European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 248–263

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review Article

The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals

Julia Christina Kasper *, Wolfgang Friess

Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Munich, Germany

ARTICLE INFO

Article history:
Available online 21 March 2011

Keywords:
Lyophilization
Freeze-drying
Modifications of the freezing step
Influence on process performance
Influence on product quality
Influence on protein stability

ABSTRACT

Lyophilization is a common, but cost-intensive, drying process to achieve protein formulations with long-term stability. In the past, typical process optimization has focused on the drying steps and the freezing step was rather ignored. However, the freezing step is an equally important step in lyophilization, as it impacts both process performance and product quality.

While simple in concept, the freezing step is presumably the most complex step in lyophilization. Therefore, in order to get a more comprehensive understanding of the processes that occur during freezing, the physico-chemical fundamentals of freezing are first summarized. The available techniques that can be used to manipulate or directly control the freezing process in lyophilization are also reviewed. In addition, the consequences of the freezing step on quality attributes, such as sample morphology, physical state of the product, residual moisture content, reconstitution time, and performance of the primary and secondary drying phase, are discussed. A special focus is given to the impact of the freezing process on protein stability.

This review aims to provide the reader with an awareness of not only the importance but also the complexity of the freezing step in lyophilization and its impact on quality attributes of biopharmaceuticals and process performance. With a deeper understanding of freezing and the possibility to directly control or at least manipulate the freezing behavior, more efficient lyophilization cycles can be developed, and the quality and stability of lyophilized biopharmaceuticals can be improved.

1. Introduction

Lyophilization also known as freeze-drying is an important and well-established process to improve the long-term stability of labile drugs, especially therapeutic proteins [1]. About 50% of the currently marketed biopharmaceuticals are lyophilized, representing the most common formulation strategy [2]. In the freeze-dried solid state, chemical or physical degradation reactions are inhibited or sufficiently decelerated, resulting in an improved long-term stability [3]. Besides the advantage of better stability, lyophilized formulations also provide easy handling during shipping and storage [1].

A traditional lyophilization cycle consists of three steps; freezing, primary drying, and secondary drying [1]. During the freezing step, the liquid formulation is cooled until ice starts to nucleate, which is followed by ice growth. This results in the separation of most of the water into ice crystals from a matrix of glassy and/or crystalline solutes [4,5]. During primary drying, the crystalline ice formed during freezing is removed by sublimation. Therefore, the chamber pressure is reduced well below the vapor pressure of ice, and the shelf temperature is raised to supply the heat removed by ice sublimation [6]. At the completion of primary drying, the product can still contain approximately 15–20% of unfrozen water, which is then desorbed during the secondary drying stage, usually at elevated temperature and low pressure, to finally allow the desired low moisture content to be achieved [7].

In general, lyophilization is a very time- and energy-intensive drying process [8]. Freezing is typically over within a few hours, while drying often requires days. Within the drying phase, however, secondary drying is relatively short (~hours) compared to primary drying (~days) [1,4]. For that reason, lyophilization cycle development has typically focused on optimizing the primary drying step, i.e. shortening the primary drying time by adjusting the shelf temperature and/or chamber pressure without influencing product quality [5,9]. Although freezing is one of the most critical stages during lyophilization, the importance of the freezing process has rather been neglected in the past [10].

* Corresponding author. Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Butenandtstrasse 5, 81377 Munich, Germany. Tel.: +49 89 2180 77085; fax: +49 89 2180 77020.
E-mail address: julia.kasper@cup.uni-muenchen.de (J.C. Kasper).

0939-6411/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2011.03.010

The freezing step is of paramount importance. Freezing itself is the major dehydration step in lyophilization [6]. Solvent water is removed from the liquid formulation in the form of a pure solid ice phase, leading to a dramatic concentration of the solutes [11,12]. Moreover, the kinetics of ice nucleation and crystal growth determine the physical state and morphology of the frozen cake and consequently the final properties of the freeze-dried product [11–13]. Ice morphology is directly correlated with the rate of sublimation in primary and secondary drying [14]. In addition, freezing is a critical step with regards to the biological activity and stability of the active pharmaceutical ingredients (API), especially pharmaceutical proteins [1].

While simple in concept, the freezing process is presumably the most complex but also the most important step in the lyophilization process [10]. To meet this challenge, a thorough understanding of the physico-chemical processes that occur during freezing is required. Moreover, in order to optimize the freeze-drying process and product quality, it is vital to control the freezing step. But the random nature of ice nucleation makes this challenging. However, several approaches have been developed to trigger ice nucleation during freezing.

The purpose of this review is to provide the reader with an awareness of the importance and complexity of the physico-chemical processes that occur during freezing. Furthermore, currently available freezing techniques are summarized, and an attempt is made to address the consequences of the freezing procedure on process performance and product quality. A special focus is given to the critical factors that influence protein stability. Understanding and controlling the freezing step in lyophilization will lead to optimized, more efficient lyophilization cycles as well as products with an improved stability.

2. Physico-chemical fundamentals of freezing

In general, freezing is defined as the process of ice crystallization from supercooled water [15]. The freezing process first involves the cooling of the solution until ice nucleation occurs. Ice crystals begin to grow at a certain rate, resulting in freeze-concentration of the solution, a process that can result in both crystalline and amorphous solids, or in mixtures [11]. The following sections (2.1–2.3) summarize the physico-chemical fundamentals of freezing.

First, the distinction between cooling rate and freezing rate should be emphasized. The cooling rate is defined as the rate at which a solution is cooled. The freezing rate is the rate of postnucleation ice crystal growth, which is largely determined by the amount of supercooling prior to nucleation [16,17]. Therefore, the freezing rate of a formulation is not necessarily related to its cooling rate [18].

2.1. Freezing phenomena: supercooling, ice nucleation and ice crystal formation

In order to review the physico-chemical processes that occur during freezing of pure water, the relationship between time and temperature during freezing is displayed in Fig. 1. When pure water is cooled at atmospheric pressure, it does not freeze spontaneously at its equilibrium freezing point (0 °C) [19]. The retention of the liquid state below the equilibrium freezing point of the solution is termed as "supercooling" [19]. Supercooling (represented by line A) always occurs during freezing, often in the range of 10–15 °C or more [12,18]. The degree of supercooling depends on the solution properties and process conditions and is defined as the difference between the equilibrium ice formation temperature and the actual temperature at which ice crystals first form

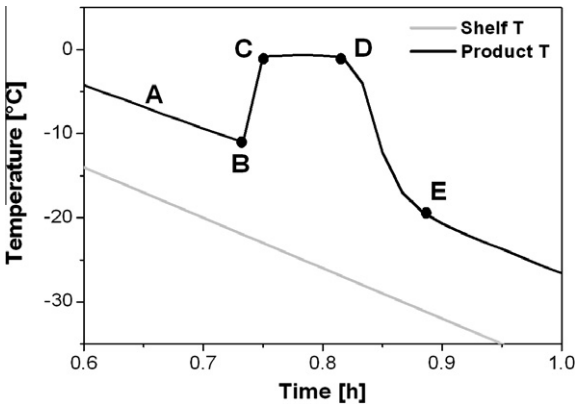

Fig. 1. Temperature profile measured with a thermocouple for a pure water sample during shelf-ramped freezing with 1 °C/min. The figure was created from own data by the authors.

[1,6,11,20]. As discussed later, it is necessary to distinguish between "global supercooling", in which the entire liquid volume exhibits a similar level of supercooling, and "local supercooling", in which only a small volume of the liquid is supercooled [14]. Supercooling is a non-equilibrium, meta-stable state, which is similar to an activation energy necessary for the nucleation process [21]. Due to density fluctuations from Brownian motion in the supercooled liquid water, water molecules form clusters with relatively long-living hydrogen bonds [22] with similar molecular arrangements as in ice crystals [11,15]. Because this process is energetically unfavorable, these clusters break up rapidly [15]. The probability for these nuclei to grow in both number and size is more pronounced at lowered temperatures [15]. Once the critical mass of nuclei is reached, ice crystallization occurs rapidly in the entire system (point B) [15,21,22]. The limiting nucleation temperature of water, referred to as the "homogeneous nucleation temperature", appears to be at about −40 °C. At this temperature, the pure water sample will contain at least one spontaneously formed active water nucleus, capable of initiating ice crystal growth [11]. However, in all pharmaceutical solutions and even in sterile-filtered water for injection, the nucleation observed is "heterogeneous nucleation". Meaning that ice-like clusters are formed via adsorption of layers of water on "foreign impurities" [6,11]. Such "foreign impurities" may be the surface of the container, particulate contaminants present in the water, or even sites on large molecules such as proteins [23–25].

Once stable ice crystals are formed, their growth proceeds by the addition of molecules to the interface [22]. However, only a fraction of the freezable water freezes immediately, as the supercooled water can absorb only 15 cal/g of the 79 cal/g of heat given off by the exothermic ice formation [12,22]. Therefore, once crystallization begins, the product temperature rises rapidly to near the equilibrium freezing point [12,26]. After the initial ice network has formed (point C), additional heat is removed from the solution by further cooling, and the remaining water freezes when the previously formed ice crystals grow [12]. Ice crystal growth is controlled by the latent heat release and the cooling rate which the sample is exposed to [22]. The freezing time is the time taken from the completed ice nucleation to the time of removal of latent heat (from point C to point D). The temperature drops when the freezing of the sample is completed (point E) [21].

The number of ice nuclei formed, the rate of ice growth, and the ice crystals' size depend on the degree of supercooling [14,20]. The higher the degree of supercooling, the higher the rate of nucleation and the faster the effective rate of freezing. This results in a high number of small ice crystals. In contrast, at a lower degree of

supercooling, one observes a lower number of large ice crystals [14,19]. The rate of ice crystal growth can be expressed as a function of the degree of supercooling [23]. For example, for water for injection showing a degree of supercooling of 10 ± 3 °C, an ice crystal growth rate of about 5.2 cm/s results [23]. In general, a slower cooling rate leads to a faster freezing rate and vice versa. Thus, in case of cooling rate versus freezing rate, it has to be kept in mind "slow is fast and fast is slow".

Nevertheless, one has to distinguish between the two basic freezing mechanisms; global supercooling and directional solidification. When global supercooling occurs, which is typically the case for shelf-ramped freezing, the entire liquid volume achieves a similar level of supercooling and solidification progresses through the already nucleated volume [12,14]. In contrast, directional solidification occurs when a small volume is supercooled, which is the case for high cooling rates, e.g., with nitrogen immersion. Here, the nucleation and solidification front are in close proximity in space and time and move further into non-nucleated solution. In this case, a faster cooling rate will lead to a faster freezing rate [12,14].

However, ice nucleation in general is a stochastically event [6,18]. Ice nucleation and, in consequence, ice crystal size distribution will differ from vial-to-vial, resulting in a large sample heterogeneity within one batch [6,14,27]. During freezing, the growth of ice crystals within one vial can also be heterogeneous, influencing intra-vial uniformity [5].

So far 10 polymorphic forms of ice have been described. However, at temperatures and pressures typical for lyophilization, the stable crystal structure of ice is limited to the hexagonal type, in which each oxygen atom is tetrahedrally surrounded by four other oxygen atoms [23]. The fact that the ice crystal morphology is a unique function of the nucleation temperature was first reported by Tammanm in 1925 [28]. In general, three different types of growth of ice crystals around nuclei can be observed in solution [15]. (i) If the water molecules are given sufficient time, they arrange themselves regularly into hexagonal crystals, called dendrites; (ii) if the water molecules are incorporated randomly into the crystal at a fast rate, "irregular dendrites" or axial columns that originate from the center of crystallization are formed; (iii) at higher cooling rates, many ice spears can originate from the center of crystallization without side branches, referred to as spherulites. However, the ice morphology depends not only on the degree of supercooling but also on the freezing mechanism. It is reported that "global solidification" creates spherulitic ice crystals, whereas "directional solidification" results in directional lamellar morphologies with connected pores [12,14]. While some solutes will have almost no effect on ice structure, other solutes can affect not only the ice structure but also its physical properties [19]. Based on Raoults's Law, the presence of solutes at high concentrations will result in a depression of the freezing point of the solution and in a faster ice nucleation because of the promotion of heterogeneous nucleation. This leads to a enormously lowered degree of supercooling [21].

2.2. Crystallization and vitrification of solutes

The hexagonal structure of ice is of great importance in the lyophilization of pharmaceutical formulations, while most solutes cannot fit in the dense structure of the hexagonal ice as it forms [23]. Consequently, the concentration of the solute constituents of the formulation is increased in the interstitial region between the growing ice crystals. This is referred to as "cryoconcentration" [11,12]. If this separation does not occur, a solid solution is formed with a greatly reduced vapor pressure and the formulation cannot be lyophilized [23]. The total solute concentration increases rapidly and is only a function of the temperature and independent of the initial concentration [4]. For example, for an isotonic saline solution a 20-fold concentration increase is reported when cooled to −10 °C, and all other components in a mixture show similar concentration increases [4]. Upon further cooling, the solution will increase to a critical concentration, above which the concentrated solution will either undergo eutectic freezing or vitrification [7].

An eutectic mixture is formed by the simple crystallization of solutes from cryoconcentrated solution [19]. For example, mannitol, glycine, sodium chloride, and phosphate buffers are known to crystallize upon freezing, if present as the major component [12]. When such a solution is cooled, pure ice crystals will form first. Two phases are present, ice and freeze-concentrated solution. The composition is determined via the equilibrium freezing curve of water in the presence of the solute (Fig. 2). The system will then follow the specific equilibrium freezing curve, as the solute content increases as more pure water is removed via ice formation. The lowest temperature at which the solute remains a liquid is the eutectic melting temperature (Teu). As the solution reaches Teu and a certain solute concentration (Ceu), the freezing curve will meet the solubility curve. Here, the freeze-concentrate is saturated and eutectic freezing, which means solute crystallization, will occur [7,19]. Only below Teu, the system can be completely solidified [19]. The Teu and Ceu are independent of the initial concentration of the solution [7]. In general, the lower the solubility of a given solute in water, the higher the Teu [19]. A general rule for multicomponent systems is that the crystallization of any component is influenced, i.e. hindered, by other components [11]. In practice, similar to the supercooling of water, only a few solutes will spontaneously crystallize at Teu [11]. Such delayed crystallization of solutes from a freezing solution is termed supersaturation and can lead to an even more extreme freeze-concentration [11]. Moreover, supersaturation can inhibit complete crystallization, leading to a meta-stable glass formation (e.g., mannitol) [12,23]. It is also possible that crystalline states exist in a mixture of different polymorphs or as hydrates [11]. For example, mannitol can exist in the form of several polymorphs (α, β and δ), and under certain processing conditions, it can crystallize as a monohydrate [11].

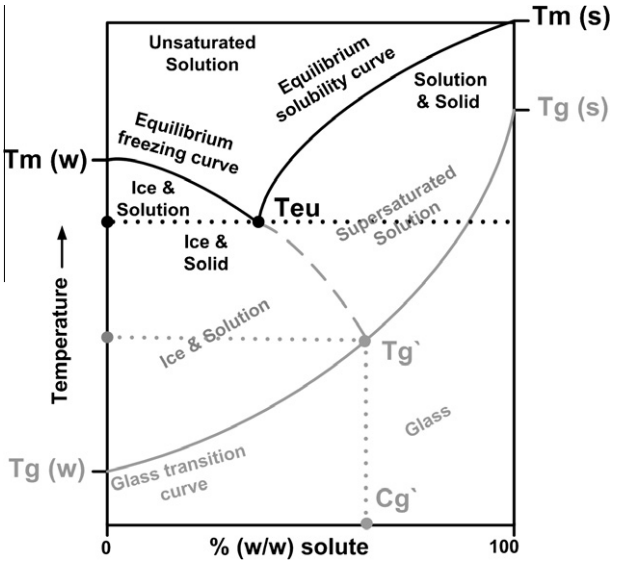

Fig. 2. State diagram for a water (w)/solute (s) system. Tm (w) and Tm (s): melting temperatures of water and solute, Teu: eutectic temperature, Tg (w) and Tg (s): glass transition temperature of water and solute and Tg′: glass transition temperature of the maximally freeze-concentrated solution. Crystallization (black drawings) of a solute occurs below Teu. In the case of vitrification (gray drawings), the solute does not crystallize at Teu, freeze-concentration proceeds and transits into a glass state at Tg. The figure was modified from Ref. [18].

The phase behavior is extremely different for polyhydroxy compounds, like sucrose, which do not crystallize at all from a freezing solution in real time [11]. The fact that sucrose does not crystallize during freeze-concentration is an indication of its extremely complex crystal structure [11]. The interactions between sugar –OH groups and those between sugar –OH groups and water molecules are closely similar in energy and configuration, resulting in very low nucleation probabilities [11]. In this case, water continues to freeze beyond the eutectic melting temperature, and the solution becomes increasingly supersaturated and viscous [11]. The increasing viscosity slows down ice crystallization until at some characteristic temperature no further freezing occurs [11]. This is called glassification or vitrification [18]. The temperature at which the maximal freeze-concentration (Cg′) occurs is referred to as the glass transition temperature (Tg′) [11,29]. This is the intersection of the freezing point depression curve and the glass transition or isoviscosity curve, shown in the "supplemented phase diagram" [30] or "state diagram" (Fig. 2) [11]. Tg′ is the point on the glass transition curve, representing a reversible change between a viscous, rubber-like liquid and a rigid glass system [19]. In the region of the glass transition, the viscosity of the freeze-concentrate changes about four orders of magnitude over a temperature range of a few degrees [19]. Tg′ depends on the composition of the solution, but is independent of the initial concentration [4,11,27]. For example, the maximal freeze-concentration of sucrose is a concentration of 72–73% [31]. In addition to Tg′, the collapse temperature (Tc) of a product is used to define more precisely the temperature at which a structural loss of the product will occur. In general, Tc is several degrees higher than Tg′ as the high viscosity of the sample close to Tg′ will prevent viscous flow [10]. The glassy state is a solid solution of concentrated solutes and unfrozen, amorphous water. It is thermodynamically unstable with respect to the crystal form, but the viscosity is high enough, in the order of 10^{14} Pa s, so that any motion is in the order of mm/year [4,11,29].

The important difference between eutectic crystallization and vitrification is that the interstitial between the ice crystal matrices in crystalline material consists of an intimate mixture of small crystals of ice and solute, whereas the interstitial region in amorphous solutes consists of solid solution and unfrozen, amorphous water [19,23]. Therefore, in crystalline material, nearly all water is frozen and can easily be removed during primary drying without requiring secondary drying [19]. However, in amorphous solutes, about 20% of unfrozen water is associated with the solid solution, which must be removed by a diffusion process during secondary drying [19]. Moreover, the Teu for crystalline material and the Tg′, respectively Tc, for amorphous material define the maximum allowable product temperature during primary drying [19]. Eutectic melting temperatures are relatively high compared to glass transition temperatures, allowing a higher product temperature during primary drying and resulting in more efficient drying processes [19]. If the product temperature exceeds this critical temperature, crystalline melting or amorphous collapse will occur. This loss of structure in the freeze-dried products termed "cake collapse" [11,19].

2.3. Phase separation and other types of freezing behavior

A property characteristic of multicomponent aqueous solutions (particularly those containing at least one polymer component) is the liquid–liquid phase separation during freezing into two liquid equilibrium phases, which are enriched in one component [11,19]. This phase separation behavior has been reported for aqueous solutions of polymers such as PEG/dextran or PVP/dextran but is also reported for proteins and excipients [32,33]. When a critical concentration of the solutes is reached, the enthalpically unfavorable interactions between the solutes exceed the favorable entropy of a solution with complete miscibility [34]. Another proposed explanation is that solutes have different effects on the structure of water, leading to phase separation [35].

Besides the separation into two amorphous phases, two other types of phase separation are stated in literature; crystallization of amorphous solids and amorphization from crystalline solids [18]. Crystallization of amorphous solids often occurs when meta-stable glasses are formed during freezing. Upon extremely fast cooling, a compound that normally would crystallize during slower freezing is entrapped as an amorphous, meta-stable glass in the freeze-concentrate [12,23]. However, with subsequent heating above Tg′, it will undergo crystallization, which is the basis for annealing during freeze-drying (see Section 3.3) [19]. Without annealing, the meta-stable glass can crystallize spontaneously out of the amorphous phase during drying or storage [18]. Amorphization from crystalline solids, that can be buffer components or stabilizers, predominantly occurs during the drying step and not during the freezing step [18,36].

Additionally, lyotropic liquid crystals, which have the degree of order between amorphous and crystalline, are reported to form as a result of freeze-concentration. However, their influence on critical quality attributes of the lyophilized product is not clarified [19]. Clathrates, also termed gas hydrates, are also known to form, especially in the presence of non-aqueous co-solvents, when the solute alters the structure of the water [23].

3. Modifications of the freezing step

As aforementioned, the ice nucleation temperature defines the size, number, and morphology of the ice crystals formed during freezing. Therefore, the statistical nature of ice nucleation poses a major challenge for process control during lyophilization. This highlights the importance of a controlled, reproducible and homogeneous freezing process. Several methods have already been developed in order to control and optimize the freezing step. Some only tend to influence ice nucleation by modifying the cooling rate, and some just statistically increase the mean nucleation temperature. Few others allow a true control of the nucleation at the desired nucleation temperature.

3.1. Shelf-ramped freezing

Shelf-ramped freezing is a conventional freezing condition that is most often employed in lyophilization [37]. Here, the filled vials are placed on the shelves of the lyophilizer, and the shelf temperature is then decreased linearly (0.1 °C/min up to 5 °C/min, depending on the capacity of the lyophilizer) with time [37,38]. Both water and ice have low thermal conductivities and large heat capacities. The thermal conductivity between vials and shelf is limited; thus, the shelf-ramped cooling rate is inherently slow [11]. In order to ensure the complete solidification of the samples, they must be cooled below Tg′ for amorphous material and, respectively, below Teu for crystalline material. Traditionally, many lyophilization cycles use a final shelf temperature of −50 °C or lower as this was the maximal cooling temperature of the freeze-drier [7]. It is now suggested to use a final shelf temperature of −40 °C if the Tg′ or Teu is higher than −38 °C, or to use a temperature 2 °C less than the Tg′ and Teu [1]. Complete solidification also requires a significant amount of time [11]. The time for complete solidification generally depends on the fill volume. The larger the fill volume, the more time is required for complete solidification [11]. Tang et al. [1] suggest that the final shelf temperature should be held for 1 h for samples with a fill depth of less than or equal to 1 cm or 2 h for samples with a fill depth of greater than 1 cm. Fill depths greater than 2 cm should be avoided. However, the holding

time should be increased proportionately when such high depths are required.

To obtain a more homogeneous freezing, the vials are often equilibrated for about 15–30 min at a lowered shelf temperature (5–10 °C) before the shelf temperature is linearly decreased [1]. The vials are either directly loaded on the cooled shelves or loaded at ambient temperatures, and the shelf temperature is then decreased to the hold temperature [1,5,9].

Two-step freezing is another modification of the shelf-ramped freezing process, where a "supercooling holding" is applied. Here, the shelf temperature is decreased from either room temperature or the preset lowered shelf temperature, to about −5 to −10 °C and held for 30–60 min. This leads to a more homogenous supercooling state across the total fill volume [1,5]. When the shelf temperature is then further decreased, relatively homogeneous ice formation is observed [5].

Although shelf-ramped frozen samples show a high degree of supercooling, ice crystal growth proceeds extremely fast when the nucleation temperature is reached and results in many small ice crystals [9,39]. The ice nucleation cannot be directly controlled when shelf-ramped freezing is applied and is therefore quite random [4]. It is not practical to manipulate the ice nucleation temperature as the cooling rates are limited inside the lyophilizer and the degree of supercooling might not change within such a small range [1,14]. A drawback of shelf-ramped freezing is the fact that different vials may become subject to different degrees of supercooling, typically about ±3 °C from the mean [4]. This results in a great variability in product quality and process performance [4].

3.2. Pre-cooled shelf method

When applying the pre-cooled shelf method, the vials are placed on the lyophilizer shelf that has already cooled to the desired final shelf temperature, e.g. −40 °C or −45 °C [1,13,14]. It is reported that the placement of samples on a pre-cooled shelf results in higher nucleation temperatures (−9.5 °C) compared to the conventional shelf-ramped freezing (−13.4 °C) [14]. Moreover, the freezing rate after ice nucleation is actually slower compared to shelf-ramped freezing due to the lowered degree of supercooling and more limited time for thermal equilibration throughout the fill volume [40]. A large heterogeneity in supercooling between vials is also observed with this method [14]. A distinct influence of the loading shelf temperature on the nucleation temperature is described in literature [13,14]. Searles et al. [14] found that the nucleation temperatures for samples placed on a shelf at −44 °C were several degrees higher than for samples placed on a −40 °C shelf. Thus, the shelf temperature should be chosen with care when using this method.

3.3. Annealing

Annealing is a hold step at a temperature above the glass transition temperature [12]. Annealing is generally performed to allow for complete crystallization of crystalline compounds and to improve inter-vial heterogeneity and drying rates [1,19]. Tang et al. [1] proposed the following annealing protocol: When the final shelf temperature is reached after the freezing step, the product temperature is increased to 10–20 °C above Tg′ but well below Teu and held for several hours. The shelf temperature is then decreased and held at the final shelf temperature. Annealing has a rigorous effect on the ice crystal size distribution [17,41] and can eliminate the interdependence between the ice nucleation temperature and ice crystal size and morphology. If the sample temperature exceeds Tg′, the system follows the equilibrium freezing curve and some of the ice melts [12,41]. The raised water content and the increased temperature enhance the mobility of the amorphous phase and all species in that phase [12]. Increased mobility of the amorphous phase enables the relaxation into physical states of lower free energy [12]. According to the Kelvin equation, ice crystals with smaller radii of curvature will melt preferentially due to their higher free energy compared to larger ice crystals [12,37,41]. Ostwald ripening (recrystallization) results in the growth of dispersed crystals larger than a critical size at the expense of smaller ones and is a consequence of these chemical potential driving forces [12,41]. Small ice crystals do not reform upon refreezing of the annealed samples, as the present large ice crystals serve as nucleation sites for addition crystallization [41]. The mean ice crystal radius rises with time$^{1/3}$ during annealing [37,41]. As a consequence of that time dependency, the inter-vial heterogeneity in ice crystal size distribution is reduced with increasing annealing time. Meaning, vials comprised of smaller ice crystals "catch up" with the vials that contained larger ice crystals at the start of annealing [12,17,37,41]. Searles et al. [41] found that when annealing multiple sheets of lamellar ice crystals with a high surface area merged to form pseudo-cylindrical shapes with a lower interfacial area. In addition to the increased ice crystal size, it was also observed that annealing opened up holes on the surface of the lyophilized cake. The hole formation is explained by the diffusion of water from melted ice crystals through the frozen matrix at the increased annealing temperature. And in the case of meta-stable glass formation of crystalline compounds, annealing facilitates complete crystallization [42]. Above Tg′, the meta-stable glass is re-liquefied and crystallization occurs when enough time is provided. Furthermore, annealing can also promote the completion of freeze-concentration (devitrification), as it allows amorphous water to crystallize [41]. This is of importance when samples are frozen too fast, and water capable of crystallization was entrapped as amorphous water in the glassy matrix. The phenomenon of annealing also becomes relevant when samples are optimally frozen, but then kept at suboptimal conditions in the lyophilizer or in a freezer before lyophilization is performed [11].

3.4. Quench freezing

Quench freezing, also referred to as vial immersion, is the process of immersing vials into either liquid nitrogen or liquid propane (ca. −200 °C) or a dry ice/acetone or dry ice/ethanol bath (ca. −80 °C), long enough for complete solidification. The vials are then placed on a pre-cooled shelf [9,16]. In this case, ice crystal formation begins on both the vial wall and bottom, where the heat-transfer media has contact [10]. This freezing method results in a lowered degree of supercooling, as well as a high freezing rate. Because the sample temperature is decreased quickly, small ice crystals are formed. Liquid nitrogen immersion has been said to induce less supercooling than slower methods [9,37,39]. However, this faster cooling method actually induces supercooling only in a small sample volume before nucleation starts and freezes by directional solidification [12,14]. While it is reported that external quench freezing might be advantageous for some applications [39], this uncontrolled freezing method promotes heterogeneous ice crystal formation and is not applicable in large-scale manufacturing [7].

3.5. Directional freezing

Directional, respectively vertical, freezing can be performed in order to generate straight, vertical ice crystallization. Ice nucleation is induced at the bottom of the vial by contact with dry ice and followed by slow freezing on a pre-cooled shelf [9]. In this case, the ice propagation is vertical, and lamellar ice crystals are formed [9].

Unidirectional solidification is a similar approach described by Schoof et al. [43] as the solidification through a gradient freezing

stage which is based on the Power-Down principle. Homogenous ice crystal morphology here occurs with a temperature gradient between the upper and the lower cooling stage of 50 K/cm.

3.6. Ice fog technique

In 1990, Rowe [44] described an ice fog technique for the controlled ice nucleation during freezing. After the vials are cooled to the desired nucleation temperature, on the lyophilizer shelf, a flow of cold nitrogen is released into the chamber. The high humidity of the chamber generates an ice fog, a vapor suspension of small ice particles. The ice fog penetrates into the vials, where it initiates ice nucleation at the solution surface [45]. Rambhatla et al. [20] successfully implemented this technique for temperature-controlled nucleation, in the range of −1 °C to −11 °C, in laboratory scale lyophilizers. The challenge in this study was the induction of ice nucleation in all vials at the same time. Because the small particle of the ice fog does not reach all the vials simultaneously, inter-vial heterogeneity occurs [20]. Therefore, Patel et al. introduced a variation in the ice fog method, in which a reduced pressure in the chamber was applied to enable a faster and more uniform freezing [45]. With this modification, a rapid ice nucleation within one minute and a uniform ice crystal structure in all vials were observed. Although it is a promising technique to control ice nucleation inside the lyophilizers, it is not yet implemented in large-scale, commercial lyophilization [20].

3.7. Electrofreezing

Electrofreezing (EF) is another method to control ice nucleation. In this method, a high voltage pulse is applied to generate an ice nucleus on a platinum electrode, which initiates ice crystallization [46,47]. The capability of high voltage to induce ice nucleation in supercooled water was first reported by Rau [48] in 1951. There are still, however, discussions about the basic mechanisms of EF, including the influence on molecular dynamics [46], bubble formation and breakdown [48], and electrolytic formation of hydrated metal-ion complexes [49]. For this external freezing method, samples are first cooled to the desired temperature. Ice nucleation is then induced by EF, and samples are further cooled. For direct EF, a simple and disposable electrode setup composed of a gold wire can be used to allow the ice nucleation of many samples at the same time (Fig. 3a) [47]. However, the presence of high amounts of excipients, especially salts, inhibits ice nucleation [46,47]. Special electrode caps (Fig. 3b), called indirect EF, were developed as a result, to achieve ice nuclei generation independent form the sample composition [47]. In these caps, the ice nucleus is formed on the platinum electrode in a separate small volume of water. It then grows through a narrow cannula and PTFE-tube into the sample [47].

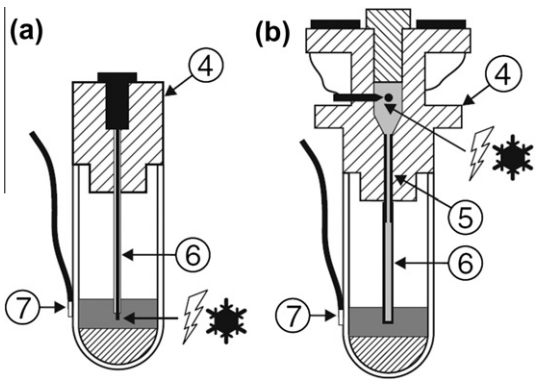

Fig. 3. Schematic drawing of the electrodes used for (a) direct and (b) indirect electrofreezing, adapted from Ref. [47].

This method only allows the parallel freezing of eight samples under identical conditions [46]. The ice nucleation temperature can be induced at the desired temperature by electrofreezing (−1.5 to −8.5 °C for indirect EF and −4.5 to −8.5 °C for direct EF) [47]. Small spherical ice crystals grow when the ice nucleation temperature is low and large plate-like ice crystals form at higher ice nucleation temperatures [47]. However, this freezing method has only been applied in modified cryotubes, and the need for individual electrodes for each sample diminishes the applicability in manufacturing.

3.8. Ultrasound-controlled ice nucleation

Nucleation can be induced by ultrasonic vibration. This was first applied in the food science field, e.g. for manufacturing of ice cream [15]. Inada et al. [50] reported that the phase change from supercooled water to ice, by ultrasonic vibration, can be actively controlled at the desired freezing temperature. The mechanisms of sono-nucleation are still being discussed [51–53]. Acoustic cavitation, which results in the formation of air bubbles in the liquid, is a key factor. In addition, during the final stage of collapse of a cavitating bubble, the equilibrium freezing temperature of water increases due to very high pressures. This results in an increased supercooling level, which is the driving force for ice nucleation.

Nakagawa et al. [54] introduced ultrasound-controlled nucleation for lyophilization of pharmaceutical proteins. An ultrasound transducer, which is connected to an ultrasound generator, is attached to an aluminum plate, which is combined with a cooling stage to cool the vials (Fig. 4). Ice nucleation is triggered with an ultrasound wave once the vials have reached a desired temperature. Samples are then continually cooled down to the final temperature to allow for complete solidification. Larger and directional ice crystals of the dendrite type were found when the sample was nucleated at higher temperatures, while smaller and heterogeneous ice crystals were formed at lower nucleation temperatures. It was also observed that ice crystal initiated by ultrasound started at the bottom of the vial and progressed to the top, resulting in the possible formation of a cryoconcentrated solution layer at the top of the sample. In comparison with the samples nucleated at the same nucleation temperature without ultrasound, no significant differences in ice morphology were observed. This indicates that the ice morphology depends only on the nucleation temperature, and not on the mode of nucleation. In a follow-up study, Hottot et al. [55] investigated the effect of ultrasound-controlled nucleation on structural and morphological properties of freeze-dried mannitol solutions. They found that a compromise between nucleation temperature level and ultrasound pulse power is necessary to get the most stable mannitol polymorph with a highly permeable cake structure [55]. Saclier et al. [53,56] found in a theoretical model and also experimentally that the size and circularity of the ice crystals depends on both supercooling and the acoustic power used. In the aforementioned studies, the controlled ice nucleation during freezing was always performed externally. Passot et al. [57] used a prototype freeze-dryer, in which one of the shelves is equipped with the ultrasound technology. In accordance, they found that the controlled nucleation by ultrasound was possible at a nucleation temperature close to the equilibrium freezing point and that the homogeneity of the whole batch (100 vials) could be improved.

A significant intra-vial heterogeneity of ice crystal distribution with smaller ice crystals at the vial bottom compared to larger ice crystals at the top was observed by applying ultrasound-induced ice nucleation [54]. Thus, Nakagawa et al. [54] applied an additional annealing step to reduce the intra-vial heterogeneity. A good mechanical and thermal contact between the more or less curved

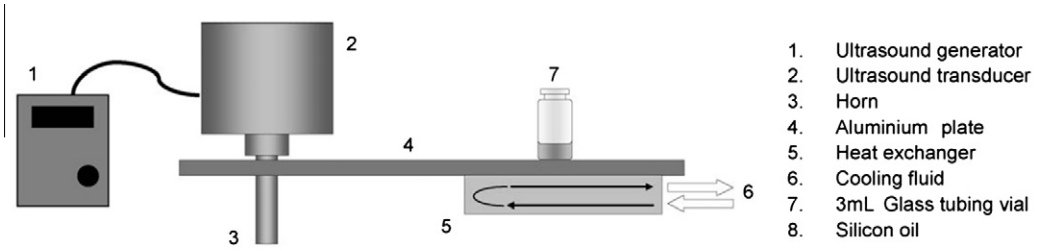

Fig. 4. Cooling stage with ultrasound system proposed by Nakagawa et al. [54].

vial bottoms and the plate surface is another challenge for successful implementation [54]. However, an advantage of ultrasound-controlled nucleation is the ability to be applied without the need for direct contact with the product and is thus chemically non-invasive [15].

3.9. Vacuum-induced surface freezing

At low pressure, evaporation of water is favored. Known as self-cooling, the associated enthalpy of evaporation reduces the local temperature in the water surface, such that the water surface freezes, and a thin film of ice is formed [58,59]. Based on this concept, Kramer et al. [59] introduced a "vacuum-induced surface freezing" technique. The vials were placed onto the pre-cooled shelves (+10 °C) of the freeze-drier, and the pressure was reduced to 1 mbar (equal to 750 mTorr). After 5 min under these conditions, a 1–3-mm-thick layer of ice was formed on the surface of the sample. In order to prevent further water loss by boiling and inhibit melting of the ice film on the surface, the chamber pressure was released to atmospheric pressure as fast as possible, and the shelf temperature was simultaneously decreased to 3–4 °C below the eutectic melting temperature of the formulation. This temperature was held for 1 h, and the shelf temperature was subsequently decreased to −40 °C. The release of the vacuum and reduction in the shelf temperature lead to the growth of dendritic ice crystals, resulting in the formation of long, chimney-like, extremely large ice crystals.

It is still unclear whether this technique can be scaled up for commercial applications. Moreover, this method has high risks of uncontrolled boiling, which can result in a "puff off" [5] when the unfrozen portion in the vial boils and blows up the frozen surface. This can influence the concentration of the sample [10] and could also influence the API. Therefore, Liu et al. [5] modified this method as follows: The temperature was held at −10 °C for equilibration before pulling a vacuum to 600 mTorr to induce freezing. In parallel, the shelf temperature was rapidly (>1 °C/min) decreased to −45 °C. They showed that the lowered initial shelf temperature is necessary because when performed at higher temperatures ice is only formed at the top of the vial. The heat uptake by evaporation is not enough to lower the temperature of the whole fill volume and therefore results in a two-layer solidification (the first from vacuum-induced freezing, the second from shelf cooling).

3.10. High-pressure shift freezing or depressurization technique

"High-pressure assisted freezing" was the first freezing method in which increased pressures were applied to promote freezing [15,60]. Under high pressure, the freezing point of water is lowered and a large number of small ice crystals are formed [61]. This method also generates smaller ice crystals compared to other conventional rapid freezing methods, such as liquid nitrogen immersion [62]. So far, this method was only applied in food science.

Later, researchers from the same group investigated a "high-pressure shift freezing" method [63,64] where the pressure is released slowly or quickly. The phase transition from liquid to solid occurs as a result of the pressure change, and instantaneous ice formation is promoted. In 2007, Gasteyer et al. [65] introduced an analogous method, referred to as "depressurization method", for the controlled ice nucleation in samples intended for lyophilization. The samples are initially cooled to the equilibrium freezing temperature in a pressurized gas atmosphere, which is subsequently de-pressurized to induce ice nucleation.

This freezing method involves several steps. The freeze-dryer is loaded, and air within the chamber is purged with a pressurization gas, e.g. argon or nitrogen. The chamber is pressurized up to less than 50 psig (approximately 2600 Torr or 3.5 bar), and the samples are cooled to and equilibrated at the desired nucleation temperature. The samples are then nucleated by depressurizing the freeze-drying chamber. After the nucleation, the samples are further cooled to the final shelf temperature. The detailed construction of a freeze-dryer, that is applicable for that purpose, is described in more detail in a follow-up patent by Rampersad et al. [66]. The system consists of a freeze-drying chamber, a gas circuit to pressurize the freeze-drying chamber, and a separate circuit for depressurization.

Bursac et al. [67] demonstrated that this freezing method could be applied in both laboratory and small commercial-scale freeze-dryers for a wide range of formulations and containers. They showed that the nucleation of aqueous samples could be well controlled within 1 °C of the equilibrium freezing point. One main advantage of this technique is that the samples are only contacted by inert gas, which is removed from the vials during lyophilization [67]. The technique can be implemented with minor additions to equipment on freeze-dryers that are designed to withstand pressures, i.e. during steam sterilization [66,67]. However, in the presence of a capacitance manometer, or if the product chamber is not intended and approved for such high pressures, the adaptation of this technique will be very cost-intensive. This required equipment has now been integrated in commercially available freeze-dryers with the "ControLyo™ nucleation on demand technology" [68].

3.11. Addition of ice nucleating agents

In general, all insoluble impurities have the potential to serve as ice nucleating agent (INA) [15]. INAs promote a heterogeneous ice nucleation process that occurs at higher temperatures compared to samples that do not contain INAs [15]. The most studied non-biogenic INA is silver iodine (AgI), which is also used for cloud-seeding and snow-making [15,69]. AgI enhances ice nucleation not only because of crystal structure's similarity to ice [70] but also because of an electric field mechanism [71]. Among the biogenic INAs, six different species of ice nucleation bacteria have been

studied in food science, of which *Pseudomonas syringae* is most widely used [15,69]. These biogenic INAs favor ice nucleation, because their structure is similar to that of the ice crystal lattice, lacks surface charge and is of high hydrophobicity [15,72,73].

Searles et al. [14] used *P. syringae* (0.001% w/v) and AgI (1 mg/ml) to alter the ice nucleation temperature during freezing of a 10% HES solution. The addition of *P. syringae* reduced supercooling (nucleation at −1.8 °C compared to −13.4 °C for control samples) and directional solidification with complete lamellar ice crystal structure [14]. Samples seeded with AgI nucleated in a temperature range between −5 °C and −7.5 °C in the center of the meniscus, where most of the AgI was concentrated and a mixture between spheroidal and lamellar ice crystal structures was formed [14]. Liu et al. [5] found in the case of high fill depth, AgI (0.1 mg per vial) limited supercooling (about −2 °C). However, this study also showed that ice grew from the bottom to the top of the vial and dendritic ice crystals were observed. Nucleation efficiency for *P. syringae* depends on the INA concentration [69]. This also seems to be the case for AgI. Searles et al. [14] also showed that vials with high particulate contamination from drying the open vials after washing in an uncontrolled laboratory environment also slightly decreased the degree of supercooling to −11.4 °C compared to −13.4 °C in conventional shelf-ramped freezing. Overall, the presence of INAs increases the average nucleation temperature but does not allow controlled nucleation, and individual vials may show a great heterogeneity. Moreover, the addition of ice nucleating agents is not of practical use for FDA-regulated and approved pharmaceutical products.

3.12. Non-aqueous co-solvents

Teagarden and Baker [74] published a comprehensive review on the potential of non-aqueous co-solvents in lyophilization. The use of non-aqueous co-solvents has both advantages and disadvantages [74]. The advantages include increased sublimation rate leading to a decreased drying time, potentially increased wettability, improved reconstitution characteristics, increased solubility and stability of some drugs in solution, and enhanced sterility assurance [74]. Operator safety concerns due to high degree of flammability or explosion potential is one disadvantage. Toxicity and regulatory issues because of residual solvent levels can also arise [74]. The most extensively used co-solvent in lyophilization is tertiary butyl alcohol, *tert*-butanol (TBA). This is due to the fact that it is 100% miscible in water shows a high freezing point (24 °C) and a high vapor pressure (26.8 mm Hg at 20 °C) [74]. Other co-solvents that do not freeze completely in commercial freeze-driers are very difficult to use and often result in unacceptable freeze-dried cakes [74]. In general, the specific effect of TBA is related to the modification of the ice crystal habit which leads to the growth of needle-shaped crystals [11]. However, the water–TBA mixture shows an extremely complex series of eutectic, peritectic, and hydration phenomena [11]. Kasraian et al. [75] suggested a phase diagram for TBA–water systems, which can be described as side-by-side placement of two simple eutectic phase diagrams: one for water–TBA hydrate with Teu at 20% TBA and one for TBA hydrate–TBA with Teu at 90% TBA. Depending on the concentration of TBA used, ice, solid TBA hydrate, or solid TBA will separate upon cooling. At concentrations lower than 20%, pure ice will form and leads to increasingly concentrated TBA solutions. TBA crystallizes as TBA hydrate when the concentration is increased to 20% TBA.

vThe size and morphology of the ice crystals depends on the amount of TBA present. In the presence of 1% TBA, the ice crystal morphology does not differ from pure water [75]. At a concentration of 3% larger, dendritic ice crystals form. Above this concentration, but still below the eutectic concentration of 20%, needle-shaped ice crystals form [75]. In accordance, Liu et al. [5] found that with the presence of 5% TBA, large needle-like ice crystals were formed and grew faster than the ice crystals in the control. It is also reported that the freezing rate also influences the size of the ice crystals with the presence of TBA. Smaller ice crystals for fast freezing and bigger ice crystals for slower freezing [76]. Moreover, the collapse temperature of e.g. sucrose, is not influenced by TBA addition (3–10% w/v) [77].

The level of residual solvent in the final product can be critical when TBA is added to the samples. It is influenced by the initial TBA concentration, the freezing rate, and the physical state of the solutes [76]. In general, crystalline samples contain very low levels of residual TBA. With the presence of an amorphous solid, the removal of TBA is hindered when used at low initial concentrations (<2%), as TBA does not crystallize but is dispersed in the amorphous phase. Complete crystallization is inhibited during fast freezing, resulting in high residual TBA levels. Annealing, which promotes TBA crystallization, lowers residual TBA levels [78]. An additional critical point is the time between filling the highly volatile solvent and freezing of the solution. During this time, some dissolved substances can be carried along the evaporating stream and recondense near the top of the vial. This time span should kept as short as possible in order to avoid dry powder spots near the neck of the vial after drying. This will also avoid reduced cake heights [74].

3.13. Others

Vial pretreatment by scoring, scratching, or roughening can also be applied to lower the degree of supercooling during freezing. Searles et al. [14] used scored vials, scratched at the bottom interior surface with a metal scribe. The produced surface defects or scraped glass particles were supposed to catalyze ice nucleation. However, only a marginal increase in ice nucleation temperature to −13.1 °C, compared to −13.4 °C for shelf-ramped frozen samples, was observed. With regard to particle contamination, this method is undesirable for pharmaceuticals. Randolph et al. [37] suggested the incorporation of ice nucleation chemistry into the vial interior. However, no successful implementation has been demonstrated yet.

4. Consequences of the freezing step on general quality attributes of biopharmaceuticals and process performance

As aforementioned, the freezing process directly influences number, size, and shape of ice crystal formation. The ice crystal properties are set early in the freezing process by the ice nucleation temperature. The properties are also influenced by the freezing rate and the time required for complete solidification and directly impact several quality attributes of the biopharmaceutical such as morphology, product uniformity, physical state, residual moisture content, or reconstitution time and also primary and secondary drying performance, as summarized in Table 1. However, the quality attributes of the biopharmaceutical and process performances are not influenced only by the process conditions. It should also be emphasized that the quality attributes are also influenced by factors such as formulation composition, fill volume and fill depth or properties of the glass vials.

4.1. Intra-vial and inter-vial uniformity

The growth of ice crystals during freezing as well as the distribution of solutes across the vial can be heterogeneous. Both are reflected by intra-vial uniformity [5]. Intra-vial heterogeneity results in unpredictable changes in sublimation rate during drying and, in the most extreme case unacceptable quality of the

Table 1
Summary of the various freezing methods, which allow or do not allow nucleation control (NC), and their impact on ice nucleation temperature (INT), freezing rate (FR), freezing type (global supercooling versus directional solidification), ice crystal morphology (ICM), specific surface area (SSA), dry layer resistance (DLR), and drying time (DT). Percentages are referred to the values obtained for shelf-ramped freezing. All trends are estimated to best knowledge. ↑↑↑ or ↓↓↓: extremely high or low, ↑↑ or ↓↓: very high or low, ↑ or ↓: high or low.

Freezing method	Description	NC	INT	FR	FT	ICM	SSA	DLR	DT	Examples
Shelf-ramped freezing	Shelf temperature is decreased linearly with 0.1–5 °C/min	No	↓↓	↑↑	Global	Very small, spherulitic	↑↑	↑↑	↑↑	[14] 10% HES: INT −13.4 °C [57] 5% sucrose: INT −7.3 °C
Two-/three-step freezing	Like shelf-ramped freezing but with holding step at approx. −10 °C and 5 °C	No	↓	↑	Global	Small, spherulitic	↑	↑	↑	[5]: 15% SBECD: DT −3%
Pre-cooled shelf method	Vials are placed on a pre-cooled shelf at −40 to −45 °C	No	↑	↓	Global/ directional	Mixed small to medium	↓	↓	↓	[14] 10% HES: INT −9.5 °C, DT −14% [57]: 5% sucrose: INT −3.5 °C, DT −18%
Annealing	Holding step above Tg′	No	–	↓↓↓[a]	Global[a]	Very large, spherulitic	↓↓	↓↓	↓↓	[41] 10% HES: DT up to −350%
Quench freezing	Immersion of the vials in liquid nitrogen or dry ice/alcohol bath	No	↑	↑↑↑	Directional	Very small, lamellar	↑↑↑	↑↑↑	↑↑↑	[108] buffer with NaCl: SSA +65%
Vertical freezing	Nucleation at vial bottom with dry ice, then cooled by shelf-ramping	No	↑	↓↓	Directional	Large, lamellar	↓↓	↓↓	↓↓	[9] DT −28%
Ice fog technique	Ice fog generates small ice particles as nucleating agents	Yes	↑↑	↓↓	Directional	Large	↓↓	↓↓	↓↓	[20] HES 5%: INT −1.5 °C, DT −8%, SSA −11%
Electrical	High voltage generates ice nucleus at electrode	Yes	↑↑	↓↓	Directional	Large, chimney-like	↓↓	↓↓	↓↓	[47] 10% HES: INT −2 °C, DT −6%
Ultrasound	Ultrasound triggered nucleation	Yes	↑↑	↓↓	Global	Large, chimney-like	↓↓	↓↓	↓↓	[54] 10% mannitol: INT −1 °C, DT −10% [57] 5% sucrose: INT −5.5 °C, DT −11%
Vacuum-induced freezing	Self-cooling of the sample on the surface due to the enthalpy of evaporation	Yes	↑↑	↓↓	Directional	Large, chimney-like	↓↓	↓	↓	[5] 15% SBECD: −9% DT [25] 2% mannitol, DT −25%
Depressurization technique	Ice nucleation as a result of pressure change	Yes	↑↑	↓↓	Directional	Large	↓↓	↓↓	↓↓	[9] 5% sucrose: SSA −50%, DT −27%, PS + 140%, INT −3 °C
AgI	Ice nucleating agent acts as "ice nucleus"	No	↑	↓	Global/ directional	Lamellar/ spherulitic	↓	↓	↓	[5] 15% SBECD: DT −6% [14] 10% HES: INT −6.1 °C, DT −29%
P. syringae	Ice nucleating agent acts as "ice nucleus"	No	↑	↓	Global	Large, lamellar	↓	↓	↓	[14] 10% HES: INT −1.8 °C, DT −60% [57] 5% sucrose: INT −1.6 °C, DT −30%
Non-aqueous cosolvent: TBA	Ice crystal habit is altered	No	–	–	Global	Large, needle-shaped	↓↓	↓↓	↓↓	[5] 15% SBECD: DT −24% [29] 5% sucrose: DT −90%
Scored vial	Surface defects catalyze ice nucleation	No	↓	↓	Global	Small, spherulitic	↑	↑	↑	[77] 10% HES: INT −13.1 °C, DT −8%

[a] For annealing not the freezing rate but the long time provided for ice crystal growth is indicated.

biopharmaceutical [5]. The lower the temperature equilibration in the sample, the more heterogeneous ice crystals form across the vial. For example, ice crystal distribution is more homogeneous in the two-step shelf-ramped frozen samples when compared to those in shelf-ramped freezing without a holding step [5]. More time is available with a slower freezing rate for the solute to concentrate ahead of the advancing freezing front. For shelf-ramped freezing, high heterogeneity in solute distribution across the vial is reported, especially at high fill volumes [5]. Patapoff et al. [9] concluded when the sample was frozen by shelf-ramping with a first fast and later slower increase in dry layer resistance that the structure of the dried cake varied in the vertical direction. Liu et al. [5] determined the vertical heterogeneity by three-section weight analysis. In shelf-ramped freezing, a highly concentrated core was found in the middle. In this study, the best intra-vial uniformity resulted from shelf-ramped two-step freezing or by addition of TBA. If the controlling parameters are not sufficiently adjusted, vacuum-induced freezing often results in two-layer solidification: one from vacuum-induced freezing and one from shelf cooling [5]. Annealing is suggested to improve intra-vial and also inter-vial heterogeneity [20,41,54]. During annealing, larger ice crystals grow at the expense of smaller ones, leading to a large and more uniform ice crystal size that is no longer dependent on ice nucleation temperature.

Inter-vial or batch uniformity is a consequence of the stochastic phenomenon of nucleation. Thus, all the vials in a batch do not have the same nucleation temperature and will not behave equally during drying [56]. Passot et al. [57] found that the ultrasound and pre-cooled shelf method allowed a significant increase in inter-vial uniformity when compared to shelf-ramped freezing. The optimal batch uniformity was obtained by the addition of a nucleating agent. Annealing also has an inter-vial homogenization effect [41]. Webb et al. [79] demonstrated that the variations in primary drying endpoints were three to four times larger for non-annealed versus annealed samples. Bursac et al. [67] showed that the depressurization technique improved batch homogeneity with a 60% decrease in standard variations between vials. Although ice nucleating agents can also improve inter-vial uniformity, the best way to produce a homogeneous batch is to directly control the ice nucleation temperature in all vials of a batch during freezing.

4.2. Sample morphology

Sublimation of the ice crystals leaves pores in the solute matrix. The texture and porosity of the final, dried biopharmaceutical product are directly fixed by the details of ice growth in the freezing process. It is proposed that the cake texture changes from a homogeneous, sponge-like structure for samples with a high degree of supercooling to a lamellar structure with a degree of orientation for samples with a low degree of supercooling. In addition to the degree of supercooling, freezing rate and time required for

complete solidification also determine ice crystal morphology. In general, freezing methods where supercooling exceeds 5 °C freeze by global supercooling and result in a dispersed spherulitic morphology. The size of the spheroidal pores correlates directly with the degree of supercooling. Directional solidification is often observed at very high cooling rates, or when ice nucleation is induced close to the equilibrium freezing point. This directional solidification shows lamellar plate morphology, and the interface velocity is a major determinant [12,14].

High freezing rate after slow cooling via shelf-ramped freezing results in very small spherulitic pores, approximately 100 μm in diameter, and a sponge-like matrix [14]. In the two-step freezing process, the samples are first equilibrated in a super-cooled state across the whole sample volume [5]. Thus, when shelf temperature is further increased, rapid and homogeneous freezing occurs and results in small, uniform, spherulitic pores. In the pre-cooled shelf method, a lower and less consistent degree of supercooling is observed [14]. As the time for temperature equilibration is limited, an enormous temperature gradient can be observed inside the sample. This leads to smaller pores at the bottom and large pores near the top. Liquid nitrogen immersion freezes by directional solidification in combination with a high freezing rate, resulting in small lamellar-oriented pores [80]. In comparison with the pre-cooled shelf method, the temperature gradient during vertical freezing is more pronounced. This forms chimney-like large lamellar structures [9]. The addition of an annealing step promotes the formation of large spherulitic pores due to Ostwald ripening [41]. Freezing by directional solidification is assumed in all methods that allow controlled nucleation at a temperature close to the equilibrium temperature and could result in large chimney-like pores. Vacuum-induced freezing leads to long parallel chimney-like pores with a diameter of approximately 200 μm [59]. The controlled nucleation via depressurization of a 5% sucrose solution resulted in an increased pore diameter of 120 μm compared to 50 μm in shelf-ramped freezing [67]. Large chimney-like pores at the top and smaller lamellar pores at the bottom were observed in samples frozen by ultrasound-induced nucleation [54]. Large lamellar, highly oriented pores were detected when electrofreezing at high nucleation temperatures [47]. In the presence of AgI, a mixed morphology of the cake is produced. A trend of dominating lamellar structures appears at the top where ice nucleation starts, while spheroidal structures appear at the bottom [14]. Supercooling is almost eliminated with the addition of *P. syringae* and thus enables a directional solidification with total lamellar morphology [14]. TBA influences the ice crystal habit and promotes the formation of large, needle-shaped pores stretching from the top to the bottom [5].

4.3. Primary and secondary drying performance

The primary drying rate or the sublimation during primary drying can be expressed by the following equation [6]:

$$\frac{dm}{dt} = \frac{Po - Pc}{Rp + Rs}$$

The mass transfer rate for the water vapor is represented by dm/dt. Po is the equilibrium vapor pressure over ice at the product temperature, while Pc is the chamber pressure. Rp is the dry product layer resistance to vapor transfer, and Rs is the resistance of the stopper. Rp is much larger than Rs, especially for samples of high concentration and high fill depth [81]. According to this equation, the sublimation rate is directly correlated to the dry layer resistance of the product, which is determined by the freezing step related pore size of the product. The smaller the pores in the solute matrix previously occupied by ice crystals, the greater the resistance to water vapor flow from the product and the slower the sublimation rate [6].

In secondary drying, the remaining unfrozen water, which can be about 20% for amorphous samples, requires diffusion through the solid matrix and a desorption step from the surface of the matrix [6]. Therefore, the sublimation rate in secondary drying strongly correlates with thickness and surface area of the interstitial matrix [57].

Low supercooling close to the equilibrium freezing point results in the formation of large ice crystals and low surface area, and thus accelerated primary drying but slower secondary drying [57]. In contrast, a high degree of supercooling during freezing results in many small crystals and larger surface area and thus slower sublimation but faster desorption [57]. Primary drying dominates for most formulations with a low solid content [6]. Searles et al. [14] showed that a 1% increase in ice nucleation temperature resulted in a 3% increase in drying times. Freezing processes that result in pronounced supercooling, like shelf-ramped freezing or two-step freezing, require a substantially extended primary drying time compared to the other available methods [5,41].

The pre-cooled shelf method can be used to reduce the drying time in cases when freezing has to be performed on the shelf of the freeze-drier without the possibility for controlled ice nucleation. Searles et al. [14] found that this method leads to an increase in drying rate by about 14% compared to shelf-ramped freezing. Similar results were obtained by Passot et al. [57], who described an approximately 18% shorter sublimation time.

However, annealing appears to be the most effective possibility to decrease drying rates in this case. Searles et al. [41] reported that annealing for only 30 min increased the primary drying rate by a factor of 3.5 because larger ice crystals were formed due to Ostwald ripening. It was also found that the formation of large holes on the cake surface of the annealed surface could additionally have facilitated sublimation. In general, higher annealing temperatures and longer annealing times correlate with a faster drying rate [12].

All drying rates are limited to maximum values that were reached when the sublimation rate is no longer controlled by mass transfer but by energy transfer [12]. However, annealing was not always found to increase the drying rate, as the physical state of the sample can also have an impact. For example, it was observed that in systems that contain a crystalline phase, such as mannitol/trehalose/sodium chloride, primary drying time was increased due to changes in the pore structure via changes in crystallinity [42].

Meanwhile, freezing by immersion in liquid nitrogen has been shown to result in very small ice crystals and large surface area, and thus decreased primary drying rates compared to shelf-ramped freezing were observed [14]. The non-vertical ice formation obtained by ice growth starting from the wall of the vial additionally contributes to the restricted ice sublimation for samples frozen by liquid nitrogen immersion [9].

The controlled nucleation via depressurization of a 5% sucrose solution resulted in a increased pore size and led to a shortened drying time by 27% according to Bursac et al. [67]. In comparison, Kramer et al. [59] showed that the vacuum-induced surface freezing method led to the formation of large, chimney-like pores, resulting in decreased primary drying times of 25% for a 2% mannitol formulation and of 15% for a 2% sucrose formulation. Conversely, Nakagawa et al. [54] reported that the controlled ice nucleation by ultrasound close to the equilibrium freezing temperature (−2 °C) increased the primary drying rate by 60% when compared to samples that nucleated at lower temperatures (−8 °C). Passot et al. [57] observed only an 18% decrease in sublimation time for samples nucleated using ultrasound. When the biogenic ice nucleation agent *P. syringae* was added to the samples, a 30% [57] and 60% [14] increase in drying rate was experienced. In this case, the faster drying rate was not only a result of the lowered degree of supercooling but also attributed to an increase in lamellar ice crystal content [14].

The addition of TBA was also found to increase the ice sublimation rate. [82] Kasraian et al. [77] found that the addition of 5% TBA to a 5% sucrose solution resulted in an approximate 10-fold decrease in product resistance and drying time. A reason for this is that TBA has the ability to modify the crystal habit of ice so that large needle-shaped ice crystals are formed and resulted in a decreased mass transfer resistance [76]. The fact that TBA itself has a high vapor pressure also plays a role in the increase of sublimation rates [74,82,83]. Daoussi et al. [83] reported a 10–30 times higher sublimation rate of a 90% TBA sample when compared to traditional aqueous formulations.

The drying performance can be influenced by the direction of ice crystal formation and by the direct correlation between drying rate and pore size. The connection between pores and the formation of a skin on the top of the cake may also have an influence on the drying performance. For example, it was found that in vertically oriented ice obtained by vertical freezing, the sublimation rate was 40% faster and drying time was 50% shorter compared to standard-frozen solutions [9]. Moreover, ice-structures formed by top-down freezing could be an additional factor for the increased drying rates with the presence of TBA [5]. With high solid content in combination with small ice crystals, the ice crystals can be completely coated by an amorphous matrix. In this case, sublimation is extremely slowed while the water vapor has to diffuse across the amorphous layer, due to the lack of connections between pores [9,11]. Moreover, during slow freezing, the solute can concentrate ahead of the advancing freezing front and in extreme cases produce an almost impermeable glassy product skin at the top of the vial [11]. Patapoff et al. [9] reported that the dry layer resistance is high as the sublimation front moves through the skin. The resistance then increased more slowly once the sublimation front passed the skin.

To generally optimize the process time of lyophilization, the product temperature should be as high as possible, while still low enough to avoid product melting or collapse. A controlled product temperature needs to be in equilibrium with the heat-transfer rate to the product and removal of heat by sublimation, which is directly correlated to the mass transfer rate of water vapor [6]. Searles et al. [41] found that the product temperature of an annealed sample was 5 °C cooler than the corresponding non-annealed sample due to an increased heat removal by the sublimation.

The mass transfer is directly correlated to product resistance. At low product resistance, the process is no longer controlled by mass transfer. It is instead controlled by heat transfer. In this case, the product temperature moves toward a temperature at which the vapor pressure of ice equals the chamber pressure. It is then insensitive to additional energy input [12]. This allows further optimizing of the drying time by increasing the shelf temperature well above Tg′ [11]. The same effect was observed when using TBA as non-aqueous co-solvent [77]. In contrast to pure sucrose samples, sucrose samples with TBA addition showed a fast sublimation rate and did not show collapse. This can be explained by the maintenance of a low product temperature due to the rapid sublimation and by the faster decrease in water content and increase in viscosity that prevented viscous flow of the sample.

4.4. Physical state of the sample

The physical state of excipients is of significant importance for lyophilized biopharmaceuticals. It can influence reconstitution time, storage stability, and protein stability and governs the risk of vial breakage. Various studies demonstrate that the freezing rate can affect the physical state of the solutes. This was comprehensively studied for mannitol samples [84–90]. For example, after fast freezing (−20 °C/min), mannitol was found to be amorphous. This amorphous form transformed to a meta-stable crystalline sate in the frozen matrix [84]. In contrast, when the sample was cooled at −2 °C/min, mannitol was crystalline [85].

The freezing process impacts also the preferred formation of different polymorphs. Izutsu et al. [86] detected a mixture of α- and β-polymorphs after slow freezing of 10% mannitol samples, and the formation of mainly the δ-polymorph after fast freezing. Cannon and Trappler [87] observed for a 70 mM mannitol sample that shelf-ramped freezing resulted mostly in the δ-polymorph with a minor presence of the α-polymorph. Freezing on a pre-cooled shelf produced mostly the α-polymorph with a minor β-content. A hold step at −20 °C during shelf-ramped freezing led to δ-polymorph formation, and after annealing at −20 °C for 1 h only the β-polymorph was detected.

Kim et al. [88] reported that the physical state is affected by the freezing rate and the mannitol concentration. Fast freezing of a 10% mannitol sample produced the δ-polymorph, whereas fast freezing of 5% mannitol resulted primarily in the β-form. In addition, Nakagawa et al. [89] found that the freezing step influences the vertical distribution of mannitol polymorphs in the sample along the direction of heat flux during freezing. The polymorphic form was also influenced by the absence or presence of protein [90]. The formation of mannitol hemihydrate was completely inhibited with the presence of protein [90].

In order not to form a meta-stable state and not to decrease the Tg′ of the formulation, annealing is essential for the crystallization of bulking agents such as glycine or mannitol [18,19]. For example, Hawe et al. [91] found that the application of an annealing step during lyophilization could increase the mannitol crystallinity in mannitol–sucrose–NaCl formulations. However, the annealing step favored the formation of mannitol hydrate, which is known to convert into the anhydrous polymorph upon storage.

4.5. Residual moisture content

All freezing methods that increase crystal size, thus decreasing the SSA, result in a limited desorption rate during secondary drying. Consequently, there is an increased residual moisture content in the final biopharmaceutical product if secondary drying time is not adequately adjusted [5]. When compared to shelf-ramped frozen samples, Liu et al. [82] observed a slight increase in the residual moisture content for 15% sulfobutylether-7-beta-cyclodextrin (SBECD) samples after annealing, two-step freezing and vacuum-induced freezing.

A twofold increase in residual moisture content of samples frozen with the ice nucleating agent AgI is correlated with a large increase in ice crystal size. Interestingly, the addition of TBA did influence the ice crystal morphology but the residual moisture content was not changed. In contrast to the pronounced increase in residual moisture content for samples containing AgI, Passot et al. [57] did not detect an increase in residual moisture content for samples with added *P. syringae*. The application of ultrasound did, however, induce nucleation, resulting in a 50% increase in the residual moisture content of 5% sucrose samples [57]. The increase in the freezing rate during shelf-ramped freezing from 0.2 to 1.0 °C/min also slightly increased the residual moisture content, which is comparable to the moisture content after freezing on pre-cooled shelves [57,59].

In accordance with Liu et al. [5], Webb et al. [79] found a slightly increased residual moisture content in annealed versus non-annealed sucrose/HES/interferon-γ samples. Kramer et al. [59] reported that for mannitol samples the residual moisture content was higher after vacuum-induced freezing in comparison with shelf-ramped freezing. However, this was not the case for sucrose and glycine samples.

4.6. Reconstitution time

There are several factors that can influence the reconstitution properties of a lyophilized sample. These include the morphology of the cake, the surface area of the cake, the presence of cake collapse or meltback, the presence of hydrophobic coatings, the homogeneity of the dry matrix, and the formation of channels between pores and the physical solid state [74]. Therefore, it is difficult to propose a general relation between freezing process and reconstitution time.

It is proposed that the changes that allow more efficient water vapor transport during drying may also improve wettability of the porous cake [12]. However, limited literature is available with regard to the correlation between freezing step and reconstitution time.

Annealing can affect reconstitution time, but the absolute influence is controversially discussed [12]. For example, Searles et al. [41] found that annealed HES samples were completely dissolved slightly faster than unannealed samples. In contrast, Webb et al. [79] reported slower dissolution for annealed HES and sucrose/HES formulations. The shorter reconstitution time observed by Searles et al. [41] upon annealing was explained by the formation of holes in the dried layers of the cake, facilitating liquid penetration. It is generally assumed that increased pore size produced by annealing increases the thickness of the matrix layers and reduces the surface area. Therefore, this process will prolong reconstitution times [41]. Based on this observation, it can be assumed that freezing methods resulting in a low surface will slow down reconstitution. However, further studies are needed to proof this assumptions.

In addition to sample morphology, the physical state of the samples could also influence reconstitution time. The amorphous form has a higher solubility compared to the crystalline form. Different polymorphic forms also have different dissolution rates [92]. Kim et al. [88] demonstrated that slow freezing of 10% mannitol resulted in a mixture of α- and β-polymorphs with a reconstitution time of 78 s while nitrogen immersion produced the δ-polymorph with a reconstitution time of 36s. This cannot be solely attributed to different dissolution rates of mannitol polymorphs, as the SSA was additionally affected by the freezing method. Modifying the reconstitution time by altering freezing process is also of high interest with regard to the high reconstitution times observed for lyophilized high-concentration protein samples [93].

5. Consequences of the freezing step on protein stability

In addition to the consequences of the freezing step on general quality attributes of biopharmaceuticals and drying performance, the freezing step will also affect protein stability. There are several factors that contribute to the detrimental effects on proteins during freezing. Most of the effects can be directly correlated to the freezing protocol used.

5.1. General stress factors during freezing

The three main stress factors that occur during freezing that could impact protein stability are cold denaturation, ice formation, and freeze-concentration [16].

The protein's free energy of unfolding typically shows a parabolic function of temperature and becomes negative not only at high but also at low temperatures. This is referred to as cold denaturation [94–96]. Cold denaturation is related only to decreased temperatures and occurs in the absence of freezing. Therefore, it must be completely differentiated from freezing denaturation [97]. Cold denaturation is reported for a high number of proteins [16]. However, the impact of cold denaturation on protein stability in lyophilization is regarded as marginal. This is because the estimated cold denaturation temperatures are often well below lyophilization temperatures and are even further reduced in the presence of saccharides and polyols. Additionally, the rate of unfolding can be sufficiently slow on the time scale of the lyophilization process. Drying can be finished before significant unfolding occurs [1,12,16,98].

The more significant changes in protein stability occur when ice crystallizes in the solution, promoting freeze-concentration and leading to a large ice–water interface. The solutes concentrate upon freeze-concentration and buffer components can crystallize, leading to a drastic pH shift. And depending on the formulation composition, liquid–liquid phase separation can occur.

Freeze-concentration of the solutes during freezing can also raise the solute concentration to a destabilizing level [32]. The rate of bimolecular degradation reactions is increased at high concentrations. However, the decrease in temperature and the increase in viscosity exhibit a counteracting effect and limit the extent of the rise in reaction rates [16]. When electrolytes are present in the protein formulation, the increase in concentration also increases the ionic strength. This will potentially destabilize proteins [16]. Here, the thermodynamic stability of the native conformation is reduced via charge-shielding effects or preferential binding of ions to the protein surface [95]. The role of freeze-concentration on protein stability has been mostly speculated based on experience with enzymes, which might noticeably differ when stabilizers are present [16]. For instance, Bhatnagar et al. [97] found no lactate dehydrogenase (LDH) degradation in ice-free sucrose solutions, whereas significant degradation was observed in the freeze-concentrate of the same composition.

All pKa and pKb values are directly affected by temperature, depending on the buffer-type, and causes the pH to change during cooling [11]. However, the selective crystallization of buffer salts during freeze-concentration results in more drastic pH shifts during freezing and is thus more detrimental with regard to protein stability. For instance, the decrease in pH can be 3 pH units or more for sodium phosphate buffers. The basic disodium salt is less soluble and has a higher eutectic point than the monosodium salt, leading to its precipitation [16]. Gomez et al. [99] observed that the extent of salt precipitation and pH decrease during non-equilibrium freezing, observed in lyophilization, are smaller than those predicted by the equilibrium freezing behavior. At extreme pH values, increased electrostatic repulsion between equal charges in proteins will induce protein unfolding or denaturation [94]. Freezing of a LDH solution in sodium phosphate buffer resulted in a pH drop from 7.5 to 4.5 and caused protein denaturation [100]. Generally, the pH shift during freezing can be minimized by the optimal choice of buffer salts, keeping the buffer concentration at a minimum, maintaining all buffer species in the amorphous state via the addition further solutes, or by adjusting the freezing method [1,16,19].

In addition to the crystallization of buffer salts during freeze-concentration, crystallization of additional solutes such as mannitol or glycine revokes their stabilizing effect on proteins [16,101]. In general, the crystallization of the solutes strongly depends on cooling rate and annealing conditions. It also depends on the presence of other co-solutes [32]. For example, inhibition of mannitol or glycine crystallization by adding sufficient co-solutes resulted in an increased LDH stabilization [85].

Liquid–liquid phase separation of amorphous solutes can also be observed as a result of freeze-concentration. Phase separation is most common when polymers like PEG, PVP, dextran, or ficoll are used as cryoprotectants [32]. Phase separation will lead to the loss of the stabilizing effect of the excipient and thus negatively influence protein stability. Izutsu and Kojima [72] reported that freeze-concentration separated combinations of proteins

(lysozyme, ovalbumin, BSA) and non-ionic polymers (ficoll, PVP) into different amorphous phases. Heller et al. [33] showed that the effects of liquid–liquid phase separation in PEG/dextran systems can be detrimental on the stability of recombinant human hemoglobin during freezing and drying. The phase separation promoted the protein partitioning into a PEG-rich and a dextran-rich phase and that the two phases differed in their lyoprotective properties [34].

The formation of large ice–water interfaces due to ice crystallization occurs during freezing. This can promote surface-induced denaturation of surface-sensitive proteins. The mechanism of protein denaturation at the ice surface is still being discussed. One hypothesis states the generation of an interfacial electrical field, via the preferential partitioning of one ionic species into the ice lattice, influences protein stability [102]. Another hypothesis states that ice formation leads to an ordering of the water molecules in the direct vicinity of the protein. When the protein adsorbs on the ice, the ordered water is unblocked and entropy increases. This provides a thermodynamic driving force for protein unfolding [16,103].

Protein denaturation at the ice surface can be reduced or prevented by addition of nonionic surfactants, which compete with proteins for adsorption at the ice–water interface [16]. Chang et al. [104] observed a strong correlation between the tendency of a protein for freeze denaturation and its tendency for surface denaturation. As the ice crystal surface area during freezing is predetermined by the freezing protocol, the stability of a surface-sensitive protein is additionally strongly influenced by the sole freezing step.

It must be kept in mind that in addition to the freezing process, the formulation strongly affects freezing-induced protein stabilization. Cryoprotectants stabilize proteins during freezing by "preferential exclusion" [105]. These solutes tend to be excluded from the surface of the protein and therefore lead to a "preferential hydration" of the protein and increases the thermoydynamic stability of the native state [105]. Other factors can also control the stabilizing effects of cryoprotectants. These factors include the role of the stabilizer in minimizing protein adsorption on the ice surface, stress occurring late in freezing and the increased viscosity of the freeze-concentrate [12]. Stabilizers may also operate, at least in part, through their ability to prevent crystallization of buffer components, thereby reducing a potential pH shift [12]. Moreover, the vitrification hypothesis is discussed, according to which protein mobility and kinetics of unfolding are reduced in the glassy state [106].

5.2. Influence of the freezing step on protein stability

The freezing procedure in lyophilization can influence the crystallization of buffer components and other solutes, liquid–liquid phase separation and also the extent of the ice–water surface.

With respect to protein denaturation at the ice–water interface, there exists a strong correlation between freezing rate and protein stability. At high freezing rates, smaller ice crystals and larger ice–water interfaces are formed, leading to a greater extent of surface-induced denaturation of surface-sensitive proteins [94]. It is therefore recommended to use freezing conditions that reduce ice crystal interfaces in order to minimize surface-induced protein destabilization. Strambini and Gabellieri [107] showed that the increase in ice crystal surface area induced changes in protein structure and protein aggregation. There are several studies demonstrating that faster freezing resulting in larger ice–water interfaces, when compared to slow freezing, caused increased protein instabilities. Sarciaux et al. [108] found a lower level of insoluble aggregates of bovine IgG in phosphate buffer with shelf-ramped freezing in comparison with liquid nitrogen immersion after lyophilization. Eckhardt et al. [109] observed the same trend for human growth hormone during freeze-thawing. Chang et al. [104] showed that slow freezing resulted in less turbidity of various proteins upon freeze-thawing as opposed to freezing in liquid nitrogen. Jiang and Nail [40] investigated the effect of different freezing methods on catalase, β-galactosidase, and LDH activity in phosphate buffers. The highest level of protein activity was observed when pre-cooled shelf (−40 °C) freezing was applied. Intermediate recovery was obtained by shelf-ramped freezing (−0.5 °C/min) and lowest recovery by liquid nitrogen immersion. In this case, no direct correlation between cooling rate and protein stability could be proposed.

This emphasizes again the importance of distinguishing between cooling rate and actual freezing rate. Although the cooling rate is slower for shelf-ramped freezing than for to the pre-cooled shelf method, the freezing rate is higher due to the higher degree of supercooling and improved thermal equilibrium throughout the sample volume. The cooling rate and the freezing rate are fast in the case of freezing by liquid nitrogen immersion, as the freezing behavior is shifted to directional solidification. Cochran et al. [38] used various methods to influence nucleation temperature during freezing and found an inverse relationship between the extent of supercooling and recovery of LDH activity after lyophilization and reconstitution.

Annealing is directly correlated with a decrease in the specific surface area and is thus known to impact protein stability. Sarciaux et al. [108] found that annealing reduced the percentage of bovine IgG aggregates from 33% to 12%, attributing to the reduction in aggregation due to the lower surface area of the annealed samples. Ironically, during annealing, the protein has at first to endure the surface denaturation stresses during "normal" freezing. The ice surface is reduced only after annealing. It seems that the unfolding due to surface denaturation is reversible in the liquid state and is only fixed after drying. This is also consistent with the finding of Sarciaux et al. [108] that there was no damage of IgG after freeze-thawing but after lyophilization when freezing occurred rapidly. Webb et al. [68] hypothesized that aggregation of recombinant human interferon during freeze-drying and spray-freeze-drying is a result of a retained internal stress in the glass that forms on freezing and not as a result of adsorption at the ice surface. However, there are some contradictions found in literature to the aforementioned relation between freezing rate, ice surface area, and protein stability. For example, Nema and Avis [110] found that fast freezing results in a reduced loss of LDH activity compared to slow freezing. They explained their finding by the fact that fast freezing minimizes the time that a protein spends in the freeze-concentrated environment where degradation reactions can take place. Additionally, Heller et al. [39] observed that damage to hemoglobin in PEG/dextran formulations can be avoided by rapidly freezing the samples in liquid nitrogen. In this case, however, protein damage was a result of phase separation, which is more pronounced during slow freezing.

On the other hand, annealing not only decreases the ice surface area but also promotes crystallization of some solutes which can be associated with a pronounced loss in protein activity. For example, Izutsu et al. [111] showed that the activity of beta-galactosidase in the presence of mannitol or inositol decreased after annealing. The same trend was observed for LDH and L-asparaginase when mannitol crystallization was promoted due to the annealing step [86]. It was also found that inhibition of crystallization of mannitol with the presence of additional solutes can improve protein stability [85]. However, even under fairly aggressive annealing conditions the protein itself can inhibit mannitol crystallization [112].

The freezing rate will also influence the selective precipitation of buffer salts, thus influencing the extent of changes in the pH during freezing. Each buffer salt has its own critical cooling rate,

above which crystallization is inhibited and will not result in a pH shift [113]. Annealing will further accentuate pH shifts and the protein will be exposed to unfavorable pH values for a increased period of time [41].

There is only limited information available on the influence of protein stability with freezing procedures other than shelf-ramped freezing and liquid nitrogen immersion. Passot et al. [57] found that the freezing method only influenced the recovery of catalase when the poor stabilizer maltodextrin was used. Under these conditions, a higher activity was observed after lyophilization and storage when the ultrasound technology or the pre-cooled shelf method was used. Bursac et al. [67] found for samples of 1 mg/ml LDH in 5% mannitol, a 22% decrease in protein activity frozen with the depressurization technique. Whereas stochastic shelf-ramped freeze-thawing results in a 39% loss of activity.

Even the storage stability of a protein can be impacted by the freezing procedure. Hsu et al. [114] observed that fast freezing during lyophilization of a recombinant tissue plasminogen activator resulted in weaker storage stability of the protein. The authors proposed that the protein diffuses during freezing into the large ice–water interface and is then less protected by the excipient during storage. This is in accordance with the finding of Patapoff et al. [9] that the aggregation rate of a therapeutic protein is decreased upon storage when an annealing step was performed during lyophilization. The pH drop during freezing can also potentially affect the storage stability of lyophilized proteins as lyophilized proteins exhibit a "pH memory" [115–117]. For example, lyophilized interleukin aggregated more rapidly when formulated in a phosphate buffer at pH 6.5 in comparison with a citrate buffer at the same pH during storage [115]. Furthermore, the crystallization of meta-stable amorphous excipients or conversion hydrates to the anhydrous polymorphs can affect storage stability of proteins. For example, mannitol crystallization might be inhibited during fast freezing, but *in situ* crystallization of the meta-stable mannitol could be facilitated under storage conditions. The protein-stabilizing effect will be lost as a result. In addition, meta-stable mannitol hydrate transfers with liberation of water under storage conditions that can be critical with regard to protein stability [91].

In summary, the stability of proteins during freezing is affected differently by varying freezing rates and depending on the present protein denaturation mechanism. These mechanisms include surface-induced denaturation, pH-induced denaturation, denaturation due to the crystallization of the stabilizing excipient or phase-separation-induced denaturation.

6. Conclusion and practical considerations

During freezing, the samples first experience supercooling until heterogeneous ice nucleation occurs. The ice nuclei then grow, leading to cryoconcentration of the sample. Eutectic crystallization or vitrification of the solutes is observed when a critical concentration is exceeded. In regard to global supercooling versus directional solidification, the ice crystal number and size is directly controlled by the degree of supercooling or by the freezing rate.

The random nature of ice nucleation is a big challenge for process control and results in vial-to-vial and batch-to-batch heterogeneity. Especially, the difference in the degree of supercooling when operating in a typical sterile manufacturing environment, containing much less foreign particles that can act as heterogeneous ice nucleation sites, is one of the biggest challenges in upscaling [20]. In this case, samples show a more pronounced degree of supercooling and result in smaller ice crystals and increased product layer resistance. Longer drying rates are also required, and there is an increased risk for product lost [1]. Freezing methods that directly control ice nucleation are therefore essential in order to counter this challenge.

Various freezing methods have been developed in order to manipulate the freezing behavior. However, only a few are capable to directly control ice nucleation and have the potential to be applied in manufacturing scale.

The freezing step is of significant importance during lyophilization because it is the main desiccation step. Moreover, the freezing procedure directly impacts ice crystal formation and thus product morphology. Freezing methods that result in a high degree of supercooling freeze by global supercooling and result in the formation of small, spherulitic ice crystals. The size of these crystals is directly correlated to the degree of supercooling. Procedures that induce ice nucleation at a low degree of supercooling freeze by global solidification. In this case, the interface velocity determines the size of the ice crystals.

Primary drying rates correlate with dried product resistance. Resistance is determined by pores size and the orientation of the pores. The formation of a skin at the top of the product can also determine resistance. Thus, freezing methods that result in the formation of large, vertically oriented ice crystals can drastically decrease primary drying time and show a decrease in product temperatures. This is due to the shift from mass transfer to heat transfer controlled product resistance, which results in more space for process optimization. However, reduction in primary drying time frequently involves the extension of the secondary drying step. Although primary drying times dominate, a compromise between primary and secondary drying rate must be found in order to minimize the total cycle length and reach the desired final moisture content.

The freezing step also affects the physical state of the excipients. Depending on the freezing rate, various polymorphs can be formed and the formation of meta-stable amorphous states or hydrate formation can be promoted. This can severely influence storage stability or lead to vial cracking during drying.

The freezing step also impacts the selective crystallization of excipients, especially in buffer components. This potentially leads to significant pH shifts, induces liquid–liquid phase separation and determines the extent of ice–water interfaces. Protein stability is therefore directly dependent on the freezing procedure. In the case of a surface-sensitive protein, slow freezing rates and the application of an annealing step could improve protein stability. For these slow freezing conditions, however, buffer crystallization and phase separation can be more pronounced. This can negatively impact protein stability. Fast freezing rates should be used and annealing should be avoided if a shift in pH, crystallization or phase separation of the stabilizing excipients is observed. Thus, process optimization and formulation development should be accomplished hand in hand. Freezing methods that allow controlled ice nucleation at temperatures close to the equilibrium freezing point are suggested. These methods result in the formation of large oriented ice crystal, increase the drying rates, improve batch uniformity, reduce meta-stable glass formation, facilitate process scale-up and minimize protein damage at the reduced ice–water interfaces. Annealing provides a promising alternative if no controlled ice nucleation method is available. The presence of additives like ice nucleating agents or TBA could also have a positive effect. However, ice nucleating agents have to face regulatory concerns.

The awareness of the complexity of the freezing process and its consequences on product quality and process performance is essential for successful lyophilization. The knowledge of how to control, or at least manipulate, the freezing step will help to develop more efficient lyophilization cycles and biopharmaceutical products with an improved stability.

Declaration of interest

The authors report no conflicts of interest and are solely responsible for the content and writing of the review.

Acknowledgments

Sarah Claus is kindly acknowledged for scientific input and valuable discussion during preparation of this manuscript. The authors would like to express their gratitude for financial support from the "Collagen Modification by Enzymatic Technologies" Cornet grant of the German Federation of Industrial Research Associations and the excellence cluster m4 Project T12: synthetic siRNA as new therapeutic platform for personalized medicine" of the Federal Ministry of Education and Research.

References

[1] X. Tang, M. Pikal, Design of freeze-drying processes for pharmaceuticals: practical advice, Pharm. Res. 21 (2004) 191–200.
[2] H.R. Constantino, Excipients of use in lyophilized pharmaceutical peptide, protein, and other bioproducts, in: H.R. Constantino (Ed.), Lyophilization of Biopharmaceuticals, AAPS Press, Arlington, VA, USA, 2004.
[3] J.F. Carpenter, M.J. Pikal, B.S. Chang, T.W. Randolph, Rational design of stable lyophilized protein formulations: some practical advice, Pharm. Res. 14 (1997) 969–975.
[4] F. Franks, Freeze-drying of bioproducts: putting principles into practice, Eur. J. Pharm. Biopharm. 45 (1998) 221–229.
[5] J. Liu, T. Viverette, M. Virgin, M. Anderson, P. Dalal, A study of the impact of freezing on the lyophilization of a concentrated formulation with a high fill depth, Pharm. Dev. Technol. 10 (2005) 261–272.
[6] M.J. Pikal, S. Rambhatla, R. Ramot, The impact of the freezing stage in lyophilization: effects of the ice nucleation temperature on process design and product quality, Am. Pharm. Rev. 5 (2002) 48–53.
[7] P. Cameron, Good Pharmaceutical Freeze-Drying Practice, Interpharm Press, Inc., Buffalo Grove, USA, 1997.
[8] G.-W. Oetjen, P. Haseley, Freeze-Drying, Wiley-VHC Verlag, Weinheim, 2004.
[9] T.W. Patapoff, D.E. Overcashier, The importance of freezing on lyophilization cycle development, Biopharm 15 (2002) 16–21.
[10] T. Jennings, The Freezing Process, in: Lyophilization, Introduction and Basic Principles, Interpharm Press, Englewood, USA, 1999.
[11] F. Franks, T. Auffret, Freeze-Drying of Pharmaceuticals and Biopharmaceuticals, RSC Publishing, Cambridge, UK, 2007.
[12] J.A. Searles, Freezing and annealing phenomena in lyophilization, in: L. Rey, J.C. May (Eds.), Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, Marcel Dekker, Inc., USA, New York, 2004.
[13] A. Hottot, S. Vessot, J. Andrieu, Freeze drying of pharmaceuticals in vials: influence of freezing protocol and sample configuration on ice morphology and freeze-dried cake texture, Chem. Eng. Process. 46 (2007) 666–674.
[14] J.A. Searles, J.F. Carpenter, T.W. Randolph, The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf, J. Pharm. Sci. 90 (2001) 860–871.
[15] G. Petzold, J. Aguilera, Ice morphology: fundamentals and technological applications in foods, Food Biophys. 4 (2009) 378–396.
[16] B.S. Bhatnagar, R.H. Bogner, M.J. Pikal, Protein stability during freezing: separation of stresses and mechanisms of protein stabilization, Pharm. Dev. Technol. 12 (2007) 505–523.
[17] J.J. Schwegman, F.C. John, L.N. Steven, Evidence of partial unfolding of proteins at the ice/freeze-concentrate interface by infrared microscopy, J. Pharm. Sci. 98 (2009) 3239–3246.
[18] J. Liu, Physical characterization of pharmaceutical formulations in frozen and freeze-dried solid states: techniques and applications in freeze-drying development, Pharm. Dev. Technol. 11 (2006) 3–28.
[19] S.L. Nail, S. Jiang, S. Chongprasert, S.A. Knopp, Fundamentals of freeze-drying, in: S.L. Nail, M.J. Akers (Eds.), Development and Manufacture of Protein Pharmaceuticals, Kluwer Academic/Plenum Publisher, New York, 2002.
[20] S. Rambhatla, R. Ramot, C. Bhugra, M. Pikal, Heat and mass transfer scale-up issues during freeze drying: II. Control and characterization of the degree of supercooling, AAPS PharmSciTech 5 (2004) 54–62.
[21] M. Akyurt, G. Zaki, B. Habeebullah, Freezing phenomena in ice–water systems, Energy Convers. Manage. 43 (2002) 1773–1789.
[22] M. Matsumoto, S. Saito, I. Ohmine, Molecular dynamics simulation of the ice nucleation and growth process leading to water freezing, Nature 416 (2002) 409–413.
[23] T. Jennings, The importance of process water, in: Lyophilization, Introduction and Basic Principles, Interpharm Press, Englewood, USA, 1999.
[24] P.W. Wilson, A.F. Heneghan, A.D.J. Haymet, Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation, Cryobiology 46 (2003) 88–98.
[25] G. Blond, Velocity of linear crystallization of ice in macromolecular systems, Cryobiology 25 (1988) 61–66.
[26] M.J. Pikal, S. Shah, D. Senior, J.E. Lang, Physical chemistry of freeze-drying: measurement of sublimation rates for frozen aqueous solutions by a microbalance technique, J. Pharm. Sci. 72 (1983) 635–650.
[27] R.H.M. Hatley, A. Mant, Determination of the unfrozen water content of maximally freeze-concentrated carbohydrate solutions, Int. J. Biol. Macromol. 15 (1993) 227–232.
[28] G.H.J.A. Tammanm, The States of Aggregation: The Changes in the State of Matter in their Dependence upon Pressure and Temperature, Van Nostrand Company, Inc., New York, 1925.
[29] V. Kett, D. McMahon, K. Ward, Thermoanalytical techniques for the investigation of the freeze drying process and freeze-dried products, Curr. Pharm. Biotechnol. 6 (2005) 239–250.
[30] A.P. MacKenzie, Non-equilibrium freezing behaviour of aqueous systems, Philos. Trans. Roy. Soc. London, Ser. B 278 (1977) 167–189.
[31] Y. Roos, M. Karel, Phase transitions of amorphous sucrose and frozen sucrose solutions, J. Food Sci. 56 (1991) 266–267.
[32] T.W. Randolph, Phase separation of excipients during lyophilization: effects on protein stability, J. Pharm. Sci. 86 (1997) 1198–1203.
[33] M.C. Heller, J.F. Carpenter, T.W. Randolph, Effects of phase separating systems on lyophilized hemoglobin, J. Pharm. Sci. 85 (1996) 1358–1362.
[34] M.C. Heller, J.F. Carpenter, T.W. Randolph, Manipulation of lyophilization-induced phase separation: implications for pharmaceutical proteins, Biotechnol. Prog. 13 (1997) 590–596.
[35] B.Y. Zaslavsky, T.O. Bagirov, A.A. Borovskaya, N.D. Gulaeva, L.H. Miheeva, A.U. Mahmudov, M.N. Rodnikova, Structure of water as a key factor of phase separation in aqueous mixtures of two nonionic polymers, Polymer 30 (1989) 2104–2111.
[36] A. Pyne, K. Chatterjee, R. Suryanarayanan, Crystalline to amorphous transition of disodium hydrogen phosphate during primary drying, Pharm. Res. 20 (2003) 802–803.
[37] T.W. Randolph, J.A. Searles, Freezing and annealing phenomena in lyophilization: effects upon primary drying rate, morphology, and heterogeneity, Am. Pharm. Rev. 5 (2002) 40–47.
[38] T. Cochran, S.L. Nail, Ice nucleation temperature influences recovery of activity of a model protein after freeze drying, J. Pharm. Sci. 98 (2009) 3495–3498.
[39] M.C. Heller, J.F. Carpenter, T.W. Randolph, Protein formulation and lyophilization cycle design: prevention of damage due to freeze-concentration induced phase separation, Biotechnol. Bioeng. 63 (1999) 166–174.
[40] S. Jiang, S.L. Nail, Effect of process conditions on recovery of protein activity after freezing and freeze-drying, Eur. J. Pharm. Biopharm. 45 (1998) 249–257.
[41] J.A. Searles, J.F. Carpenter, T.W. Randolph, Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg′ in pharmaceutical lyophilization, J. Pharm. Sci. 90 (2001) 872–887.
[42] X. Lu, M.J. Pikal, Freeze-drying of mannitol–trehalose–sodium chloride-based formulations: the impact of annealing on dry layer resistance to mass transfer and cake structure, Pharm. Dev. Technol. 9 (2004) 85–95.
[43] H. Schoof, J. Apel, I. Heschel, G. Rau, Control of pore structure and size in freeze-dried collagen sponges, J. Biomed. Mater. Res. 58 (2001) 352–357.
[44] T.D. Rowe, A technique for the nucleation of ice, in: International Symposium on Biological Product Freeze-Drying and Formulation, 1990.
[45] S. Patel, C. Bhugra, M. Pikal, Reduced pressure ice fog technique for controlled ice nucleation during freeze-drying, AAPS PharmSciTech 10 (2009) 1406–1411.
[46] A. Petersen, H. Schneider, G. Rau, B. Glasmacher, A new approach for freezing of aqueous solutions under active control of the nucleation temperature, Cryobiology 53 (2006) 248–257.
[47] A. Petersen, G. Rau, B. Glasmacher, Reduction of primary freeze-drying time by electric field induced ice nucleus formation, Heat Mass Transfer 42 (2006) 929–938.
[48] W. Rau, Eiskeimbildung durch Dielektrische Polarisation, Z. Naturforsch. 6 (1951) 649–657.
[49] T. Hozumi, A. Saito, S. Okawa, K. Watanabe, Effects of electrode materials on freezing of supercooled water in electric freeze control, Int. J. Refrig. 26 (2003) 537–542.
[50] T. Inada, X. Zhang, A. Yabe, Y. Kozawa, Active control of phase change from supercooled water to ice by ultrasonic vibration 1. Control of freezing temperature, Int. J. Heat Mass Transfer 44 (2001) 4523–4531.
[51] X. Zhang, T. Inada, A. Yabe, S. Lu, Y. Kozawa, Active control of phase change from supercooled water to ice by ultrasonic vibration 2. Generation of ice slurries and effect of bubble nuclei, Int. J. Heat Mass Transfer 44 (2001) 4533–4539.
[52] X. Zhang, T. Inada, A. Tezuka, Ultrasonic-induced nucleation of ice in water containing air bubbles, Ultrason. Sonochem. 10 (2003) 71–76.
[53] M. Saclier, R. Peczalski, J. Andrieu, A theoretical model for ice primary nucleation induced by acoustic cavitation, Ultrason. Sonochem. 17 (2010) 98–105.
[54] K. Nakagawa, A. Hottot, S. Vessot, J. Andrieu, Influence of controlled nucleation by ultrasounds on ice morphology of frozen formulations for pharmaceutical proteins freeze-drying, Chem. Eng. Process. 45 (2006) 783–791.
[55] A. Hottot, K. Nakagawa, J. Andrieu, Effect of ultrasound-controlled nucleation on structural and morphological properties of freeze-dried mannitol solutions, Chem. Eng. Res. Des. 86 (2008) 193–200.

[56] M. Saclier, R. Peczalski, J. Andrieu, Effect of ultrasonically induced nucleation on ice crystals' size and shape during freezing in vials, Chem. Eng. Sci. 65 (2010) 3064–3071.
[57] S. Passot, I.C. Trelea, M. Marin, M. Galan, G.J. Morris, F. Fonseca, Effect of controlled ice nucleation on primary drying stage and protein recovery in vials cooled in a modified freeze-dryer, J. Biomech. Eng. 131 (2009) 074511–074515.
[58] K. McDonald, D.-W. Sun, Vacuum cooling technology for the food processing industry: a review, J. Food Eng. 45 (2000) 55–65.
[59] M. Kramer, B. Sennhenn, G. Lee, Freeze-drying using vacuum-induced surface freezing, J. Pharm. Sci. 91 (2002) 433–443.
[60] P.D. Sanz, L. Otero, C. de Elvira, J.A. Carrasco, Freezing processes in high-pressure domains, Int. J. Refrig. 20 (1997) 301–307.
[61] B. Li, D.-W. Sun, Novel methods for rapid freezing and thawing of foods – a review, J. Food Eng. 54 (2002) 175–182.
[62] M.N. Martino, L. Otero, P.D. Sanz, N.E. Zaritzky, Size and location of ice crystals in pork frozen by high-pressure-assisted freezing as compared to classical methods, Meat Sci. 50 (1998) 303–313.
[63] L. Otero, P.D. Sanz, High-pressure shift freezing. Part 1. Amount of ice instantaneously formed in the process, Biotechnol. Prog. 16 (2000) 1030–1036.
[64] P.P. Fernández, L. Otero, B. Guignon, P.D. Sanz, High-pressure shift freezing versus high-pressure assisted freezing: effects on the microstructure of a food model, Food Hydrocolloids 20 (2006) 510–522.
[65] T.H. Gasteyer, R.R. Sever, B. Hunek, N. Grinter, M.L. Verdone, Lyophilization system and method, in: Patent Application Publication, United States, 2007.
[66] B.M. Rampersad, R.R. Sever, B. Hunek, T.H. Gasteyer, Freeze-dryer and method of controlling the same, in: Patent Application Publication, United States, 2010.
[67] R. Bursac, R. Sever, B. Hunek, A practical method for resolving the nucleation problem in lyophilization, Bioprocess Int. (2009) 66–72.
[68] <http://www.spscientific.com/ControLyoTechnology> (accessed 12.02.10).
[69] N. Cochet, P. Widehem, Ice crystallization by *Pseudomonas syringae*, Appl. Microbiol. Biotechnol. 54 (2000) 153–161.
[70] B. Vonnegut, The nucleation of ice formation by silver iodide, J. Appl. Chem. 18 (1947) 593–595.
[71] M. Gavish, J. Wang, M. Eisenstein, M. Lahav, L. Leiserowitz, The role of crystal polarity in alpha-amino acid crystals for induced nucleation of ice, Science 256 (1992) 815–818.
[72] K.-i. Izutsu, S. Kojima, Freeze-concentration separates proteins and polymer excipients into different amorphous phases, Pharm. Res. 17 (2000) 1316–1322.
[73] L.R. Maki, E.L. Galyan, M.-M. Chang-Chien, D.R. Caldwell, Ice nucleation induced by *Pseudomonas syringae*, Appl. Environ. Microbiol. 28 (1974) 456–459.
[74] D.L. Teagarden, D.S. Baker, Practical aspects of lyophilization using non-aqueous co-solvent systems, Eur. J. Pharm. Sci. 15 (2002) 115–133.
[75] K. Kasraian, P.P. DeLuca, Thermal analysis of the tertiary butyl alcohol-water system and its implications on freeze-drying, Pharm. Res. 12 (1995) 484–490.
[76] S. Wittaya-Areekul, S.L. Nail, Freeze-drying of tert-butyl alcohol/water cosolvent systems: effects of formulation and process variables on residual solvents, J. Pharm. Sci. 87 (1998) 491–495.
[77] K. Kasraian, P.P. DeLuca, The effect of tertiary butyl alcohol on the resistance of the dry product layer during primary drying, Pharm. Res. 12 (1995) 491–495.
[78] S. Wittaya-Areekul, G.F. Needham, N. Milton, M.L. Roy, S.L. Nail, Freeze-drying of tert-butanol/water cosolvent systems: a case report on formation of a friable freeze-dried powder of tobramycin sulfate, J. Pharm. Sci. 91 (2002) 1147–1155.
[79] S.D. Webb, J.L. Cleland, J.F. Carpenter, T.W. Randolph, Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-γ, J. Pharm. Sci. 92 (2003) 715–729.
[80] S.D. Webb, J.L. Cleland, J.F. Carpenter, T.W. Randolph, Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-γ, J. Pharm. Sci. 92 (2003) 715–729.
[81] M.J. Pikal, M.L. Roy, S. Shah, Mass and heat transfer in vial freeze-drying of pharmaceuticals: role of the vial, J. Pharm. Sci. 73 (1984) 1224–1237.
[82] J. Oesterle, F. Franks, T. Auffret, The influence of tertiary butyl alcohol and volatile salts on the sublimation of ice from frozen sucrose solutions: implications for freeze-drying, Pharm. Dev. Technol. 3 (1998) 175–183.
[83] R. Daoussi, S. Vessot, J. Andrieu, O. Monnier, Sublimation kinetics and sublimation end-point times during freeze-drying of pharmaceutical active principle with organic co-solvent formulations, Chem. Eng. Res. Des. 87 (2009) 899–907.
[84] R.K. Cavatur, N.M. Vemuri, A. Pyne, Z. Chrzan, D. Toledo-Velasquez, R. Suryanarayanan, Crystallization behavior of mannitol in frozen aqueous solutions, Pharm. Res. 19 (2002) 894–900.
[85] A. Pyne, K. Chatterjee, R. Suryanarayanan, Solute crystallization in mannitol–glycine systems—implications on protein stabilization in freeze-dried formulations, J. Pharm. Sci. 92 (2003) 2272–2283.
[86] K. Izutsu, S. Yoshioka, T. Terao, Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying, Chem. Pharm. Bull. 42 (1994) 5–8.
[87] A.J. Cannon, E.H. Trappler, The influence of lyophilization on the polymorphic behavior of mannitol, PDA J. Pharm. Sci. Technol. 54 (2000) 13–22.
[88] A.I. Kim, M.J. Akers, S.L. Nail, The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute, J. Pharm. Sci. 87 (1998) 931–935.
[89] K. Nakagawa, W. Murakami, J. Andrieu, S. Vessot, Freezing step controls the mannitol phase composition heterogeneity, Chem. Eng. Res. Des. 87 (2009) 1017–1027.
[90] X. Liao, R. Krishnamurthy, R. Suryanarayanan, Influence of processing conditions on the physical state of mannitol—implications in freeze-drying, Pharm. Res. 24 (2007) 370–376.
[91] A. Hawe, W. Frieß, Impact of freezing procedure and annealing on the physico-chemical properties and the formation of mannitol hydrate in mannitol-sucrose–NaCl formulations, Eur. J. Pharm. Biopharm. 64 (2006) 316–325.
[92] P. Hiwale, A. Amin, L. Kumar, A.K. Bansal, Variables affecting reconstitution time of dry powder for injection, Pharm. Technol. 2 (July) (2008).
[93] S.J. Shire, Z. Shahrokh, J. Liu, Challenges in the development of high protein concentration formulations, J. Pharm. Sci. 93 (2004) 1390–1402.
[94] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (2000) 1–60.
[95] E.Y. Chi, S. Krishnan, T.W. Randolph, J.F. Carpenter, Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation, Pharm. Res. 20 (2003) 1325–1336.
[96] K. Dill, Dominant forces in protein folding, Biochemistry 29 (1990) 7133–7155.
[97] B.S. Bhatnagar, M.J. Pikal, R.H. Bogner, Study of the individual contributions of ice formation and freeze-concentration on isothermal stability of lactate dehydrogenase during freezing, J. Pharm. Sci. 97 (2008) 798–814.
[98] X.C. Tang, M.J. Pikal, The effect of stabilizers and denaturants on the cold denaturation temperatures of proteins and implications for freeze-drying, Pharm. Res. 22 (2005) 1167–1175.
[99] G. Gomez, M.J. Pikal, N. Rodriguez-Hornedo, Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions, Pharm. Res. 18 (2001) 90–97.
[100] T.J. Anchordoquy, J.F. Carpenter, Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state, Arch. Biochem. Biophys. 332 (1996) 231–238.
[101] D. Piedmonte, C. Summers, A. McAuley, L. Karamujic, G. Ratnaswamy, Sorbitol crystallization can lead to protein aggregation in frozen protein formulations, Pharm. Res. 24 (2007) 136–146.
[102] E.J. Workman, S.E. Reynolds, Electrical phenomena occurring during the freezing of dilute aqueous solutions and their possible relationship to thunderstorm electricity, Phys. Rev. 78 (1950) 254.
[103] W. Norde, Adsorption of proteins from solution at the solid–liquid interface, Adv. Colloid Interface Sci. 25 (1986) 267–340.
[104] B.S. Chang, B.S. Kendrick, J.F. Carpenter, Surface-induced denaturation of proteins during freezing and its inhibition by surfactants, J. Pharm. Sci. 85 (1996) 1325–1330.
[105] J.F. Carpenter, T. Arakawa, J.H. Crowe, Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying, Dev. Biol. 74 (1992) 225–238 (discussion 238-229).
[106] M.J. Pikal, Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics, in: L. Rey, J.C. May (Eds.), In Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, Marcel Dekker, Inc., New York, 2004, pp. 63–107.
[107] G.B. Strambini, E. Gabellieri, Proteins in frozen solutions: evidence of ice-induced partial unfolding, Biophys. J. 70 (1996) 971–976.
[108] J.-M. Sarciaux, S. Mansour, M.J. Hageman, S.L. Nail, Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying, J. Pharm. Sci. 88 (1999) 1354–1361.
[109] B.M. Eckhardt, J.Q. Oeswein, T.A. Bewley, Effect of freezing on aggregation of human growth hormone, Pharm. Res. 8 (1991) 1360–1364.
[110] S. Nema, K.E. Avis, Freeze–thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants, J. Parenter. Sci. Technol. 47 (1993) 76–83.
[111] K. Izutsu, S. Yoshioka, T. Terao, Decreased protein-stabilizing effects of cryoprotectants due to crystallization, Pharm. Res. 10 (1993) 1232–1237.
[112] D. Dixon, S. Tchessalov, A. Barry, N. Warne, The impact of protein concentration on mannitol and sodium chloride crystallinity and polymorphism upon lyophilization, J. Pharm. Sci. 98 (2009) 3419–3429.
[113] B.S. Chang, C.S. Randall, Use of subambient thermal analysis to optimize protein lyophilization, Cryobiology 29 (1992) 632–656.
[114] C.C. Hsu, H.M. Nguyen, D.A. Yeung, D.A. Brooks, G.S. Koe, T.A. Bewley, R. Pearlman, Surface denaturation at solid–void interface—a possible pathway by which opalescent participates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures, Pharm. Res. 12 (1995) 69–77.
[115] B.S. Chang, G. Reeder, J.F. Carpenter, Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist, Pharm. Res. 13 (1996) 243–249.
[116] X.M. Lam, H.R. Costantino, D.E. Overcashier, T.H. Nguyen, C.C. Hsu, Replacing succinate with glycolate buffer improves the stability of lyophilized interferon-[gamma], Int. J. Pharm. 142 (1996) 85–95.
[117] H.R. Costantino, K. Griebenow, R. Langer, A.M. Klibanov, On the pH memory of lyophilized compounds containing protein functional groups, Biotechnol. Bioeng. 53 (1997) 345–348.