the product is placed on (e.g. in a vial). After lowering the product temperature to values below temperature of complete solidification of all components (for pharmaceutical formulations represented by T_g') sublimative drying is fostered by applying vacuum. The necessary energy input for solid to gaseous phase transition (i.e. from ice to water vapor) is provided via controlling the temperature of the shelves in a way that ensures the desired product temperature at the given vacuum. After sublimation is completed, in a secondary drying phase the temperature of the shelves is increased to higher values to remove non-frozen water via desorption from the product to reach desired residual water content. Summing up, the freeze-drying process is operated by the control of (shelf) temperature and vacuum (also denoted as chamber pressure).

While for conventional freeze-drying people have a good understanding of the limits on how the process can be controlled to reach desired product temperatures and final product characteristics (e.g. [17]), for aggressive freeze-drying this information has never been determined so far. The following section presents the results of studies about the influence of different combinations of process parameters (shelf temperature, chamber pressure, time) on final product characteristics (physical and protein stability) and drying kinetics of primary and secondary drying step. In order to keep the focus on the influence of process parameters, a straightforward number of formulations has been chosen representing both, purely amorphous as well as partially crystalline products.

2.1 INFLUENCE OF PROCESS VARIATIONS ON PHYSICAL CHARACTERISTICS

The influence of applied shelf temperature and chamber pressure were evaluated in a two-step study. As a first step, based on the collapse-drying process of Schersch [18] (originally adapted from [19]), the dependence of collapse behavior from applied chamber pressure was studied, using a purely amorphous product (5% wt/wt sucrose solution). As it was already shown by Schersch, purely amorphous sugar solutions result in sort of a foamed product upon freeze-drying by the collapse-drying protocol. However, the formation of foam from the initially micro-porous structure of the frozen product implied a tremendous change in structure, that raised the question whether this process is well controllable or not. Especially spattering of the low viscous collapsing matrix within the vial or even out of the vial seems to be a highly probable scenario, which would be unacceptable keeping in mind the use of such a freeze-drying technology for production of biopharmaceuticals.

2.1.1 SUCROSE-BASED LYOPHILIZATES

The behavior of collapsing material regarding spattering or other uncontrollable behavior during aggressive freeze-drying was investigated. A 5% (wt/wt) sucrose solution (in 10mM sodium succinate buffer pH 5.5; 2R glass vials, 1 ml fill) was subjected to a number of different aggressive freeze-drying protocols based on variations of aggressive freeze-drying cycle 1 (cf. Table 3.4, page 55) and using a FTS LyoStarII freeze-drier. In order to simulate at least a medium load of the freeze-drier, one of the three shelves of the freeze-drier was loaded with filled vials (594 vials). To avoid a bias in resulting characteristics due to edge effects, the outmost two rows of vials were not used for analysis. As it was known that chamber pressure plays a major role for drying behavior, only this parameter was adjusted to different levels in the first study (cf. Table 4.1).

Table 4.1: Parameters used for aggressive freeze-drying of 5% (wt/wt) sucrose and resulting product temperatures during primary drying (PD)

ID	chamber pressure	shelf temperature	drying time	product temp. PD
	mbar	°C	hours	°C
0.5	0.5	45	24	-24.3
1	1	45	24	-17.2
2	2	45	24	-11.5
4	4	45	24	-0.7

As expected, the different drying protocols resulted in products of different macroscopic appearance. Pictures of representative samples can be found in Figure 4.1.

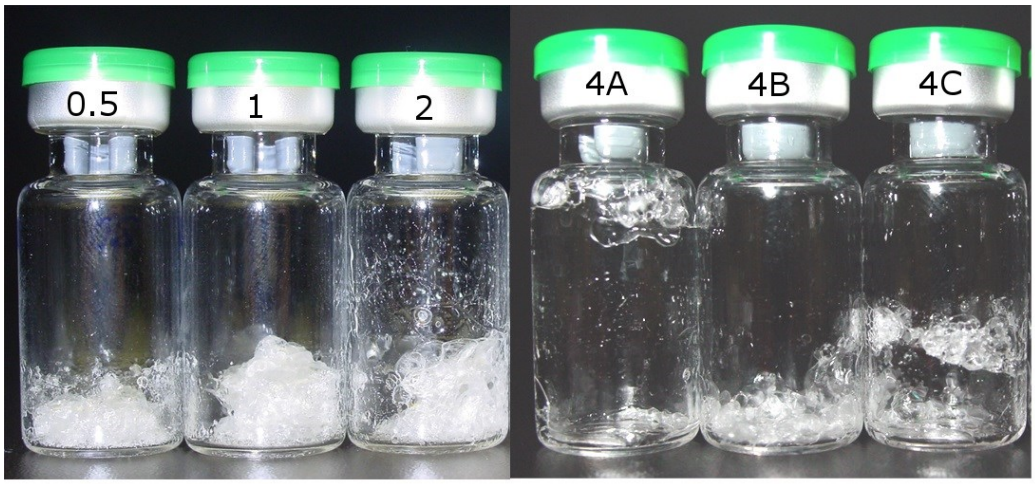

Figure 4.1: Macroscopic appearance of collapsed lyophilizates produced at different chamber pressures (initial solution 5% (wt/wt) sucrose in 10mM sodium succinate buffer; numbers indicate applied chamber pressure in mbar; 4A – 4C: 4 mbar, due to inconsistent appearance 3 vials shown)

Chamber pressures of 0.5 mbar and 1 mbar resulted in foamed products, neither showing spattered material at the inner walls of the vial, nor at the stopper (throughout the whole batch, >50 vials visually inspected). Samples dried at higher chamber pressure of 2 mbar and above consistently showed spattered material all over the inner wall of the vial and at the inside of the stopper. Whereas at 4 mbar chamber pressure, the material even splashed out of the stopper during drying and the single vials of the batch showed inconsistent appearance throughout the batch. Characteristic product temperature data of 5% (wt/wt) sucrose subjected to aggressive freeze-drying at different chamber pressures are shown in Figure 4.2. The product temperature during primary drying is characterized by a plateau, significantly lower than the applied shelf temperature during the first few hours of drying (due to so called self-cooling of the material as a result of sublimation: phase transition from solid to liquid "consumes" the energy provided by the shelves). If there is no more ice to be sublimed, the product temperature increases to a level close to the shelf temperature. Depending on the applied chamber pressure, sublimation took place at different product temperatures. Low chamber pressure resulted in comparable low product temperature, while higher chamber pressure resulted in higher product temperatures (cf. Table 4.1 and Figure 4.2). Compared to conventional freeze-drying, where sublimation is completed within around 30 hours for the identical setting (data not shown), the time needed to remove ice by sublimation is extremely short. Counting from the start of drying (indicated by vacuum pull-down and start of the heating ramp), sublimation is completed within maximum 6 hours.

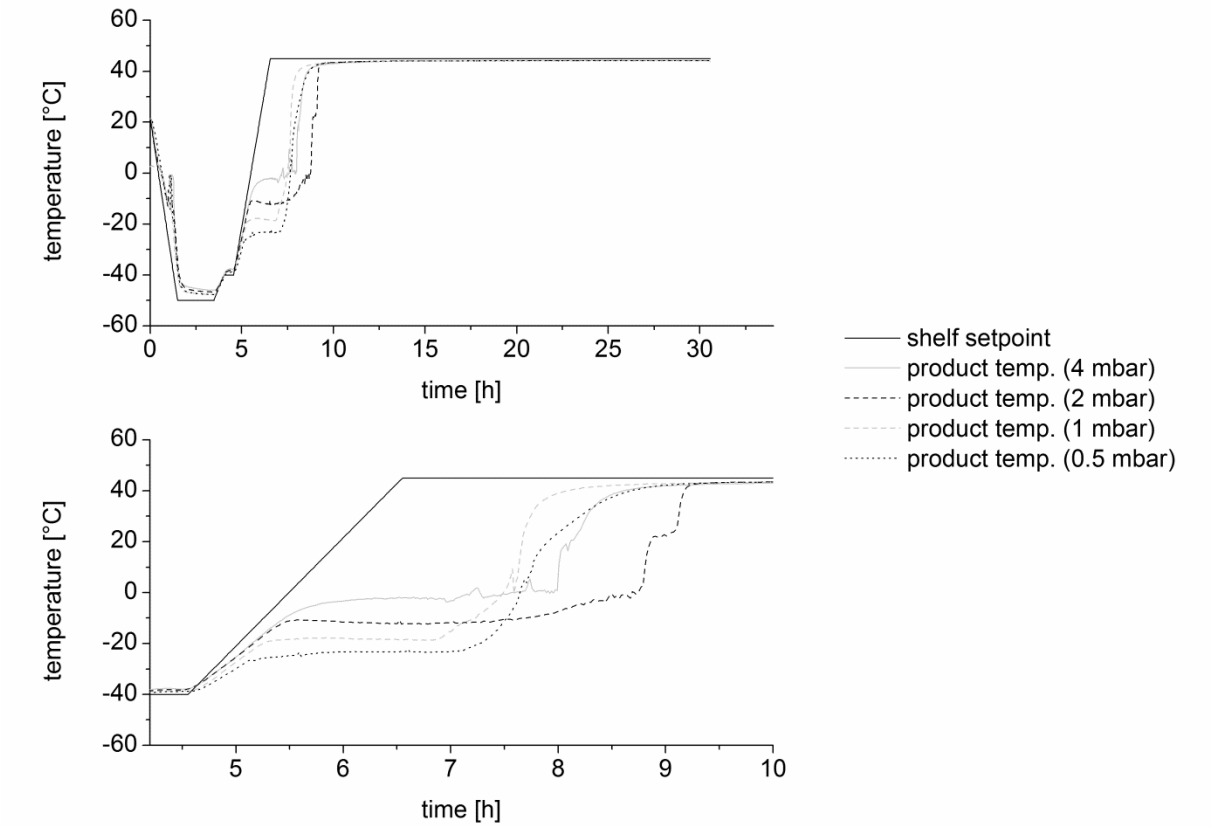

Figure 4.2: Product temperatures 5% (wt/wt) sucrose solution (in 10mM sodium succinate buffer pH 5.5; 2R glass vials, 1 ml fill) aggressive freeze-dried at different chamber pressures. Upper graph shows the complete process, lower graph shows enlarged detail of product temperatures during early part of aggressive freeze-drying

Most reasonable, the different product temperatures, which result from the different chamber pressures (cf. Table 4.1), directly influence the viscosity of the collapsing material. Higher chamber pressures result in higher product temperatures and therefore decreased viscosity. Using equation (1.7) (see page 20) and assuming the applicability also to viscous material around 30 K above the glass transition temperature, for a chamber pressure of 4 mbar – and therefore product temperature of -0.7 °C – the viscosity of the collapsing sucrose is roughly 3.7 million times lower than at the glass transition. Even for a product temperature of -24.3 °C (chamber pressure of 0.5 mbar) the viscosity is about 180 times lower than at the glass transition. As there is a high flow of water vapor from the product at lower viscosity, the chance for the product to be carried off to the inner vial wall or even through the stopper is increased compared to lower viscosities (i.e. lower product temperatures as a result of lower chamber pressures).

Residual moisture data (cf. Figure 4.3) show that for a chamber pressure of 0.5 mbar up to 2 mbar there is residual water content of about 2.2% and no significant difference in residual water content. On the contrary, a chamber pressure of 4 mbar resulted in higher residual water content of 3.3%. Obviously, the non-controlled spattering of the vials during drying hinders effective removal of residual water. Additionally, the standard deviation between three analyzed vials was consistently higher for samples that showed spattering of material (2 mbar and 4 mbar chamber pressure). This is taken as an

indication for inhomogeneous drying behavior of single vials within one batch for cycles that apply higher chamber pressures. Consequently, the application of chamber pressure of 0.5 mbar as well as 1 mbar resulted in lyophilizates that dry controllable (i.e. no spattering or other uncontrollable drying behavior) and homogeneously throughout the batch.

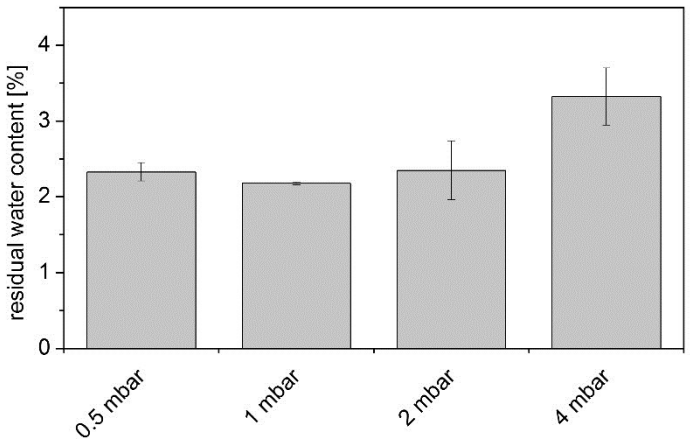

Figure 4.3: Residual water content of aggressively freeze-dried 5% (wt/wt) sucrose (in 10mM sodium succinate buffer pH 5.5; 2R glass vials, 1 ml fill; n=3).

From a more general perspective, also the residual water content of samples from cycles utilizing chamber pressures of 0.5 and 1 mbar are comparably high for a freeze-dried pharmaceutical formulation. Especially, if the high drying temperature of 45 °C and the comparably long time of about 18 hours at product temperatures close to 45 °C (cf. upper part of Figure 4.2) are taken into consideration. Although the target residual water content for pharmaceutical protein formulations has to be determined for every product separately, an often recommended aim is a residual water content of below 2% [6]. Due to controllable drying behavior and lowest residual moisture content, from this study a chamber pressure of 1 mbar (at 45 °C shelf temperature) seems to be most feasible for aggressive freeze-drying of 5% sugar solution.

2.1.2 TREHALOSE-BASED LYOPHILIZATES

The knowledge gained from the experimental series using 5% (wt/wt) sucrose solution was used as a starting point for a second study that was initiated to investigate the influence of chamber pressure, shelf temperature and process time on the physical characteristics of resulting lyophilizates. In order to maximize the knowledge on a design space for aggressive freeze-drying towards formulations containing a bulking agent, besides 5% (wt/wt) trehalose two formulations containing phenylalanine as bulking agent were included (cf. Table 4.2). Trehalose was chosen instead of sucrose to extend the study to the second of the mainly used amorphous stabilizers in freeze-dried pharmaceutical products [8].

Table 4.2: Formulations used to characterize the influence of process conditions on the resulting characteristics of aggressively freeze-dried model formulations

ID	trehalose	phenylalanine	potassium phosphates	mab_T1
	mg/ml	mg/ml	mg/ml	mg/ml
tre	50		2.4	1.0
tre:phe 10:0.5	47.6	2.4	2.4	1.0
tre:phe 10:3	38.5	11.5	2.4	1.0

Formulation tre:phe 10:0.5 exemplifies a formulation at 5% (wt/wt) solid content, containing a low amount of bulking agent, while formulation tre:phe 10:3 represents a formulation at a high content of bulking agent. Preliminary tests have shown that tre:phe 10:3 can be considered as similar to a formulation of trehalose and mannitol at a ratio of 1:3 regarding physico-chemical characteristics like residual moisture, glass transition temperature and specific surface area (compare section 3 of CHAPTER 5, page 113pp.). To concurrently investigate the influence of different cycle conditions on the stability of an incorporated protein, mab_T1 was added in a concentration of 1 mg/ml. Results referring to the stability of mab_T1 are shown in section 2.3 of this chapter.

For this study, samples were freeze-dried in a Christ 2-12D freeze-drier (at approx. 1/2 load) utilizing the different freeze-drying protocols shown in Table 4.3.

Table 4.3: Cycles used for aggressive freeze-drying of 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe 10:3

ID	chamber pressure	shelf temperature	drying time
	mbar	°C	hours
0.1_30_8	0.1	30	8
0.1_30_24	0.1	30	24
0.1_60_8	0.1	60	8
0.1_60_24	0.1	60	24
1_45_8	1	45	8
1_45_24	1	45	24
2_45_15	2	45	15
2_45_24	2	45	24
4_30_8	4	30	8
4_30_24	4	30	24
4_60_8	4	60	8
4_60_24	4	60	24

The range of chamber pressure was set from 0.1 mbar, representing a pressure frequently used for conventional freeze-drying, to 6 mbar representing the theoretical upper limit of sublimation (triple point of water). However, during preliminary tests a stepwise reduction of chamber pressure set point to 4 mbar was necessary, due to the fact that above 4 mbar the samples showed meltback right after start of the heating ramp indicating product temperatures exceeding the melting temperature of the formulation (around 0 °C).

Shelf temperature was varied from 30 °C, regarded as low shelf temperature for aggressive freeze-drying, up to 60 °C, representing the maximum shelf temperature the utilized freeze-drier is capable. As it was known that primary drying of 5% (wt/wt) trehalose solution is completed within maximum 6 hours (comparable to 5% (wt/wt) sucrose solution, data not shown) 8 hours were set as lower limit for drying time. The upper limit of drying time was set to 24 hours considering the idea that aggressive freeze-drying should represent a fast freeze-drying process compared to conventional freeze-drying. In order to reduce the number of necessary freeze-drying runs, samples of 8 and 24 hours drying time were produced within one freeze-drying cycle. To remove samples after 8 hours drying time, the freeze-drier was vented with nitr ogen, samples were removed and the freeze-drier was evacuated again to complete the 24 hours drying for the remaining samples. As primary drying was completed for all samples after 8 hours, indicated by product temperatures close to the shelf temperature, the final structures of the lyophilizates were already settled.

Product temperature

Table 4.4 shows the product temperatures of the three different formulations during primary drying. While product temperatures approach the shelf temperature after finishing sublimation, during sublimation (i.e. primary drying) of aggressive freeze-drying the product temperatures resides from 33.4 K up to 87.9 K below the shelf temperature (difference between Ts and Tp).

Table 4.4: Product temperatures during primary drying of 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe 10:3

ID	5% (wt/wt) trehalose	tre:phe 10:0.5	tre:phe 10:3
	°C	°C	°C
0.1_30	-28.8	-26.7	-27.7
0.1_60	-27.9	-26.8	-20.7
1_45	-17.7	-16.4	-12.3
2_45	-11.5	-8.4	-7.5
4_30	-3.7	-4.1	-3.4
4_60	-3.7	-2.5	-1.7

Regarding the influence of shelf temperature and chamber pressure on the product temperature, an interesting trend could be shown. Although there is a difference of 30 K (30 °C versus 60 °C) between the shelf temperatures applied in different cycles, neither for low chamber pressure (0.1 mbar) nor for high chamber pressure (4 mbar) trehalose as well as tre:phe 10:0.5 did show a considerable difference in resulting product temperatures. On the other hand, comparing product temperatures of samples from aggressive freeze-drying cycles applying identical shelf temperatures of e.g. 30 °C but different chamber pressures, it was shown that a low chamber pressure resulted in low product temperatures, while a high chamber pressure resulted in high product temperatures. For example at a chamber pressure of 0.1 mbar the product temperature of 5% (wt/wt) trehalose exhibits -28.8 °C while at the same shelf temperature but with 4 mbar chamber pressure the product temperature reaches -3.7 °C. The same clear trend holds true for both,

the low as well as high bulking agent containing formulation. However, while the low bulking agent containing formulation exhibits product temperatures very similar to the formulation containing no bulking agent, tre:phe 10:3 exhibits a slightly higher product temperature especially at 0.1 as well as 1 mbar chamber pressure. This particular formulation contains phenylalanine (=bulking agent) in an amount, preventing the macroscopic collapse of the lyophilizate (compare also Figure 4.4) as well as resulting in higher specific surface area (compare also Figure 4.5) and therefore indicating a higher resistance of the already dried layer against the passing water vapor (i.e. product resistance R_P; compare equation (1.3)). By a higher product resistance the local pressure at the sublimation front is increased and consequently the product temperature increased.

Generally, it can be stated that the product temperature during sublimation (i.e. primary drying) of aggressive freeze-drying is mainly determined by the applied chamber pressure as it is the case in conventional freeze-drying, too.

Macroscopic appearance

Having a look at the products from the different formulations and aggressive freeze-drying cycles it revealed quite different macrosopic appearance of the samples (cf. Figure 4.4). The appearance of the samples exhibits a broad range from no visuable sings of collapse to completely collapsed with spattering through the stopper. While 5% (wt/wt) trehalose and tre:phe 10:0.5 showed a very similar appearance for all applied cycles, the higher content of phenylalanine in formulation tre:phe 10:3 rendered this formulation more resistively against macroscopic collapse.

Figure 4.4: Macroscopic appearance of samples subjected to different aggressive freeze-drying protocols (24 hours drying time). A: 5 % (wt/wt) trehalose, B: tre:phe 10:0.5, C: tre:phe 10:3; I: 0.1 mbar_30 °C, II: 4 mbar_30 °C, III: 1 mbar_45 °C, IV: 2 mbar_45 °C, V: 0.1 mbar_60 °C, VI: 4 mbar_60 °C

As expected for the aggressive conditions of the applied freeze-drying cycles, the samples containing no or a low amount of bulking agent (tre, tre:phe 10:0.5) showed macroscopic detectable collapse for all of the applied shelf temperatures and chamber pressures. While for chamber pressures of 1 mbar and above, there could be observed fully collapsed appearance for 5% (wt/wt) trehalose (foam-like appearance) and tre:phe 10:0.5 (sponge-like appearance), a chamber pressure of 0.1 mbar resulted in samples that showed only slight shrinkage. At that chamber pressure, even the application of shelf temperature of 60 °C was not able to increase the product temperatures to an extent that would cause fully collapsed lyophilizates. To gain data about the critical product temperature of the utilized formulations, the glass transition temperature of the maximally freeze-concentrated solution (T_g') was determined by DSC analysis. Moreover, also the collapse temperatures of the formulations were determined using freeze-drying microscopy (FDM). Table 4.5 shows the corresponding results. Both 5% (wt/wt) trehalose and tre:phe 10:0.5 showed comparable glass transition and collapse

temperatures. Comparing product temperatures of both formulations T_g' and T_c^{micro} it becomes apparent that for a chamber pressure of 0.1 mbar the product temperatures are in the range of those critical product temperatures. Therefore, the viscosity of the samples is not low enough to cause full collapse, but some degree of micro-collapse resulting in apparent shrinkage. For increasing chamber pressures of 1 mbar and 2 mbar, also the product temperature increases to values considerably higher than T_g' and T_c^{macro}, consequently resulting in fully collapsed products. At a chamber pressure of 4 mbar all formulations showed macroscopic collapse, tre and tre:phe 10:05 even foamed out of the stopper as already observed for aggressive freeze-drying of 5% (wt/wt) sucrose at 4 mbar (see above). The higher content of phenylalanin in tre:phe 10:3 resulted in an appearance indicating collapse at a late stage of primary drying as a considerable piece of already dried cake was elevated by material collapsing at the bottom of the vial.

Table 4.5: Glass transition temperature of the maximally freeze-concentrated solution (T_g') as well as collapse temperatures (T_c^{micro}, T_c^{macro}) of 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe 10:3

Formulation	T_g'	T_c^{micro}	T_c^{macro}
	°C	°C	°C
5% (wt/wt) trehalose	-30.8	-30.5	-28.2
tre:phe 10:0.5	-30.5	-30.0	-28.5
tre:phe 10:3	-29.3	-20.3	-10.2

In contrast to the formulations containing no or a low amount of phenylalanine as bulking agent (tre, tre:phe 10:0.5), up to chamber pressures of 2 mbar, tre:phe 10:03 resulted in macroscopically elegant cakes independent from shelf temperature. Comparing again the critical temperatures Tg', T_c^{micro}, and T_c^{macro} with the product temperatures of tre:phe 10:3 at the different chamber pressure from the different cycles, it becomes apparent that up to a chamber pressure of 2 mbar the product temperature is below or in the vicinity of T_c^{macro}. Contrary to the formulations tre and tre:phe 10:0.5 the amount of phenylalanine contained in tre:phe 10:3 result in a remarkable difference of 10.1 K between T_c^{micro} and T_c^{macro} according to FDM. Moreover T_c^{micro} is 9 K higher than T_g' .This phenomenon can be attributed to the formation of a crystalline phenylalanine dominated matrix within the product, which is able to withstand higher temperatures and to which amorphous sugar is thought to collapse on (compare also [20]). So while the product temperature exceed T_g' and/or T_c^{micro}, T_c^{macro} is not exceeded to a degreee causing macroscopically detectable collapse.

Specific Surface Area (SSA)

To confirm the observation from assesment of macroscopic appearance of the aggressive freeze-dried samples, also the specific surface area (SSA) was evaluated. As shown by Schersch [18], the determination of the SSA is a sensitive and valuable tool to detect even slight changes in the structure of a product that is not discenarbly visible. Especially the occurance of micro-collapse in aggressive freeze-dried products can be determined by reduced SSA compared to products that are freeze-dried conventionally. SSA of freeze-dried samples was determined by BET gas adsorption (cf. Figure 4.5). For

comparison to non-collapsed products, also conventionally freeze-dried samples were analyzed (freeze-dried according to cycle 3, shown in Table 3.8 on page 57).

Comparing the SSA of sample from aggressive freeze-drying to conventionally freeze-dried ones (cf. Figure 4.5), the formulation with high bulking agent content (tre:phe 10:3) showed a significant reduction of SSA from 3.3 m²/g (conventional) to at most 2.2 m²/g (aggressive, 0.1 mbar, 30°C). Increasing chamber pressure – and therefore increasing product temperature – resulted in decreasing SSA down to 1.1 m²/g (2 mbar, 45°C), although the macroscopic appearance showed no visible signs of collapse. These data confirm the assumption that micro-collapse occurred during aggressive freeze-drying. Micro-collapse was caused by product temperatures above T_g' and T_c^{micro}. For samples of tre:phe 10:3 that showed macroscopic collapse, the SSA is further reduced to around 0.4 m²/g.

Regarding SSA, formulations containing no or a low amount of phenylalanine are comparable. Obviously, the low amount of bulking agent does not influence the drying behavior regarding SSA. Comparing the slightly shrunken samples aggressively freeze-dried at 0.1 mbar with conventionally freeze-dried samples, a reduction from 2.1 m²/g to about 1.0 m²/g could be noticed, indicating micro-collapse of that products. Fully collapsed samples of tre and tre:phe 10:5 exhibits SSA of 0.2 m²/g and below.

According to macroscopic appearance and specific surface area data, no change in structure of product occurred during the period between 8 hours and 24 hours drying time (data not shown). Therefore, the additional drying time can be regarded as dominated by secondary drying mechanisms.

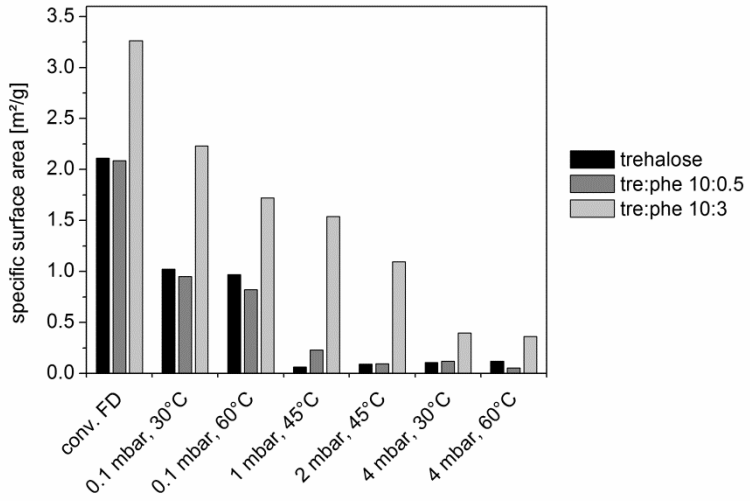

Figure 4.5: Specific surface area of samples subjected to different aggressive freeze-drying protocols (24 hours drying time).

Reconstitution time

Since the highly porous structure of freeze-dried products generally allows for a fast rehydration of such products, a reduced porosity is also considered to worsen the rehydration behavior of collapsed products [21, 22]. The products from aggressive freeze-drying of the three different formulations were rehydrated with water and the time until complete dissolution was determined (cf. Figure 4.6). Despite collapsed structure of the samples freeze-dried at higher chamber pressures, all samples were completely rehydrated within below 50 seconds, independently from formulation composition or process parameters applied. Having a closer look at the samples that showed extreme collapse, including spattering out of the vials (i.e. freeze-dried at 4 mbar), the reconstitution time of both formulations containing phenylalanine seems to be slightly prolonged compared to the samples showing no gross collapse (i.e. freeze-dried at 0.1 mbar). However, the formulation containing no phenylalanine (5% (wt/wt) trehalose) did also show full collapse with spattering out of the vial, if subjected to aggressive freeze-drying at high chamber pressures, but rehydrates within 14 seconds. Based on the specific surface area as quantitative method for the degree of collapse, no correlation between degree of collapse and reconstitution time could be established. Furthermore, no influence of shelf temperatures on the reconstitution time could be detected. In addition, the reconstitution times after 8 hours drying time are comparable to those determined after 24 hours drying time. The only parameter that has a slight influence on the reconstitution time is the applied chamber pressure during aggressive freeze-drying. However, the small increase in reconstitution time is potentially a side effect of the collapse behavior of those samples, which is partly accompanied by spattering of the material within the complete interior surface of the vial as well as out of the vial.

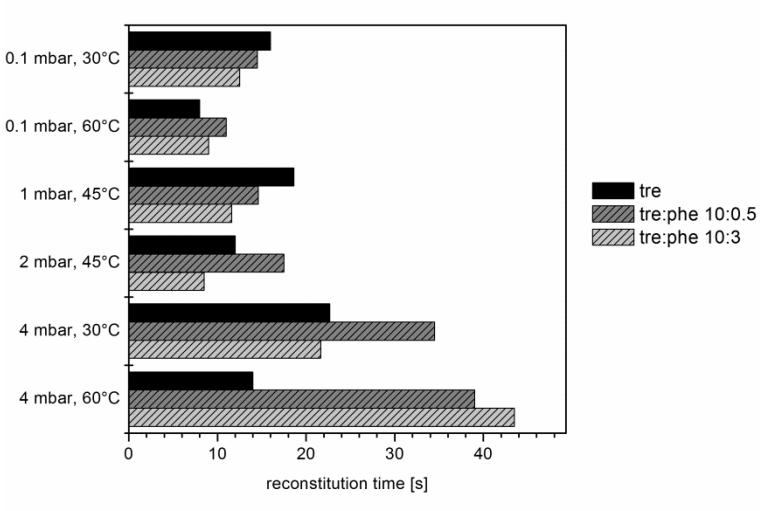

Figure 4.6: Reconstitution time of aggressive freeze-dried samples (24 hours drying time)

Residual water content

The residual water content of the lyophilizates is summarized in Figure 4.7. Samples aggressively freeze-dried from 5% (wt/wt) trehalose at a (low) chamber pressure of

0.1 mbar exhibited a residual water content of 1.6% (Ts=30 °C) or 0.7% (Ts=60 °C), respectively, after only 8 hours drying. After 24 hours drying, these sample species ended up at 0.3% (Ts = 60 °C) or 0.6% (Ts = 30 °C) water content. As outlined above, these samples exhibited only micro-collapse as they were aggressively freeze-dried at 0.1 mbar. On the other hand, high chamber pressure of 4 mbar resulted in higher residual water content. As expected, higher shelf temperature (60 °C, 2.1%) resulted in lower water content compared to lower shelf temperature (30 °C, 7.3%). Even after 24 h drying, the fully collapsed samples exhibit residual water contents of up to 4.1% (4 mbar, 30 °C). It was shown above that drying at high chamber pressures results in full collapse and drastically reduced SSA. Nevertheless, by applying a shelf temperature of 45 °C also the collapsed samples could be dried to below 2% within 24 h. By a shelf temperature of 60 °C, the collapsed trehalose samples even achieved 1.1% water content. Summarizing the findings for trehalose, a residual water content of well below 2% can be achieved within 24 h by high shelf temperature (e.g. 60 °C). If a low chamber pressure (e.g. 0.1 mbar) is utilized, after 8 h drying a water content of below 2% can be reached, even with comparably low shelf temperature of 30 °C.

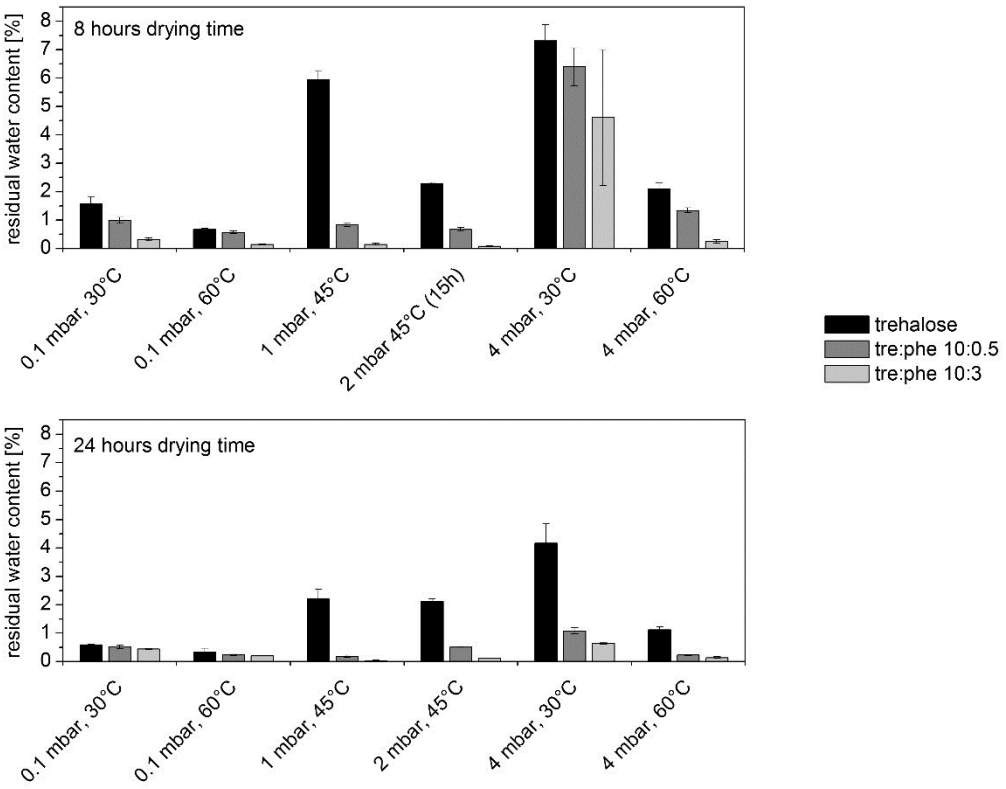

Figure 4.7: Residual water content of aggressive freeze-dried 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe 3 formulation. Upper graph after 8 hours drying time, lower graph after 24 hours drying time (n=3).

As a second model, a formulation containing a low amount of bulking agent (tre:phe 10:0.5) was investigated (results cf. Figure 4.7). Using chamber pressures up to 2 mbar, already after 8 h drying time, low residual water contents of below 1% were achieved. For a chamber pressure of 4 mbar, the residual moisture was highly dependent on the shelf temperature. While a shelf temperature of 60 °C resulted in 1.3% residual

water, for Ts = 30 °C 6.4% residual water was determined.
After a drying time of 24 h, overall low residual water contents of 0.5% and below were determined for almost all tre:phe 10:0.5 products. Only the samples freeze-dried at 4 mbar, exhibited 1.1% residual water. Obviously, a shelf temperature of 30 °C was too low to reach lower residual water content in that fully collapsed material. Like already observed for the trehalose samples, higher shelf temperature result in lower residual water content.
Comparing the water content of tre:phe 10:0.5 and trehalose samples, the samples with a low amount of bulking agent exhibited drastically reduced water content regardless of the the collapsed structure. If shelf temperatures of 45 °C and higher were applied, the lyophilizates could be dried to below 1.3% within 8 h. As will be shown below (cf. page 85pp.), this improvement of drying behavior was achieved without the formation of of crystalline (non-collapsed) cake strucutre. The collapsed tre:phe 10:0.5 lyophilizates remained fully amorphous.

Finally, also a formulation containing a high amount of bulking agent (i.e. phenylalanine, tre:phe 10:3) was studied. The same trends as for the formulation containing a low amount of phenylalanine could be noticed (cf. Figure 4.7): up to a chamber pressure of 2 mbar, very low residual water contents of 0.3% and below were achieved already after 8 h drying. While for a chamber pressure of 4 mbar and Ts = 60 °C a low residual water content of 0.3% was determined, too, pc = 4 mbar and a shelf temperature of 30 °C resulted in 4.6% residual water.
After 24 h drying, all products exhibit residual water contents of 0.6% and below. Interestingly, the collapsed samples from aggressive freeze-drying at 4 mbar and 60 °C exhibits slightly lower residual water content (0.1%) compared to the macroscopic elegant samples aggressively freeze-dried at 0.1 mbar and 60 °C (0.2%). A possible explanation for this difference could be a small difference in product temperature during secondary drying. Chamber pressure plays an important role for the heat transport into the product via convection, while higher chamber pressures provide more convective heat transport. For a chamber pressure of 4 mbar an average product temperature of 56.3 °C was recorded during drying between 8 and 24 hours, while for a chamber pressure of 0.1 mbar a product temperature of 51.4 °C was recorded.

Summing up the findings on the residual water content, for all three types of formulation at a certain chamber pressure (0.1 or 4 mbar) a significantly lower residual water content is achieved for higher shelf temperatures. In addition, a considerable influence of the SSA on the residual water content was detected, which is already known for conventionally freeze-dried lyophilizates [23]. While products exhibiting a higher specific surface area can be dried fast to low residual moisture (i.e. within a drying time of 8 hours), collapsed samples, naturally exhibiting a lower SSA, needed more time to reach low residual water contents. If aggressively freeze-dried under conditions that causes collapse (i.e. chamber pressure at or above 1 mbar), the formulation containing no bulking agent (5% (wt/wt) trehalose) could be dried only to 1.1%. As the level of chamber pressure plays an important role for the formation of the structure of the lyophilizate during aggressive freeze-drying, besides the shelf temperature and the drying time in contrast to conventional freeze-drying, also chamber pressure plays a major role for the level of residual water content in the final product.

Glass transition temperature

All aggressively freeze-dried samples revealed a detectable glass transition, characterized by a shift of the baseline in the heat signal obtained by differential scanning calorimetry (DSC, example curves of samples aggressively freeze-dried at 2 mbar and 45 °C shown in Figure 4.8). Therefore, it can be concluded that amorphous material is present in the dried products. The glass transition temperatures are shown in Figure 4.9.

The use of a (low) chamber pressure of 0.1 mbar resulted in high glass transition temperatures of samples from all formulations (no / low / high bulking agent content). After only 8 h drying at Ts=30 °C, Tgs of 84.4 °C (5% (wt/wt) trehalose) up to 100.2 °C (tre:phe 10:3) were achieved. Applying a shelf temperature of 60 °C, Tgs of 93.8 °C to 106.1 °C were reached, respectively. Using higher chamber pressures (≥ 1 mbar), samples of 5% (wt/wt) trehalose showed comparably low glass transition temperatures of for example 31.2 °C for 4 mbar and 30 °C. Also Tgs of samples that contain bulking agent were slightly affected by a chamber pressure of 4 mbar, but exhibited high values for 1 mbar and 2 mbar. As the residual water content of all samples is lower after a drying time of 24 hours, the corresponding glass transition temperatures are consequently higher.

However, although the glass transition temperatures of the collapsed trehalose samples are significantly lower compared to the formulations containing a low or high amount of bulking agent, after 24 hours drying time they show values higher than 50 °C. At the same time, formulations containing bulking agent (phenylalanine) exhibit considerably high glass transition temperatures already at a low concentration and already after a drying time of 8 hours.

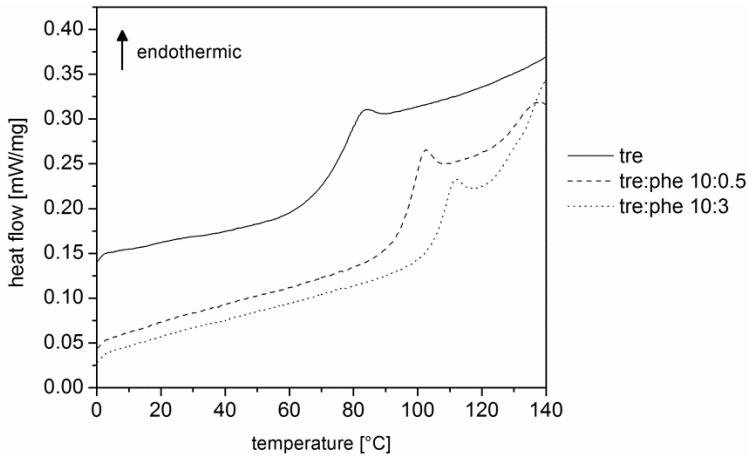

Figure 4.8: DSC example curves of aggressive freeze-dried 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe3 formulation (process parameters: 2 mbar chamber pressure, 45 °C shelf temperature, 24 hours drying time).

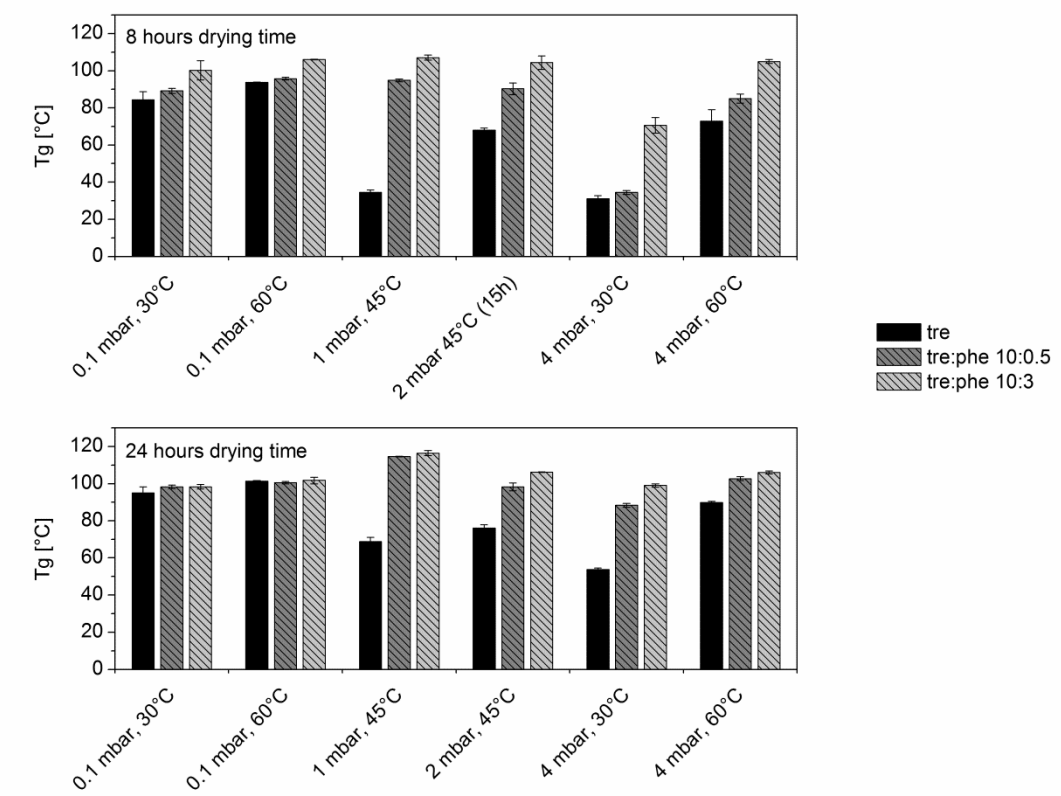

Figure 4.9: Glass transition temperatures of aggressive freeze-dried 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe3 formulation. Upper graph after 8 hours drying time, lower graph after 24 hours drying time (n=3).

In general, increased storage stability is attributed to higher glass transition temperature [24-26]. Since the Kauzmann temperature T_K (theoretical intersection of enthalpy of a supercooled liquid and the crystalline state (for detailed description see e.g. [27]), lies about 50 K below Tg, it is suggested by some authors to aim for a glass transition temperature of 50 K above the planned storage temperature [5, 28, 29]. Usually, a difference of 30 K between the intended storage temperature and the glass transition temperature is regarded as sufficient to achieve stability during storage. Since (aggressive) freeze-drying aims for products that are storable at ambient conditions (25 °C), a glass transition of at least 55 °C needs to be achieved.

As water acts as plasticizer of an amorphous matrix [30], the glass transition temperature is inversely proportional to residual water content. Consequently, aggressively freeze-dried samples of higher water content exhibit glass transition at lower temperature. Figure 4.10 shows the correlation of glass transition temperature and residual water content of the aggressively freeze-dried samples. A linear regression of data from all formulations revealed a slope of -11.0 K $\%^{-1}$ (R^2=0.92). These data imply that with a decrease of 1% in water content an increase of about 11 K in the glass transition temperature is to be expected. Interpolation according to the regression revealed that for achieving a Tg of 55 °C a water content of 4.4% is necessary. To achieve a Tg of 75 °C a water content of about 2.6% needs to be reached.

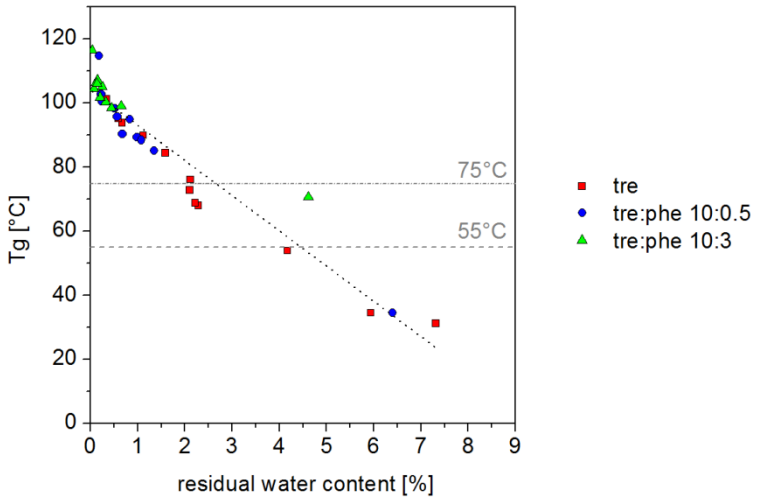

Figure 4.10: Comparison of glass transition temperatures and residual water content of aggressively freeze-dried 5% (wt/wt) trehalose, tre:phe 10:0.5, and tre:phe 10:3 formulation. Data of 8 h and 24 h drying are included. The dotted line represents a linear regression of Tg vs. water content of sample from all three formulations.

Table 4.6 gives an overview of process conditions that are able to produce lyophilizates, potentially offering storage stability at room temperature (i.e. 25 °C, Tg ≥ 55 °C or even Tg ≥ 75 °C).

Besides aggressive freeze-drying at 4 mbar chamber pressure and 30 °C shelf temperature, all investigated process conditions were able to produce lyophilizates of Tg ≥ 55 °C within 8 hours drying time – independent from the amount of added bulking agent (no, low, high). Most of the samples even reached glass transition temperatures of ≥ 75 °C, which is most remarkably for collapsed tre:phe 10:0.5 samples. Despite the collapsed structure, these lyophilizates could be dried within 8 hours drying time to Tgs of ≥ 75 °C if shelf temperatures ≥ 45 °C were used.

Table 4.6: Overview of process conditions of aggressive freeze-drying that are able to produce samples with a glass transition temperatures of ≥ 55 °C or ≥ 75 °C (pc = chamber pressure, Ts = shelf temperature; collapsed samples are marked by an asterisk)

ID	pc	Ts	trehalose				tre:phe 10:0.5				tre:phe 10:3			
	mbar	°C	8 h drying		24 h drying		8 h drying		24 h drying		8 h drying		24 h drying	
			Tg ≥55 °C	Tg ≥75 °C	Tg ≥55 °C	Tg ≥75 °C	Tg ≥55 °C	Tg ≥75 °C	Tg ≥55 °C	Tg ≥75 °C	Tg ≥55 °C	Tg ≥75 °C	Tg ≥55 °C	Tg ≥75 °C
0.1_30	0.1	30	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
0.1_60	0.1	60	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
1_45	1	45	**no***	**no***	yes*	**no***	yes*	yes*	yes*	yes*	yes	yes	yes	yes
2_45	2	45	yes*	**no***	yes*	yes*	yes*	yes*	yes*	yes*	yes	yes	yes	yes
4_30	4	30	**no***	**no***	**no***	**no***	**no***	**no***	yes*	yes*	yes*	**no***	yes*	yes*
4_60	4	60	yes*	**no***	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes*

*collapsed samples

Crystallinity

As already outlined above, DSC data showed that all aggressively freeze-dried samples indicated substantial amounts of amorphous material, most probably dominated by amorphous trehalose. To have a closer look on the molecular structure, X-Ray powder diffraction (XRD) was used to characterize the influence of chamber pressure, shelf temperature, and drying time on the dried products. Especially potential crystallization of trehalose or phenylalanine during aggressive freeze-drying was monitored, as the crystallization of excipients in freeze-dried product is considered as potentially harmful for the incorporated molecules of interest [31, 32]. Distinct peaks in the diffractograms from XRD analysis were regarded as attributes of a crystalline structure. Figure 4.11 shows diffractograms of freeze-dried samples of the three formulations according to the different aggressive freeze-drying protocols (drying time of 24 hours). In addition, samples after 8 hours drying were analyzed as well. Since they exhibit comparable XRD patterns to samples after 24 hours drying time, those data are not shown. All lyophilizates are regarded as fully amorphous as no peaks were detectable in samples of 5% (wt/wt) trehalose. Obviously, no crystallization occurred during collapsing of the samples, although the mobility is high according to low viscosity. Having a closer look to the formulation containing a high amount of phenylalanine (tre:phe 10:3), several distinct peaks are discernible for all applied process conditions.

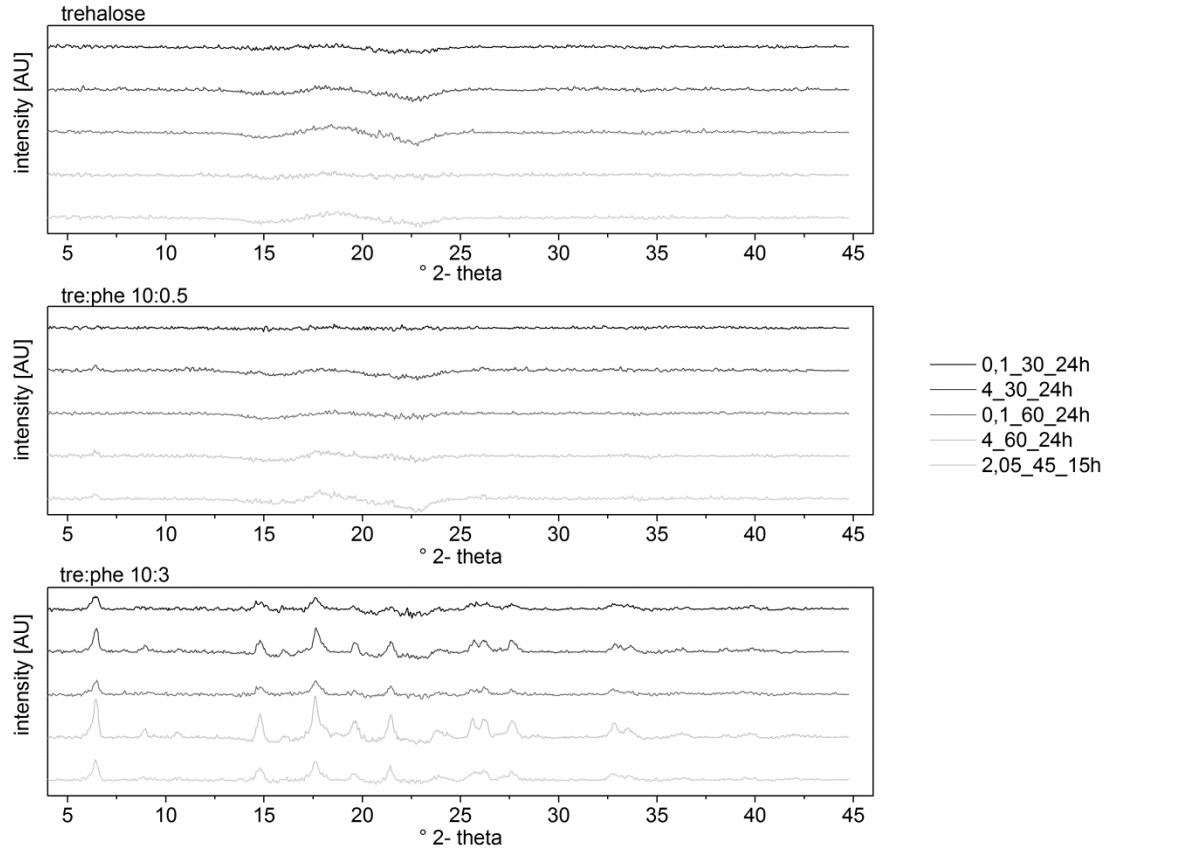

Figure 4.11: X-Ray Powder diffraction patterns of samples subjected to aggressive freeze-drying at different conditions. Top: 5% (wt/wt) trehalose, middle: tre:phe 10:0.5, bottom: tre:phe 10:3.

According to the International Centre for Diffraction Data (ICDD) database, the detected characteristic peaks at 6.4°, 14.7°, 17.4°, and 21.3° 2-theta can be attributed to crystalline L-phenylalanine hydrate. Although XRD was used as qualitative method, slight differences can be detected comparing the diffractograms of products from different aggressive freeze-drying cycles, especially for samples freeze-dried from formulations containing a high amount of phenylalanine. At low chamber pressure of 0.1 mbar, the intensity of all characteristic peaks is less pronounced compared to samples from cycles applying a high chamber pressure of 4 mbar. An intermediate chamber pressure of 2 mbar seem to result in intensities comparable to 4 mbar chamber pressure. Taking the macroscopic appearance of the product from high chamber pressure into consideration, there seems to be a connection between complete collapse and a higher degree of crystallization. Most probably, during aggressive freeze-drying at high chamber pressure, there is sufficient mobility in the system allowing molecular rearrangement resulting in crystallization of phenylalanine hydrate. This trend seems to be also valid for the formulation containing a low amount of phenylalanine (tre:phe 10:0.5), as only the samples subjected to higher chamber pressure (and which were therefore collapsed) showed a small peak at 6.4 °2-theta indicating a minute amount of crystalline phenylalanine in the respective products. Comparing the intensity of the peak at 6.4 °2-theta to the specific heat of an endothermic event detected by DSC around 140 °C (cf. right graph of Figure 4.12), this endothermic event can be probably assigned to the melt of phenylalanine hydrate.

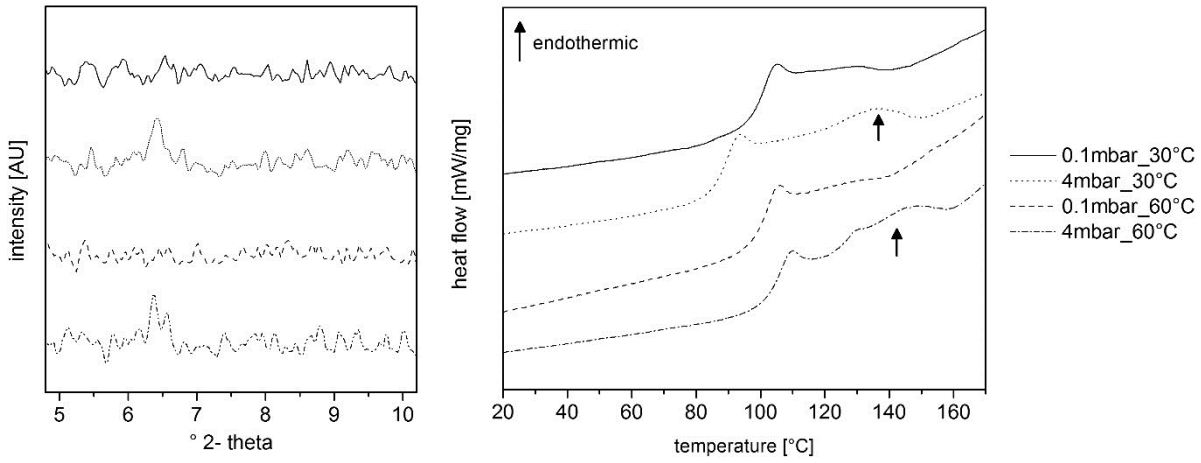

Figure 4.12: Enlarged XRD patterns (left) and DSC curves of the respective samples (right) of samples of aggressively freeze-dried tre:phe 10:0.5 formulation using different drying protocols (arrows highlight an endothermic event assigned to phenylalanine hydrate melting).

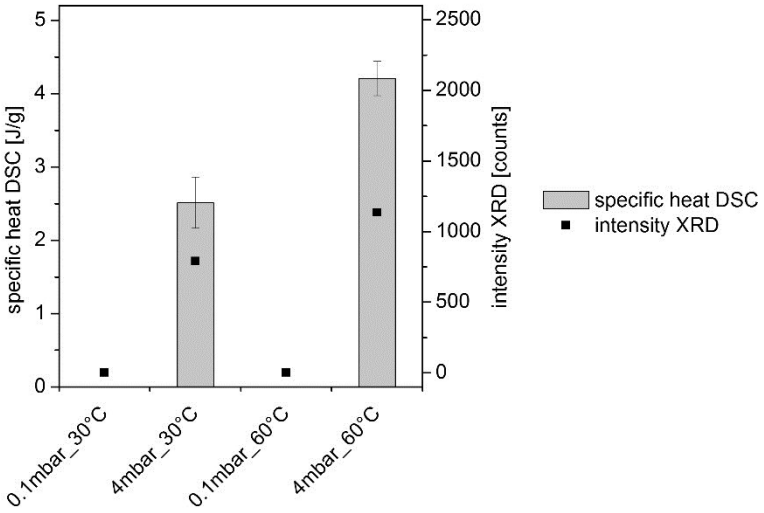

Figure 4.13: Comparison of specific heat of phenylalanine hydrate melting (n=3) and intensity of the XRD intensity at 6.4 ° 2-theta for the formulation containing low amount of phenylalanine (tre:phe 10:0.5).

Confirming the results of the XRD measurements, no endothermic event could be observed from the DSC curves of samples aggressively freeze-dried at low chamber pressure. These products are regarded as fully amorphous. Relating the intensity of the XRD peak at 6.4 °2-theta to the amount of specific heat of melting of phenylalanine hydrate determined by DSC (cf. Figure 4.13), it was noticed that the occurrence of a detectable XRD peak is accompanied by a detectable DSC event. Comparing the samples freeze-dried at high chamber pressure, for a shelf temperature of 60 °C, slightly higher peak intensity (XRD) as well as a slightly higher specific heat was detected compared to freeze-dried at 30 °C shelf temperature, suggesting a somewhat higher amount of phenylalanine hydrate present in the sample. However, the correlation of peak intensity from XRD measurement and the amount of specific heat from DSC analysis seems not to be feasible in every case, as the melting endotherm of the formulation containing a high amount of phenylalanine did not show a higher specific heat, although the intensity of the XRD peak at 6.4 ° 2-theta is increased more than 4-fold (data not shown). As the amount of specific heat for a potential melting of phenylalanine hydrate is small and therefore susceptible to integration error, the presence or absence of characteristic peaks in a XRD diffractogram are considered as more representative for the presence or absence of crystalline material in a sample than a DSC scan.

2.1.3 Conclusions

The influence of process parameters on the physico-chemical characteristics of aggressively freeze-dried products were outlined in the section above. Aggressive freeze-drying aims to generate dry lyophilizates, which exhibit product characteristics allowing storage at room temperature. For most of the molecules to be stabilized in a lyophilizate a (partly) amorphous matrix is inevitable for stabilization. Therefore, glass transition temperatures play a major role. Moreover, a low specific surface area improves the storage stability of e.g. embedded proteins, as recently shown by some authors [10, 33, 34].

Trehalose-based formulations (5% wt/wt) that contain no bulking agent exhibited a collapsed appearance with low specific surface area upon freeze-drying at a chamber pressure of ≥ 1 mbar. Due to the low specific surface area, the collapsed samples could

not be dried to below 2% residual water content within 8 h drying time, not even by applying a high shelf temperature of 60 °C. After 24 h at 60 °C, the collapsed trehalose lyophilizates reached a water content of below 2% (namely 1.1%). A shelf temperature of 30 °C or 45 °C was not sufficient to decrease the water content below 2% within 24 hours. On the other hand, also the lyophilizates exhibiting a water content slightly above 2% showed high glass transition temperatures. Based on an intended storage temperature of 25 °C, a glass transition temperature of 55 °C is considered as benchmark to be met. Collapsed trehalose lyophilizates can be dried to a Tg > 55 °C within either 8 h drying at 60 °C shelf temperature, or 15 h at 45 °C. Drying at 30 °C shelf temperature did not result in sufficiently high glass transition. Overall, the samples produced at 4 mbar chamber pressure showed promising physico-chemical characteristics. However, collapsing material spattered out of the vials during drying. Spattering is regarded as uncontrollable drying behavior. Hence, aggressive freeze-drying of disaccharide formulations, at least for the applied solid content (5% wt/wt), is not feasible at a chamber pressure of 4 mbar.

Although shelf temperatures of 30 °C or 60 °C were utilized, aggressive freeze-drying of trehalose at a chamber pressure of 0.1 mbar did not result in collapse, but slightly shrinked appearance. The non-collapsed microstructure is also reflected in comparably high specific surface area of these samples. These lyophilizates exhibited low water content and high glass transition after 8 h drying at 60 °C and 30 °C, which is attributed to effective desorption of water from the large surface area during the secondary drying phase.

Generally, high Tg at rather high water content can be attributed to the inherently high glass transition temperature of trehalose. For formulations based on other disaccharides (e.g. sucrose) of inherently lower Tg than trehalose, low residual water content is a necessary prerequisite to achieve high glass transition temperatures. To realize storable collapsed lyophilizates of disaccharide formulations by aggressive freeze-drying, medium chamber pressure (1-2 mbar), high shelf temperatures (≥ 45 °C) and drying times of ≥ 24 h are recommended. If one aims for production of non-collapsed lyophilizates within 8 h drying time, the use of low chamber pressure (≤ 0.1 mbar) and high shelf temperature (e.g. 60 °C) is advised.

From a general perspective, the amorphous state is preferable for successful stabilization of e.g. therapeutic proteins. As shown above, collapsed sugar lyophilizates retain their amorphous structure independent from process conditions. However, these collapsed samples are difficult to dry to low residual water content within the intended short drying times of aggressive freeze-drying. For improvement of drying, still maintaining the fully amorphous structure, the addition of a small quantity of phenylalanine (i.e. a bulking agent) was evaluated (tre:phe 10: 0.5). XRD analysis of aggressively freeze-dried lyophilizates revealed that the use of a higher chamber pressure of ≥ 2 mbar results in small crystalline fractions in the lyophilizates, which were not observed in samples produced at lower chamber pressure (≤ 1 mbar). Consequently, for producing fully amorphous lyophilizates of the sugar:phenylylanine formulation, a chamber pressure of ≤ 1 mbar is recommendable.

Although phenylalanine was added, aggressive freeze-drying resulted in similar appearance and specific surface area for the different freeze-drying conditions as for pure

trehalose lyophilizates. The samples dried at low chamber pressure exhibited a non-collapse structure. These samples were dried to ≤ 1% water content and a Tg of ≥ 85 °C within 8 h drying time at shelf temperatures of ≥ 30 °C. On the other hand, the use of a chamber pressure of 1 mbar resulted in collapsed lyophilizates. In contrast to the trehalose samples, those collapsed lyophilizates reached a water content of ≤ 1% within 8 h drying time (Ts=45 °C). Hence, by the addition of a small quantity of phenylalanine to the sugar-based formulation **collapsed, dry, and fully amorphous lyophilizates** could be achieved within 8 h drying time.

A common strategy in formulation design is the addition of substantial amounts of bulking agents to freeze-drying formulations. Bulking agents are intended to crystallize during the freezing step. Hence, at least partially crystalline lyophilizates result from freeze-drying of formulations containing a bulking agent. In this study, trehalose was combined with phenylalanine at a ratio of 10:3 (overall 5% solid content) to generate a partly crystalline system.

XRD analysis of the aggressively freeze-dried samples showed the presence of crystalline L-phenylalanine hydrate independent from process parameters used. The presence of crystalline material is reflected in the appearance of the lyophilizates after drying. For chamber pressures of 0.1 mbar to 2 mbar, the aggressively freeze-dried samples exhibited a non-collapsed appearance and also large specific surface areas. The use of 4 mbar chamber pressure resulted in sort of collapse (elevation of the already dried cake by collapsing material at the bottom of the cake). But also for these samples comparably large SSA was detected.

Because of the large SSA, the partly crystalline lyophilizates were dried rapidly. Already after 8 h drying time the lyophilizates exhibited residual water contents of <0.5%. Only the lyophilizates dried at 4 mbar and 30 °C could not be dried to such low water content within 8 h (4.6%). As indicated by DSC, a substantial amount of amorphous material (mainly trehalose) is present in all samples. Due to the low water content of the samples, the glass transition temperatures of all lyophilizates (besides 4 mbar, 30 °C) ranged above 95 °C.

Overall, partly crystalline formulations can be aggressively freeze-dried to low water content and high Tg by using chamber pressures of 0.1 – 2 mbar and shelf temperatures of 30-60 °C within 8 h drying time.

2.2 INFLUENCE OF PROCESS VARIATIONS ON DRYING KINETICS

To gain more information about the kinetics of water loss during aggressive freeze-drying, a number of experiments were conducted utilizing a sample thief to extract samples from a running freeze-drying run. In a first experimental series at several time points, pre-weighted samples were extracted and the loss of mass (i.e. water by sublimation) and/or residual water content was determined. As during primary drying phase weight loss over time showed a sigmoidal shape, data were fitted using a Boltzman function[1] and the first derivative was calculated representing the sublimation rate (in g/h).

[1] Boltzman function: $y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2$

In order to compare the kinetics of primary (i.e. sublimative) drying of aggressive to conventional freeze-drying, two cycles were operated employing 5% sucrose (in 10 mM sodium succinate buffer pH 5.5; filling volume 1 ml, glass type 1 DIN 2R vials). Aggressive freeze-drying was operated at 1 mbar chamber pressure and 45 °C shelf temperature for 24 h of drying time (compare cycle 1_45 from Table 4.3) resulting in fully collapsed products. For conventional freeze-drying of the same samples 0.09 mbar chamber pressure and -20 °C shelf temperature were applied for primary drying for 30 h. An additional secondary drying step at 5 °C shelf temperature and 0.09 mbar (7 h) was included. Conventional freeze-drying resulted in elegant lyophilizates. Contrary to the water loss profile of aggressive freeze-drying, water loss of conventional freeze-drying followed a less pronounced sigmoidal profile, which was fitted by a sigmoidal logistic function (type 3)[2]. Sublimation rate was calculated by the first derivative of the fitted data and expressed in g/h. Comparing now weight loss profiles and sublimation rates of aggressive vs conventional freeze-drying (cf. Figure 4.14), it becomes obvious that aggressive freeze-drying showed a dramatically faster weight loss compared to conventional freeze-drying. While for aggressive freeze-drying sublimation is completed within 4 h of drying time, indicated by weight loss reaching a plateau level, for conventional freeze-drying 30 h of drying time were needed to complete sublimation. Expressed in sublimation rates (cf. Figure 4.14), during aggressive freeze-drying water loss by sublimation reached a maximum of 0.67 g/h, against a maximum of 0.047 g/h for conventional freeze-drying.

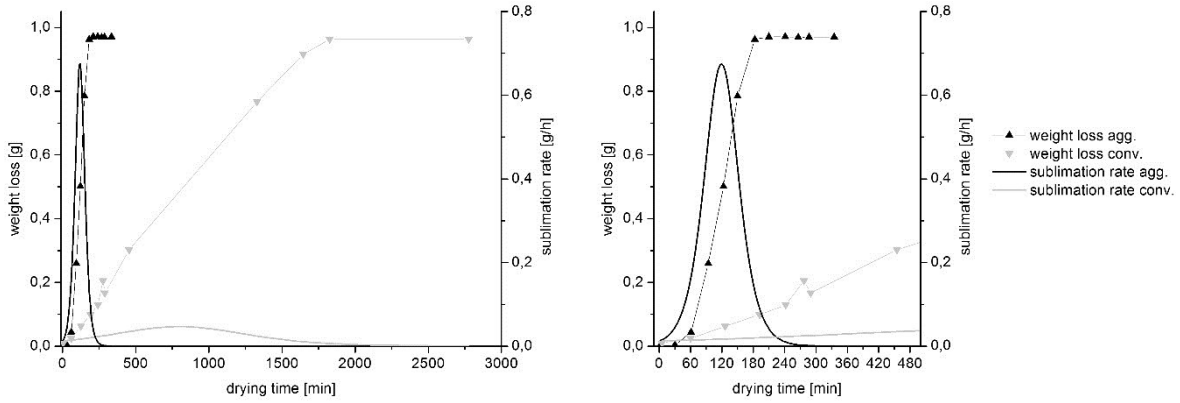

Figure 4.14: Weight loss data as well as calculated sublimation rates of either aggressively or conventionally freeze-dried 50mg/ml sucrose solution (left: overview, right: first 6 h of drying phase enlarged).

To confirm the weight loss data and sublimation rates of the samples extracted by a sample thief, both cycles were additionally featured by a freeze-balance (Martin Christ, Osterode am Harz, Germany). Basically, the freeze-drying balance showed comparable but slightly faster weight loss profiles (data not shown). Due to construction of the balance, only one vial without further shielding against heat input by radiation was weighted. Thus, the sublimation behavior (i.e. weight loss) of the vials extracted by the sample thief from a shelf completely covered with filled vials are regarded as more representative for all vials.

[2] Slogistic3 function: $y = \frac{a}{1+be^{-kx}}$

As mentioned above, during secondary phase of both drying cycles further samples were extracted using a sample thief and the residual water content was determined. Figure 4.15 shows the respective data. Secondary drying is governed by desorptive drying of the already settled material structure. The sample extracted from the aggressive freeze-drying cycle showed sort of an asymptotic decrease of residual water content over drying time. While within about 7 h the sample could be dried from 7.0% to 2.0% residual water content, further 16 h resulted in a decrease of just 0.5% to a final residual water content of 1.5% – although the product temperature was comparably high (above 40 °C). As besides the applied temperature, the specific surface area available for desorption plays a major role for secondary drying [35], the secondary drying behavior of the fully collapsed samples from aggressive freeze-drying can be attributed to their low specific surface area of below 0.1 m²/g. On the other hand, conventional freeze-drying at 5 °C shelf temperature resulted in a decrease from 8.9% to 2.2% residual water content within 15.8 h. Although there are not enough data available to conclude a profound drying kinetic curve for conventionally freeze-drying, the secondary drying kinetic of conventional freeze-drying is known to follow an asymptotic behavior as well [35]. An overlay of water loss of aggressively and conventionally freeze-dried samples (cf. right graph in Figure 4.15) suggest that secondary drying of both species follows a similar profile. However, it needs to be mentioned that aggressive freeze-drying utilized 45 °C shelf temperature, while SD of conventionally freeze-drying was accomplished at 5 °C.

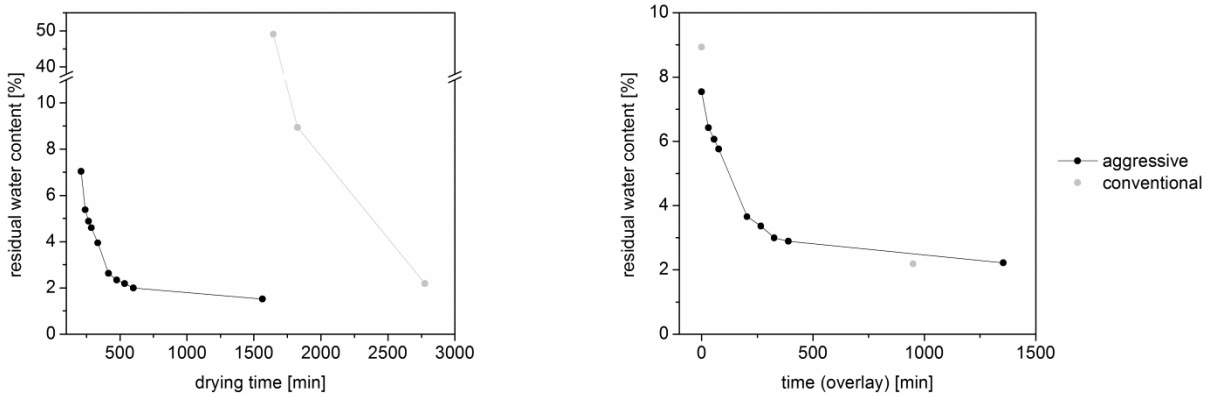

Figure 4.15: Secondary drying kinetic expressed as residual water content over time of either aggressively or conventionally freeze-dried 5% sucrose (left: residual water content over drying time; right: overlay of residual water content over time).

In order to evaluate the influence of process parameters on the drying kinetics for further characteristic model formulations, the formulations according to Table 4.2 were used, representing a formulation containing no bulking agent (5% (wt/wt) trehalose), a low amount of bulking agent (tre:phe 10:0.5), as well as a high amount of bulking agent (tre:phe 10:3).

To get an idea, whether the addition of bulking agent changes the drying kinetics dramatically, in a first step the three model formulations were subjected to aggressive freeze-drying at 1 mbar chamber pressure and 45 °C shelf temperature for 24 h of drying time (compare cycle 1_45 from Table 4.3). During the cycle samples were extracted by a sample thief. As shown in Figure 4.16, weight loss during primary drying of 5% (wt/wt)

trehalose is comparable to 5% (wt/wt) sucrose. Moreover, the addition of a low amount of phenylalanine as bulking agent (formulation tre:phe 10:0.5) showed no influence on primary drying kinetic, too. The addition of a high amount of phenylalanine (tre:phe 10:3) however, resulted in a slight shift of the weight loss profile indicating a slightly slower primary drying kinetic. Taking also the slightly higher product temperature into consideration (cf. Table 4.4), the slower primary drying can be attributed to the higher product resistance of the cakes.

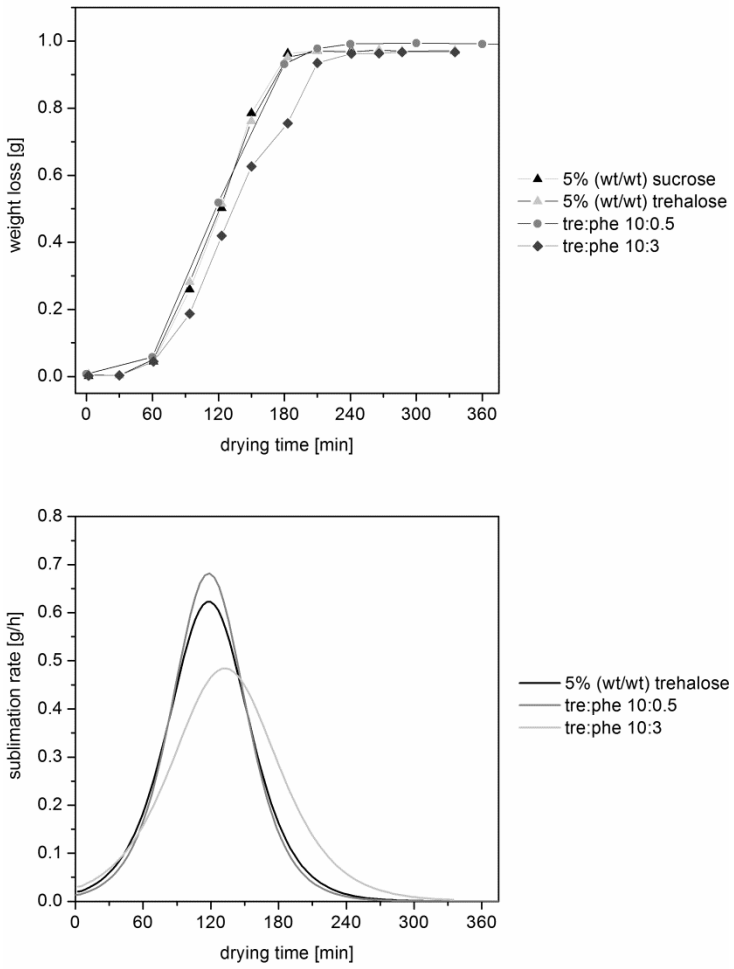

Figure 4.16: Primary drying kinetics of aggressive freeze-drying. Primary drying kinetics are expressed as weight loss (upper graph) and sublimation rate (lower graph) (process 1mbar, 45 °C, 24h).

As already outlined above, samples containing a high amount of bulking agent did not show macro-collapse under the applied process conditions. Regarding secondary drying kinetics, the effect of bulking agent on the drying kinetics is significantly more pronounced (cf. Figure 4.17). All formulations showed an asymptotic progress of secondary drying. The initial steep decrease of residual moisture after finishing the primary drying is more pronounced for higher amounts of phenylalanine. 5% (wt/wt) trehalose showed a similar progress of drying like 5% (wt/wt) sucrose at overall slightly higher residual water content.

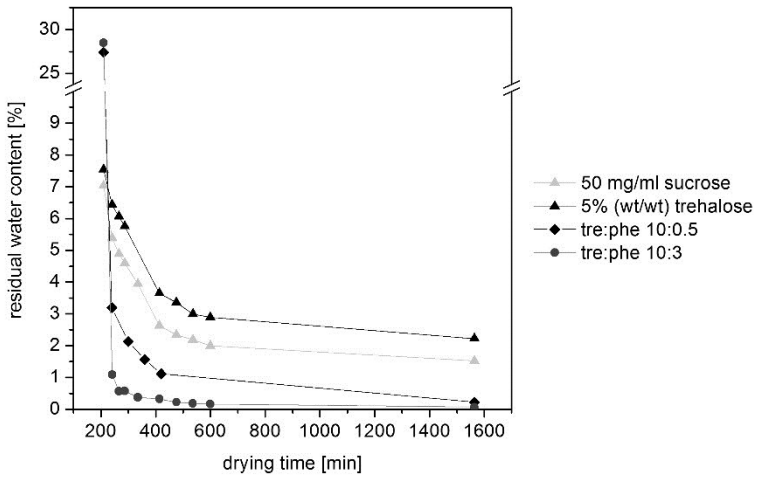

Figure 4.17: Secondary drying kinetics of aggressive freeze-drying expressed as residual water content (different formulations, process 1mbar, 45 °C, 24 h).

To reduce the experimental effort for the different cycles, primary drying kinetics were determined as average values. For this purpose, the endpoint of sublimation was determined by the product temperature, reaching shelf temperature and the average sublimation rate was calculated. Independent from formulation, within every single aggressive freeze-drying cycle, the product temperature reached the level of the shelf temperature within a narrow timeframe (less than 30 minutes) indicating a comparable primary drying behavior of all three formulations (data not shown) as already observed from weight loss data (see above). Although resulting in collapsed (5% (wt/wt) trehalose and tre:phe 10:0.5) or non-collapsed (tre:phe 10:3) products, the primary drying behavior differs only slightly. Therefore, in order to elucidate the influence of shelf temperature and chamber pressure on the primary drying kinetics, the product temperature data of the three formulations were averaged. Hence, the average sublimation rates displayed in Figure 4.18 represent also an average of the three formulations. For comparison, the average sublimation rate of a conventionally freeze-dried 5% trehalose sample is shown in Figure 4.18, too.

Compared to the conventional cycle, a drastic increase of the sublimation rate was noticed for each of the aggressive cycles. According to the correlation between product temperature and sublimation rate [35], an increase in chamber pressure (i.e. higher product temperature, cf. Table 4.4) results in higher sublimation rate as expected. However, a further increase of chamber pressure to above 1 mbar did not result in a further increase of sublimation rate, although the product temperatures still increase. Overall, the highest sublimation rates were noticed for chamber pressures of 1 mbar and 2 mbar. As expected, the sublimation rate at a distinct chamber pressure (low and high) was influenced by the shelf temperature, with higher sublimation rates for higher shelf temperatures.

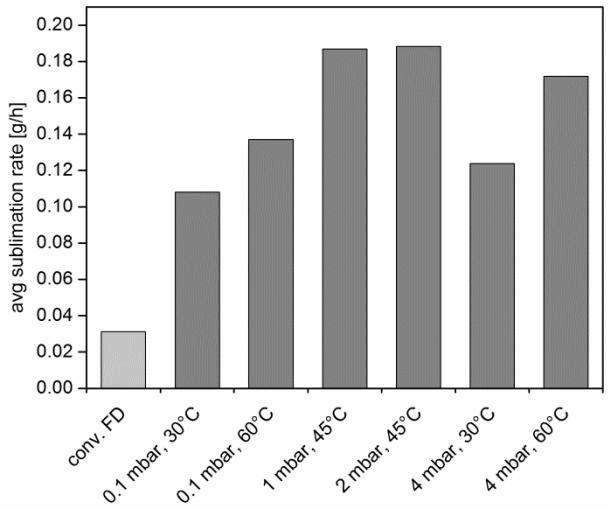

Figure 4.18: Averaged sublimation rates of trehalose-based samples subjected to different aggressive freeze-drying conditions. For comparison, the sublimation rate of conventional freeze-drying is displayed, too.

The drying behavior during secondary drying is mainly influenced by the structure formed during primary drying. As shown above, the samples resulting from the different formulations exhibited different secondary drying kinetics (cf. Figure 4.15), which depend on the specific surface area of the lyophilizates. Larger surface enables a better desorption of water from the lyophilizate. Since the temperature has a major influence on desorption, too, for higher shelf temperatures faster drying is to be expected. Figure 4.19 displays the residual water content over drying time. In this study, no sample thief was used to extract samples. Therefore, only a small number of data points are available. Nevertheless, this rough description of secondary drying showed that higher temperature result in faster drying as it was expected. The effect of low chamber pressure (0.1 mbar), which also seems to enhance secondary drying, is accounted to the larger SSA of the samples produced at a chamber pressure of 0.1 mbar. This assumption is underlined by investigations on the influence of chamber pressure on the drying kinetics of conventionally freeze-dried lyophilizates [23].

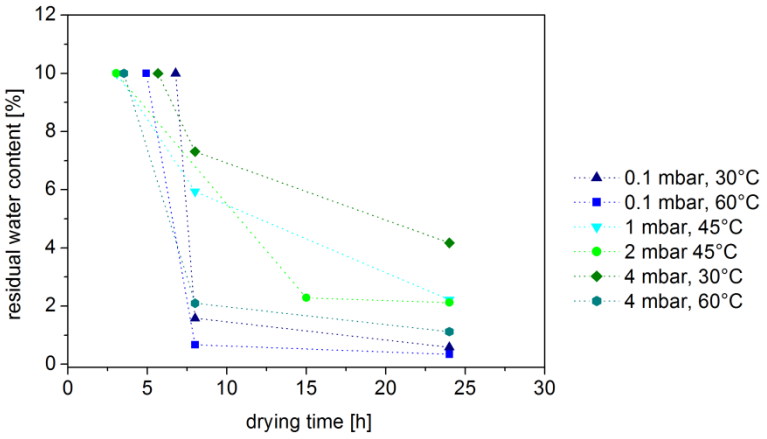

Figure 4.19: Secondary drying kinetics of aggressive freeze-drying expressed as residual water content (5% trehalose at different process parameters; a residual water content of 10% is estimated as entry value for secondary drying).

2.3 Influence of Process Variations on Protein Stability

The ability to stabilize a sensitive molecule is the major interest of freeze-drying. To evaluate the influence of variations of process parameters on the stabilization of a sensitive molecule, the recombinant monoclonal human IgG1 antibody mab_T1 was selected as a suitable model. Own observations during a pre-test to select a proper model revealed its susceptibility freeze-drying stress. To investigate the influence of shelf temperature, chamber pressure, and drying time on the stability of mab_T1, the formulations shown in Table 4.7 were subjected to the aggressive freeze-drying cycles displayed in Table 4.3 (page 73, except the cycle at 1 mbar, which was not included). The three different formulations can be regarded as representative for typical disaccharide based freeze-drying formulations containing (i) no bulking agent (5% (wt/wt) trehalose), (ii) a low amount of phenylalanine as bulking agent (tre:phe 10:0.5), or (iii) a high amount of phenylalanine as bulking agent (tre:phe 10:3).

Table 4.7: Formulations used to characterize the influence of process conditions on the stability of mab_T1 in aggressively freeze-dried lyophilizates.

ID	mab_T1	trehalose	phenylalanine	potassium phosphates
	mg/ml	mg/ml	mg/ml	mg/ml
tre	1.0	50		2.4
tre:phe 10:0.5	1.0	47.6	2.4	2.4
tre:phe 10:3	1.0	38.5	11.5	2.4

Since mab_T1 is especially prone to aggregation if not proper stabilized, the aggregation behavior was investigated by HP-SEC, subvisible particle count (by light obscuration

LO), and turbidity. While HP-SEC is utilized to analyze samples regarding soluble protein aggregates, subvisible particle counts and turbidity was employed to detect non-soluble protein aggregates.

2.3.1 NO BULKING AGENT

Figure 4.20 shows the influence of shelf temperature, chamber pressure, and drying time on the formation of subvisible particles ≥ 1 µm during aggressive freeze-drying of 1 mg/ml mab_T1 in a 5% (wt/wt) trehalose formulation. Overall, low amounts of subvisible particles ≥1 µm were detected for the collapsed lyophilizates. Neither an impact of the different chamber pressures (2 mbar or 4 mbar), nor the different shelf temperatures (30 °C, 45 °C, 60 °C) were noticed. For a shelf temperature of 30 °C and 45 °C a minor tendency of increased svp counts for increasing drying time was observed.

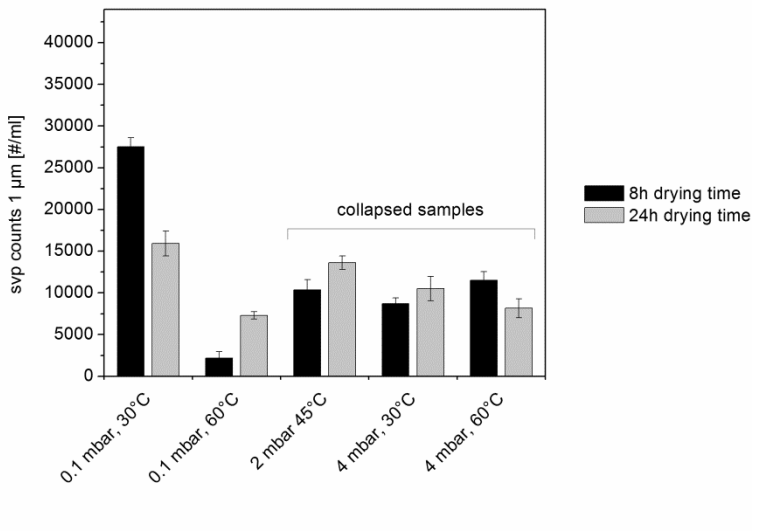

Figure 4.20: Sub-visible particle counts of particles ≥1 µm of samples aggressively freeze-dried from 5% (wt/wt) trehalose formulation containing 1 mg/ml mab_T1 (analyzed by LO; svp counts before freeze-drying around 1,000 particles ≥1 µm per ml; n=3).

Although the samples freeze-dried at Ts=30 °C and pc=0.1 mbar exhibited a non-collapses appearance, the highest amount of insoluble aggregates (i.e. svp) was detected. Since mab_T1 is susceptible to surface stress, the higher specific surface area of non-collapsed samples offers an explanation for more pronounced aggregation. As shown in Figure 4.5, the non-collapsed samples exhibit a SSA of about 1 m²/g, while the collapsed samples range below 0.15m²/g. Interestingly, the lowest amount of svp was detected in non-collapsed samples freeze-dried at pc=0.1 mbar and Ts=60° C for 8 h. These samples had a large SSA, too. Obviously, the high shelf temperature result in a better stabilization of mab_T1 despite the large SSA. An annealing effect, which would reduce molecular mobility [36], is suggested as potential explanation. The increase of svp in those samples after 24 h drying suggest that high temperatures should be used only for short drying cycles of non-collapsed lyophilizates.

The overall trends regarding aggregation detected by subvisible particle formation were underlined by comparable trends in turbidity data (cf. Figure 4.21). Since turbidity

is also sensitive for particles smaller than 1 µm, obviously aggregates in the size range below 1 µm were formed during aggressive freeze-drying.

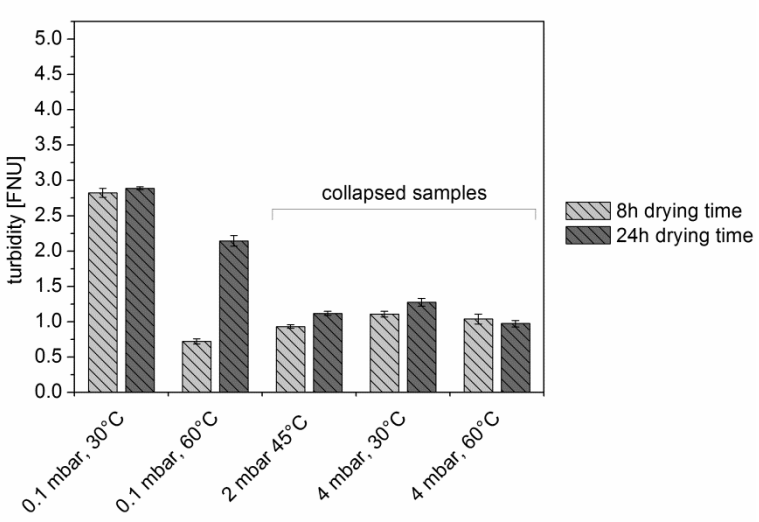

Figure 4.21: Turbidity of samples aggressively freeze-dried from 5% (wt/wt) trehalose formulation containing 1 mg/ml mab_T1 (analyzed by nephelometry; turbidity before freeze-drying around 0.7 FNU; n=3).

Table 4.8 shows the HP-SEC results. Throughout all analyzed samples, no decrease in monomer content was detected, indicating no formation of soluble aggregates (dimer or higher molecular weight species) or fragmentation of monomer. Comparing aggressively freeze-dried samples to a sample prior to freeze-drying, a slight tendency of lower monomer recovery for higher shelf temperatures seems to exist. However, because of overlapping standard deviations the differences between the species are regarded negligible.

Table 4.8: Monomer content and monomer recovery of mab_T1 (c=1mg/ml) in lyophilizates aggressively freeze-dried from a 5% (wt/wt) trehalose formulation using various conditions (analyzed by HP-SEC; monomer content prior to freeze-drying 97.5%; n=3).

process parameters			monomer content		monomer recovery	
chamber pressure	shelf temperature	drying time	mean	SD	mean	SD
mbar	°C	hours	%	%	%	%
0.1	30	8	98.3	0.3	98.9	1.9
		24	97.9	0.3	99.5	2.6
	60	8	97.7	0.1	97.3	0.9
		24	97.8	0.0	96.5	1.0
2	45	8	97.9	0.0	98.3	0.9
		24	97.9	0.1	97.0	0.8
4	30	8	97.9	0.0	100.4	0.7
		24	98.0	0.0	98.2	0.9
	60	8	97.9	0.0	96.7	0.9
		24	98.0	0.0	99.2	1.4

To assess the mab_T1 stability data also in the light of the physico-chemical characteristics of the lyophilizates aggressively freeze-dried from 5% (wt/wt) trehalose formulation, the data shown in section 2.1.2 are taken into consideration.

Although the samples showed some differences in physico-chemical characteristics, overall a good stabilization of mab_T1 was observed. After 24 h drying, collapsed samples (freeze-dried at 2 or 4 mbar chamber pressure) showed slightly less aggregation than non-collapsed lyophilizates (freeze-dried at 0.1 mbar chamber pressure). Besides different appearance and SSA, also a range of residual water contents and glass transition temperatures were observed for samples aggressively freeze-dried using different process parameters. Nevertheless, no impact on the stabilization of mab_T1 could be noticed. It is concluded that a broad range of aggressive freeze-drying process parameters can be used to generate lyophilizates of 5% (wt/wt) trehalose that can successfully stabilize a sensitive molecule like mab_T1.

2.3.2 Low amount of phenylalanine (tre:phe 10:0.5)

The stability of mab_T1 in the aggressively freeze-dried products based on the formulation containing a low amount of phenylalanine as bulking agent (tre:phe 10:0.5) was evaluated. For products aggressively freeze-dried at 0.1 mbar chamber pressure a very low amount of subvisible particles ≥1µm was detected independent from shelf temperature (cf. Figure 4.22). Higher chamber pressures of 4 mbar resulted in slightly higher amounts of subvisible particles. Additionally, for higher chamber pressure a tendency to higher svp counts for longer drying times was detected.

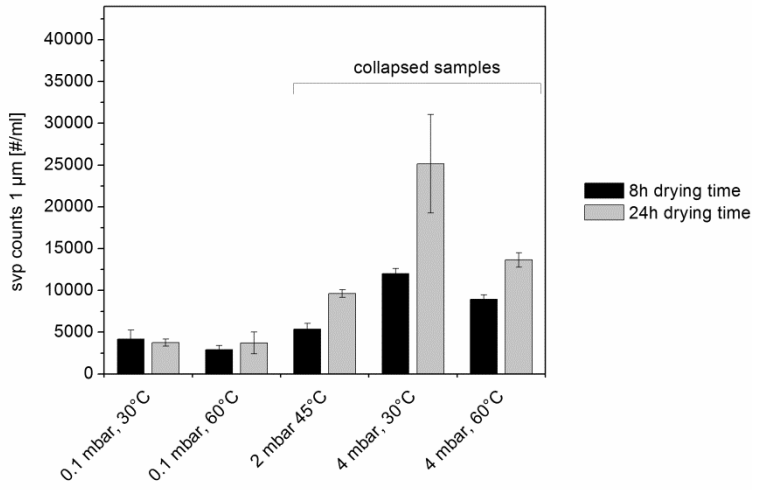

Figure 4.22: Sub-visible particle counts of particles ≥1 µm of samples aggressively freeze-dried from tre:phe 10:0.5 formulation, representing a formulation containing a low amount of bulking agent, using various conditions (analyzed by LO; svp counts before freeze-drying around 1000 particles ≥1 µm per ml; n=3).

Figure 4.23 shows the turbidity of the analyzed lyophilizates. An increase of turbidity with increasing drying time could also be observed for the samples freeze-dried at low chamber pressure. Also the lyophilizates freeze-dried at 4 mbar and 30 °C exhibited a substantial increase. As increasing svp counts as well as turbidity indicate an increase in

non-soluble particles, increasing drying time seems to be connected to increasing aggregation of mab_T1. While overall the majority of the samples exhibit comparable low svp counts as well as low turbidity, samples freeze-dried applying 4 mbar chamber pressure and 30 °C shelf temperature showed remarkable formation of non-soluble aggregates of mab_T1.

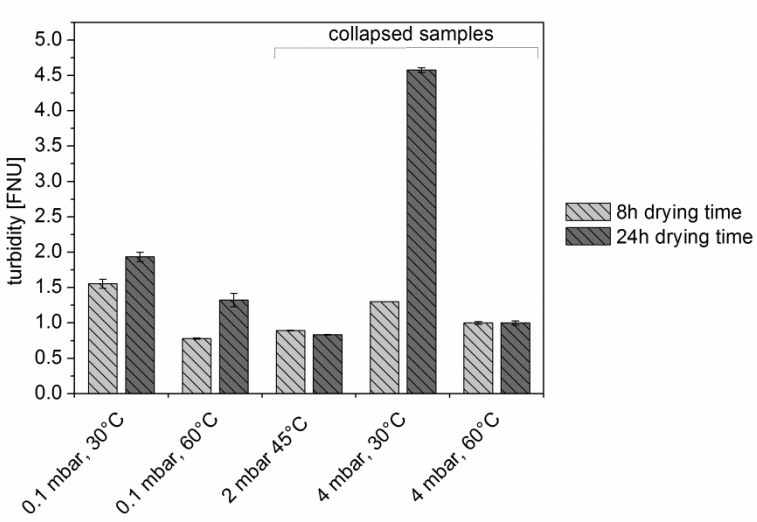

Figure 4.23: Turbidity of samples aggressively freeze-dried using various conditions from formulation containing trehalose and a low amount of phenylalanine as bulking agent (tre:phe 10:0.5) (analyzed by nephelometry; turbidity before freeze-drying around 0.7 FNU; n=3).

Regarding the formation of soluble aggregates, HP-SEC data revealed no decrease in monomer content (cf. Table 4.9) indicating no formation of soluble aggregates. Having a look at monomer recovery of the samples aggressively freeze-dried compared to the formulation prior to freeze-drying, no clear trend could be observed. While for low chamber pressure (0.1 mbar) a higher shelf temperature resulted in decreased monomer recovery, for high chamber pressure (4 mbar) a higher shelf temperature led to higher monomer recovery. As there is no extensive formation of aggregates, neither soluble nor non-soluble, the cause for fluctuating recoveries remained unclear.

Table 4.9: Monomer content and monomer recovery of samples aggressively freeze-dried from tre:phe 10:0.5 formulation using various conditions (analyzed by HP-SEC; monomer content prior to freeze-drying 97.4%; n=3).

process parameters			monomer content		monomer recovery	
chamber pressure	shelf temperature	drying time	mean	SD	mean	SD
mbar	°C	hours	%	%	%	%
0.1	30	8	97.9	0.0	98.1	2.2
		24	97.8	0.1	99.7	1.8
	60	8	97.8	0.1	95.7	2.2
		24	97.7	0.1	94.2	1.9
2	45	8	97.8	0.1	98.7	2.1
		24	97.9	0.1	96.8	2.1
4	30	8	97.8	0.1	98.1	2.0
		24	98.0	0.2	95.6	2.2
	60	8	98.0	0.1	102.0	2.4
		24	98.0	0.1	101.4	2.1

2.3.3 HIGH AMOUNT OF PHENYLALANINE (TRE:PHE 10:3)

To complete the picture, in addition to the formulations containing no bulking agent or a low amount of phenylalanine as bulking agent, also a formulation containing a high amount of phenylalanine (tre:phe10:3) was investigated regarding the influence of process parameters on the stability of incorporated mab_T1. As already outlined above, this formulation can be regarded as comparable to a formulation containing for example trehalose and mannitol at a ratio of 1:2 (at 5% solid content) concerning physico-chemical characteristics during and after aggressive freeze-drying.

The subvisible particle counts, representing non-soluble particles, are shown in Figure 4.24. Samples freeze-dried at low chamber pressure (0.1 mbar) exhibited a slight tendency that a higher shelf temperature of 60 °C resulted in less aggregation compared to 30 °C shelf temperature. This trend is even more pronounced after a drying time of 24 h. Contrary to this tendency, at high chamber pressure of 4 mbar higher shelf temperature resulted in increased aggregation compared to 30 °C shelf temperature. Overall, high chamber pressure resulted in more aggregation. Samples from the process that applied medium chamber pressure of 2 mbar and medium shelf temperature of 45 °C, however, did not show aggregation of mab_T1.

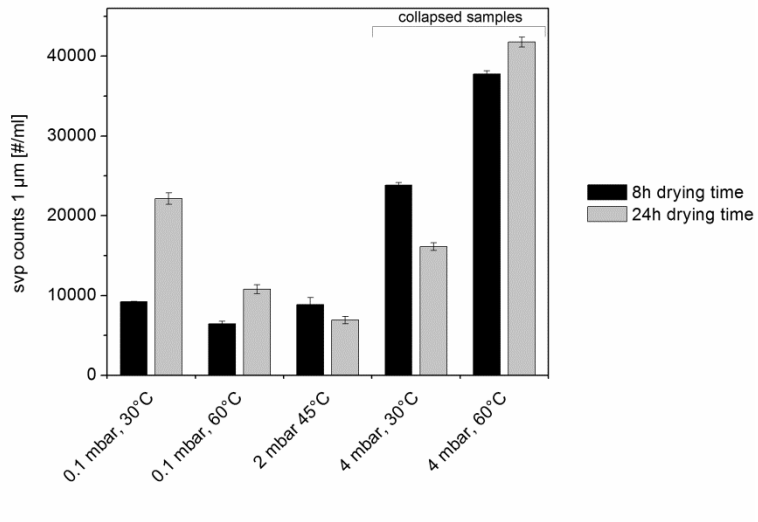

Figure 4.24: Sub-visible particle counts of particles ≥1 µm of samples aggressively freeze-dried from tre:phe 10:3 formulation, representing a formulation containing a high amount of bulking agent, using various conditions (analyzed by LO; svp counts before freeze-drying around 1000 particles ≥1 µm per ml; n=3).

In addition, turbidity of that product did not show increased values compared to the formulation prior to freeze-drying (cf Figure 4.25). Interestingly, the samples aggressively freeze-dried at low chamber pressure, which showed lower svp counts as lyophilizates dried at high chamber pressures, exhibited higher turbidity values. Considering the physico-chemical characteristics, the difference in specific surface area (cf. Figure 4.5, page 78) is thought to foster different aggregation mechanisms leading to different sized aggregates (turbidity also accounts for aggregates smaller than 1 µm).

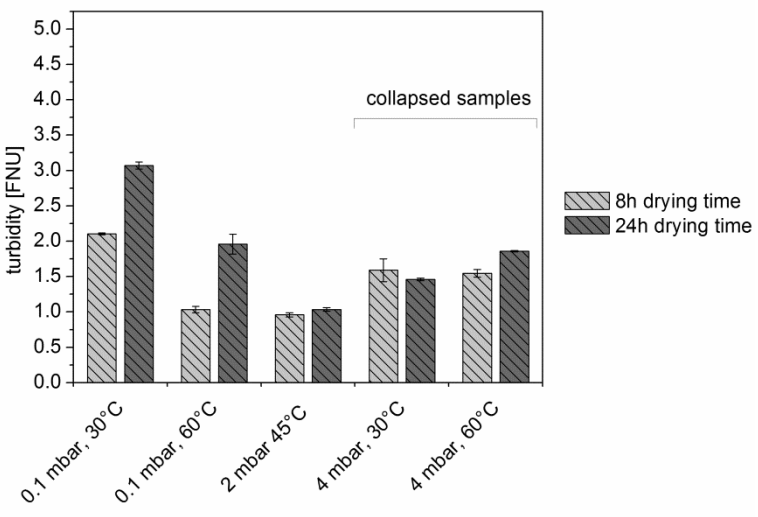

Figure 4.25: Turbidity of samples aggressively freeze-dried using various conditions from formulation containing trehalose and a high amount of phenylalanine as bulking agent (tre:phe 10:3) (analyzed by nephelometry; turbidity before freeze-drying around 0.7 FNU; n=3).

The monomer content of the aggressively freeze-dried samples remained unchanged compared to the formulation prior to freeze-drying (cf. Table 4.10). None of the analyzed

products did show aggregation to soluble aggregates, the monomer content remained unchanged after aggressive freeze-drying. A decreased recovery was noticed only for the samples freeze-dried at 0.1 mbar chamber pressure and 60 °C shelf temperature suggesting a degradation of mab_T1.

Table 4.10: Monomer content and monomer recovery of samples aggressively freeze-dried from tre:phe 10:3 formulation using various conditions (analyzed by HP-SEC; monomer content prior to freeze-drying 97.5%; n=3).

process parameters			monomer content		monomer recovery	
chamber pressure	shelf temperature	drying time	mean	SD	mean	SD
mbar	°C	hours	%	%	%	%
0.1	30	8	98.0	0.4	99.0	3.2
		24	98.0	0.2	102.0	3.2
	60	8	97.8	0.0	96.2	3.4
		24	97.8	0.0	93.7	2.9
2	45	8	98.1	0.0	98.8	2.9
		24	98.0	0.0	97.6	3.1
4	30	8	97.8	0.0	98.1	3.6
		24	97.7	0.0	98.4	3.1
	60	8	97.9	0.1	98.1	3.5
		24	97.8	0.1	97.5	2.7

2.3.4 CONLCUSIONS

Aggressive freeze-drying of the formulation containing no bulking agent (5% (wt/wt) trehalose) at high chamber pressure and high shelf temperature resulted in excellent stabilization of mab_T1 in collapsed lyophilizates. The non-collapsed lyophilizates that result from low chamber pressure exhibited aggregation of the incorporated mab_T1, especially if long drying times were used.

Formulations that contain a low amount phenylalanine as bulking agent (tre:phe 10:0.5) showed best stabilization of mab_T1 in collapsed lyophilizates that result from medium chamber pressure and medium shelf temperature. Lyophilizates freeze-dried at 60 °C shelf temperature provided sound stabilization, too, if the drying time is kept short. Thereby, no difference between non-collapsed and collapsed samples was noticed.

Aggressive freeze-drying of the formulation with the high bulking agent content (tre:phe 10:3) is accompanied by a worse stabilization of mab_T1. Non-collapsed (dried at low pc) and collapsed samples (dried at high pc) showed aggregation of mab_T1 indicated by increased concentrations of subvisible particles. In addition, a clear trend of increased aggregation for prolonged drying time was noticed. The poor stabilization of mab_T1 in lyophilizates containing a high content of bulking agent is attributed to the high specific surface area of these products (compare section 2.1.2).

Overall, the chamber pressure indirectly influences the stability of mab_T1, mainly by the resulting structure of the lyophilizates (collapsed=low specific surface area, non-

collpased=high specific surface area). Short drying time is preferable in every case, but with respect to low residual water content only applicable to formulations that contain a bulking agent. The applied shelf temperature seems to play a two-sided role for stabilization of mab_T1. For non-collapsed samples (due to high bulking agent content or low chamber pressure), high shelf temperatures resulted in less aggregation than low shelf temperatures. Since these lyophilizates have a comparable large specific surface area, a negative effect of higher temperatures would have been expected. Although the specific surface area plays a major role for stabilization, a correlation between SSA, shelf temperature, and stability of mab_T1 cannot be stated. Probably, high shelf temperature resulted in relaxation of the amorphous part in the lyophilizates.

Independent from drying time for all formulations a medium chamber pressure of 2 mbar and a medium shelf temperature of 45 °C resulted in good preservation of mab_T1. These settings seem to be preferable regarding protein stabilization during aggressive freeze-drying.

3. SUMMARY AND CONCLUSIONS

In this chapter, the influence of different settings for aggressive freeze-drying on the final product characteristics as well as on the drying kinetics were studied. Furthermore, the implications for the stabilization of an incorporated model protein (mab_T1, monoclonal IgG antibody) was evaluated. In order to get an idea, in which range the process parameters need to be controlled for successful operation of aggressive freeze-drying, typical freeze-drying formulations that contain an amorphous stabilizer (sucrose or trehalose) were aggressively freeze-dried. The main process parameters (shelf temperature, chamber pressure, and process time) were controlled at different levels. To account for the common strategy to add bulking agents to freeze-drying formulations, two formulations that contain phenylalanine were included in the studies.

In a first step, the chamber pressure was varied between 0.5 and 4 mbar and the drying behavior as well as final product characteristics of a 5% sucrose solution were determined. The variation of chamber pressure imposed a considerable influence on the product temperature during sublimation. In all of the applied cycles, the product temperature of the sucrose samples was above the collapse temperature. While low chamber pressure resulted in comparably low product temperatures, high chamber pressure caused high product temperatures as expected. The collapsed sugar formulation spattered out of the vials as one major consequence of high product temperatures. It is concluded that at 4 mbar chamber pressure the drying behavior is not controllable. Samples generated at lower chamber pressure (up to 1 mbar) did not experience this phenomenon. Therefore, for sucrose formulations (without bulking agent) a maximal chamber pressure of 1 mbar is advised.

The difference in collapse behavior did not influence the extremely fast sublimation of water during the primary drying step. Already after 6 h drying, the frozen ice was sublimated and the primary drying completed. As usual in freeze-drying, the water content was reduced by a secondary drying step (in this case 18 h). Except for the lyophilizates freeze-dried at 4 mbar, after secondary drying an overall comparable residual water content was detected. The water content was at a comparably high level (around 2.2%), known for collapsed samples based on pure sugar formulations. In order to generate lyophilizates of lower water content, the use of additional excipients is regarded inevitable (unless the drying time is drastically prolonged).

In the second step of the study, in addition to varying the chamber pressure, the shelf temperature and process time were altered, too. The utilized formulations were based on trehalose and complemented by two formulations, containing phenylalanine as bulking agent. In order to evaluate the capability of the lyophilizates produced with the different aggressive freeze-drying processes to stabilize proteins, a monoclonal IgG1 antibody (mab_T1) was included in the formulations.

While in the first step of the study the chamber pressure was varied between 0.5 mbar and 4 mbar, the range was expanded down to 0.1 mbar for the trehalose based formulations. As expected, for medium and high chamber pressure (above 1 mbar) also the trehalose based lyophilizates collapsed during aggressive freeze-drying. The formulations, which comprise no or a low amount of bulking agent, spattered out of the vial. On the other hand, if freeze-dried at 0.1mbar, even the formulations containing no bulking agent

exhibited a slightly shrunken but overall acceptable appearance - despite high shelf temperatures of up to 60 °C. The marked differences for different chamber pressure can be explained by the influence of the chamber pressure on the product temperature. The chamber pressure is the major determinant for the product temperature and therefore collapse behavior and final structure. Moreover, it was noticed that the sublimation is faster for higher chamber pressures due to the higher product temperatures. Because of the major influence on the collapse behavior, the chamber pressure strongly influences the physico-chemical characteristics of the lyophilizates regarding e.g. specific surface area, water content, or glass transition temperatures. The evaluation of mab_T1 stability in the collapsed and non-collapsed samples, which were produced at the different chamber pressures, revealed that a medium to high pressure is preferable for stabilization. Most probably, the low specific surface area of these lyophilizates offered best stabilization to mab_T1.

The influence of shelf temperature was investigated in the range of 30 °C to 60 °C. The variation of the shelf temperature revealed higher sublimation rates for higher shelf temperature. The effect was less pronounced than expected, however, it can be explained by the low influence of the shelf temperature on the product temperature. Although the shelf temperature varied in a range of 30 K, the product temperature remained almost unchanged. In contrast to the minor influence of shelf temperature on the sublimation, a variation of the shelf temperature affected the effectiveness of secondary drying. Both, the residual water content and glass transition temperature were influenced. A higher shelf temperature resulted in lower water content and higher Tg. Regarding the influence of shelf temperature on the stability of the incorporated antibody mab_T1, an interesting phenomenon was observed. High shelf temperature resulted in less aggregation than low shelf temperature.

Drying time was varied between 8 h and 24 h. While appearance, crystallinity, and specific surface area remained unchanged, increased drying time resulted in considerably reduced residual water content and higher Tg as expected. Concerning the stability of the embedded antibody mab_T1, the data showed that pro-longed drying time increased aggregation.

Summarizing the data on the influence of variation of process conditions on the resulting products from aggressive freeze-drying, an overall good to excellent stabilization of mab_T1 can be stated. An optimum level of stabilization was achieved by samples of low specific surface area (< 0.5 m^2/g) and fully amorphous structure. Although lyophilizates of very different physico-chemical characteristics were generated by the different shelf temperatures, chamber pressures, and drying times, at least sufficient stabilizations of the sensitive model protein could be ensured. Therefore, it is concluded that aggressive freeze-drying can be performed in a wide range of shelf temperatures (30 °C – 60 °C) and chamber pressures (0.1 mbar – 4 mbar). Since increased drying times result in decreasing stabilization, short cycles (8 hours drying time) are preferable in terms of protein stabilization. To produce lyophilizates of sufficiently low residual water content and low specific surface area by short drying cycles, the addition of low amounts of phenylalanine to sugar-based formulations (ratio 10:0.5, e.g. tre:phe 10:0.5) was the key to success. Due to on the one hand excellent preservation of mab_T1 and on the other hand sound physico-chemical characteristics, medium chamber pressure of 1 to 2 mbar and medium shelf

temperature of 45 °C is advised for aggressive freeze-drying of sugar-based protein formulations.

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CHAPTER 5 THE INFLUENCE OF FORMULATION COMPOSITION ON PHYSICO-CHEMICAL CHARACTERISTICS OF AGGRESSIVELY FREEZE-DRIED LYOPHILIZATES

1. INTRODUCTION

The systematic study about the influence of structural collapse on the stability of pharmaceutical proteins by Schersch and coworkers has undoubtedly shown that even a severe change in product structure (referred to as complete structural collapse) does not necessarily result in instability of incorporated proteins [1]. Even more remarkably, they have shown that proteins in collapsed formulations showed an at least comparable or even a superior stability during storage at rather harsh conditions (50 °C for 6 months) compared to samples freeze-dried conventionally [2, 3]. Based on those findings, and the overall growing interest in applying high product temperatures during freeze-drying [4-7], and in order to save time and resources, the investigation of the robustness and potentially universal use of aggressive freeze-drying to different types of protein formulations is of high interest. In chapter 4 of this thesis it could be shown that aggressive freeze-drying is a well controllable and reproducible technique to achieve products of suitable characteristics for protein stabilization.

To extend the knowledge about the influence of formulation design on physico-chemical characteristics of aggressively freeze-dried lyophilizates, in this chapter the range of selected freeze-drying formulations was extended. As already outlined in the general introduction (cf. pages 11 and 12), for most pharmaceutical formulations, the use of an amorphous excipient is obligatory for the stabilization of incorporated sensitive molecules. Amorphous excipients act as water replacement in the dry state [8-11] and provide a vitrified glassy matrix of reduced mobility [12-14]. In order to increase the mechanic strength of the resulting dry product cake, crystalline components are added frequently (see e.g. [15]). Taking that into consideration, all formulations evaluated in in this chapter are based on a considerable amount of an amorphous excipient (e.g. a disaccharide). Since formulation strategy for freeze-drying formulations emphasizes the importance of bulking agents [16], the utilization of bulking agents for aggressive freeze-drying was scrutinized closely. Due to a number of circumstances (e.g. necessary dose), the protein concentration in a biopharmaceutical drug can cover a considerable range [17]. Therefore, the effect of protein concentration on resulting physico-chemical characteristics was evaluated, too. Beyond different protein concentrations, freeze-drying formulations exhibit also an overall different content of dissolved solids, usually in form of stabilizing excipients. To account for the effect of different solid contents on resulting product characteristics, formulations covering a range of solid contents were aggressively freeze-dried and investigated.

The aggressively freeze-dried products were evaluated with respect to key product qualities. Major physico-chemical characteristics of aggressive freeze-dried products like residual water content and glass transition temperature were determined. Furthermore,

important characteristics like specific surface area analysis and crystallinity were monitored.

2. TYPE OF DISACCHARIDE

As already outlined in more detail in the general introduction, both sucrose and trehalose are the most prominent stabilizers used to protect valuable molecules in freeze-dried products [17]. Therefore, it is of major interest to evaluate the influence of sugar selection on the physico-chemical characteristics of products from aggressive freeze-drying. For this purpose, 5% (wt/wt) solutions of sucrose as well as trehalose were aggressively freeze-dried in 2R vials with 1ml fill using the protocol shown in Table 3.4 (page 55) in an FTS LyoStar II freeze-drier. Aggressive freeze-dried samples were analyzed regarding appearance, residual water content, glass transition temperature, specific surface area, as well as molecular structure (crystallinity).

Figure 5.1: Macroscopic appearance of products aggressively freeze-dried samples from 5% (wt/wt) sugar solutions (process settings: pc=1 mbar, Ts=45 °C). Left picture shows sucrose based samples, right picture trehalose based.

Figure 5.1 shows the macroscopic appearance of aggressively freeze-dried samples from either 5% (wt/wt) sucrose (left picture) or 5% (wt/wt) trehalose solution (right picture). Both sugars showed a comparable, fully collapsed appearance, which can be described as foam-like structure. The apparent volume of the foam-like structures equals the initial filling height. Observations of the appearance in the course of drying revealed that this foam-like structure is formed at the late stage of sublimative drying following a pronounced shrinkage of the drying product.

The comparable structure of samples of either sugar is also reflected in the pictures attained by a digital microscope at a magnification of 100x (cf. Figure 5.2). As the samples could not be removed from the vial without destruction, the pictures show fragments of the freeze-dried material. However, despite of fragmentation, the original glassy structure of the samples could be displayed, showing that the sugars formed a transparent glass. The red scale bar represents 250 μm, which is about one magnitude larger than the range of typical pore sizes for non-collapsed samples.

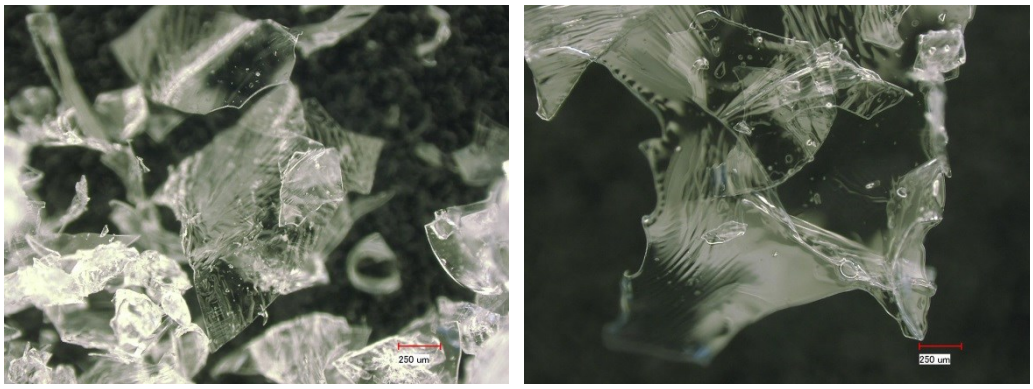

Figure 5.2: Microscopic appearance (100x magnification) of aggressively freeze-dried samples from 5% (wt/wt) sugar solutions (process settings: pc=1 mbar, Ts=45 °C). Left picture shows sucrose based samples, right picture trehalose based.

Although the samples were subjected to a comparably long aggressive freeze-drying cycle of 24 h at 45 °C shelf temperature, the residual water content of samples from both sugars is still comparably high (cf. Figure 5.3, left graph).

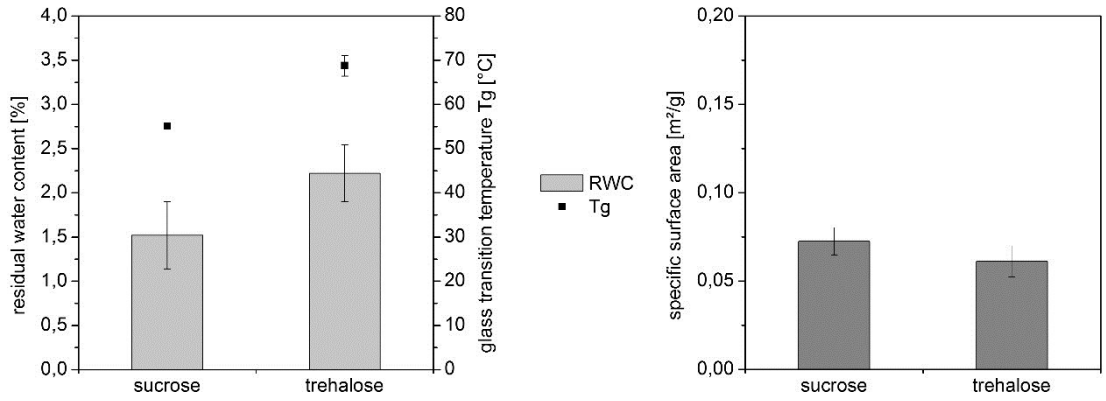

Figure 5.3: Physico-chemical characteristicss of samples aggressively freeze-dried from 5% (wt/wt) sucrose or 5% (wt/wt) trehalose solution (process settings: pc=1 mbar, Ts=45 °C). Left graph shows residual water content (grey bars) and glass transition temperature (black squares), right graph shows specific surface area.

While a residual water content of 1.5% was detected for sucrose based samples, trehalose based samples ended up at 2.2%. From conventional freeze-drying it is known that trehalose exhibits higher residual water contents if subjected to similar freeze-drying processes. As especially drying by desorption within the secondary drying step is dependent from the available surface for desorption, specific surface of the collapsed lyophilizates was determined (cf. Figure 5.3, right graph). No apparent difference was detected for the different sugars. Both, sucrose as well as trehalose based samples showed a very low specific surface area compared to conventionally freeze-dried lyophilizates, which exhibit SSA of around 1 to 2 m²/g (data not shown). Regarding glass transition temperature, the aggressively freeze-dried products showed glass transition temperature of 55.1 °C (sucrose) and 68.8 °C (trehalose), respectively (cf. Figure 5.3, left graph).

During aggressive freeze-drying of sucrose and trehalose solution, there is considerable mobility in the drying matrix resulting in collapsed appearance after the process. Therefore, also crystallization of the sugars seemed so be possible in the course of collapse. To figure out, whether crystallization plays a role, the dried samples were analyzed using powder X-Ray diffraction (XRD). The absence of peaks in the diffractograms (cf. Figure 5.4) revealed the fully amorphous structure of samples from both sugars. This is further assisted by the absence of melting peaks in the DSC thermograms.

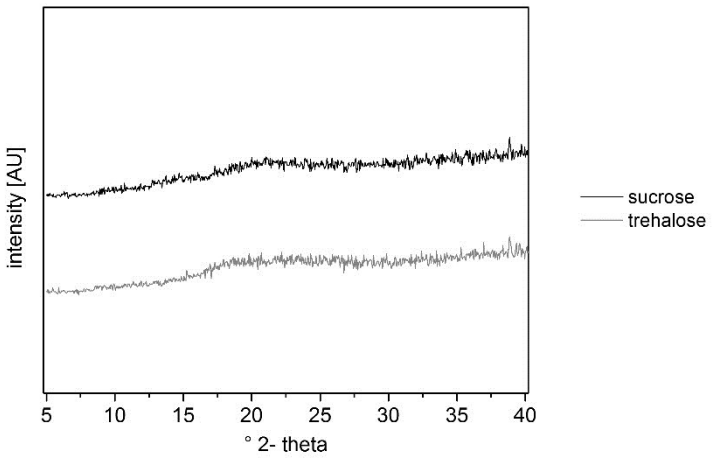

Figure 5.4: XRD diffraction pattern of samples aggressively freeze-dried from 5% (wt/wt) sucrose or 5% (wt/wt) trehalose solution (process settings: pc=1 mbar, Ts=45 °C).

Usually, freeze-drying of pharmaceutical formulations is designed to form highly porous product structures, which then readily dissolves in a diluent. One major concern regarding collapsed lyophilizates is a prolonged reconstitution time due to the less porous structure [18]. Therefore, the rehydration behavior of collapsed trehalose and sucrose samples were checked by the addition of high-purified water. Despite the drastically reduced specific surface area compared to conventionally freeze-dried lyophilizates, trehalose based samples were fully rehydrated after 18 ± 3 s, sucrose based samples within 22 ± 5 s. The overall short rehydration can partly be assigned to the very good solubility of sucrose and trehalose in water.

Summing up the influence of type of disaccharide on physico-chemical characteristics of aggressively freeze-dried lyophilizates, sucrose and trehalose resulted in comparable products with regard to appearance, crystallinity, and specific surface area. Small differences were detected for the glass transition temperatures as well as residual water content. In the context of potential protein stabilization, the fully amorphous nature as well as high glass transition temperature renders both sugars suitable excipients for aggressive freeze-drying. In addition, the low specific surface area could improve the stabilization of especially surface sensitive proteins. On the other hand, comparable high residual water contents could potentially foster for example degradation of incorporated biopharmaceuticals. However, usually the addition of a protein to the amorphous sugar matrix results in sort of a bulking effect and therefore lower water content in protein containing lyophilizates.

3. ADDITION OF BULKING AGENT

A common strategy of conventional freeze-drying to form a mechanical strong product cake is to add excipients to a freeze-drying formulation that crystallizes during freezing [17, 19]. Mannitol represents one of these bulking agents that is frequently used [15]. In addition, amino acids have been shown to act as bulking agent [20, 21]. While usually bulking agents are used in excess to amorphous stabilizers regarding weight to weight ratios [15], amino acids have been shown to act at lower weight to weight ratios [30]. In order to evaluate the influence of bulking agent on the physico-chemical characteristics, a number of different weight-to-weight ratios of mannitol or phenylalanine to sucrose or trehalose were subjected to aggressive freeze-drying in 2R vials with 1ml fill according to the protocol shown in Table 3.4 (page 55) by using a FTS LyoStar II freeze-drier. Table 5.1 shows the formulations used within that study.

Table 5.1: Formulations to characterize the influence of bulking agent on the physico-chemical characteristics of aggressively freeze-dried products.

formulation	lysozyme	sucrose	trehalose	mannitol	phenylalanine
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc	1	50.0			
suc:man 10:2	1	41.7		8.3	
suc:man 1:1	1	25.0		25.0	
suc:man 2:10	1	8.3		41.7	
suc:phe 10:0.5	1	47.6			2.4
suc:phe 10:1	1	45.5			4.5
suc:phe 10:2	1	41.7			8.3
suc:phe 10:3	1	38.5			11.5
tre	1		50.0		
tre:man 10:2	1		41.7	8.3	
tre:man 1:1	1		25.0	25.0	
tre:man 2:10	1		8.3	41.7	
tre:phe 10:0.5	1		47.6		2.4
tre:phe 10:1	1		45.5		4.5
tre:phe 10:2	1		41.7		8.3
tre:phe 10:3	1		38.5		11.5

The ratio between sugar and either bulking agent was varied from low to high contents. Due to differences in water solubility of mannitol and phenylalanine, high and low bulking agent content is represented by different absolute concentrations of either substance. In order not to compromise the evaluation by a potential additional effect of the solid content on the physico-chemical characteristics, the solid content was kept at 51 mg/ml for all formulations. To account for the fact that generally a formulation is designed to protect a protein in a pharmaceutical formulation, additionally lysozyme was

added to the formulation. As in this study the physico-chemical characteristics were of interest, no characterization of the stability of lysozyme was conducted.

Product temperature

In order to characterize the drying behavior of the different formulations regarding product temperature, thermocouples were placed in one vial of each formulation. It was of special interest, in which way the addition of bulking agent influences the product temperature especially during the primary drying stage. Figure 5.5 displays exemplary curves for trehalose based formulations containing both, low and high amount of mannitol or phenylalanine. Both formulation containing a low amount of either mannitol or phenylalanine showed a product temperature course comparable to the formulation containing no bulking agent (tre), characterized by a clear plateau around -20 °C, which is reached after an overall process time of 5.5 h. After the end of sublimation at around 7 h process time, the product temperatures tended towards the shelf temperature of 45 °C. The product temperature of the formulation containing a low amount of phenylalanine (tre:phe 10:0.5) showed a smooth characteristic. Both, the formulation containing no bulking agent (tre) or a low amount of mannitol (tre:man 10:2) showed a somehow serrated characteristics towards the end of primary drying, which is attributed to the slightly different collapsing behavior of those formulations compared to tre:phe 10:0.5 (compare macroscopic appearance below).

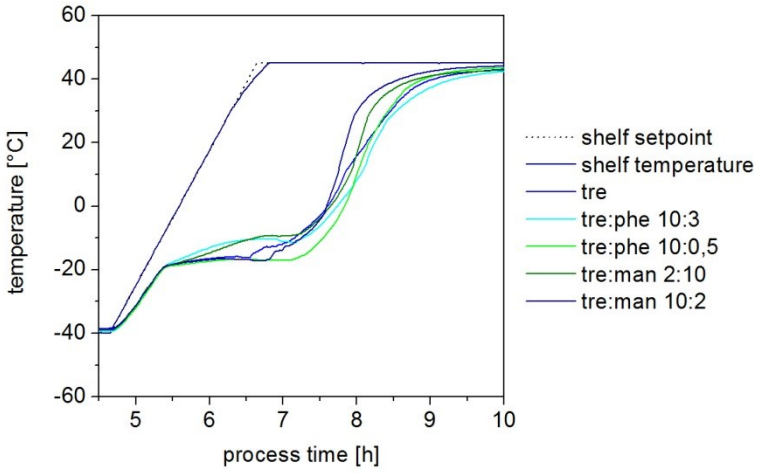

Figure 5.5: Product temperatures of trehalose-based formulations containing both, a high and low amount of mannitol or phenylalanine; primary drying section between 5 and 10 h drying time is shown (solid content 5% (wt/wt); 2R glass vials, 1 ml fill; process settings: pc=1 mbar, Ts=45 °C).

The formulations containing a high amount of bulking agent (tre:phe 10:3 and tre:man 2:10) showed a somewhat different behavior regarding product temperature. Product temperatures of both formulations showed no clear plateau phase during primary drying phase, but a steady, slight increase in the overall smoothly shaped curves. Taking the macroscopic appearance and the specific surface area (see below) into consideration, this steady increase in product temperature during primary drying can be attributed to increasing product resistance due to growing dry layer that have to be passed

by the water vapor formed at the progressing sublimation front. Increasing resistance consequently lead to increasing pressure at the sublimation front resulting in increasing product temperature. Formulation of medium bulking agent content (e.g. tre:man 1:1, tre:phe 10:1) also showed a slight rise in product temperature during primary drying, however, less pronounced compared to the formulation containing a high amount of bulking agent. Sucrose based formulations showed similar product temperature course (data not shown).

For all formulations, the mean product temperature during primary drying (cf. Table 5.2) was calculated by average of the recorded product temperatures between 5.5 and 6.75 h overall process time. For all formulations, an increase in bulking agent content resulted in an increase in mean product temperature during primary drying phase. The lack of a distinct plateau of product temperature of formulations containing a high amount of bulking agent is reflected in the higher standard deviation of the calculated mean product temperature, too. Comparing the mean product temperatures to the glass transition temperatures of the maximally freeze-concentrated solutions (Tg', cf. Table 5.2) showed that product temperature were at least 15 °C higher than Tg'. Therefore, for all formulations some sort of collapse was expectable. Interestingly, increasing amount of mannitol result in a slight decrease in Tg', while increasing amounts of phenylalanine resulted in slightly increasing Tg'.

Table 5.2: Product temperatures during primary drying and glass transition temperatures of the maximally freeze-concentrated solution of bulking agent containing formulations.

formulation	Tg'		product temperature primary drying	
	mean	SD	mean	SD
	°C	°C	°C	°C
suc	-33.8	0.2	-16.2	0.9
suc:man 10:2	-35.4	0.1	-17.4	0.2
suc:man 1:1	-38.2	0.4	-15.9	1.2
suc:man 2:10	-40.6	0.1	-13.8	2.7
suc:phe 10:0.5	-32.9	0.2	-17.4	0.3
suc:phe 10:1	-32.3	0.0	-17.5	0.7
suc:phe 10:2	-30.8	0.2	-14.9	2.0
suc:phe 10:3	-30.1	0.2	-13.2	2.4
tre	-31.3	0.2	-16.3	1.2
tre:man 10:2	-34.3	0.2	-17.2	0.5
tre:man 1:1	-37.9	0.1	-16.7	1.5
tre:man 2:10	-40.0	0.2	-13.7	2.8
tre:phe 10:0.5	-31.0	0.1	-17.4	0.6
tre:phe 10:1	-30.6	0.2	-16.6	1.6
tre:phe 10:2	-29.5	0.2	-15.5	2.0
tre:phe 10:3	-28.3	2.5	-12.8	2.3

Macroscopic and microscopic appearance

Figure 5.6 shows the macroscopic appearance of the aggressively freeze-dried formulations. Regarding the sugar, on which the formulations were based on the appearance was overall comparable between sucrose and trehalose. Minor different appearance was

noticed for the formulation containing no bulking agent. Both samples were fully collapsed. While the sucrose formulations showed a foamy appearance, as already shown above for samples containing no protein, the trehalose formulation exhibited a less foamy structure, but rather a flat layer at the bottom of the vial. All other formulations showed an appearance that was independent from the sugar they were based on. Hence, in the following descriptions it is not differentiated between samples from either sugar.

Figure 5.6: Macroscopic appearance of aggressively freeze-dried formulations containing different amounts of bulking agents (mannitol or phenylalanine), showing an overall comparable appearance between sucrose and trehalose (see text for sample description; process settings: pc=1 mbar, Ts=45 °C).

The addition of a low amount of mannitol to the sugar based formulations (suc:man 10:2 ≙ sm2 in Figure 5.6; tre:man 10:2 ≙ tm2) resulted in no change in appearance compared to the pure sugar formulations. Although they also showed collapsed appearance, the formulations containing a low amount of phenylalanine (suc:phe10:0.5 ≙ sp0.5; tre:phe 10:0.5 ≙ tp0.5) exhibited a more spongy structure. The samples from formulations containing a medium content of phenylalanine (suc:phe10:1 ≙ sp1; tre:phe 10:1 ≙ tp1) showed signs of pronounced micro-collapse expressed as macroscopically visible pores. In addition, they showed some shrinkage in cake height. A medium content of mannitol (suc:man10:10 ≙ sm10; tre:man 10:10 ≙ tm10) as well as a medium to high content of phenylalanine (suc:phe10:2 ≙ sp2; tre:phe 10:2 ≙ tp2) resulted in an acceptable product cake with slight shrinkage in height. The addition of a high amount of mannitol (suc:man10:50 ≙ sm50; tre:man 10:50 ≙ tm50) or phenylalanine (suc:phe10:3 ≙ sp3; tre:phe 10:3 ≙ tp3) resulted in an elegant cake with no signs of collapse or shrinkage.

Regarding the macroscopic appearance, mannitol and phenylalanine showed comparable trends, however, the overall amounts in the formulations were quite different. Phenylalanine forms a macroscopically non-collapsed product already at a much lower amount compared to mannitol. From a general perspective, the application of medium to high amount of bulking agent hinders macroscopically visible collapse even throughout aggressive freeze-drying, which resulted in product temperatures considerably higher than the glass transition temperature of the maximally freeze-concentrated solution (Tg').

In order to have a closer look to the microscopic structure of the aggressively freeze-dried samples, scanning electron microscopy (SEM) was utilized. Figure 5.7 and Figure 5.8 show exemplarily the SEM pictures of the sucrose-based samples. The microscopic structure of the trehalose-based samples appeared similar. Both, the sample containing no or a low amount of mannitol exhibited a structure of huge flakes under the REM. The flake-like structure can be addressed to the brittle nature of the macroscopically foam-like structure of the lyophilizates (compare Figure 5.6). For the preparation of the sample prior to REM the foam-like structure needed to be removed from the vial. During that procedure, the brittleness of the thin material layers made it impossible not to partly destroy the structure. Nevertheless, both fully collapsed samples (suc, suc:man 10:2) did not show the typical highly porous structure of common lyophilizates. The addition of a low amount of mannitol did not result in a change of the structure observable by SEM.

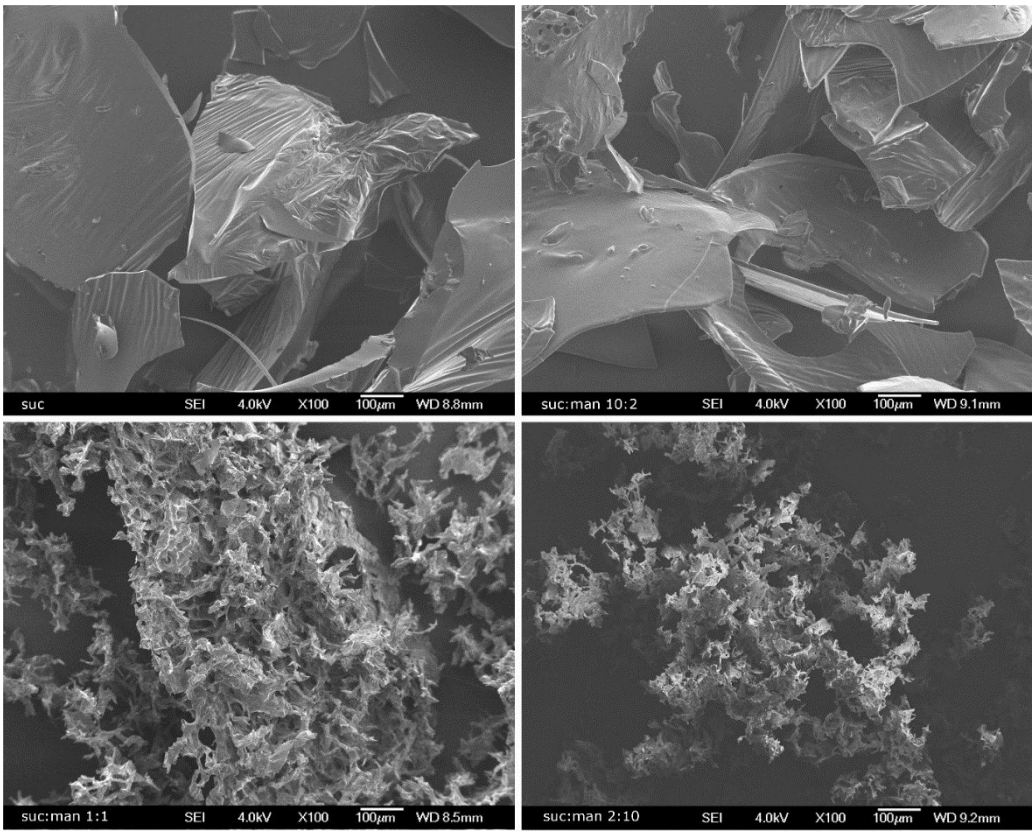

Figure 5.7 Exemplarily SEM pictures of the sucrose-based samples with the bulking agent suc:man; top left picture (suc) and top right picture (suc:man 10:2) are fully collapsed samples with huge flakes. Bottom left picture (suc:man 1:1) and bottom right picture (suc:man 2:10) display formulations containing a medium or high level of mannitol and exhibiting a porous structure.

An increase of the amount of mannitol to medium level resulted in a porous microscopic structure. Compared to conventionally freeze-dried samples, this structure appeared somehow denser. Larger magnification (not shown) revealed a structure, that can be described as sort of mixture of crystalline and smooth structures. This somehow coated appearance can be attributed to the mixture of (partly) crystalline mannitol and amorphous sucrose. Samples freeze-dried from the highest amount of mannitol (suc:man 2:10) exhibited a highly porous structure, which seems to be dominated by crystalline structures.

While low amounts of mannitol did not result in a change in microscopic structure compared to the samples containing no bulking agent, already low amounts of phenylalanine (suc:phe 10:0.5) resulted in a dense but not flake-like structure, which best can be described as a dense macro-porous network (cf. Figure 5.8).

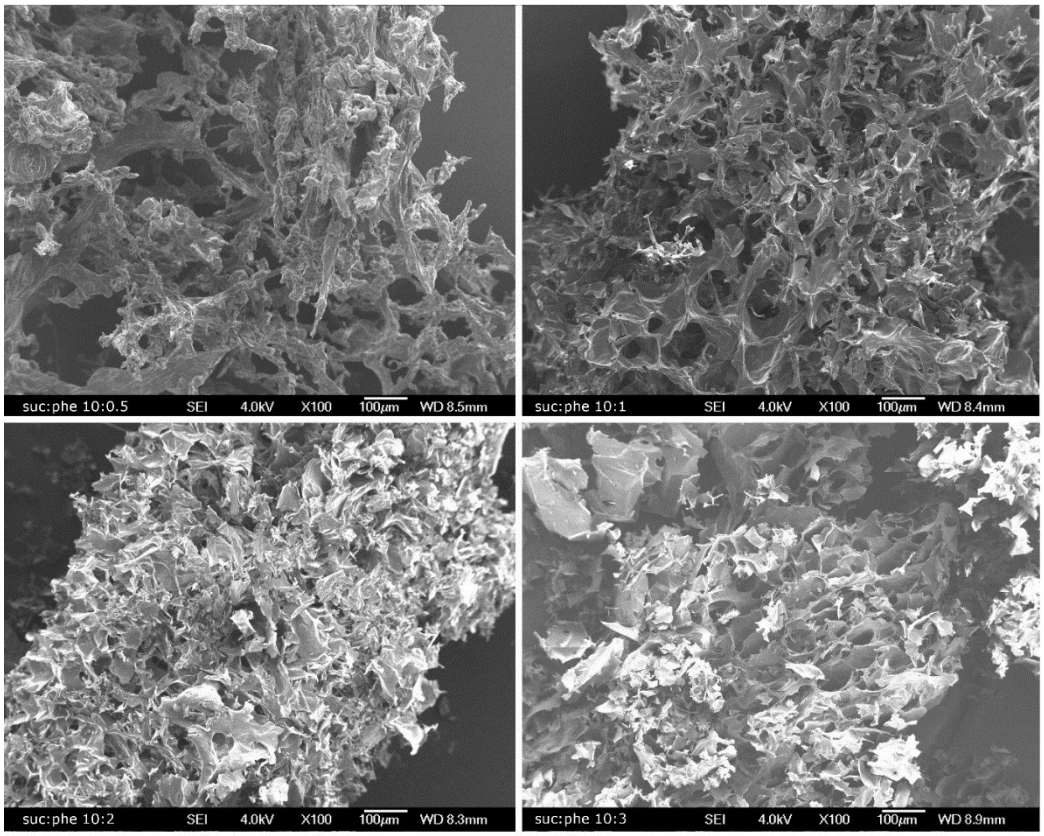

Figure 5.8: Exemplarily SEM pictures of the sucrose-based samples containing phenylalanine as bulking agent. Top left picture low amount of phenylalanine (suc:phe 10:0.5), top-right suc:phe 10:1, bottom left suc:phe 10:2 , bottom right suc:phe 10:3.

Increasing the phenylalanine content resulted in a stepwise reduction of pore sizes and formation of the micro-porous structure characteristic for lyophilizates. While for suc:phe 10:1 and 10:2 signs of micro-collapse can be distinguished (large pores with surrounding massive "walls"), the addition of high amounts of phenylalanine did not show that phenomenon. Increasing amounts of phenylalanine – as well as mannitol – resulted in increased brittleness of the porous structure on a microscopic level.

Residual water content

As already outlined above, aggressive freeze-drying of pure sugar formulations resulted in residual water contents of 1.5% and above due to the dense structure of the collapsed lyophilizates, which hinders an effective secondary drying. As the addition of bulking agent changed the macroscopic appearance of aggressively freeze-dried formulations dramatically, a closer look to the achieved residual water content is of interest. Figure 5.9 displays the residual water content of the lyophilizates aggressively freeze-dried from the formulations shown in Table 5.1. Without the addition of a bulking agent, the samples exhibited comparable high residual water contents of 1.9% (suc) and 3.7% (tre). The addition of a low amount of mannitol did not result in a significant change compared to the samples with no bulking agent. In contrast, by adding low amounts of phenylalanine to the sugar based formulations, a residual water content of 0.8% (suc:phe 10:0.5) or even 0.2% (tre:phe 10:0.5) was achieved. Medium to high amounts of phenylalanine resulted in residual water contents of below 0.1%, while for medium and high amounts of mannitol, residual water contents of between 0.5% and 0.2% were achieved. In other words, the addition of either bulking agent drastically decreased the residual water content of the aggressively freeze-dried lyophilizates.

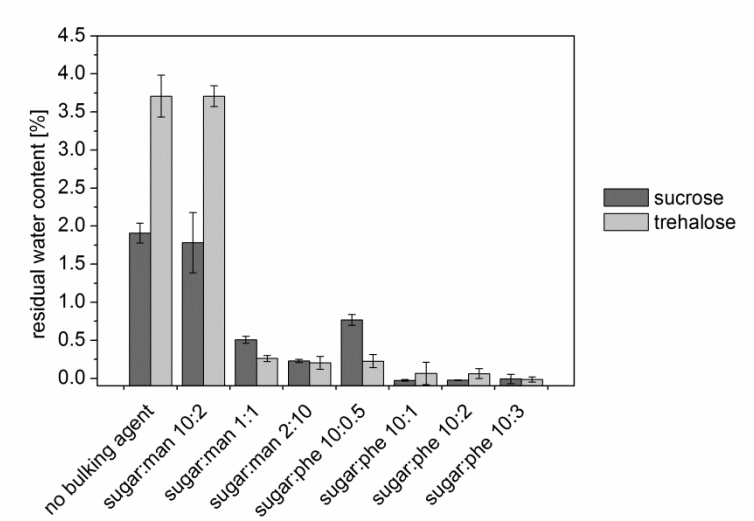

Figure 5.9: Effect of bulking agent on the residual water content for sugar based aggressively freeze-dried lyophilizates (n=3).

It is noteworthy that the samples, freeze-dried from the formulations containing a low amount of phenylalanine, exhibited low residual water contents, although they showed collapsed appearance. Overall, as phenylalanine decreased the residual water content already at comparable low concentrations, this bulking agent seems to be superior to mannitol in this case concerning decreasing residual water content.

Specific surface area

The desorptive drying mechanism, which governs the kinetics of secondary drying and therefore the final residual water content, are inherently dependent from the specific

surface. Higher SSA is expected to result in lower residual moisture and vice versa. Beyond the description of the appearance of lyophilizates subjected to aggressive freeze-drying, SSA data allow to quantify the degree of collapse. Hence, the specific surface area of the aggressively freeze-dried lyophilizates was analyzed using BET gas adsorption. Figure 5.10 shows the determined specific surface areas. Overlooking all data for phenylalanine containing lyophilizates, a slight tendency of higher SSA for trehalose based samples could be noticed. Very low SSA was detected for formulations containing no bulking agent (below 0.1 m^2/g). While the addition of low amounts of mannitol had no effect on the SSA (still below 0.1 m^2/g), low amounts of phenylalanine resulted in slightly increased SSA of 0.26 m^2/g for the trehalose based lyophilizates. In contrast, the same amount of phenylalanine did not increase the SSA of the sucrose based formulation. A medium amount of mannitol resulted in increased SSA of 0.43 m^2/g (suc:man 1:1) and 0.73m^2/g (tre:man 1:1), respectively. A comparable effect was detected for the formulations, containing a medium amount of phenylalanine (sugar:phe 10:1). Generally, increasing amounts of phenylalanine resulted in a stepwise increase in SSA up to 1.11 and 1.30 m^2/g for the formulations containing phenylalanine at a ratio of 10:2. In contrast to mannitol, further increase of phenylalanine amount did not result in further increase in SSA. The formulations containing high amounts of mannitol ended up at 3.46 m^2/g (suc:man 2:10) and 3.36 m^2/g (tre:man 2:10).

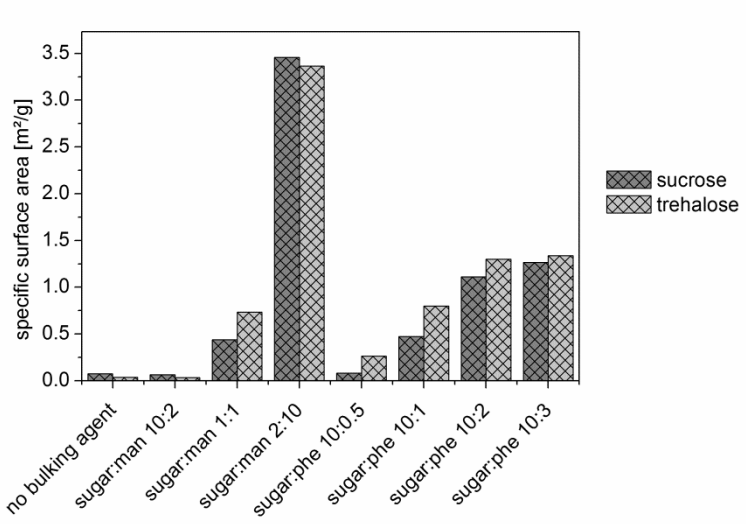

Figure 5.10: Effect of bulking agent on the specific surface area of sugar based aggressively freeze-dried lyophilizates.

Overall, the determined specific surface areas support the observations of the macroscopic appearance of the different formulations. Macroscopically collapsed samples showed very low specific surface areas. The addition of bulking agents resulted in a stepwise increase of the specific surface area, which is also noticeable macroscopically. Samples containing a medium amount of bulking agent showed a non-collapsed appearance but shrinkage of the product cake (sugar:man 1:1; sugar:phe 10:1), which is detectable as distinct decrease of SSA compared to formulations containing high amounts of bulking agent. The latter showed shrinkage neither.

Glass transition temperature

As freeze-drying of protein formulations is performed to achieve a sound stabilization of an embedded protein during an extended period of storage, the glass transition temperature of the underlying excipient matrix is of importance. Usually, higher glass transition temperatures are regarded as superior for storage stability. Figure 5.11 shows the glass transition temperatures (Tg) determined by DSC. Trehalose is known to inherently exhibit higher glass transition temperatures at comparable residual water contents.
The lyophilizates aggressively freeze-dried from the formulations containing no bulking agent exhibited glass transition temperatures of around 50 °C (suc 49.5 °C; tre 53.1 °C), which is regarded as high enough to provide considerably storage stability. The addition of even small amount of phenylalanine increased the glass transition temperature especially for trehalose based samples to above 80 °C (except tre:phe 10:1). In addition, the glass transition temperatures of sucrose were increased by the addition of phenylalanine, however, not in a similar manner compared to trehalose. The addition of mannitol revealed unexpected results, since only the formulation containing a medium amount of mannitol showed an increase in glass transition temperature. For both formulations, containing a high or a low amount of mannitol, a decrease in glass transition temperature was detected for formulations based on both, sucrose and trehalose. Possibly, a considerable amount of mannitol remained amorphous and decreased the glass transition temperature of the mixture, while for the formulation containing a medium amount of mannitol a larger amount of mannitol crystallized. Even the comparably low residual water content of sugar:man 2:10 did not result in increased Tg. Regarding the connection between glass transition temperature and residual water content, partly crystalline lyophilizates are generally prone to a phenomenon which could minimize the effect of lower residual water contents towards higher Tg's. Assuming that there is no water embedded in the crystalline structure of a bulking agent, the residual water content of a partly crystalline lyophilizates is predominantly present as part of the amorphous mixture [22]. Comparing an amorphous and a partly crystalline lyophilizate of the same residual water content and same solid content, in the partly crystalline lyophilizate the residual water content of the amorphous phase must be consequently higher as in this sample less amorphous material contains all the water. In turn, in a fully amorphous sample the water is distributed in a larger amount of material, i.e. the water "concentration" is lower, resulting in higher glass transition temperature.

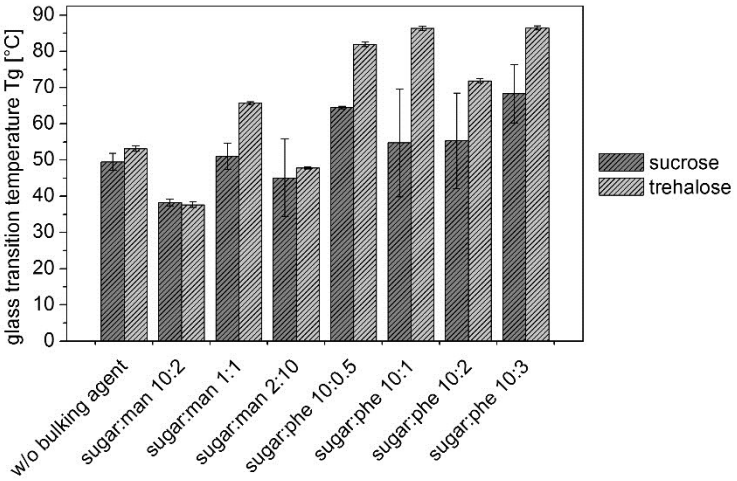

Figure 5.11: Effect of bulking agent on the glass transition temperature of sugar based aggressively freeze-dried lyophilizates (n=3).

Crystallinity

To address the molecular structure of the samples aggressively freeze-dried from the formulations containing different amount of phenylalanine or mannitol (cf. Table 5.1), Powder X-Ray diffraction (XRD) was utilized. The resulting diffraction patterns are shown in Figure 5.12. The samples from both formulations containing no bulking agent (suc, tre) showed no distinct diffraction peaks. Therefore, they are regarded as fully amorphous. Also the addition of low to medium amounts of phenylalanine (sugar:phe 10:0.5 and sugar:phe 10:1) did not result in discernible diffraction. Hence, these samples are considered fully amorphous, too. The addition of high amounts of phenylalanine in trehalose based formulations (10:2 and 10:3) resulted in small but discernible diffraction peaks. According to the International Centre for Diffraction Data (ICDD) database, those diffraction patterns could be identified as L-phenylalanine hydrate (especially based on the most distinct peak at 6.4° 2-theta). The peak around 38.8° 2-theta result from the copper sample holder. While the addition of the highest amount of phenylalanine to the sucrose based formulation (i.e. formulation suc:phe 10:3) resulted also in discernible peaks, XRD of formulation with the second highest content of phenylalanine (suc:phe 10:2) showed an amorphous pattern.

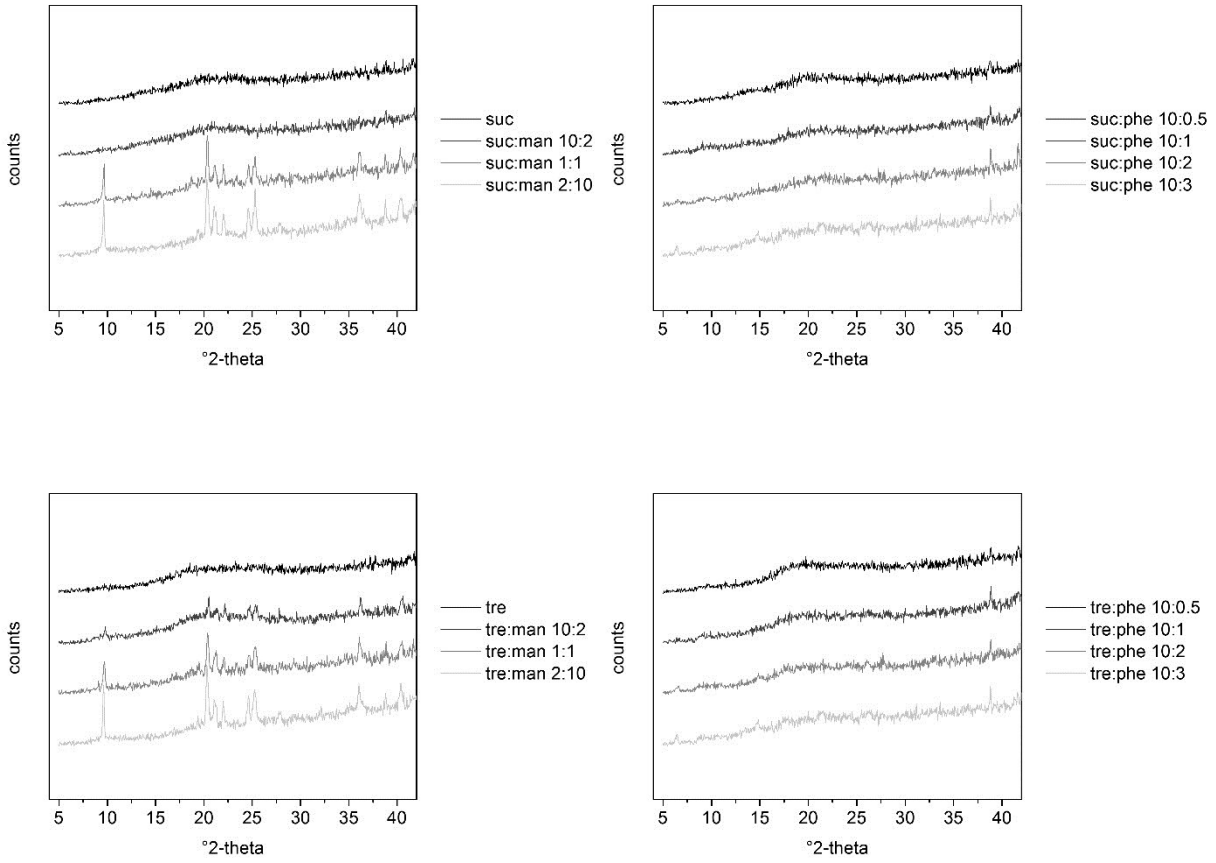

Figure 5.12: Effect of bulking agent on the diffraction pattern of sugar based aggressively freeze-dried lyophilizates.

While low amounts of mannitol resulted in fully amorphous structure for sucrose based samples, trehalose based samples showed small, but discernible diffraction peaks (cf. Figure 5.12). Independent from sugar, medium and high amounts of mannitol resulted in clear diffraction pattern underlining the presence of considerable amounts of crystalline structures in those samples. According to literature (JCPDS database [23-25]), the diffraction pattern detected from the formulation containing medium to high mannitol amounts, can be attributed to the δ-polymorph of mannitol (characteristic peaks at 9.7°, 20.4, and 24.6° 2-theta). Neither peaks characteristic for the α-polymorph (13.6°, 17.2° 2-theta), nor for the β-polymorph (14.6°, 16.8°, 23.4° 2-theta), nor for the hydrate form (17.9° 2-theta) of mannitol were detectable. Therefore, solely the δ-polymorph is formed during aggressive freeze-drying of the formulation containing medium and high amounts of mannitol.

Summary

It was shown that the addition of bulking agent (mannitol or phenylalanine) to either sucrose or trehalose based formulations influence physico-chemical characteristics of aggressively freeze-dried lyophilizates. As expected, increasing weight-to-weight ratios resulted in increased influence of the respective bulking agent. It needs to be mentioned that the applied absolute amount of either mannitol or phenylalanine was quite different.

While phenylalanine act as suitable bulking agent already at weight-to-weight ratios of 10:2, mannitol needs to be applied at least at weight-to-weight ratios of 1:1.

With increasing amounts of bulking agent, the macroscopic appearance changed from collapsed for low amounts of mannitol and phenylalanine to no visible signs of collapsed for high amount of either bulking agent. Medium amounts of bulking agent resulted in some shrinkage and macroscopically discernible larger pores (esp. for phenylalanine).

The observations regarding the influence of bulking agent on the macroscopic appearance were supported by the determined specific surface areas. Increasing amounts of mannitol or phenylalanine resulted in increasing specific surface areas underlining the change in structure at a microscopic level. By increasing amounts of either bulking agent, the microscopic structure addressed by scanning electron microscopy tended from flake-like structure towards the appearance known from conventionally freeze-dried, which is characterized by a highly porous structure composed of small pores and thin interstitial walls between the pores. Assessing the specific surface area had shown that changes in structure, which can be attributed to micro-collapse, can be clearly identified if present. Obviously, for increasing amounts of bulking agent the crystalline part of the matrix prevents macro- or even micro-collapse. The evidence for the at least partly crystalline structure of the bulking agents at medium to high contents have been shown by powder X-ray diffraction analysis. Mannitol gave a strong crystalline signal of the stable δ-polymorph while phenylalanine crystallized in the hydrate form. Overall, there seems to be a slight tendency of sucrose seeming to depress crystallization slightly more effective.

An increase of the amount of bulking agent resulted in a decrease in residual water content. This effect can also be clearly ascribed to the increase in specific surface area with increasing bulking agent content, since increase in SSA makes secondary drying mechanism more effective. Consequently, glass transition temperatures were increased for increasing amounts of phenylalanine. Although, increasing amounts of mannitol resulted in decreased residual water contents, the glass transition temperatures did not show an increase as well. Mannitol was utilized in far higher weight-to-weight ratios (bulking agent to sugar) compared to phenylalanine to achieve comparable effects regarding physico-chemical characteristics. Therefore, it is reasonable that the glass transition temperatures did not increase since the residual water content is distributed only in the amorphous phase.

The product temperatures of all formulations were remarkably above the glass transition temperature of the maximally freeze-concentrated solution (Tg'). Therefore, at least micro-collapse necessarily occurred in all formulations during the primary drying stage. The differences in the microscopic structure (i.e. specific surface area), which are depending on the amount of bulking agent in the formulation, resulted in slightly different product temperatures, too. Overall, higher amount of bulking agent resulted in slightly higher product temperatures. This effect can be attributed to the higher resistance of the non-collapsed samples aggressively freeze-dried from high bulking agent containing formulations.

Comparing both bulking agents utilized in this study, phenylalanine resulted in products with superior characteristics compared to mannitol. For the stabilization of a pharmaceutical API (e.g. a protein), usually an amorphous lyophilizate with low residual water content and high glass transition temperature represents the aimed goal [17]. As pure sugar formulations result in time consuming freeze-drying cycles in order to achieve low

residual water contents and acceptable appearance of the product, bulking agents (most often mannitol) are included to improve the drying behavior. However, especially for very sensitive molecules, the formation of additional surfaces by crystallization of a bulking agent within the lyophilizates can be detrimental. A formulation, showing full amorphous structure, low residual moisture, high glass transition temperature, and that can be freeze-dried fast, would be worth striving for. Leaving out the macroscopic appearance, aggressively freeze-dried formulations based on sucrose or trehalose and low amounts of phenylalanine provides all desired qualities. The addition of higher amounts of phenylalanine also results in macroscopic elegant cakes, however, at the costs of forming crystalline material in the lyophilizate.

4. AMINO ACIDS AS BULKING AGENTS

Inspired by the efficient benefit of phenylalanine as bulking agent for aggressive freeze-drying of sugar-based formulations, a number of further amino acids were investigated regarding their suitability to act as bulking agent. Amongst the selection of amino acids, glycine is the most prominent representative, which is reported to be used as bulking agent in some formulations [15]. Based on either sucrose or trehalose, the formulations shown in Table 5.3 were subjected to aggressive freeze-drying in 2R vials with 1ml fill according to the protocol shown in Table 3.4 (page 55) by using a FTS LyoStar II freeze-drier. Since phenylalanine has shown favorable physico-chemical characteristics at a sugar to phenylalanine ratio of 10:0.5 (see previous section), this weight-to-weight ratio was also applied for the investigation of the further amino acids. The formulations were composed at a total solid content of 50 mg/ml.

The resulting lyophilizates were characterized regarding macroscopic appearance, specific surface area, residual water content, glass transition temperature, and molecular structure. In addition, the drying behavior of the different formulations was followed by recording the product temperatures in order to check for potential differences in the product temperatures during the quasi steady state within the primary drying phase. Moreover, product temperature data allows for a rough determination of the endpoint of primary drying and therefore an estimate of drying kinetics.

Table 5.3: Formulations to characterize the physico-chemical characteristics of aggressively freeze-dried formulations containing various amino acids.

formulation	sucrose	trehalose	arginine	glycine	histidine	isoleucine	leucine	methionine	tryptophan
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc:arg	47.5		2.5						
suc:gly	47.5			2.5					
suc:his	47.5				2.5				
suc:iso	47.5					2.5			
suc:leu	47.5						2.5		
suc:met	47.5							2.5	
suc:trp	47.5								2.5
tre:arg		47.5	2.5						
tre:gly		47.5		2.5					
tre:his		47.5			2.5				
tre:iso		47.5				2.5			
tre:leu		47.5					2.5		
tre:met		47.5						2.5	
tre:trp		47.5							2.5

Product temperature

Having a look at the product temperatures firstly, all formulations showed similar product temperatures during primary drying (cf. Table 5.4), which range around -17.5 °C. The mean product temperatures were calculated by average of the temperature during the plateau, which is characteristic for the sublimation phase of freeze-drying.

Table 5.4: Product temperatures during primary drying of aggressive freeze-drying of sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5.

	product temperature primary drying			
amino acid	sucrose based		trehalose based	
	mean	SD	mean	SD
	°C	°C		
arginine	-16.5	0.3	-17.0	0.5
glycine	-18.0	0.5	-17.4	0.4
histidine	-17.8	0.3	-17.2	0.9
isoleucine	-17.6	0.3	-16.7	1.2
leucine	-17.7	0.2	-17.1	0.9
methionine	-17.5	0.4	-17.6	0.5
tryptophan	-17.8	0.4	-17.1	0.5

No apparent differences were detected for the different formulations. Product temperatures were comparable to sucrose- and trehalose-based formulations that contain a low amount of bulking agent (mannitol or phenylalanine; compare Table 5.2). It needs to be mentioned that latter samples additionally contained 1 mg/ml lysozyme. Since also a 5% (wt/wt) sucrose formulation, which contained no protein or bulking agent, exhibited a product temperature of -17.2 °C (compare Table 4.1, page 69) the addition of low

amounts of bulking agents or protein does apparently not influence the product temperature. In addition, the endpoint of primary drying (characterized by the increase of the product temperature towards shelf temperature) is comparable for all formulations (data not shown). The progress of the product temperature during the aggressive freeze-drying process is similar but slightly different for the different formulations. While the formulations containing arginine, glycine, histidine, and tryptophan showed the jagged progress comparable to aggressively freeze-drying of pure sugar solutions, the formulations containing isoleucine, leucine, and methionine showed a smooth progress, which is characteristic for formulations containing e.g. sugar and phenylalanine at a ratio of at least 10:0.5.

Macroscopic appearance

The macroscopic appearance of the lyophilizates are displayed in Figure 5.13. The samples that were aggressively freeze-dried from formulations containing arginine, glycine, histidine, and tryptophan exhibited a collapsed, foam-like appearance comparable to samples aggressively freeze-dried from pure sugar formulations.

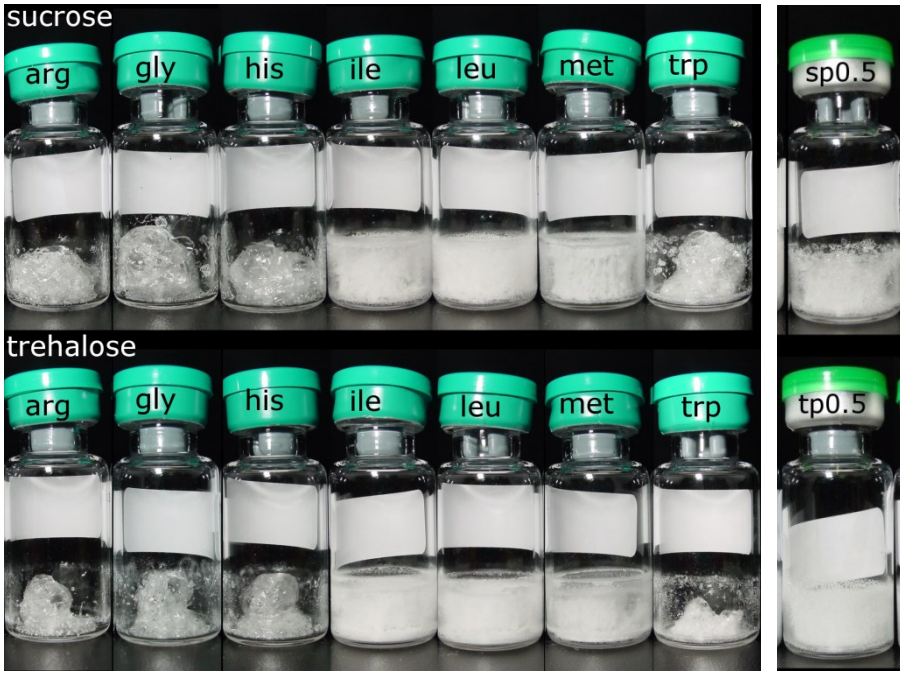

Figure 5.13: Macroscopic appearance of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5 (upper row sucrose based, lower row trehalose based). For comparison suc:phe 10:0.5 (sp5) and tre:phe 10:0.5 (tp0.5) is shown.

In contrast, lyophilizates containing isoleucine, leucine, and methionine showed a collapsed but more structured, sponge-like appearance. Obviously, the addition of arginine, glycine, histidine, and tryptophan in low concentrations had no effect on the drying behavior (i.e. product temperature and macroscopic appearance), while isoleucine, leucine and methionine affected the drying behavior and result. Regarding macroscopic appearance, latter samples were similar to the lyophilizates aggressively freeze-dried from sugar:phenylalanine (10:0.5) formulations.

Residual water content

The residual water content of the aggressively freeze-dried samples was analyzed via Karl-Fischer titration. By analogy to macroscopic appearance, the residual water content of the samples containing arginine, glycine, histidine, and tryptophan exhibit high values comparable to the samples containing no amino acid (cf. Figure 5.14). Sucrose based samples showed residual water contents of around 2%, while trehalose based samples ended up at of 2.8% and above.

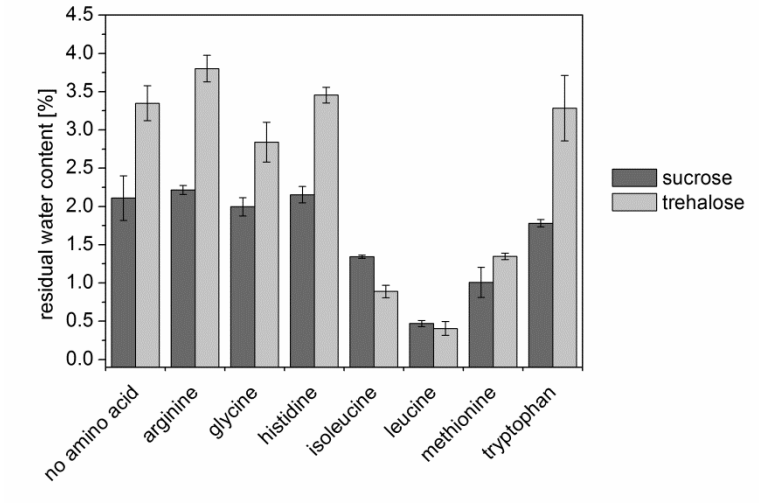

Figure 5.14: Residual water content of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5.

The addition of isoleucine, leucine, and methionine on the contrary resulted in markedly decreased residual water contents, most pronounced for the formulations containing leucine. Latter formulations exhibited residual water contents of 0.5% (sucrose based) and 0.4% (trehalose based). However, also the reduction to 1.3% / 0.9% by isoleucine and 1.0% / 1.3% by methionine expresses a substantial potential of those amino acids to act as bulking agent in the capacity of drying enhancer.

Glass transition temperature

Since water acts as plasticizer of the amorphous matrix, glass transition temperatures are inherently linked to the residual water content. As expected, the group of samples exhibiting no reduction of residual water content did not alter the glass transition temperature compared to the pure sugar formulations. Sucrose-based formulation showed glass transition temperatures of 47.2 °C to 50.2 °C, trehalose-based samples of 57.3 °C to 61.3 °C (cf. Figure 5.15). Amino acids that showed an impact on the residual water content (iso, leu, met), resulted in an increase of glass transition temperatures. While there is only a slight increase for sucrose-based samples, trehalose-based samples exhibited a steep increase of larger 20 K to glass transitions to above 88 °C.

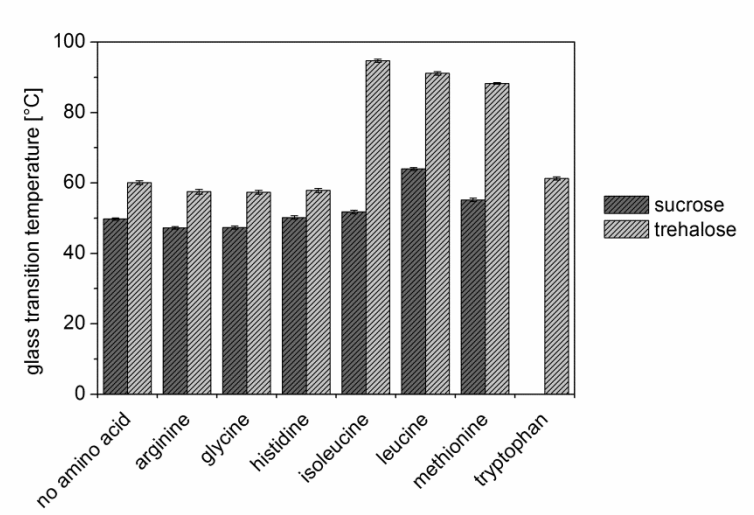

Figure 5.15: Glass transition temperature of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5 (n=3; suc:trp missing due to failure during measurement).

Specific surface area

In the previous section (3) it was shown that for increasing amounts of bulking agent the specific surface area was increased as well. Assisted by the link between higher SSA and lower residual water content it was concluded that higher SSA is the major determinant for decreased residual water content. Therefore, the specific surface areas of the aggressively freeze-dried lyophilizates containing different amino acids were evaluated.

Figure 5.16 shows the results of BET gas adsorption analysis. Keeping in mind a SSA of around 1 m^2/g of conventionally freeze-dried sugar formulations, low SSA of about 0.1 m^2/g were detected, underlining the collapsed nature of all samples. For trehalose-based samples, a clear trend of slightly increased SSA (0.13 to 0.16 m^2/g) of lyophilizates containing isoleucine, leucine, or methionine is detectable as expected. However, sucrose-based samples did not show a similar clear trend. On the contrary, suc:arg exhibited the overall higher SSA for sucrose based samples (0.16 m^2/g), which is regarded as outlier, however. Although the macroscopic appearance was quite different, material of similar SSA was formed during aggressive freeze-drying.

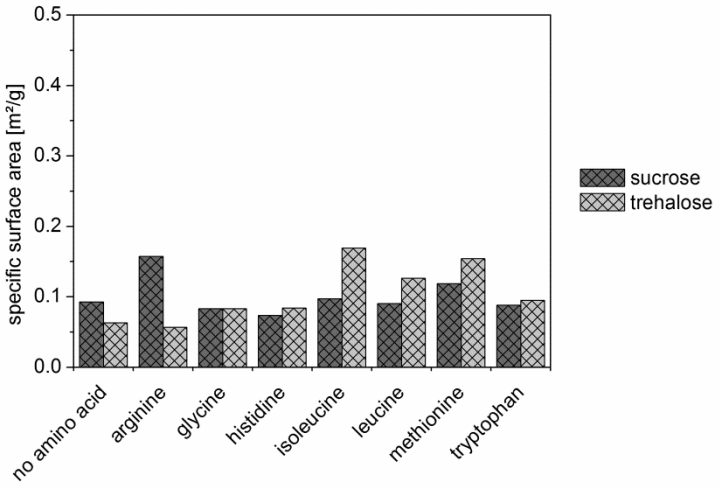

Figure 5.16: Specific surface area of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5.

Crystallinity

To complete the picture, samples of all aggressively freeze-dried formulations were analyzed utilizing powder X-ray diffraction (XRD). The resulting diffraction patterns are shown in Figure 5.17.

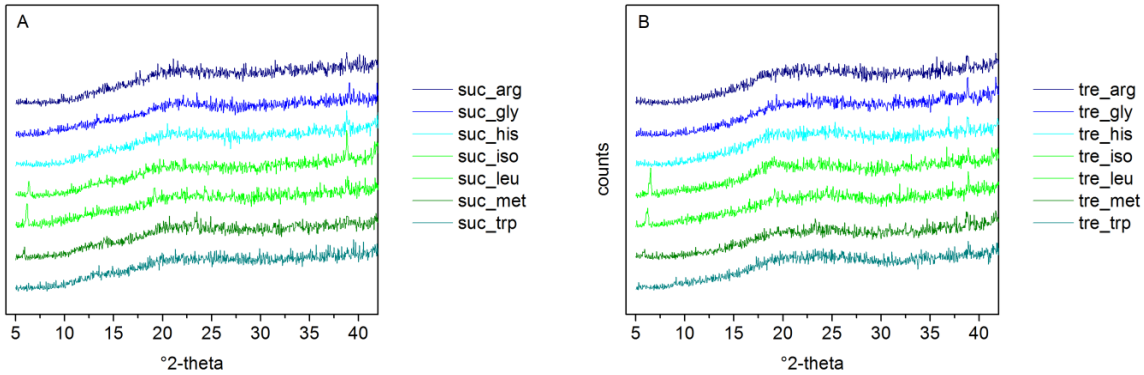

Figure 5.17: Diffraction patterns of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5 (A: sucrose-based formulations, B: trehalose-based formulations).

All samples show a predominantly amorphous structure expressed by an amorphous halo and the lack of pronounced diffraction peaks. However, by having a closer look, at least one characteristic diffraction peak can be observed and definitely identified for each of the amino acids L-isoleucine (6.4° 2-theta), L-leucine (6.1° 2-theta), and L-methionine (5.8° 2-theta). According to XRD, all other amino acid containing formulations showed purely amorphous structure.

Summary

The investigation concerning the ability of different amino acids to act as bulking agent at low sugar to amino acid weight-to-weight ratio revealed that L-isoleucine, L-leucine, and L-methionine are able to change the resulting physico-chemical characteristics of aggressively freeze-dried model formulations based on either sucrose or trehalose. By the addition of either one of the before mentioned amino acids, the appearance and the residual water content could be influenced. Especially for trehalose-based samples, a remarkable increase of glass transition temperature was detected, as well. Although a remarkable change of the macroscopic appearance from foamy collapsed to sponge-like collapsed could be discerned, the specific surface areas were hardly increased. Compared to amino acids that did not show any effect on the physico-chemical characteristics (L-arginine, L-glycine, L-histidine, L-tryptophan) at the investigated low concentrations, the above mentioned three amino acids at least partly crystallized during aggressive freeze-drying. Hence, the most probable explanation for the effect on the physico-chemical characteristics offers the crystalline state of these amino acids. Comparing the substance properties of all investigated amino acids, the combination of high hydrophobicity and low water solubility seems to result in crystallization of iso, leu, and met. Interestingly, although having comparable substance properties like leucine and methionine, phenylalanine did not crystallize during aggressive freeze-drying as shown above.

5. SOLID CONTENT OF THE FORMULATION

The formulations investigated so far and shown above have been fixed to an overall solid content of 50mg/ml, which represents a solid content frequently used for protein formulations. Nevertheless, in general the solid content of a protein formulation can be varied due to various requirements (e.g. stabilization, tonicity). To account for the effect of different solid contents on the resulting product from aggressive freeze-drying, a range of formulations of solid contents of 25 mg/ml up to 150 mg/ml was aggressively freeze-dried. Pure sugar (sucrose or trehalose) formulations or mixtures of sugar and phenylalanine (at a ratio of 10:0.5) were utilized, respectively. The formulations used for this study can be found in Table 5.5. 1 ml aliquots of the respective formulations were filled in 2R vials and subjected to the aggressive freeze-drying protocol shown in Table 3.5 (page 56) in a FTS LyostarII freeze-drier (process settings: pc=1 mbar, Ts=45 °C). The resulting lyophilizates were analyzed regarding physico-chemical characteristics (macroscopic appearance, residual water content, glass transition temperature, specific surface area, molecular structure/crystallinity).

Table 5.5: Formulations to characterize the physico-chemical characteristics of aggressively freeze-dried formulations of different solid contents.

formulation	sucrose	trehalose	phenylalanine	solid content
	mg/ml	mg/ml	mg/ml	mg/ml
suc_25	25			25
suc_50	50			50
suc_75	75			75
suc_100	100			100
suc_150	150			150
tre_25		25		25
tre_50		50		50
tre_75		75		75
tre_100		100		100
tre_150		150		150
suc:phe_25	23.81		1.19	25
suc:phe_50	47.62		2.38	50
suc:phe_75	71.43		3.57	75
suc:phe_100	95.24		4.76	100
suc:phe_150	142.86		7.14	150
tre:phe_25		23.81	1.19	25
tre:phe_50		47.62	2.38	50
tre:phe_75		71.43	3.57	75
tre:phe_100		95.24	4.76	100
tre:phe_150		142.86	7.14	150

Macroscopic appearance

Figure 5.18 displays the macroscopic appearance of the samples aggressively freeze-dried from various solid contents. As already experienced from other studies (compare above) both, sucrose and trehalose, showed a comparable, collapsed appearance, which best can be described as foamy matrix. For trehalose at a low solid content of 25 mg/ml the formation of a foamy matrix during aggressive freeze-drying seems to be hindered. This sample resulted in a flat rigid layer, still showing small solid bubbles on the surface of the dried material. Overall, trehalose seems to exhibit slightly less voluminous formation of foamy structure. Independent from choice of sugar, increasing solid content resulted in increased apparent volume of the foamy matrix. Obviously, by increasing the amount of sugar, literally more material can participate in the formation of the interstitial walls of the matrix and therefore the volume of the whole matrix is increased. Figure 5.18 also shows the macroscopic appearance of formulations containing phenylalanine at a sugar to phenylalanine weight-to-weight ratio of 10:0.5. In contrast to the formulations containing no phenylalanine, a change in solid content did not result in a massive change of the apparent volume of the aggressively freeze-dried sample.

Figure 5.18: Macroscopic appearance of formulations of different solid contents of the initial solution (solid content in mg/ml showed as numbers on the vial). Formulations contain either no bulking agent (left) or phenylalanine at a sugar to phenylalanine weight-to-weight ratio of 10:0.5 (right).

However, especially for sucrose-based formulations a solid content of 25 and 50 mg/ml resulted in sort of shrinkage of the samples expressed as less contact between the inner walls of the vial and the dried sample. This phenomenon can be observed for tre:phe_25, too. Independent from sugar or solid content, the samples exhibited a sponge-like structure with macroscopically clearly visible pores. Lower solid contents seem to exhibit a somewhat more filigree structure compared to samples of higher solid contents. As all samples showed an appearance not comparable to conventionally freeze-dried samples, they are regarded as collapsed.

Product temperature

The product temperatures detected at the plateau during sublimation (primary drying) are listed in Table 5.6. Neither for sucrose formulations nor for sucrose-based formulations containing phenylalanine, the variation of solid content resulted in a significant change of the product temperature. For all formulations the product temperatures were measured at around -18 °C. While the product temperature of trehalose formulation also ranged round -18 °C, tre:phe formulations exhibited slightly higher product temperatures of about -16 °C (cf. Table 5.6). However, due to the limited number of thermocouples available, only the product temperature of the lowest and highest solid content was monitored for trehalose and trehalose based samples. As shown above, increased resistance of the already dry product matrix against passing water vapor can be accounted

for increased product temperatures. Since the resistance of the product matrix is connected to the microscopic structure of the product, specific surface area data could support the idea outlined above.

Table 5.6: Product temperatures during primary drying of aggressive freeze-drying of sugar-based formulations from various solid contents.

solid content	product temperature primary drying							
	sucrose		suc:phe 10:0.5		trehalose		tre:phe 10:0.5	
	mean	SD	mean	SD	mean	SD	mean	SD
mg/ml	°C	°C	°C	°C	°C	°C	°C	°C
25	-18.1	0.2	-17.9	0.3	-18.7	0.2	-15.3	0.3
50	-17.9	0.3	-17.2	0.3	*	*	*	*
75	-18.4	0.2	-17.8	0.3	*	*	*	*
100	-18.0	0.4	-17.2	0.2	*	*	*	*
150	-18.1	0.2	-17.9	0.3	-17.3	0.4	-16.7	0.2

*not determined due to limited number of thermocouples

Specific surface area

Figure 5.19 shows the specific surface area data of the aggressively freeze-dried samples. Focusing first on the formulation that exhibited slightly higher product temperatures (tre:phe), in fact, consistently higher SSA could be detected for the different samples of that formulation compared to all other samples at the respective solid content. Moreover, increasing solid content resulted in decreased specific surface area. Starting from 0.34 m^2/g for a solid content of 25 mg/ml, the SSA is stepwise decreased to 0.10 m^2/g for a solid content of 150 mg/ml. This decrease in SSA for increasing solid contents would underline the slightly higher product temperature of tre:phe_25 compared to tre:phe_150. On the other hand, also markedly higher SSA of suc:phe_25 compared to all other sucrose or suc:phe samples did not affect the resulting product temperature.

In contrast to the tre:phe samples, for suc:phe lyophilizates a steep decrease of the SSA (from 0.24 to 0.09 m^2/g) was detected for an increase of solid content from 25 mg/ml to 50 mg/ml. A further increase to 150 mg/ml resulted in no further decrease in SSA.

For sucrose and trehalose formulations, a change in solid content between 50 mg/ml and 150mg/ml did not result in a change of SSA. The available amount of suc_25 and tre_25 was not sufficient to result in a measurement that met the criteria for a BET fit. Therefore, data for these formulations are missing.

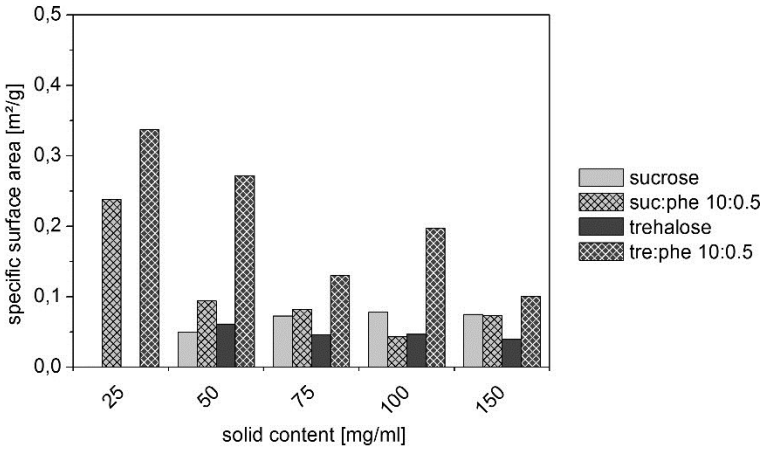

Figure 5.19: Specific surface area of aggressively freeze-dried sugar-based formulations of various solid content of the initial solution (sugar-based formulations with and without addition of phenylalanine at a ratio of 10:0.5 are shown).

Residual water content

As shown in section 3 of this chapter, the addition of low amounts of phenylalanine to sucrose and trehalose resulted in drastically reduced residual water content for a solid content of 50 mg/ml. It was of interest, if the same effect can be observed for the investigated range of solid contents from 25 to 150 mg/ml, which represents a typical range for pharmaceutical protein formulations [15].

The results of Karl-Fischer titration of aggressively freeze-dried samples are shown in Figure 5.20. In this study, a comparably short aggressive freeze-drying protocol (overall 8 h drying time) was used. Hence, the samples containing no phenylalanine exhibited high residual water contents. Independent from solid content, for sucrose samples a residual water content of around 3% was detected. In contrast, trehalose samples revealed a decrease of residual water content for increasing solid content. Starting from around 6% residual water content for a solid content of 25 mg/ml and 50 mg/ml, a stepwise decrease to 3.6% (75 mg/ml), to 2.4% (100 mg/ml), and to 1.9% (150 mg/ml) was detected. Obviously, the water was removed more effectively for higher solid contents compared to lower solid contents.

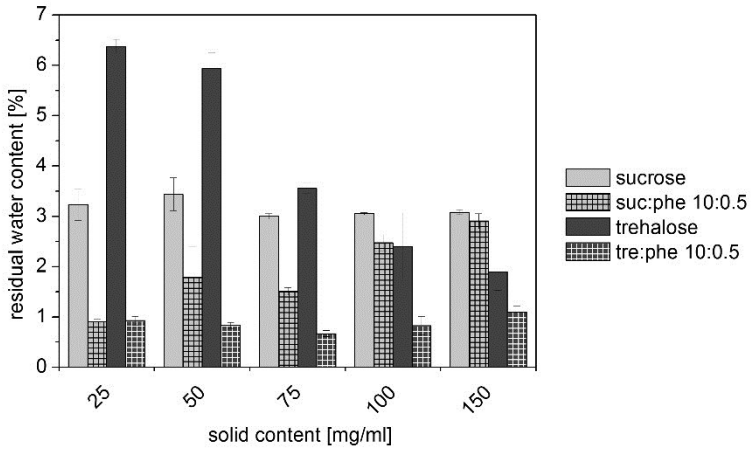

Figure 5.20: Residual water content of aggressively freeze-dried sugar-based formulations of various solid content of the initial solution (sugar-based formulations with and without addition of phenylalanine at a ratio of 10:0.5 are shown).

As already observed in section 3, the addition of phenylalanine at a weight-to-weight ratio of 10:0.5 result in a steep decrease of residual water content. For sucrose-based samples (suc:phe) at a solid content of 25 mg/ml, the residual water content is decreased from 3.2% to 0.9% for example. Remarkably, increasing solid content of suc:phe resulted in an increase in residual water content, ending up at residual water contents comparable to the samples without phenylalanine for the highest solid content (150 mg/ml). In contrast to suc:phe samples, independent from the solid content, the tre:phe samples showed a comparable residual water content of 0.7% to 1.1%.

Overall, there is no clear trend regarding the influence of solid content on the residual water content. While the residual water content of aggressively freeze-dried sucrose lyophilizates is unaffected by the solid content, higher solid contents resulted in lower residual water of aggressively freeze-dried trehalose lyophilizates. The addition of phenylalanine to the sugar based formulations changed the picture. While for low solid content the residual water content is reduced drastically compared to the sugar formulations, sucrose-based lyophilizates containing phenylalanine exhibited an increase in residual water content for increasing solid content. On the other hand, trehalose-based samples exhibited comparable, low residual water contents for all solid contents investigated.

Glass transition temperature

The analysis of the glass transition temperatures (Tg) of the aggressively freeze-dried samples revealed trends, which fit to the results of the residual water content of the samples. Since water acts as plasticizer on the amorphous matrix, usually higher residual water contents are linked to lower glass transition temperatures and vice versa. Table 5.7 lists the glass transition temperatures analyzed via DSC.

As expected from the comparable residual water contents, DSC analysis showed no influence of solid content on Tg of aggressively freeze-dried sucrose samples. For sucrose-based samples that contain phenylalanine, a decrease of Tg was noticed for increasing solid contents. Aggressively freeze-dried trehalose lyophilizates, however, exhibited an increase of Tg for increasing solid contents. Independent from solid content, tre:phe

samples overall showed high glass transition temperatures of above 90°C. Only the lyophilizates freeze-dried from 150 mg/ml solid content exhibited a lower glass transition, which can be attributed to the higher water content of these species.

Overall, it can be summarized, that the inversely proportional link between (e.g. high) glass transition temperatures and (e.g. low) residual water content can be established for all analyzed samples. The addition of phenylalanine increased the glass transition temperature pronouncedly. While for higher solid contents this effect vanished for sucrose-based samples, for trehalose-based samples (containing phenylalanine) it still was at a solid content of 150 mg/ml the Tg is 25.7 K above the Tg of the trehalose samples.

Table 5.7: Glass transition temperatures of aggressively freeze-dried sugar-based formulations from various solid contents analyzed by differential scanning calorimetry (DSC, n=3).

	glass transition temperature							
solid content	sucrose		suc:phe 10:0.5		trehalose		tre:phe 10:0.5	
	mean	SD	mean	SD	mean	SD	mean	SD
mg/ml	°C	°C	°C	°C	°C	°C	°C	°C
25	41.3	0.8	62.4	0.6	36.6	0.6	94.3	0.2
50	43.9	1.1	54.0	0.8	43.5	2.0	94.8	0.7
75	43.8	1.0	56.1	1.1	48.8	1.0	97.3	0.8
100	45.4	1.3	49.3	1.1	56.2	3.1	91.2	2.3
150	43.2	0.8	48.8	0.0	51.1	0.4	76.8	0.7

Crystallinity

In order to characterize the morphology of the aggressively freeze-dried samples, they were analyzed using X-Ray powder diffraction (XRD). The resulting diffraction patterns are shown in Figure 5.21. Since for none of the samples any distinct diffraction peak could be observed (small peak at 38.95° 2-θ observed for suc_25 and tre_25 can be unequivocally attributed to the background copper sample holder), all samples are regarded as fully amorphous. For sucrose and suc:phe, as well as tre and tre:phe, the characteristic amorphous halo of the respective sugar can be observed. Obviously, the variation of solid content did not result in a change of the morphology of the aggressively freeze-dried samples.

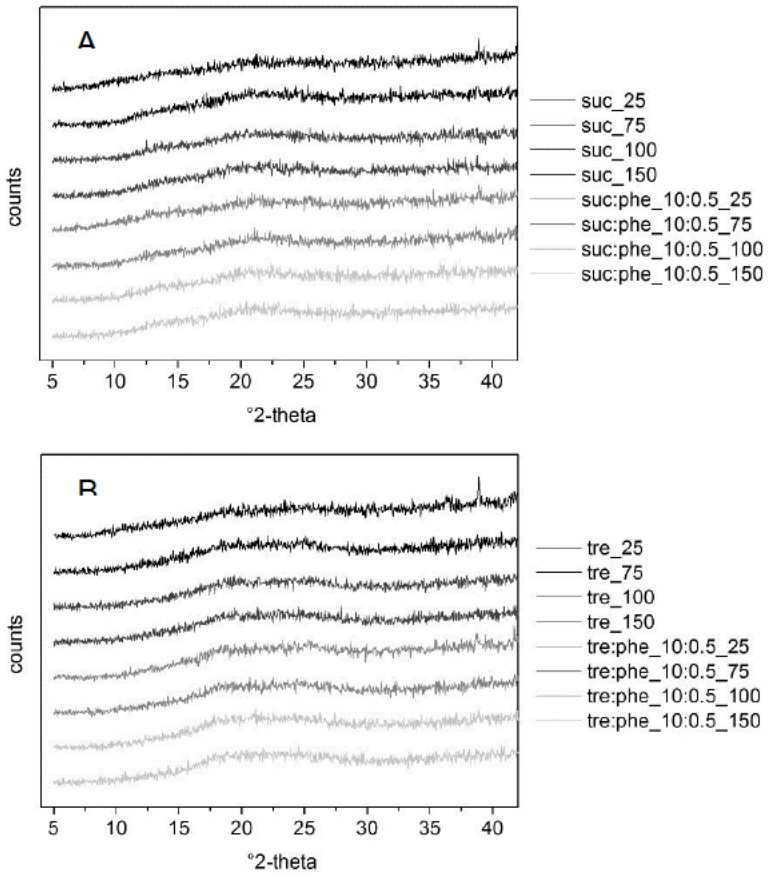

Figure 5.21: XRD diffraction pattern of aggressively freeze-dried sugar-based formulations of various solid content of the initial solution (sugar-based formulations with and without addition of phenylalanine at a ratio of 10:0.5 are shown; A: sucrose-based formulations, B: trehalose-based formulations).

Summary

The investigation of the formulations, which were subjected to aggressive freeze-drying, revealed that a number of physico-chemical characteristics are influenced by the solid content of the initial solution. An overview of the physico-chemical data is given in Table 5.8.

The overall foamy structure of collapsed sucrose and trehalose is maintained for the different solid contents (except 25 mg/ml). However, the volume of the foamy structure increased by increasing solid content (identical initial filling height). Showing inherently a different, but still collapsed, sponge-like appearance, the samples containing phenylalanine did not show a change in "cake" volume, but rather an apparent increase in the density of the structure. This phenomenon resulted in a slight increase of the product temperature during sublimation especially for the tre:phe samples, whereas in general the product temperature is rather unaffected by the solid content.

Table 5.8: Overview of physico-chemical characteristics of aggressively freeze-dried sugar-based formulations of various solid content of the initial solution (sugar-based formulations with and without addition of phenylalanine at a ratio of 10:0.5 are shown).

solid content	sucrose			suc:phe 10:0.5			trehalose			tre:phe 10:0.5		
	Tg (mean ± SD)	water content (mean ± SD)	SSA	Tg mean ± SD	water content (mean ± SD)	SSA	Tg (mean ± SD)	water content (mean ± SD)	SSA	Tg (mean ± SD)	water content (mean ± SD)	SSA
mg/ml	°C	%	m²/g	°C	%	m²/g	°C	%	m²/g	°C	%	m²/g
25	41.3 ± 0.8	3 2 ± 0.3	n.a.	62.4 ± 0.6	0.9 ± 0.0	0.24	36.6 ± 0.6	6.4 ± 0.1	n.a.	94.3 ± 0.2	0.9 ± 0.1	0 34
50	43.9 ± 1.1	3.4 ± 0 3	0 05	54.0 ± 0.8	1.8 ± 0.6	0.09	43.5 ± 2.0	5.9 ± 0.3	0.06	94.8 ± 0.7	0.8 ± 0.1	0 27
75	43.8 ± 1.0	3.0 ± 0.1	0 07	56.1 ± 1.1	1.5 ± 0.1	0.08	48.8 ± 1.0	3.6 ± 0.1	0.05	97.3 ± 0.8	0.7 ± 0.1	0.13
100	45.4 ± 1.3	3.1 ± 0.0	0 08	49.3 ± 1.1	2.5 ± 0.2	0.04	56.2 ± 3.1	2.4 ± 0.7	0.05	91.2 ± 2.3	0.8 ± 0 2	0 20
150	43.2 ± 0.8	3.1 ± 0.0	0 08	48.8 ± 0.0	2.9 ± 0.1	0.07	51.1 ± 0.4	1.9 ± 0.4	0.04	76.8 ± 0.7	1.1 ± 0.1	0.10

n.a.: due to low amount of sample BET SSA analysis not possible

Regarding the specific surface area, the aggressive freeze-dried samples exhibited low SSA compared to conventionally freeze-dried samples. While the SSA of sucrose and trehalose samples were not affected by the solid content, both, suc:phe as well as tre:phe showed a decrease in SSA for increasing solid content. Since the apparent volume of the "cake" did not increase, it is conclusive that the increase in solids (i.e. simply more material) result in a structure possessing thicker interstitial structure between the large pores.

As it is the native purpose of drying to reduce the water content, the residual water content is a key parameter also for aggressive freeze-drying. Both disaccharides used in this study represent the most frequently used stabilizers in freeze-dried pharmaceutical protein formulations. The residual water contents of the lyophilizates without phenylalanine are regarded as comparably high (> 2%) for freeze-dried products. This is owed to the short drying time of only 6 h at 45 °C shelf temperature. Although both sugars are closely related, the resulting lyophilizates showed quite different trends regarding the influence of solid content on the residual water content. All lyophilizates aggressively freeze-dried from sucrose formulations of various solid contents showed comparable residual water content. In contrast, trehalose lyophilizates exhibited decreased residual water contents for higher solid contents. In general, the residual water content is linked to the specific surface area of a lyophilizate, since the surface area available for desorption directly influences the amount of water that is removed under comparable conditions (temperature, pressure). Surprisingly, the SSA data of trehalose samples did not show an increase in SSA for increasing solid content, which would explain the lower water content of samples of higher solid content. On the other hand, the macroscopic appearance of samples of lower solid content indicates a somewhat denser structure compared to higher solid contents (see Figure 5.18). However, the macroscopic structure seems not to offer a comprehensive explanation for the substantial decrease of the residual water content. The

formation of a crystalline (bulk) structure would offer a possible explanation for better drying. Though, neither XRD diffractions nor DSC melting endotherms were noticed, which would indicate the presence of crystalline trehalose.

The addition of phenylalanine to the still sugar dominated formulations (phenylalanine added at a weight-to-weight ratio of 10:0.5) resulted in a substantial decrease of water content of samples at low solid content. The addition of phenylalanine obviously resulted in an effect within the excipient matrix, which is similar to the effect of a bulking agent. Nevertheless, XRD and DSC indicate the absence of crystalline material. Hence, at the low contents used in that study, phenylalanine did not crystallize, but act as a drying enhancer anyway. For increasing solid content, different trends of sucrose-based and trehalose-based samples were observed. The residual water content of trehalose-based samples remained low for the different solid contents, whereas the residual water content of sucrose-based samples was increased with increasing solid content. Apparently, for increasing solid content the effect of phenylalanine as drying enhancer is diminished for sucrose-based lyophilizates. On the other hand, for trehalose-based samples the unknown effect works over the whole range of investigated solid contents.

The trends outlined for the residual water content are assisted by the glass transition temperatures of the lyophilizates (naturally inversely proportional). Especially the formulations containing trehalose and phenylalanine exhibited high glass transition temperatures. Based on DSC and XRD data, all aggressively freeze-dried lyophilizates formulations are fully amorphous independently from solid content.

Based on the physico-chemical characterization of the lyophilizates aggressively freeze-dried from formulations of different solid contents, the characteristics comparable to samples analyzed elsewhere (compare CHAPTER 4, page 95) (low residual water content, high glass transition temperature, low SSA, amorphous nature) give reason to expect good stabilizing properties for biopharmaceuticals over the whole range of solid contents analyzed.

6. PROTEIN CONCENTRATION

Besides the composition of the excipient matrix, which is usually mainly based on stabilizers like disaccharides, and the freeze-drying process utilized, also the concentration of the molecule to be stabilized can potentially affect the characteristics of the resulting dried product. Therefore, in this study the effect of an increased protein concentration of 10 mg/ml compared to a protein concentration of 1 mg/ml was investigated. For this study, mab_T1 was utilized as model protein, which was added to a sugar-based excipient mixtures of a solid content of 50 mg/ml (cf. Table 5.9). 1ml aliquots of the respective formulations were filled in 2R vials and subjected to the aggressive freeze-drying protocol shown in Table 3.5 (page 56) in a FTS LyostarII freeze-drier. Besides the appearance of the aggressively freeze-dried samples, the resulting residual water content as key quality was investigated. In addition, glass transition temperatures and specific surface area of the lyophilizates were determined.

Table 5.9: Formulations used to study the protein stabilization in aggressively freeze-dried formulations of different solid contents.

formulation	mab_T1	sucrose	trehalose	phenylalanine	solid content
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc_1	1	50			51
suc_10	10	50			61
tre_1	1	50			51
tre_10	10	50			61
suc:phe_1	1	47.62		2.38	51
suc:phe_10	10	47.62		2.38	61
tre:phe_1	1		47.62	2.38	51
tre:phe_10	10		47.62	2.38	61

Macroscopic appearance

Figure 5.22 displays the macroscopic appearance of the aggressively freeze-dried samples. As already shown above, the addition of phenylalanine to formulations containing 1 mg/ml mab_T1 changed the macroscopic appearance from a foam-like to a rather sponge-like structure (left picture). The increased protein concentration of 10 mg/ml resulted in a sponge-like structure for the sucrose and trehalose based samples, too (right picture). For the sucrose lyophilizate, still some foamy parts were observed. In addition, also the appearance of the sucrose sample containing phenylalanine was slightly changed to a somewhat less coherent structure. Overall, the increase in protein concentration from 1 to 10 mg/ml resulted in minor changes in the appearance. Especially trehalose-based samples seem to exhibit similar appearance for both protein contents. Comparing the macroscopic appearance of trehalose samples containing 1 mg/ml or 10 mg/ml mab_T1 to the appearance of samples without added protein, it is noticeable that the samples containing no protein exhibited a comparable foam-like structure very similar to the su-

crose samples. Upon addition of only 1 mg/ml mab_T1 the appearance of trehalose samples exhibited a reduced volume and a less pronounced foamy structure compared to sucrose lyophilizates, which were sort of unaffected by the addition of protein.

Figure 5.22: Macroscopic appearance of lyophilizates aggressively freeze-dried from sugar based formulations containing 1 mg/ml (left picture) or 10 mg/ml (right picture) mab_T1 (suc: sucrose; sp: sucrose:phenylalanine 10:0.5; tre: trehalose; tp: trehalose:phenylalanine 10:0.5).

Residual water content

Since the addition of the higher amount of protein (10 mg/ml) showed an effect on the observed appearance of especially the sucrose lyophilizates, it can be expected that also other physico-chemical characteristics were changed by the increase of the protein content in the aggressively freeze-dried formulations. Figure 5.23 shows the residual water content of the analyzed samples, for comparison of sucrose and trehalose samples, also the residual water content of lyophilizates without protein is shown. While the residual water content of sucrose lyophilizates is hardly affected by the addition of 1 mg/ml protein, trehalose lyophilizates showed an increase in residual water content from 3.4% to 5.7%. The increase in residual water content of the latter sample is attributed to a change in the lyophilizate structure. The sample without protein showed a foam-like structure of a certain volume, while the addition of 1 mg/ml mab_T1 resulted in a decrease in volume and a dense structure of reduced volume. Obviously, this dense nature of the lyophilizate renders the desorptive drying more difficult.

A further increase of the protein concentration from 1 mg/ml to 10 mg/ml mab_T1, a significant increase in residual water content was detected. Only trehalose samples showed a slight decrease of water content (from 5.7% to 4.5%). The residual water content of tre:phe increased from 0.6% to 1.7%, and of suc:phe from 0.9% to 2.1%, respectively. In addition, the RWC of sucrose samples was increased from 2.8% to 5.2%.

In conventional freeze-drying, a higher protein concentration usually results in better drying (by a bulking effect of the protein). As shown above, by adding a higher concentration of protein, the macroscopic appearance was changed. Hence, also the microscopic structure is likely to be different. To account of the structure on the microscopic level, the specific surface area of the aggressively freeze-dried lyophilizates was analyzed using BET gas adsorption.

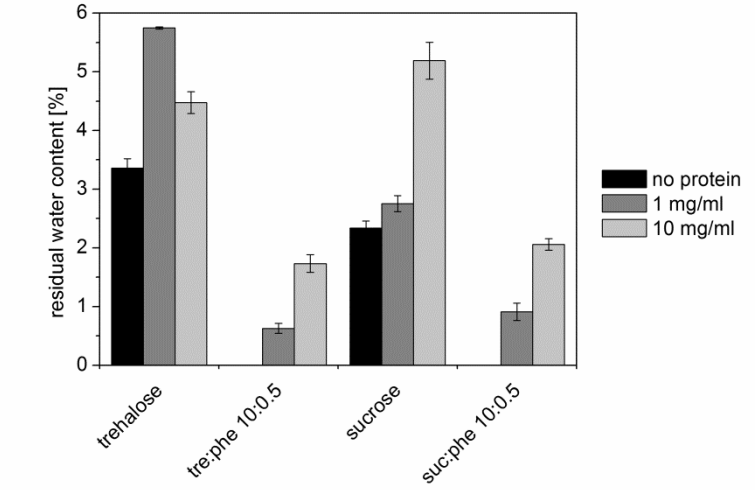

Figure 5.23: Residual water content of lyophilizates aggressively freeze-dried from sugar based formulations containing 1 mg/ml or 10 mg/ml mab_T1. For trehalose and sucrose lyophilizates also residual water content of samples containing no protein are shown.

Specific surface area

Figure 5.24 illustrates the determined SSA of the lyophilizates. Generally, the samples containing phenylalanine exhibited higher SSA compared to the samples aggressively freeze-dried from sugar formulations. However, the overall low SSA for all samples underlines the collapsed nature of these lyophilizates. Besides the trehalose samples, which seems to represents sort of a specialty, all samples showed a decrease of SSA for increased protein content. Obviously, the addition of 1 mg/ml and 10 mg/ml mab_T1 changed the microscopic structure toward a structure of less specific surface. Since the residual water of the lyophilizates usually depends on the effectiveness of secondary drying, a reduced SSA conclusively explains the increased residual water content for samples of higher protein content. It is known, however, that for higher protein contents than 10 mg/ml the protein itself works as bulking agent, represented by higher SSA and lower residual moistures for high protein concentrations [6].

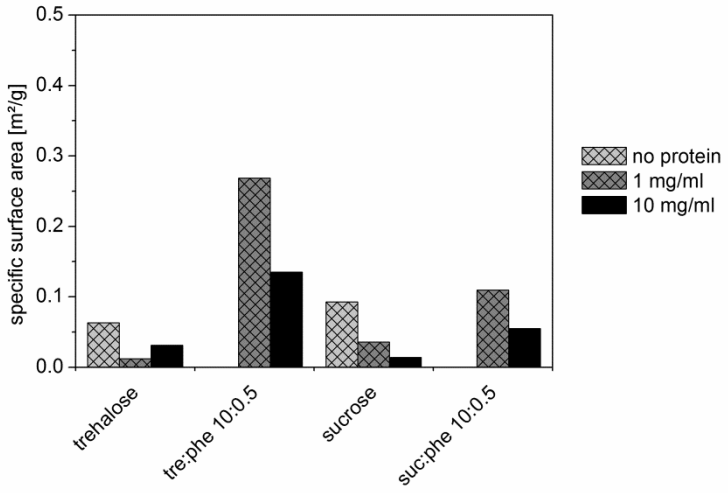

Figure 5.24: Specific surface area of lyophilizates aggressively freeze-dried from sugar based formulations containing no protein (only data for trehalose and sucrose available), 1 mg/ml or 10 mg/ml mab_T1.

Glass transition temperature

Figure 5.25 displays the glass transition temperatures of the aggressively freeze-dried samples containing 1 mg/ml or 10 mg/ml mab_T1 analyzed by DSC.

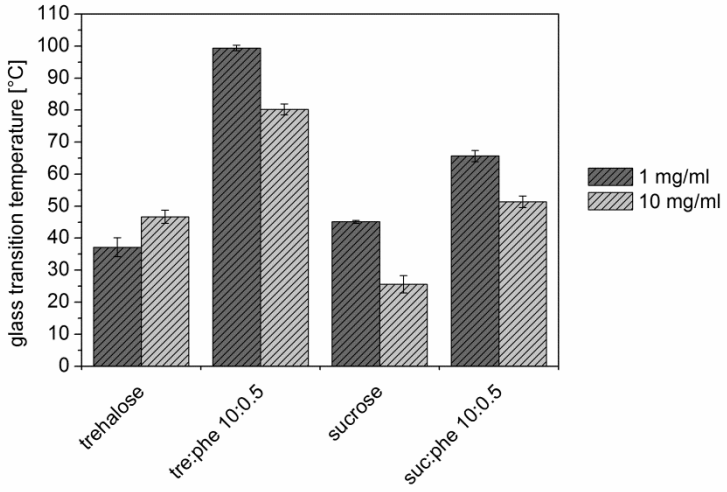

Figure 5.25: Glass transition temperatures of lyophilizates aggressively freeze-dried from sugar based formulations containing 1 mg/ml or 10 mg/ml mab_T1.

For samples containing phenylalanine, higher glass transition temperatures were detected compared to sugar lyophilizates. An increase of the protein content from 1 mg/ml to 10 mg/ml resulted in a decrease of the Tg for all samples, except the trehalose lyophilizate. While both samples containing phenylalanine and 10 mg/ml mab_T1 still exhibited relatively high glass transition temperatures of 80.2 °C and 51.4 °C, respectively, Tg of sucrose and trehalose lyophilizates were detected at 46.7 °C and 25.6 °C. It is obvious that lower glass transition temperatures are connected to higher residual water content (compare Figure 5.23). Generally, the Tg of lyophilizates containing 10 mg/ml protein and

phenylalanine are regarded as high enough for a considerable storage stability for incorporated sensitive molecules. On the other hand, lyophilizates aggressively freeze-dried from formulations containing no phenylalanine and 10 mg/ml mab_T1 exhibited rather low Tg. Therefore, the very short aggressive freeze-drying protocol would need to be prolonged in order to reach lower residual water contents and thus higher glass transition temperatures for the sugar formulations without phenylalanine.

Summary

To get an idea of the resulting physico-chemical characteristics of lyophilizates with moderately increased protein concentration (from 1 mg/ml to 10 mg/ml mab_T1), formulations based on sucrose or trehalose were aggressively freeze-dried with the short protocol. With a drying time of 8 h (2 h ramp to 45 °C and 6 h at 45 °C shelf temperature), this protocol had been developed for 1 mg/ml protein concentration and led to dry products for sugar to phenylalanine weight-to-weight ratios of 10:0.5 with residual water contents of 1% and below. The addition of higher amounts of protein revealed that the structure of the resulting collapsed lyophilizates was changed compared to a protein content of 1 mg/ml. This change in structure is partly macroscopically visible. Moreover, the analysis of the specific surface area of the resulting lyophilizates revealed a reduction of SSA, indicating a condensed microscopic structure. Since a denser structure renders secondary (desorptive) drying mechanisms less effective, the residual water contents are higher compared to samples of a protein content of 1 mg/ml. In consequence, the glass transition temperatures decreased. As protein itself is able to generate a bulking structure in lyophilizates [6], also samples containing no phenylalanine were included in the study. They exhibited no reduction of the (inherently high) residual water content, but rather an increase. Glass transition temperature followed that trend in the opposite direction. Obviously, the amount of protein that was applied in this study was not high enough to enable the formation of a bulking structure by the protein. In order to achieve residual water levels of around 1%, the duration of the drying protocol would need to be prolonged.

7. SUMMARY AND CONCLUSIONS

The present studies aimed to investigate the influence of formulation design on physico-chemical characteristics of aggressively freeze-dried lyophilizates. For this purpose, a range of freeze-drying formulations were selected, especially with respect to formulations that contain bulking agents in different quantities. Since for the majority of sensitive molecules, which are intended to be stabilized by freeze-drying, the utilization of an amorphous excipient is inevitable for stabilization, the investigated formulations are based on the most common cryo-/lyo-protectans, namely sucrose and trehalose.

In a first step, formulations comprising only sucrose or trehalose (in buffered aqueous solutions) were aggressively freeze-dried. The resulting lyophilizates from both sugars were amorphous and exhibited a foam-like collapsed appearance of low specific surface area. Due to the low SSA, the lyophilizates showed comparably high residual water content of above 1.5% (trehalose > sucrose) although freeze-dried at high shelf temperature (45 °C) for 24 h. Nevertheless, acceptable glass transition temperatures of above 50 °C were achieved (trehalose > sucrose).

In addition to amorphous stabilizers, freeze-drying formulations frequently contain bulking agents to improve the appearance and decrease drying time. The stepwise addition of bulking agents (mannitol, phenylalanine) to aggressive freeze-dried formulations resulted in decreasing degree of collapse, both, visible as well as detected by SSA measurements. Moreover, increasing amounts of bulking agents resulted in stepwise decreased residual water content and in case of phenylalanine also an increase of the glass transition temperature of the amorphous phase. XRD analysis revealed that the change in physico-chemical characteristics could be attributed to crystallization of the bulking agent.

Despite exhibiting collapse (i.e. low SSA) and a fully amorphous structure, formulations containing sugar and a low amount of phenylalanine (ratio 10:0.5) showed low residual water contents and high Tg. Since low SSA is reported to be potentially preferably for the stabilization of proteins in dried matrices [2, 26, 27], this special formulation are regarded as highly interesting for protein stabilization.

Since phenylalanine has turned out as promising excipients for freeze-drying, the ability of other amino acids to improve drying at a low sugar to amino acid weight-to-weight ratio (10:0.5) was investigated. In this study arginine, glycine, histidine, isoleucine, leucine, methionine, and tryptophan were used. Aggressive freeze-drying of the sugar/amino acid formulations revealed that isoleucine, leucine, and methionine are able to decrease the residual water content. Especially for trehalose-based samples, a remarkable increase of glass transition temperature was detected, as well. Moreover, the samples collapsed to a sponge-like appearance, which was somewhat different to the foam-like appearance of collapsed pure sugar samples. Nevertheless, the specific surface areas were hardly increased. Lyophilizates that contain arginine, glycine, histidine, or tryptophan showed no effect of the amino acids on the physico-chemical characteristics compared to pure sugar samples. XRD showed that isoleucine, leucine, and methionine crystallized during aggressive freeze-drying. Hence, the crystalline state of these amino acids

offer the most probable explanation for the effect on the physico-chemical characteristics. Since the intended use of a bulking agent is the formation of a crystalline "backbone" of a lyophilizate, these amino acids are generally regarded as promising bulking agents. However, compared to phenylalanine, none of the investigated amino acids exhibited a comparable effect on the physico-chemical characteristics: Phenylalanine improved the physico-chemical characteristics but remained fully amorphous.

In a separate study, the influence of different solid contents of the formulations (25 - 150 mg/ml) on the physico-chemical properties was evaluated. Both, sucrose and trehalose formulations were studied. Additionally, formulations that contain phenylalanine at a ratio of 10:0.5 (sugar:phe) were included in the study. All of the aggressively freeze-dried lyophilizates exhibited a fully amorphous structure regardless of solid content.

To achieve low water content (and consequently high glass transition temperatures), the most promising results of a formulation without phenylalanine was achieved for the trehalose samples of 150 mg/ml, which showed low SSA and comparably low water content. However, to achieve water content of ≤ 1% and high Tg, the use of trehalose and a low amount of phenylalanine (10:0.5) is inevitable. The (low) residual water content of tre:phe samples hardly changed for the different solid contents, although the SSA was stepwise decreased for higher solid contents. As low SSA is preferable for protein stabilization, higher solid contents are advisable. Regarding water content and SSA, for suc:phe 10:0.5 formulations the preferred solid content should be between 50 – 75 mg/ml.

The addition of a higher amount of protein (10 mg/ml) to sucrose and trehalose formulations with and without low amounts of phenylalanine was evaluated. Analysis of the specific surface area of the resulting lyophilizates revealed a reduction of SSA. Hence, the resulting residual water contents are higher compared to samples of a protein content of 1 mg/ml. Consequently, also the glass transition temperatures were lower. It is concluded that the amount of protein influences the formation of the collapsed structure and was not high enough to enable the formation of a bulking structure by the protein. Nevertheless, the formulations that contained phenylalanine (tre:phe 10:0.5, suc:phe 10:0.5) exhibited acceptable water content of ≤ 2%. Moreover, the tre:phe 10:0.5 samples exhibited a Tg of ≥ 80 °C, indicating promising properties for storage stabilization of a protein.

Finally, it can be summarized that aggressive freeze-drying is applicable to a wide range of sugar-based formulations. Both, sucrose and trehalose, which are the major excipients for protein stabilization, served as sound foundation of aggressively freeze-dried lyophilizates. Amongst all investigated lyophilizates, trehalose-based samples that contain phenylalanine at a ratio of 10:0.5 exhibited superior potential for protein stabilization: low water content, high Tg, low SSA, and fully amorphous structure.

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CHAPTER 6 THE INFLUENCE OF FORMULATION COMPOSITION ON PROTEIN STABILITY IN AGGRESSIVELY FREEZE-DRIED LYOPHILIZATES

1. INTRODUCTION

The overall aim of freeze-drying is to stabilize sensitive molecules against degradation during storage. In pharmaceutical products, most often a protein is the molecule to be stabilized [1]. As recently shown by Schersch et al., structural collapse of a lyophilizate does not necessarily result in instability of incorporated proteins [2]. Moreover, they have shown that proteins in collapsed formulations can show a superior stability during storage compared to samples freeze-dried conventionally [3, 4]. Overall, there is a growing interest in applying high product temperatures during freeze-drying [5-8] in order to save time and resources. Since high product temperatures can only be achieved by aggressive freeze-drying, further investigation of the robustness and potentially universal use of aggressive freeze-drying to different types of protein formulations is of high interest. In CHAPTER 5 of this thesis it was shown for a broad range of formulations that aggressive freeze-drying resulted in suitable physico-chemical characteristics for protein stabilization.

The formulations studied in this chapter were based on an amorphous excipient (e.g. a disaccharide), which is obligatory for the stabilization of incorporated proteins (amorphous excipients act as water replacement in the dry state [9-12] and provide a vitrified glassy matrix of reduced mobility [13-15]). Since freeze-drying formulations often contain bulking agents [1], the impact of this usually crystalline components on protein stability in aggressively freeze-dried lyophilizates was examined. The major focus of the study was to evaluate aggressive freeze-drying for formulations of 50 mg/ml excipient concentration and 1 mg/ml protein. However, also excipient concentrations of 25 mg/ml to 150 mg/ml and 10 mg/ml protein concentration (the protein concentration in a biopharmaceutical drug can cover a considerable range [16]) were investigated.

The utmost goal of freeze-drying is the stabilization of a valuable molecule. Inherently, the stability of this molecule is the major product quality. In order to cover a significant range of marketed biopharmaceutical proteins [17], an recombinant monoclonal IgG1 antibody (mab_T1) was used as model protein. Mab_T1 is usually stored as refrigerated liquid bulk solution due to its susceptibility to freezing and drying stresses. Freeze-drying without proper stabilization in terms of suitable excipients and an appropriate freeze-drying process consequently result in substantial instability, ending up in the formation of considerable amounts of aggregates. Therefore, mab_T1 is considered as suitable model to reveal even minor changes towards non-ideal stabilization.

2. TYPE OF DISACCHARIDE

Sucrose and trehalose have turned out as preferable choice for stabilizing incorporated proteins [18]. In order to evaluate, whether both sugars act as suitable stabilizers during aggressive freeze-drying, too, formulations containing 1 mg/ml mab_T1 and either 50mg/ml sucrose or trehalose (compare Table 6.1) were subjected to aggressive freeze-drying. 1ml aliquots of the respective formulations were filled in 2R vials and subjected to the aggressive freeze-drying protocol shown in Table 3.5 (page 56, pc=1 mbar, 6 h at Ts=45 °C,) in a Martin Christ 2-12D freeze-drier.

Table 6.1: Formulations to characterize the influence of type of disaccharide on the protein stability of mab_T1 (formulations based on 10 mM potassium phosphate buffer pH 6.4).

formulation	mab_T1	sucrose	trehalose
	mg/ml	mg/ml	mg/ml
suc	1	50	
tre	1		50

Sub-visible particles and turbidity

Figure 6.1 shows the subvisible particle counts ≥1 μm (assessed by LO) and the turbidity of reconstituted samples. Before freeze-drying, the sucrose- or trehalose-based formulation exhibited a subvisible particle content of below 4000 particles ≥ 1 μm per ml. The overall low amounts of subvisible particles were not changed during aggressive freeze-drying, neither for sucrose-based samples nor for trehalose-based ones. In addition, particles of ≥ 10 μm as well as ≥ 25 μm were not increased during aggressive freeze-drying. The detected concentrations are far below the limit of the Ph. Eur. (6000 ≥ 10 μm and 600 ≥ 25 μm per container, in this case per ml, since filling volume was 1 ml). Per ml sucrose-based samples contained 20±8 particles ≥ 10 μm and 2±2 particles ≥ 25 μm, trehalose-based samples 27±26 particles ≥ 10 μm and 1±2 particles ≥ 25 μm (cf. Figure 6.1, right graph). The left graph in Figure 6.1 also displays the turbidity of the formulations before and after freeze-drying. While the turbidity of freeze-dried sucrose samples seems to be slightly increased to 1.30 ± 0.19 FNU compared to 0.96 ± 0.08 FNU before freeze-drying, the turbidity of trehalose based samples were apparently unchanged (0.87 ± 0.02 FNU before vs. 1.07 ± 0.33 FNU after freeze-drying).
Overall, no substantial changes were detected for subvisible particles and turbidity before and after freeze-drying. It is concluded that both, sucrose and trehalose successfully stabilized mab_T1 against aggregation to non-soluble aggregates. For comparison, also a formulation containing mab_T1 without stabilizing disaccharide (i.e. mab_T1 in 10mM potassium phosphate buffer) was aggressively freeze-dried using the same aggressive freeze-drying protocol. The resulting products exhibited subvisible particle counts of around 50,000 particles ≥ 1μm per ml and a turbidity of about 30 FNU (data not shown).

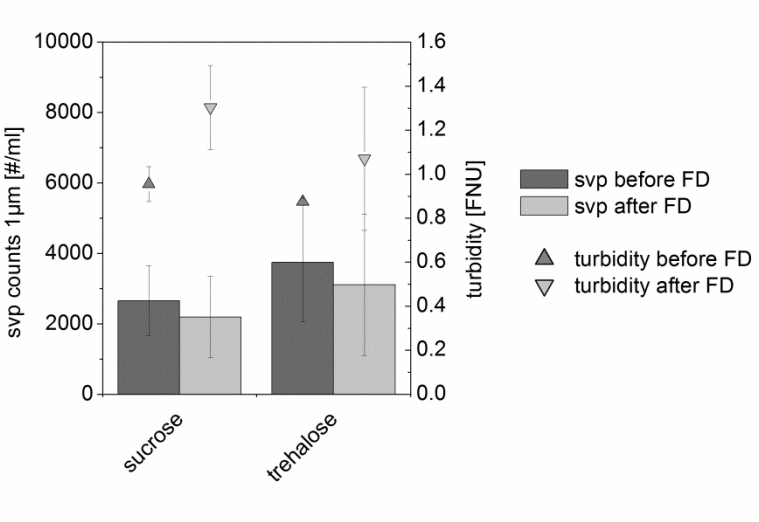

Figure 6.1: Sub-visible particle count ≥1µm (bars) and turbidity (triangles) before and after aggressive freeze-drying (sugar based formulations containing 1 mg/ml mab_T1).

To account for soluble aggregates, the reconstituted samples were analyzed using HP-SEC. The results are summarized in Table 6.2. Comparing samples regarding the amount of high molecular weight species (HMWS) or low molecular weight species (LMWS) to the formulations before freeze-drying, no substantial increase could be detected. Moreover, the monomer content was unchanged after aggressive freeze-drying. To account for a potential loss of protein, the detected monomer amount (integrated area under the curve) of freeze-dried samples were compared to the monomer amount prior to freeze-drying. Since neither this monomer recovery was decreased, nor instability indicating HMWS or LMWS were increased, aggressive freeze-drying did not alter the samples regarding aggregation or fragmentation of mab_T1.

Table 6.2: HP-SEC data to characterize the influence of type of disaccharide on the protein stability of mab_T1 (formulations based on 10 mM potassium phosphate buffer pH 6.4).

	HMWS content		LMWS content		monomer content		monomer recovery
	before freeze-drying	after freeze-drying	before freeze-drying	after freeze-drying	before freeze-drying	after freeze-drying	
formulation	%	%	%	%	%	%	%
suc	1.4 ± 0.1	1.7 ± 0.1	1.1 ± 0.2	0.8 ± 0.2	97.3 ± 0.3	97.6 ± 0.5	101.2 ± 0.4
tre	1.3 ± 0.0	1.7 ± 0.1	1.3 ± 0.0	0.8 ± 0.1	97.6 ± 0.1	97.5 ± 1.3	100.4 ± 0.7

The conformational stability of mab_T1 after aggressive freeze-drying was assessed by FT-IR spectroscopy. The secondary structure of mab_T1 was unaffected, since no change was detected in the FT-IR spectra after freeze-drying.

Summing up the results concerning the influence of type of disaccharide on the stability of mab_T1 during aggressive freeze-drying, no difference could be noticed between

sucrose and trehalose. Both sugars are able to stabilize mab_T1 during aggressive freeze-drying successfully.

3. ADDITION OF BULKING AGENT

As shown in CHAPTER 5, aggressive freeze-drying of formulations containing no bulking agent resulted in high residual water contents due to the reduced specific surface area (compare page 109). In order to reduce the residual water content without being forced to prolong the aggressive freeze-drying cycle, the addition of bulking agents like mannitol seemed to be feasible. In fact, it could be shown that the addition of a certain amount of bulking agent reduces the residual water content of the resulting lyophilizates dramatically (see page 113). In the previous section it was shown that formulations of sucrose and trehalose without containing a bulking agent stabilized mab_T1 successful during aggressive freeze-drying. Now, the influence of adding bulking agents to sugar based formulations on the stability of mab_T1 was studied. In a first step, each, low and high amounts of mannitol, glycine and phenylalanine were added to sugar-based formulation according to the overview given in Table 6.3. Aliquots of 1ml of the formulations were filled into DIN 2R glass vials (type 1) and aggressively freeze-dried according to the protocol shown in Table 3.5 (page 56) in a Martin Christ 2-12D freeze-drier. For comparison, the same formulations were subjected to a conventional / moderate freeze-drying cycle in the same configuration (vials, filling volume, freeze-drier). For freeze-drying of these samples, the protocol shown in Table 3.8 (page 57; pc=0.07 mbar, primary drying 60 h at Ts=-30 °C, secondary drying 10 h at Ts=10 °C) was utilized. Since the focus of the investigation was set on the influence of formulation composition on the stability of incorporated mab_T1 of aggressively freeze-dried lyophilizates, not all results from the moderately freeze-dried samples are shown in the following.

In a second step, the lyophilizates from aggressive freeze-drying, that exhibit a low residual water content and a glass transition of above 50 °C, were subjected to an indicative stability study at 2-8 °C, 30 °C, and 50 °C for up to 6 months. In Table 6.3 the formulations that were included in the stability study are highlighted by an asterisk. The same formulations were also moderately freeze-dried and stored. Mab_T1 was added to all formulations at a concentration of 1 mg/ml. Since mab_T1 was known to aggregate upon freeze-drying unless suitable stabilized, aggregation behavior was characterized investigating subvisible particle counts, turbidity, and HP-SEC. In addition, the secondary structure of mab_T1 was monitored via FT-IR of selected reconstituted samples.

Table 6.3: Formulations used to study the influence of bulking agent on the protein stability of mab_T1 of aggressively freeze-dried products (formulations based on 10 mM potassium phosphate buffer pH 6.4).

formulation	mab_T1	sucrose	trehalose	mannitol	glycine	phenylalanine
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc:man 10:3	1	38.46		11.54		
suc:man 10:30*	1	12.50		37.50		
suc:gly 10:3	1	38.46			11.54	
suc:gly 10:30*	1	12.50			37.50	
suc:phe 10:0.5*	1	47.62				2.38
suc:phe 10:3*	1	38.46				11.54
tre:man 10:3	1		38.46	11.54		
tre:man 10:30*	1		12.50	37.50		
tre:gly 10:3	1		38.46		11.54	
tre:gly 10:30*	1		12.50		37.50	
tre:phe 10:0.5*	1		47.62			2.38
tre:phe 10:3*	1		38.46			11.54

* subjected to indicative stability study

3.1 PRODUCT CHARACTERISTICS AFTER AGGRESSIVE FREEZE-DRYING

3.1.1 APPEARANCE AND RECONSTITUTION

Prior to analysis, the samples were reconstituted. All of the aggressively freeze-dried lyophilizates were fully reconstituted within 35 second at the maximum (see Table 6.4). Besides tre:gly, all lyophilizates from moderate freeze-drying exhibited increased reconstitution times. Obviously, aggressive freeze-drying did not result in prolonged reconstitution times. Contrary to the literature ([19]), even the samples exhibiting a collapsed appearance (marked by an asterisk in Table 6.4 and displayed in Figure 6.2) did rather show a decrease and not an increase of reconstitution time.

Table 6.4: Reconstitution times of lyophilizates of bulking agent containing samples, subjected to aggressive or moderate freeze-drying.

formulation	aggressive freeze-drying		moderate freeze-drying	
	sucrose	trehalose	sucrose	trehalose
	seconds	seconds	seconds	seconds
sugar:man 10:3	21 ± 1 *	27 ± 8 *	55 ± 5	55 ± 5
sugar:man 10:30	17 ± 1	23 ± 6	35 ± 5	47 ± 7
sugar:gly 10:3	14 ± 2 *	19 ± 4 *	39 ± 4	15 ± 4
sugar:gly 10:30	15 ± 1	21 ± 3	46 ± 3	14 ± 1
sugar:phe 10:0.5	29 ± 8 *	35 ± 1 *	32 ± 3	43 ± 8
sugar:phe 10:3	29 ± 4	32 ± 2	57 ± 2	49 ± 4

*lyophilizates showed a collapsed appearance

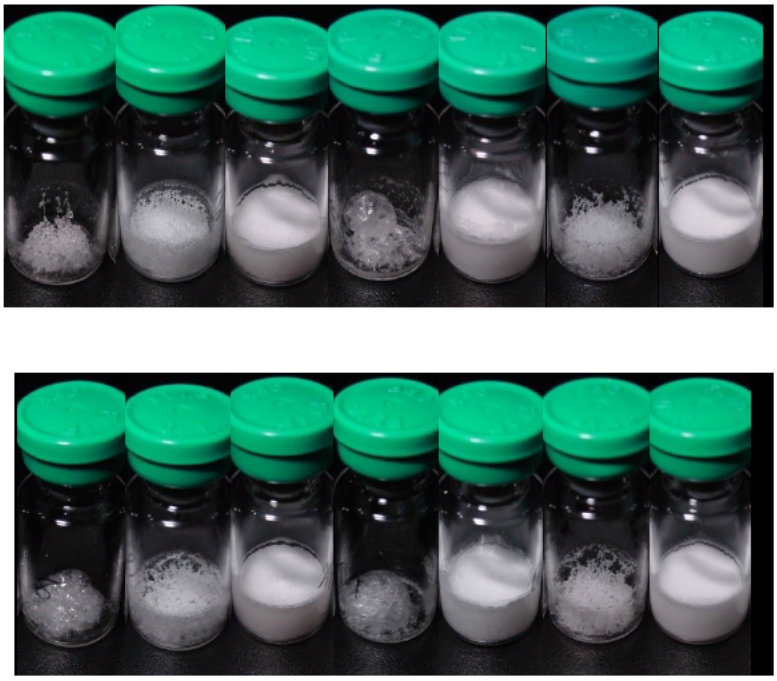

Figure 6.2: Macroscopic appearance after freeze-drying (top row trehalose based, bottom row sucrose based; from left to right: sugar, sugar:phe 10:0.5, sugar:phe 10:3, sugar:gly 10:3, sugar:gly 10:30, sugar:man 10:3, sugar:man 10:30).

3.1.2 PROTEIN STABILITY

Soluble aggregates

The reconstituted samples were analyzed by HP-SEC in order to detect soluble protein aggregates. High molecular weight species (HMWS) were quantified by the area under the curve. Figure 6.3 shows the HMWS content before and after aggressive freeze-drying. Most of the analyzed formulations showed no apparent increase of HMWS species after aggressive freeze-drying. Both formulations containing mannitol in higher quantities (suc:man 10:30, and tre:man 10:30) showed a slight increase of HMWS after freeze-drying. Slightly increased aggregation indicates insufficient stabilization of mab_T1 in formulations with high amounts of mannitol.

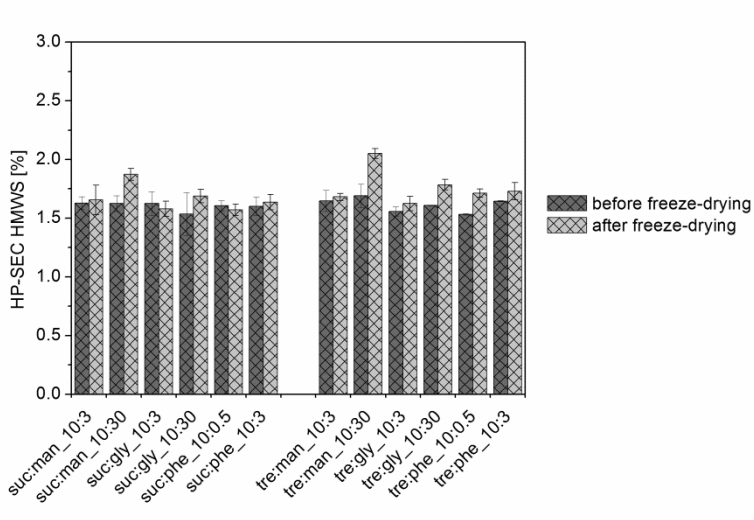

Figure 6.3: HMWS of mab_T1 detected by HP-SEC of formulations containing bulking agent in different quantities. The results of samples analyzed before and after aggressive freeze-drying are shown.

In addition to the amount of HMWS, the percentage recovery of mab_T1 monomer was calculated from the HP-SEC data (cf. Figure 6.4). Overall, a monomer recovery of > 97% was detected. No clear trend could be established comparing the monomer recovery of the different lyophilizates. For trehalose-based samples, a somewhat reduced monomer recovery was detected for samples containing a high amount of mannitol or phenylalanine. In sucrose-based samples for the formulation containing a low amount of mannitol (suc:man 10:3) a slightly reduced monomer recovery was detected. Regarding soluble aggregates (HMWS) the recovery of mab_T1 monomer, an overall good stabilization of mab_T1 during aggressive freeze-drying was achieved by the applied formulations.

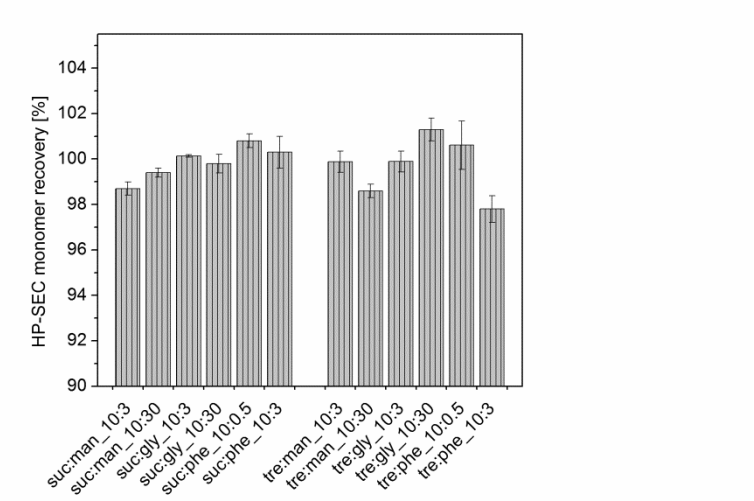

Figure 6.4: Monomer recovery of mab_T1 detected by HP-SEC. The monomer content (in term of area under the curve) of reconstituted aggressively freeze-dried samples was compared to the monomer content of the formulations before freeze-drying.

Non-soluble aggregates

The reconstituted lyophilizates were further analyzed regarding subvisible particle (svp) content and turbidity. While subvisible particles were detected in the range of ≥ 1 µm, turbidity values also allow for quantification of smaller non-soluble aggregates. Figure 6.5 and Figure 6.6 show the svp counts and turbidity values of samples freeze-dried aggressively or moderately, respectively. Aggressive freeze-drying of both formulations containing a high amount of mannitol as bulking agent (suc:man 10:30 and tre:man 10:30) exhibited a drastic increase of subvisible particles (from around 3,000 particles/ml to above 80,000 particles/ml). In addition, the turbidity of those samples was increased from around 1 FNU before freeze-drying to 5.4 FNU and 2.7 FNU (suc:man 10:30 and tre:phe 10:30, respectively). It is concluded that suc:man 10:30 and tre:man 10:30 were not able to completely stabilize mab_T1 against aggregation during aggressive freeze-drying. Moderately freeze-dried samples of those formulations also showed an increase in subvisible particle level and turbidity (cf. Figure 6.6).

Aggressive freeze-drying of all other formulations resulted in low subvisible particle counts of particles ≥ 1 µm (cf. Figure 6.5). A small increase of particles were observed for the formulations that contain a high amount of phenylalanine (suc:phe 10:3 and tre:phe 10:3). Those samples exhibited an increased turbidity, too. Although the svp count of the formulations containing a high amount of glycine is unchanged, the turbidity is markedly increased (independent from sugar). The increase of turbidity indicates the formation of non-soluble aggregates smaller than 1 µm.

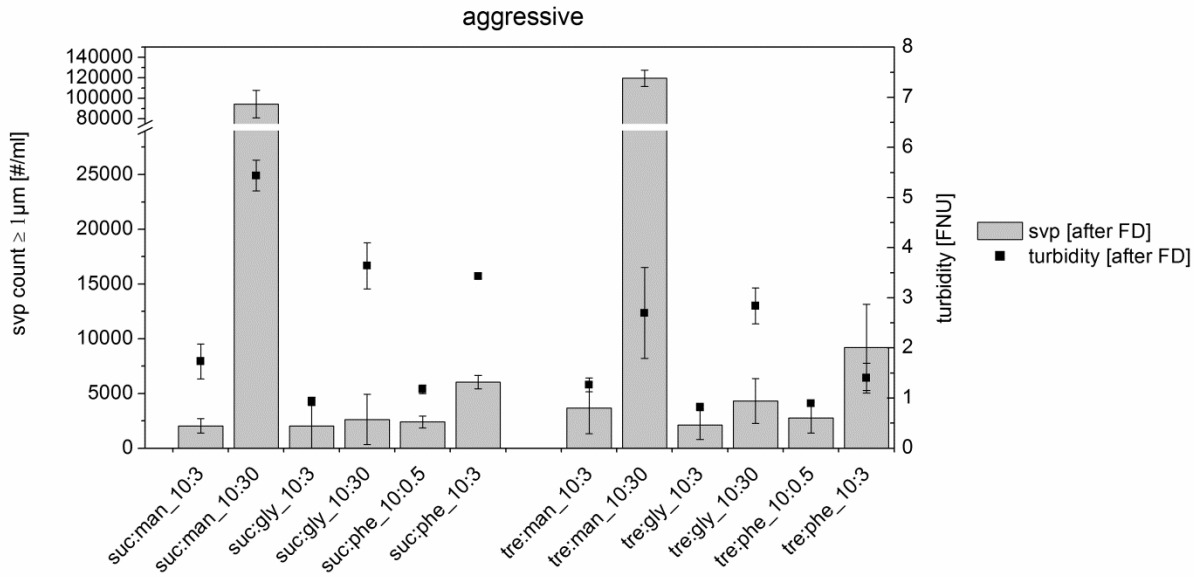

Figure 6.5: Subvisible particle counts (particles ≥ 1µm) as well as turbidity of aggressively freeze-dried of formulations containing bulking agent in different quantities. The subvisible particle counts of the formulations before freeze-drying ranged around 3,000 particles ≥ 1µm per ml. Turbidity values of the formulation before freeze-drying ranged around 1 FNU.

Summarizing, it was observed that formulations containing high amount of bulking agents (and were therefore macroscopically not collapsed) showed a tendency towards

instability, which is indicated by the formation of aggregates. On the other hand, lyophilizates that have been freeze-dried from formulations containing low amounts of bulking agents (sugar:man 10:3, sugar:gly 10:3, augar:phe 10:0.5) exhibited no signs of aggregation.

Comparing the aggregation of mab_T1 (svp, turbidity) of aggressive versus moderate freeze-drying, samples containing a low amount of bulking agent showed comparable svp counts and turbidity (compare Figure 6.6). For lyophilizates that contained a low amount of phenylalanine, the samples from moderate freeze-drying even showed a slightly higher turbidity compared to the samples from aggressive freeze-drying.

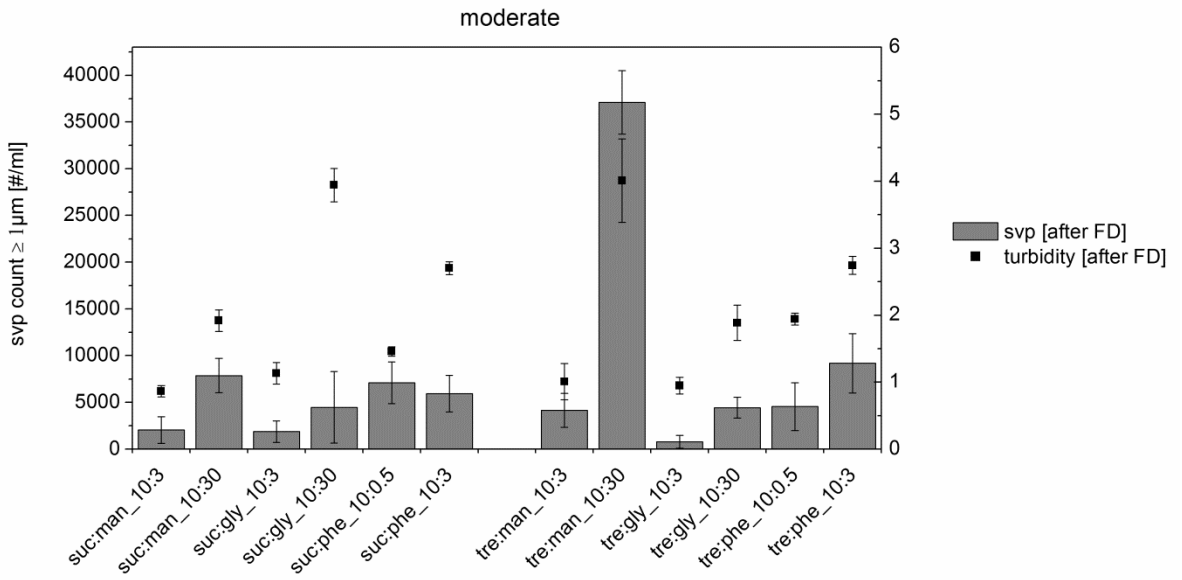

Figure 6.6: Subvisible particle counts (particles ≥ 1µm) as well as turbidity of moderately freeze-dried of formulations containing bulking agent in different quantities. The subvisible particle counts of the formulations before freeze-drying ranged around 3,000 ≥ 1µm per ml. Turbidity values of the formulation before freeze-drying ranged around 1 FNU.

Secondary structure

The secondary structure of a protein is an important criterion to judge potential instability. To assess the secondary structure of mab_T1, second derivative FT-IR spectroscopy of the amide I region was utilized. Generally, the secondary structure of IgG proteins consist of around 70% β-sheet structures, while the remaining structures are of unordered nature [20, 21]. The 2nd derivative spectra of the formulations were checked for the appearance of additional bands at wavenumbers of 1620-1625 cm^{-1} and 1680-1700 cm^{-1}, which are indicative for hydrogen bonded intermolecular β-sheet structures [22-24]. In addition, band broadening or shifts of peaks are regarded as (unwanted) changes in the secondary structure of mab_T1.

Figure 6.7 displays the second derivative FT-IR spectra of bulking agent containing sucrose- and trehalose-based formulations before and after aggressive freeze-drying. Formulations containing glycine could not be investigated due to interfering FT-IR spectra of mab_T1 and glycine. The major peak at 1639 cm^{-1} is indicative for intramolecular β-

sheet structures [20]. A slightly more pronounced band at 1690 cm^{-1}, indicating a tendency towards unfolding, was identified for suc:man 10:3, suc:phe 10:3, tre:man 10:30. For tre:man 10:30 additionally a minute shift of the main peak towards lower wavenumbers was noticed.

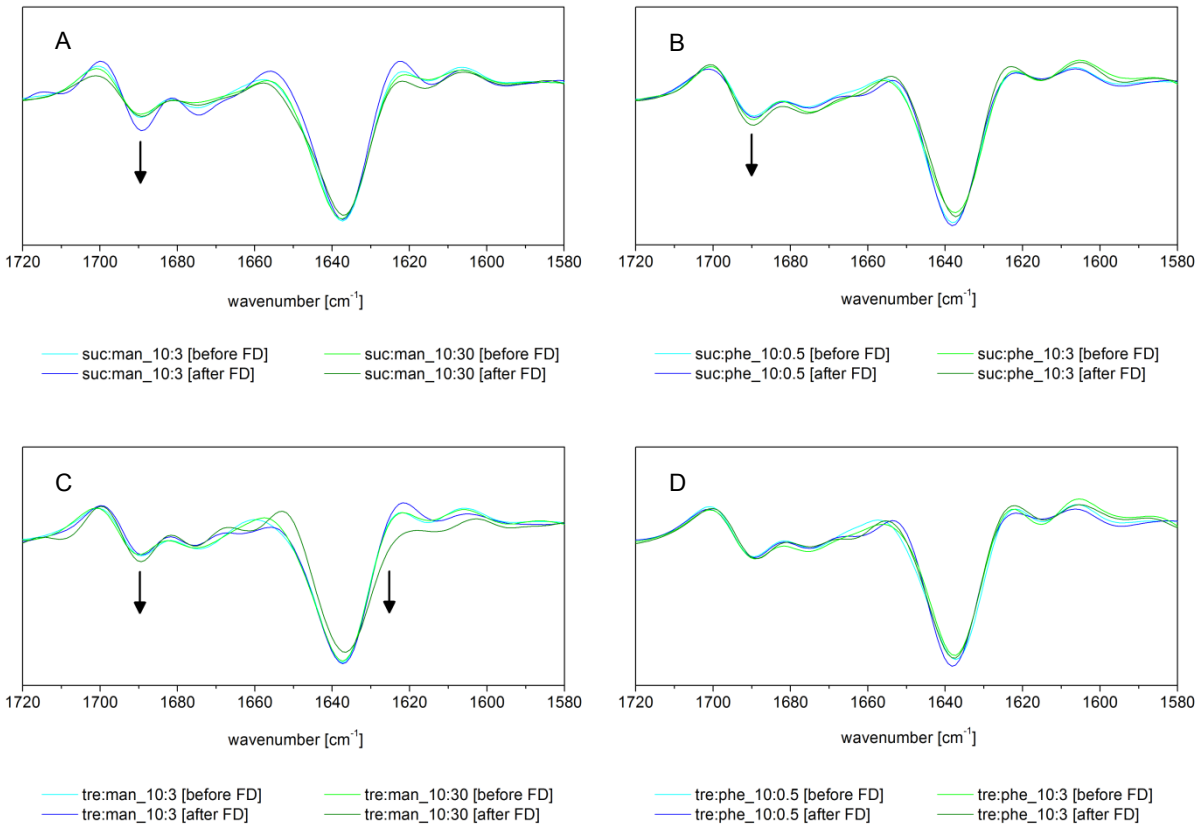

Figure 6.7: 2nd derivative FT-IR spectra (amide I band) before and after aggressive freeze-drying. The sugar-based formulations contain bulking agent in low and high quantities (A: suc:man; B: suc:phe; C: tre:man; D: tre:phe; arrows highlight small changes in the spectra).

Overall, no drastic change in the FT-IR spectra was discernible. Formulations containing low and high amounts of bulking agents did not show a clear trend towards instability. Hence, the influence of bulking agent on the stability of the secondary structure of mab_T1 is regarded as negligible.

Summary

As shown above, the addition of high amounts of bulking agents to either sucrose- or trehalose-based formulation subjected to aggressive freeze-drying resulted in increased aggregation of mab_T1. On the other hand, formulations containing low amounts of bulking agents showed excellent stabilization of mab_T1. In order to evaluate whether the differences in the ability to stabilize mab_T1 can be attributed to the physico-chemical characteristics of the lyophilizates, the residual water content, the specific surface area, and the crystallinity of the samples were analyzed.

3.1.3 PHYSICO-CHEMICAL CHARACTERISTICS

Residual water content

As shown in the precious chapter (compare page 113), the addition of increasing amounts of bulking agents decrease the residual water content of the resulting lyophilizates. Figure 6.8 depicts the residual water content of samples from aggressive freeze-drying of the formulations listed in Table 6.3. While formulations containing a high amount of bulking agent (mannitol, glycine, or phenylalanine) exhibit low residual water contents of below 0.5%, the lyophilizates containing a low amount of mannitol or glycine exhibited high residual water contents. The formulations containing a low amount of phenylalanine exhibited low to medium residual water content of 0.75 ± 0.05% for the trehalose-based formulations and 1.69 ± 0.47% for the sucrose-based formulations, respectively. Besides the slight difference between suc:phe 10:0.5 and tre:phe 10:0.5, the residual water contents of sucrose- and trehalose- based lyophilizates were comparable.

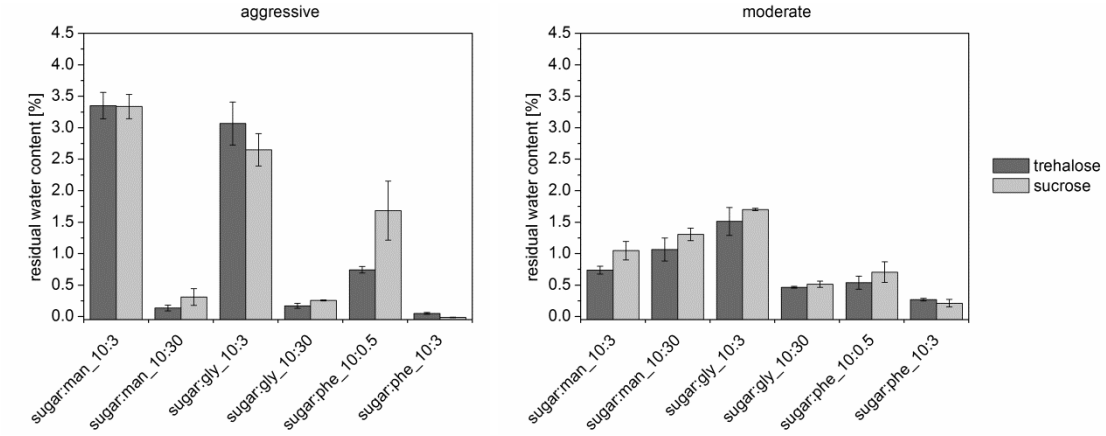

Figure 6.8: Residual water content of lyophilizates after aggressive or moderate freeze-drying (overall drying time: aggressive 8 h, moderate 77 h). The sugar-based formulations contain bulking agent in low and high quantities.

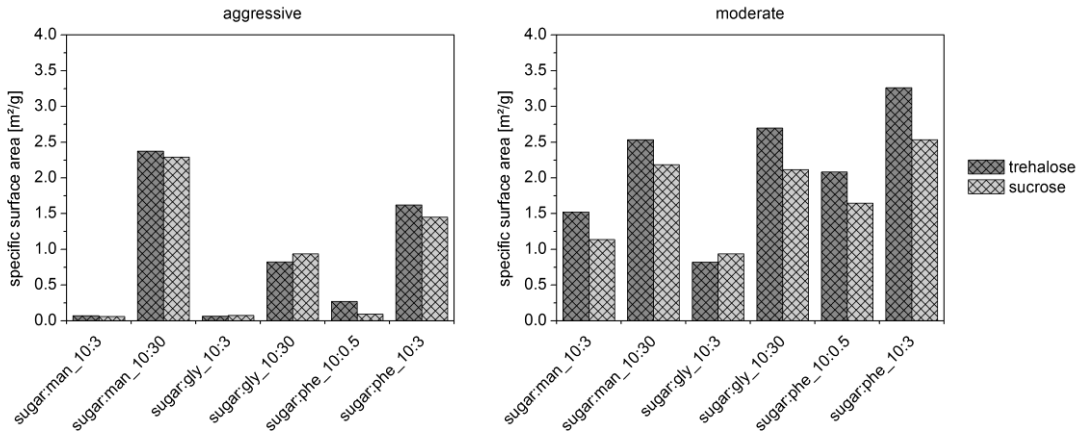

Figure 6.9: Specific surface area of lyophilizates after aggressive or moderate freeze-drying. The sugar-based formulations contain bulking agent in low and high quantities.

Taking the macroscopic appearance (Figure 6.2) and the specific surface area (cf. Figure 6.9) into consideration, the collapsed nature and therefore drastically decreased SSA

hinders an effective secondary drying of the formulations containing mannitol or glycine at a low concentration. To reach comparable residual water contents for latter formulations, it would have been necessary to extend the drying time of the aggressive freeze-drying process. The process was designed to freeze-dry the trehalose-based formulation containing a low amount of phenylalanine to a residual water content of below 1%. The same target was set for the design of the moderate freeze-drying cycle. The residual water contents of the moderately freeze-dried lyophilizates are shown in Figure 6.8, they ranged from 0.49% (suc:phe 10:3) to 1.96% (suc:gly 10:3). No apparent difference could be detected between sucrose- and trehalose-based samples. For the trehalose-based formulation containing a low amount of bulking agent, a residual water content of 0.81% was determined. Overall, the differences between the different formulations of low and high bulking agent content were less pronounced compared to aggressive freeze-drying. This can be attributed to less pronounced differences in the SSA of the different lyophilizates resulting from moderate freeze-drying (cf. Figure 6.9). However, also for moderately freeze-dried samples a higher SSA of samples containing higher amounts of bulking agent can be distinguished. Comparing sucrose- and trehalose-based lyophilizates, a minor tendency of lower SSA for sucrose-based samples could be noticed.

Specific surface area and protein stability

Since the SSA of dried products is known to play an important role for the stabilization of incorporated molecules [25, 26], the SSA of aggressive freeze-dried samples is compared to the turbidity, which was identified as the most sensitive tool for instability of mab_T1. The left graph in Figure 6.10 depicts the relationship of SSA and turbidity of aggressively freeze-dried samples. Comparing the SSA and the turbidity, samples of higher SSA exhibit higher turbidity. In other words, aggressively freeze-dried samples of higher SSA resulted in more pronounced aggregation (=instability) of mab_T1 compared to samples of low SSA. Consequently, it can be stated that higher amounts of bulking agents result in inferior stabilization of mab_T1.

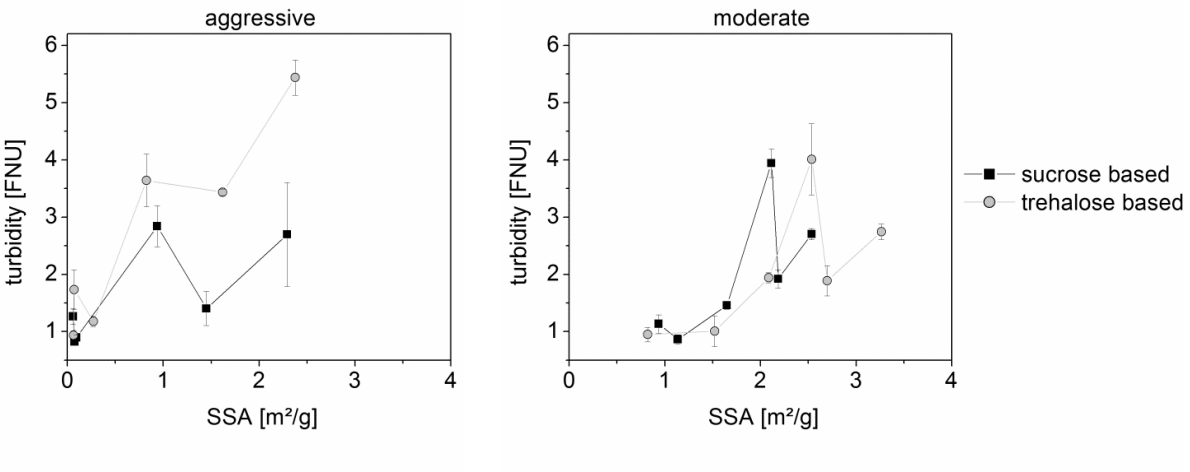

Figure 6.10: Turbidity versus SSA of aggressively and moderately freeze-dried lyophilizates. Sucrose- and trehalose-based samples of different bulking agent contents are displayed.

Moderately freeze-dried samples of higher SSA exhibited higher turbidity values (cf. right graph in Figure 6.10), too. However, while for aggressively freeze-dried samples an increased turbidity was notice for a SSA of ≥ 0.5 m²/g, moderately freeze-dried lyophilizates showed an increase only at a SSA of ≥ 1.5 m²/g. Hence, high specific surface area by itself does not necessarily result in aggregation of mab_T1 (=turbidity). Potentially, a combination of (high) SSA and (high) temperature during drying causes instability.

Crystallinity

Usually, the incorporation of a protein in an amorphous matrix is regarded as necessary for successful stabilization [27]. The addition of bulking agents result in the formation of at least partially crystalline structures, which potentially cause instability of the incorporated protein molecules. The crystallinity was analyzed using X-Ray powder diffraction (XRD). Figure 6.11 displays the diffraction patterns from XRD analysis. In general, no difference in the spectra of sucrose- or trehalose-based samples was noticed. The type of sugar did not affect the crystallinity.

No distinct peaks were observable from the diffraction patterns of lyophilizates aggressively freeze-dried from formulations containing low amounts of glycine or phenylalanine (peak at 38.8° 2-θ originates from copper sample holder). These samples are regarded as fully amorphous according to the characterization by XRD.

Samples that contain high amounts of glycine or phenylalanine formed crystalline structures during freeze-drying indicated by distinct peaks. The diffraction peaks of glycine containing samples can be attributed to β-glycine [28]. As phenylalanine was used in a lower amount (10:3) compared to glycine (10:30), the peaks were smaller in those species. Reference diffraction patterns of the International Centre for Diffraction Data (ICDD, formerly JCPDS database) were utilized to identify the peaks detected for the formulations containing a high amount of phenylalanine. The peaks at 6.5°, 15.1°, 17.5°, and 21.6° 2-θ are characteristic for phenylalanine-hydrate.

To avoid the formation of metastable mannitol polymorphs, formulations containing mannitol should be subjected to a thermal treatment after freezing (annealing). Since aggressive freeze-drying aims for short process times, annealing was not included in the performed freeze-drying process. Hence, the potential formation of metastable mannitol hemihydrate was accepted. Samples containing a low amount of mannitol exhibited diffraction peaks at 9.6° and 17.9° 2-θ that can be attributed to mannitol hemihydrate [29]. Moderately freeze-dried lyophilizates of the same formulation exhibited no diffraction peaks (Figure 6.11, right graphs). If high amounts of mannitol are used, a number of diffraction peaks were observed. Besides mainly δ-mannitol, also mannitol hemihydrate was noticed. On the contrary, aggressive freeze-drying of formulations containing a high amount of mannitol resulted in lyophilizates containing no mannitol hemihydrate (absence of the characteristic peak at 17.9° 2-θ). As the freezing steps of both cycles, aggressive as well as moderate, were identical, obviously a recrystallization (to the stable δ-modification) of mannitol occurred during the drying phase of aggressive freeze-drying.

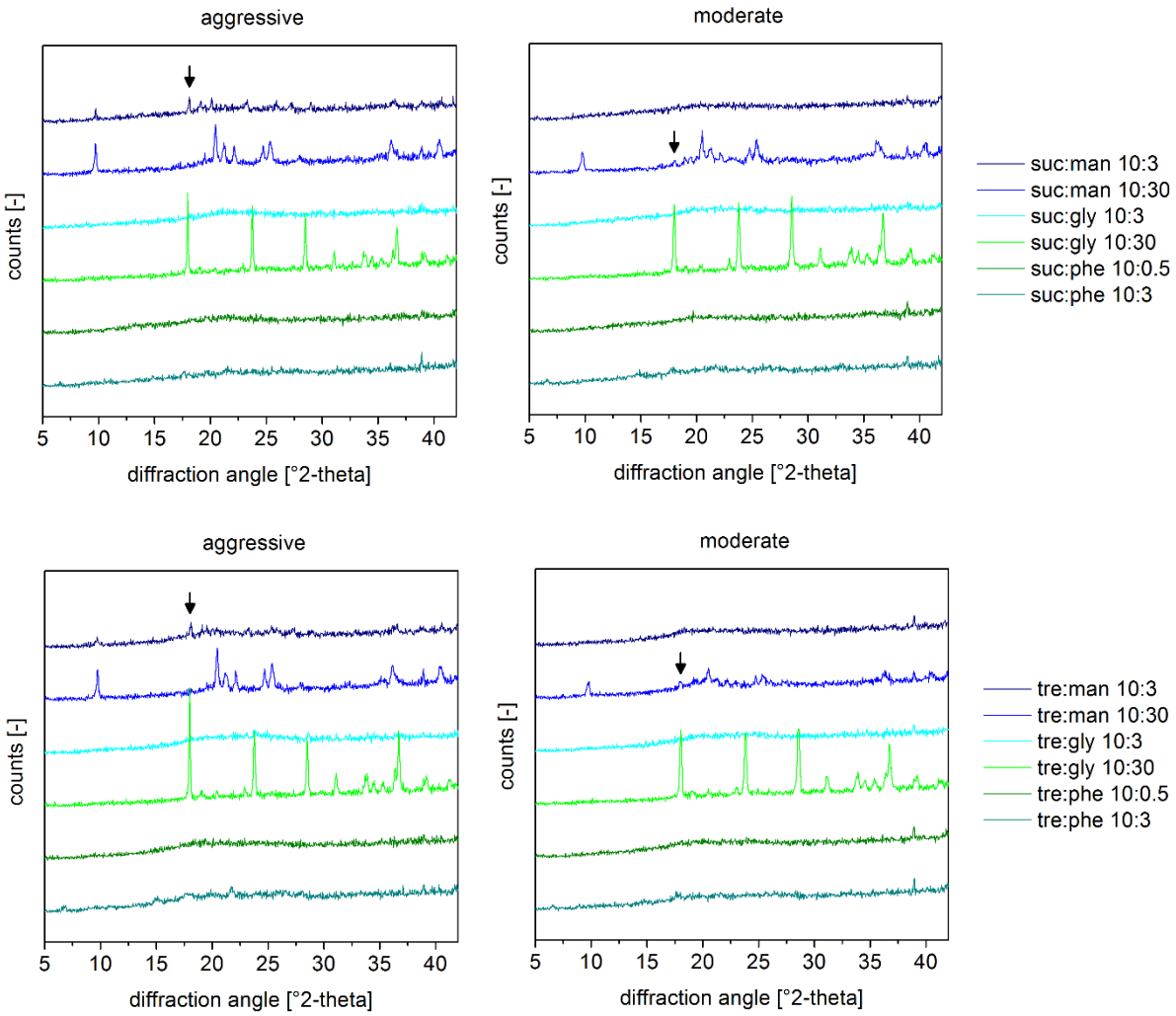

Figure 6.11: Diffraction patterns of lyophilizates after aggressive or moderate freeze-drying. The sugar-based formulations contain bulking agent in low and high quantities.

Regarding the influence of bulking agents on the stability of mab_T1 in aggressively freeze-dried lyophilizates, the presence of crystalline material seems to increase the aggregation of mab_T1. As shown above, the aggressively freeze-dried samples, which contain a high amount of mannitol, glycine, or phenylalanine, showed increased subvisible particle counts and turbidity. As the formation of a crystalline bulking structure is accompanied by an increased SSA, instability of mab_T1 cannot be exclusively accounted to crystallinity in the lyophilizates. Most probably, the large SSA, which results from the crystallization of the bulking agent, is responsible for the slightly less successful stabilization of mab_T1.

3.1.4 Summary

Summing up, the results regarding the influence of bulking agents on the ability of aggressively freeze-dried formulations to stabilize mab_T1 have shown that most of the formulations exhibited an overall good preservation of mab_T1 stability. No difference could be noticed between the sugars utilized as main stabilizers, implicating that both are suitable candidates for freeze-drying formulations for aggressive freeze-drying. Three

bulking agents (mannitol, glycine, and phenylalanine) were added to sucrose- or trehalose-based formulations in low and high amounts. While all samples of the formulations containing a low amount of either bulking agent showed very good stabilization of mab_T1, the addition of higher amounts of bulking agent resulted in slightly worse stabilization.
Since aggressive freeze-drying of formulations that contain low amounts of mannitol or glycine resulted in high residual water contents, the use of higher amounts is recommendable in order to reduce the residual water content. On the other hand, it has turned out that formulations applying high amounts of bulking agent resulted in partly crystalline lyophilizates, which exhibits high SSA und therefore somewhat higher risks for instability. Therefore, either shorter drying time (i.e. higher residual water content) or a medium amount of glycine or mannitol (i.e. lower SSA) seems to be feasible for a good combination of low residual water content and sound protein stabilization.
Phenylalanine was utilized as a third bulking agent in this investigation. For the formulations containing a high amount of phenylalanine the same observation as for mannitol and glycine were made. The aggressively freeze-dried lyophilizates exhibit slightly less successful preservation of mab_T1 stability due to the facts outlined above. However, the formulations applying low amount of phenylalanine showed an excellent preservation of mab_T1 stability and low residual water content / fully amorphous structure. Overall, these lyophilizates (suc:phe 10:0.5 and tre:phe 10:0.5) have emerged as preferable choice for generating stable products of mab_T1.

3.2 PRODUCT CHARACTERISTICS AFTER STORAGE

3.2.1 PROTEIN STABILITY

Some of the formulations investigated above were subjected to an indicative stability study under accelerated conditions. For that purpose, selected samples were stored at 2-8 °C, 30 °C, and 50 °C for up to 6 months. Suitable formulations were selected by their glass transition temperature. A glass transition temperature of above 50 °C was regarded as necessary. Figure 6.12 depicts the glass transition temperatures of lyophilizates aggressively freeze-dried from the formulations denoted in Table 6.3. Inherently, trehalose-based samples exhibited higher glass transition temperatures compared to sucrose-based samples.

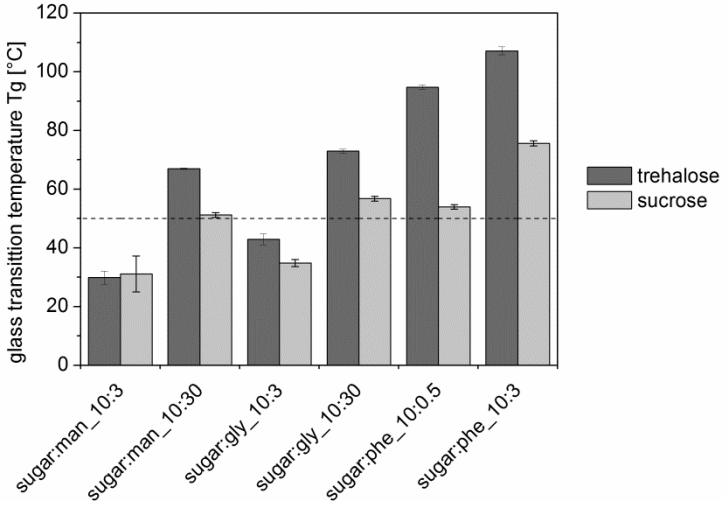

Figure 6.12: Glass transition temperatures of lyophilizates after aggressive freeze-drying. The sugar-based formulations contain bulking agent in low and high quantities.

Based on the threshold of 50 °C, the formulations containing a high amount of mannitol, glycine, or phenylalanine were chosen. Additionally, the formulations containing a low amount of phenylalanine fulfilled the criterion and were included in the storage stability study, too. In order to compare the aggressively freeze-dried samples to conventionally freeze-dried ones, the identical formulations were moderately freeze-dried and subjected to the same storage conditions. Major focus was set on the aggregation indicating analytical methods. Besides soluble aggregates (HP-SEC) and non-soluble aggregates (subvisible particle counts, turbidity), also the secondary structure of mab_T1 (2nd derivative FT-IR) was assessed. To possibly track down potential instabilities of mab_T1 to physico-chemical changes, also residual water content (Karl-Fischer titration), specific surface area (BET gas adsorption), and crystallinity (XRD) were evaluated.

Soluble aggregates

Soluble aggregates of mab_T1 are represented by high molecular weight species (HMWS) that were quantified by HP-SEC. Figure 6.13 displays the HMWS of reconstituted samples after storage over 10 and 26 weeks at the different storage conditions. On the left-hand side, sucrose-based samples are shown and on the right-hand side trehalose-based samples.

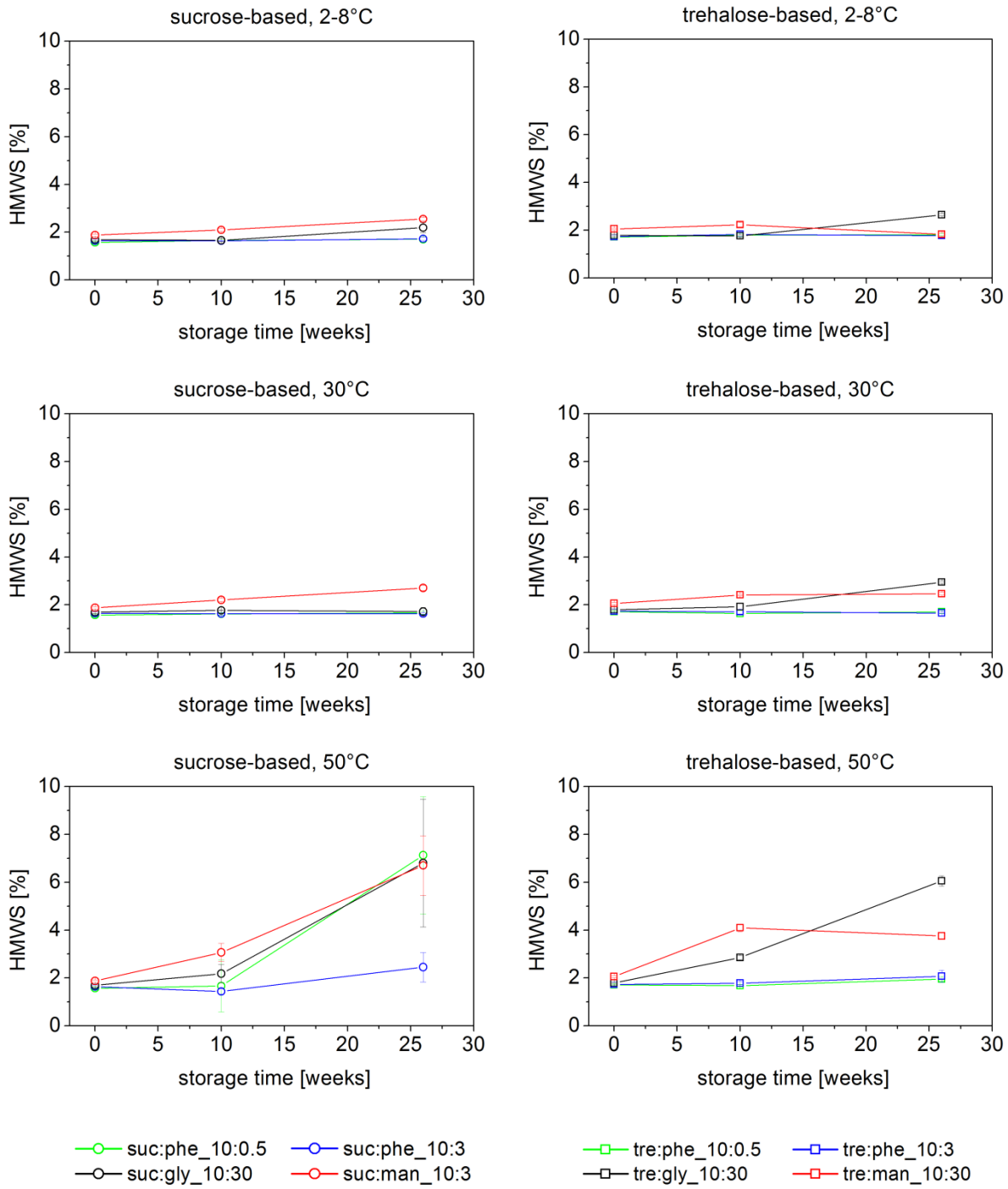

Figure 6.13: High molecular weight species (HMWS) of mab_T1 after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks.

At refrigerated storage of **2-8 °C** no increase of HMWS was noticed for all formulations that contain phenylalanine as bulking agent. For the sucrose-based formulations, containing mannitol or glycine, a slight increase of HMWS from 1.7% to 2.2% (gly) and 1.9% to 2.6% (man) was detected after 26 weeks of storage. The HMWS content of the trehalose-based formulation containing glycine was slightly increased, too, while tre:man did not show an increase.

During storage at **30 °C** a similar behavior was observed. The lyophilizates composed of sucrose and glycine exhibited a slight increase of HMWS, while the lyophilizates composed of sucrose and mannitol exhibited no increase. Both, trehalose-based glycine and mannitol lyophilizates showed a minor increase after 26 weeks at 30 °C. The samples containing phenylalanine exhibit no increase of the low HMWS content over the whole storage period of 26 weeks.

At a storage temperature of **50 °C** all sucrose-based samples showed an increase of HMWS of up to 7.1% indicating substantial aggregation of mab_T1. Only the formulation containing a high amount of phenylalanine exhibited just a minor increase. Although the increase of trehalose-based mannitol and glycine samples is slightly less pronounced compared to the sucrose-based formulations, both species exhibited a distinct increase of HMWS during storage at 50 °C. Trehalose-based lyophilizates containing phenylalanine and stored at 50 °C did not show any increase over the whole storage period of 26 weeks. Obviously, both formulations stabilized mab_T1 pretty well against formation of soluble aggregates.

Overall, especially trehalose-based lyophilizates that contain phenylalanine as bulking agent showed excellent stabilization of mab_T1 during the indicative stability study.

Samples of aggressive and moderate freeze-drying were compared for a storage temperature of 50 °C (the HMWS of all moderately freeze-dried and stored samples can be found in Table 9.5 in the appendix). Figure 6.14 depicts HMWS of lyophilizates stored for 26 weeks at 50 °C. Samples moderately freeze-dried from formulations containing mannitol and glycine showed substantial formation of HMWS. It is remarkable that all aggressively freeze-dried samples exhibited a comparable or even lower HMWS content compared to moderately freeze-dried samples. Only the moderately freeze-dried suc:phe 10:0.5 formulation exhibited lower HMWS content than the aggressively freeze-dried sample. Interestingly, the former sample was completely collapsed after storage (initially non-collapsed appearance).

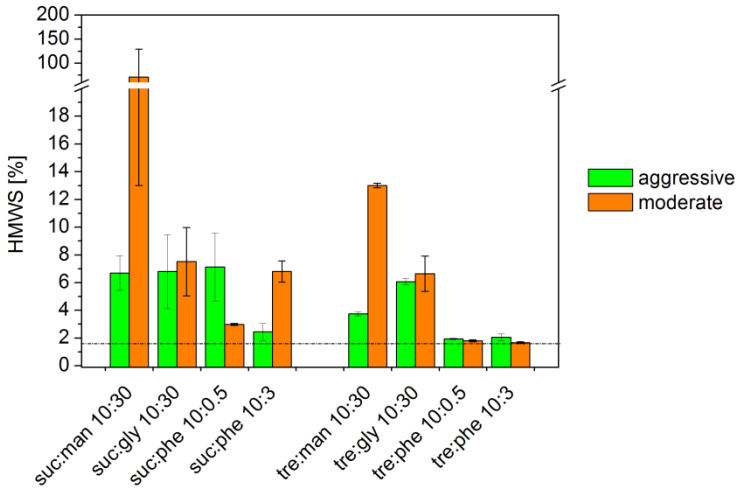

Figure 6.14: Comparison of high molecular weight species (HMWS) after storage over 26 weeks at 50 °C. Green bars represent aggressively freeze-dried samples and orange bars moderately freeze-dried samples, respectively. The dotted line displays the average HMWS content after freeze-drying, i.e. before storing the samples.

Overall, the trehalose-based lyophilizates, which contain phenylalanine (tre:phe 10:0.5, tre:phe 10:3), were able to completely prevent the formation of HMWS. These formulations revealed an excellent stabilization of mab_T1.

The recovery of mab_T1 monomer was calculated from the integrated HP-SEC chromatograms. The monomer content before aggressive freeze-drying was set as 100%. A decrease of monomer recovery is attributed to a loss in protein due to instability of mab_T1 (e.g. aggregation). The monomer recoveries of the different samples after storage at the different temperatures are summarized in Table 6.5.

After freeze-drying, tre:man 10:30 and tre:phe 10:3 already showed a slight decrease of monomer recovery. While for tre:phe 10:3 no further decrease during storage for 26 weeks was detected, the recovery of tre:man 10:30 further decreased for storage temperatures of 30 °C and 50 °C. Overall, the stored samples showed a monomer recovery of above 88%. As expected, lower recovery was detected after 26 weeks at 50 °C. Comparing the different formulations, aggressively freeze-dried and stored suc:gly 10:30, tre:gly 10:30, tre:phe 10:0.5, and tre:phe 10:3 showed good monomer recovery of ≥ 95%.

Table 6.5: Monomer recovery of aggressively freeze-dried samples after storage.

aggressive	HP-SEC monomer recovery (%)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	99.4 ± 0.2	99.2 ± 0.2	105.1 ± 0.3	98.0 ± 0.4	98.2 ± 0.5	94.8 ± 0.5	89.0 ± 1.7
suc:gly 10:30	99.8 ± 0.4	100.3 ± 0.2	107.1 ± 1.8	100.8 ± 0.4	100.1 ± 0.4	97.0 ± 0.3	96.7 ± 0.3
suc:phe 10:0.5	100.8 ± 0.3	101.8 ± 0.6	96.9 ± 1.6	101.3 ± 0.6	95.5 ± 0.4	69.0 ± 23.2	90.3 ± 1.0
suc:phe 10:3	100.3 ± 0.7	100.8 ± 1.0	94.7 ± 0.8	100.5 ± 0.7	95.9 ± 0.7	101.5 ± 2.1	92.8 ± 0.7
tre:man 10:30	98.6 ± 0.3	97.8 ± 0.3	98.5 ± 0.3	96.5 ± 0.5	96.8 ± 0.4	92.4 ± 0.7	88.0 ± 0.5
tre:gly 10:30	101.3 ± 0.5	101.7 ± 0.3	100.1 ± 0.3	100.4 ± 0.6	100.4 ± 0.4	98.4 ± 0.4	95.4 ± 0.4
tre:phe 10:0.5	103.9 ± 1.1	103 ± 0.9	96.7 ± 0.7	102.7 ± 0.8	97.1 ± 0.3	101.8 ± 1.1	96.2 ± 0.2
tre:phe 10:3	97.8 ± 0.6	98 ± 0.5	95.8 ± 0.7	97.6 ± 0.6	95.7 ± 0.3	95.3 ± 0.3	94.9 ± 0.2

Again, the lyophilizates from aggressive freeze-drying were compared to the samples from moderate freeze-drying. In order to benchmark both species for the most challenging storage conditions, the monomer recovery after 26 weeks at 50 °C is compared (cf. Figure 6.15) (the monomer recovery of all moderately freeze-dried and stored samples can be found in Table 9.6 in the appendix). While the monomer recovery of all aggressively freeze-dried samples ranged above 90%, the monomer recovery of several moderately freeze-dried samples was drastically decreased. Sucrose-based formulations containing mannitol or glycine exhibited a recovery of only 4% and 56%, respectively. The monomer recovery of moderately freeze-dried formulations containing mannitol (69%) or glycine (90%) was decreased, too. All formulations containing phenylalanine as bulking agent exhibited a monomer recovery of ≥ 98%.

As already observed for HMWS content, the trehalose-based formulations, which contain phenylalanine (tre:phe 10:0.5, tre:phe 10:3), exhibited excellent stabilization of mab_T1 also in terms of monomer recovery.

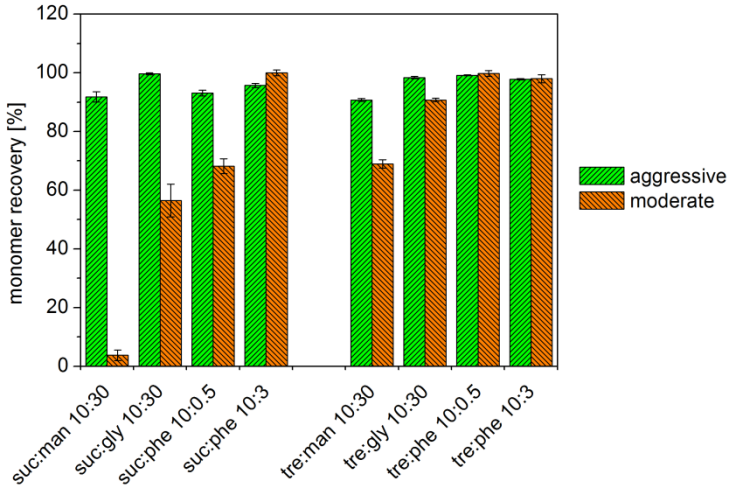

Figure 6.15: Comparison of monomer recovery after storage for 26 weeks at 50 °C. Green bars represent aggressively freeze-dried samples and orange bars moderately freeze-dried samples, respectively.

Non-soluble aggregates

The formation of non-soluble aggregates during storage of the aggressively freeze-dried samples was monitored by analyzing the turbidity as well as the subvisible particle concentration. Figure 6.16 depicts the turbidity values for the different storage temperature over storage time. On the left-hand side, sucrose-based samples are shown and on the right-hand side trehalose-based samples.

At a storage temperature of 2-8 °C, the sucrose- and trehalose-based lyophilizates containing glycine or phenylalanine (low or high amount) showed no change in turbidity. As outlined above, the samples, which apply high amounts of bulking agent (sugar:gly 10:30 and sugar:phe 10:3) exhibited a higher turbidity already right after aggressive freeze-drying. Because no increase in turbidity was detectable, it was concluded that mab_T1 was stabilized against further aggregation. Lyophilizates from both formulations that contain mannitol showed a slight increase in turbidity during storage at 2-8 °C. These samples exhibited the highest turbidity level already right after aggressive freeze-drying. Although stored at low temperature, in the course of storage, additional aggregation occurred in theses lyophilizates.

At a storage temperature of 30 °C the same trend could be observed for the formulations containing mannitol: a slight increase in turbidity compared to the value after aggressive freeze-drying. Evidently, the formulations containing mannitol did not protect mab_T1 against aggregation. Storage of the sucrose-based formulation containing glycine revealed a considerable increase of turbidity after 26 weeks at 30 °C, too. The trehalose-based samples applying glycine as bulking agent only showed a minimal increase of turbidity.

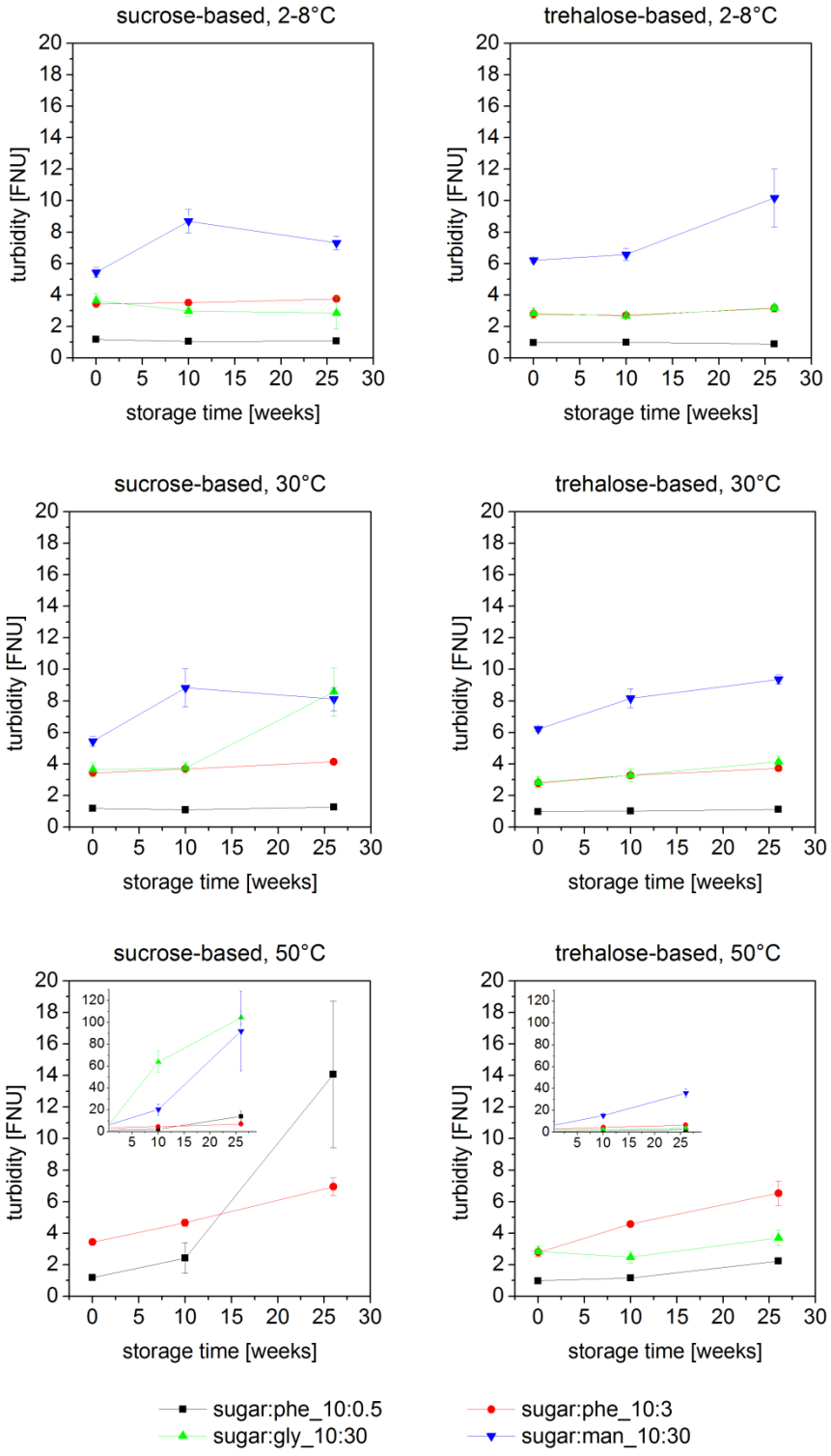

Figure 6.16: Turbidity after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

For sucrose- and trehalose-based lyophilizates, which contain a high amount of phenylalanine, a minimal increase of turbidity could be noticed, too. Both formulations that contain a low amount of phenylalanine exhibited a low turbidity even after 26 weeks at 30 °C. Apparently, these formulations were able to protect mab_T1 completely against aggregation.

Storage at 50 °C confirmed the trends already observed for a storage temperature of 30 °C. Formulations based on **sucrose** and containing mannitol or glycine revealed a dramatic increase of turbidity to up to > 80 FNU (see inserted plot in the lower left graph in Figure 6.16). Obviously, at that storage temperature, these formulations were unable to stabilize mab_T1 against aggregation. The formulations containing phenylalanine showed a considerable increase of turbidity, too. The lyophilizates containing a low amount of phenylalanine showed an even more distinct increase compared to the formulation applying a high amount of phenylalanine.

Focusing on the **trehalose** based lyophilizates stored at 50 °C, the samples containing mannitol as bulking agent showed a dramatic increase of turbidity. No successful stabilization of mab_T1 was achieved in these lyophilizates. Also the samples, which contain a high amount of phenylalanine (tre:phe 10:3) exhibited a considerable increase of turbidity. Aggregation of mab_T1 was not completely prevented. Both, the formulation containing glycine and a low amount of phenylalanine, respectively, only exhibited a slight increase in turbidity. Evidently, even during storage for 26 weeks at 50 °C, these formulations were able to prevent aggregation almost completely.

Overall, the sugar-based samples containing phenylalanine at low content (suc:phe 10:0.5, tre:phe 10:0.5) revealed exceptional stabilization of mab_T1. While sucrose-based lyophilizates prevented aggregation upon storage at up to 30 °C, trehalose-based samples exhibited excellent stability even at up to 50 °C.

For comparison, the turbidity of moderately freeze-dried and subsequently stored samples was analyzed (cf Figure 6.17). Overall, for storage at 2-8 ° and 30 °C similar trends were observed as for aggressively freeze-dried samples. Storage of the moderately freeze-dried lyophilizates at 50 °C revealed a considerable increase of turbidity in all lyophilizates. Amongst the utilized bulking agents, phenylalanine revealed the lowest increase in turbidity. **However, the excellent stabilization of mab_T1, as observed in aggressively freeze-dried samples, was not achieved by any of the moderately freeze-dried lyophilizates.**

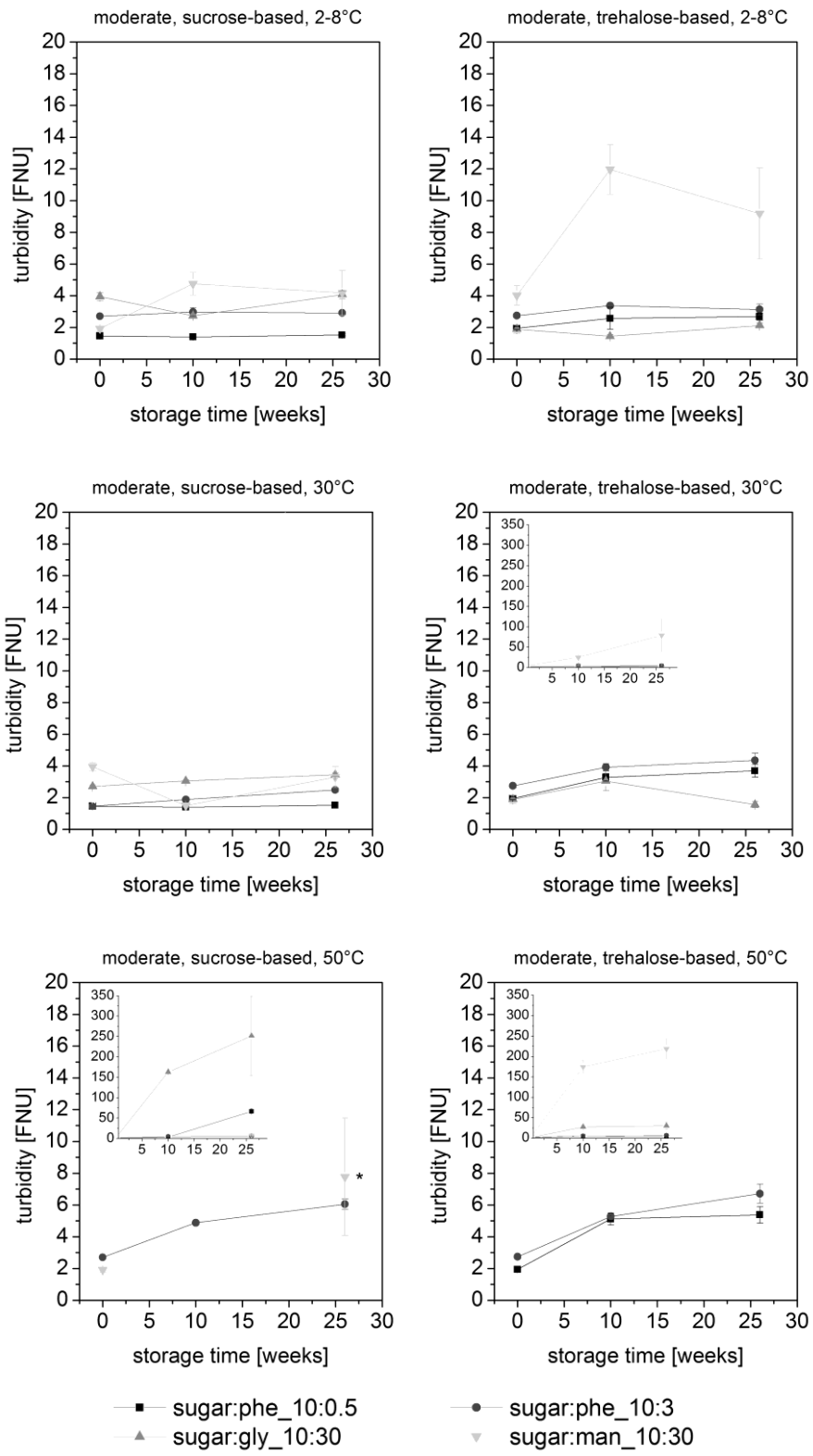

Figure 6.17: Turbidity after storage of moderately freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1; asterisk marks a not fully dissolvable sample).

In addition to the turbidity, the subvisible particle concentration of the stored lyophilizates was assesed. While turbidity is rather indicative for non-soluble protein aggregates, also in the size range below 1 μm subvisible particle counts reveals non-soluble aggregates of ≥ 1 μm. Figure 6.18 displays the svp counts of aggressively freeze-dried samples subjected to 26 weeks storage at 2-8 °C, 30 °C, or 50 °C. In general, comparable trends could be observed as delineated for the turbidity values shown above.

The mannitol containing samples exhibited svp counts, which are around 10 times higher already after freeze-drying. As expected, in these samples the svp concentration increased upon storage at 30 °C and 50 °C. Interestingly, the suc:man 10:30 lyophilizates exhibited a decrease of svp count after storage at 30 °C. Since the turbidity of these lyophilizates did not show a comparable decrease, the result of the samples after 26 weeks at 30 °C is regarded as outlier.

Based on either sucrose or trehalose, the formulations containing glycine or phenylalanine exhibited a constantly low svp level for storage temperatures of 2-8 °C and 30 °C. Obviously, these lyophilizates completely prevented aggregation of mab_T1 to non-soluble aggregates.

Storing the samples at 50 °C revealed worse stabilization of mab_T1 in sucrose-based lyophilizates that contain glycine (suc:gly 10:30) or a low amount of phenylalanine (suc:phe 10:0.5). In contrast, the respective trehalose based lyophilizates (tre:gly 10:30, tre:phe 10:0.5) showed excellent stabilization. Evidently, these formulations were able to stabilize mab_T1 successfully against aggregation even if stored 26 weeks at 50 °C.

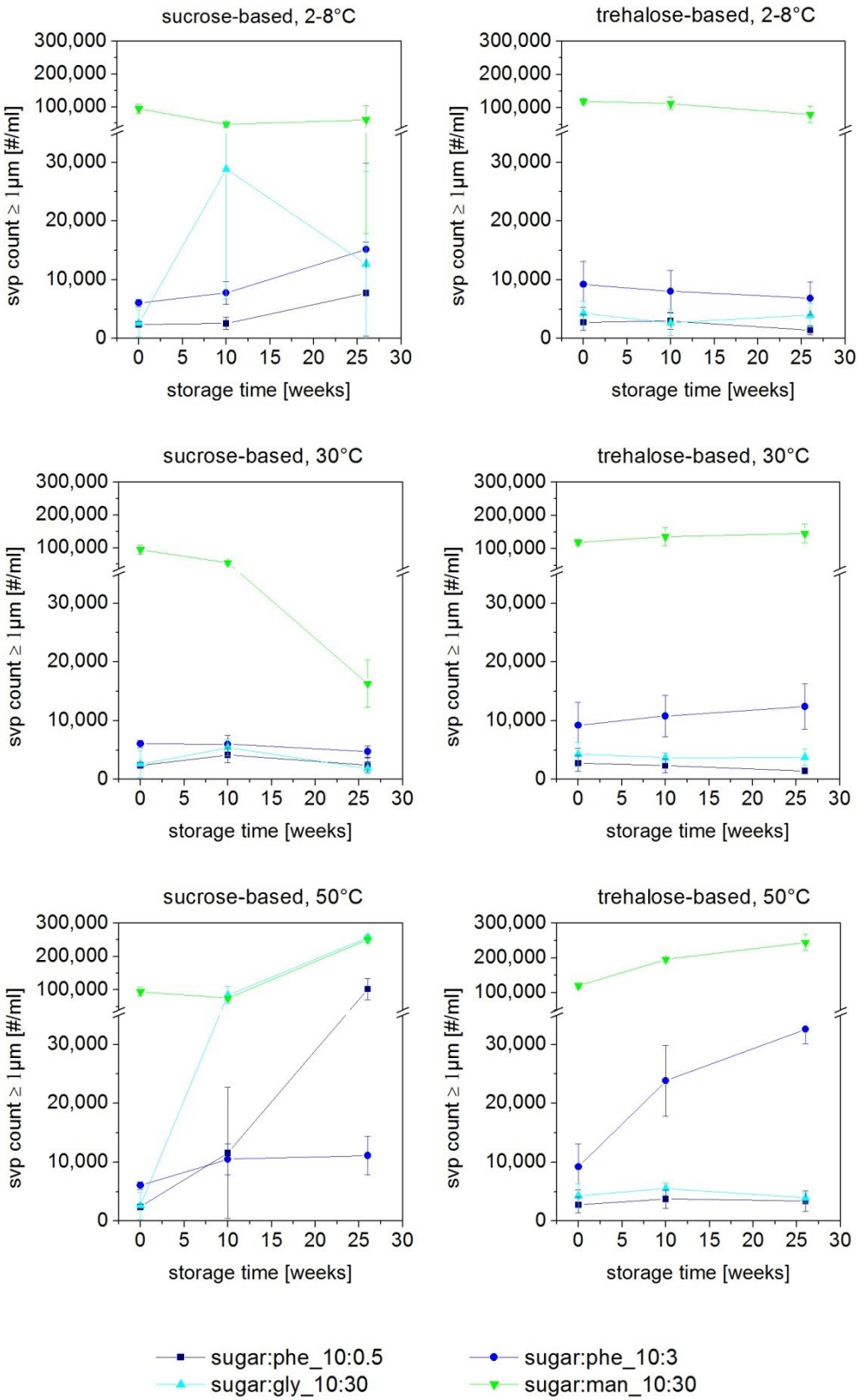

Figure 6.18: Sub-visible particle counts ≤1 µm after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

Table 6.6: Subvisible particle counts of aggressively freeze-dried lyophilizates. Subvisible particle of ≥10µm and ≥25 µm of samples stored at 2-8°C, 30°C, or 50°C are listed, respectively.

	subvisible particles ≥ 10µm and ≥ 25µm (#/ml)													
	after freeze-drying		2-8°C storage				30°C storage				50°C storage			
			10 weeks		26 weeks		10 weeks		26 weeks		10 weeks		26 weeks	
	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
suc:man 10:30	67 ± 31	5 ± 5	417 ± 256	16 ± 19	77 ± 33	1 ± 2	114 ± 35	7 ± 7	114 ± 29	3 ± 3	1549 ± 933	112 ± 51	9110 ± 9363	105 ± 100
suc:gly 10:30	29 ± 14	5 ± 3	137 ± 120	0 ± 0	18 ± 11	0 ± 0	58 ± 28	2 ± 2	17 ± 10	1 ± 2	33 ± 12	6 ± 4	618 ± 185	7 ± 6
suc:phe 10:0.5	86 ± 10	11 ± 5	19 ± 6	1 ± 1	13 ± 10	1 ± 2	30 ± 12	3 ± 3	15 ± 6	1 ± 1	143 ± 140	8 ± 11	1328 ± 466	21 ± 23
suc:phe 10:3	40 ± 5	1 ± 2	97 ± 43	3 ± 4	19 ± 9	1 ± 2	93 ± 49	1 ± 2	36 ± 18	1 ± 2	165 ± 49	2 ± 4	61 ± 32	4 ± 6
tre:man 10:30	411 ± 263	53 ± 49	40 ± 14	0 ± 1	50 ± 19	1 ± 2	67 ± 16	0 ± 1	139 ± 35	0 ± 0	132 ± 41	2 ± 2	95 ± 34	4 ± 3
tre:gly 10:30	13 ± 7	0 ± 1	8 ± 8	0 ± 1	11 ± 6	0 ± 1	40 ± 20	1 ± 1	21 ± 10	0 ± 0	23 ± 6	2 ± 2	6 ± 4	0 ± 0
tre:phe 10:0.5	34 ± 9	0 ± 1	24 ± 16	2 ± 2	9 ± 6	1 ± 1	17 ± 6	1 ± 1	17 ± 15	3 ± 4	38 ± 21	1 ± 1	19 ± 9	1 ± 2
tre:phe 10:3	101 ± 61	5 ± 4	83 ± 72	0 ± 1	31 ± 15	1 ± 2	81 ± 51	1 ± 2	51 ± 14	1 ± 2	372 ± 137	1 ± 2	76 ± 28	1 ± 1

In addition to determining svp of ≥ 1 µm, larger species of ≥ 10 µm and ≥ 25 µm were assessed according to the guideline of the European Pharmacopeia (Ph.Eur. 2.9.19).

The detected amounts of svp ≥ 10 µm and ≥ 25 µm are listed in **Fehler! Verweisquelle konnte nicht gefunden werden.**. Besides the trehalose-based sample containing mannitol stored at 50 °C for 26 weeks, none of the analyzed lyophilizates reached the limit of 6,000 particles ≥ 10 µm. Overall, the svp counts of svp ≥ 25 µm was low (typically below 10 particles per ml) and no sample reached 600 particles ≥ 25µm.

Overall, the svp counts of particles ≥ 10µm underline the observations for turbidity and svp of ≥ 1µm. For a storage temperature of up to 30 °C no apparent increase in the svp counts ≥ 10 µm were noticed. Storing the samples at 50 °C revealed the formation of increase numbers of particles in the lyophilizates composed of sucrose and either mannitol, glycine, or a low amount of phenylalanine (suc:man 10:30, suc: gly 10:30, suc:phe 10:0.5). It appears that in these samples comparably large mab_T1 aggregates were formed.

In contrast to most of the sucrose-based lyophilizates, samples based on trehalose did not show an increase in the svp level of particles ≥ 10 µm. Although smaller particles (≥ 1 µm) were increased in the samples containing mannitol and glycine, no increase in larger aggregates (≥ 10 µm) was detectable.

In order to pin down the subvisible particle counts of aggressively freeze-dried samples after storage, moderately freeze-dried samples are subjected to the identical storage conditions and further, the svp counts were analyzed. Table 6.6 depicts the svp counts of particles ≥ 1 µm. The overall trends are comparable to those observed for the turbidity of stored moderately freeze-dried samples (compare Figure 6.17).

Table 6.6: Subvisible particle counts of moderately freeze-dried lyophilizates. Subvisible particle of ≥1µm of samples stored at 2-8 °C, 30 °C, or 50 °C are listed.

moderate	subvisible particle counts ≥ 1µm (#/ml)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	7,871 ± 1,831	45,627 ± 21,378	7,363 ± 3,093	100,757 ± 32,951	69,956 ± 6,053	n.d.	31,625 ± 16,885
suc:gly 10:30	4,475 ± 3,819	3,144 ± 1,087	1,248 ± 776	8,002 ± 3,438	2,832 ± 881	386,5163 ± 204,069	173,250 ± 24,968
suc:phe 10:0.5	7,108 ± 2,216	2,736 ± 1,265	1,959 ± 760	8,015 ± 2,214	3,051 ± 1,502	38,167 ± 28,708	256,346 ± 5,609
suc:phe 10:3	5,934 ± 1,941	6,584 ± 778	3,912 ± 971	7,617 ± 703	4,723 ± 939	20,361 ± 5,322	35,419 ± 2,659
tre:man 10:30	37,097 ± 3,397	13,4582 ± 5,123	49,157 ± 20,344	458,222 ± 103,443	159,650 ± 15,623	2,908,793 ± 254,374	200,344 ± 8,801
tre:gly 10:30	4,435 ± 1,107	22,525 ± 5,172	2,900 ± 1,106	67,583 ± 11,718	1,841 ± 528	109,483 ± 7,572	190,980 ± 25,623
tre:phe 10:0.5	4,551 ± 2,551	3,873 ± 1,373	3,272 ± 844	2,790 ± 704	5,589 ± 1,957	9,545 ± 468	11,863 ± 1,658
tre:phe 10:3	9,179 ± 3,169	6,944 ± 1,265	6,130 ± 1,819	7,986 ± 1,559	13,468 ± 2,705	19,790 ± 2,077	15,861 ± 1,246

n.d.: not determined

Besides the formulations containing mannitol, all moderately freeze-dried lyophilizates showed low svp contents for a storage temperature of up to 30 °C (and 26 weeks storage). For a storage temperature of 50 °C all moderately freeze-dried samples showed an increase in svp content ≥1 µm. While trehalose-based lyophilizates that contain phenylalanine (tre:phe 10:0.5, tre:phe 10:30) showed a minor increase of svp level, all lyophilizates containing mannitol or glycine exhibited a tremendous increase of svp level after 26 weeks at 50 °C.

Summing up the results regarding aggregation in aggressively freeze-dried lyophilizates, the data have shown excellent stabilization of mab_T1 in trehalose-based samples, which contain phenylalanine (tre:phe). Especially the lyophilizates that contain a low amount of phe (tre:phe 10:0.5) revealed superior stabilization, even after six months storage at 50 °C. Also sucrose-based lyophilizates containing phenylalanine showed admirable stabilization for storage up to 30 °C. On the other hand, independent from sugar, diminished stability was observed for lyophilizates containing mannitol or glycine. Compared to moderate freeze-drying, equal or even better storage stability of mab_T1 was achieved by aggressive freeze-drying.

Secondary Structure

As already outlined above, the secondary structure of proteins is susceptive to changes if not properly stabilized. To assess the secondary structure of mab_T1 in the course of storage, the samples were analyzed, utilizing the 2nd derivative FT-IR spectra of reconstituted samples. Since the optical assessment of the spectra does not allow for quantitative comparison, the overlap of spectra was calculated as described by Prestrelski et al. [30] and expressed by a correlation coefficient. The 2nd derivative spectra of samples after freeze-drying and storage were compared to the spectra of the formulations before freeze-drying. A reference sample of mab_T1 solution, which was subjected to a heating ramp in the temperature controlled Bio-ATR cell of the FT-IR spectrometer, exhibited a correlation coefficient of 0.985 at 70 °C, 0.645 at 80 °C and 0.080 at 90 °C. While for a correlation coefficient of 0.985 optically still a good overlap of spectra was attested, a correlation coefficient of 0.645 represent a clearly discernible change in the 2nd derivative FT-IR spectra (spectra not shown).

Figure 6.19 displays the correlation coefficients of aggressively and moderately freeze-dried samples after freeze-drying and storage. Overall, high correlation coefficients of > 0.985 were determined for aggressively freeze-dried samples (tre:man 10:30 is regarded as outlier since the stored samples of this formulation showed high correlation coefficients). Due to interfering absorbance of glycine, sample containing this bulking agent could not be assessed by FT-IR spectroscopy. Compared to 2-8 °C and 30 °C storage temperature, for all analyzed formulations it was noticed that a storage temperature of 50°C resulted in a slight decrease of the correlation coefficient, indicating minor changes of the secondary structure in these lyophilizates. Comparing sucrose- and trehalose-based formulations, no apparent difference could be observed. In contrast to the aggregation to soluble and non-soluble aggregates (expressed as HWMS, turbidity and svp count), mannitol exhibited higher correlation coefficients compared to both formulations containing phenylalanine. It is reported that aggregation can occur without detectable

changes in the secondary structure [31, 32]. Comparing the lyophilizates from aggressive freeze-drying to those from moderate freeze-drying, the moderately freeze-dried sucrose-based samples exhibited lower correlation coefficients, i.e. the protection of the secondary structure is less effective. While tre:man 10:30 also showed slightly worse preservation of secondary structure, both formulations containing phenylalanine demonstrated well conservation of the secondary structure.

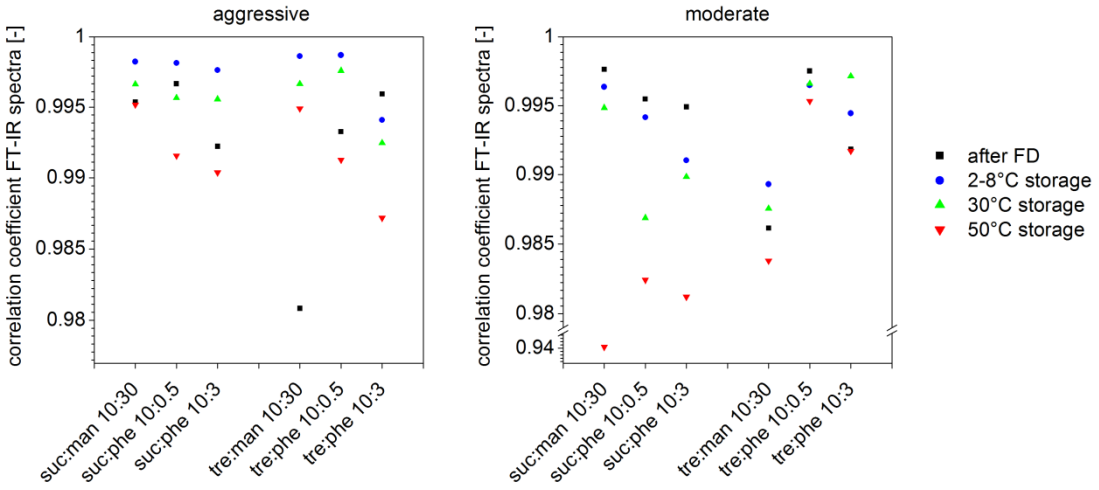

Figure 6.19: Correlation coefficient of FT-IR spectra after freeze-drying and storage over 10 weeks at different temperatures. For calculation of the correlation coefficient, spectra derived from reconstituted samples were compared to the FT-IR spectra of the respective formulations before freeze-drying.

3.2.2 Physico-chemical characteristics after storage

In order to evaluate, whether the trends observed for the stability of mab_T1 in formulations containing bulking agents can be ascribed to physico-chemical characteristics of the respective lyophilizates, the residual water content, glass transition temperatures, specific surface area, and crystallinity were assessed.

Macroscopic appearance

The macroscopic appearance of the trehalose-based samples showed no change after storage over 26 weeks (cf. Figure 6.20). Sucrose-based samples exhibited no change of appearance at a storage temperature of up to 30 °C, too (cf. Figure 6.21). However, storage at 50 °C revealed a change in color from white to brown for the samples containing glycine or a low amount of phenylalanine. In addition, the latter lyophilizates exhibited pronounced shrinkage of the already initially collapsed material. Obviously, these samples underwent non-enzymatic browning via the Maillard reaction (brown pigments are formed by a multi-step reaction between reducing sugars and amino groups [33]).

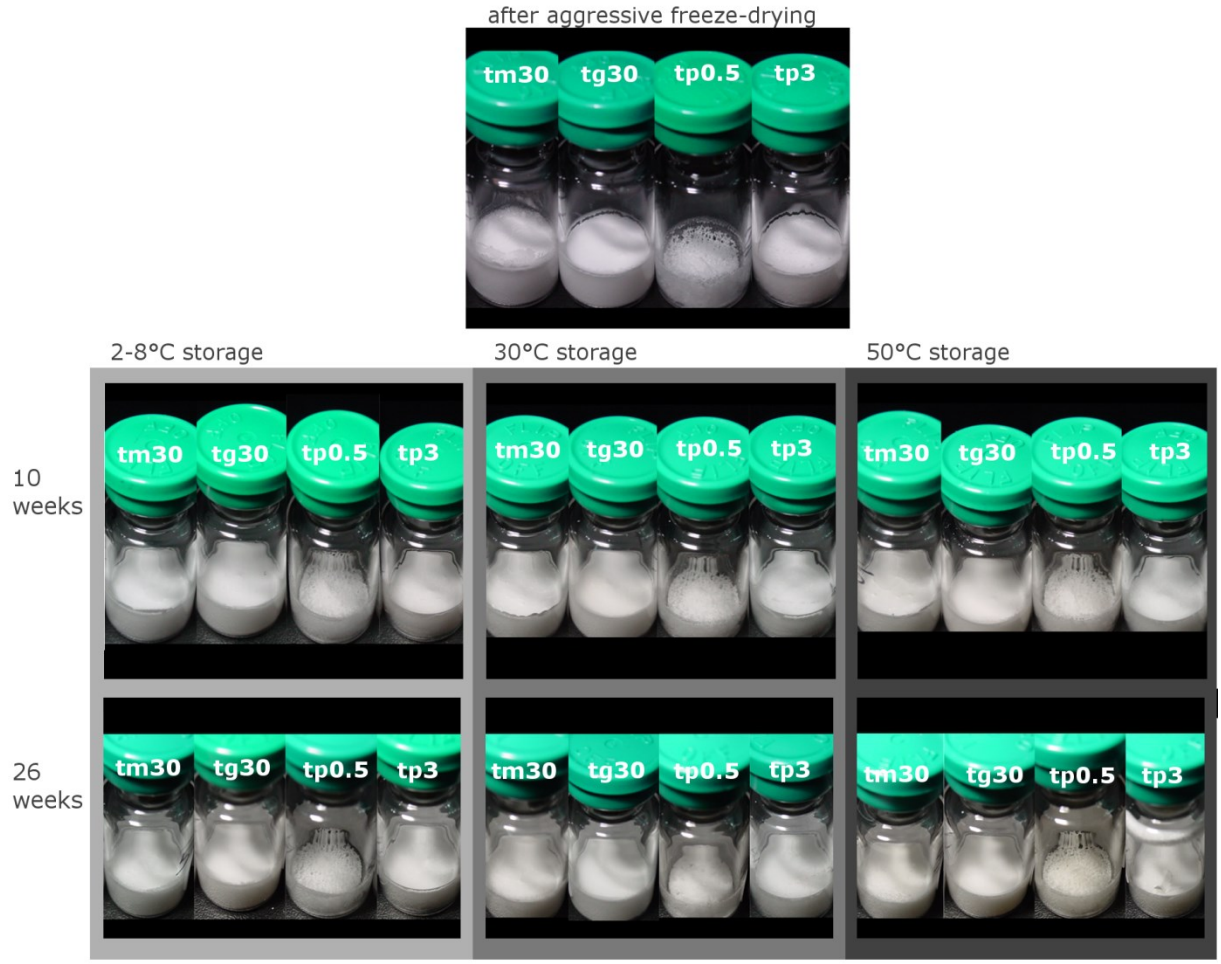

Figure 6.20: Macroscopic appearance after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (trehalose-based formulations contain bulking agents and 1 mg/ml mab_T1; tm30 ≙ tre:man 10:30, tg30 ≙ tre:gly 10:30, tp0.5 ≙ tre:phe 10:0.5, tp30 ≙ tre:phe 10:3).

Freeze-dried sucrose is reported to be prone to non-enzymatic browning [34]. Sucrose itself has non-reducing characteristics and cannot participate in the Maillard reaction directly. Hence, prior to reacting with the amino groups of glycine or phenylalanine sucrose was obviously hydrolyzed to fructose and glucose. However, neither DSC thermograms exhibited melting endotherms at 150 °C (glucose, [35]) or 120 °C (fructose, [36]) nor XRD diffraction data showed a characteristic peak at 17.2 ° 2-θ (cf. Figure 6.24). Apparently, in terms of thermal properties and diffraction the excess of sucrose hides the rather minor amounts of glucose and fructose.

Figure 6.21: Macroscopic appearance after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (sucrose-based formulations contain bulking agents and 1 mg/ml mab_T1; sm30 ≙ suc:man 10:30, sg30 ≙ suc:gly 10:30, sp0.5 ≙ suc:phe 10:0.5, sp30 ≙ suc:phe 10:3).

The macroscopic appearance of moderately freeze-dried samples after storage at up to 30°C for 26 weeks revealed no change in the elegant cake structure compared to initially after freeze-drying (data not shown). Storing the samples at 50°C resulted in discoloration (browning) of the sucrose-based lyophilizates that contain glycine or a low amount of phenylalnine as bulking agent (cf. Figure 6.22). Obviously, non-enzymatic browning happened to these samples, too. The formulation containing sucrose and a low amount of phenylalanine (suc:phe 10:0.5) additionally exhibited pronounced shrinkage of the cake to a dense yellowish-brown puck.

While the aggressively freeze-dried lyophilizates that contain sucrose and mannitol did not show any signs of discoloration after storage at 50°C, the same formulations moderately freeze-dried revealed discoloration, too. No matter whether aggressively or moderately freeze-dried, the formulation containing sucrose and a high amount of phenylalanine (suc:phe 10:3) remained unchanged regarding the color of the lyophilizates (compare Figure 6.21 and Figure 6.22).

Due to the difference in the bond energy of the disaccharide bond of sucrose (> 115 kJ/mol) and trehalose (< 4 kJ/mol), sucrose is more prone to split into glucose and fructose [37]. As expected, aggressively as well as moderately freeze-dried trehalose-based samples showed no discoloration after storage at 50°C – with one exception. The moderately

freeze-dried samples containing glycine as bulking agent exhibited a slight, but discernible discoloration towards a shiny red-brown color. Apparently, although trehalose is comparable robust against hydrolysis, in these samples a certain degree of hydrolysis occurred, which is followed by non-enzymatic browning.

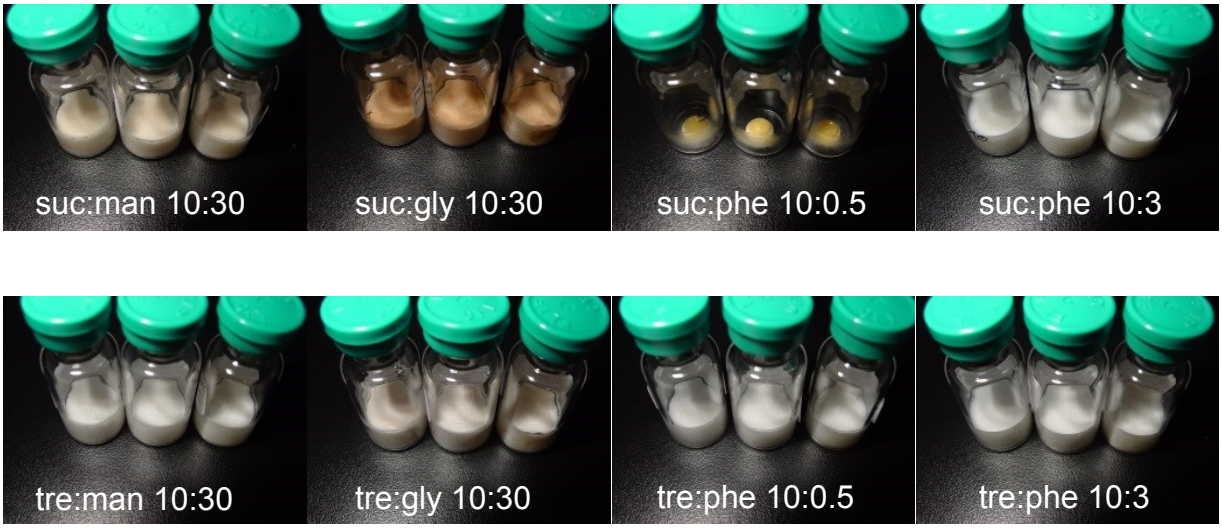

Figure 6.22: Macroscopic appearance after storage of moderately freeze-dried samples at 50 °C for 26 weeks (sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

Non-enzymatic browning is reported to be a diffusion controlled reaction, which is therefore related to the viscosity of the material [38]. As the viscosity drops dramatically at the glass transition, lyophilizates stored above their glass transition temperature are reported to show extensive non-enzymatic browning [38]. In the present study, the sucrose-based samples showing browning when stored in the vicinity to the glass transition temperature (assessed by DSC, cf. Table 6.7). Hence, the necessary mobility for hydrolysis of sucrose and the reaction cascade resulting in non-enzymatic browning is plausible in these lyophilizates.

The aggressively freeze-dried samples containing mannitol did not exhibit non-enzymatic browning upon storage, although they showed comparable glass transition temperatures (close to the storage temperature of 50°C). Hence, the amino acids utilized as bulking agents (glycine, phenylalanine) are considered as main reaction partners of glucose / fructose. The sucrose-based formulation that contain a high amount of phenylalanine (suc:phe 10:3) revealed no change in color. These samples showed considerably high glass transition temperatures throughout the stability study. Obviously, low mobility prevented a chemical reaction.

Glass transition temperature

Generally, the glass transition temperatures of all aggressively freeze-dried samples exhibited no substantial change when storing the samples at 2-8°C, 30°C, 50°C (cf. Table 6.7). As expected, trehalose-based samples showed higher Tg than sucrose-based ones. Especially the lyophilizates that contain phenylalanine (tre:phe) exhibited considerably

high glass transition temperatures of ≥ 90 °C even after storage at 50 °C for 26 weeks (i.e. 6 months). As shown above, these phe containing lyophilizates also offered superior stabilization of mab_T1. Obviously, the high glass transition temperatures allow storage at high temperatures with full stabilization of mab_T1.

Table 6.7: Glass transition temperatures after storage of **aggressively** freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

aggressive	glass transition temperature (°C)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	51.2 ± 0.8	47.8 ± 0.9	46.7 ± 1.9	49.0 ± 2.2	51.8 ± 1.3	51.6 ± 2.8	47.4 ± 0.1
suc:gly 10:30	56.8 ± 0.8	58.4 ± 2.6	38.8 ± 0.4	57.1 ± 1.8	56.3 ± 2.2	49.4 ± 1.2	48.5 ± 1.4
suc:phe 10:0.5	54 ± 0.8	50.9 ± 2.2	56.5 ± 4.3	62.6 ± 4.3	55.8 ± 1.2	58.3 ± 1.6	60.1 ± 1.6*
suc:phe 10:3	75.6 ± 0.9	76.4 ± 1.1	71.5 ± 7.5	73.8 ± 2.0	68.8 ± 1.5	71.0 ± 0.8	71.0 ± 1.5
tre:man 10:30	67 ± 0.2	65.3 ± 2.2	66.5 ± 1.7	66.2 ± 0.7	63.0 ± 4.4	63.7 ± 1.6	64.2 ± 0.1
tre:gly 10:30	73 ± 0.7	73.6 ± 0.7	72.7 ± 2.5	70.7 ± 1.8	65.6 ± 1.9	69.7 ± 2.1	66.6 ± 0.4
tre:phe 10:0.5	94.8 ± 0.7	98.7 ± 1.4	100.9 ± 0.4	96.2 ± 1.1	98.3 ± 0.2	96.2 ± 2	95.1 ± 2.4
tre:phe 10:3	107.1 ± 1.4	105.7 ± 0.7	108.8 ± 1.3	102.2 ± 0.9	100.6 ± 5.2	99.2 ± 1.2	99.5 ± 0.1

*only detectable in one of three analyzed samples, the other two samples showed melting of crystalline sucrose

Also after moderate freeze-drying, the samples containing phenylalanine as bulking agent revealed considerably higher Tg compared to samples applying mannitol or glycine as bulking agent (cf. Appendix, Table 9.2).

The sucrose-based samples containing mannitol or glycine revealed low glass transition temperatures. Nevertheless, these samples were put to all storage conditions in order to compare moderately and aggressively freeze-dried lyophilizates of all formulations also at the highest storage temperature of 50°C. In contrast to the constant glass transition temperatures of aggressively freeze-dried samples throughout the storage study (compare Table 6.7), the moderately freeze-dried lyophilizates revealed a considerable decrease of the glass transition temperatures for all formulations. As expected, storing the suc:man 10:30 lyophilizates roughly 20°C above their glass transition temperature resulted in crystallization of sucrose. In the stored samples no glass transition was detectable. The DSC thermogram revealed melting of crystalline sucrose instead. In addition, XRD revealed the characteristic peaks of crystalline sucrose (compare Figure 9.2). Interestingly, also the formation of α-mannitol was evidently in these samples.

Residual water content

It is a well-known phenomenon that under accelerated storage conditions (i.e. raised temperatures) the residual water content of the lyophilizates can show an increased over time, which is caused e.g. by water vapor permeability of the stopper [39, 40].

Overall, aggressively freeze-dried sucrose- and trehalose-based samples are comparable (cf. Figure 6.23). By aggressive freeze-drying, low initial water content was

achieved. Only the sucrose-based lyophilizates that contain a low amount of phenylalanine showed slightly higher water contents. In addition, they exhibited high standard deviations, which are attributed to inhomogeneity of the water content within the batch. Trehalose-based samples containing phenylalanine have turned out as excellent stabilizer of mab_T1 (see 3.2.1). These lyophilizates exhibited low water content, too. Also the collapsed tre:phe 10:0.5 samples could be dried to < 1%.

The stored samples exhibited no increase of water content throughout the 26 weeks storage period. Even for storage at 50 °C, constant water content was observed (cf. Figure 6.23). Hence, the aggressively freeze-dried lyophilizates provide a robust foundation for storage-stable products.

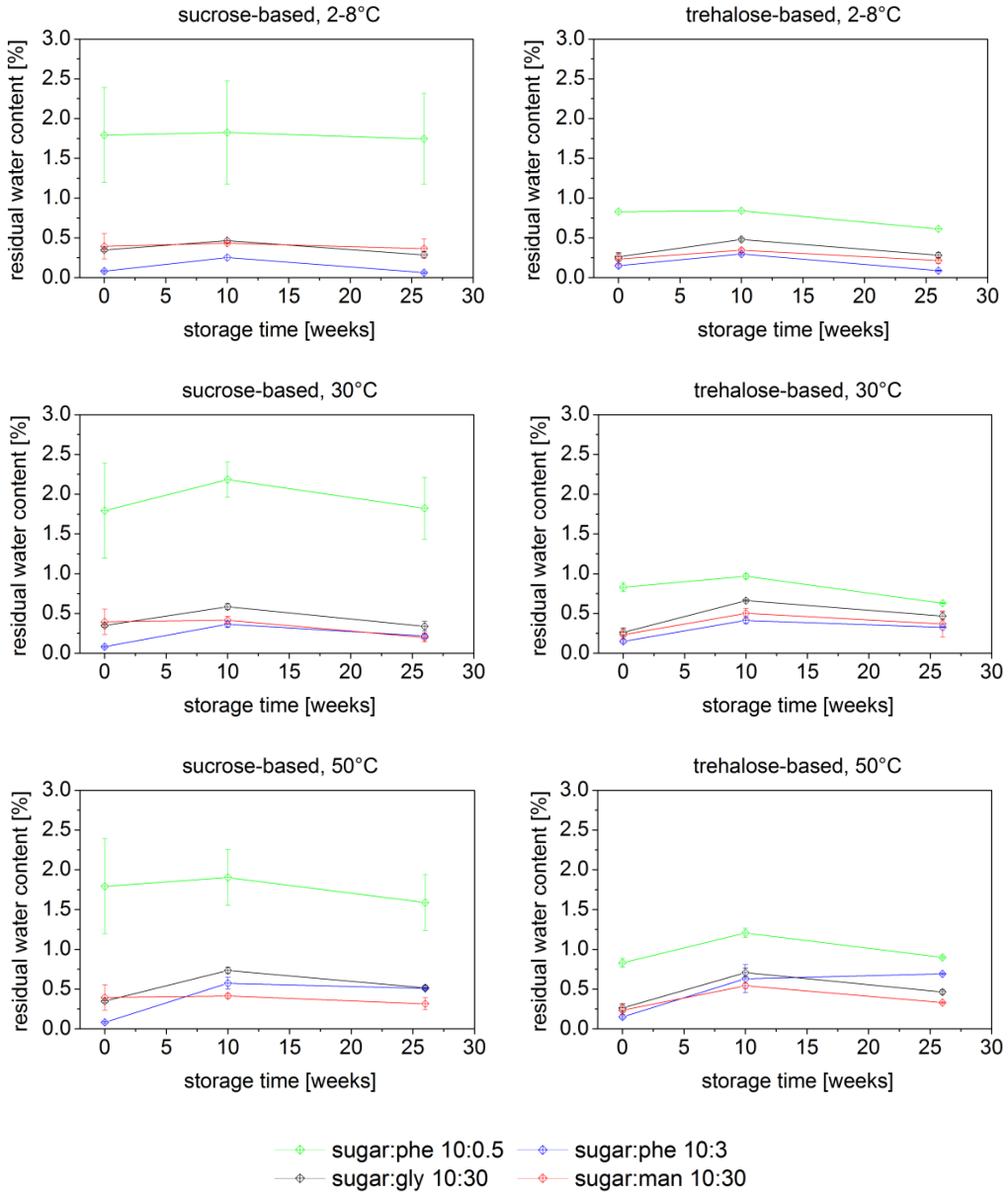

Figure 6.23: Residual water content after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

The minor increase of water content, which is noticed for aggressively freeze-dried suc:man 10:30 samples, is ascribed to water consumption by chemical reactions. This theory is underlined by the considerable decrease of RWC in moderately freeze-dried suc:man samples (compare Appendix, Figure 9.1). The decrease of water is accompanied by a drastic decrease of remaining monomer (i.e. recovery, compare Figure 6.15). Most probably, glycation of mab_T1 occurred in these samples.

Overall, moderately freeze-dried samples exhibited a considerable increase of water content, which was manifested already after 10 weeks at 30°C or 50°C (compare Appendix, Figure 9.1). No further change was noticed between 10 and 26 weeks of storage.

Specific surface area

It was shown in CHAPTER 5 (page 113 pp.) that the addition of substantial amounts of bulking agent resulted in macroscopically non-collapsed lyophilizates, even if aggressive conditions were utilized for freeze-drying (i.e. 45 °C shelf temperature and 1 mbar chamber pressure). These results were underlined in this study. Depending on the nature and amount of bulking agent, a range of specific surface areas (SSA) was noticed after aggressive freeze-drying (see Table 6.8).

Samples, that contain mannitol, glycine, or a high amount of phenylalanine (suc:phe 10:3, tre:phe 10:3) exhibited an intact cake. However, compared to moderately freeze-dried samples (cf. Table 6.9) substantial lower SSA was observed for aggressively freeze-dried lyophilizates of the same formulation. Evidently, micro-collapse occurred during aggressive freeze-drying.

The lyophilizates containing a low amount of phenylalanine (suc:phe 10:0.5, tre:phe 10:0.5) showed very low SSA of 0.09 m^2/g and 0.27m^2/g. These samples collapsed during aggressive freeze-drying due to the high product temperature.

Table 6.8: Specific surface area after storage of **aggressively** freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (macroscopically collapsed samples in bold).

formulation	specific surface area (m^2/g)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	2.29	2.32	2.31	2.28	2.28	2.46	2.38
suc:gly 10:30	0.94	0.77	0.91	0.72	0.82	0.73	0.82
suc:phe 10:0.5	**0.09**	**0.08**	**0.10**	**0.12**	**0.09**	**0.07**	**0.06**
suc:phe 10:3	1.45	1.50	1.30	1.54	1.54	1.43	1.51
tre:man 10:30	2.38	2.26	2.21	2.37	2.33	2.23	2.18
tre:gly 10:30	0.83	0.79	0.79	0.88	0.95	0.81	0.90
tre:phe 10:0.5	**0.27**	**0.19**	**0.24**	**0.30**	**0.26**	**0.26**	**0.26**
tre:phe 10:3	1.62	1.51	1.64	1.46	1.63	1.57	1.79

Table 6.9: Specific surface area after storage of **moderately** freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (macroscopically collapsed samples in bold).

formulation	specific surface area (m^2/g)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	2.19	2.42	2.27	2.64	2.89	1.78	2.11
suc:gly 10:30	2.12	2.45	2.26	2.34	2.10	0.91	1.00
suc:phe 10:0.5	1.65	1.71	1.54	1.55	1.53	**0.01**	**0.02**
suc:phe 10:3	2.54	2.73	2.56	2.57	2.85	2.40	2.38
tre:man 10:30	2.54	2.56	2.94	2.77	3.47	1.99	2.19
tre:gly 10:30	2.70	2.86	2.99	2.47	2.70	1.63	1.66
tre:phe 10:0.5	2.09	1.89	1.92	2.01	1.90	1.92	2.14
tre:phe 10:3	3.26	3.53	3.31	2.96	3.37	3.27	3.08

Some of the lyophilizates that were moderately freeze-dried exhibited a considerable decrease of SSA indicating (micro-) collapse during storage (cf. Table 6.9). Both, the sucrose- as well as trehalose-based lyophilizates containing glycine as bulking agent revealed a distinct decrease of the SSA upon storage at 50°C. The SSA of the suc:phe 10:0.5 samples stored at 50 °C were drastically decreased, too. These samples also exhibited a fully collapsed appearance (cf. Figure 6.22, p. 182).

On the contrary, **none of the aggressively freeze-dried samples exhibited a change of the SSA. Although the storage temperature of 50 °C was close to the glass transition temperature of some samples (compare Table 6.7), no changes of the microscopic structure was noticed after 6 months**. This correlates well with the visual impression of the macroscopic appearance (compare Figure 6.20 and Figure 6.21, page 180). As expected from the high glass transition temperatures, the trehalose-based lyophilizates containing phenylalanine showed no change in SSA during storage (tre:phe 10:0.5, tre:phe 10:3).

Crystallinity

The crystallinity of aggressively freeze-dried samples was analyzed utilizing XRD. Figure 6.24 depicts the resulting diffractograms. The formulations containing glycine, mannitol, or a high amount of phenylalanine did not reveal any apparent alteration of the XRD pattern in the course of storage (independent from sugar or storage temperature). Indicated by distinct diffraction peaks, all of these samples revealed a partly crystalline structure, which remained unchanged during storage.

Lyophilizates composed of sucrose or trehalose and a low content of phenylalanine exhibited a fully amorphous structure after aggressive freeze-drying. The trehalose-based lyophilizates (tre:phe 10:0.5) remained fully amorphous for all storage conditions after 26 weeks. For a storage temperature of 30°C, also the sucrose-based lyophilizates (suc:phe 10:0.5) revealed a fully amorphous structure. However, upon storage at 50°C for 26 weeks these samples exhibited a distinct diffraction pattern, which can be attributed to crystalline sucrose. Obviously, high mobility in these samples, which were stored close to the Tg, fostered crystallization of sucrose from the amorphous matrix.

Moderate freeze-drying of formulations that contain glycine or a high amount of phenylalanine showed a partly crystalline structure comparable to samples from aggressive freeze-drying (data see appendix, Figure 9.2). Independent from temperature, no change of crystallinity was observed after 26 weeks storage. Lyophilizates, that contain a low amount of phenylalanine (suc:phe 10:0.5, tre:phe 10:0.5), remained fully amorphous throughout the storage period.

In contrast, distinct alteration in the crystallinity and polymorphism upon storage was observed in the lyophilizates that contain mannitol as bulking agent. The different crystalline structures, which appeared for the different storage temperatures, suggest that in a first step α-mannitol is formed (characteristic peaks at 13.6°, 17.2°, and 18.7° 2-θ). Storing these samples at 50°C additionally resulted in crystallization of sucrose, indicated by characteristic peaks at 11.7° , 13.1°, 19.6°, and 31.9° 2-θ.

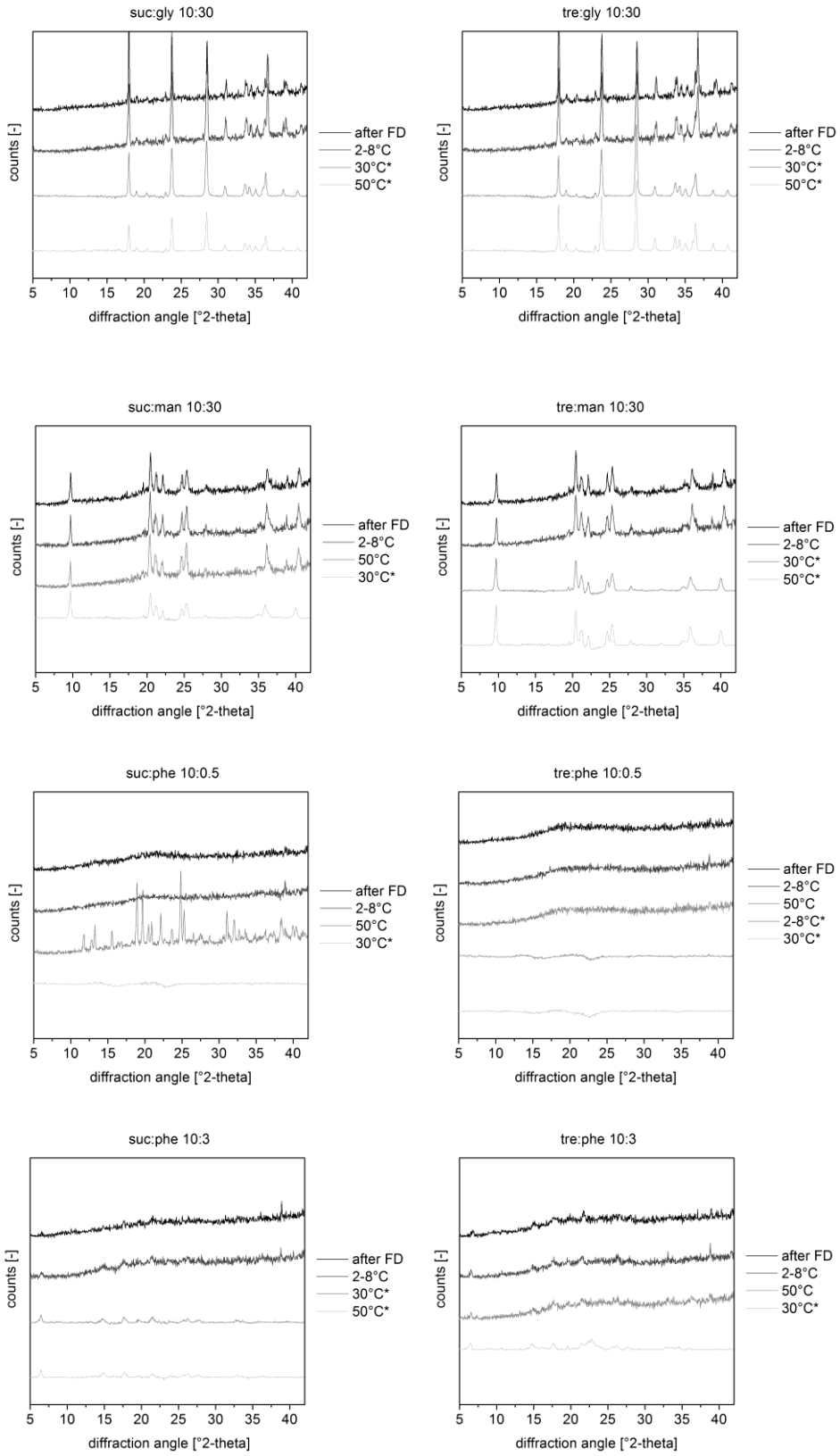

Figure 6.24: XRD diffraction patterns after storage of aggressively freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1; diffraction patterns marked by * were measured on an alternative device).

3.2.3 SUMMARY

During the accelerated storage stability study (6 months at 2-8°C, 30°C and 50°C) of sugar-based lyophilizates that contain bulking agents, several trends were observed.

Especially storage of samples at 50°C revealed pronounced differences of the formulations in the ability to stabilize incorporated mab_T1. **The formulations based on trehalose and utilizing phenylalanine as bulking agent, exhibited excellent stabilization of mab_T1 throughout the whole storage period**. On the other hand, especially formulations containing mannitol failed in protection of mab_T1 against aggregation. Insufficient stabilization of a mAb in mannitol containing lyophilizates from aggressive freeze-drying was already observed by Schersch et al. [3].

Already after aggressive freeze-drying both, the sucrose- as well as the trehalose-based lyophilizates that contain mannitol, revealed aggregation of mab_T1. In the course of storing the samples at 30°C or above, further aggregation was detected, which can be probably attributed to the comparably low glass transition temperature. At a storage temperature of 50°C the samples are stored in the vicinity of their Tg, allowing for increased mobility. The same phenomenon was observed for sucrose-based samples that contain only a low amount of phenylalanine. Finally, storage of these samples at 50°C (i.e. close to their initial Tg) resulted in substantial non-enzymatic browning. The pronounced formation of soluble and non-soluble aggregates, detected for these samples, can be attributed to chemical degradation to a serious extend. Non-enzymatic browning was observed in the sucrose-based samples that contain glycine as well. The discoloration of these samples is accompanied by distinct increase of non-soluble aggregates.

Comparing the data for a storage temperature of 30°C, which is at least 20 K below the initial glass transition temperatures of the aggressively freeze-dried lyophilizates, formulations that contain glycine or phenylalanine revealed complete preservation of mab_T1 throughout the storage period of 6 months.

Compared to the equivalents from moderate freeze-drying, the aggressively freeze-dried samples performed at least equal in terms of protein stabilization. Considering the physico-chemical characteristics, the aggressively freeze-dried samples showed less change of important properties. While moderately freeze-dried samples showed an increase in residual water content and hence a decrease of glass transition temperatures over time, aggressive freeze-drying resulted in lyophilizates that remained unchanged even upon storage at 50°C. Moderately freeze-dried lyophilizates that contain mannitol or glycine showed low Tg, which potentially explains the worse performance in terms of protein stabilization of these samples especially if stored at 50°C. This underlines the importance of high glass transition temperatures for sound storage stability. Low Tg allows for increased mobility, resulting in non-enzymatic browning. Instability in mannitol containing samples is also indicated by crystallization (sucrose-based) or a change in mannitol polymorphism (sucrose- and trehalose-based). In addition, while overall the residual water content is rather increased during storage, the samples containing mannitol as bulking agent exhibited a decrease upon storage at 30°C and 50°C. This is considered as a further hint for chemical reactions in these lyophilizates.

In the end, the study on the storage stability revealed that lyophilizates, which were aggressively freeze-dried from sugar-based formulations of high bulking agent content (mannitol, glycine, phenylalanine), exhibited a non-collapsed appearance, but were less

successful in stabilizing the sensitive model mab_T1. Due to the formation of a crystalline structure, these products were able to withstand macroscopic collapse. However, the macroscopic intact cake exhibits a large specific surface area, which diminishes the stabilization. Less successful stabilization was also shown for the moderately freeze-dried lyophilizates, all of which exhibited non-collapsed appearance and large SSA. Moreover, the correlation between SSA and stabilization was also shown for other dried products in the literature [41].

On the other hand, outstanding storage stability of mab_T1 was achieved by aggressively freeze-dried lyophilizates that contain trehalose and phenylalanine at a ratio of 10:0.5. These samples showed a collapsed structure of low specific surface area. Moreover, they were fully amorphous and exhibited high glass transition temperature. Despite the low SSA, these samples could be dried to low water contents within 12 h by the aggressive protocol (i.e. 4 h freezing and 8 h drying).

Considerations on the reason for differences in stabilization unveiled two facts: On the one hand, the formulations containing mannitol exhibited the highest SSA and worst stabilization. On the other hand, at high storage temperatures of 50°C trehalose-based samples of lower SSA (low amount of phenylalanine) performed better than tre:phe 10:3 presenting higher SSA. Finally, this led to the conclusion that high SSA serves most probably as a key instability factor. Consequently, for most effective stabilization of proteins in lyophilizates, low SSA should by aspired by freeze-drying.

3.3 AMINO ACIDS AS BULKING AGENTS

As outlined above, a number of amino acids have been evaluated regarding their potential to act as bulking agent in sugar-based formulations intended for aggressive freeze-drying. Based on either sucrose or trehalose, the formulations shown in Table 6.10 were subjected to aggressive freeze-drying in 2R vials with 1 ml fill according to the protocol shown in Table 3.4 (page 55) using a FTS LyoStar II freeze-drier. Since phenylalanine has shown favorable physico-chemical characteristics at a sugar to phenylalanine ratio of 10:0.5 (see previous section), this weight-to-weight ratio was also applied for the investigation of the further amino acids. To assess the ability to stabilize an incorporated protein, mab_T1 was added to the formulations at a concentration of 1 mg/ml. The formulations were composed at a total solid content of 51 mg/ml. Amongst the selection of amino acids, glycine is the most prominent representative, which is reported to be used as bulking agent in some formulations [18].

The physico-chemical characteristics of the resulting lyophilizates were already described (see CHAPTER 5, page 125pp.) regarding macroscopic appearance, specific surface area, residual water content, glass transition temperature, and crystallinity. In this section, the integrity of mab_T1 is evaluated by HP-SEC, analyzing the samples regarding their monomer/dimer content and recovery of monomer after aggressive freeze-drying. In addition, the potential formation of non-soluble aggregates is followed by the turbidity and sub-visible particle concentration.

Table 6.10: Formulations to characterize the ability of combinations of sugars and amino acids to stabilize mab_T1 during aggressive freeze-drying.

formulation	mab_T1	sucrose	trehalose	arginine	glycine	histidine	isoleucine	leucine	methionine	tryptophan
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc:arg	1	47.5		2.5						
suc:gly	1	47.5			2.5					
suc:his	1	47.5				2.5				
suc:ile	1	47.5					2.5			
suc:leu	1	47.5						2.5		
suc:met	1	47.5							2.5	
suc:trp	1	47.5								2.5
tre:arg	1		47.5	2.5						
tre:gly	1		47.5		2.5					
tre:his	1		47.5			2.5				
tre:ile	1		47.5				2.5			
tre:leu	1		47.5					2.5		
tre:met	1		47.5						2.5	
tre:trp	1		47.5							2.5

Before analyzing the lyophilizates regarding protein integrity, the samples were reconstituted with highly purified water and the reconstitution time was determined. Table

6.11 depicts the reconstitution times that were noticed for the different formulations. All samples were completely dissolved in less than 20 seconds. Sucrose-based samples seem to exhibit even slightly lower reconstitution times compared to trehalose-based samples. Except the lyophilizate containing isoleucine, all sucrose-based samples were reconstituted within 10 seconds. Taking the macroscopic appearance into consideration (compare Figure 6.25, all samples exhibited a collapsed appearance) the fact that these collapsed lyophilizates were readily reconstituted is remarkable and confounds the reports on generally prolonged reconstitution times, which were attributed to collapsed lyophilizates [19].

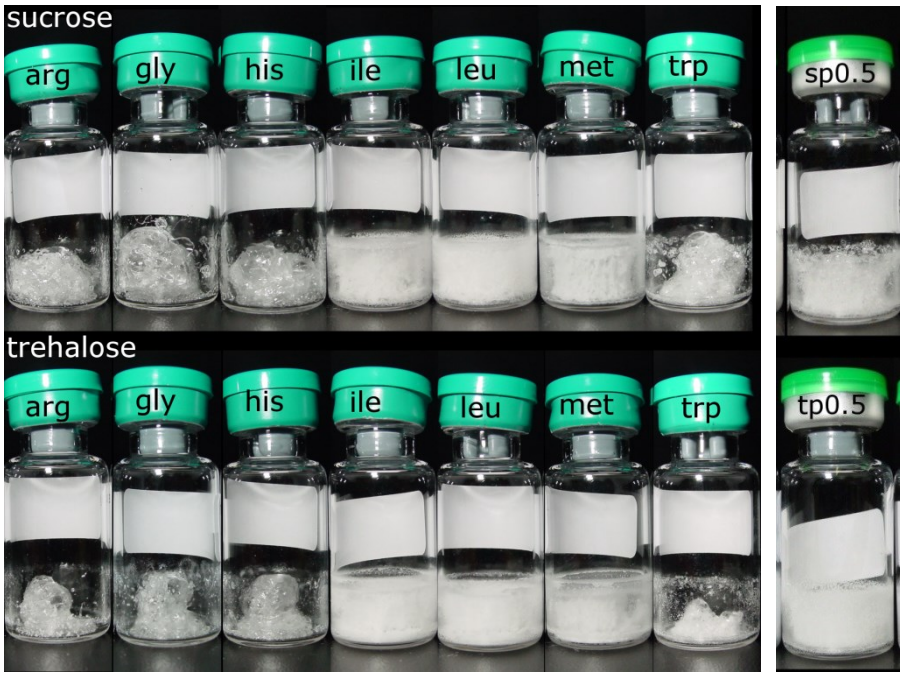

Figure 6.25: Macroscopic appearance of aggressively freeze-dried sugar-based formulations containing different amino acids at a sugar to amino acid ratio of 10:0.5 (upper row sucrose based, lower row trehalose based). For comparison suc:phe 10:0.5 (sp5) and tre:phe 10:0.5 (tp0.5) is shown.

Table 6.11: Reconstitution times (n=6) of sugar-based lyophilizates containing amino acids at a ratio of 10:0.5 and 1 mg/ml mab_T1.

amino acid	sucrose	trehalose
	seconds	seconds
arginine	7 ± 1	18 ± 3
glycine	9 ± 1	17 ± 2
histidine	8 ± 1	15 ± 2
isoleucine	17 ± 3	9 ± 1
leucine	7 ± 1	10 ± 3
methionine	7 ± 1	12 ± 3
tryptophan	8 ± 1	14 ± 2

HP-SEC analysis of formulations prior to aggressive freeze-drying, revealed a monomer content of 97.6%. The monomer content of the reconstituted samples was throughout comparable to the formulations prior to aggressive freeze-drying (cf. Table 6.12). Also the protein recovery of reconstituted samples was calculated. As shown in Table 6.12, no apparent loss of monomer was noticed (overall high monomer recovery of > 98.5%). Although trehalose-based samples seem to exhibit minimally less monomer recovery, due to overlapping standard deviations, no trend could be derived. Neither the formation of high molecular weight species (HMWS) nor loss in monomer could be noticed for the aggressively freeze-dried samples. Thus it it concluded that mab_T1 was stabilized against aggregation to soluble aggregates by all of the investigated formulations. Obviously, the excess amount of stabilizing amorphous disaccharide (sucrose or trehalose) renders the stabilization of mab_T1 highly effective.

Table 6.12: Monomer content and recovery of mab_T1 (1 mg/ml) aggressively freeze-dried from sugar-based formulations containing amino acids at a ratio of 10:0.5. (n=3).

	HP-SEC			
amino acid	sucrose based		trehalose based	
	monomer content	monomer recovery	monomer content	monomer recovery
	mean ± SD	mean ± SD	mean ± SD	mean ± SD
	%	%	%	%
arginine	97.64 ± 0.06	104.8 ± 4.2	97.56 ± 0.02	103.0 ± 1.8
glycine	97.79 ± 0.04	100.5 ± 2.8	97.77 ± 0.02	98.8 ± 2.1
histidine	97.80 ± 0.01	100.5 ± 1.6	97.57 ± 0.02	99.9 ± 1.7
isoleucine	97.86 ± 0.01	100.8 ± 1.1	97.63 ± 0.01	98.7 ± 1.7
leucine	97.77 ± 0.01	99.2 ± 1.3	97.63 ± 0.01	98.3 ± 1.3
methionine	97.75 ± 0.01	98.5 ± 1.4	97.67 ± 0.00	99.5 ± 1.1
phenylalanine	97.43 ± 0.50	100.8 ± 0.3	97.33 ± 0.55	100.6 ± 1.1
tryptophan	97.90 ± 0.01	99.2 ± 1.2	97.65 ± 0.01	99.3 ± 1.6

The formulations before freeze-drying exhibited low subvisible particle counts (around 2,000 to 3,000 particles ≥ 1 µm per ml) and low turbidity values (around 0.8 FNU). Analysis of the reconstituted aggressively freeze-dried lyophilizates revealed comparably low svp content and turbidity for samples that contain arginine, glycine, histidine, tryptophan, or phenylalanine. No increase was observed for sucrose-based (cf. Figure 6.26) or trehalose-based (Figure 6.27) samples. On the contrary, the samples containing isoleucine, leucine, or methionine exhibited an increase in turbidity and/or svp content, clearly indicating aggregation of mab_T1 in these formulations. The most pronounced increase of aggregates was detected for the samples the contain isoleucine.

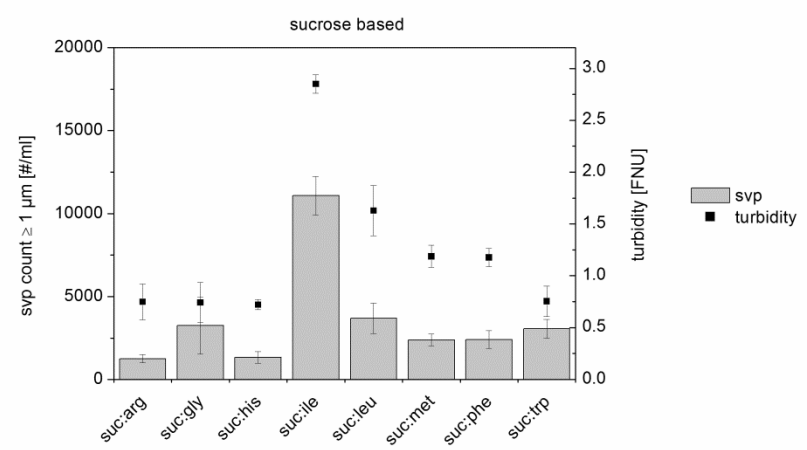

Figure 6.26: Subvisible particle counts (particles ≥ 1µm) and turbidity of lyophilizates aggressively freeze-dried from sucrose-based formulations containing amino acids at a ratio of 10:0.5 (subvisible particle counts before freeze-drying ranged around 3,000 particles ≥ 1µm per ml, turbidity values before freeze-drying around 0.8 FNU).

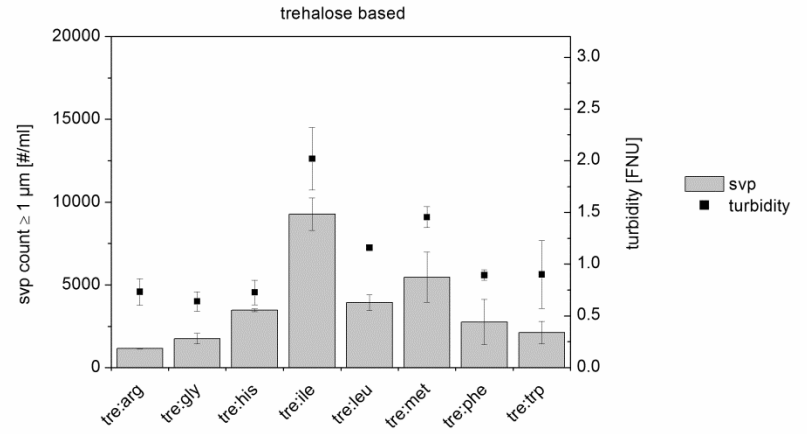

Figure 6.27: Subvisible particle counts (particles ≥ 1µm) and turbidity of lyophilizates aggressively freeze-dried from trehalose-based formulations containing amino acids at a ratio of 10:0.5 (subvisible particle counts before freeze-drying ranged around 3,000 particles ≥ 1µm per ml, turbidity values before freeze-drying around 0.8 FNU).

Taking the physico-chemical characteristics into consideration, the lyophilizates containing isoleucine, leucine, methionine, or phenylalanine showed a number of characteristics that differ from the other formulations. For comparison, the physico-chemical and integrity data of the trehalose-based samples are summarized in Table 6.13 (overall similar trends were observed for sucrose-based samples).

Table 6.13: Summary amino acid screening.

amino acid	appear-ance	SSA	crystallin-ity	water content	Tg	monomer content	svp	turbidity
		m^2/g		%	°C	%	≥1µm/ml	FNU
arginine	collapsed	0.06	amorphous	3.8	57.5	97.6	1161	0.73
glycine	collapsed	0.08	amorphous	2.8	57.3	97.8	1755	0.64
histidine	collapsed	0.08	amorphous	3.5	57.9	97.6	3470	0.73
isoleucine	collapsed	0.17	partly crys-talline	0.9	94.7	97.6	9269	2.02
leucine	collapsed	0.13	partly crys-talline	0.4	91.1	97.6	3925	1.16
methionine	collapsed	0.15	partly crys-talline	1.3	88.3	97.7	5462	1.46
phenylala-nine	collapsed	0.27	amorphous	0.8	94.8	97.3	2759	0.90
tryptophan	collapsed	0.09	amorphous	3.3	61.3	97.7	2114	0.90

The appearance of these lyophilizates exhibited a different collapsed nature (see Figure 6.25) compared to the lyophilizates containing arginine, glycine, histidine, or tryptophan. The collapsed structure is underlined by the low specific surface area of all lyophilizates. Slightly higher SSA was noticed in the samples that contain isoleucine, leucine, methionine, and phenylalanine. As shown by XRD (compare also Figure 5.17), isoleucine, leucine and methionine formed crystalline structures (although they are utilized in low amounts compared to the amorphous sugar matrix). This increase of SSA is accompanied by crystallization of the incorporated amino acids in ile, leu, and met samples. As indicated by an increase of subvisible particles and turbidity, in these partly crystalline samples the ability to stabilize incorporated mab_T1 was diminished.

On the contrary, the phenylalanine containing lyophilizates remained fully amorphous. Although these samples exhibited a slightly higher SSA (compared to arg, gly, his, and trp), an excellent stabilization of mab_T1 was achieved.

The lyophilizates containing arg, gly, his, and trp showed an excellent stabilization during aggressive freeze-drying, too. However, these amino acids had no (decreasing) effect on the high residual water content of aggressively freeze-dried lyophilizates. Hence, the glass transition temperatures were considerably lower. Due to the high water content and low Tg, a successful stabilization during storage is questionable. Nevertheless, it was shown that admixing non-crystallizing amino acids to formulations intended for aggressive freeze-drying did not cause instability of an embedded protein.

In summary, only phenylalanine at low concentrations was able to decrease the water content of sugar-based lyophilizates, remain fully amorphous, and successfully stabilized embedded mab_T1.

4. SOLID CONTENT OF THE FORMULATION

Most of the formulations investigated in this work were designed to contain a solid content of 50 mg/ml, which is representative for the range of commonly used freeze-drying formulations. Usually, the solid content is mainly composed of excipients (cryo-/ lyoprotectant, bulking agent). However, depending on the requirements regarding the stability of the incorporated molecule of interest, a range of different solid contents are reported for freeze-dried products [18].

The effect of different solid contents on the resulting physico-chemical characteristics of lyophilizates from aggressive freeze-drying was already outlined in CHAPTER 5 (page 131 pp.). To address additionally the influence of different solid contents on the ability to stabilize an incorporated protein, a set of formulations of solid contents bracketing the usual range of solid contents for sugar-based formulations (25 mg/ml and 150 mg/ml) was aggressively freeze-dried.

The utilized formulations contain a disaccharide and phenylalanine (at a ratio of 10:0.5). In other studies it was shown that at a solid content of 50 mg/ml the resulting in lyophilizates provide excellent preservation of protein stability (compare section 3.2.1). Since sucrose and trehalose are the most often used disaccharides in freeze-drying formulations [18], both were employed. The formulations used for this study can be found in Table 6.14. 1 ml aliquots of the respective formulations were filled in 2R vials and subjected to the aggressive freeze-drying protocol shown in Table 3.4 (page 55, Table 3.5) in a FTS LyostarIII freeze-drier. The resulting lyophilizates were analyzed regarding the ability to prevent aggregation of mab_T1 during aggressive freeze-drying.

Table 6.14: Formulations to characterize the ability of aggressively freeze-dried formulations of different solid contents to stabilize incorporated mab_T1 (formulations were prepared in 10 mM potassium phosphate buffer, pH 6.4).

formulation	mab_T1	sucrose	trehalose	phenylalanine	excipient content without buffer
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
suc:phe_25	1	23.81		1.19	25
suc:phe_150	1	142.86		7.14	150
tre:phe_25	1		23.81	1.19	25
tre:phe_150	1		142.86	7.14	150

Although the samples exhibited a collapsed appearance (cf. Figure 6.28), they readily rehydrate and were fully reconstituted within less than 30 seconds. The time of reconstitution is depicted in Table 6.15. Compared to the sucrose-based lyophilizates, which were reconstituted in less than 10 seconds, the trehalose-based samples showed slightly slower reconstitution. However, the influence of the sugar or the solid content on the reconstitution time is regarded as negligible since the differences for the samples are in a narrow frame.

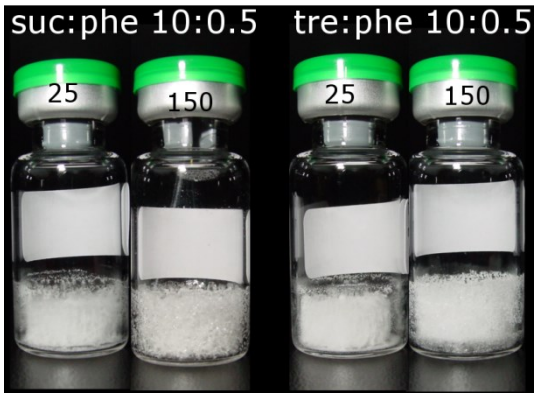

Figure 6.28: Macroscopic appearance of formulations of different solid contents of the formulation (solid content in mg/ml showed as numbers on the vial). Formulations contain either no bulking agent (left) or phenylalanine at a sugar to phenylalanine weight-to-weight ratio of 10:0.5 (right).

Table 6.15: Reconstitution times (n=6) of sugar-based lyophilizates of low and high solid content (the formulations contain a low amount of phenylalanine and 1 mg/ml mab_T1).

formulation	reconstitution time
	seconds
suc:phe_25	7 ± 1
suc:phe_150	7 ± 1
tre:phe_25	27 ± 3
tre:phe_150	17 ± 3

To account for soluble aggregates, the reconstituted samples were analyzed by HP-SEC and compared to the formulations before aggressive freeze-drying. In all samples, three different species could be determined from the HP-SEC chromatograms. In the samples prior to freeze-drying, besides the dominant monomer peak (around 97.8%), also minor fractions of high molecular weight species (HMWS, around 1.6%) and low molecular weight species (LMWS, around 0.6%) were detected. Figure 6.29 shows the monomer content of the formulations before and after freeze-drying.

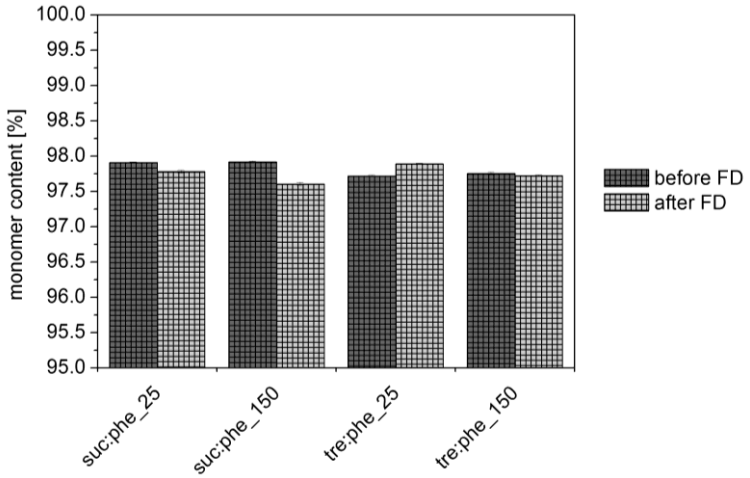

Figure 6.29: Monomer content of mab_T1 (1 mg/ml, n=3) in sugar-based lyophilizates of low and high solid content (the formulations contain a low amount of phenylalanine).

No considerable decrease of monomer was noticed. Thus, independent from sugar and solid content, the formulations were able to prevent formation of soluble aggregates (and fragments) of mab_T1.

Figure 6.30 displays the monomer recovery. Both sucrose-based formulations showed a complete recovery of monomer (> 99%) independent from solid content. The trehalose-based lyophilizates exhibited a slightly lower monomer recovery of > 90%. The samples of low solid content exhibited slightly lower monomer recovery. Since no formation of HMWS was detectable, the decrease in monomer is attributed to aggregation towards non-soluble aggregates. Hence, the concentration of subvisible particles (svp) and the turbidity was assessed.

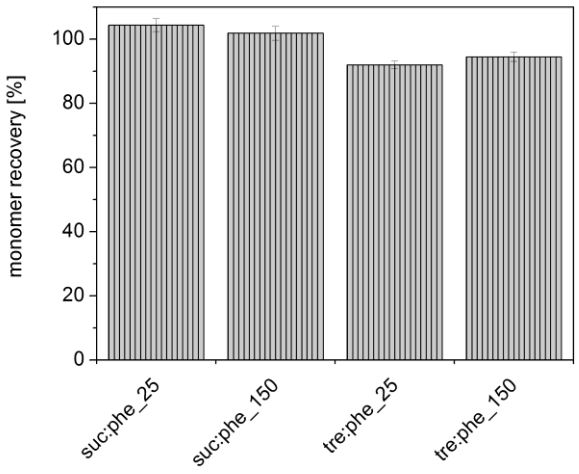

Figure 6.30: Monomer recovery of mab_T1 (n=3) in sugar-based lyophilizates of low and high solid content (the formulations contain a low amount of phenylalanine).

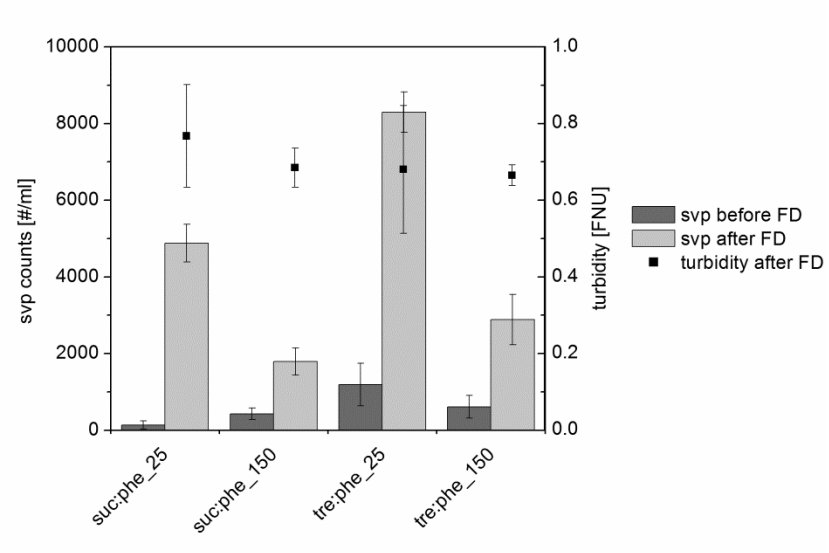

Figure 6.31: Subvisible particle counts (particles ≥ 1µm) and turbidity of aggressively freeze-dried samples of low and high solid content containing 1 mg/ml mab_T1 (the turbidity of the formulations before freeze-drying ranged around 0.6 FNU).

The svp concentration was assessed by light obscuration. In addition, turbidity of reconstituted samples was determined by static light scatting at 90°. Figure 6.31 depicts the svp concentrations of particles ≥ 1 µm per ml and the turbidity. The amount of particles of ≥ 10 µm and ≥ 25 µm are listed in Table 6.16.

Compared to the formulations before freeze-drying, all of the reconstituted lyophilizates exhibited an increase of the svp content. The samples of high solid content (150 mg/ml) showed only a minor increase, which can be attributed to the lab environment. A more pronounced increase was observed for the lyophilizates of low solid content (25 mg/ml). Obviously, in these samples non-soluble aggregates of mab_T1 were formed due to insufficient stabilization. In contrast to the investigations on the influence of bulking agent on the stability of mab_T1 in aggressively freeze-dried lyophilizates (see section 3.1.2, page 156 pp.), no increase of turbidity was detected in any of the samples (cf. Figure 6.31).

Table 6.16: Subvisible particle counts (particles ≥ 10 µm and ≥ 25 µm) of aggressively freeze-dried samples of low and high solid content containing 1 mg/ml mab_T1.

formulation	subvisible particles (#/ml)			
	before freeze-drying		after freeze-drying	
	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
suc:phe_25	8 ± 8	2 ± 4	137 ± 21	17 ± 4
suc:phe_150	16 ± 5	2 ± 0	47 ± 8	1 ± 1
tre:phe_25	14 ± 8	3 ± 3	129 ± 20	12 ± 6
tre:phe_150	6 ± 4	2 ± 2	46 ± 12	7 ± 2

Summing up the findings concerning the influence of solid content of aggressively freeze-dried sugar-based formulations that contain phenylalanine (at a ratio of 10:0.5), lyophilizates of high solid content (150 mg/ml) were able to prevent aggregation of mab_T1 during aggressive freeze-drying. For a low solid content (25 mg/ml) mab_T1 was slightly less successful protected against aggregation. Overall, no difference between sucrose and trehalose-based samples was observed.

Considering the physico-chemical data (cf. Table 6.17, for details see section 5 of CHAPTER 5, page 131 pp.), the worse stabilization in lyophilizates of low solid content can be attributed to the larger specific surface area. Samples of 25 mg/ml exhibited a SSA of 0.34 m^2/g, while for 150 mg/ml a SSA of 0.10 m^2/g was determined.

Based on the stability data for samples of 50 mg/ml and the comparable physico-chemical characteristics of lyophilizates of solid contents between 50 and 150 mg/ml, a good storage stability is probable for tre:phe 10:0.5 formulations of ≥ 50 mg/ml solid content.

Table 6.17: Overview of physico-chemical characteristics of aggressively freeze-dried samples of low and high solid content containing 1 mg/ml mab_T1.

formulation	appearance	SSA	water content	Tg	crystallinity
		m^2/g	%	°C	
suc:phe_25	collapsed	0.24	0.91 ± 0.04	62.4 ± 0.6	amorphous
suc:phe_150	collapsed	0.07	2.90 ± 0.15	48.8 ± 0.1	amorphous
tre:phe_25	collapsed	0.34	0.93 ± 0.09	94.3 ± 0.2	amorphous
tre:phe_150	collapsed	0.10	1.10 ± 0.12	76.8 ± 0.7	amorphous

5. PROTEIN CONCENTRATION

In order to get an insight, whether aggressive freeze-drying is applicable to protein formulations of higher protein concentration, selected formulations containing 10 mg/ml mab_T1 were aggressively freeze-dried. Either sucrose or trehalose was utilized as major excipient. Additionally, two sugar-based formulations that contain a low amount of phenylalanine were selected. The utilized formulations are depicted in Table 6.18.

For successful stabilization, a certain stabilizer to protein ratio is necessary [42]. It was shown above that for aggressive freeze-drying a sugar to protein ratio of 50 to 1 performed well in protecting mab_T1. It was decided, to maintain the concentration of the excipients at 50 mg/ml, which consequently resulted in a reduction of the sugar to protein ratio of 5 to 1, which is recommended as lowest advisable ratio for stabilization[42].

1 ml aliquots of the respective formulations were filled in 2R vials and subjected to the aggressive freeze-drying protocol shown in Table 3.4 (page 55 Table 3.5) in a FTS LyostarIII freeze-drier. The resulting lyophilizates were analyzed with regard to aggregation of mab_T1. Soluble aggregates were assessed using HP-SEC, non-soluble aggregates were evaluated utilizing subvisible particle detection by light obscuration and turbidity by static light scattering. Additionally, the reconstitution time was monitored.

Table 6.18: Formulations to characterize the ability to stabilize mab_T1 at protein concentration of 10 mg/ml during aggressively freeze-drying (formulations were prepared in 10 mM potassium phosphate buffer, pH 6.4).

formulation	mab_T1	sucrose	trehalose	phenylalanine
	mg/ml	mg/ml	mg/ml	mg/ml
suc	10	50		
suc:phe	10	47.62		2.38
tre	10		50	
tre:phe	10		47.62	2.38

Despite the collapsed appearance (cf. Figure 6.32), the lyophilizates dissolved fast and were completely reconstituted with ≤ 20 seconds. Table 6.19 displays the reconstitution times of the samples. No apparent difference was noticed for the different formulations. Neither the type of disaccharide nor the addition of phenylalanine in low concentration imposed an influence on the reconstitution time.

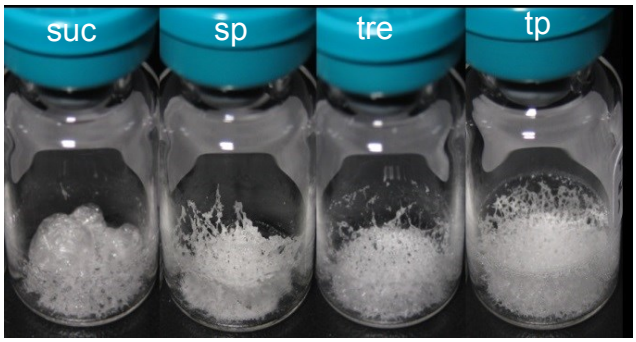

Figure 6.32: Macroscopic appearance of lyophilizates aggressively freeze-dried from sugar based formulations containing 1 mg/ml (left picture) or 10 mg/ml (right picture) mab_T1 (suc: sucrose; sp: sucrose:phenylalanine 10:0.5; tre: trehalose; tp: trehalose:phenylalanine 10:0.5).

Table 6.19: Reconstitution time of sugar-based aggressively freeze-dried lyophilizates that contain 10mg/ml mab_T1 (suc:phe and tre:phe samples contain phenylylanine at a ratio of 10:0.5; n=6).

formulation	reconstitution time
	seconds
suc	18 ± 1
suc:phe	15 ± 1
tre	19 ± 2
tre:phe	10 ± 3

The monomer content was assessed by HP-SEC, and the recovery of monomer after aggressive freeze-drying was calculated (cf. Figure 6.33).

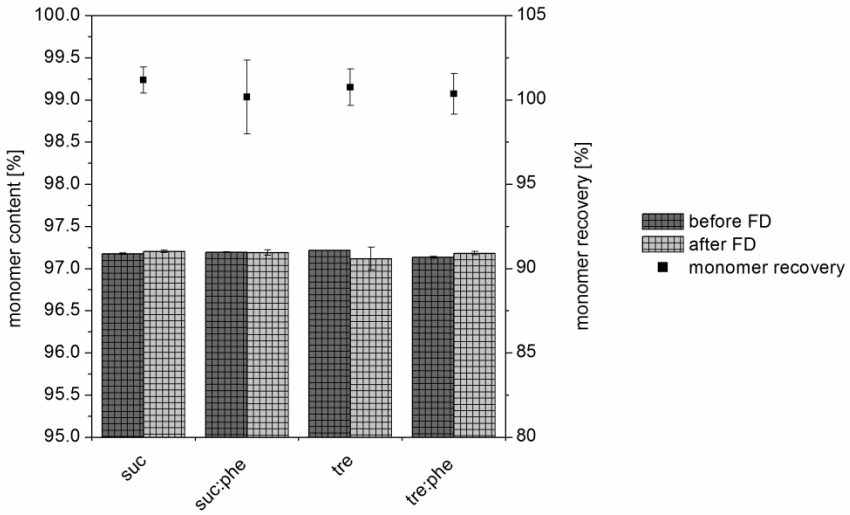

Figure 6.33: Monomer content and monomer recovery of sugar-based lyophilizates containing 10mg/ml mab_T1 (suc:phe and tre:phe samples contain phenylalanine at a ratio of 10:0.5; n=3).

For none of the investigated formulations a decrease in monomer content could be determined. No change of high molecular weight species (HMWS, e.g. mab_T1 dimer or

oligomer) and low molecular weight species (LMWS, e.g. mab_T1 fragments) in the reconstituted freeze-dried samples were observed (data not shown). A monomer recovery of around 100% was observed (cf. Figure 6.33).

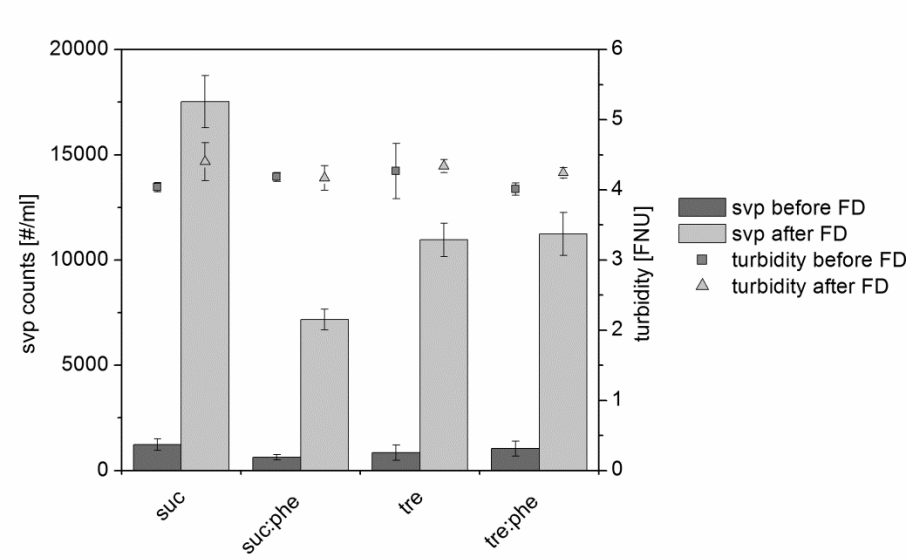

Figure 6.34: Subvisible particle counts (particles ≥ 1µm) and turbidity of aggressively freeze-dried samples containing 10 mg/ml mab_T1 (suc:phe and tre:phe samples contain phenylalanine at a ratio of 10:0.5; n=3).

Table 6.20: Subvisible particle counts (particles ≥ 10 µm and ≥ 25 µm) and turbidity of aggressively freeze-dried samples containing 10 mg/ml mab_T1 (suc:phe and tre:phe samples contain phenylalanine at a ratio of 10:0.5; n=3).

formulation	subvisible particles (#/ml)			
	before freeze-drying		after freeze-drying	
	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
sucrose	14 ± 4	1 ± 2	276 ± 23	12 ± 5
suc:phe	16 ± 13	8 ± 11	104 ± 12	5 ± 2
trehalose	7 ± 2	1 ± 2	181 ± 18	7 ± 3
tre:phe	8 ± 3	0 ± 0	148 ± 25	6 ± 4

The svp content and the turbidity of the samples were monitored. Particles of ≥ 1 µm, ≥ 10 µm, and ≥ 25 µm were investigated. As usual, the formulations were filtered (0.22µm PVDF membrane) prior to freeze-drying. Although the formulations were filtered, the comparably high turbidity of the mab_T1 bulk material could not be significantly reduced. Obviously, a certain population of the particles causing turbidity exhibited a particle size allowing them to pass the 0.22 µm filter. Figure 6.34 depicts the svp content and the turbidity before and after aggressive freeze-drying. The particle content of particles ≥ 10 µm and ≥ 25 µm were listed in Table 6.20.

Summary

After aggressive freeze-drying, no distinct change of the turbidity values were detected (cf. Figure 6.34). However, an increase of svp concentration, most pronounced for particles ≥ 1 µm, was detected for all formulations after aggressive freeze-drying. Nevertheless, samples that showed substantial instability exhibited svp concentrations of

100,000 particles ≥1 µm/ml (compare e.g. section 3.2.1, page 165 pp.). Since for lyophilizates at 10 mg/ml protein concentration below 20,000 particles ≥1 µm/ml were detected, still a good stabilization of mab_T1 could be achieved. While no difference was noticed between the formulations based on trehalose, the formulation applying sucrose as major excipient exhibited a slightly higher svp content compared to the sucrose-based formulation containing a low amount of phenylalanine. However, no clear correlation can be established between stabilization and physico-chemical characteristics (compare Table 6.21, details of physico-chemical characteristics see CHAPTER 5, page 141 pp.).

Table 6.21: Overview of physico-chemical characteristics of aggressively freeze-dried samples containing 10 mg/ml mab_T1 (suc:phe and tre:phe samples contain phenylalanine at a ratio of 10:0.5; water content and Tg n=3).

formulation	appearance	SSA	water content	Tg	crystallinity
		m^2/g	%	°C	
sucrose	collapsed	0.01	5.19 ± 0.31	25.6 ± 2.7	amorphous
suc:phe 10:0.5	collapsed	0.05	2.06 ± 0.10	51.4 ± 1.8	amorphous
trehalose	collapsed	0.03	4.48 ± 0.19	46.7 ± 2.1	amorphous
tre:phe 10:0.5	collapsed	0.14	1.73 ± 0.15	80.2 ± 1.7	amorphous

Comparing pure sucrose and trehalose lyophilizates, no distinct differences of residual water content or specific surface area were detected. Adding a low amount of phenylalanine drastically reduced the water content and increased the Tg. Both formulations, which contain phenylalanine (suc:phe 10:0.5 vs. tre:phe 10:0.5) showed comparable water content and SSA. Hence, in terms of SSA, crystallinity, and water content sucrose-based and trehalose-based formulations resulted in comparable (collapsed) lyophilizates.

Although the sucrose(-based) formulations exhibited a considerably lower glass transition temperature (Tg) compared to the trehalose(-based) samples, a connection to the slightly worse stabilization of mab_T1 in sucrose lyophilizates cannot be plausibly explained by that fact, since the samples were not stored at temperatures above the Tg.

Summing up the results regarding the ability of aggressive freeze-drying to stabilize a protein at a concentration of 10 mg/ml, it was shown that the utilized model protein (mab_T1) could be stabilized against aggregation to soluble aggregates. Four different formulations, based on either trehalose or sucrose, have been demonstrated a full preservation of monomer content and showed a complete recovery of monomer after aggressive freeze-drying. Although the detection of subvisible particles revealed an increase of particle concentration after aggressive freeze-drying, pronounced aggregation of mab_T1 to non-soluble aggregates was prevented. Obviously, the amount of mab_T1 monomers participating in aggregation to svp is too low to result in a detectable decrease in monomer. Moreover, assuming that a certain (very low) percentage of mab_T1 aggregates during freeze-drying, for samples of higher protein concentration, a higher svp concentration is expectable. Therefore, aggressive freeze-drying of protein formulations at a sugar to protein ratio of 5 to 1 is regarded as feasible.

6. SUMMARY AND CONCLUSIONS

In the present study, the influence of the formulation design on the protein stabilization in aggressively freeze-dried lyophilizates was investigated. The recombinant monoclonal IgG1 antibody mab_T1, which is sensitive to freezing and drying stresses, was used as model protein. For the majority of sensitive molecules, which are intended to be stabilized by freeze-drying, using an amorphous excipient is necessary for stabilization. Thus, the investigated formulations are based on the most common cryo-/lyo-protectants sucrose and trehalose.

As a first step, formulations without bulking agents were studied. Despite the collapsed appearance and rather high water content, the samples of trehalose and sucrose (at 50 mg/ml) completely stabilized mab_T1 during aggressive freeze-drying.

It was shown in the previous chapter that adding bulking agents to aggressively freeze-dried lyophilizates can improve the physico-chemical characteristics (water content, Tg). Now, the impact of adding bulking agents on the protein stability was investigated.

Three bulking agents (mannitol, glycine, or phenylalanine) were added to sucrose- or trehalose-based formulations in low and high amounts. Previous test have shown that bulking agents allow **extremely short aggressive freeze-drying cycles of 12 h (including freezing)**. Hence, the formulations were aggressively freeze-dried utilizing such a short protocol. All samples containing a low amount of either bulking agent showed very good stabilization of mab_T1. However, neither a low amount of mannitol nor glycine was able to reduce the residual water content to a satisfactory degree. On the other hand, the application of high amounts of bulking agents successfully decreases the residual water content. However, the addition of higher amounts of bulking agent resulted in slightly worse stabilization of mab_T1 especially mannitol deteriorated the stability. Most probably, the high SSA of these lyophilizates imposed substantial stress to the protein. **Again, the formulations containing a low amount of phenylalanine (tre:phe 10:0.5, suc:phe 10:0.5) stand out by excellent stabilization and low residual water content.**

Due to their promising qualities, the lyophilizates composed of sucrose/trehalose and high amounts of glycine, mannitol, or phenylalanine, were subjected to a storage stability study at 30°C and 50°C (and a control at 2-8°C) for up to 6 months. Additionally, lyophilizates that contain a low amount of phenylalanine were included in this study. As a control, moderately freeze-dried samples of the respective formulations were stored at identical conditions, too. **In contrast to moderately freeze-dried samples, the lyophilizates from aggressive freeze-drying containing glycine or phenylalanine revealed no relevant change in any of the physico-chemical characteristics upon storage at 30°C**. However, storage of sucrose-based mannitol samples revealed physico-chemical instability by the formation of α-mannitol.

Regarding the preservation of protein stability, the aggressive freeze-dried samples exhibited better stabilization of mab_T1 during 6 months storage. Sugar-based formulations with glycine and phenylalanine exhibited sound stabilization of mab_T1. Lyophilizates that contain mannitol exhibited worst stabilization, even if stored at 2-8°C. In gen-

eral, the sucrose-based lyophilizates with glycine and phenylalanine showed good stabilization of mab_T1, if stored not higher than 30°C. At 50 °C storage, also these samples showed some aggregation after 6 months. In contrast, even if stored at 50°C, mab_T1 was well stabilized for 6 months by the trehalose-based formulations containing glycine or phenylalanine. **Especially the formulations that contain a low amount of phenylalanine (tre:phe 10:0.5) completely prevented aggregation of mab_T1. The superior stabilization in theses samples is ascribed to the unique physico-chemical properties: Fully amorphous structure despite adding phenylalanine, low SSA due to collapse during aggressive freeze-drying and low water content / high glass transition temperature despite the collapsed appearance.**

Based on the remarkable stability achieved by the addition of low amounts of phenylalanine to collapsed sugar-based lyophilizates, **other amino acids were evaluated**, too. Arginine, glycine, histidine, isoleucine, leucine, methionine, or tryptophan were added to sucrose- or trehalose-based formulations and aggressively freeze-dried. As shown in CHAPTER 5, isoleucine, leucine, and methionine are able to reduce the residual water content due to crystallization and increase of SSA. Now, the evaluation of the ability to protect mab_T1 against aggregation revealed increased aggregation in the formulations that contain isoleucine, leucine, or methionine. It is concluded that the increased SSA, due to crystallization of the amino acids, diminishes the ability to stabilize incorporated mab_T1 in aggressive freeze-dried lyophilizates. The other amino acids which were used (arg, gly, his, trp) exhibited good stabilization of mab_T1, however did no show an improvement of water content and Tg compared to pure sugar lyophilizates. Thus, phenylalanine stands out by combining suitable physico-chemical properties and excellent protein stabilization upon combination with trehalose or sucrose.

In a further study, the **effect of low or high solid content on the ability to stabilize** mab_T1 was investigated. Since suc:phe 10:0.5 and tre:phe 10:0.5 have shown superior stabilization, the solid content of these formulation was varied from 25 mg/ml to 150 mg/ml. While for samples of ≥ 50 mg/ml aggregation of mab_T1 was prevented, slightly less successful stabilization was observed for low solid contents (25 mg/ml). Worse stabilization can be attributed to larger SSA of latter samples.

A supplementary investigation aimed to evaluate the capability to stabilize mab_T1 at 10 mg/ml. Besides sucrose and trehalose, also suc:phe 10:0.5 and tre:phe 10:0.5 were utilized at 50 mg/ml excipient concentration. Although especially the lyophilizates from sucrose and trehalose showed rather high water content, mab_T1 was effectively protected. As already observed for formulations of 1 mg/ml mab_T1 concentration, adding phenylalanine considerably decreased the water content without increasing the SSA. This phenomenon was observed for a protein concentration of 10 mg/ml, too. The sugar-based lyophilizates containing phenylalanine revealed excellent stabilization. Therefore, it is concluded that proteins can be stabilized by aggressive freeze-drying also at concentrations at least up to 10 mg/ml.

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CHAPTER 7 APPLICABILITY OF AGGRESSIVE FREEZE-DRYING FOR STABILIZATION OF VARIOUS PROTEINS

1. INTRODUCTION

The systematical investigations of Schersch e.t al concerning the influence of collapse of lyophilizates on the product quality has shown that even sensitive model proteins are not affected by collapse during freeze-drying [1]. Furthermore, the same authors showed that collapsed lyophilizates exhibited an admirable stability during storage at accelerated conditions (i.e. elevated temperatures) and even have advantage over conventionally freeze-dried lyophilizates of comparable residual water level upon storage [2, 3]. However, due to the collapsed nature, and therefore drastic reduced specific surface area of the lyophilizates, secondary drying mechanisms are slowed down dramatically [4], resulting in the necessity to keep the collapsed samples prolonged times at high temperatures to achieve acceptable residual water level [1]. This fact holds true especially for lyophilizates "collapse-dried" from freeze-drying formulations that contain disaccharides like sucrose or trehalose without further excipients like bulking agents. Supporting the findings of Schersch et al [1] in chapter 5 of this thesis was shown, that the addition of substantial amounts of bulking agents like mannitol or glycine prevents the occurrence of macroscopic collapse even if aggressive protocols are utilized to freeze-dry the samples. Phenylalanine has turned out as high potential bulking agent especially for aggressively freeze-dried lyophilizates containing a model antibody (mab_T1). Finally, it was shown that aggressive freeze-drying resulted in lyophilizates of excellent stabilization properties. Although major stabilization concepts of freeze-drying, namely water replacement [5-8] and vitrification [9-11], are considered as aunique concept to stabilize embedded proteins, the considerable number of different freeze-dried formulations [12] emphasizes (slightly) different requirements for stabilization of different molecules / proteins. Hence, it is of substantial interest to evaluate, whether promising results obtained from aggressive freeze-drying of the model antibody mab_T1 can be extended to other proteins. In order to explore the potential of aggressive freeze-drying to be no longer an unwanted aberration during freeze-drying, but become a suitable alternative to conventional freeze-drying, a number of further proteins were included in sugar based formulations and subjected to aggressive freeze-drying. Besides a second recombinant monoclonal IgG antibody (mab_T2), a recombinant human granulocyte colony-stimulating factor (GCSF) was studied. Furthermore, L-lactic dehydrogenase was used as supplementary model protein due to its well-documented susceptibility to freeze-drying stresses [13-24].

2. MAB_T2

As shown in CHAPTER 5, aggressive freeze-drying of sucrose- and treahalose-based formulations containing no bulking agent resulted in high residual water contents (compare page 109). The addition of a certain amount of bulking agent reduced the residual water content of the resulting lyophilizates dramatically (see page 113). In CHAPTER 6 it was shown that formulations of sucrose and trehalose, that contain a low amount of phenylalanine, provided excellent stabilization of mab_T1 successful during aggressive freeze-drying and storage. Other bulking agents (mannitol, glycine) exhibited less successful stabilization. Larger specific surface area in combination with crystallization of the bulking agents has turned out as major cause for instability of mab_T1. For the actual study, the investigated formulations contained polysorbate 20 to account for surface stress. Mab_T2 was chosen as model protein, which was aggressively freeze-dried in formulations with and without bulking agents (cf. Table 7.1). Mannitol, glycine, or phenylalanine were utilized at a low or high sugar (sucrose or trehalose) to bulking agent weight-to-weight ratio.

Aliquots of 1ml of the formulations were filled into DIN 2R glass vials (type 1) and aggressively freeze-dried according to the protocol shown in Table 3.5 (page 56) in a Martin Christ 2-12D freeze-drier. The focus of the investigation was set on the influence of formulation composition on the stability of incorporated mab_T2 in aggressively freeze-dried lyophilizates. Aggregation of mab_T2 was characterized by subvisible particle counts, turbidity, and HP-SEC. In addition, the secondary structure of selected reconstituted samples was monitored via FT-IR.

Table 7.1: Formulations to evaluate the ability to stabilize mab_T2 during aggressive freeze-drying (formulations based on 10 mM sodium citrate buffer pH 5.4).

formulation	mab_T2	sucrose	trehalose	mannitol	glycine	phenylalanine	polysorbate 20
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	%
suc	1	50.00					0.004
suc:man 10:3	1	38.46		11.54			0.004
suc:man 10:30	1	12.50		37.50			0.004
suc:gly 10:3	1	38.46			11.54		0.004
suc:gly 10:30	1	12.50			37.50		0.004
suc:phe 10:0.5	1	47.62				2.38	0.004
suc:phe 10:3	1	38.46				11.54	0.004
tre	1		50.00				0.004
tre:man 10:3	1		38.46	11.54			0.004
tre:man 10:30	1		12.50	37.50			0.004
tre:gly 10:3	1		38.46		11.54		0.004
tre:gly 10:30	1		12.50		37.50		0.004
tre:phe 10:0.5	1		47.62			2.38	0.004
tre:phe 10:3	1		38.46			11.54	0.004

As expected, the lyophilizates containing no or a low amount of bulking agent showed a collapsed appearance (see appendix, Figure 9.3). The samples that contain a high amount of bulking agent showed an elegant cake appearance. All lyophilizates readily rehydrated within one minute (cf. Table 7.2).

Table 7.2: Reconstitution time of aggressively freeze-dried lyophilizates containing 1 mg/ml mab_T2 and different amounts of bulking agents (n=3).

formulation	reconstitution time	
	sucrose	trehalose
	seconds	seconds
sugar	16 ± 1	37 ± 3
sugar:man 10:3	35 ± 6	26 ± 3
sugar:man 10:30	13 ± 1	13 ± 2
sugar:gly 10:3	17 ± 2	16 ± 1
sugar:gly 10:30	10 ± 0	16 ± 4
sugar:phe 10:0.5	48 ± 8	32 ± 2
sugar:phe 10:3	35 ± 2	27 ± 3

The results of the size exclusion chromatography are summarized in Figure 7.1. In the formulations before freeze-drying low amounts of HMWS (≤ 0.4%) were observed. After aggressive freeze-drying, a low, but significant increase of aggregates was observed for the samples that contain a high amount of mannitol. These samples also exhibited a decreased monomer recovery. Besides the tre:man 10:3 lyophilizates, all collapsed samples revealed no increase of HMWS or decrease of monomer recovery. Also the elegant samples containing a high amount of glycine or phenylalanine showed good stabilization of mab_T2.

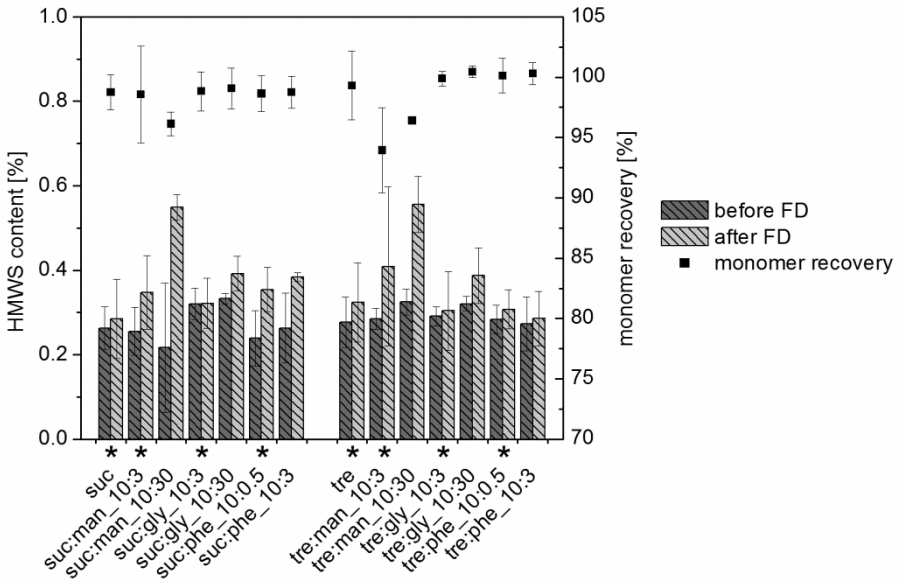

Figure 7.1: HMWS and monomer recovery of mab_T2 determined by HP-SEC of formulations containing bulking agent in different quantities. The results of samples analyzed before and after aggressive freeze-drying are shown (collapsed samples are marked by an asterisk).

The subvisible particle count (particles ≥ 1µm/ml) and turbidity values of all lyophilizates revealed no increase after aggressive freeze-drying (cf. Figure 7.2). Collapsed and non-collapsed samples completely prevented the formation of non-soluble aggregates of mab_T2.

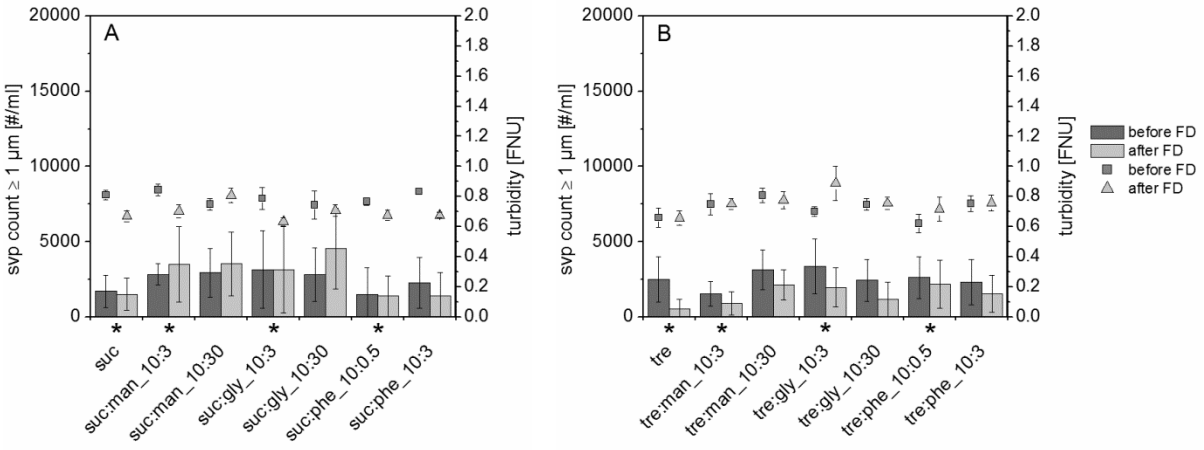

Figure 7.2: Subvisible particles ≥ 1µm/ml and turbidity of formulations containing 1 mg/ml mab_T2 and bulking agent in different quantities. The results of samples analyzed before and after aggressive freeze-drying are shown (A: sucrose-based samples, B: trehalose-based samples; bars represent svp, squares and triangles represent turbidity; collapsed samples are marked by an asterisk).

The secondary structure of mab_T2 was analyzed utilizing the 2nd derivative FT-IR spectra of reconstituted samples. The optical assessment of the spectra does not allow for quantitative comparison (exemplary suc:phe and tre:phe spectra see Figure 7.3). Hence, the overlap of spectra was calculated as described by Prestrelski et al. [25] and expressed by a correlation coefficient (a correlation coefficient of 1 represents complete overlap, for a correlation coefficient of 0.985 optically still a good overlap of spectra was observed). Overall, high correlation coefficients of ≥ 0.985 were determined for aggressively freeze-dried samples (cf. Figure 7.4). Sucrose-based samples revealed slightly better correlation of the spectra than trehalose-based ones. Apparently, the secondary structure of mab_T2 was slightly changed after aggressive freeze-drying in most of the trehalose-based lyophilizates. Only the samples that contain a low amount of phenylalanine (tre:phe 10:0.5) exhibited a high correlation coefficient of ≥ 0.995 indicating no change.

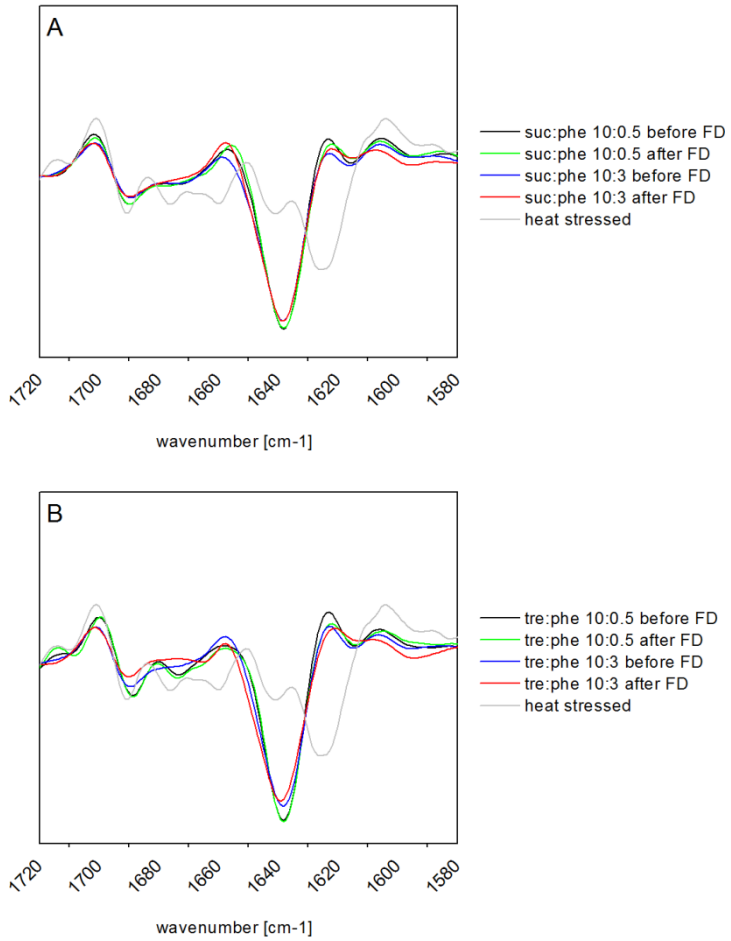

Figure 7.3: 2nd derivative FT-IR spectra (amide I band) before and after aggressive freeze-drying. The sugar-based formulations contain phenylalanine at low and high quantities (A: sucrose-based; B: trehalose-based; for comparison the spectrum of a heat stressed mab_T2 sample is shown).

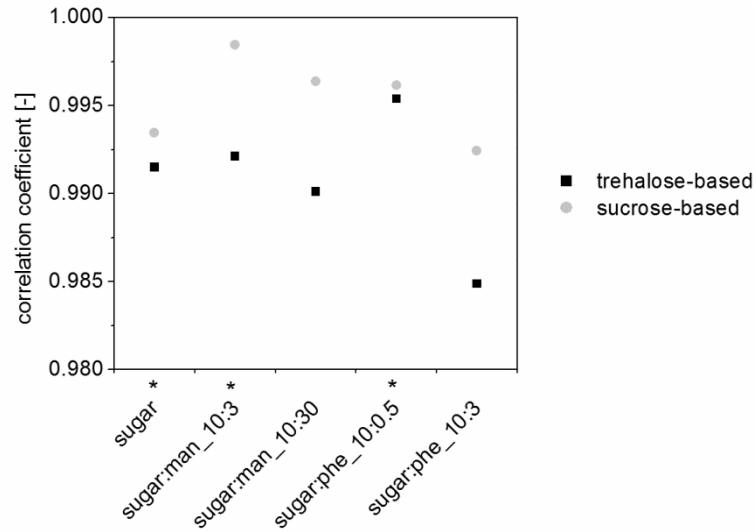

Figure 7.4: Correlation coefficient of 2nd derivative FT-IR spectra of aggressively freeze-dried formulations containing 1 mg/ml mab_T2 and bulking agent in different quantities. For calculation of the correlation coefficient, spectra derived from reconstituted samples were compared to the respective formulations before freeze-drying (corr. coefficient of a heat stressed samples was 0.682).

Summarizing, the results determined for the stabilization of mab_T2 in aggressively freeze-dried lyophilizates have shown that collapsed samples exhibited better stabilization than non-collapsed samples. The non-collapsed samples resulted from formulations of high bulking agent content. As already observed for the stabilization of mab_T1 (compare CHAPTER 6), the formation of partly crystalline samples of high SSA reduces the ability to protect a protein. The overall slightly worse stabilization of mab_T2 is attributed to the same mechanism, too. However, for mab_T2 overall less aggregation compared to mab_T1 was noticed. This is ascribed to the presence of polysorbate 20 in all formulations. Also for the stabilization of mab_T2, the combination of sucrose/trehalose and a low amount of phenylalanine (at a ratio of 10:0.5) revealed superior potential.

3. GRANULOCYTE COLONY-STIMULATING FACTOR (GCSF)

In CHAPTER 6 and the section above it was shown that monoclonal antibodies can be successfully stabilized by aggressive freeze-drying. To investigate the potential of aggressive freeze-drying also for smaller proteins, Granulocyte Colony-Stimulating Factor (GCSF) was used as model. Non-glycosylated GSCF is marketed mainly as liquid formulation (several Filgrastim products), while glycosylated GCSF is available as a freeze-dried product (Lenograstim in Granocyte®). In Granocyte® GSCF is formulated with arginine, mannitol, phenylalanine, methionine, and polysorbate 20 [26]. Since sugar-based formulations have revealed high potential for excellent stabilization, GCSF was aggressively freeze-dried in the formulations listed in Table 7.3. The formulations were based on a 10 mM sodium citrate buffer at pH 4.0.

Table 7.3: Formulations to evaluate the ability to stabilize GCSF during aggressively freeze-drying (formulations based on 10 mM sodium citrate buffer pH 4.0).

formulation	GCSF	sucrose	trehalose	mannitol	glycine	phenylalanine	polysorbate 20
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	%
suc	1	50.00					0.004
suc:man 10:3	1	38.46		11.54			0.004
suc:man 10:30	1	12.50		37.50			0.004
suc:gly 10:3	1	38.46			11.54		0.004
suc:gly 10:30	1	12.50			37.50		0.004
suc:phe 10:0.5	1	47.62				2.38	0.004
suc:phe 10:3	1	38.46				11.54	0.004
tre	1		50.00				0.004
tre:man 10:3	1		38.46	11.54			0.004
tre:man 10:30	1		12.50	37.50			0.004
tre:gly 10:3	1		38.46		11.54		0.004
tre:gly 10:30	1		12.50		37.50		0.004
tre:phe 10:0.5	1		47.62			2.38	0.004
tre:phe 10:3	1		38.46			11.54	0.004

Aliquots of 1 ml of the formulations were filled into DIN 2R glass vials (type 1) and aggressively freeze-dried according to the protocol shown in Table 3.5 (page 56) in a Martin Christ 2-12D freeze-drier. The focus of the investigation was set on on the stability of incorporated GCSF in aggressively freeze-dried lyophilizates. Aggregation was characterized by subvisible particle counts, turbidity, and HP-SEC. In addition, the secondary structure of selected reconstituted samples was monitored via FT-IR.

For formulations that contain a high amount of bulking agent (suc:man 10:30, suc:gly 10:30, suc:phe 10:3, tre:man 10:30, tre:gly 10:30, tre:phe 10:3), aggressive freeze-drying resulted in non-collapsed lyophilizates, whereas samples with no or a low amount of bulking agent showed a collapsed appearance (suc, suc:man 10:3, suc:gly 10:3, suc:phe 10:0.5, tre, tre:man 10:3, tre:gly 10:3, tre:phe 10:0.5; pictures see appendix, Figure 9.5). Nevertheless, all samples were reconstituted within less than one minute (see appendix for details, Table 9.5).

Size exclusion chromatography revealed overall low HMWS of less than 1%. However, the aggressively freeze-dried samples exhibited some increase of HMWS indicating aggregation of GSCF. Interestingly, the non-collapsed samples showed a higher content of HWMS compared to collapsed samples. Regarding monomer recovery, no relevant difference was observed between the samples. However, the monomer recovery of only 90 - 95% indicates a loss, which is not only reflected in the HMWS.

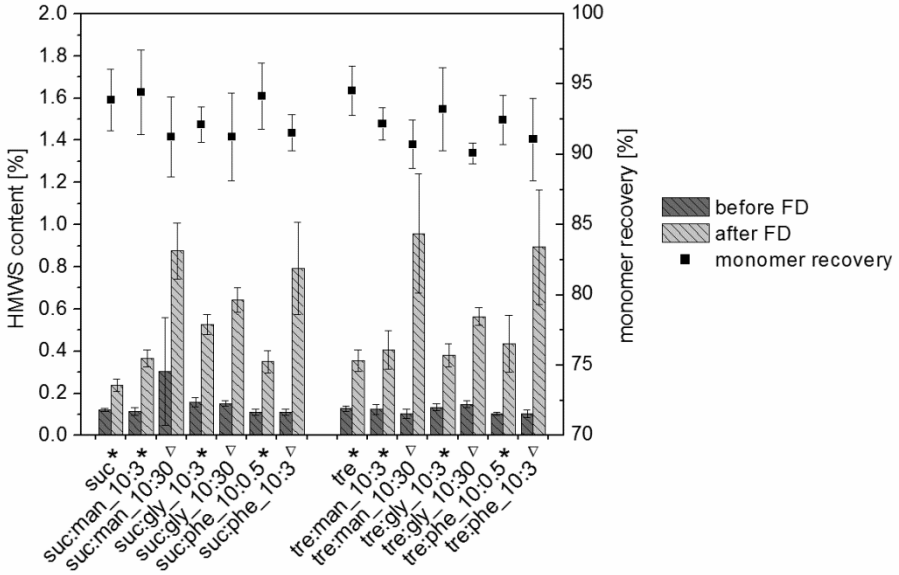

Figure 7.5: HMWS and monomer recovery of GCSF determined by HP-SEC of formulations containing bulking agent in different quantities. The results of samples analyzed before and after aggressive freeze-drying are shown (collapsed samples are indicated by an asterisk in the axis annotation, non-collapse samples are marked by a triangle in the axis annotation).

Analyzing the lyophilizates regarding their subvisible particle concentration (≥ 1 µm/ml) and turbidity revealed comparably high particle concentration already before freeze-drying (cf. Figure 7.6). After aggressive freeze-drying, especially the non-col-

lapsed samples exhibited a considerable increase of svp concentration, indicating aggregation of GCSF. Besides the samples containing a low amount of glycine, the collapsed samples showed no increase of svp content. However, the turbidity of most of the collapsed samples was increased. Although the increase of turbidity was less pronounced for non-collapsed samples, GCSF was not completely stabilized against aggregation. The sucrose-based samples exhibited slightly better stabilization than the trehalose-based samples. Best stabilization of GCSF was achieved by the sucrose-based samples containing a low amount of mannitol or phenylalanine (suc:man 10:3, suc:phe 10:0.5).

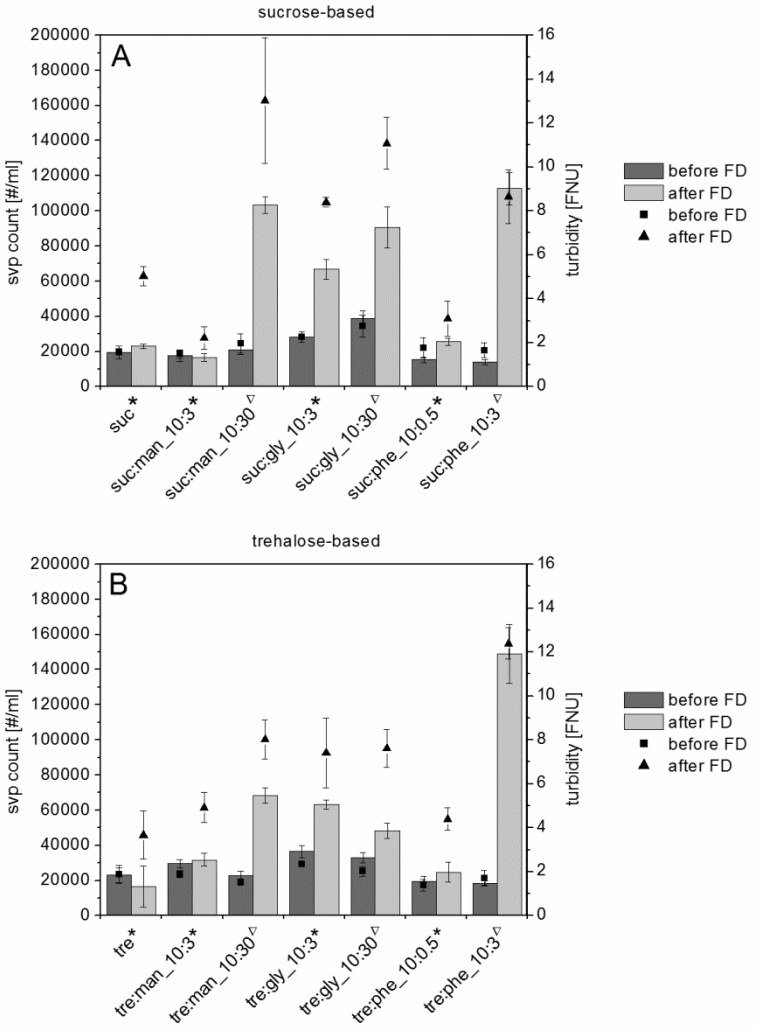

Figure 7.6: Subvisible particles ≥ 1µm/ml and turbidity of formulations containing 1 mg/ml GCSF and bulking agent in different quantities. The results of samples analyzed before and after aggressive freeze-drying are shown (A: sucrose-based samples, B: trehalose-based samples; bars represent svp, squares and triangles represent turbidity; collapsed samples are indicated by an asterisk in the axis annotation, non-collapse samples are marked by a triangle in the axis annotation).

The secondary structure of GCSF was analyzed by 2nd derivative FT-IR spectra. The overlap of spectra was calculated as described by Prestrelski et al. [25] and expressed by

a correlation coefficient. A correlation coefficient of 1 would indicate a comparable secondary structure. For a heat stressed solution of GSCF (at 10 mg/ml), a correlation coefficient of 0.921 was determined.

Figure 7.7 displays the correlation coefficients of the analyzed samples (due to inferring spectra, glycine could not be analyzed). The trehalose-based samples showed overall high correlation coefficients. Good preservation of secondary structure was determined for the collapsed samples without bulking agent or with a low content of phenylalanine. Sucrose-based samples exhibited slightly lower correlation coefficients. Especially the lyophilizates containing a low amount of phenylalanine exhibited less successful preservation of the secondary structure.

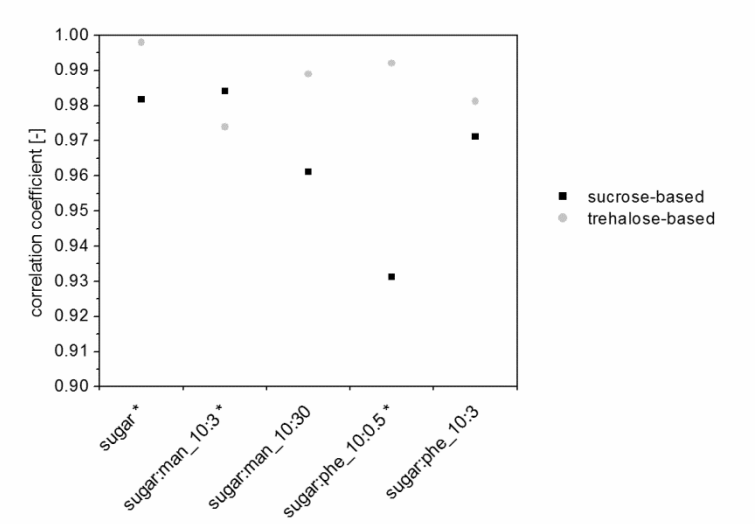

Figure 7.7: Correlation coefficient of 2nd derivative FT-IR spectra of aggressively freeze-dried formulations containing 1 mg/ml GCSF and bulking agent in different quantities. For calculation of the correlation coefficient, spectra derived from reconstituted samples were compared to the respective formulations before freeze-drying (corr. coefficient of a heat stressed samples was 0.921).

Summarizing, aggressive freeze-drying of sugar-based formulations resulted in acceptable stabilization of GCSF. Comparing non-collapsed and collapsed samples, the latter were superior in preventing aggregation. Overall, best stabilization was achieved by formulations without a bulking agent (50mg/ml sucrose or trehalose), which had a collapsed structure after aggressive freeze-drying. Also the collapsed tre:phe 10:0.5 lyophilizates revealed good stabilization. Nevertheless, also in collapsed samples, no complete stabilization of GCSF was achieved. However, the comparably high subvisible particle concentration before freeze-drying indicate a potential instability of GCSF already in the initial formulations, which could be caused by a non-ideal buffer system. As this study highlighted the potential of collapsed lyophilizates to stabilize GCSF, further investigations are advised, which take the buffer into consideration.

4. L-LACTIC DEHYDROGENASE (LDH)

During the studies on the stabilization of antibodies during aggressive freeze-drying (mab_T1, see CHAPTER 6; mab_T2, see section 2 in this chapter) sugar-based formulations that contain phenylalanine had shown excellent stabilization. Hence, it was conclusive to evaluate the ability of these formulations to stabilize also a further sensitive model. L-lactic dehydrogenase (LDH) represents one of the most frequently utilized models to evaluate the impact of freeze-drying on protein stability. Although LDH is not a pharmaceutical for therapeutic use, it is able to reveal also minor insufficient stabilization during freeze-drying [13-24]. As the trehalose-based formulations have shown superior stabilization during storage also at elevated temperatures, these formulations were chosen for the actual study (cf. Table 7.4). Phenylalanine was added at a low (10:0.5) and high (10:3) weight-to-weight ratio.

Table 7.4: Formulations to evaluate the ability to stabilize L-lactic dehydrogenase during aggressively freeze-drying (formulations based on 10 mM sodium phosphate buffer pH 7.5).

formulation	LDH	trehalose	phenylalanine
	mg/ml	mg/ml	mg/ml
tre:phe 10:0.5	0.75	47.62	2.38
tre:phe 10:3	0.75	38.46	11.54

To benchmark aggressive freeze-drying to the conventional technique, the same formulations were moderately freeze-dried (using the protocol shown in Table 3.7, page 57; chamber pressure 0.04 mbar, shelf temperature primary drying at -40 °C/-20 °C, secondary drying at 10 °C). Since the impact of drying time was of interest, two aggressive freeze-drying protocols were used. The first protocol applies a hold time of 24 h at 45 °C (overall process time 30 h 20 min, for details see Table 3.4, page 55), the second protocol operates a hold time of only 6 h at 45 °C (overall process time 12 h 20 min, details in Table 3.5, page 56). A chamber pressure of 1 mbar is used in both cycles. Aliquots of 1 ml of the formulations were filled into DIN 2R glass vials (type 1) and freeze-dried in a FTS LyoStar II freeze-drier.

The samples were analyzed after freeze-drying and subjected to an indicative stability study at 50 °C for 6 months (for comparison samples were stored at 2-8 °C, too). Key physico-chemical characteristics of the lyophilizates were monitored (water content, glass transition temperature, crystallinity, specific surface area). The characterization of the protein stability was focused on the aggregation of LDH. The samples were characterized by subvisible particle counts, turbidity, and HMWS (HP-SEC). Additionally, the catalytic activity of selected reconstituted samples was monitored via an enzymatic assay.

4.1 PHYSICO-CHEMICAL CHARACTERISTICS

Aggressively freeze-dried samples (by either protocol) containing a low amount of phenylalanine (tre:phe 10:0.5) had a collapsed appearance (data not shown, compare e.g.

CHAPTER 6, Figure 6.2, page 156). Samples containing a high amount of phenylalanine (tre:phe 10:3) exhibited an elegant appearance.

The residual water content after freeze-drying and after 6 months storage was determined by Karl-Fischer titration (cf. Figure 7.8). As expected, the collapsed samples (tre:phe 10:0.5), aggressively freeze-dried by the short protocol, initially exhibited the highest water content. Nevertheless, the water content of 1.1% is considered as sufficiently low as starting point for a storage stability study. The non-collapsed samples (tre:ph 10:3) reached water contents of < 0.2% regardless, which protocol was used. As expected, after storage over 6 months a (moderate) increase was observed for all samples (caused e.g. by diffusion of water through the stopper), which is more pronounced at 50 °C storage. Only the tre:phe 10:0.5 lyophilizates, aggressively freeze-dried by the short protocol, exhibited an apparent decrease, which potentially could be explained by consumption of water due to crystallization of amorphous phenylalanine to phenylalanine hydrate. However, neither the DSC thermogram nor the XRD diffraction pattern revealed the presence of crystalline phenylalanine hydrate in these samples. Hence, the reason for a decrease of water content remained unclear and the data must be considered as an outlier.

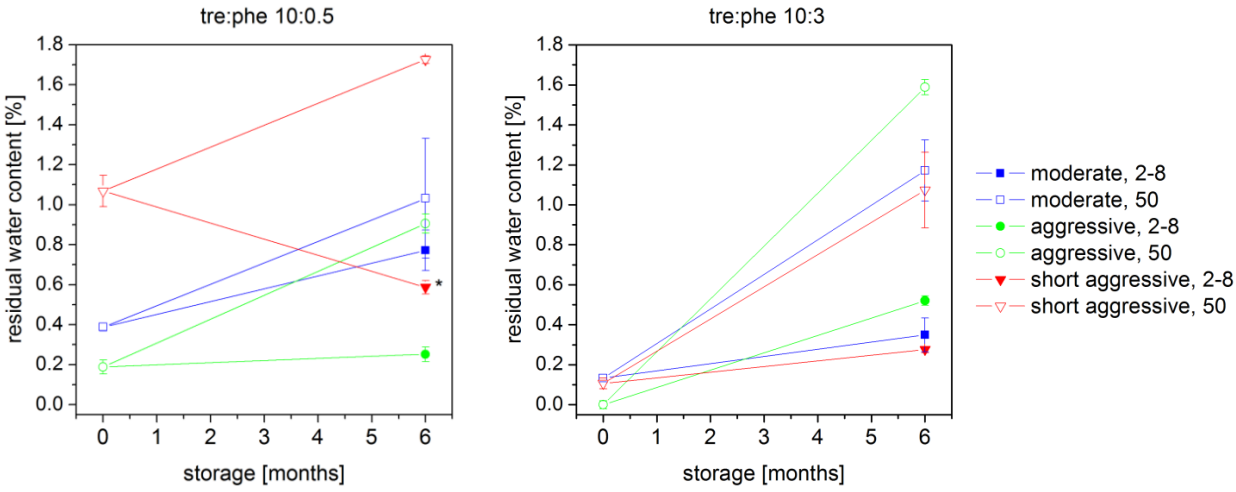

Figure 7.8: Residual water content after freeze-drying and storage of lyophilizates at 2-8 °C and 50 °C for 6 months (the trehalose-based formulations contain a low or high amount of phenylalanine and 0.75 mg/ml LDH; the sample marked by an asterisk is regarded as aberration).

Table 7.5 lists the glass transition temperatures (Tg) detected by DSC measurements. Collapsed samples (tre:phe 10:0.5) exhibited a high Tg of > 90 °C, non-collapsed samples of even > 100 °C. The initially high Tg of collapsed and non-collapsed lyophilizates were only slightly decreased after storage (in accordance with the increase of water content). Based on the high Tg, good preservation of protein stability is expected.

Table 7.5: Glass transition temperatures after freeze-drying and storage of lyophilizates at 2-8 °C and 50 °C for 6 months (the trehalose-based formulations contain a low or high amount of phenylalanine and 0.75 mg/ml LDH).

	tre:phe 10:0.5			tre:phe 10:3		
	after FD	26 weeks stored		after FD	26 weeks stored	
storage temperature	n.a.	2-8°C	50°C	n.a.	2-8°C	50°C
	Tg ± SD [°C]	Tg ± SD [°C]	Tg ± SD [°C]	Tg ± SD [°C]	Tg ± SD [°C]	Tg ± SD [°C]
moderate	116.0 ± 0.9	100.6 ± 0.6	96.0 ± 1.9	113.4 ± 1.1	104.2 ± 1.0	98.7 ± 0.3
aggressive	114.7 ± 0.2	112.4 ± 0.3	100.4 ± 1.0	116.5 ± 1.3	106.1 ± 1.2	92.9 ± 1.0
short aggressive	90.1 ± 5.9	103.1 ± 0.4	87.1 ± 2.0	114.3 ± 4.2	104.4 ± 7.8	99.2 ± 1.2

n.a. not applicable

Analysis of the crystallinity (by XRD) after freeze-drying revealed fully amorphous structure of all tre:phe 10:0.5 lyophilizates (cf. Figure 7.9 A). Figure 7.9 depicts the diffraction pattern after storing the lyophilizates for 6 months at 2-8 °C or 50 °C. Both, the non-collapsed samples from moderate freeze-drying and the collapsed samples from the aggressive cycles retained their fully amorphous structure (cf. Figure 7.9 C).

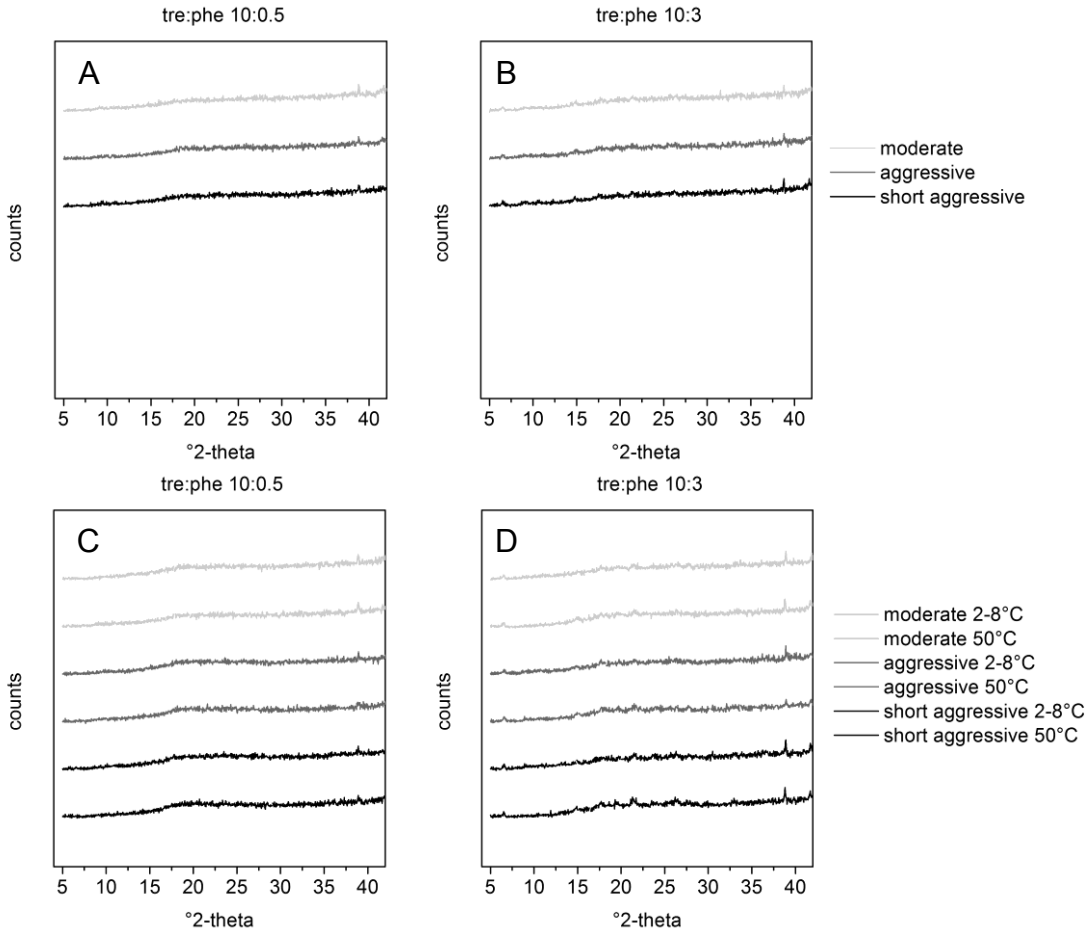

Figure 7.9: Diffraction patterns (by XRD) of moderately or aggressively freeze-dried lyophilizates. Samples after freeze-drying (A, B) and storage at 2-8 °C or 50 °C for 6 months (C, D) are shown. The trehalose-based formulations contain a low (tre:phe 10:0.5) or high (tre:phe 10:3) amount of phenylalanine and 0.75 mg/ml LDH (the peak at 38.8° 2-theta results from the copper sample holder).

All of the samples containing a high amount of phenylalanine exhibited a partly crystalline structure after freeze-drying (cf. Figure 7.9 B).

The diffraction pattern that was identified as crystalline L-phenylalanine hydrate using reference spectra of the International Centre for Diffraction Data (ICDD) database (characteristic peaks at 6.4°, 14.7°, 17.4°, and 21.3° 2-theta; cf. Figure 7.9 D). Also for these lyophilizates, no change (e.g. recrystallization) was observed throughout storage over 6 months.

The specific surface area (SSA) of the lyophilizates was determined by krypton gas adsorption (according to Brunauer-Emmett-Teller, BET). As expected, the tre:phe 10:0.5 samples, which were aggressively freeze-dried and exhibited a collapsed appearance, showed a drastically reduced SSA compared to non-collapsed moderately freeze-dried samples (cf. Figure 7.10 A). No change was noticed after storing samples at 2-8 °C or 50 °C for 6 months. Hence, no (further) collapse occurred during storage.

Comparing samples of low phenylalanine content to samples of high phenylalanine content, latter samples showed higher SSA without exception (cf. Figure 7.10 A vs. B). Although all tre:phe 10:3 samples (moderate/aggressive/short aggressive) exhibited a comparable macroscopic appearance, a reduced SSA was noticed for the aggressively freeze-dried samples (cf. Figure 7.10 B). Obviously, aggressive freeze-drying resulted in micro-collapse, which is macroscopically not observable. Comparing samples of the aggressive and short aggressive cycle, slightly lower SSA was noticed for former samples. As the aggressive cycle utilized a hold time of 24 h at 45 °C (compared to 6 h in the short cycle), ongoing micro-collapse would offer a reasonable explanation. On the other hand, after storing the samples for 6 months, a comparable SSA was observed for both species.

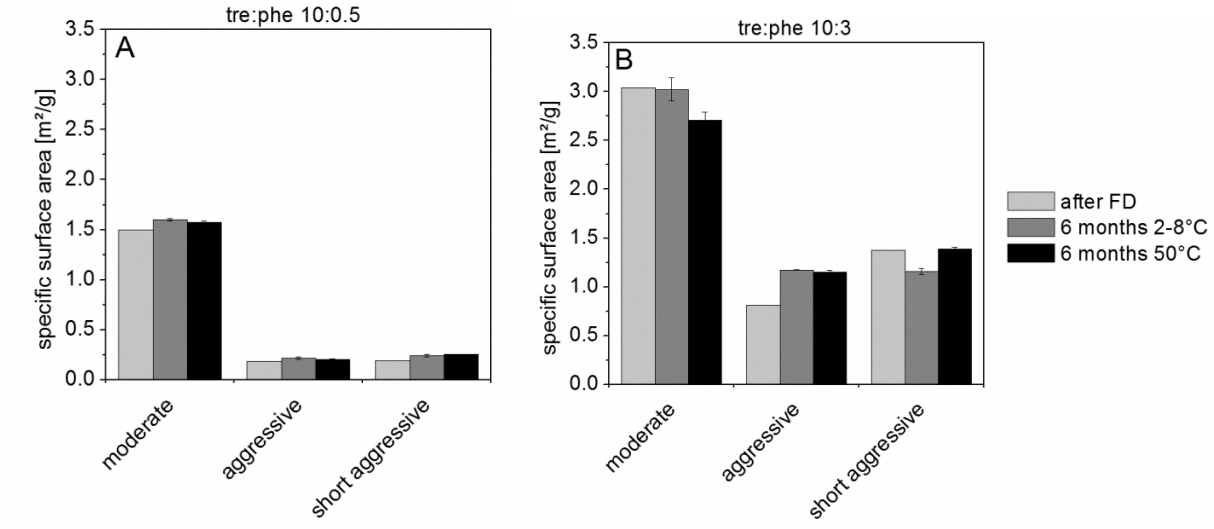

Figure 7.10: Specific surface area of lyophilizates after freeze-drying and storage of lyophilizates at 2-8 °C and 50 °C for 6 months (the trehalose-based formulations contain a low or high amount of phenylalanine and 0.75 mg/ml LDH).

4.2 PROTEIN STABILITY

HP-SEC was utilized to assess high molecular weight species (HWMS) of LDH in samples after freeze-drying and storage (cf. Figure 7.11). Before freeze-drying a HWMS content of 0.6 ± 0.09% (tre:phe 10:0.5) and 0.90 ± 0.01% (tre:phe 10:3) was determined. Independent from drying cycle (moderate, aggressive, short aggressive), a minor increase was noticed for the tre:phe 10:0.5 samples after freeze-drying. No increase was detected for tre:phe 10:3 samples.

After storing the samples for 6 months only a minimal increase was observed all samples of the three different cycles, even in case the samples were stored at 50 °C. No difference was noticed between collapsed and non-collapsed samples. Obviously, also in collapsed lyophilizates, which were generated by aggressive freeze-drying of the tre:phe 10:0.5 formulation, good stabilization of LDH against aggregation to HMWS was achieved.

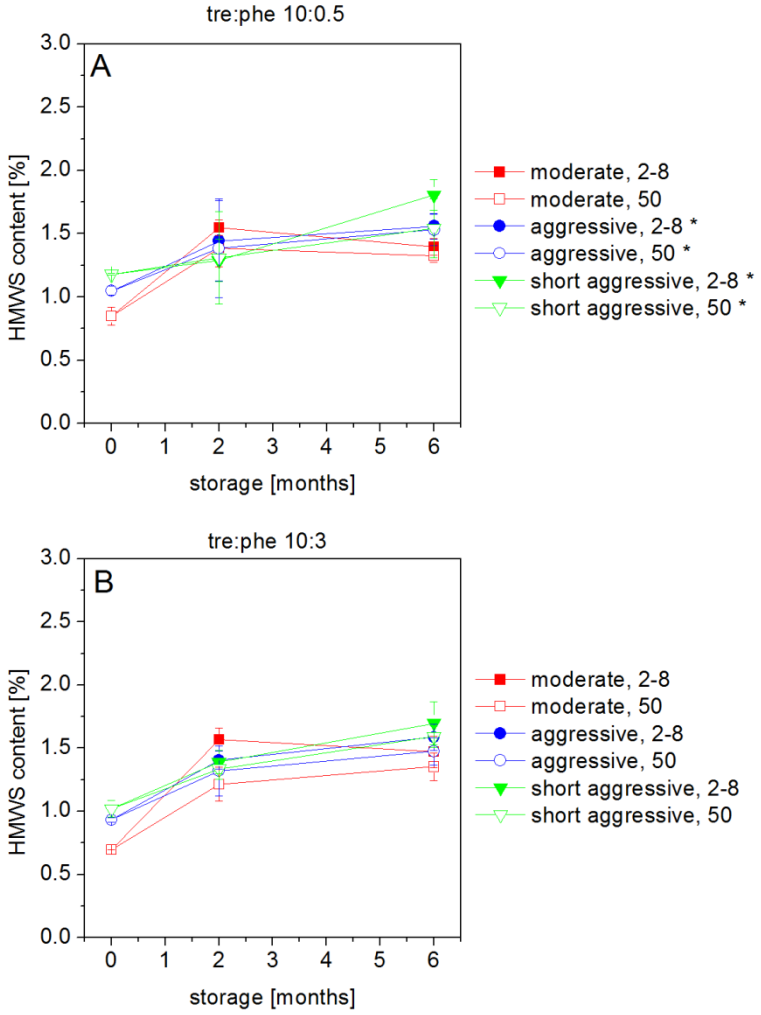

Figure 7.11: HP-SEC high molecular weight species of LDH in lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months(the trehalose-based formulations contain a low (A) or high amount (B) of phenylalanine and 0.75 mg/ml LDH; before freeze-drying a HWMS content of 0.6 ± 0.09% (tre:phe 10:0.5) and 0.90 ± 0.01% (tre:phe 10:3) was determined; initially collapsed samples are marked by an asterisk).

The concentration of subvisible particles, which represents non-soluble LDH aggregates, was assessed by light obscuration (cf. Figure 7.12). Before freeze-drying concentrations of ≤ 2000 particles ≥ 1 µm/ml were detected for both formulations.

After freeze-drying for all samples containing a high amount of phenylalanine a drastic increase of svp was observed (cf. Figure 7.12 B). Moderate and aggressive freeze-drying resulted in pronounced aggregation in these non-collapsed samples. During storage at 2-8 °C or 50 °C, no further increase of the svp concentration ≥ 1 µm was observed. In contrast, the svp of ≥ 10 µm and ≥ 25 µm exhibited a considerable increase during storage (see appendix, Table 9.7) indicating ongoing aggregation toward larger aggregates.

The lyophilizates that contain a low amount of phenylalanine showed a two-sided result (cf. Figure 7.12 A): While the moderately freeze-dried (non-collapsed) samples also exhibited severe aggregation, the aggressively freeze-dried (collapsed) samples showed no substantial increase. **Apparently, in the collapsed samples aggregation during freeze-drying was prevented**. During storage, the collapsed lyophilizates showed a minor increase of svp concentration, which is slightly more pronounced for 50 °C storage temperature. Comparing lyophilizates of the two aggressive cycles, upon storage at 50 °C samples of the short cycle showed a slightly higher svp concentration after 6 months. However, a svp concentration < 30,000 particles ≥ 1 µm/ml is regarded as suitable level for samples stored at 50°C. Regarding svp of ≥ 10 µm and ≥ 25 µm a comparable, slight increase was noticed for samples of both cycles (see appendix, Table 9.6).

The trends concerning aggregation to non-soluble aggregates were underlined by the turbidity of reconstituted samples (see Figure 9.7 in the appendix for details). After freeze-drying, non-collapsed samples, regardless whether produced by moderate freeze-drying of tre:phe 10:0.5 or aggressive/moderate freeze-drying of tre:phe 10:3, exhibited an increase of turbidity from < 1 FNU to > 5 FNU. For the collapsed tre:phe 10:0.5 lyophilizates no change of turbidity was observed after aggressive freeze-drying.

After storage of the samples for 6 months, moderately freeze-dried samples exhibited no further increase of the turbidity (i.e. no further aggregation occurred). In contrast, the aggressively freeze-dried tre:phe 10:3 samples revealed a further increase of turbidity, more pronounced in case stored at 50 °C. Obviously, the aggressive conditions during drying fostered further aggregation of LDH in non-collapsed lyophilizates during storage. On the other hand, tre:phe 10:0.5 samples showed no increase after storage of 6 months at 50°C, underlining the conclusion that aggregation of LDH was prevented in these collapsed lyophilizates.

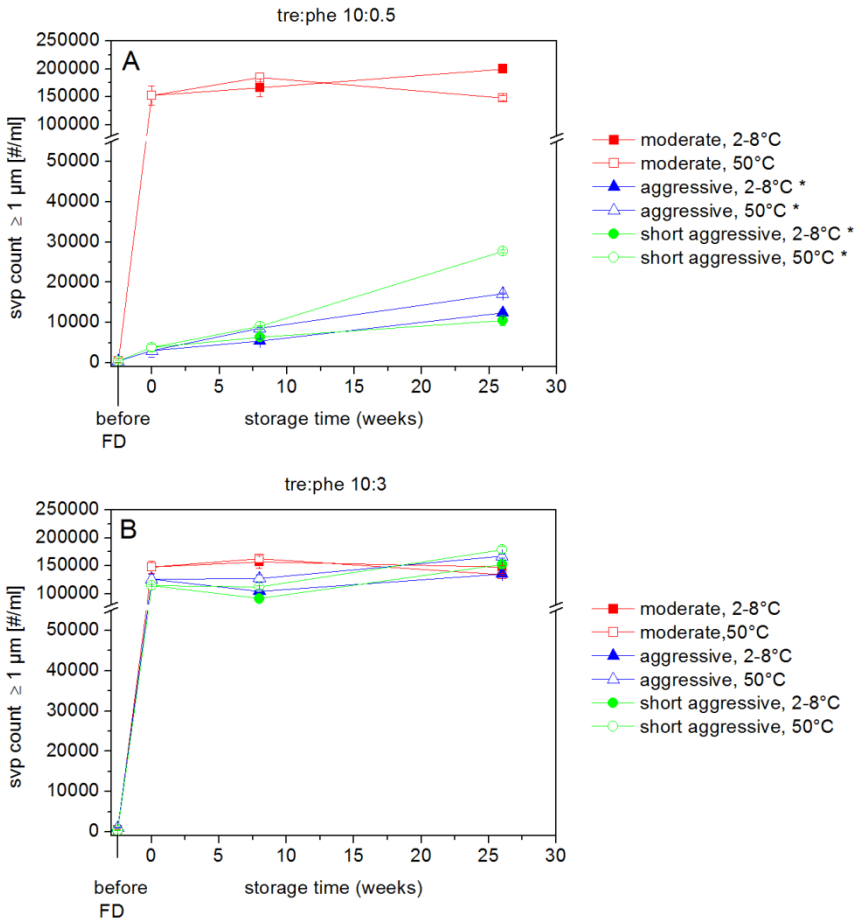

Figure 7.12: Subvisible particle (svp) concentration of LDH in lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months(the trehalose-based formulations contain a low (A) or high amount (B) of phenylalanine and 0.75 mg/ml LDH; initially collapsed samples are marked by an asterisk).

The enzymatic activity of LDH was tested before and after freeze-drying, and after storing samples for 6 months at 2-8 °C or 50 °C (cf. Figure 7.13). Despite their collapsed structure, aggressively freeze-dried tre:phe 10:0.5 samples showed comparable recovery of activity to non-collapsed samples. The collapsed lyophilizates produced by the short cycle exhibited a slightly lower recovery. However, analysis at the end of the storage study of 6 months revealed > 90% recovery of activity for the latter samples. Hence, apparently lower activity after freeze-drying is ascribed to an experimental aberration in the enzymatic assay. **Overall, excellent preservation of LDH activity was noticed for collapsed tre:phe 10:0.5 samples stored at 50 °C for 6 months.**

Although the high svp concentration of non-collapsed samples indicates instability of LDH, the enzymatic activity was not affected. The tre:phe 10:3 samples from moderate and aggressive freeze-drying revealed > 90% recovery of activity at the end of the storage study at 50°C. Also the non-collapsed lyophilizates were able to preserve LDH activity.

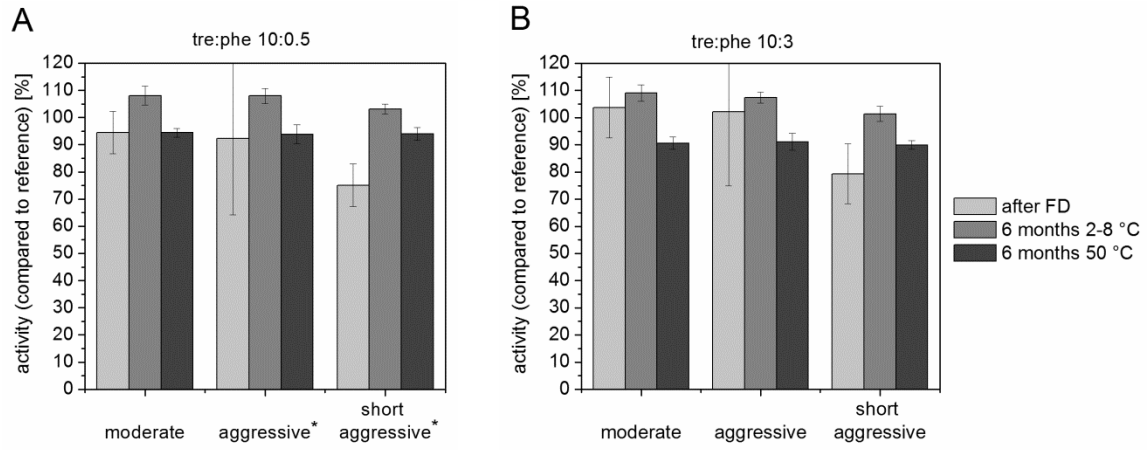

Figure 7.13: Enzymatic activity of LDH in lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months(the trehalose-based formulations contain a low (A) or high amount (B) of phenylalanine and 0.75 mg/ml LDH; initially collapsed samples are marked by an asterisk).

The data outlined above have shown superior stabilization of LDH in aggressively freeze-dried lyophilizates, which collapsed during drying. These lyophilizates were generated from a trehalose-based formulation that contained a low amount of phenylalanine (tre:phe 10:0.5). Most remarkably, the collapsed samples showed excellent preservation of LDH stability even after 6 months at 50°C. Lyophilizates of non-collapsed appearance, regardless whether produced by moderate freeze-drying of tre:phe 10:0.5 or moderate/aggressive freeze-drying of the formulation with high phenylalanine content (tre:phe 10:3), revealed less successful stabilization especially against aggregation.

Comparing the results from protein stability and physico-chemical evaluation of the lyophilizates produced by either moderate or aggressive freeze-drying, no correlation was found between residual water content and protein stability. Usually, higher water content is regarded as inferior for protein stabilization [27]. However, the collapsed samples from aggressive freeze-drying, which exhibited higher water contents (compare Figure 7.8), did not show inferior but superior stabilization of LDH. Also very low water content would be a conceivable scenario for worse stabilization [28, 29]. However, the aggressively freeze-dried tre:phe 10:0.5 samples, which exhibited a lower water content than the moderately freeze-dried ones, showed better stabilization. Hence, it is concluded that water content did not play the major role for the differences observed in protein stabilization. No correlation between glass transition temperatures and stability was observable, too.

All lyophilizates based on the tre:phe 10:0.5 formulation showed a fully amorphous structure (compare Figure 7.9), but the non-collapsed samples from moderate freeze-drying exhibited as poor stabilization as the partly crystalline samples based on tre:phe 10:3. Hence, a fully amorphous structure by itself did not guarantee good protein stabilization.

Since LDH is reported to be a surface sensitive protein [16] a correlation of instability and SSA seemed reasonable. Relating aggregation of LDH (i.e. turbidity) to the specific surface area of the lyophilizates uncovered that aggregation was observed in all non-collapsed samples, which had a high SSA (cf. Figure 7.14). Even if moderate conditions were used, the high SSA of non-collapsed tre:phe 10:0.5 samples caused aggregation. Compared to the moderately freeze-dried tre:phe 10:3 samples, the SSA was considerably

lower in the aggressively freeze-dried non-collapsed samples. However, similar turbidity was observed. It is concluded that in case of non-collapsed lyophilizates aggressive conditions result in instability at lower SSA compared to moderate conditions.

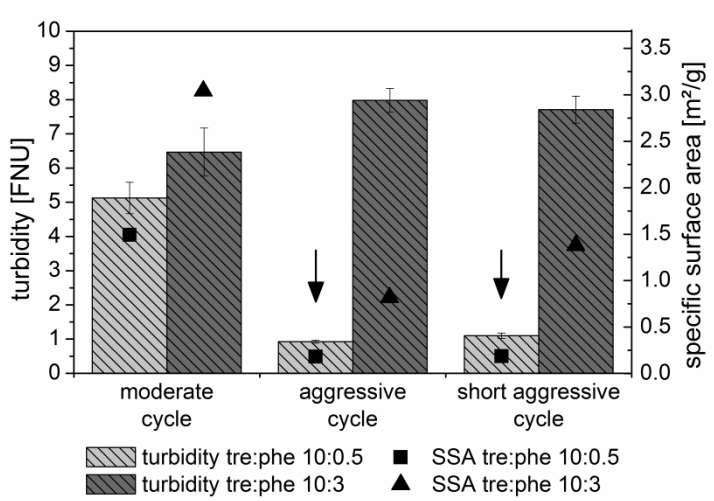

Figure 7.14: Comparison of turbidity and specific surface area of after freeze-drying. The trehalose-based formulations contain a low (tre:phe 10:0.5) or high amount (tre:phe 10:3) of phenylalanine and 0.75 mg/ml LDH (collapsed lyophilizates are marked by an arrow).

In contrast to the non-collapsed lyophilizates, the aggressively freeze-dried tre:phe 10:0.5 samples, which exhibited low SSA due to collapse, showed no increase of aggregation after freeze-drying (cf. Figure 7.14). These collapsed samples also showed excellent stabilization of LDH during storage at 50°C. The superior stability of LDH in collapsed lyophilizates, which was found in this study, supports the findings of Schersch et al [1, 2]. Low SSA is concluded to be the major prerequisite for successful stabilization.

5. SUMMARY AND CONCLUSIONS

In this chapter, the investigation on the applicability of aggressive freeze-drying to three different model proteins was investigated. Mab_T2 (IgG antibody), GCSF, and LDH (from rabbit muscle) were utilized to evaluate the stabilization of proteins in aggressively freeze-dried lyophilizates. As a benchmark, moderately freeze-dried samples were investigated, too.

Aggressive freeze-drying of formulations that contain no or only a low amount of bulking agent resulted in collapsed samples. Non-collapsed samples were achieved either by adding larger amounts of bulking agents to the formulations, or by moderate freeze-drying. The addition of larger amounts of bulking agents resulted in partially crystalline lyophilizates, which were able to withstand structural collapse. Consequently, these samples exhibit a large surface area.

Since LDH is known as surface sensitive model protein [16] it was expected that a possible correlation between stabilization and SSA would be disclosed by investigating

samples of high and low SSA. In fact, for non-collapsed samples of large SSA worst stabilization of LDH after freeze-drying and storage was observed. Collapsed samples of low SSA, however, revealed excellent stabilization. These samples could be stored for 6 months at 50 °C without drastic sings of instability. Thus, it is concluded, that the combination of high SSA and aggressive drying conditions is the major stress for incorporated LDH.

Overall, mab_T2 was well stabilized in all aggressively freeze-dried lyophilizates. Collapsed samples even showed slightly superior prevention of aggregation. The overall good stabilization also in non-collapsed samples is ascribed to the presence of polysorbate 20 in all formulations. The combination of high SSA and aggressive drying conditions was shown to be the major stress for incorporated LDH (compare above). Hence, due to the partly crystalline structure and large SSA of non-collapsed maa_T2 samples, worse stabilization of mab_T2 is probable if no polysorbate would be used in the formulation. On the other hand, during formulation development for aggressive freeze-drying the addition of polysorbate or other surfactants could be a promising strategy to counteract surface stress.

Comparing non-collapsed and collapsed samples of GCSF, the latter were superior in preventing aggregation. However, also in collapsed samples no complete stabilization of GCSF was achieved (indicated by aggregation). As already in the initial formulations aggregation was observed, incomplete stabilization is partly attributed to a non-ideal buffer. It was beyond the scope of this investigation to screen for another buffer. Nevertheless, this study highlighted the potential of collapsed lyophilizates to stabilize GCSF.

Overall, the most remarkable finding was that all of the three proteins were better stabilized in collapsed than in non-collapsed lyophilizates. Obviously, the high SSA of non-collapsed samples affected the stability of incorporated protein. The importance of low SSA for dried protein formulations was already highlighted by Abdul-Fattah et al [30, 31]. In the present studies, it was shown that collapsed lyophilizates, which had a low SSA, are the preferred choice for stabilization of incorporated proteins. Especially lyophilizates that were composed of trehalose and phenylalanine at a weight-to-weight ratio of 10:0.5 presented superior ability to stabilize various proteins.

6. REFERENCES

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CHAPTER 8 FINAL SUMMARY AND CONCLUSION

The objective of this thesis was to investigate whether aggressive freeze-drying can catch up with conventional freeze-drying as a robust and reliable drying technique for sensitive biopharmaceuticals. In previous work it was shown that collapse of lyophilizates does not have a negative effect on protein stability during freeze-drying [1], but even revealed better stabilization during storage [2]. This was taken as a starting point for a sound investigation on aggressive freeze-drying. In order to broaden knowledge on how to apply aggressive freeze-drying, the potential design space for major process parameters (temperature, pressure, and time) was investigated. The drying kinetics of sublimation (primary drying) and desorption (secondary drying) were closely monitored to gain understanding on how to perform and potentially optimize aggressive freeze-drying. To account for the main strategies in formulation design for freeze-dried products, lyophilizates of sugar-based formulations with or without bulking agents were intensively characterized after aggressive freeze-drying. As for the first time a systematic characterization of the design space of aggressive freeze-drying was performed, key physico-chemical parameters (e.g. water content, crystallinity) were precisely inspected. The resulting samples were judged regarding the potential to ensure protein stabilization based on the concepts of water replacement [3-6] and vitrification [7-9]. Beyond the physico-chemical characteristics of lyophilizates, the ability to stabilize incorporated proteins was the major determinant to assess aggressive freeze-drying.

In **CHAPTER 1**, a comprehensive introduction is given. **CHAPTER 2** summarizes the objectives of the thesis, the utilized materials and methods are outlined in **CHAPTER 3**.

The influence of process parameters (shelf temperature, chamber pressure, process time) on drying kinetics, physico-chemical characteristics, and protein stabilization of aggressively freeze-dried lyophilizates was studied in **CHAPTER 4**. The monoclonal IgG1 antibody mab_T1 was utilized as model protein, which represents the currently most important class of protein pharmaceuticals. Additionally, mab_T1 is susceptive to freeze-drying stresses if not suitably stabilized. Formulations were based on either sucrose or trehalose, which are superior cryo-and lyoprotectants for proteins [10, 11]. The selection of formulations was completed by two formulations containing phenylalanine as bulking agent (low and high concentration).

Chamber pressures of 0.1 mbar to 4 mbar and shelf temperatures of 30 °C to 60 °C were investigated. The drying time was varied from 8 h to 24 h. For all conditions, very high sublimation rates were observed. Within maximum 6 h sublimation was completed.

The lyophilizates of high phenylalanine content revealed a non-collapsed appearance regardless of the process parameters. If a chamber pressure of 0.1 mbar was used, also the formulations without bulking agent exhibited a non-collapsed appearance even if 60 °C shelf temperature were utilized. This is caused by the considerable influence of the chamber pressure on the product temperature (during sublimation). For pressures of 0.1 mbar to 4 mbar the product temperature ranged from -28.8 °C to -1.7 °C, respectively. The lyophilizates containing no or a low amount of a bulking agent collapsed during the

primary drying if a chamber pressure of ≥ 1 mbar was utilized. No increase of the product temperature was observed, Hence, collapse did not result in restriction of water vapor flow (flow hindrance would result in an increase of Tp). Obviously, the high mobility of the collapsing matrix allows unimpeded water vapor flow. At chamber pressures of 4 mbar, the samples that contain no or a low amount of bulking agent partly spattered to the stopper/out of the vials. Although this behavior is regarded as unacceptable/uncontrollable, they were characterized as well.

For conventional freeze-drying a correlation of sublimation rate and product temperature has been described [12]. For aggressive conditions, which were studied in this work, a higher sublimation rates was observed for higher product temperature in the range between -28 °C to -15 °C. However, above -15 °C no further increase of sublimation rate was noticed.

All samples that contain no bulking agent revealed a fully amorphous structure for either process condition. The formulations containing a low amount of phenylalanine revealed a fully amorphous structure in case freeze-dried at ≤ 2 mbar. For 4 mbar chamber pressure, a small fraction of crystalline phenylalanine hydrate was found in these lyophilizates (also detected in samples of high bulking agent content). Most probably, the high mobility (due to high product temperature) in the collapsing lyophilizates allowed for crystallization.

Our investigations revealed that chamber pressure is the major determinant for the product temperature and therefore for collapse behavior and final structure. Nevertheless, as expected, higher shelf temperatures between 30 °C and 60 °C resulted in higher sublimation rates due to higher energy input. Interestingly, the product temperatures were hardly affected by shelf temperature. Despite high temperatures were used, also collapsed samples (which contained no bulking agent) showed comparably high water contents after 24 h (i.e. around 18 h secondary drying). If a low amount of phenylalanine was added to the sugar based formulation (10:0.5 wt:wt ratio), regardless of the collapsed structure, low water content of <1% was achieved already after 8 h drying.

Summarizing the influence of process conditions of aggressive freeze-drying on protein stabilization, an overall good to excellent stabilization of mab_T1 (recombinant monoclonal IgG1 antibody) was observed. Evaluating the stability in the collapsed and non-collapsed samples revealed that collapsed samples consistently showed superior stabilization. Non-collapsed lyophilizates, which resulted from either low chamber pressure or high bulking agent content, revealed an increase of aggregated species. Comparing the physico-chemical properties of these lyophilizates to non-collapsed ones, the major difference was the considerably higher specific surface of latter samples. In case of high bulking agent content, additionally the partly crystalline structure seems to be detrimental to mab_T1 stability. As collapsed lyophilizates were produced by medium to high chamber pressure, it is concluded that values of ≥ 1 mbar are preferable for stabilization. As for 4 mbar the collapse behavior was not controllable (spattering), the chamber pressure is advised to be in the range between 1 and 2 mbar for aggressive freeze-drying. In addition, also higher shelf temperatures resulted in slightly better stabilization, indicating a possible annealing effect, which is described to improve stabilization of embedded proteins [13-15]. As high shelf temperatures also caused lower water content, a shelf temperature of ≥ 45 °C is desirable for aggressive freeze-drying. Overall, an optimum level of stabilization was achieved by samples of low specific surface area and fully amorphous

structure. Monitoring of the stability of mab_T1 over drying time revealed slightly less successful stabilization for longer drying time. Hence, aggressive cycles should by as short as possible. **To produce fully amorphous lyophilizates of sufficiently low residual water content and low specific surface area by short drying cycles, the addition of low amounts of phenylalanine to sugar-based formulations (ratio 10:0.5) is the key to success.**

In **CHAPTER 5** and **CHAPTER 6** the influence of formulation composition on physico-chemical characteristics (chapter 5) and protein stability (chapter 6) of aggressively freeze-dried lyophilizates were studied. The recombinant monoclonal IgG1 antibody mab_T1, which is sensitive to freezing and drying stresses, was used as model protein.

As non-reducing disaccharides are effective cryo-and lyoprotectants, the majority of freeze-dried antibody drugs are based on either sucrose or trehalose [16]. Hence, these sugars were chosen as backbone of the investigated formulations. A range of freeze-drying formulations was selected, especially with respect to formulations that contain bulking agents (mannitol, glycine or phenylalanine) in different quantities. In a first step, formulations without bulking agents were studied. Despite the collapsed appearance and high water content, the samples of trehalose and sucrose (at 50 mg/ml) completely stabilized mab_T1 during aggressive freeze-drying.

Previous test have shown that bulking agents allow extremely short aggressive freeze-drying cycles of 12 h (including freezing). Such a short cycle was now utilized for drying the formulations that contain bulking agents. All samples containing a low amount of either bulking agent showed very good stabilization of mab_T1. However, neither a low amount of mannitol nor glycine was able to reduce the residual water content to a satisfactory degree.

As already observed by Schersch at al [1], a stepwise addition of mannitol to aggressive freeze-dried formulations resulted in a decreasing degree of collapse, both, visible as well as detected by SSA measurements. The non-collapsed appearance was reflected by comparably high specific surface areas. In the present work, a similar effect was shown in case phenylalanine or glycine was used as bulking agent. Remarkably, a comparable bulking effect (in terms of appearance and SSA) was observed at a ten times lower sugar to phenylalanine ratio (i.e. at 10:3 instead of 10:30 for mannitol or glycine). The bulking effect of glycine (10:30) and phenylalanine (10:3) could be attributed to crystallization (detected by XRD). Although improving especially the drying towards low water contents, the addition of higher amounts of bulking agent resulted in slightly worse stabilization of mab_T1, especially mannitol deteriorated the stability. Obviously, the partly crystalline structure and the high SSA of these lyophilizates imposed some stress to the protein.

Formulations containing sugar and a low amount of phenylalanine (tre:phe 10:0.5, suc:phe 10:0.5) revealed a collapsed structure. Intriguingly, despite these samples exhibited collapse (i.e. low SSA) and were fully amorphous, they could be dried to low residual water contents and high Tg. Moreover, these lyophilizates stood out by excellent stabilization of mab_T1.

Consequently, these collapsed lyophilizates composed of sucrose/trehalose and a low amount of phenylalanine were subjected to a storage stability study at 30°C and 50°C (and a control at 2-8°C) for up to 6 months. Additionally, aggressively freeze-dried non-

collapsed lyophilizates that contain high amounts of glycine, mannitol, or phenylalanine were included in this study. For comparison, moderately freeze-dried samples of the respective formulations were stored at identical conditions. Summarizing, it was found that even if stored at 50°C, mab_T1 was completely stabilized for 6 months by the trehalose-based lyophilizates that contain a low amount of phenylalanine (tre:phe 10:0.5). In case samples of suc:phe 10:0.5 were stored, excellent preservation was achieved for storage up to 30 °C. The glass transition temperatures of the sucrose-based lyophilizates were in the vicinity of 50 °C. Hence, high mobility reduced the ability for stabilization. Although acceptable stabilization was achieved, none of the moderately freeze-dried samples reached a comparable stability as the collapsed lyophilizates. In addition, the aggressively freeze-dried non-collapsed samples (due to high bulking agent content) revealed ongoing aggregation of mab_T1 in the course of storage. Obviously, the high SSA of non-collapsed resulted in inferior storage stability.

The superior stabilization of suc:phe 10:5 and tre:phe 10:0.5 samples is ascribed to the unique physico-chemical properties: Fully amorphous structure despite adding phenylalanine, low SSA due to collapse during aggressive freeze-drying and low water content / high glass transition temperature despite the collapsed appearance. In the light of literature, the superior protein stabilization by amorphous matrices of low SSA [2, 17, 18] is reasonable. Based on the data, even room temperature storage is feasible. Hence, these special formulations are regarded as first choice for protein stabilization in aggressively freeze-dried lyophilizates.

In order to evaluate, whether other amino acids feature a comparable potential as phenylalanine, arginine, glycine, histidine, isoleucine, leucine, methionine, and tryptophan were added to sugar-based formulations at a weight-to-weight ratio of 10:0.5. Aggressive freeze-drying of the sugar/amino acid formulations resulted in collapsed lyophilizates with low SSA. Compared to lyophilizates containing no amino acid, a decrease of water content was observed for isoleucine, leucine, and methionine. For trehalose-based samples also a remarkable increase of glass transition temperature was detected. XRD revealed that contrary to phenylalanine, isoleucine, leucine, and methionine crystallized during freeze-drying. Evaluating of the ability to protect mab_T1 against aggregation revealed increased aggregation in the formulations that contain isoleucine, leucine, or methionine. It is concluded that crystallization of the amino acids diminishes the ability to stabilize incorporated mab_T1 in aggressive freeze-dried lyophilizates. The other amino acids used (arg, gly, his, trp) exhibited good stabilization of mab_T1, however no improvement of water content and Tg compared to pure sugar lyophilizates was achieved. Isoleucine, leucine, and methionine exhibit a negative hydropathy index [19]. Hence, the effect on the physico-chemical lyophilizate characteristics is ascribed to the hydrophobic nature of the side chains of these amino acids, which fosters crystallization in aqueous solutions. Although a negative hydropathy index is also reported for phenylalanine [19], no crystallization was detectable. **It is concluded that phenylalanine exhibits a unique effect on the physico-chemical lyophilizate characteristics: It drastically improves the drying of collapsed lyophilizates and remains fully amorphous. Thus, phenylalanine stands out by combining suitable physico-chemical properties and excellent protein stabilization upon combination with trehalose or sucrose.**

In a separate study it was shown that in the range between 25 and 150 mg/ml sucrose and trehalose formulation with and without phenylalanine (ratio 10:0.5) resulted in collapsed lyophilizates of low SSA and fully amorphous structure. For pure sugar lyophilizates, a slight tendency of lower water content was observed for higher solid contents. For samples that contain phenylalanine, a decrease of SSA with increasing solid content was noticed. While for samples of ≥ 50 mg/ml aggregation of mab_T1 was prevented, slightly less successful stabilization was observed for low solid contents (25 mg/ml). Worse stabilization is attributed to larger SSA of latter samples.

The addition of a higher amount of protein (10 mg/ml mab_T1) resulted in a reduction of the specific surface area and a slight increase of residual water contents (compared to lyophilizates of 1 mg/ml). Although especially the lyophilizates from sucrose and trehalose showed rather high water content, mab_T1 was effectively protected. As already observed for formulations of 1 mg/ml mab_T1 concentration, adding phenylalanine considerably decreased the water content without increasing the SSA. The sugar-based lyophilizates containing phenylalanine revealed excellent stabilization. Therefore, it is concluded that proteins can be stabilized by aggressive freeze-drying also at concentrations at least up to 10 mg/ml.

In **CHAPTER 7**, the applicability of aggressive freeze-drying to three different model proteins was investigated. Mab_T2 (monoclonal IgG1 antibody), GCSF, and LDH was utilized to evaluate the stabilization of proteins in aggressively freeze-dried lyophilizates.

LDH is known as a surface sensitive model [20]. Thus, it was expected that a possible correlation between stabilization and the specific surface area, which was found for mab_T1, would be substantiated. The first choice formulation of the mab_T1 investigations (tre:phe 10:0.5) was chosen and compared to the formulation containing a high amount of phenylalanine(tre:phe 10:3), which did not collapse during aggressive freeze-drying. In fact, for non-collapsed samples of large SSA insufficient stabilization of LDH after freeze-drying and storage was observed. Collapsed tre:phe 10:0.5 samples of low SSA, however, revealed excellent stabilization. These lyophilizates could be stored for 6 months at 50 °C without showing instability. It is concluded that the combination of high SSA and aggressive drying conditions is the major stress for incorporated proteins.

For the investigation on mab_T2 and GCSF stabilization sucrose/trehalose-based formulations with low and high amounts of mannitol, glycine, and phenylalanine were aggressively freeze-dried (12 h protocol). Independent, whether embedded in collapsed or non-collapsed lyophilizates, mab_T2 was well stabilized in all aggressively freeze-dried lyophilizates. The combination of high SSA and aggressive drying conditions in non-collapsed lyophilizates did not cause major instability. Collapsed samples even showed slightly superior prevention of aggregation. The good stabilization also in non-collapse samples is ascribed to the presence of polysorbate 20 in all formulations. The addition of polysorbate or other surfactants could be a promising strategy for a formulation development also for aggressive freeze-drying.

Comparing non-collapsed and collapsed samples of GCSF, the latter were superior in preventing aggregation. However, also in collapsed samples, no complete stabilization of GCSF was achieved. As already in the initial formulations aggregation was observed, incomplete stabilization is partly attributed to a non-ideal buffer system. It was beyond

the scope of this investigation to screen for another buffer. Nevertheless, this study highlighted the potential of collapsed lyophilizates to stabilize GCSF.

Overall it was found that LDH, mab_T2, and GCSF were better stabilized in collapsed than in non-collapsed lyophilizates. Obviously, the low SSA of collapsed samples is preferable for a whole range of proteins supporting the observations on the importance of low SSA for dried protein formulations [17, 21]. In the present studies, it was show that collapsed lyophilizates of low SSA are the preferred choice for stabilization of incorporated proteins. Especially lyophilizates that were composed of trehalose and phenylalanine at a weight-to-weight ratio of 10:0.5 presented superior ability to stabilize various proteins.

Having an overall look on the results of this thesis, aggressive freeze-drying turned out as controllable, reproducible, and very fast technique to generate lyophilizates. By selecting appropriate process conditions, elegant or collapsed lyophilizates can be produced by aggressive freeze-drying of common protein formulations – even if no bulking agents are used. Despite beeing usually regarded as not acceptable, compared to elegant samples, collapsed lyophilizates have shown superior potential to stabilize sensitive proteins during storage. It has turned out that the low specific surface area of collapsed samples (in combination with a fully amorphous structure) can result in excellent stability. However, collapsed lyophilizates (of formulations that contain no bulking agent) were difficult to dry to low water contents within the short time of aggressive protocols (typically $\leq$ 24 h). The use of small amounts of phenylalanine, which were added to sucrose- or trehalose-based formulations at a weight-to-weight ratio of 10:0.5 (sugar:phe) emerged as a superior formulation strategy for aggressive freeze-drying. These formulations could by aggressively freeze-dried to fully amorphous, collapsed lyophilizates within 12 h and were able to stabilize therapeutics monoclonal antibodies (IgG1) for 6 months even at 50°C storage.

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CHAPTER 9 APPENDIX

1. ADDITIONAL TABLES AND FIGURES

Table 9.1: HP-SEC data to characterize the influence of type of disaccharide on the protein stability of mab_T1 (formulations based on 10 mM potassium phosphate buffer pH 6.4)

	HMWS content		LMWS content		monomer content		monomer recovery
	before freeze-drying	after freeze-drying	before freeze-drying	after freeze-drying	before freeze-drying	after freeze-drying	
formulation	%	%	%	%	%	%	%
suc:man 10:3	1.36±0.04	1.66±0.12	1.30±0.08	0.95±0.22	97.34±0.35	97.39±0.21	100.58±0.27
suc:man 10:30	1.35±0.06	1.87±0.05	1.46±0.25	0.8±0.03	97.19±0.32	97.32±0.08	99.37±0.2
suc:gly 10:3	1.35±0.08	1.58±0.07	1.29±0.05	0.79±0.13	97.36±0.04	97.63±0.08	101.97±0.05
suc:gly 10:30	1.28±0.15	1.69±0.06	1.24±0.00	0.8±0.08	97.49±0.08	97.51±0.71	99.83±0.39
suc:phe 10:0.5	1.34±0.04	1.57±0.05	1.23±0.05	0.73±0.08	97.43±0.49	97.7±0.21	100.77±0.34
suc:phe 10:3	1.33±0.07	1.64±0.07	1.23±0.06	0.84±0.15	97.44±1.12	97.53±0.25	100.3±0.67
tre:man 10:3	1.37±0.08	1.68±0.03	1.14±0.05	0.93±0.04	97.49±0.26	97.39±0.63	101.77±0.44
tre:man 10:30	1.41±0.08	2.05±0.04	1.23±0.01	0.94±0.06	97.36±0.32	97.01±0.32	98.63±0.31
tre:gly 10:3	1.30±0.03	1.62±0.06	0.93±0.24	0.92±0.03	97.77±0.37	97.46±0.5	101.77±0.43
tre:gly 10:30	1.34±0.00	1.79±0.04	1.14±0.16	1±0.05	97.52±0.39	97.22±0.59	101.27±0.48
tre:phe 10:0.5	1.28±0.00	1.71±0.04	1.21±0.19	0.95±0.09	97.44±0.33	97.33±0.55	103.86±1.07
tre:phe 10:3	1.37±0.00	1.73±0.07	1.02±0.18	0.94±0.11	97.60±0.02	97.33±1.15	97.79±0.55

Table 9.2: Glass transition temperatures after storage of **moderately** freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1)

moderate	glass transition temperature (°C)						
		2-8°C		30°C		50°C	
	after FD	10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
suc:man 10:30	30.5 ± 6.4	22.3 ± 2.2	20.2 ± 2	19.6 ± 0.1	17.1 ± 0.4	n.d.	n.d.
suc:gly 10:30	40.3 ± 3.5	40.8 ± 1.7	38.9 ± 1.3	36.3 ± 0.8	33.8 ± 0.4	31.4 ± 2.7	31.8 ± 2
suc:phe 10:0.5	67.5 ± 0.3	64.6 ± 1	60.1 ± 2.6	59.7 ± 1.9	64.6 ± 0.6	58.8 ± 0.4	55.1 ± 0.7
suc:phe 10:3	73.7 ± 0.6	66.6 ± 3.5	70.4 ± 0.1	59.5 ± 1.5	46.3 ± 6.2	56.4 ± 7.9	56.8 ± 3.1
tre:man 10:30	50.3 ± 2.7	45.4 ± 2.6	34.3 ± 3.8	37.9 ± 2.2	32.8 ± 4.8	37.7 ± 1.1	40.6 ± 0.6
tre:gly 10:30	59 ± 0.1	57.6 ± 0.3	53.2 ± 0.8	51.4 ± 0.6	77.1 ± 10	46 ± 0.9	46.9 ± 0.4
tre:phe 10:0.5	96.7 ± 1.1	94.6 ± 0.6	95.5 ± 0.7	87.6 ± 0.9	84.4 ± 0.4	82 ± 0.5	83.7 ± 1.2
tre:phe 10:3	103.1 ± 1.2	99.7 ± 0.8	100.2 ± 0.9	92.6 ± 1.1	74.9 ± 7	85.6 ± 2	83.1 ± 0.5

n.d.: no glass transition detected

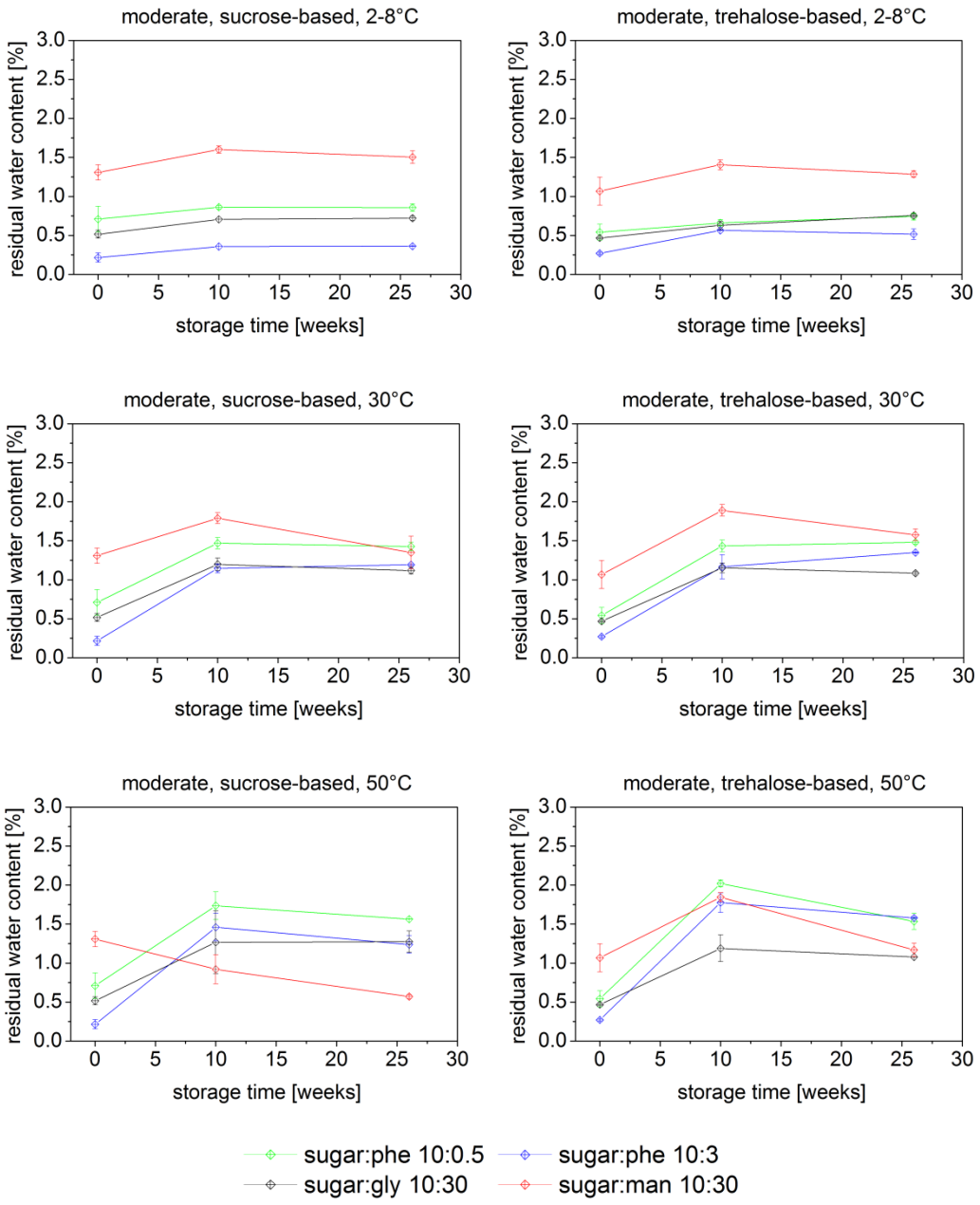

Figure 9.1: Residual water content after storage of moderately freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for up to 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1).

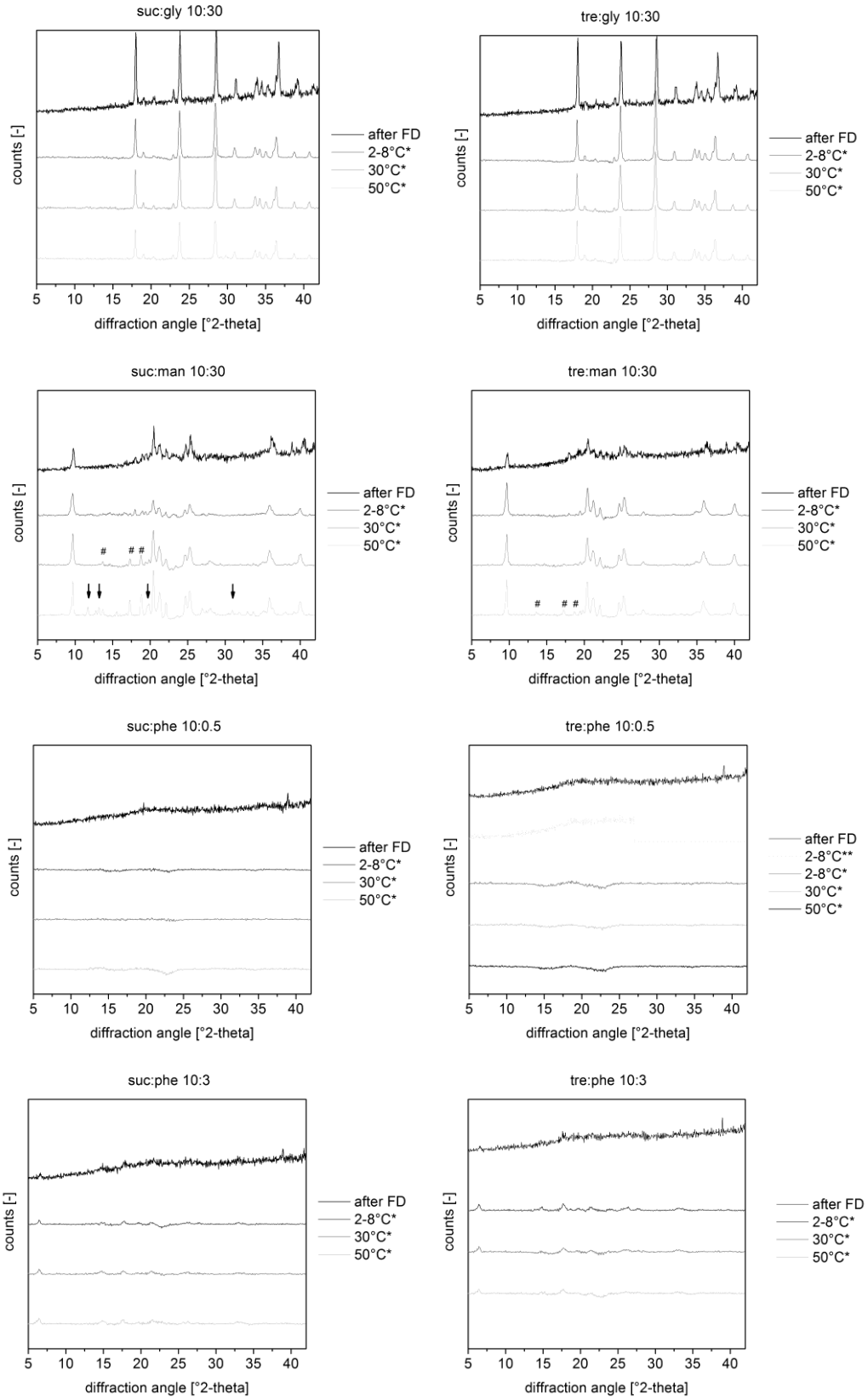

Figure 9.2: XRD diffraction patterns after storage of moderately freeze-dried samples at 2-8 °C, 30 °C, and 50 °C for 26 weeks (the sugar-based formulations contain bulking agents and 1 mg/ml mab_T1; # indicates diffractions of α-mannitol, diffractions of sucrose are highlighted by arrows; diffraction patterns marked by * were measured on an alternative device)

Table 9.3: HMWS detected by HP-SEC of moderately freeze-dried lyophilizates after storage for 26 weeks at 2-8 °C, 30 °C, or 50 °C. For comparison, HWMS before and after freeze-drying are shown. The sugar-based formulations contain bulking agents and 1.0 mg/ml mab_T1 (n=3).

formulation	before FD	after FD	after 26 weeks		
			2-8 °C	30 °C	50 °C
suc:man 10:30	1.3 ± 0.2	1.8 ± 0.1	1.7 ± 0.0	2.4 ± 0.0	71.1 ± 58.1
suc:gly 10:30	1.3 ± 0.2	1.8 ± 0.1	1.8 ± 0.0	2.6 ± 0.4	7.5 ± 2.5
suc:phe 10:0.5	1.3 ± 0.2	1.8 ± 0.1	1.7 ± 0.0	1.9 ± 0.4	3.0 ± 0.1
suc:phe 10:3	1.3 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.0	6.8 ± 0.8
tre:man 10:30	1.4 ± 0.1	1.9 ± 0.0	1.7 ± 0.0	2.9 ± 0.0	13.0 ± 0.2
tre:gly 10:30	1.4 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.9 ± 0.1	6.6 ± 1.3
tre:phe 10:0.5	1.4 ± 0.1	1.8 ± 0.1	1.6 ± 0.0	1.8 ± 0.1	1.8 ± 0.1
tre:phe 10:3	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.1

Table 9.4: Monomer recovery (detected by HP-SEC) of moderately freeze-dried lyophilizates after storage for 26 weeks at 2-8 °C, 30 °C, or 50 °C compared to monomer content after freeze-drying. The sugar-based formulations contain bulking agents and 1.0 mg/ml mab_T1 (n=3).

formulation	after 26 weeks		
	2-8 °C	30 °C	50 °C
suc:man 10:30	100.9 ± 0.6	99.9 ± 0.4	3.7 ± 1.8
suc:gly 10:30	101.3 ± 0.5	99.9 ± 0.2	56.5 ± 5.6
suc:phe 10:0.5	101.6 ± 0.5	101.5 ± 0.5	68.2 ± 2.5
suc:phe 10:3	101.3 ± 0.6	101.4 ± 0.6	100.0 ± 1.0
tre:man 10:30	100.7 ± 0.5	98.2 ± 0.2	69.0 ± 1.4
tre:gly 10:30	101.2 ± 0.4	100.7 ± 0.4	90.8 ± 0.6
tre:phe 10:0.5	101.7 ± 0.6	101.2 ± 0.5	99.8 ± 1.0
tre:phe 10:3	100.6 ± 1.3	100.8 ± 1.3	98.0 ± 1.4

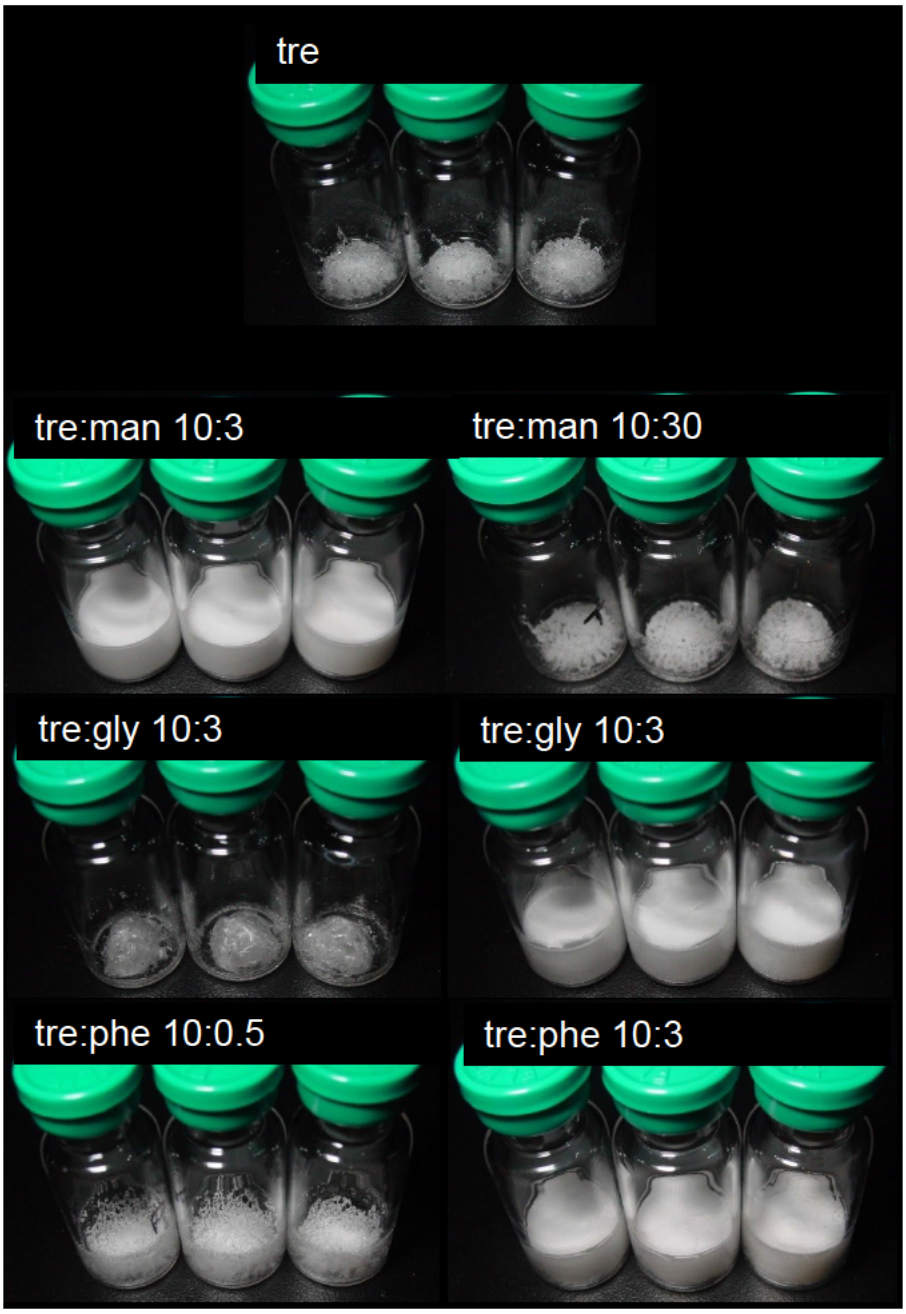

Figure 9.3: Macroscopic appearance of aggressively freeze-dried trehalose-based lyophilizates containing 1 mg/ml mab_T2.

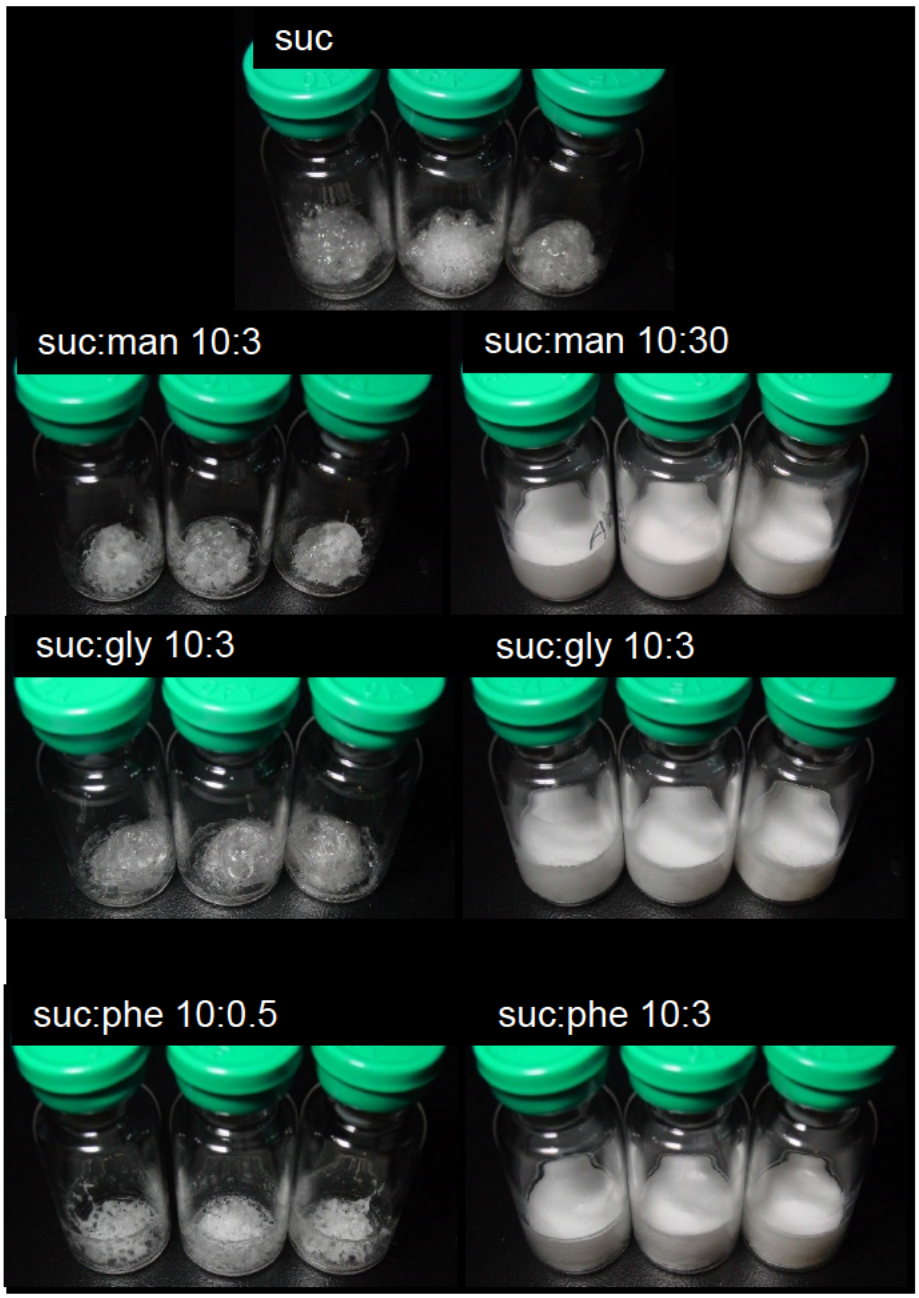

Figure 9.4: Macroscopic appearance of aggressively freeze-dried sucrose-based lyophilizates containing 1 mg/ml mab_T2.

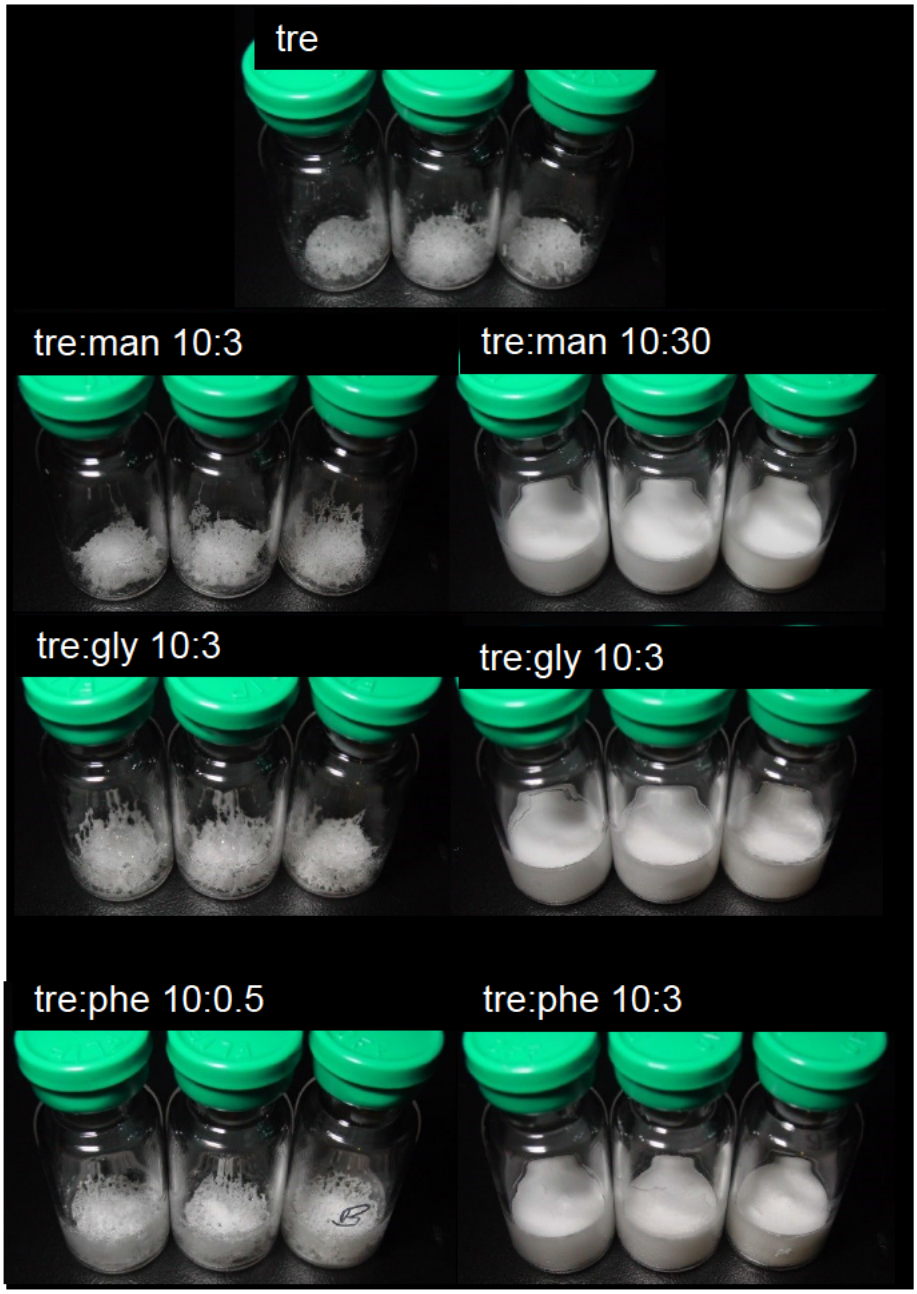

Figure 9.5: Macroscopic appearance of aggressively freeze-dried trehalose-based lyophilizates containing 1 mg/ml GCSF.

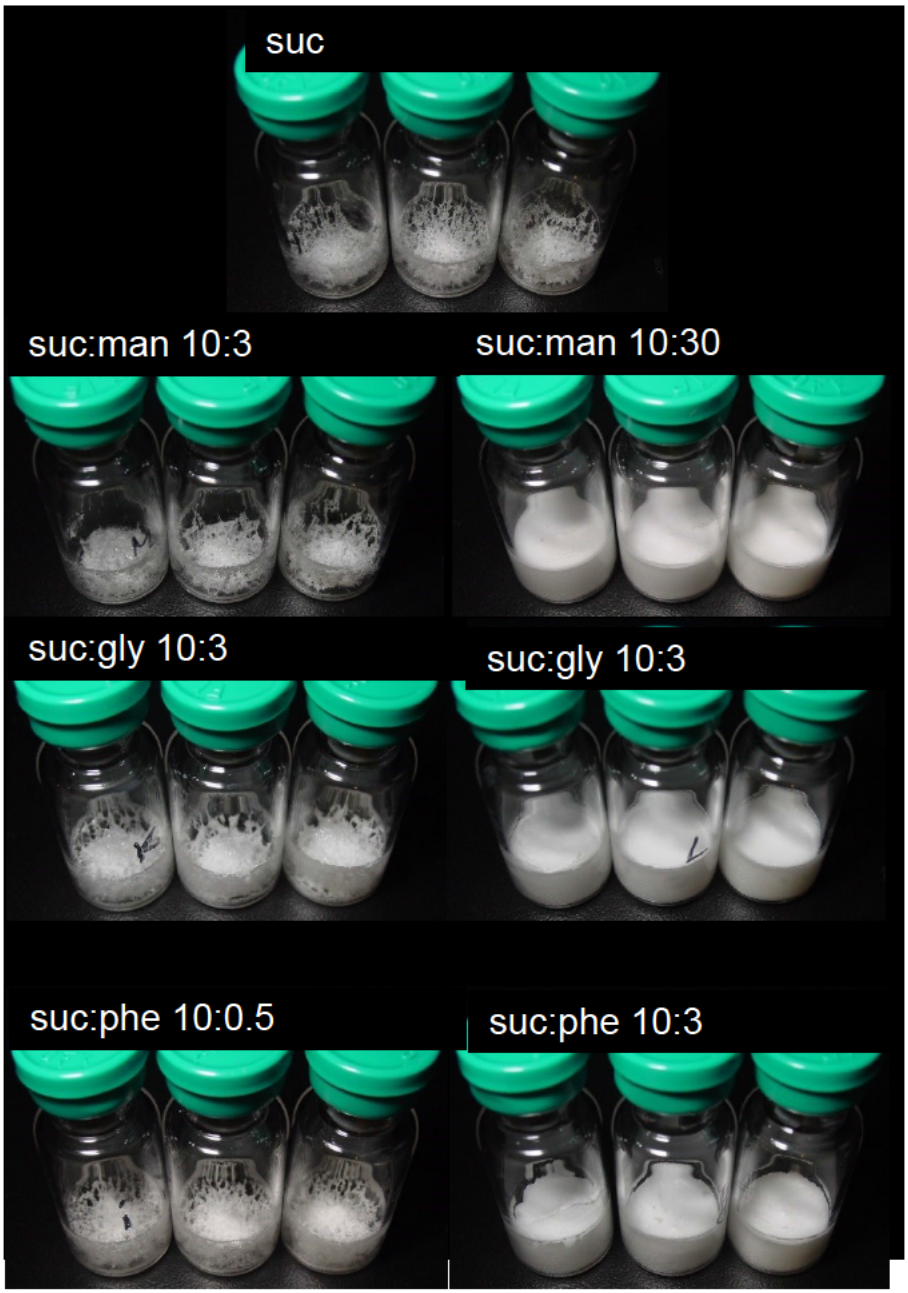

Figure 9.6: Macroscopic appearance of aggressively freeze-dried sucrose-based lyophilizates containing 1 mg/ml GCSF.

Table 9.5 Reconstitution time of sugar-based aggressively freeze-dried lyophilizates containing 1 mg/ml GCSF and different amounts of bulking agents (n=3).

formulation	reconstitution time	
	sucrose	trehalose
	seconds	seconds
sugar	27 ± 7	43 ± 2
sugar:man 10:3	24 ± 3	23 ± 2
sugar:man 10:30	14 ± 4	14 ± 2
sugar:gly 10:3	17 ± 2	17 ± 3
sugar:gly 10:30	10 ± 2	18 ± 1
sugar:phe 10:0.5	30 ± 5	26 ± 6
sugar:phe 10:3	33 ± 5	29 ± 4

Table 9.6: Subvisible particle (svp) concentration of LDH in lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months. The trehalose-based formulations contain a low amount of phenylalanine (tre:phe 10:0.5) and 0.75 mg/ml LDH (n=3; before freeze-drying 7 ± 0 ≥ 10 µm and 0 ± 0 ≥ 10 µm svp per ml)

	moderate				aggressive*				short aggressive*			
	2-8°C storage		50°C storage		2-8°C storage		50°C storage		2-8°C storage		50°C storage	
	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
after FD	47 ± 27	2 ± 1	47 ± 27	2 ± 1	10 ± 0	3 ± 1	10 ± 0	3 ± 1	10 ± 4	2 ± 1	10 ± 4	2 ± 1
8 weeks	2903 ± 180	10 ± 7	3036 ± 155	9 ± 2	44 ± 1	4 ± 1	66 ± 1	2 ± 2	75 ± 2	2 ± 2	55 ± 5	6 ± 2
26 weeks	4891 ± 113	14 ± 4	1303 ± 71	10 ± 5	149 ± 25	9 ± 4	192 ± 10	7 ± 3	129 ± 15	3 ± 3	189 ± 18	6 ± 2

Table 9.7: Subvisible particle (svp) concentration of LDH in lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months. The trehalose-based formulations contain a low amount of phenylalanine (tre:phe 10:0.5) and 0.75 mg/ml LDH (n=3; before freeze-drying 13 ± 5 ≥ 10 µm and 2 ± 3 ≥ 10 µm svp per ml)

	moderate				aggressive				short aggressive			
	2-8°C storage		50°C storage		2-8°C storage		50°C storage		2-8°C storage		50°C storage	
	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
after FD	21 ± 2	0 ± 1	21 ± 2	0 ± 1	51 ± 22	0 ± 0	51 ± 22	0 ± 0	24 ± 4	2 ± 2	24 ± 4	2 ± 2
8 weeks	2207 ± 144	6 ± 4	2188 ± 987	10 ± 3	1503 ± 55	72 ± 3	854 ± 34	37 ± 3	1328 ± 68	76 ± 16	167 ± 17	2 ± 2
26 weeks	1602 ± 57	13 ± 3	1170 ± 41	17 ± 10	1736 ± 44	21 ± 11	1866 ± 77	46 ± 13	1866 ± 20	13 ± 0	1140 ± 50	51 ± 8

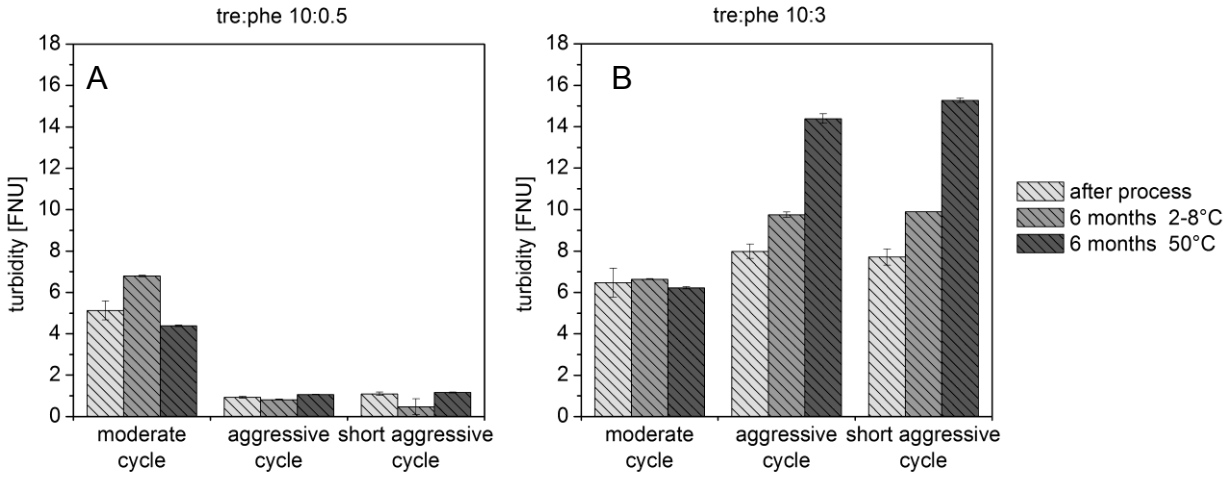

Figure 9.7: Turbidity of lyophilizates after freeze-drying and storage at 2-8 °C and 50 °C for 6 months(the trehalose-based formulations contain a low (A) or high amount (B) of phenylalanine and 0.75 mg/ml LDH).

2. LIST OF FIGURES

3. LIST OF TABLES