# Recent advances and persistent challenges in the design of freeze-drying process for monoclonal antibodies

Hassana HSEIN[1], Julie AUFFRAY[1,2], Thierry NOEL[2], Pierre TCHORELOFF[1]

[1] Univ. Bordeaux, CNRS, Arts et Metiers Institute of Technology, Bordeaux INP, INRAE, I2M Bordeaux, F-33400 Talence, France

2 Univ. Bordeaux, CNRS, Microbiologie Fondamentale et Pathogénicité, UMR 5234, Bordeaux, France.

**Keywords**

freeze-drying, monoclonal antibodies, optimization, process parameters, product quality attributes, stability

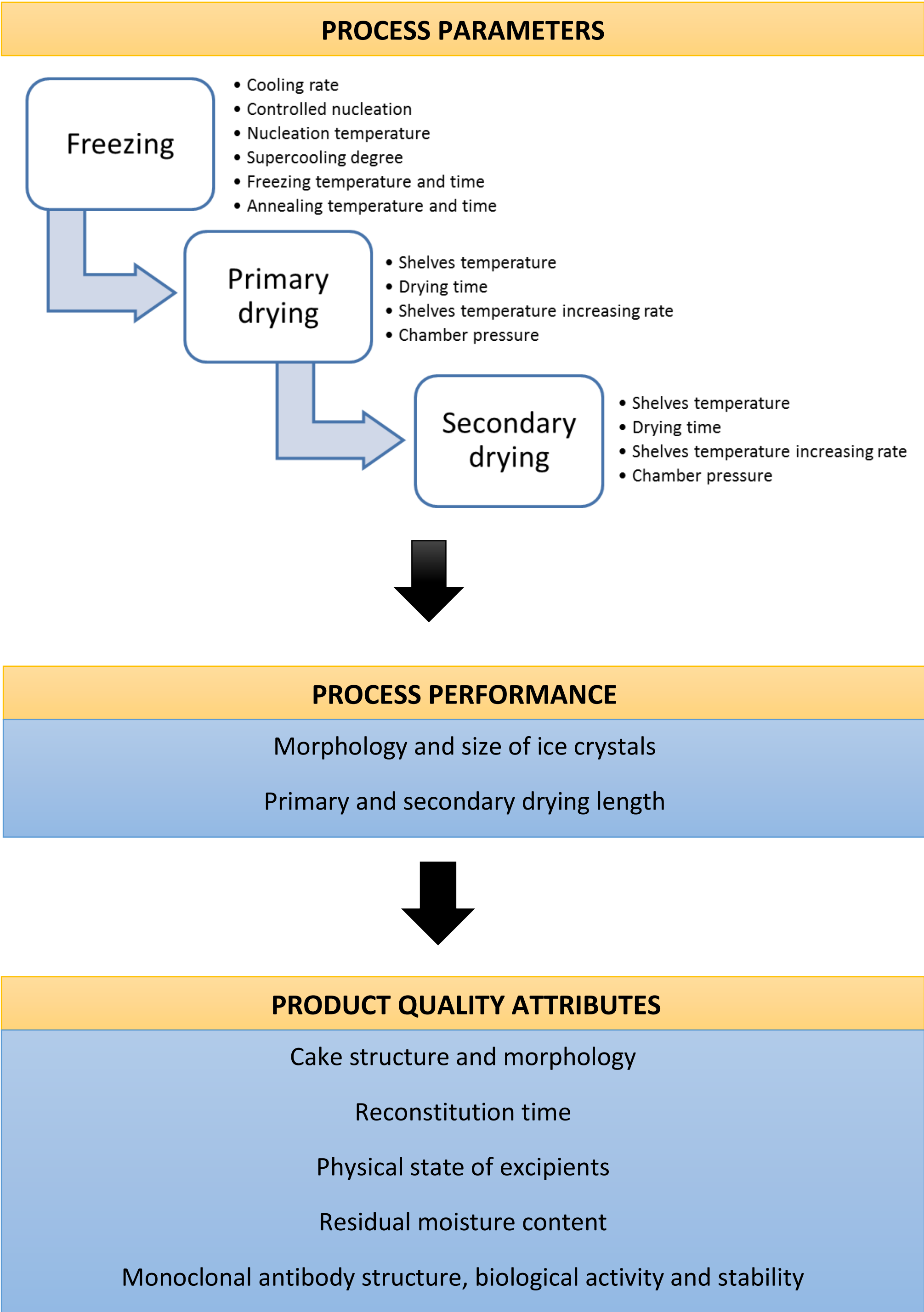
**List of abbreviations**

CIN: controlled ice nucleation
Fab: antigen binding fragment
Fc: crystallizable fragment
FD: freeze-drying
HPBCD: 2-hydropropyl-betacyclodextrine
IgG: immunoglobulin G
mAb: monoclonal antibody
mAbs: monoclonal antibodies
Pc: chamber pressure
RM: residual moisture content
SSA: specific surface area
Tc: collapse temperature
Teu: eutectic temperature
T'g: glass transition temperature of the maximally freeze-concentrated solution
Tp: product temperature
Ts: shelves temperature

## Abstract

Monoclonal antibodies constitute nowadays an important therapeutic class and the number of approved molecules for clinical uses continues to increase, achieving considerable part of the therapeutic market. Yet, the stability in solution of these biopharmaceuticals is often low. That's why freeze-drying has been and remains the method of choice to obtain monoclonal antibodies in the solid state and to improve their stability. The design of freeze-drying process and its optimization are still topical subjects of interest and the pharmaceutical industry is regularly challenged by the requirements of quality, safety and efficiency set by the regulatory authorities. These requirements imply a deep understanding of each step of the freeze-drying process, developing techniques to control the critical parameters and to monitor the quality of the intermediate and the final product. In addition to quality issues, the optimization of the freeze-drying process in order to reduce the cycle length is of great interest since freeze-drying is known to be an energy-expensive and time-consuming process. In this review, we will present the recent literature dealing with the freeze-drying of monoclonal antibodies and focus on the process parameters and strategies used to improve the stability of these molecules and to optimize the FD process.

## Graphical abstract



# 1. Introduction

Since 1975 and the discovery of the hybridoma technology for monoclonal antibody (mAb) production by Köhler and Milstein (Köhler and Milstein 1975), the development of mAbs therapies continues to revolutionize medicine in all its fields such as oncology, dermatology, hematology, neurology or immune mediated disease... The increasing number of approved monoclonal antibodies for clinical use as well as in research and development shows the importance of these therapeutic biomolecules. In fact, the Food and Drug Administration approved the 100$^{th}$ mAb in 2021 (Mullard 2021), 35 years after the first marketed Orthoclone OKT3 mAb. It has been estimated that by 2022, the mAbs market will reach 130 to 200 billion dollars (Grilo and Mantalaris 2019). Recently, the great improvements in mAbs identification, selection and production techniques allowed the fast development of new promising covid therapies such as REGEN-COV™ (casirivimab et imdevimab), which already obtained an emergency use authorization by the Food and Drug administration (Taylor et al. 2021).

The advent of mAbs in therapeutic field is essentially due to their specific recognition of biological targets correlated with a good safety profile. The safety profile has been significantly improved by decreasing the immunogenicity after administration thanks to the evolution of mAbs types from murine to chimeric, then to humanized and finally to human antibodies (Chiu et al. 2019). Target recognition and specificity are linked to the tridimensional structure and to the amino-acids sequence of these glycoproteins macromolecules, particularly to the crystallizable fragment (Fc) and the two antigen-binding fragment (Fab) of immunoglobulin G (IgG, the major isotype of antibodies) (figure 1). The Fab fragments are essentially implicated in the antigen recognition and in the therapeutic specificity through different mechanisms of action, either by their neutralizing function or by their antagonist function after binding to the epitope. The Fc fragment is implicated in activating the immune system either by interacting with Fc-receptors on (innate) immune cells or by the recognition of proteins of the complement system. Due to the fundamental link between the mAb molecular structure and the target recognition, it is crucial to conserve the tridimensional structural integrity all over the mAb life cycle from formulation, process, and storage to the administration to patient (Wang et al. 2007; Chiu et al. 2019).

Although antibodies are great potential biotherapies, their formulation and routes of administration remain a great challenge. In fact, all of the approved mAb except Ranibizumab are administered to patients via the parenteral route either by intravenous, subcutaneous or intramuscular injection. The choice of parenteral route is essentially due to the high molecular weight which limits their absorption via the epithelial cells and to the proteinaceous structure which makes them susceptible to enzymes degradation when administered by alternative routes of administration. For parenteral route, liquid formulations, are often preferred from an industrial standpoint due to the lower cost and easier production compared to other pharmaceutical forms.

However, mAbs in liquid formulations are prone to chemical and physical instabilities such as hydrolysis, oxidation, deamidation, isomerization or even aggregation (figure 1). They are also sensitive to degradation at high temperature (Le Basle et al. 2020; Butreddy et al. 2021). In order to limit mAbs instabilities in aqueous solutions, freeze-drying process has been the method of choice to obtain dried pharmaceutical form of mAbs with more than 40% of marketed monoclonal antibodies available in lyophilized form.

Freeze-drying consists in a 3-step process where the solution is first frozen (freezing), then ice crystals are removed by sublimation (primary drying) while the final step is the desiccation (secondary drying) of residual interstitial water. These freeze-dried formulations ensure long term stability and better storage conditions for the mAbs. Despite these advantages, multiple stresses may occur during freeze-drying such as crystallization, pH changes, dehydration stress, ionic strength changes, interfacial stress (ice-liquid) or ice crystal formation (figure 1). These stresses encountered by molecules during freezing and drying steps may lead to the denaturation/unfolding of proteins and can be deleterious for maintaining mAbs biological activity. For this reason, it is crucial to choose the adequate excipients during formulation and the optimal process parameters to ensure cryo and lyoprotection as well as to limit aggregation. A large panel of excipients is available for formulation optimization and their role and mechanism of protection are already well defined and fully reviewed (Bjelošević et al. 2020). We note the addition of sugars, polyols, amino acids, surfactants, buffering agents and collapse temperature modifiers as main formulation excipients. A summary of commonly used excipients in the literature and in marketed freeze-dried mAbs products is presented in table 1 with the description of their respective role and critical characteristics. Moreover, since freeze-drying is a time and energy consuming complex process, it necessitates a deep

comprehension of all parameters in order to be optimized. Defining operating parameters for freezing and sublimation steps determines process length, cake's appearance, physico-chemical and mechanical properties of freeze-dried product as well as biological activity of mAbs.

Therefore, the objective of this short review is to provide an updated state of the art of the optimization of mAbs lyophilization process. We will focus on the importance of cooling rate, nucleation and annealing in the freezing step. Afterwards, we will discuss the choice of shelves temperatures, ramp rate and chamber pressure in the sublimation step. We will finish by a short description of the process parameters used during the desiccation step.

## 2. Freezing

### 2.1. General aspects to be considered

Besides being the first step in a freeze-drying (FD) process (figure 2), freezing is also a determinant one. Freezing consists in cooling the aqueous solution distributed as individual doses in vials or as bulk placed in trays below its thermodynamic freezing point until ice nucleation occurs. Then, crystal growth will take place resulting in freeze-concentration of the solution (Bursac et al., 2009). Upon further cooling, the concentration of the solution will increase to a critical concentration, above which the concentrated solution will either undergo eutectic freezing or vitrification (Kasper and Friess 2011a; Cameron 2013). Even if it seems a simple step, freezing is decisive to successfully run a freeze-drying process because it will determine the size and morphology of ice crystals that influence the sublimation rate during primary drying and the final quality attributes of the product (cake microstructure, porosity, specific surface area…) (Searles et al. 2001a). The size and morphology of ice crystals are essentially correlated to the cooling rate and specifically to the nucleation temperature and the degree of supercooling. The degree of supercooling is the temperature difference between the thermodynamic freezing point and the actual temperature at which ice nucleation occurs. Forming small ice crystals means that water vapor will flow through narrow pores due to increased ice surface area. Thus, the product resistance to flow of vapor will increase, resulting in prolonged primary drying time (Capozzi and Pisano 2018; Pisano et al. 2019). It is estimated that for every 1°C increase in nucleation temperature, drying time decreases by 1 to 3% (Searles et al. 2001a). Taking into consideration the importance of ice

nucleation temperature and ice crystal size during the freezing step, several recent studies focused on the prediction of ice crystal size with mathematical models followed by experimental validation to simulate the cake's pore size (Pisano and Capozzi 2017; Arsiccio et al. 2017; Arsiccio et al. 2019; Harguindeguy et al. 2021; Harguindeguy et al. 2022) and to optimize the primary drying step (Arsiccio and Pisano 2018). In addition to these advanced models and subsequent construction of the QbD processes, several technologies were developed to control nucleation temperature to improve the freeze-drying process performance and its scalability resulting in a control of the final product quality attributes (Geidobler and Winter 2013; Pisano 2019).

## 2.2. Stresses during freezing step

In addition to influencing process performance, freezing is also known to impact the stability of incorporated biomolecules and protein activity (Arsiccio, Giorsello, et al. 2020; Oddone et al. 2020; Arsiccio, Matejtschuk, et al. 2020; Fang et al. 2020; Susa et al. 2021). This is true for monoclonal antibodies which are prone to stress during freezing due to phase separations and the formation of multiple interfaces between the biomolecule and ice crystals leading to physical or chemical instabilities. We can cite, for example, ionic strength change, pH shift due to salt crystallization, increase in protein-protein interactions which may lead to aggregation, mechanical stress due to ice crystal formation and to interfacial stress (ice-liquid) (Emami et al. 2018; Butreddy et al. 2021). Amongst all these effects, ice/freeze-concentrate interface is considered as the utmost origin of protein instability during freezing and different potential mechanisms of interaction between the proteins and the ice-water interface have been reported (Arsiccio, McCarty, et al. 2020; Arsiccio and Pisano 2020). It is possible to avoid degradation and to determine crystals morphology during freezing by selecting the right formulation and by a proper choice of the process parameters.

Taking into consideration all these elements, we will review which freezing strategies and process parameters have been adopted in monoclonal antibodies lyophilization studies. We can cite three main strategies consisting in choosing an appropriate cooling rate (conventional shelf-ramped freezing or fast cooling rate), controlling ice nucleation or choosing an annealing step.

### 2.3. Slow or fast cooling rate

#### 2.3.1. Main features

The common strategies to achieve desirable cooling rate are the protocol of shelf ramped freezing protocol, the placement of the samples on a precooled shelves or the immersion in liquid nitrogen.

Shelf-ramped freezing is the conventional freezing method and the most frequently used in freeze-drying (Randolph and Searles 2002). Vials are introduced on the shelves at room temperature or at 0-5°C if samples are unstable at room temperature and thenshelves temperatures are decreased linearly with time at a predefined speed (0.1 up to 5°C/min) depending on the freeze-dryer capacity (Randolph and Searles 2002; Kasper and Friess 2011a). Shelves temperature is usually decreased below T'g for amorphous formulation and below Teu for formulations containing crystalline excipients. Even though final freezing temperature of -50°C or lower are traditionally chosen, it is recommended to be 2°C lower than T'g or Teu (Kasper and Friess, 2011a). The holding time at the final freezing temperature depends on the fill depth volume in the vial and varies from 1 to 4h (Pieters et al. 2012). An initial hold step at temperatures higher than the nucleation temperature (0 up to 5°C) is frequently added to freezing protocol to ensure an homogeneous temperature within the vials. Conventional shelf-ramped freezing protocol is considered as a slow cooling rate method since the thermal conductivity between the vials and the shelves is limited. As demonstrated in many studies, a slow cooling rate results in a heterogeneous degree of supercooling leading to variability in the drying behavior during primary drying. This variability is a barrier for the development of a robust freeze-drying process. Considering process performance, a slow cooling rate during shelf-ramped freezing protocol leads to the formation of large ice crystals with smaller specific surface area (SSA) (Izutsu 2018; Pisano et al. 2019). The relationship between SSA and ice crystal size is direct for amorphous cakes but for crystalline products, the SSA is mainly dictated by the roughness of the pore walls which depends on the obtained polymorph(s) (Oddone et al. 2017). One established advantage for large ice crystals is decreasing primary drying time due to larger pores which reduces resistance to water vapor flow during sublimation on the detriment of secondary drying step which will be longer due to a slower desorption rate (Oddone et al. 2017; Arsiccio et al. 2019).

. On the contrary to conventional shelf-ramped freezing protocol, precooled shelves strategy results in higher nucleation temperatures and lower degree of supercooling compared to the

conventional shelf-ramped freezing but is not a freezing strategy of choice due to significant supercooling heterogeneity between vials (Tang and Pikal 2004; Kasper and Friess 2011a). Faster cooling rates e.g. quenching in liquid nitrogen often result in a higher degree of supercooling and a smaller ice crystal size (Kasper and Friess 2011a).

### 2.3.2. Slow or fast cooling rate in the case of mAbs formulations?

The most recurrent protocol found in the literature for mAbs FD is the conventional shelf-ramped freezing protocol with a hold step at 5°C for 30 min (Colandene et al. 2007; Park et al. 2013; Awotwe-Otoo et al. 2015; Kulkarni et al. 2020). Another frequent intermediate step during the shelf-ramped freezing is an additional hold step of 30–60 min at a shelf temperature of about -5 to -10°C or (Awotwe-Otoo et al. 2015; Kulkarni et al. 2020). The aim of this two-step shelf-ramped freezing is to achieve partial temperature equilibration in the samples. Another important point to consider during shelf-ramped freezing is the random aspect of ice nucleation. Uncontrolled ice nucleation temperature can have negative repercussions on the product quality attributes such as variability in cake appearance and reduced stability of mAbs (Awotwe-Otoo et al. 2013). Cooling rate during shelf-ramped freezing step has an impact on cake's SSA. Smaller SSA reduces the formation of ice-water interface and induces smaller ice-frozen concentrated surface area. This will decrease the protein alteration during freezing (Arsiccio and Pisano 2020; Butreddy et al. 2021) by decreasing the risk of adsorption. Since the freezing step can perturb the protein conformation and determines the texture and quality of freeze-dried products (Hottot et al. 2007), faster cooling rates by placing vials on precooled shelves or quenching in liquid nitrogen, dry ice/acetone or dry ice/ethanol bath were investigated. Arsiccio et al. (2019 and 2020) demonstrated by a model-based approach that the choice of an adequate cooling rate depends on each protein and specifically on its instability mechanisms. Proteins with high bulk stability (eg. lactate deshydrogenase) are more prone to denaturation at the ice-water interface while poorly stable proteins in solution are prone to cold denaturation and are subsequently sensitive to the duration of the freezing step. Therefore, a slow cooling rate is recommended for the first case (denaturation at the ice surface) while a high cooling rate is privileged for cold-denaturation prone proteins.

Miller et al studied the frozen state stability of mAb and found that aggregates formation correlated with low cooling rate. They demonstrated that the fast freezing of a model

monoclonal antibody in a trehalose-based formulation resulted in a better stability than a slow cooling rate with less aggregation even for frozen samples stored above T'g. They also showed that the solute distribution was more uniform in vessels when a fast cooling rate was applied. This more homogeneous distribution of proteins and trehalose through the tank was due to the formation of ice dendrites resulting in the entrapment of the solutes in micropockets in the interdendritic spaces, limiting their transport by convection and diffusion (Miller et al. 2013). In contrary, Skrabanja et al reported that fast freezing by immersion in $CO_2$/acetone of a mAb formulation resulted in an increased aggregation compared to slow freezing (Skrabanja et al. 1994). Sarciaux et al. also showed that the rapid cooling by quenching in liquid nitrogen of bovine IgG in a phosphate buffer resulted in a higher level of insoluble aggregates in comparison with shelf-ramped freezing after lyophilization. This observation was in accordance with the surface area measurements and environmental electron microscopic data displaying a higher surface area of freeze-dried solids after fast cooling (Sarciaux et al. 1999). These contradictory results concerning the formation of insoluble aggregates are probably due to the difference in formulations (protein concentrations, use of a buffering agent) and to the fact that the whole freeze-drying process was completed in the study of Sarciaux et al. study while only the freezing step with a storing at temperature above T'g was realized in the study of Miller et al. Finally, the cooling rate also influences the reconstitution time. Beech et al demonstrated that a mAb formulation at a concentration of 50 $mg.mL^{-1}$ cooled down at the slow rate of 0.5 °C/min had shorter reconstitution times than formulations frozen in liquid nitrogen despite having the greater surface area (Beech et al. 2015). This result corroborates other studies which concluded that an increase in specific surface area of the formulation did not decrease the reconstitution time (Awotwe-Otoo et al. 2013; Geidobler and Winter 2013). Authors explained this result by the size and morphology of pores evaluated by mercury intrusion porosimetry and by scanning electron microscopy (Beech et al. 2015). The total pore volume was lower for cakes obtained by quench freezing, which may be partially caused by closed pores in the lyophilized material. In addition, the samples prepared by applying a slow cooling rate, presented a more open network of larger spherical pores compared to the quench cooled cakes with narrower cylindrical pores which can explain this reconstitution time difference. Nevertheless, this conclusion has to be taken with caution since the reconstitution time depends on several factors such as the protein type and

concentration, the inclusion of an annealing step as well as the morphology and size of the pores.

## 2.4. Controlled ice nucleation

### 2.4.1. Key elements

The onset of ice nucleation is a random event which can result in longer lyophilization cycles, increased manufacturing costs and vial-to-vial heterogeneity (Bursac et al. 2009).. Studies demonstrated that controlled ice nucleation (CIN) results in an homogeneous ice nucleation at a lower degree of supercooling (Kasper and Friess 2011b; Awotwe-Otoo et al. 2013) and impacts the primary drying rate, the specific surface area as well as many product quality attributes (Konstantinidis et al. 2011; Pisano 2019). In addition, CIN may be an asset to improve scale up from development to production scale by reducing vial to vial nucleation temperature heterogeneity and primary drying time (Vollrath et al. 2018). Amongst the different documented controlled ice nucleation techniques (Geidobler and Winter 2013; Pisano 2019), there are three different techniques available on the market:

- The ice fog technique which was first introduced by (Rowe 1990) and improved afterwards, Patel et al (Patel et al. 2009). Commercial systems have been developed and we can cite FreezeBooster® (Millrock Technology), LyoCoN (Martin Christ), and VERISEQ® nucleation system (IMA life/Linde) as commercially available ice fog systems (Geidobler et al. 2012; Brower et al. 2015).
- The high pressure shift freezing /depressurization technique (Geidobler and Winter 2013). Using this technology, we can cite ControLyo™ (SP Scientific), and a recently published method from OPTIMA pharma (Vollrath et al. 2019).
- The vacuum-induced surface freezing (Geidobler et al. 2012). For instance, SynchroFreeze (Hof) rely on a partial vacuum technique to induce ice nucleation (Luoma et al. 2020). In addition, Christ and Telstar developed a new vacuum- induced technology for controlled nucleation under the name of LyoCoN and "Lyonuc" brand respectively (telstar.com; martinchrist.de). Recently, Regis et al. demonstrated that surface treatment of glass vials impacts the application of vacuum-induced surface freezing. Hydrophobic coatings promoted boiling and blow-up phenomena while hydrophilic coatings increased the risk of fogging. In the case of hydrophobic coatings,

the addition of surfactant and degasification step prior to nucleation allowed to significantly reduce bubbling for a successful induction of nucleation (Regis et al. 2021).

For additional information, advances in CIN techniques and their influence on process parameters and protein activities were recently reviewed and discussed by Pisano 2019.

### 2.4.2. Interest of controlled ice nucleation for mAb's formulations

Controlling the nucleation temperature is gaining a high attention in the field of monoclonal antibodies freeze drying as an approach to develop robust and economical lyophilization cycles.

Awotwe et al (2013), compared classical shelf-ramped method of freezing with CIN by studying the impact of these two freezing methods on the quality attributes of a lyophilized monoclonal antibody and on the process performance. For this purpose, an IgG3 antibody at 1 mg.mL$^{-1}$ final concentration and formulated with sucrose and polysorbate 20 was used as a model antibody. For CIN, authors used a fully integrated Technology named the ControLyo$^{TM}$ to enable the pressurization and depressurization of the chamber (Awotwe-Otoo et al. 2013). The mechanism that induces the nucleation by rapid depressurization is still hypothetical. In comparison to the conventional freezing method, CIN resulted in a uniform nucleation and a reduced degree of supercooling leading to a higher sublimation rate due to the formation of larger ice crystals reducing thereby the primary drying time by 19%. The study also showed that CIN improved product quality attributes (better cake morphology and appearance, improved vial-to-vial homogeneity, reduced reconstitution time and specific surface area). However, the residual moisture content (RM) was higher for batches freeze-dried with a CIN step necessitating a longer secondary drying time to achieve similar values as for the batches freeze-dried with random nucleation (Awotwe-Otoo et al. 2013). In a further study, same authors demonstrated that CIN improved the stability of an IgG1 monoclonal antibody used at different concentrations (1 mg.mL$^{-1}$ and 20 mg.mL$^{-1}$) and formulated with sucrose and polysorbate 20 in comparison to uncontrolled nucleation in terms of mAb glycation. The degree of glycation measured by boronate affinity chromatography was lower for CIN lyophilizates and was dependent on the storage conditions of the lyophilizates. Lyophilized samples stored at 5°C/60% RH resulted in lower levels of glycation compared with samples that were stored at 25°C/60% RH. It is important to note that observed levels of glycation in both freezing methods did not affect the secondary structure of the mAb (Awotwe-Otoo et al.

2015). Gitter et al recently conducted a study to compare two market-dominating controlled nucleation methods, specifically ice fog and depressurization techniques (Gitter et al. 2018a). Three different formulations containing a model mAb IgG1 ($5 g.L^{-1}$) based on amorphous (sucrose, trehalose), semi-crystalline (mannitol:sucrose 4:1) and tween 80 excipients were studied. For comparison purposes, a classical freeze-drying protocol including the same process steps except CIN was also executed. The results of this study showed that both CIN techniques are comparable with respect to drying process performance and product quality attributes. However, some of the studied parameters were different between CIN and classical freezing protocols. We note a higher supercooling and random nucleation for the classical shelf-ramped freezing method compared to CIN and a higher primary drying time. In the random protocol, nucleation started at the bottom of the vial and then propagated up to the surface while in both CIN protocols, nucleation started at the surface and propagated down-to the bottom. Regarding the lyophilizate's quality attributes, trehalose and sucrose samples obtained after the random nucleation freezing protocol presented a lower RM than those obtained by the CIN protocol. The lower RM for random nucleated samples is correlated to the higher SSA which accelerate the desorption rate during the secondary drying step. In contrast, a larger SSA was observed for mannitol:sucrose formulations when CIN was applied with slightly different residual moisture content. Reconstitution time was shorter for trehalose and mannitol:sucrose formulations when CIN was applied but not for sucrose-based lyophilizates while turbidity, X-ray diffraction patterns and soluble aggregates results were similar for all freeze-drying protocols.

Concerning mAb stability, Vollrath et al (2018) demonstrated that CIN had neither a positive nor a negative impact on the product's physico-chemical stability of two IgG1 antibodies compared to random nucleation. Antibodies were formulated at low and high concentrations with addition of sucrose and in the presence or absence of polysorbate. The authors applied the CIN technique described by (Geidobler et al. 2012) consisting in two steps starting by a well-controlled depressurizing step followed by a repressurization step through the venting valve of the condenser. As expected, the lyophilizates obtained by CIN presented a lower SSA and aRM than randomly nucleated lyophilizates. An important conclusion in this study was that polysorbate promoted sucrose crystallization in both low concentration antibody studies during storage. In addition, formulations containing polysorbate presented lower particles level after reconstitution with no further advantage of CIN on stability (Vollrath et al. 2018).

Furthermore, Vollrath et al 2019, compared the impact of three different ice fog methods at two nucleation temperature (-3°C and -10°C) on different mAbs formulation. The chosen ice fog methods were: the VERISEQ® nucleation system, the Millrock FreezeBooster® and the method published by Geidobler et al 2012, which vary in ice fog generation temperature and induction pressure. The study shows an improved vial to vial homogeneity during the primary drying step for CIN cycles regardless of the method used, compared to random nucleation cycles which can be advantageous for the scale-up drying process. However, this advantage was no longer perceivable when complete drying cycles were performed. They also demonstrated that, when CIN is applied, the nucleation temperature is not the only factor determining the SSA and product properties and that the selected excipient and the mAbs concentration are to be considered. Luoma et al (2020) recently compared three mechanistically different CIN techniques during the freezing step of lyophilization, which are the depressurization, partial vacuum, and ice fog techniques. For this study, mAb at 10 and 100 $mg.mL^{-1}$ protein concentrations, formulated with 240 mM sucrose in a 20 mM histidine hydrochloride buffer at pH 5.8 and containing 0.004% (w/v) or 0.04% (w/v) polysorbate 20 were used. The tudy showed that comparable solid state properties and stressed stability behaviors were obtained with these three techniques but highlighted the fact that each technique requires different levels of process development and installation considerations. In addition, nucleation robustness limitations related to formulation or vial format were identified for the partial vacuum and depressurization methods (Luoma et al. 2020). Finally, all these studies demonstrate the positive impacts of CIN techniques (see table 2) on process performance and many product quality attributes but no evidence of improved mAb stability is presented.

## 2.5. Annealing step

### 2.5.1. Fundamentals

Annealing is a common freezing strategy used when bulking agent such as mannitol or glycine is added to the formulation. The main annealing objective is to ensure a complete crystallization of the bulking agent or any crystallizing excipient in the formulation, improving thereby the product stability. Annealing is a thermal treatment following the initial freezing step and consists in increasing shelves temperature above T'g but below the ice melting temperature of the formulation matrix and holding it for a specified period of time. Annealing

will lead to grow larger ice crystals at the detriment of smaller ice crystals denoted as the Ostwald ripening mechanism (Searles et al. 2001b; Randolph and Searles 2002; Esfandiary et al. 2016). Obtaining larger ice crystals through annealing is supposed to lead to lower product resistance to sublimation and then to reduce primary drying time. Another reported benefit for annealing step is the improvement of batch homogeneity by reducing vial to vial heterogeneity during drying (Searles et al. 2001b; Lu and Pikal 2004). Despite the numerous annealing advantages, this thermal treatment can have negative impacts. First, a special attention should be paid for formulations containing crystallizable buffer components, such as sodium phosphate, because annealing may both emphasize pH shifts and expose the proteins to unfavorable pH conditions for longer periods of time (Searles et al. 2001b). In addition, as mentioned for CIN, larger ice crystals corroborate with a lower SSA leading to a higher RM in final product, necessitating longer secondary drying time.

### 2.5.2. Annealing during freeze-drying step of mAbs formulations

Performing annealing in the freezing step may improve the stabilization of protein against process induced stresses by reducing the interfacial area of the frozen system (Butreddy et al. 2021)

Applying an annealing step for mAbs freeze-drying, is predominantly performed when mannitol or other bulking agent are used in the formulation for stability issues. Park et al. (2013) lyophilized an anti-streptavidin IgG1 at 5 mg.mL$^{-1}$ with different formulations based on 4% mannitol with or without sucrose at different pH. Annealing was performed during the freezing step by increasing the shelf temperature from -45°C to −15°C at 0.2°C/min then holding it at −15°C for 240 min before lowering again the temperature to −45°C at 0.2°C/min and holding it for 120 min before starting primary drying. The solid state study showed that sucrose was amorphous while mannitol crystallized to the same form in all formulations. The extent of mannitol crystallization was dependent on the concentration of sucrose and protein added to the formulation. Different characterization tests showed that mAbs stayed in the amorphous state and confirmed the necessity to maintain the amorphous form of mannitol or sucrose to emphasize mAbs storage stability. In fact, the conservation of the native structure of the protein in the solid state and the improvement of IgG1 stability were observed for formulations containing amorphous sucrose and amorphous mannitol and sucrose (Park et al. 2013). In another study, Sarciaux et al observed that annealing a bovine IgG formulation

reduced the aggregate percentage from 33 to 12% in the final product. This decrease in aggregation was attributed to a lower surface area fir the annealed samples (Sarciaux et al. 1999). This result corroborates with a recent study of Lim et al who showed that the monomer content of freeze-dried samples of a model protein was higher when an annealing step was added to the process and concluded that annealing can be helpful to inhibit protein aggregation. They also showed that annealing leads to more homogeneous cake structure, decreased water content, reduced duration of freeze-drying process and to the preservation of the protein stability. However, different polymorphs of mannitol were observed and the transition temperature of the excipients depended on the annealing process (Lim et al. 2018). Finally, in a recent study, Wang et al. compared the effect of annealing and ice-fog CIN (using a VERISEQ® system), carried out at the same temperature, on the quality attributes and process performance of a 50 $mg.mL^{-1}$ mAb formulation. A lyophilization cycle based on shelf-ramped freezing was used as a control. Results showed that CIN yielded in the shortest primary drying and reconstitution time as well as to the lowest cake resistance and specific surface area. Moisture content was the lowest for shelf-ramped freezing and the highest for CIN. Annealing results were between those of shelf-ramped freezing and CIN for all the parameters cited above (Wang et al. 2022).
To conclude on the freezing step, a summary of freezing protocols and their influence on the freeze-drying process parameters and on product quality attributes is presented in table 2.

## 3. Primary drying

### 3.1. General aspects to be considered

Primary drying corresponding to sublimation is the second and longest step of the freeze-drying cycle (figure 2). During this stage, frozen free water is removed by sublimation through an appropriate combination of shelf temperature (Ts) and chamber pressure (Pc) values. The choice of Ts and Pc directly impacts the sublimation rate and determines the product temperature (Tp) which is one of the most critical process quality attributes (Tang and Pikal 2004; Hedoux 2013).

Concerning the sublimation rate, primary drying is the most time and energy consuming step of the lyophilization (Hedoux 2013). For example, it is established that increasing Tp by 1°C, can reduce the primary drying cycle time by 13% (Tang and Pikal 2004). Recently, a detailed

energy and economic analysis of the various freeze-drying steps was published and estimated that this stage represents 65% of the operational cost of a total freeze-drying cycle (Stratta et al. 2020).

The target and optimum Tp to achieve during primary drying, is dependent on the formulation and is set based on the following product critical temperatures: the glass transition temperature in the frozen state (T'g) for amorphous systems and the collapse temperature (Tc) which is generally 2°C above T'g or the eutectic temperature (Teu) for crystalline systems. It cannot be directly selected.

The major risk of overpassing product critical temperatures is the occurrence of structural collapse which can lead to a variety of changes in the cake properties, for both esthetical and stability considerations. It was thus established that keeping the Tp 2 to 3°C below T'g during conventional freeze-drying process, allowed to ensure optimum freeze-drying conditions and keep intact the microscopic organization in the cake in order to maintain the stability of biopharmaceutics (Tang and Pikal 2004). .

In practice, identifying adequate combinations of the critical process parameters such as Ts and Pc while achieving adequate product quality attributes and an optimal sublimation rate in order to optimize the drying cycle for the industry is proved to be a great challenge. Trial and error approaches are usually used to design an optimized primary drying process but the resulting recipe is often very far from being optimal with a considerable potential for improvement. In this context, the pharmaceutical sector investigated different approaches to determine the primary drying cycle parameters such as the design space, model-based tools and inline vs offline methods. These approaches were also driven by the regulatory authorities publishing the guidance for industry PAT (process analytical technology) like the US-FDA in 2004 encouraging to implement a quality by design approach in order to ensure the quality of the product and the robustness of process. Many PAT tools have been developed and investigated for the in-line and off-line monitoring of the freeze-drying process (Awotwe Otoo et al. 2014; Bosca et al. 2016; Fissore 2017; Fissore et al. 2018). The key element and most explored strategy considering a "quality by design" approach is the building of a design space. The design space of the primary drying step represents a multidimensional combination of the boundaries of critical process parameters (Ts, Pc, Tp) for which the product quality is not impaired and is frequently correlated to the determination of heat and mass transfer. The

design space is usually obtained by mathematical models, experimental investigations or a combination of a statistical design of experiments with mathematical models (Fissore et al. 2011; Patel and Pikal 2013; Assegehegn et al. 2020; Carfagna et al. 2020; Juckers et al. 2022). Even if these tools and approaches are numerous, shelf temperature (Ts) and chamber pressure (Pc) were and still remain the main process parameters taken into account in the optimization of the primary drying step whatever the API added to the formulation. However, a specific attention should be given for biomolecule formulations and especially mAb formulations since an inadequate choice of process parameters will not only influence the cake appearance and morphology but can also compromise the mAb structure, activity and stability.

In the next paragraph, we will discuss the strategies and the choice of process parameters implicated in the optimization of monoclonal antibodies freeze-drying.

## 3.2. Primary drying optimization for mAb formulations

### 3.2.1. Energy input

#### 3.2.1.1. Using the shelves temperature

In addition to the impact on sublimation rate described above, process parameters chosen for primary drying step have a great impact on the product quality attributes of mAbs formulations. A specific attention should be given for mAbs formulations since inadequate choice of process parameters will not only influence the cake appearance and morphology but can compromise the mAbs structure, activity and stability.

Traditionally, Ts for therapeutic protein and mAbs freeze-drying are set at values allowing to keep Tp below T'g during primary drying in order to avoid the collapse of the dried substance. This classical conservative primary drying strategy usually corresponds to long primary drying cycles and compromises the optimization of the primary drying step, which can last up to several days. This issue led scientists to study the effect of collapse on protein stability. With respect to antibodies, Shersh et al. studied the effect of collapse on IgG1 antibodies at a concentration of 4 $mg.mL^{-1}$ by applying either conservative or aggressive freeze-drying cycles. For the conservative cycle, Ts was set at temperatures allowing to get Tp significantly lower than formulation's T'g. For the aggressive cycle, a Ts rising up to 45°C during primary drying was used then causing collapse. In order to compare the influence of collapse on mAbs stability, formulations with totally amorphous

(disaccharides) or partially crystalline (disaccharides/mannitol) excipients were investigated. Authors did not observe any difference in aggregation rate between collapsed or non-collapsed conditions, the level of monomers being greater than 98% regardless of the collapsed formulations thus suggesting excellent physical stability of antibodies. In addition, no change was detected for the secondary and tertiary structure of mAbs indicating the conservation of the chemical stability of antibodies which is inextricably connected to their biological activity. This result shows no effect of collapse on the stability of antibodies (Schersch et al. 2010). Even further, a higher degree of stabilization in collapsed cakes compared to non-collapsed has been assessed in relation to the physical stability of antibodies after long-term storage (Schersch et al. 2012).

Moreover, another study (Depaz et al. 2016) interested in the optimization of the primary drying step, discredited the "consensus" suggesting that keeping Tp below the value of T'g is necessary to avoid collapse and to improve mAbs stability. The study explored the behavior of the lyophilizate as a function of Tp. By working on completely amorphous formulations of IgG1 or IgG4 antibodies at different concentrations (5, 25, 50 and 100 mg.mL$^{-1}$), it has been demonstrated that working at Tp levels exceeding T'g does not systematically lead to collapse and that the different drying conditions had no impacts on IgG chemical and physical stability as well as on the product quality. Furthermore, cases of micro-collapse were correlated with protein concentration in the formulation i.e for any given protein, the window between T'g and Tc increases with increasing protein concentration and thus give some leeway to reach a Tp above T'g but below Tc (Colandene et al. 2007; Depaz et al. 2016). Indeed, Horn et al. recently described the possibility of applying an aggressive primary drying cycle on a formulation with low and high mAb concentrations (2, 7.5 and 80 mg.mL$^{-1}$) with a mixture of amorphous and crystalline excipients. During this cycle, Tp were significantly higher than the T'g value of sucrose while it remained below the eutectic temperature of mannitol, which crystallizes crystallized during the process. The crystallization of mannitol thus maintained an elegant cake structure by acting as a scaffold for the dry substance while the amorphous matrix (sucrose) acted as protein stabilizer. The addition of this crystalline compound in a studied precise weight ratio allows to target much higher product temperatures than those used in conventional cycles (-39°C in the conservative cycle and -10°C in the aggressive cycle) and to considerably reduce the duration of the primary drying step from 47 hours to 7 hours (Horn et al. 2018).

Using an even more "aggressive" primary drying process, Colandene et al. studied the impact of a product temperature above Tc on a totally amorphous formulation of IgG at 80 $mg.mL^{-1}$. Samples were analyzed and compared to samples freeze-dried under "conventional" conditions (below the T'g). In addition to significantly reduced primary drying time, results showed that neither the activity of the antibody (evaluated by bioassay), nor the aggregation rate (evaluated by sec-HPLC) presented any differences. Minor secondary structural changes were identified when the cakes were analyzed by Fourrier transform infrared spectroscopy. Nevertheless, the aggressive cycle was not implicated in these modifications since the same variations were observed after lyophilization by the conventional cycle (Colandene et al. 2007).

Similar conclusions concerning aggressive cycles were made by Bjelosevic et al. who have shown that selecting Ts at 30°C and thus reaching a Tp 9°C above the $T_c$ value for some formulations didn't induce macro-collapse and didn't affect the stability of antibodies. In addition, this aggressive cycle resulted in a 54% reduction of the primary drying time (from 48 to 22 h) compared to Ts monitored at -20°C in a conventional drying cycle (Bjelošević et al. 2018). The Tc value and the glass transition temperature depend on the water content of the sample. As water content decreases with the progress of primary drying, the viscosity increases, and therefore, collapse is partially stopped and the effective Tc increases (Pikal and Shah 1990). This can explain why Tp higher than Tc (initially measured at the maximal freeze concentrated mixture) can be reached without inducing collapse.

More recently, the development of a freeze-drying cycle of antibody formulations in a single drying step, i.e. combing the sublimation and the secondary drying stage (desorption), has been proposed (Pansare and Patel 2019). Pansare et al. proposed a process that involves a single Ts (≥ 40°C) during drying steps. This led to maintain Tp above Tc while keeping the same pressure during the drying cycle and reduced process time of at least 40%. This drastic process didn't have any impact on the physical or chemical stability of the antibody. Concerning the cake appearance, the study showed that adding a crystallizing excipient to the formulation improved the product appearance and that partial collapse and shrinkage were only observed for formulations containing only amorphous excipients and low c mAb concentrations (≤25 $mg.mL^{-1}$). In another aggressive FD study (Haeuser et al. 2019) carried out at 10 or 30°C Ts for primary drying step (reducing the cycle time by 50%), adding 2-hydropropyl-betacyclodextrine (HPBCD) to sucrose formulation contributed to provide an elegant cake for mAb at low

concentrations (10 mg.mL$^{-1}$). In addition, mAbs stability was comparable to the conservative FD cycle and mAbs presented a good stability for 3 months during storage at 40°C. Some internal cracks were observed by µ-computed tomography depending on the formulation and cycle parameters (Haeuser et al. 2019).

**3.2.1.2. Effect of ramp rate temperature**

In addition to the choice of optimal Ts and Pc during the primary drying step, the temperature ramp rate is also an important parameter which can impact the development of a freeze-drying cycle.

Recent studies by Ohori et al. were carried out in order to characterize the impact of this unique parameter in a freeze-drying cycle. It was thus demonstrated that low ramp rates (<0.33°C/min) were correlated with cases of collapse whereas an increase of this rate (>0.44° C/min) made it possible to minimize the collapses in a trehalose formulation. More precisely, using a fast ramp rate is considered as optimal for FD process when high Ts (i.e aggressive cycles) are applied during primary drying (Ohori and Yamashita 2017; Ohori et al. 2018). Furthermore, cases of collapse occurring during the primary drying stage due to too low temperature ramp rates could lead to an increase in the residual humidity which would be detrimental for the stability of protein formulations (Ohori et al. 2018).

Consistent with these results, Horn et al. did not show a negative impact on product attributes when the drying ramp rate was increased from 0.5°C to 1°C/ min (Horn et al. 2018). In contrast, Pansare et al., observed more shrinkages when the ramp rate was 0.5°C/min compared to 0.1°C/min in both conservative and aggressive antibodies FD cycles (Pansare and Patel 2019). Thus, the authors do not all agree on the effects of the speed of increasing ramp rate temperatures, without a clear explanation being able to be retained.

Moreover, the drying ramp rate parameter can influence the duration of the drying cycle time and may be optimized for this end. For example, a reduction of 4h (Horn et al. 2018) and 2h (Haeuser et al. 2019) of cycle times was respectively attributed to an increase in the ramp rate from 0.5 ° C to 1 ° C and from 0.2 ° C to 1 ° C.

**3.2.1.3. Using electromagnetic radiation at microwave frequencies**

The drying stage in the lyophilization process can also be provided by other means than convection and conduction (heated shelves). Heat supply via the use of microwaves is a process which is commonly used for freeze-drying in the food industry since it is a process which maintains the appearance of the product (color, taste, texture) and significantly reduces time of the lyophilization process (Fan et al. 2019).

The heat transfer is generated by the capacity of polar compounds (such as water, amino acids, sugars…) to absorb the electromagnetic microwaves and to convert this energy into heat. The application of such a drying system on antibodies and vaccines was presented for the first time at the CPPR Freeze Drying of Pharmaceuticals & Biologics Conference in 2014. Gitter et al., compared the impact of drying by a conventional FD method to FD using microwaves on different antibody formulations. They highlighted that the process using microwaves could be favorable for the development of the lyophilization process of antibodies. In addition to reducing the drying cycle time by more than 75%, drying by this system did not negatively impact the stability properties of mAbs even after storage at high temperature (Gitter et al. 2018b; Gitter et al. 2019). Nevertheless, differences in the residual moisture level between the samples were observed which can be explained by a poor uniformity in the dissipation of heat in the frozen matrix. This heterogeneity in batches, already mentioned in the literature, represents the greatest limitation of this process. By using another microwave device such as a solid state semiconductor (which unlike magnetron, can be directly controlled by the manipulator during the process) the inter-batch heterogeneity was considerably reduced (Gitter et al. 2019). However, this heterogeneity and increased residual moisture level did not impact the stability of various glass forming antibody formulations (trehalose, sucrose, arginine) nor their crystallization tendency. Thus, the recent application of microwave FD methods for antibody lyophilization, is very promising for the optimization of the process and still needs to be developed.

#### 3.2.2. Pressure

Pressure is the second process parameter governing primary drying step by improving the heat and mass transfers. During drying, sublimation occurs by applying a Pc well below the ice vapor pressure of crystallized water in order to enhance the sublimation rate. Pressure value between ¼ and ½ of the equilibrium vapor pressure of ice at the desired

product temperature is generally applied (Colandene et al. 2007). The following equation can be used to set the $P_c$ value (Tang and Pikal 2004):

$$P_c = 0.29 \cdot 10^{(0.019 \cdot \mathrm{T_p})} \qquad \text{equation 1}$$

where *P*c is the chamber pressure and *T*p is the product temperature

The driving force of sublimation is proportional to the difference between the equilibrium ice vapor pressure and Pc i.e. the higher Pc, the higher the sublimation rate.

$$\frac{dm}{dt} = \frac{P_{ice} - P_c}{R_p + R_S} \qquad \text{equation 2}$$

where, *dm*/*dt* is ice sublimation rate (g/hour per vial), $P_{ice}$ is the equilibrium vapor pressure of ice at the sublimation interface temperature (Pa or any pressure unit), and $R_p$ and $R_s$ are the dry layer and stopper resistance, respectively, to water vapor transport from the sublimation interface (Pa·h/g).

Keeping in mind that primary drying is the longest step in a FD cycle, optimization of the cycle duration by adapting the Pc can be considered. Nonetheless, as mentioned for Ts, Pc is indirectly related to Tp. Thereby, increasing Pc will increase Tp and may cause collapses and damages. Conversely, too low Pc lead to risks of contamination with volatile stopper components and pump oil as well as heterogeneity in heat transfer between vials (Tang and Pikal 2004). Thereby, it is established that a moderate pressure in the chamber varying between 100 and 150 mTorr (0.13 and 0.2 mbar respectively) is required to obtain the best homogeneity of heat transfer. Consistent with this proposal, Haeuser et al. studied the effect of increasing the pressure chamber from 0.1 mbar to 0.2 mbar during an aggressive primary drying (i.e., Tp > T'g) of an HPBCD/sucrose/mAb formulation. After increasing Pc, an increase in Tp was registered and cases of micro-collapse on the dry structure were speculated but no effect on the cycle time reduction was shown (Haeuser et al. 2019). Conversely, in another study the duration of the "aggressive" primary drying of a formulation containing IgG/sucrose/glycine was reduced by 0.5 h by applying the same variation of Pc (0.1 to 0.2 mbar) (Bjelošević et al. 2018). In addition, increasing the pressure chamber from 0.1 mbar to 0.3 mbar reduced the cycle time by 3.5 h without causing collapses although the product temperature was increased by 3°C (Bjelošević et al. 2018). The occurrence of collapse after modifying Pc has to be taken with caution because it is dependent on the formulation and the critical temperatures of the API and chosen excipients.

More drastically, chamber pressures of 1.1 mbar and 2.2 mbar were used for “fast primary drying” of a mAb/mannitol/sucrose/glycerol formulation. Although being well above the recommended pressures, elegant cakes without collapses were obtained. Pc variation from 1.1 mbar to 2.2 mbar increased the product temperature by 1.4°C and resulted in the reduction of the primary drying time from 9.5 h to 6.3 h gaining a considerable amount of time (3.2 h) (Horn et al. 2018). This result may be explained by the fact that increasing the pressure in the chamber will generally increase the heat transfer to the product, and thus, increasing the mass transfer to the condenser.

# 4. Secondary drying

## 4.1. General aspects to be considered

Secondary drying is the last step of a freeze-drying process and consists of removing unfrozen bound water by desorption. In order to allow desorption to take place, the shelf temperature is increased at a specified ramp rate and chamber pressure is either decreased or kept at the same set value as during the primary drying step (figure 2). The main objective of monitoring and optimizing the process parameters during secondary drying is to limit the risk of collapse and to control final residual moisture content in the final freeze-dried product.

Typically, the beginning of secondary drying takes place when the Tp reaches or exceeds the shelf temperature. The products temperature is usually monitored by thermocouples placed in the vials at different shelf position. Since the vials containing thermocouples are not representative of the entire batch because of the bias in freezing and sublimation behaviors, other methods were developed. Another sensitive, reliable, and robust method to determine the start point for secondary drying is the comparative pressure measurement. Indeed, most freeze-driers are equipped with pirani and capacitance manometers sensors. The pirani vacuum gauge is a thermal conductivity-type sensor and so the apparent pressure measured by the pirani gauge depends on the gas composition and decreases when water vapor in the chamber decreases. On the contrary, a capacitance manometer indicates the absolute vacuum and is not affected by water vapor in the system. At the end of primary drying, the pressure value given by the pirani manometer sharply decreases to converge with the value given by the capacitance manometer. Therefore, waiting until the Pirani reading reaches the capacitance manometer reading is usually recommended and considered as a good practice

before starting the secondary drying step (Nail et al. 2017). Many other methods exists to monitor the end of primary drying and are described and reviewed by (Patel et al. 2010; Nail et al. 2017). We can cite (i) the dew point monitor (electronic moisture sensor), (ii) the process of $H_2O$ concentration from tunable diode laser absorption spectroscopy (TDLAS), (iii) the lyotrack (gas plasma spectroscopy), (iv) the condenser pressure and (v) the pressure rise test (manometric temperature measurement (MTM) or variations of this method). The chosen method to determine the primary drying endpoint impacts the residual moisture content in the samples at the end of the primary drying step. RM represents generally 8 to 20% of the sample depending on the solid state (Patel et al. 2010).

Four process parameters have to be chosen to conduct and optimize the secondary drying phase and they are: the final shelf temperature, the temperature ramp rate, the chamber pressure and the hold time. Ts is the main process variable during secondary drying. The desorption rate seems to be is independent of the chamber pressure (Pikal et al. 1990; Siew 2018) in the range of commonly used chamber pressure (0.05-0.40 mbar) (Assegehegn et al. 2021) probably because the rate-limiting step in the secondary drying is diffusion of water through the glassy mixture of partly-dried solids (Siew 2018). Therefore, the pressure value set during the primary drying can be used also for secondary drying. The temperature ramp rate depends on the formulation and chosen excipients. For amorphous formulations, a slow ramp rate (i.e. 0.1–0.15 °C/min) has to be considered in order to avoid collapse because Tg is still relatively low at the beginning of the desorption due to a relatively high residual moisture content at this step (Tang and Pikal 2004). Tg evolves gradually as the water is desorbed and higher shelves temperatures can be then applied. In contrast, higher ramp rates (0.3–0.4 °C/min) are generally safe for crystalline formulations for which the risk of collapse is absent (Tang and Pikal 2004). Furthermore, protein formulation temperatures above 50 °C and the extension of the time t>6 h are not advisable.

### 4.2. Specific aspects related to mAbs

As seen above, the main objective of secondary drying is to control the residual humidity of the final product. High residual moisture content may be detrimental for protein stability since water molecules act as reaction precursor or as plasticizer increasing molecular mobility. Usually, a RM below 1% is the target value (Schneid et al. 2011) for protein formulation since

many degradation reactions are considered to be slowed down below this level (Horn et al. 2018). However, very low level of RM may lead to so-called overdrying which is detrimental for protein stability specifically for long term storage and then should be avoided. In fact, some studies showed that optimal stability for some biopharmaceutical molecules is obtained for RM values between 1 and 3% (Schneid et al. 2011). Few studies evaluated the optimization of secondary drying parameters for mAb formulations are published.

For the ramp rate to use, Heljo et al showed that freeze drying a polyclonal bovine serum IgG (1 $mg.mL^{-1}$) in a trehalose formulation at different ramp rate (0.08, 0.5 and 1°C/min) did not result in changes in protein stability (Heljo et al. 2013). The stability was studied by measuring (i) in vitro binding activity after freeze-drying with ELISA, (ii) IgG monomer recovery with asymmetric flow field flow fractionation and (iii) average particle size and polydispersity with dynamic light scattering (DLS).

## 5. Conclusion

Freeze-drying of monoclonal antibodies formulations is a frequently used process to improve mABs stability and long term storage. The number of studies conducted in order to understand the influence of process parameters and formulation strategies on freeze-dried mABs samples is in continuous progress since several years. Eventhough a classical process design is still frequently applied (shelf-ramped freezing, quenching in liquid nitrogen, choosing Ts lower than T'g), recent studies show the possibility of improving the process control by controlling ice nucleation during the freezing step and reducing the primary drying step by setting Ts at values greater than T'g without compromising final product quality attributes. However, results are sometimes contradictory since differences in the experimental protocols can be present between different papers evaluating the same process parameter. One established conclusion in all papers is that multiple formulation and process parameters have to be taken into consideration in order to successfully run a controlled freeze-drying process for mABs and to improve their stability. Finally, a summary of the advantages and disadvantages of the different freeze-drying protocols cited in this review is presented in table 3.

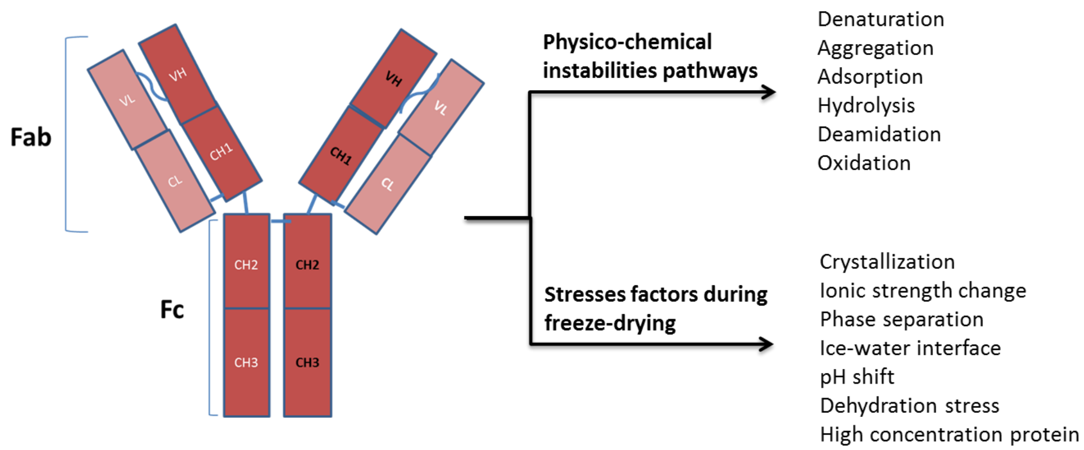



Figure 1: Immunoglobulin G (Ig G) structure and subsequent physico-chemical instabilities pathways and stress factors during freeze-drying

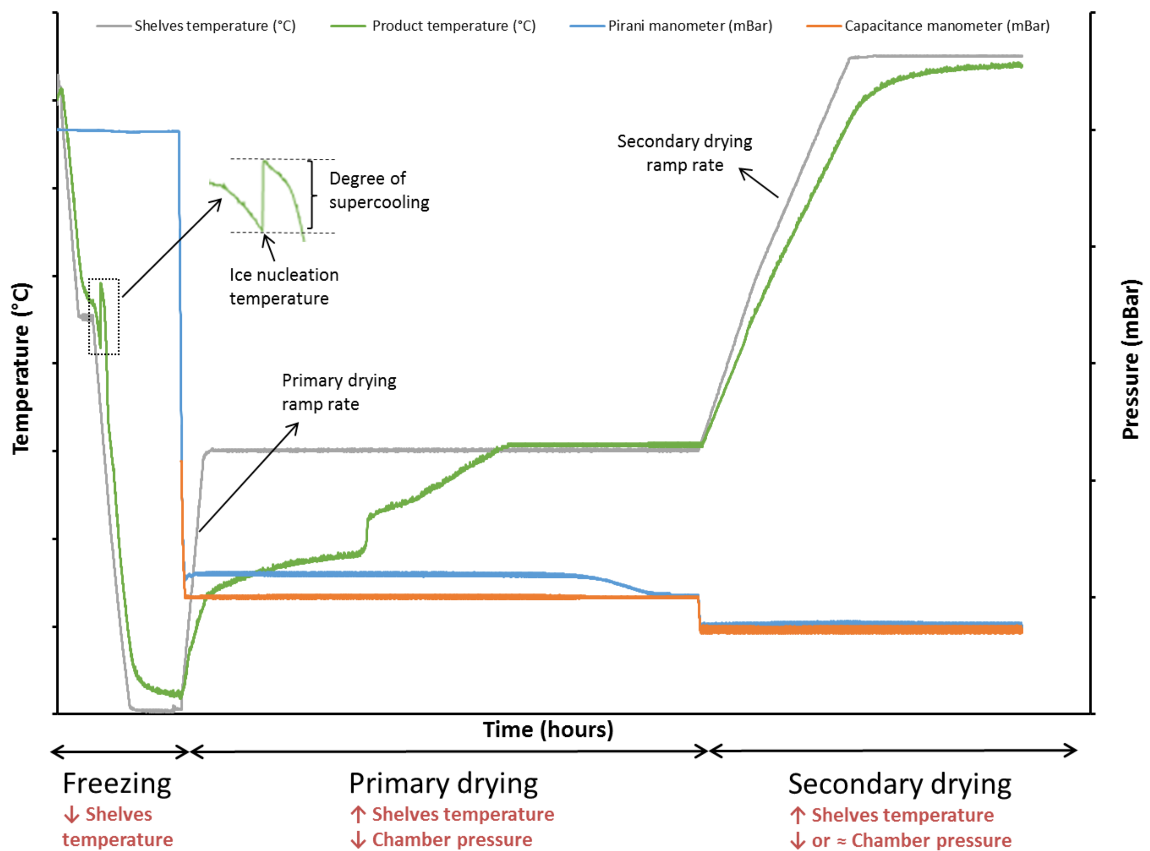



Figure 2: Standard freeze-drying cycle including freezing, primary drying and secondary drying steps (personal data).

Table 1 : Types of excipients used for monoclonal antibodies freeze-drying with their respective role and critical temperatures (Wang 2000; Baheti et al. 2010; Cui et al. 2017; Mensink et al. 2017; Bjelošević et al. 2020)

| Types of excipients | | Role | Critical temperature | | |
|---|---|---|---|---|---|
| | | | Tg (°C) | Tg' (°C) | Tc (°C) |
| **Dissaccharides** | Trehalose | > Acts as stabilisers : prevent physical and chemical degradation of biomolecules during lyophilization as a cryoprotectant (during the freezing phase) and as a lyoprotectant (during the drying phase) | 111 | -32 | -29 |
| | Sucrose | > Stabilization is based on two mechanisms vitrification and water replacement theory | 60-65 | -32 | -31 |
| **Polymers** | Hydroxylpropyl-β-cyclodextrin | > Stabilizer: maintain protein stability during freezing, drying, storage and reconstitution<br>> Cryoprotectants or lyoprotectants in a concentration range between 1 and 10% (w/v)<br>> Increases the Tg of dried materials thus improving stability<br>> Prevents protein unfolding and aggregation at interfaces by acting as a surfactant when added at low concentrations (0.1%; w/v) | 108 | -12.1 | -9.2 to -5.8 |
| | Polyethylene glycol PEG 200, 300, 400, 600, 3350 and 4000 | > Allows shorter drying process due to increased product temperatures<br>> Ensures stability of the protein<br>> Prevents phase separation | | | |
| | Polyvinylpyrrolidone (low-molecular weight) | > Inhibits crystallisation of buffer salts<br>> Acts as stabilizer when used in combination with sugars | 180 | -22.4 (PVP K40) | -23.9 to -15.4 |
| **Polyols** | Mannitol | > Acts as bulking agent : provides an elegant appearance to the lyophilised cake and contributes to the adequate morphology of the cake<br>> Enables aggressive primary drying (Tp > Tg' or Tc) | 13 | -32; -27 | |
| **Amino acids** | Glycine | > Acts as bulking or buffering agent<br>> Possess synergistic stabilizing properties with sugars, and mainly with sucrose | | -37 | |
| | Histidine | > Acts as buffer and cryo/lyo-protectant when maintained in an amorphous state<br>> Acts as an antioxidant | 37 | -32 | |
| | Arginine | > Can prevent protein–protein interactions and aggregation due to its weak binding to the protein surface<br>> Improves protein solubility and decreases formulation viscosity which is of great interest for highly concentrated formulations | | | |
| | Lysine | > Acts as buffering agent | 68 | | |
| **Surfactants** | Polysorbate 20<br>Polysorbate 80<br>Poloxamer 188 | > Limit biomolecules aggregation by adsorbing to hydrophobic surfaces of proteins and prevents adsorption of proteins at ice-water interfaces<br>> Facilitate the reconstitution step by adsorbing at the air/water interface | | | |
| **Buffering agent** | Acetate buffer<br>Citrate buffer<br>Histidine buffer<br>Phosphate buffer<br>Succinate buffer<br>Tris base buffer | > pH adjusting agents : maintain a constant pH of the solution during the freeze-drying cycle, storage and reconstitution<br>> Maintain pH near phyiological values to avoid side effect after application and near the value at which the protein has greatest stability | 11 | -54<br>-41<br>-33<br>-45<br><br>-51 | |
| **Tc modifiers** | Dextran 1 kDa<br>Dextran 40 kDa<br>Gelatin<br>Ficoll<br>Hydroxyethyl starch | > Reduce the time of the lyophilization cycle Exhibit high critical temperature values making it possible to work at higher freezing and drying temperatures and thus | 151.8<br>223.2<br><br><br>>110 | -22.1<br>-12.3<br>-9<br>-19<br>-12 | -10<br>-10<br>-8<br>-20<br>> -5 |

Tg: glass transition temperature, T'g: glass transition temperature of the maximally freeze-concentrated solution, Tc: collapse temperature

Table 2 : summary of freezing protocols and their influence on process parameters and on freeze-dried quality attributes

| | Degree of supercooling | Size of ice crystals | Ice Nucleation temperature | Sublimation rate | Residual moisture | Secondary drying time | Cake apparence | Reconstitution time |
|---|---|---|---|---|---|---|---|---|
| **Slow cooling rate** | ↓ | ↑ | Random | ↑ | NC | NC | + | ↓ |
| **Fast cooling rate** | ↑ | ↓ | Random | ↓ | NC | NC | ++ | ↑ |
| **Controlled ice nucleation** | ↓ | ↑ | Uniform | ↑ | ↑ | ↑ | ++ | ↓ |
| **Annealing step** | NC | ↑ | Uniform | ↑ | ↑ | ↑ | ++ | ↓ |

Table 3 : Advantages and disadvantages of the different freeze-drying protocols recently used for freeze-drying monoclonal antibody formu lations.

| Freeze-drying step | | Advantages | Disadvantages |
|---|---|---|---|
| **Freezing** | Slow cooling rate | High size of ice crystals<br>Increase in sublimation rate<br>Reduction of reconsitution time | Heterogeneous degree of supercooling and ice nucleation temperature |
| | Fast cooling rate | Better cake appearance | Decrease in sublimation rate |
| | Controlled ice nucleation<br>Annealing step | Uniform nucleation<br>Increase in sublimation rate<br>Improve product quality attributes | Increase residual moisture and secondary drying time |
| **Primary drying** | Agressive primary drying (Tp > Tg / Tp > Tc) | Increase in sublimation rate<br>Drastic reduction of the primary drying time | Risks of collapse but not systematic (formulation-dependent) |
| | Single drying step | | |
| | Fast drying ramp | | |
| | High chamber pressure (> 0,2 mbar) | Primary drying time reduction | |
| | Microwaves assisted freeze-drying | Drastic reduction of  the primary drying time | Heterogeneity in the residual moisture content between samples |

## References


Arsiccio A, Barresi AA, Pisano R. 2017. Prediction of Ice Crystal Size Distribution after Freezing of Pharmaceutical Solutions. Cryst Growth Des. 17(9):4573–4581. https://doi.org/10.1021/acs.cgd.7b00319

Arsiccio A, Giorsello P, Marenco L, Pisano R. 2020. Considerations on Protein Stability During Freezing and Its Impact on the Freeze-Drying Cycle: A Design Space Approach. J Pharm Sci. 109(1):464–475. https://doi.org/10.1016/j.xphs.2019.10.022

Arsiccio A, Matejtschuk P, Ezeajughi E, Riches-Duit A, Bullen A, Malik K, Raut S, Pisano R. 2020. Impact of controlled vacuum induced surface freezing on the freeze drying of human plasma. Int J Pharm. 582:119290. https://doi.org/10.1016/j.ijpharm.2020.119290

Arsiccio A, McCarty J, Pisano R, Shea J-E. 2020. Heightened Cold-Denaturation of Proteins at the Ice–Water Interface. J Am Chem Soc. 142(12):5722–5730. https://doi.org/10.1021/jacs.9b13454

Arsiccio A, Pisano R. 2018. Application of the Quality by Design Approach to the Freezing Step of Freeze-Drying: Building the Design Space. J Pharm Sci. 107(6):1586–1596. https://doi.org/10.1016/j.xphs.2018.02.003

Arsiccio A, Pisano R. 2020. The Ice-Water Interface and Protein Stability: A Review. J Pharm Sci. 109(7):2116–2130. https://doi.org/10.1016/j.xphs.2020.03.022

Arsiccio A, Sparavigna AC, Pisano R, Barresi AA. 2019. Measuring and predicting pore size distribution of freeze-dried solutions. Dry Technol. 37(4):435–447. https://doi.org/10.1080/07373937.2018.1430042

Assegehegn G, Brito-de la Fuente E, Franco JM, Gallegos C. 2021. Understanding and optimization of the secondary drying step of a freeze-drying process: a case study. Dry Technol. 39(8):1003–1017. https://doi.org/10.1080/07373937.2020.1739065

Awotwe-Otoo D, Agarabi C, Read EK, Lute S, Brorson KA, Khan MA. 2015. Product and process understanding to relate the effect of freezing method on glycation and aggregation of lyophilized monoclonal antibody formulations. Int J Pharm. 490(1–2):341–350. https://doi.org/10.1016/j.ijpharm.2015.03.056

Awotwe-Otoo D, Agarabi C, Read EK, Lute S, Brorson KA, Khan MA, Shah RB. 2013. Impact of controlled ice nucleation on process performance and quality attributes of a lyophilized monoclonal antibody. Int J Pharm. 450(1–2):70–78. https://doi.org/10.1016/j.ijpharm.2013.04.041

Beech KE, Biddlecombe JG, van der Walle CF, Stevens LA, Rigby SP, Burley JC, Allen S. 2015. Insights into the influence of the cooling profile on the reconstitution times of amorphous lyophilized protein formulations. Eur J Pharm Biopharm. 96:247–254. https://doi.org/10.1016/j.ejpb.2015.07.029

Bjelošević M, Seljak KB, Trstenjak U, Logar M, Brus B, Ahlin Grabnar P. 2018. Aggressive conditions during primary drying as a contemporary approach to optimise freeze-drying cycles of biopharmaceuticals. Eur J Pharm Sci. 122:292–302. https://doi.org/10.1016/j.ejps.2018.07.016

Bjelošević M, Zvonar Pobirk A, Planinšek O, Ahlin Grabnar P. 2020. Excipients in freeze-dried biopharmaceuticals: Contributions toward formulation stability and lyophilisation cycle optimisation. Int J Pharm. 576:119029. https://doi.org/10.1016/j.ijpharm.2020.119029

Brower J, Lee R, Wexler E, Finley S, Caldwell M, Studer P. 2015. New Developments in Controlled Nucleation: Commercializing VERISEQ® Nucleation Technology. In: Varshney D, Singh M, editors. Lyophilized Biol Vaccines Modality-Based Approaches [Internet]. New York, NY: Springer; [accessed 2021 Oct 15]; p. 73–90. https://doi.org/10.1007/978-1-4939-2383-0_5

Bursac R, Sever RR, Hunek H. 2009. A Practical Method for Resolving the Nucleation Problem in Lyophilization. BioProcess Int [Internet]. [accessed 2020 May 25]. https://bioprocessintl.com/manufacturing/formulation/a-practical-method-for-resolving-the-nucleation-problem-in-lyophilization-185124/

Butreddy A, Janga KY, Ajjarapu S, Sarabu S, Dudhipala N. 2021. Instability of therapeutic proteins - An overview of stresses, stabilization mechanisms and analytical techniques involved in lyophilized proteins. Int J Biol Macromol. 167:309–325. https://doi.org/10.1016/j.ijbiomac.2020.11.188

Cameron P, editor. 2013. Good Pharmaceutical Freeze-Drying Practice. Boca Raton: CRC Press. https://doi.org/10.1201/b14360

Capozzi LC, Pisano R. 2018. Looking inside the ‘black box’: Freezing engineering to ensure the quality of freeze-dried biopharmaceuticals. Eur J Pharm Biopharm. 129:58–65. https://doi.org/10.1016/j.ejpb.2018.05.020

Chiu ML, Goulet DR, Teplyakov A, Gilliland GL. 2019. Antibody Structure and Function: The Basis for Engineering Therapeutics. Antibodies. 8(4):55. https://doi.org/10.3390/antib8040055

Colandene JD, Maldonado LM, Creagh AT, Vrettos JS, Goad KG, Spitznagel TM. 2007. Lyophilization Cycle Development for a High-Concentration Monoclonal Antibody Formulation Lacking a Crystalline Bulking Agent. J Pharm Sci. 96(6):1598–1608. https://doi.org/10.1002/jps.20812

Depaz RA, Pansare S, Patel SM. 2016. Freeze-Drying Above the Glass Transition Temperature in Amorphous Protein Formulations While Maintaining Product Quality and Improving Process Efficiency. J Pharm Sci. 105(1):40–49. https://doi.org/10.1002/jps.24705

Emami F, Vatanara A, Park E, Na D. 2018. Drying Technologies for the Stability and Bioavailability of Biopharmaceuticals. Pharmaceutics. 10(3):131. https://doi.org/10.3390/pharmaceutics10030131

Esfandiary R, Gattu SK, Stewart JM, Patel SM. 2016. Effect of Freezing on Lyophilization Process Performance and Drug Product Cake Appearance. J Pharm Sci. 105(4):1427–1433. https://doi.org/10.1016/j.xphs.2016.02.003

Fan K, Zhang M, Mujumdar AS. 2019. Recent developments in high efficient freeze-drying of fruits and vegetables assisted by microwave: A review. Crit Rev Food Sci Nutr. 59(8):1357–1366. https://doi.org/10.1080/10408398.2017.1420624

Fang R, Bogner RH, Nail SL, Pikal MJ. 2020. Stability of Freeze-Dried Protein Formulations: Contributions of Ice Nucleation Temperature and Residence Time in the Freeze-Concentrate. J Pharm Sci. 109(6):1896–1904. https://doi.org/10.1016/j.xphs.2020.02.014

Geidobler R, Mannschedel S, Winter G. 2012. A New Approach to Achieve Controlled Ice Nucleation of Supercooled Solutions During the Freezing Step in Freeze-Drying. J Pharm Sci. 101(12):4409–4413. https://doi.org/10.1002/jps.23308

Geidobler R, Winter G. 2013. Controlled ice nucleation in the field of freeze-drying: Fundamentals and technology review. Eur J Pharm Biopharm. 85(2):214–222. https://doi.org/10.1016/j.ejpb.2013.04.014

Gitter JH, Geidobler R, Presser I, Winter G. 2018a. A Comparison of Controlled Ice Nucleation Techniques for Freeze-Drying of a Therapeutic Antibody. J Pharm Sci. 107(11):2748–2754. https://doi.org/10.1016/j.xphs.2018.07.019

Gitter JH, Geidobler R, Presser I, Winter G. 2018b. Significant Drying Time Reduction Using Microwave-Assisted Freeze-Drying for a Monoclonal Antibody. J Pharm Sci. 107(10):2538–2543. https://doi.org/10.1016/j.xphs.2018.05.023

Gitter JH, Geidobler R, Presser I, Winter G. 2019. Microwave-Assisted Freeze-Drying of Monoclonal Antibodies: Product Quality Aspects and Storage Stability. Pharmaceutics. 11(12). https://doi.org/10.3390/pharmaceutics11120674

Grilo AL, Mantalaris A. 2019. The Increasingly Human and Profitable Monoclonal Antibody Market. Trends Biotechnol. 37(1):9–16. https://doi.org/10.1016/j.tibtech.2018.05.014

Haeuser C, Goldbach P, Huwyler J, Friess W, Allmendinger A. 2019. Be Aggressive! Amorphous Excipients Enabling Single-Step Freeze-Drying of Monoclonal Antibody Formulations. Pharmaceutics. 11(11):616. https://doi.org/10.3390/pharmaceutics11110616

Harguindeguy M, Stratta L, Fissore D, Pisano R. 2021. Investigation of the Freezing Phenomenon in Vials Using an Infrared Camera. Pharmaceutics. 13(10):1664. https://doi.org/10.3390/pharmaceutics13101664

Harguindeguy M, Stratta L, Fissore D, Pisano R. 2022. Combining Mathematical Modeling and Thermal Infrared Data in the Freezing of Pharmaceutical Liquid Formulations. Ind Eng Chem Res. 61(12):4379–4389. https://doi.org/10.1021/acs.iecr.1c04595

Hedoux A. 2013. Lyophilisation de produits pharmaceutiques et biopharmaceutiques : Dossier complet | Techniques de l'Ingénieur. In: Tech Ing [Internet]. [place unknown]; [accessed 2021 Oct 15]; p. PHA 2 026-1-14. https://www.techniques-ingenieur.fr/base-documentaire/biomedical-pharma-th15/mise-en-forme-des-medicaments-et-autres-produits-de-sante-42611210/lyophilisation-de-produits-pharmaceutiques-et-biopharmaceutiques-pha2026/

Heljo VP, Harju H, Hatanpää T, Yohannes G, Juppo AM. 2013. The effect of freeze-drying parameters and formulation composition on IgG stability during drying. Eur J Pharm Biopharm. 85(3):752–755. https://doi.org/10.1016/j.ejpb.2013.03.033

Horn J, Schanda J, Friess W. 2018. Impact of fast and conservative freeze-drying on product quality of protein-mannitol-sucrose-glycerol lyophilizates. Eur J Pharm Biopharm. 127:342–354. https://doi.org/10.1016/j.ejpb.2018.03.003

Hottot A, Vessot S, Andrieu J. 2007. Freeze drying of pharmaceuticals in vials: Influence of freezing protocol and sample configuration on ice morphology and freeze-dried cake texture. Chem Eng Process Process Intensif. 46(7):666–674. https://doi.org/10.1016/j.cep.2006.09.003

Izutsu K. 2018. Applications of Freezing and Freeze-Drying in Pharmaceutical Formulations. In: Iwaya-Inoue M, Sakurai M, Uemura M, editors. Surviv Strateg Extreme Cold Desiccation Adapt Mech Their Appl [Internet]. Singapore: Springer; [accessed 2021 Oct 15]; p. 371–383. https://doi.org/10.1007/978-981-13-1244-1_20

Kasper JC, Friess W. 2011a. The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. Eur J Pharm Biopharm. 78(2):248–263. https://doi.org/10.1016/j.ejpb.2011.03.010

Kasper JC, Friess W. 2011b. The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. Eur J Pharm Biopharm. 78(2):248–263. https://doi.org/10.1016/j.ejpb.2011.03.010

Köhler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256(5517):495–497. https://doi.org/10.1038/256495a0

Konstantinidis AK, Kuu W, Otten L, Nail SL, Sever RR. 2011. Controlled nucleation in freeze-drying: Effects on pore size in the dried product layer, mass transfer resistance, and primary drying rate. J Pharm Sci. 100(8):3453–3470. https://doi.org/10.1002/jps.22561

Kulkarni SS, Patel SM, Bogner RH. 2020. Reconstitution Time for Highly Concentrated Lyophilized Proteins: Role of Formulation and Protein. J Pharm Sci. 109(10):2975–2985. https://doi.org/10.1016/j.xphs.2020.05.029

Le Basle Y, Chennell P, Tokhadze N, Astier A, Sautou V. 2020. Physicochemical Stability of Monoclonal Antibodies: A Review. J Pharm Sci. 109(1):169–190. https://doi.org/10.1016/j.xphs.2019.08.009

Lim JY, Lim DG, Kim KH, Park S-K, Jeong SH. 2018. Effects of annealing on the physical properties of therapeutic proteins during freeze drying process. Int J Biol Macromol. 107:730–740. https://doi.org/10.1016/j.ijbiomac.2017.09.041

Lu X, Pikal MJ. 2004. 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(1):85–95. https://doi.org/10.1081/pdt-120027421

Luoma J, Ingham E, Lema Martinez C, Allmendinger A. 2020. Comparison of Techniques to Control Ice Nucleation during Lyophilization. Processes. 8(11):1439. https://doi.org/10.3390/pr8111439

Miller MA, Rodrigues MA, Glass MA, Singh SK, Johnston KP, Maynard JA. 2013. Frozen-State Storage Stability of a Monoclonal Antibody: Aggregation is Impacted by Freezing Rate and Solute Distribution. J Pharm Sci. 102(4):1194–1208. https://doi.org/10.1002/jps.23473

Mullard A. 2021. FDA approves 100th monoclonal antibody product. Nat Rev Drug Discov. 20(7):491–495. https://doi.org/10.1038/d41573-021-00079-7

Nail S, Tchessalov S, Shalaev E, Ganguly A, Renzi E, Dimarco F, Wegiel L, Ferris S, Kessler W, Pikal M, et al. 2017. Recommended Best Practices for Process Monitoring Instrumentation in Pharmaceutical Freeze Drying—2017. AAPS PharmSciTech. 18(7):2379–2393. https://doi.org/10.1208/s12249-017-0733-1

Oddone I, Arsiccio A, Duru C, Malik K, Ferguson J, Pisano R, Matejtschuk P. 2020. Vacuum-Induced Surface Freezing for the Freeze-Drying of the Human Growth Hormone: How Does Nucleation Control Affect Protein Stability? J Pharm Sci. 109(1):254–263. https://doi.org/10.1016/j.xphs.2019.04.014

Oddone I, Barresi AA, Pisano R. 2017. Influence of controlled ice nucleation on the freeze-drying of pharmaceutical products: the secondary drying step. Int J Pharm. 524(1):134–140. https://doi.org/10.1016/j.ijpharm.2017.03.077

Ohori R, Akita T, Yamashita C. 2018. Effect of temperature ramp rate during the primary drying process on the properties of amorphous-based lyophilized cake, Part 2: Successful lyophilization by adopting a fast ramp rate during primary drying in protein formulations. Eur J Pharm Biopharm. 130:83–95. https://doi.org/10.1016/j.ejpb.2018.06.010

Ohori R, Yamashita C. 2017. Effects of temperature ramp rate during the primary drying process on the properties of amorphous-based lyophilized cake, Part 1: Cake characterization, collapse temperature and drying behavior. J Drug Deliv Sci Technol. 39:131–139. https://doi.org/10.1016/j.jddst.2017.03.013

Pansare SK, Patel SM. 2019. Lyophilization Process Design and Development: A Single-Step Drying Approach. J Pharm Sci. 108(4):1423–1433. https://doi.org/10.1016/j.xphs.2018.11.021

Park J, Nagapudi K, Vergara C, Ramachander R, Laurence JS, Krishnan S. 2013. Effect of pH and Excipients on Structure, Dynamics, and Long-Term Stability of a Model IgG1 Monoclonal Antibody upon Freeze-Drying. Pharm Res. 30(4):968–984. https://doi.org/10.1007/s11095-012-0933-z

Patel SM, Bhugra C, Pikal MJ. 2009. Reduced Pressure Ice Fog Technique for Controlled Ice Nucleation during Freeze-Drying. AAPS PharmSciTech. 10(4):1406. https://doi.org/10.1208/s12249-009-9338-7

Patel SM, Doen T, Pikal MJ. 2010. Determination of End Point of Primary Drying in Freeze-Drying Process Control. AAPS PharmSciTech. 11(1):73–84. https://doi.org/10.1208/s12249-009-9362-7

Pieters S, De Beer T, Kasper JC, Boulpaep D, Waszkiewicz O, Goodarzi M, Tistaert C, Friess W, Remon J-P, Vervaet C, Vander Heyden Y. 2012. Near-Infrared Spectroscopy for In-Line Monitoring of Protein Unfolding and Its Interactions with Lyoprotectants during Freeze-Drying. Anal Chem. 84(2):947–955. https://doi.org/10.1021/ac2022184

Pikal MJ, Shah S. 1990. The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy phase. Int J Pharm. 62(2):165–186. https://doi.org/10.1016/0378-5173(90)90231-R

Pikal MJ, Shah S, Roy ML, Putman R. 1990. The secondary drying stage of freeze drying: drying kinetics as a function of temperature and chamber pressure. Int J Pharm. 60(3):203–207. https://doi.org/10.1016/0378-5173(90)90074-E

Pisano R. 2019. Alternative Methods of Controlling Nucleation in Freeze Drying. In: Ward KR, Matejtschuk P, editors. Lyophilization Pharm Biol New Technol Approaches [Internet]. New York, NY: Springer; [accessed 2022 Aug 10]; p. 79–111. https://doi.org/10.1007/978-1-4939-8928-7_4

Pisano R, Arsiccio A, Nakagawa K, Barresi AA. 2019. Tuning, measurement and prediction of the impact of freezing on product morphology: A step toward improved design of freeze-drying cycles. Dry Technol. 37(5):579–599. https://doi.org/10.1080/07373937.2018.1528451

Pisano R, Capozzi LC. 2017. Prediction of product morphology of lyophilized drugs in the case of Vacuum Induced Surface Freezing. Chem Eng Res Des. 125:119–129. https://doi.org/10.1016/j.cherd.2017.07.004

Randolph TW, Searles JA. 2002. Freezing and annealing phenomena in lyophilization: Effect upon primary drying rate, morphology, and heterogeneity. Am Pharm Rev. 5:40–46.

Regis F, Arsiccio A, Bourlès E, Scutellà B, Pisano R. 2021. Surface Treatment of Glass Vials for Lyophilization: Implications for Vacuum-Induced Surface Freezing. Pharmaceutics. 13(11):1766. https://doi.org/10.3390/pharmaceutics13111766

Rowe TD. 1990. A technique for the nucleation of ice. In: Geneva, Switzerland.

Sarciaux J-M, Mansour S, Hageman MJ, Nail SL. 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J Pharm Sci. 88(12):1354–1361. https://doi.org/10.1021/js980383n

Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. 2010. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: Stability after freeze-drying. J Pharm Sci. 99(5):2256–2278. https://doi.org/10.1002/jps.22000

Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. 2012. Systematic Investigation of the Effect of Lyophilizate Collapse on Pharmaceutically Relevant Proteins, Part 2: Stability During Storage at Elevated Temperatures. J Pharm Sci. 101(7):2288–2306. https://doi.org/10.1002/jps.23121

Schneid SC, Gieseler H, Kessler WJ, Luthra SA, Pikal MJ. 2011. Optimization of the secondary drying step in freeze drying using TDLAS technology. AAPS PharmSciTech. 12(1):379–387. https://doi.org/10.1208/s12249-011-9600-7

Searles JA, Carpenter JF, Randolph TW. 2001a. The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf. J Pharm Sci. 90(7):860–871. https://doi.org/10.1002/jps.1039

Searles JA, Carpenter JF, Randolph TW. 2001b. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine T(g)' in pharmaceutical lyophilization. J Pharm Sci. 90(7):872–887. https://doi.org/10.1002/jps.1040

Siew A. 2018. Freeze-Drying Process Optimization: Determining the right process conditions for a freeze-drying cycle requires an understanding of the effect of each step on the drug product. Pharm Technol Eur. 30(5):14–16.

Skrabanja AT, de Meere AL, de Ruiter RA, van den Oetelaar PJ. 1994. Lyophilization of biotechnology products. PDA J Pharm Sci Technol. 48(6):311–317.

Susa F, Bucca G, Limongi T, Cauda V, Pisano R. 2021. Enhancing the preservation of liposomes: The role of cryoprotectants, lipid formulations and freezing approaches. Cryobiology. 98:46–56. https://doi.org/10.1016/j.cryobiol.2020.12.009

Tang X, Pikal MJ. 2004. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 21(2):191–200. https://doi.org/10.1023/b:pham.0000016234.73023.75

Taylor PC, Adams AC, Hufford MM, de la Torre I, Winthrop K, Gottlieb RL. 2021. Neutralizing monoclonal antibodies for treatment of COVID-19. Nat Rev Immunol. 21(6):382–393. https://doi.org/10.1038/s41577-021-00542-x

Vollrath I, Friess W, Freitag A, Hawe A, Winter G. 2018. Does controlled nucleation impact the properties and stability of lyophilized monoclonal antibody formulations? Eur J Pharm Biopharm Off J

Arbeitsgemeinschaft Pharm Verfahrenstechnik EV. 129:134–144. https://doi.org/10.1016/j.ejpb.2018.05.025

Vollrath I, Friess W, Freitag A, Hawe A, Winter G. 2019. Comparison of ice fog methods and monitoring of controlled nucleation success after freeze-drying. Int J Pharm. 558:18–28. https://doi.org/10.1016/j.ijpharm.2018.12.056

Wang J, Searles JA, Torres E, Tchessalov SA, Young AL. 2022. Impact of Annealing and Controlled Ice Nucleation on Properties of A Lyophilized 50 mg/ml MAB Formulation. J Pharm Sci.:S0022-3549(22)00214–3. https://doi.org/10.1016/j.xphs.2022.05.016

Wang W, Singh S, Zeng DL, King K, Nema S. 2007. Antibody Structure, Instability, and Formulation. J Pharm Sci. 96(1):1–26. https://doi.org/10.1002/jps.20727

Websites:

https://www.telstar.com/controlled-nucleation-more-than-just-a-homogenization-tool/, [accessed 2022 sept 22]

https://www.martinchrist.de/en/freeze-drying/controlled-nucleation-lyocon, [accessed 2022 sept 22]