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Review article

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ABSTRACT

Freeze-drying is the most widespread method to preserve protein drugs and vaccines in a dry form facilitating their storage and transportation without the laborious and expensive cold chain. Extending this method for the preservation of natural biomaterials and cells in a dry form would provide similar benefits, but most results in the domain are still below expectations. In this review, rather than consider freeze-drying as a traditional black box we "break it" through a detailed process thinking approach. We discuss freeze-drying from process thinking aspects, introduce the chemical, physical, and mechanical environments important in this process, and present advanced biophotonic process analytical technology. In the end, we review the state of the art in the freeze-drying of the biomaterials, extracellular vesicles, and cells. We suggest that the rational design of the experiment and implementation of advanced biophotonic tools are required to successfully preserve the natural biomaterials and cells by freeze-drying. We discuss this change of paradigm with existing literature and elaborate on our perspective based on our new unpublished results.

1. Introduction

Biomaterials can be defined as "*natural or synthetic materials that are suitable for introduction into living tissue especially as a part of medical device*". While natural biomaterials self-evidently comprise cells or their derivatives, synthetic biomaterials include non-natural polymers and even metals as in the case of artificial joints. Combined with cells, biomaterials have introduced a new dimension into multiple research areas such as reparative treatments, wound healing, and cancer treatment [1–5]. However, besides the advantages of combinatory treatments, biomaterials and cells introduce challenges in their long-term preservation: Typically, natural biomaterials and cells are cryopreserved in −80 °C ultra-freezers or liquid nitrogen tanks for their long-term storage. Even though widely accepted and commonly used, these cryopreservation methods require constant maintenance to keep the valuable materials intact. Power failure or failure to add liquid nitrogen can result in the loss of samples, working hours, and money. Maintaining large sample libraries cryopreserved does not only occupy large space with freezers and back-up freezers but also creates continuous costs in the terms of used electricity and liquid nitrogen. The cryopreserved materials are also exposed to the risk of cross-contamination when stored in liquid nitrogen [6]. Furthermore, the logistics of cryopreserved substances might be challenging, as can be seen with for example the vaccine for SARS-COV-2 [7].

Freeze-drying is a pliable method to counter these challenges. As a desiccation method, freeze-drying is especially suitable for the preservation of heat-sensitive substances such as protein drugs and vaccines, to formulate drugs for parenteral administration or to manufacture scaffolds. The freeze-drying process can be divided into three main steps: 1) Freezing (solidification of a sample), 2) primary drying (sublimation of frozen water) and 3) secondary drying (removal of unfrozen water) (Fig. 1). Due to the highly energetic nature of the constitutive processes, freeze-drying causes compositional, thermal, and pressure changes in the proximity of the biomaterials and cells to preserve, rendering the processed samples vulnerable to denaturation, osmotic imbalance, and mechanical stress. Studying the conditions the biological materials are subjected to during the freeze-drying process requires following the changes in chemical, physical, and mechanical environments locally which is extremely challenging. Tackling these hurdles requires approaches such as process analytical technology (PAT), and biophotonic tools like Raman and near-infrared (NIR) spectroscopy.

The lack of applied advanced detection technologies and the success

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with simpler substances, like proteins, has led into a situation where old manners are directly applied into more labile formulations. The impact of the processing steps on the composition and physicochemical conditions around cells has been, until now, mostly done indirectly by comparing the viability and/or integrity of the biological species before and after processing. If these results are of indisputable relevance, they provide limited information to rationalize freezing and drying processes. It is a black box! Accepting the black box promotes trial-and-error approaches when designing the freeze-drying process for new natural biomaterials or cells.

We discuss in this review the advances and bottlenecks of freeze-drying of natural biomaterials and cells while incorporating Quality by Design and PAT perspectives in our discussion. We believe that addressing the sample through changes in chemical, physical, and mechanical environments provides new insights into freeze-drying as a process while offering new tools to optimize the conservation of complex natural biomaterial and cell entities.

2. Quality by design and design spaces in freeze-drying of biomaterials and cells

Unfortunately, for many decades the formulation and process optimization have based on trial-and-error in drug development. To implement a more structured approach to process optimization, the International Conference of Harmonization has introduced new guidelines for science-based manufacturing of final dosage forms [11–13]. The new concept of manufacturing drugs is gathered under the umbrella of Quality-by-Design (QbD) ideology. QbD is defined as"*A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.*" [11] The flowchart of necessary steps required for systematic implementation of QbD is presented in Fig. 2.

The first step in the QbD process is to define the Quality Target Product Profile (QTPP), meaning *a prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account the safety and efficacy of the drug product.* [11]. The next step is to define Critical Material Attributes

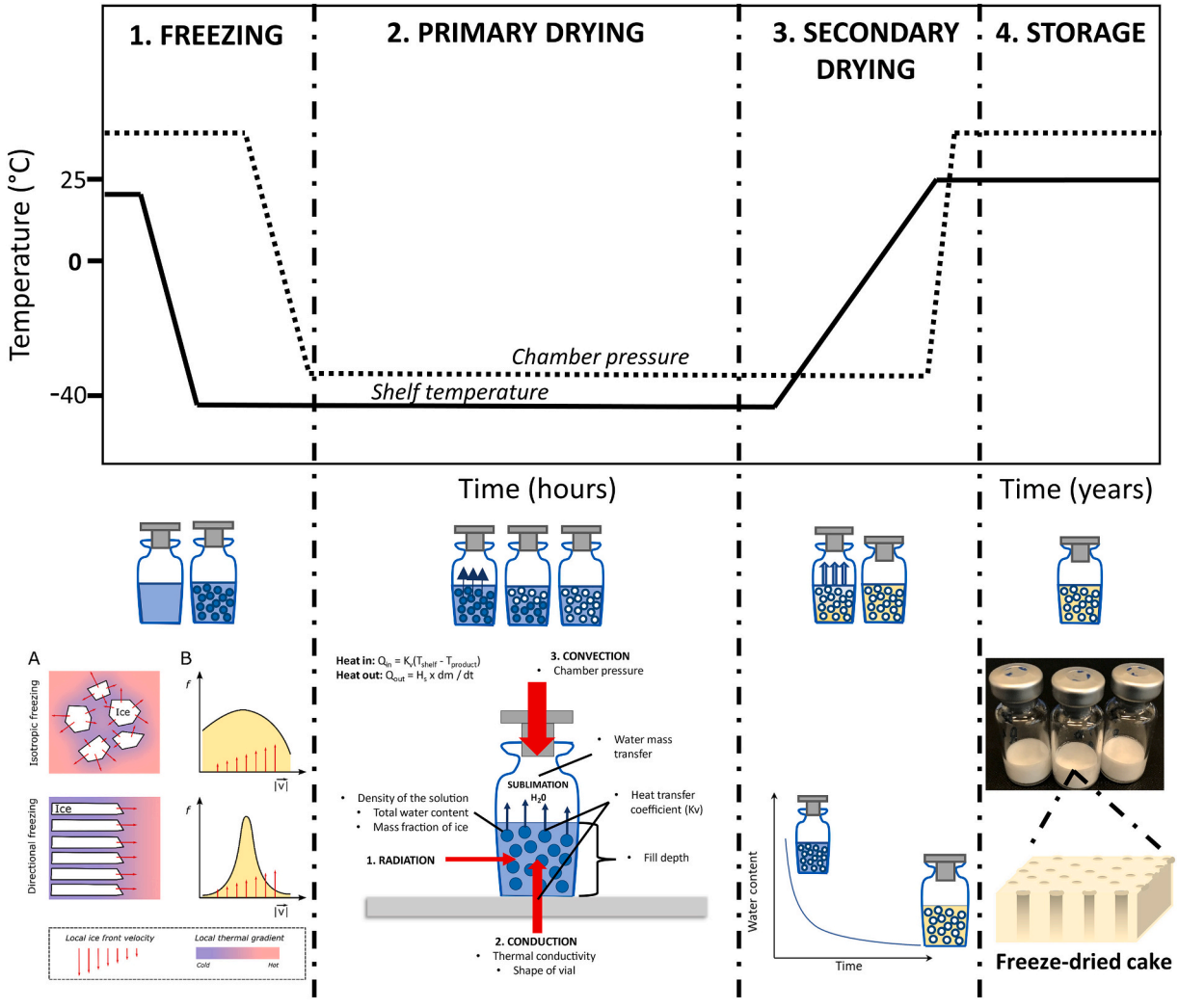


Fig. 1. Freeze-drying is a multistep process in which the sample is first frozen (1. Freezing), then the frozen water (ice) is sublimated under vacuum (2. Primary drying), after which temperature is elevated and unfrozen water is desorbed (3. Secondary drying). The product is a porous dry product that can be stored unfrozen preferably at room temperature for years (4. Storage). Rational design of the experiment and careful control of each step is required to obtain the final product of high quality. In the freeze-drying process, the most important parameters that can be controlled are the shelf temperature of the freeze-drying machine, pressure in the freeze-drying chamber, and time used for the freeze-drying process. Since the temperature of the sample determines the rate of sublimation, which directly correlates to the time required to obtain a dry enough sample, controlling and monitoring the temperature of the sample during the entire process is highly important. Modified from: Franks (2008) [8], Pikal et al. (1984) [9], and Kawasaki et al. (2019) [10].

Fig. 2. The schematic flow-chart of Quality-by-Design ideology. First, critical quality attributes, critical process parameters, and critical material attributes must be defined, and their relationships clarified. Second, risk assessment is performed. Third, design of experiment is conducted by scientifically determining the relationships between process factors. Risk assessment and quality target product profile are constantly reflected to each other to optimize the outcome.

(CMAs) and Critical Process Parameters (CPPs) which affect Critical Quality Attributes (CQAs) and ultimately to the QTPP. Typically, the definition of attributes and parameters is done by a multidisciplinary team of experts in a process initiated by the identification of relevant CMAs, CPPs, and CQAs. To facilitate the task, various standardized quality risk management tools such as Ishikawa chart [14], Failure Mode Effects Analysis [15], Fault Tree Analysis, Risk Ranking, and Filtering are commonly utilized. To summarize, typically risks are identified, analyzed, and evaluated with the following questions: I) What might go wrong? II) What is the likelihood it will go wrong? III) What are the consequences (severity)? Based on the risk assessment, decisions have to be made (Risk Control) whether the risk needs to be reduced or even eliminated due to its severity and probability (Risk Reduction) or is the risk in accepted level, harmless and unlikely (Risk Acceptance). As a final result of the risk assessment, CMAs and CPPs which have to be controlled, and CQAs which have to be monitored have been identified.

The next task in QbD approach is to figure out what are the relationships of selected CMAs and CPPs on CQAs and subsequently to the QTPP. This can be done scientifically using Design of Experiment (DoE) meaning *a structured, organized method for determining the relationship between factors affecting a process and the output of that process* [11]. The DoE exercise results in statistically significant relationships of CMAs and CPPs on CQAs that can be modeled using Principal Component Analysis, Partial Least Square Projection, or other more sophisticated modeling tools like Artificial Neural Networks. Reliable DoE models create the foundation of establishing Design Space (DS), in which *the multidimensional combination and interaction of input variables (*e.g.*, material attributes) and process parameters that have been demonstrated to provide assurance of quality* [11]. DS is just one space in the spectrum of specific spaces to seek the deeper process understanding in QbD thinking. Multidimensional space consists of multiple spaces inside each other, and the largest space is an operational space, typically defined by the constraints of specifications of equipment like temperature range, pressure/vacuum range, load capacity, and dimensions of the equipment. Inside operational space is Knowledge Space (KS), and the boundaries of KS are defined by a priori information of process or equipment, or boundaries can be derived from fundamental equations describing the performance of process or equipment. Practically, in freeze-drying, choked flow conditions can be calculated as a function of vacuum and shelf temperature settings and the dimensions of freeze-dryer [16]. Inside of KS is the DS, which defines the CMAs and CPPs yielding the required quality of the product. From the process control perspective, often inside of DS is defined Control Space (CS) defining boundaries that trigger control actions in CMAs and CPPs, if CQA(s) crosses the said boundaries.

DS and KS are subjected to continuous verification and improvement with the gained data and process understanding. Several control strategies based on empirical (DoE) or first principle models to keep CQAs within DS exist, but they are out of the scope of this review. The successful implementation of the QbD approach to the pharmaceutical processes requires monitoring that CQAs stay within the DS, achievable with PAT tools used in real-time and in-line manner/fashion. Commonly used PAT tools for freeze-drying are covered in Chapter 4. It is worth noting already here that no previous study of freeze-drying biomaterials and cells utilized QbD ideology in their experiments. We provide here suggestions on how the QbD ideology can be useful in the freeze-drying of biomaterials and cells, and how a change of paradigm is needed. The state of the art of freeze-drying of biomaterials and cells is discussed in more detail in chapter 5.

2.1. *Freezing: Solidification of sample*

In the freezing step, the temperature of the sample is lowered below its freezing point to solidify the sample. The freezing of water solution, or crystallization in general, consists of two steps: 1) nucleation and 2) the growth of nuclei to the macroscopic crystals. The driving force of nucleation is mainly defined by supercooling. As the temperature drops below the freezing point of the solution, the probability to form thermodynamically favorable ice clusters, which act as nuclei for ice crystallization, grows. There are several ways to induce ice nucleation in the samples to reduce variation between ice nucleation temperatures [17].

Upon nucleation, ice crystals begin to grow. In the context of freezing biological entities, ice crystal growth is a double-edged sword. On the one hand, it allows the creation of ice crystals that will define the biomaterial's macro-porosity, essential for fluid and gas diffusion and cell colonization. On the other hand, ice growth poses a major concern in terms of the integrity of the biological entities. Moreover, the freezing step affects directly the following steps of the freeze-drying cycle - larger supercooling (lower nucleation temperature) favors faster formation of nuclei (homogenous nucleation) which leads to the larger number of smaller crystals with large surface area. This causes longer primary drying time due to the high dry layer resistance but higher unfrozen water desorption rate during secondary drying [18]. On the contrary, smaller supercooling (high nucleation temperature) leads to the opposite phenomenon (heterogenous nucleation) and the smaller number of larger crystals which shortens the primary drying time and lengthens the secondary drying time. In addition, different vials face uneven cooling conditions that cause vial-to-vial and batch-to-batch variability in practice (for more detailed description, please refer to Pisano et al. (2019) [19]). Due to the profound influence on the final product and the whole freeze-drying process, freezing is the first critical step that needs to be optimized and controlled systematically.

In freeze-drying, the final product must be elegant cake and collapsed cakes are generally disposed of due to their unpreserved structure, potentially prolonged reconstitution times, and decreased stability [20]. To obtain an elegant cake, one key issue is to prevent the collapse of the cake during the freeze-drying process. Collapse

temperature (Tc) is the temperature in which a sample being freeze-dried will collapse and it can be determined experimentally for example, by direct microscopic observation or by determining the glass transition temperature of maximally freeze-concentrated sample (Tg') by thermal analysis [21]. Determining Tc is more laborious than Tg' and, in general, Tc is 1–2 °C higher than Tg', which means that Tg' can be used as a trustworthy estimation for Tc.

Since virtually all chemical species are insoluble in ice [22], as the formation of the solid ice continues, the amount of free water decreases resulting in the increased concentration of any solute present in the sample. The freeze-concentration of solutes increases the viscosity of the solution until no more solid ice can form. At such point, the sample has gone through glass transition and this temperature is called Tg', the glass transition temperature of the maximally freeze-concentrated sample. To optimize the freeze-drying process, and to avoid a trial-and-error approach, it is necessary to determine the Tc or Tg' and to monitor the temperature of the sample throughout the whole freeze-drying process to ensure that the temperature of the sample does not exceed Tc. Freeze-drying cycle run at a temperature higher than Tc may lead to a collapse of the samples, while lower temperatures will lead to a longer, and thus more expensive, cycle.

In polymers, in particular biopolymers, such as polysaccharides, Tg' is defined by the intersection between the cryoscopic depression curve and the composite Tg defined between ice and the solute. [23]. However, the Tg is an eminently kinetic phenomenon whereas the cryoscopic depression is purely thermodynamic. The consequence is that Tg' will depend on the velocity of freezing as shown in Qin et al. 2020 [24]. These considerations are coherent with the particularly relevant place that the cooling rate has played in domains such as cryobiology. In fact, the control over the cooling rate has been instrumental in determining if the freezing is accompanied by ice nucleation and growth leading to phase separation (slow freezing) or vitrification in which case no phase separation is observed (high cooling rates).

In the last two decades, the emergence of a materials processing technique that fully relies on the freezing step as the driving force to shape materials – ice templating, also called freeze casting – has led to the perception that thermal control over the samples' environment provided insufficient control over the local ice nucleation and growth [25]. The mechanical performance of materials prepared by ice templating depends strongly on the inner porosity of the material that is generated from the sublimation of ice crystals. Aiming at homogenizing the size and spatial distribution of the pores – but also as a manner to mimic specific natural materials such as nacre [26–28] – the fabrication technique has evolved to integrate spatial control over the freezing process. This has been done either by designing freezing setups that can exhibit controlled thermal gradients. As a result, control over the ice crystal growth led to structures with the preferential alignment of the pores along the predefined thermal gradient.

The equivalent advances took place in the context of cryobiology – under the name of directional freezing – where the application of controlled linear thermal gradients to freeze biological entities has led to some interesting results in cell cryopreservation [29–32]. However, beyond the relative success of cryopreservation protocols based on directional freezing, controlling the thermal gradient enables to decorrelate nucleation and growth during freezing, a key step in rationalizing freezing. Ishiguro and Rubinsky (1994) [33] pioneered the microscopic observation of the interaction between the ice front during freezing and red blood cells in suspension opening a pathway to the in-situ observation of freezing events [24,34,35]. The microscopic observation of freezing events has systematically shown that controlling the ice front velocity is central to predict the fate of the suspended matter during freezing. These results strengthen the importance of separating nucleation from growth during freezing. In fact, in traditional or isotropic freezing, where cooling rate is controlled rather than the thermal gradient, samples tend to cool down more homogeneously with slight local fluctuations, which leads the freezing process to occur mostly dictated by the nucleation step, since the whole sample (or at least a large volume fraction of the sample) will be supercooled at the moment the freezing starts. Such type of freezing event is generally poorly controlled in terms of the temperature at which it occurs – homogeneous nucleation being a stochastic process dependent on the formation of energetically favorable ordered water clusters – and in terms of the local velocity of the front. On the contrary, freezing samples based on a controlled gradient creates a limited supercooling volume within the sample (where nucleation will take place) followed by ice crystal's growth in the rest of the sample that is controlled by the thermal gradient (Fig. 3**)**. This strategy enables to fix the exact temperature at which the ice front will interact with the liquid front and to precisely control the ice front velocity. Additionally, since in directional freezing growth is favored with respect to nucleation, samples are less exposed to heterogeneous nucleation due to the action of suspended matter than in isotropic freezing. Although there are presently no commercial freezing setups for directional freezing, the different approaches used to apply it have been recently described by Qin et al. (2021) [28].

Arsiccio and Pisano (2018) used mechanistic Computational Fluid Dynamics (CFD) modeling to predict temperature profiles during the freezing [36]. From the temperature profile, they were able to calculate the freezing front velocity and the temperature gradients within the freezing zone. From that data, it is possible to calculate ice crystal sizes during freezing. When ice crystal sizes are known the DS of freezing can be established in order to get desired primary and secondary drying conditions. Later Arsiccio et al. (2020) illustrated how to create DS with two model proteins with different denaturation mechanisms during freezing [37]. The denaturation mechanism of the first protein was driven by the ice-water interface and for the second one, cold denaturation was the main denaturation mechanism. They used mechanistic CFD modeling to create the DS for both scenarios. For the first model protein, DS aimed to minimize the surface area of the ice-water interface with slow cooling. For the second model protein, the DS of the fast cooling rate was defined to decrease the duration of freezing.

QbD ideology can be applied for freeze-drying of biomaterials and cells to optimize freeze-drying in general, for example, to shorten the freeze-drying cycle. QbD ideology can be also applied specifically for biomaterials and cells, by first recognizing CPP and CMA of each step of the freeze-drying process. For example, CPPs for the freezing step include freezing rate, ice front velocity, and CMA include Tg' and viscosity. Learning from the freeze-drying of proteins, CFD modeling could be utilized to understand the ice front velocity and temperature gradients and to control the ice crystal sizes.

2.2. *Primary drying: Removing frozen water*

Primary drying is the main step of drying and usually the longest phase in the freeze-drying process, thus another critical step of formulation and process optimization. Also in the case of an uncontrolled freezing step, primary drying is the source of heterogenous sublimation rate which rises a challenge to define DS. During the primary drying step, the frozen solid water (ice) is sublimated under a vacuum. The driving force of sublimation is the vapor pressure difference (more precisely the temperature difference) between the frozen water and the surface of the condenser [8]. Sublimation requires heat, which is provided to the sample through three different ways: 1) radiation, 2) conduction, and 3) convection **(**Fig. 4**)**. The heat provided has to be high enough to facilitate sublimation of ice (the latent heat of sublimation of frozen ice), but the temperature of the sample must stay below Tg' to prevent collapsing of the freeze-drying cake. It is important to keep the chamber pressure under the saturation vapor pressure of ice during the whole process to facilitate the sublimation of ice. Lower pressure increases the vapor pressure difference between the condenser and ice but decreases remarkably heat flow through convection, which slows down the sublimation.

Van Bockstala et al. (2018) studied following Good Modeling

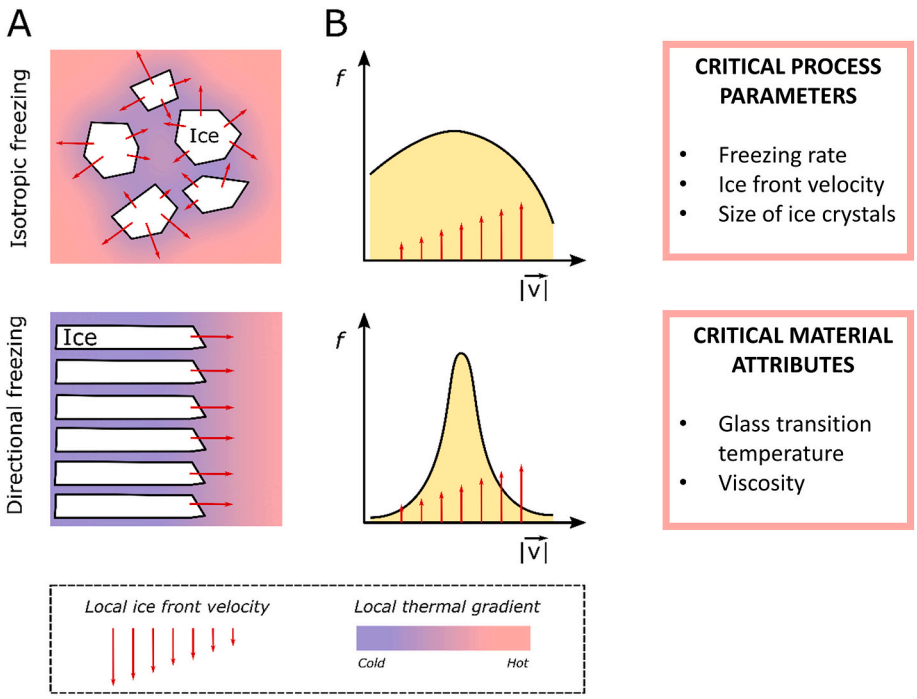


Fig. 3. The boundary conditions applied during isotropic or directional freezing determine the local thermal gradient and the distribution of local ice front velocities. A) Schematic representation of the local thermal gradient developed during isotropic and directional freezing. The purple to salmon background indicates the thermal gradient and the red arrows depict the direction and local ice front velocity. B) Simplified representation of the ice front velocity distribution depending on the type of freezing conditions. Examples of critical process parameters and material attributes in the freezing step are listed right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

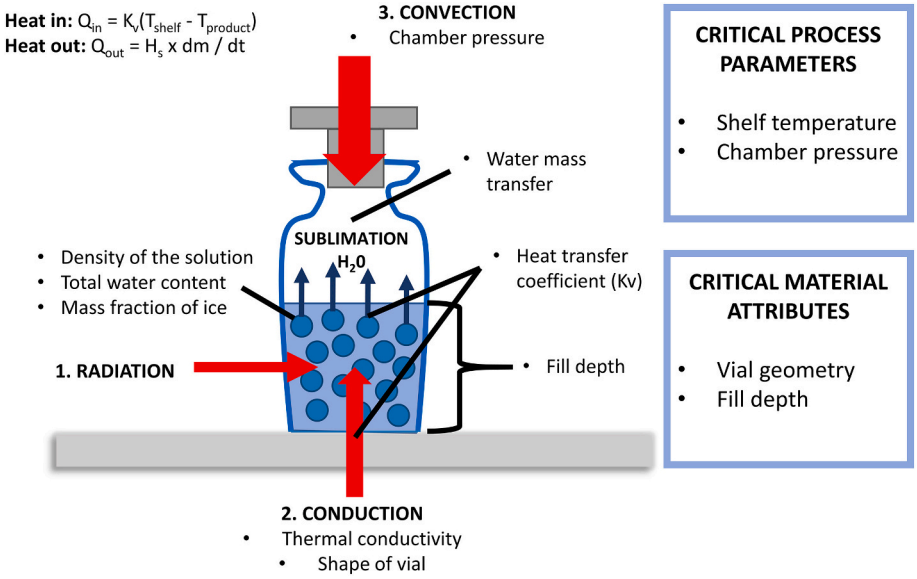


Fig. 4. The heat flow (red arrows), mass transfer (blue arrows), and factors affecting the time required for sublimation (bullet points) during primary drying. Heat-flow by convection and conduction vary depending on the pressure in the freeze-drying chamber and geometry of the freeze-drying vial, respectively. Examples of critical process parameters and material attributes in the primary drying step are listed right. Q_{in} = Energy supplied for sample. K_v = heat transfer coefficient from shelf to the sample. T_{shelf} = Shelf temperature. $T_{product}$ = product temperature. Q_{out} = Energy required to sublimate water. H_s = latent heat of sublimation. dm / dt = mass transfer of ice over time. Modified from Franks 2008 [8] and Pikal et al. 1984 [9]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Practice the most important process parameters during primary drying [38]. They used Global Sensitivity Analysis (GSA) to take into account uncertainty process parameters. They used both a regression-based and variance-based GSA in their mechanical CFD model input parameters on the output parameters of sublimation rate and the temperature at the sublimation front. Models were validated with DoE. They concluded that the mechanical CFD model together with GSA were in good agreement with experimental DoE validation studies. The advantage of this kind of approach is that uncertainty can be taken into account in models and consequently the Risk of Failure, for example, cake collapse, can be calculated. Another advantage of mechanistic modeling with uncertainty estimation is that since freeze-drying is a transient process, dynamic DS can be established. Earlier Mortier et al. (2016) has used the same approach and they highlighted that the Risk of Failure acceptance level 0.01% yielded zero defects in the quality of end-product [39].

Shelf temperature and chamber pressure can be recognized as CPP and vial geometry fill depth as CMA when applying QbD for the primary step of freeze-drying. In addition, as described in the previous chapter, valuable information can be obtained by GSA also when freeze-drying biomaterials and cells. Furthermore, with correct risk assessment, the risk of collapse of biomaterials and cells during primary drying, can be reduced.

2.3. Secondary drying and storage: Removing unfrozen water and storing vials

Typically, secondary drying aims to remove any unfrozen water still present in the sample (Fig. 5) [18]. Although water is usually present as a hydrate in crystalline hydrate or an amorphous solid, the amount of unfrozen water can still be remarkable (20–50%) particularly for some

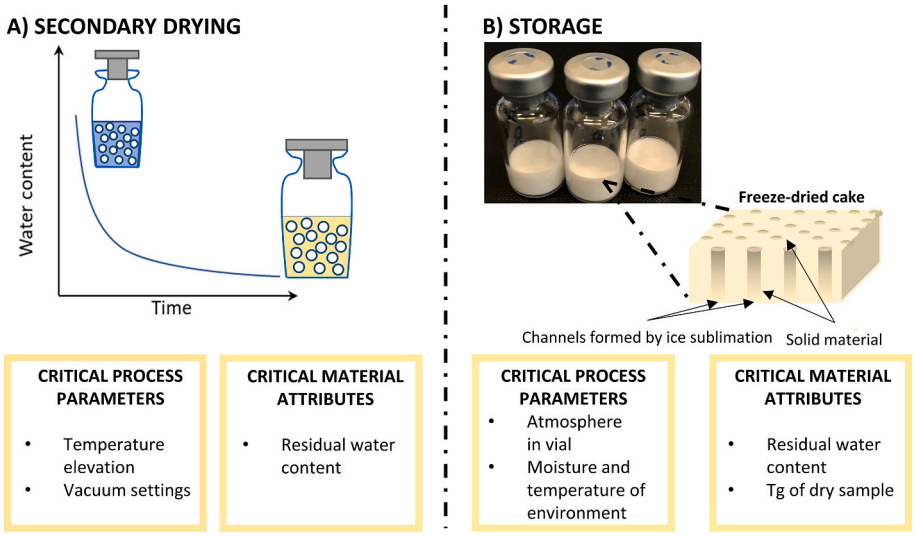


Fig. 5. A) During secondary drying the non-frozen water of the sample is removed with elevated temperature in a freeze-drying chamber. The residual water content of biomaterials and cells must be optimized because the driest possible sample is not always the best. Critical process parameters and critical material attributes in the secondary drying step are listed below. B) The aim of freeze-drying is to obtain an elegant freeze-drying cake that can be preferably stored at room temperature and which is rapid to reconstitute. Critical process parameters and critical material attributes in the storage step are listed below.

amorphous formulations [8]. Secondary drying can be performed at elevated temperatures because the ice has been removed during the primary drying and the risk of melting or collapsing is minimal. However, especially with the amorphous materials, temperature elevation is preferably performed with a slow ramp ($\leq$ 1 °C/min) to avoid collapsing. The residual water contents of samples after secondary drying are generally recommended to be 0.5–3% [8], however, some biological samples require higher residual water contents to be reconstitutable [40]. The role of residual water is discussed more in chapter 5. The secondary drying is also a critical step to optimize due to practical influence on the final product. As an example of the process optimization in secondary drying, Assegehegn (2020) created the DS for optimal residual water content (1.3–1.8% (*w*/w)) and the Tg (+60–68 °C) of dry end product using DoE [41]. Their input variables were secondary drying temperature and time, and vacuum setting. They concluded that a high vacuum setting (0.05 mbar) increases the desorption rate of water. Despite the improved heat transfer with a lower vacuum (higher chamber pressure), the desorption rate is lower, due to increased vapor flow through the pore structure which decreases the drying rate significantly. The most optimal secondary drying temperature and time were + 37 °C and 12 h, respectively. Verification run with these settings confirmed that desired output values were achieved. Optimal residual water content is extremely important to successfully reconstitute freeze-dried biomaterials and cells and creating a DS for optimal residual water content would be useful. In secondary drying, the rate of temperature elevation and vacuum settings can be recognized as CPP and residual water content as CMA.

Finally, after secondary drying, the vials are closed in the desired atmosphere which is usually dry nitrogen. Storage temperature for the freeze-dried samples should be below their glass transition temperature (Tg) where their stability is higher due to lower mobility and no collapsing occurs [42]. For economical and practical reasons, product storage at room temperature can be considered one of the driving reasons to freeze-dry material. During storage, the moisture and temperature of the surroundings and atmosphere in the vial can be considered as CPP, while residual water content and followed by that Tg of the dry sample, can be recognized as CMA.

3. Changes in chemical, physical and mechanical environments during freeze-drying

Freezing of water, and sublimation of ice are the most important phase transitions of water during freeze-drying. They affect the chemical, physical, and mechanical environments of the biomaterials and cells. Changes in the previously mentioned environments can be fatal unless addressed by choosing for example the correct excipients, freezing method, and freeze-drying cycle. In this chapter we discuss and review the chemical, physical, and mechanical environments around the cells and biomaterials during the freeze-drying and how to prevent the damage caused by changes in the environments.

3.1. Chemical environment

The chemical environment is composed of the excipients and solvents used in the freeze-drying process. In addition to protective and bulk-forming agents, excipients are required to control the physicochemical properties of the formulations such as pH, osmolarity, and ionic strength, with buffers and tonicity modifiers. Furthermore, the biomaterials and cells themselves affect the chemical environment.

During the freezing step of freeze-drying, the concentrations of solutes increase as the volume of the solvent decreases (Fig. 6). This results in chemical gradients between the cells or biomaterials and their external solution. In the common case where the solvent is water, its activity will alternate throughout the freezing and drying steps, resulting in a chemical environment with extremely limited water activity. In addition, the transition of the solvent molecules from the liquid to the solid phase may promote solute concentration and subsequent precipitation caused by reaching its solubility limit [43]. However, the precipitation during the process happens seldom and it can be easily avoided by choosing the correct excipients. The freeze concentration may affect the pH, in particular, if buffer components precipitate [43]. However, as with other solutes, this phase separation during freeze concentration is rare. Other concerns related to the shifts in pH rise from choosing a volatile buffer. If the chosen buffer, such as acetic acid, is volatile and the buffer sublimates during the primary drying, changes in the pH of the sample will be observed after reconstitution.

Freeze-drying affects the chemical bonds, such as hydrogen bonds, between the active ingredient, solute, and solvents. Removing the water and thus the hydrogen bonds water has formed with the biomaterial or cells, can result in not reconstitutable samples if not addressed by excipients.

3.2. Physical environment

By physical environment, we describe the physical parameters which change during freeze-drying. Such parameters are thermal gradients, osmotic pressure, and pressure caused by the ice during freezing (Fig. 6).

Especially cells and cell-derived biomaterials, such as extracellular

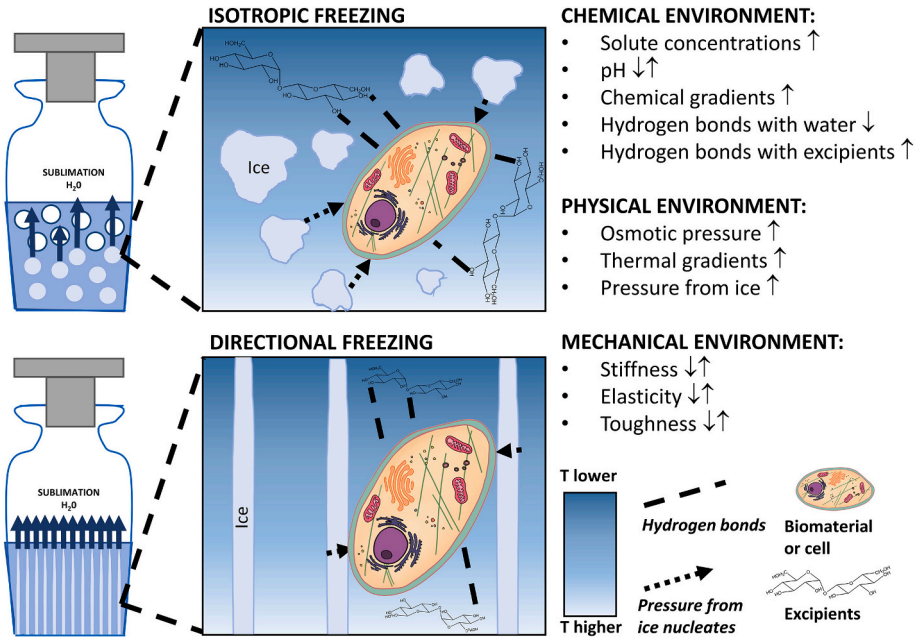


Fig. 6. Changes in chemical, physical, and mechanical environments around the biomaterials and cells during freeze-drying. Changes in the chemical environment are related to concentration changes during freezing and drying. The solute concentration increases during freezing when the amount of liquid water decreases. This affects also pH and hydrogen bonds formed with the biomaterial or cells and water. In the physical environment osmotic pressure increases during freezing and there will be thermal gradients during the whole freeze-drying cycle. The mechanical environment describes how the mechanical properties of biomaterials and their exterior change during freeze-drying. Changes in the mechanical environment are hard to analyze during the freezing and drying, but the arrows indicate that the mechanical properties can vary in any direction after freeze-drying if the freeze-drying and reconstitution were unsuccessful. These changes can have dramatic results in the applicability of the biomaterials or the survival of the cells.

vesicles (EVs), require isotonic conditions. Due to osmosis, the water will flow out of cells, if the extracellular environment is hypertonic and the cells will shrink. Conversely, if the extracellular environment is hypotonic, the water will flow inside the cells and the cells will swell. During freeze-drying, especially in the freezing step, there will be drastic changes in osmotic pressure that can damage the cells and induce denaturation of proteins [43]. Even within the slow freezing domain, different cooling rates promote different osmotic imbalances leading to the characteristic bell-shaped curves of cell viability vs. cooling rate [44].

Thermal gradients will occur during the freezing step and drying step [8,9]. Thermal gradients can cause challenges in controlling the temperature which can result in cryodamage for the biomaterials and cells. Furthermore, strong thermal gradients can lead to collapsing of the sample if the collapsing temperature is exceeded.

Pressure induces apoptosis and necrosis at 200 MPa and 300 MPa in mammalian cells, respectively [45]. Avoiding pressure-induced apoptosis and necrosis can improve the recovery of cells after freeze-drying and reconstitution. Controlling the ice front velocity is one way to maintain the pressure caused by ice nucleates below the critical pressure [35]. Controlling the geometry of the thermal gradient applied can also help to address problems related to the forces exerted during freezing.

3.3. Mechanical environment

Mechanical properties, such as the stiffness of the material can change during freeze-drying (Fig. 6). These changes determine the final form and elastic properties of the freeze-dried biomaterials. When cells are present, these changes may be dramatic since the biological response of cells may be directly linked to the surrounding mechanics [46–48]. Changes in the mechanical environment may impair the growth of cells – therefore proper formulating and control over the freezing and drying steps are important to counter changes in the mechanical properties of the freeze-dried samples.

To summarize, we believe that the preservation of complicated natural biomaterials and cells by freeze-drying requires a wide understanding of the chemical, physical, and mechanical environments surrounding the subject of interest. It is vital to not only adjust each environment individually but to consider how changes in one affect all of them. Until now, the trial and error approach is prevalent, but we suggest that more careful design and consideration are fundamental to succeed in the freeze-drying of biomaterials and cells with even better results.

3.4. Role of excipients in different environments

Saccharides, polysaccharides, proteins, and alcohols have different properties that might pave the way for success in freeze-drying (Table 1). The excipients can be categorized as stabilizers and bulking agents and act mainly as kinetic stabilizers or as thermodynamic stabilizers [49]. The idea of kinetic stabilization also referred to as the glass dynamic hypothesis, explains that excipients form a highly viscous amorphous matrix that reduces the rate of degradation reactions. Thermodynamic stabilization also referred to as the water replacement hypothesis, states that excipients form hydrogen bonds that replace the hydrogen bonds water had with the samples. However, neither of these theories alone fully explains, how excipients protect samples, which underlines the importance of considering both theories in the freeze-drying process.

Trehalose is the most common excipient in the freeze-drying of biomaterials and cells, and it can be used as a bulking agent and as a stabilizer in freeze-drying systems. Trehalose (MW = 378.33 g/mol, dihydrate) is a non-reducing disaccharide formed by two glucose units that are linked together by α-(1 1)-linkage. Tg' of trehalose and water solution is −28 °C [8], and Tg of pure, dry, amorphous trehalose varies between 75 and 120 °C [50]. Variation is observed probably due to different residual moisture contents in trehalose. Trehalose is more commonly found as dihydrate than anhydrate. Trehalose is highly hydrophilic (Log P -3.77, T = 20 °C) [51], which can make the use of trehalose as a stabilizer for cells challenging.

Trehalose forms a highly viscous matrix during freeze-drying and provides hydrogen bonds to substances being freeze-dried. Trehalose is used by organisms that can tolerate desiccation [52]. Contrary to reducing sugars, such as lactose and glucose, non-reducing sugars do not react through the Maillard reaction (browning reaction) with basic amino acids of proteins which makes non-reducing saccharides more attractive for freeze-drying processes [49]. However, the potential of the Maillard reaction does not prevent the usage of reducing sugars as excipients.

Table 1
Summary of the number of the publications found so far and the chemical properties of the natural origin excipients used in the freeze-drying of extracellular vesicles (EVs) and cells. For a comprehensive list of excipients used in the freeze-drying of proteins and small molecules, please refer to Gervasi et al. (2018) and Baheti et al. (2015) [53,54]. Data obtained from PubChem, FooDB, and polymerdatabase unless otherwise stated.

Excipient	N:o of art	Group	MW	HBD	HBA	PSA	pKa	LogP	Tg' (water solution)	Tg
Trehalose dihydrate	17	Disaccharide	378.3	10	13	192	11.91	-3.77	−28 [8]	75–120 [50]
HSA, BSA	*5*	*Protein*	*~66 kDa*				/	/	/	/
Mannitol	3	Sugar alcohol	182.2	6	6	121	13.5	−3.1	/	13 [55]
Sucrose	3	Disaccharide	342.3	8	11	190	12.6	−3.7	−32 [8]	52–76 [50]
Glycerol	2	Alcohol	92.1	3	3	60.7	14.4	−1.76	−109.6 [56]	−93 [57]
Dextran	2	*Polysaccharide*	>1000 da	11	16	277	11.78	−3.30	−10 [54]	61.7–101.7 [58]
Sodium Alginate	1	*Polysaccharide*	216.1 (monomer)	4	7	130	3.21	−1.9	−80 [59]	/
EGCG	1	Polyphenol	458.4	8	11	197	7.99	2.38	/	163 [60]
Glucose	1	Monosaccharide	180.2	5	6	110	12	−3.1	/	21.9 [61]
Lactose	1	Disaccharide	342.3	8	11	190	11.25	−3	/	84
Maltose	1	Disaccharide	342.3	8	11	190	11.25	−3	−31 [62]	/
NFC	1	*Polysaccharide*	162.1 (monomer)	/	/	/	/	/	/	/
HES	1	*Polysaccharide*	580.6 (monomer)	17	17	267	/	/	/	/
PVP	1	*Polyetherketone*	111.4 (monomer)	0	1	20.3	/	/	/	169.9
DMSO	1	Sulfoxide	*78.14*	0	2	36.3	35.1.	−1.35	−120 [63]	/
PEG	1	*Polyglycol*	44.05 (monomer)	2	2	40.5	15.1	−1.36	/	

MW: Molecular weight; HBD: Hydrogen bond donors; HBA: hydrogen bond acceptors; PSA: Polar surface area; HSA: Human serum albumin; BSA: Bovine serum albumin; EGCG: Epigallocatechin gxallate; NFC: Nanofibrillated cellulose; HES: Hydroxyethyl starch; PVP: Polyvinylpyrrolidone; DMSO: Dimethyl sulfoxide; PEG: Polyethylene glycol

Biopolymers act in general as bulking agents in the freeze-drying process. Biopolymers may also have a positive impact on the critical temperatures, mainly Tg' and Tg, of the freeze-drying formulations making their usage justifiable.

Human serum albumin (HSA) and bovine serum albumin (BSA) are proteins, and they are widely used excipients in the freeze-drying of cells. Albumin of either origin is commonly present also in the cryopreservation media of cells, as media is supplemented generally with fetal bovine serum or human serum. Considering the freeze-drying, albumin increases the Tg of dry samples which makes the storage of freeze-dried biomaterial samples or cells feasible at room temperature [64]. However, the protective mechanism of albumin is still unclear.

Mannitol is a sugar alcohol that is widely used as a bulking agent in freeze-drying formulations. It crystallizes during the freeze-drying process and provides mechanical strength and improves the elegancy of the freeze-drying cake.

Dextran is a branched polysaccharide formed from glucose monomers. Dextran is a commonly used excipient in the freeze-drying of small molecules as a bulking agent and as Tg' modifier because it has a relatively high Tg', −10 °C [54]. Dextran has been also studied in the freeze-drying formulation and a rehydration solution with cells [65,66].

Glycerol is a polyol and commonly used cryoprotective agent which prevents the formation of harmful ice crystals. Glycerol might prove useful especially in the freeze-drying of the cells. However, glycerol decreases Tg' especially with high concentrations hindering its usability in freeze-drying.

DMSO is a sulfoxide and the most commonly used cryoprotectant with the cells. DMSO prevents intracellular and extracellular ice crystal formation and protects the cells from the damage caused by freezing. However, DMSO decreases Tg' and causes similar problems in freeze-drying as glycerol [63]. In addition, DMSO is toxic to cells that cause problems in clinical applications, and requires washing of the cells after preservation.

Polyvinylpyrrolidone (PVP) has been used in the freeze-drying of red blood cells as an excipient [67]. PVP decreased the formation of ice crystals and increased the extent of vitrification during freezing. However, high PVP concentration damaged the cells probably due to high osmotic pressure and decreased the recovery of intact red blood cells. Polyethylene glycol (PEG) can prevent the damage caused by ice crystals and improve the uptake of water in the reconstitution after freeze-drying. However, with EVs PEG caused aggregation potentially due to interactions with the EVs [68].

Nanofibrillated cellulose is a biomaterial produced from the nature origin polysaccharide cellulose which is formed from glucose monomers. The fibers of nanofibrillated cellulose have a high length to width ratio: width is on the nanometer scale and length in the micrometer scale. Nanofibrillated cellulose forms highly viscous hydrogels with water and it has a wide range of applications in the biomedical field. This biomaterial can be used for example for 3D cell culturing [69]. We studied the potential of nanofibrillated cellulose as an excipient in the freeze-drying of 3D cultured cells and showed that nanofibrillated cellulose provides mechanical strength and acts as a bulking agent in the freeze-drying process and during storage [70]. In addition, nanofibrillated cellulose acts as hygroscopic material [71] which improved the uptake of water at the reconstitution phase.

4. Analysis of freeze-drying process and environments with biomaterials and cells

In the previous chapter, we have introduced the concept of different environments and changes in them during freeze-drying. However, recognizing these environments is not enough, but also understanding how to analyze them, is important. We have divided the analysis of the whole freeze-drying process into three major steps: 1) designing formulation, 2) optimizing the freeze-drying cycle, and 3) evaluating product during storage.

Traditionally, the formulation, freeze-drying cycle, and preservation of the sample have been studied offline before and after the freeze-drying process. We suggest that the utilization of process analytical technology (PAT) is required in order to freeze-dry the biomaterials and cells with a higher yield. PAT is defined by European Medicine Agency [72] and the Food and Drug Administration [73] as a *system for designing and controlling manufacturing through timely measurements* (i.e. *during processing) of critical quality and performance attributes for raw and in-process materials and also processes with the goal of ensuring final product quality*. Recently, vibrational spectroscopic techniques as Raman and infrared (IR) spectroscopies have gained interest as PAT tools to characterize the freeze-drying product and freeze-drying cycle. Vibrational spectroscopic techniques are non-invasive, widely applicable, low-cost, quick time-to-result analytical methods. Although vibrational spectroscopic techniques provide a set of powerful tools to describe samples during the freeze-drying cycle, they are often limited to an average image with poor local resolution. This is one reason why in situ analyses are as well performed to obtain comprehensive information on the freeze-drying process. The most important methods and instruments that are potential for characterizing different environments at different

steps are summarized in Fig. 7.

4.1. Analysis of the formulation before freeze-drying

The formulation should protect the biomaterials and cells from the harmful changes in the different environments during freeze-drying. Adjusting the formulation to be suitable for the biomaterials and cells and choosing the excipients to protect them during the freeze-drying and storage, are the most important steps in the formulation phase.

Even though sounding self-evident, basic characterization of the formulation is important. For the biomaterials and cells, measuring the osmolality of the formulation with an osmometer, such as freezing point depression osmometer, and pH with the pH meter are extremely important. The mechanical properties of the formulation, such as stiffness and elasticity, can be determined with a rheometer and optimized for the desired application. In the formulation phase, sample-specific reference measurements, such as the viability of cells or the viscosity of hydrogel, are recommended. Then after freeze-drying and reconstitution, the success of the freeze-drying process can be evaluated by comparing the results to the unprocessed sample.

The formulation affects not only the biomaterials or cells but also design of the freeze-drying cycle. In other words, the formulation can be optimized to be suitable for the freeze-drying cycle for example by adjusting excipients to increase Tg'. Commonly, designing the freeze-drying cycle includes the utilization of characterization instruments to analyze temperatures necessary for the process [74]. Differential scanning calorimetry (DSC) and freeze-drying microscope (FDM) can be used to analyze important phase transition temperatures of the sample before freeze-drying [75]. DSC is used to analyze changes in the crystal structure, eutectic formation, and amorphous characters of the formulation [74,76]. FDM consists of a microscope attached to a freeze-drying chamber and it is designed to analyze important phase transition temperatures for freeze-drying [77,78]. The advantage of FDM is the possibility to detect a specific collapse temperature of the formulation and to evaluate in real-time the sublimation rate of the ice. In addition, the effects of crystallization and annealing on the formulation can be recorded.

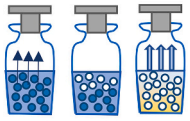
4.2. Analysis of biomaterials and cells during the freeze-drying cycle and its steps

After starting the freeze-drying cycle, the temperature of shelves in the freeze-drying chamber, condenser temperature and sample temperature must be recorded. In addition, following the pressure inside the

FORMULATION

ENVIRONMENT	*Chemical*	*Physical*	*Mechanical*
PROPERTY	1. Osmolality 2. pH	1. Freezing point 2. Tg'	1. Stiffness 2. Elasticity 3. Toughness
METHODS AND INSTRUMENTS	1. Osmometer 2. pH meter	1. DSC/FDM 2. DSC/FDM	1-3. Rheometer

FREEZE-DRYING CYCLE



ENVIRONMENT	*Chemical*	*Physical*	*Mechanical*
PROPERTY	1. Water content	1. Temperature 2. Pressure in chamber 3. Physical state of sample	
METHODS AND INSTRUMENTS	1. NIR spectroscopy	1. Thermometer 2. Manaometer 3. Raman spectroscopy	

STORAGE

ENVIRONMENT	*Chemical*	*Physical*	*Mechanical*
PROPERTY	1. Osmolality 2. pH 3. Residual water content	1. Tg	1. Porosity 2. Pore size 3. Stiffness 4. Elasticity 5. Toughness
METHODS AND INSTRUMENTS	1. Osmometer 2. pH meter 3. KF titrator, TGA, NIR spectroscopy	1. DSC	1-2. SEM /cSAX/ microtomography 3-5. Rheometer

Fig. 7. Characterization methods suitable for detecting changes in the chemical, physical, and mechanical environments during different phases of the freeze-drying process. First, analyzing properties of the formulation categorized into different environments and optimizing the formulation. Second, detecting changes in the environments during the freeze-drying cycle and optimizing the freeze-drying cycle. Third, observing changes in the environments during storage.

freeze-drying chamber and condenser is required. Nail et al. (2017) have presented recommended best practices for process monitoring instrumentation in pharmaceutical freeze-drying and they introduce common methods to follow the temperatures and pressures during the freeze-drying cycle with different thermometers and manometers, respectively [79]. However, in this review, in situ methods and biophotonic label-free detection methods are introduced to gain more detailed insights about the freezing step and how to study the samples during the freeze-drying cycle.

4.2.1. *In situ analysis of freezing step*

In situ analysis of the freezing process elaborates in detail, how the samples are *locally* influenced during freezing. In this context, *locally* means it is possible to discriminate between ice-rich zones and zones formed in between the ice crystals.

The challenge in characterizing the samples locally during freezing is that it requires a particularly controlled freezing process. Ishiguro and Rubinsky have addressed this problem using a directional freezing setup [33]. In their report, they investigated the mechanical interactions developed during freezing red blood cells by placing a glass slide containing a cell suspension over a controlled thermal gradient. Pushing the glass slide over the thermal gradient enabled to visualize the growth of ice crystals and their interaction with the suspended cells. While it was impossible to withdraw quantitative data from the local analysis, the observation of microscopy images allowed to establish the initial relationship between the ice front velocity and the fate of cells after freezing. In particular, the distribution of cells between the interstitial zones i.e. in between ice crystals and trapped within ice crystals seemed particularly sensitive to the ice front velocity. This approach was recently extended to probe the interaction of suspended droplets and particles with a moving freezing front [34,80]. By coupling a confocal microscope to a controlled temperature gradient, the authors were able to describe the interaction of the suspended particles with the moving ice front as well as the compaction occurring laterally by the ice crystals over the segregated zones. The same setup was later applied to study the interaction between living yeast cells and the moving ice front on alginate solution. The image analysis of the segregated zones, coupled with calorimetric data, allowed to describe the evolution of the solute concentration along the ice growing direction, establishing experimentally the local concentration evolution endured by cells during freezing [24]. As with previous reports within this section, this work highlighted the primordial role of ice front velocity in the cells' local environment and survival. Interestingly, a relatively modest change in ice front velocity from 10 to 50 μm s^{-1} resulted in a drop in local alginate concentration in between ice crystals from 40 to 20 wt%. While these changes in ice front velocity did not change the distribution of cells between the interstitial space and ice crystals it dramatically impacted cell viability.

A more fundamental take on the local environment produced during freezing has been unveiled using time-resolved Small Angle X-Ray Scattering on synchrotrons. By studying the self-assembly of amphiphilic molecules in the liquid phase during freezing, Albouy and colleagues were able to describe the self-assembly changes occurring below 0 °C [81]. Starting with a P123 copolymer in solution – in between the beam and the detector – and upon application of a thermal gradient, the authors could show that the concentration variation imposed by the freezing front determined an evolution of amphiphilic molecules from a diluted, disordered state to form, consecutively micelles, an hcp phase of micellar aggregates, and reaching eventually a 2D-hexagonal phase of cylindrical micelles. Although P123 molecules are not strictly comparable to the molecules that define many of the biological membranes such as phospholipids, the ability to modulate the arrangement of systems directed by weak interactions during freezing, raises important questions on the stability of biological structures such as lipid vesicles during freezing. Recently, Baccile et al. have reported the study of lipid lamellar phases during ice templating using a comparable freezing setup. The particularity of the chosen lipid phase consisted of large changes in a lamellar period that varied with the local pressure in between the lipid lamellae [35]. Measuring the variations in the lamellar period provided instrumental in quantifying the internal pressure formed in between ice crystals during freezing. Their results describe, for the first time, the remarkably high-pressure values (between 1.5 and 3 kbar) endured during freezing in absence of standard cryoprotection agents.

Despite their fundamental nature, and as a consequence, despite their distance to the actual freeze-drying conditions, these results provide much-needed guidelines to understand the multiple phenomena occurring during freezing. The results are especially relevant when it comes to systems whose integrity depends on weak interactions as with many biologicals. It is worth pointing out that in all cases, the strict definition of a thermal gradient is necessary to ensure the whole freezing process is dully observed. An extension of these principles to the whole freeze-drying process would certainly clarify many of the underlying modifications occurring throughout the multi-step process and establish full complementarity with the PAT analysis.

4.2.2. *Raman and near-infrared spectroscopies in monitoring of freeze-drying cycle*

In recent years, Raman and near-infrared (NIR) spectroscopies have been applied in-line in freeze-drying gaining profitable information [82,83] for the characterization and the monitoring of freeze-drying of protein, liposomes, active pharmaceutical ingredients (API), and excipients (Fig. 8**).** Both techniques display unique advantages; both are label-free, fast, non-destructive, and operator-independent.

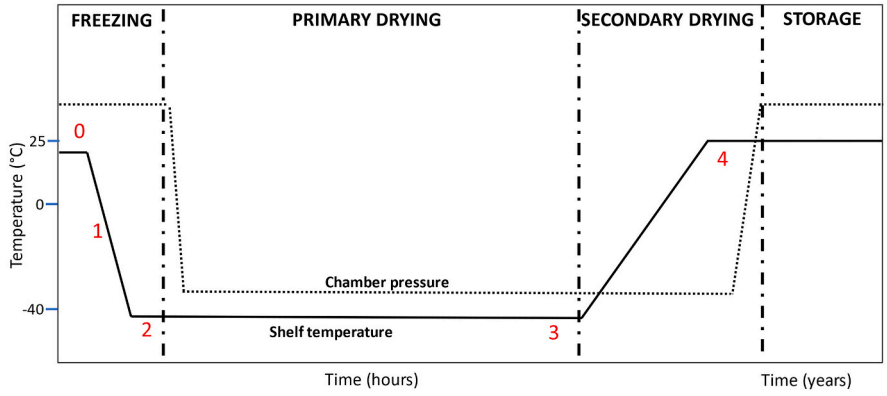
Raman spectroscopy is based on Raman scattering, a 2-photon process, where a photon emitted by a source of monochromatic light interacts with the sample. This interaction increases the virtual energy state for a short period before the second photon with either lower or higher energy is emitted. Raman spectroscopy has been successfully applied as an in-line PAT tool to monitor the starting of ice nucleation and product crystallization, freezing and primary drying endpoint, and the evolution of the product solid state during the entire process. The second derivative of the amide III (1200–1380 cm-1) region of the Raman spectra can underline how the water content affects the freeze-drying cycle as well as the stability of protein and, potentially it can be used as a tool for long term stability studies [84].

Infrared spectroscopy is based on the interaction of infrared radiation with matter, where the frequency of the radiation absorbed by the molecules matches the vibrational frequency. Near-infrared (NIR) signal (10,000–4000 cm-1) originates from the excitations of higher quanta transitions, mostly first overtones and binary combinations.

NIR spectroscopy reveals little chemical composition information, but it is sensitive to a multitude of compounds and molecular interactions. NIR spectroscopy outperforms Raman spectroscopy when it comes to the monitoring of the drying process and is preferred in monitoring the drying process and H-bond formation [85]. NIR spectroscopy is especially useful when monitoring the water contents in the samples and it can be used to quantify the residual water content.

Overall, in-line Raman spectroscopy appears to be superior to NIR spectroscopy regarding the detection of endpoint of water to ice conversion, the physical state, and the onset of API and excipients crystallization. However, it appears obvious that NIR and Raman spectroscopies are complementary techniques and the simultaneous use of both techniques during the monitoring of freeze-drying is the best solution for the process control.

It is worth noting that besides the above-described advantages, NIR and Raman spectroscopies have some limitations. Firstly, freeze-drying is a stochastic process. Inhomogeneity on the temperature of vials and, thus, ice nucleation has been observed. Therefore, controlling the process by data obtained from only one vial can be misleading [85]. Secondly, NIR and Raman spectroscopies can interfere with each other if placed on the same vial, especially during the primary drying where the frozen product reflects the NIR radiation that can interfere with Raman



N:o	Event	Major quality attribute
0	Liquid formulation before freeze-drying	**Raman**: Aqueous spectra
1	Ice nucleation	**Raman**: Ice crystal formation at 215cm^{-1}
1-2	End of the ice crystallization Potential product crystallization	**Raman**: Spectra of amorphous form. Spectra ascribable to specific polymorphs (polymers) or secondary structure (proteins) **NIR**: Ice signal overhelm the product signal
2-3	Ice crystal sublimation	**Raman**: Signal at 215 cm^{-1} decrease. Spectra is stable at the end of primary drying (temperature and pressure) **NIR**: Ice signal decrease and product band appears
3-4	Desorbtion of unfrozen water	**Raman**: Absorbed water signal is too weak to be quantified **NIR**: Evolution of the spectra confirm the progression of the secondary drying. Residual water content can be quantified. After the secondary drying the spectra is stable

Fig. 8. Raman and NIR/FTIR spectroscopies can be applied in-line as possible process analysis technologies (PAT) in the freeze-drying process. Numbers correspond to different steps in the freeze-drying cycle.

scattering and its detection resulting in a saturation of Raman spectra [83]. Thirdly, information obtained from NIR and Raman spectroscopies is affected by where the light source is focused. This can result for example in late detection of ice sublimation if the focus is on the bottom of the vial. Moreover, IR beam illuminates in most cases a large part of the vial, which can explain why it is difficult to discriminate product crystallization and the end of the water to ice conversion, while Raman spectroscopy, with a smaller focal point, detects events on a specific area. Furthermore, the NIR beam can penetrate through the samples gathering information on the inner composition.

The above-mentioned issues can be partially overcome by a careful experimental design. Nowadays, a multichannel NIR spectrometer allows the operator to obtain information simultaneously by different vials [86,87]. Multichannel Raman spectrometers have been also developed although they have not been used in freeze-drying yet [88].

4.3. *Analysis of freeze-dried biomaterials and cells during storage*

After the freeze-drying cycle, the obtained product must be characterized to ensure successful freeze-drying. In addition, monitoring the products during the storage periods, preferably with non-invasive methods, can be profitable.

Successful freeze-drying is ensured by measuring the residual water content of the sample with for example thermogravimetry, Karl Fischer titration, or gravimetric scaling based on loss on drying. Residual water plays a major role in the freeze-drying of biomaterials and cells and the standard guidelines of approximately 0.5–3% of residual water may not be valid [40]. Monitoring residual water content after freeze-drying and during storage can be also performed with NIR spectroscopy [86]. Gravimetric scaling and NIR spectroscopy can be performed without destroying the sample. However, Karl Fischer titration or thermogravimetry can be more precise methods and they can be used for example for calibration and validation of NIR spectroscopy [86].

In the future, applying a mathematic model to predict residual water contents from the obtained data, would be highly beneficial to improve the success of freeze-drying of biomaterials and cells. Before we can apply the prediction to the actual process, we have to understand 1) the optimal residual water concentration and 2) where the water is precisely located i.e. is the water bound water of the membranes and proteins or bulk water in the protective matrix. Before setting up a mathematical model, in-line residual water content monitoring with NIR provides valuable information [86].

Another important characterization method for freeze-dried biomaterials and cells after freeze-drying is scanning electron microscopy (SEM) [89]. SEM can be utilized to evaluate the morphology and surface structure of the freeze-dried samples. However, SEM is an invasive method, and the samples are destroyed after the characterization.

Vibrational spectroscopy techniques can be applied to optimizing the long-term storage conditions of the freeze-dried samples. Raman spectroscopy can be applied to evaluate the secondary structure of proteins, H-bonding, and the physical status of the samples during storage. With NIR spectroscopy information about the physical status and water absorption are obtained. In addition to the NIR, Fourier-transform infrared spectroscopy is utilized to detect water absorption and protein structure.

Taken together, traditionally utilized devices, such as DSC and FDM, and tools to follow the freeze-drying cycle, such as manometers and thermometers, are fundamental. However, combining the already proven equipment with advanced biophotonic tools and in situ analysis of biomaterials and cells, is necessary.

5. State of the art in freeze-drying of biomaterials and cells

The relevance of biomaterials in tissue engineering, drug delivery devices, or as cell culture platforms for toxicology studies along with the

present increase in cell therapies has sparked a need for newer, more robust ways to shape, preserve and vectorize these material-cell-assemblies [90,91]. One particularly attractive system is the one containing extracellular vesicles (EVs). EVs have recently gained a lot of attention as they have potential applications as drug carriers even across the blood-brain barrier, as biomarkers, or as therapeutics themselves [92–94]. However, there are still obstacles to overcome, such as storage and transportation, especially considering the natural biomaterials, such as EVs, and cells. In this chapter, we review biomaterial scaffold fabrication by freeze-drying and preservation of EVs and cells by freeze-drying.

5.1. Non-living scaffold materials

Macroporous biomaterials elaborated via freeze-drying of biopolymers have gained considerable attention in surgical procedures for their role as hemostats [95] and bone defect fillers [96]. An important number of these materials is nowadays commercially available and validated by different national and international drug agencies [97]. Also, hybrid formulations, that combine biopolymers and hydroxyapatite (and other inorganic moieties) have been freeze-dried to obtain macro-porous composite materials relevant in the context of bone replacement [98–101]. Despite their satisfactory biocompatibility properties, little control over the porosity of the material i.e. pore size distribution, orientation, etc., was evidenced. The need for further textural control in freeze-dried biopolymers was realized by reports suggesting that the morphology of pores could be a determinant factor in tissue growth [102]. The integration of ice templating, formerly described for the fabrication of lightweight ceramics, in the elaboration of biomimetic materials [103] and biomaterials [27,104,105] opened a pathway to further increase the control over the texture of macro-porous biomaterials [25]. The aligned porosity enabled effective liquid transport properties [106] when compared to randomly oriented porosity developed by isotropic freezing [107,108]. These materials favor cell colonization [109] nutrients exchange, and, importantly, due to facilitated gas diffusion, they provide simple platforms to overcome the oxygen diffusion limitations that hinder extensive cell colonization in hydrogels. The list of ice-templated biopolymers used for 3D cell culture is vast, ranging from type I collagen [110] to gelatin [111,112] and numerous polysaccharides [113,114].

Beyond the specific biological response to biopolymer hemostatic foams, bone filling materials, or 3D cell culture systems, freeze-dried and ice-templated foams display particularly efficient mechanical behavior, making them particularly suitable for applications where lightweight yet robust behavior is required [115]. Changing the chemical, physical, and mechanical environments before freeze-drying results in different scaffolds (Fig. 9).

5.2. Freeze-drying extracellular vesicles improves their clinical usability

EVs are nano-sized (< 1000 nm) biological vesicles that are produced by all cells according to current understanding [116]. They participate in a myriad of actions in normal physiological or pathological events [117]. The use of EVs in diagnostics and as drug delivery vehicles are currently highly appreciated research topics [118–120]. EVs could be used as carriers for targeted drug delivery of small drugs, RNA, and viruses [121–123]. Liposomes are the closest artificial nanoparticles to EVs and their successful freeze-drying remains a topic of interest in the pharmaceutical field. Freeze-drying can be utilized to preserve liposomes in dry form at room temperature or to formulate them for different administration routes. Freeze-drying of liposomes has been recently reviewed by Franzé et al. (2018) [124] and earlier by Chen et al. (2010) [125]. In this review, we focus on the freeze-drying of EVs.

The effect of freeze-drying on EVs is commonly studied by comparing various parameters to fresh EVs or EVs stored at −80 °C, which is the standard way to store them (Table 2**)**. The number of EVs, their size distribution, and morphology are the most common descriptors to characterize the success of freeze-drying [68,126–129]. In addition to those, the functionality of freeze-dried EVs, such as their effect on cell proliferation and cell internalization, was studied in some of the studies (Table 2). Regardless of the importance of studying the endpoint results and comparing them to the fresh samples, all reported experiments lack in-line and on-line analysis. As we stated already in the introduction, this leads to considering freeze-drying as a black box. Taken together, it is important to study the quality of the product after freeze-drying and reconstitution, but that does not replace the importance of process control and in-line and on-line analysis.

Freeze-drying of EVs requires excipients such as trehalose (the most frequently used) to prevent the vesicles' aggregation and destruction (Table 2). EVs freeze-dried with trehalose resembled EVs stored at −80 °C by their size, morphology, and concentration [68,127–129]. In addition, trehalose protected proteins and RNA molecules inside EVs [68,129]. Furthermore, EVs freeze-dried with trehalose and stored at room temperature were reported to have similar in vivo pharmacokinetics as EVs stored at −80 °C [129].

Frank et al. (2018) reported that they studied trehalose, mannitol, and polyethylene glycol (PEG) as lyoprotectants in the freeze-drying of EVs. Mannitol concentrations over 4% and PEG at all concentrations resulted in increased particle concentrations and aggregation of EVs, respectively, leaving trehalose as the most promising lyoprotectant with the concentration range of 0.5–5%. Mannitol and PEG provide mechanical strength and form the bulk for the samples. However, it seems that providing mechanical support for the EVs is insufficient alone to protect them from stress caused by freeze-drying. Nevertheless, mechanical support might be beneficial during storage and transportation.

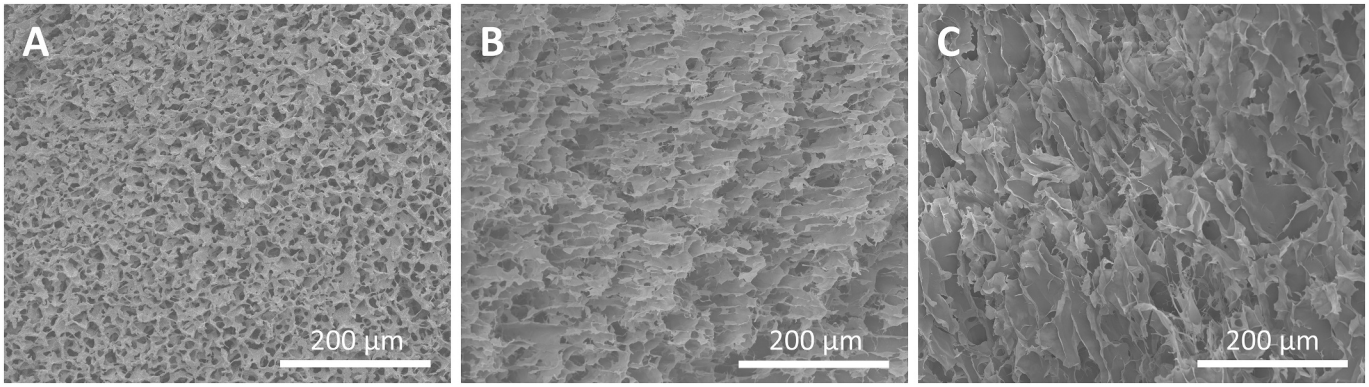


Fig. 9. Changing chemical, physical, and mechanical environments of biomaterials prior to freeze-drying affects their characteristics in the dried and reconstituted state. A) Freeze-dried nanofibrillated cellulose with excipients, B) freeze-dried nanofibrillated cellulose without excipients and C) freeze-dried chemically modified anionic nanofibrillated cellulose without excipients. Unpublished data.

Table 2
Excipients and freeze-drying methods used for extracellular vesicles and their characterization after freeze-drying. Note that no studies included PAT tools to analyze extracellular vesicles.

EV origin	Excipients	Freeze-drying method	Evaluation	Ref
MSC HUVEC A549	No excipients OR 0.5–4% trehalose OR 0.5–5% mannitol OR 5–20% PEG	Freezing is not reported. Drying by benchtop freeze-dryer overnight.	Size Number/ concentration Morphology Enzymatic activity	[68]
sBL1BL6	1.8% trehalose (50 mM)	Rapid freezing with liquid nitrogen. Drying by benchtop freeze-dryer overnight.	Polydisperse index Morphology Zeta potential Protein contents RNA contents Effect on cell proliferation In vivo pharmacokinetics	[129]
Murine MSC	0.95% trehalose (25 mM) with or without protease inhibitors	Uncontrolled freezing in -80 °C. Drying by benchtop freeze-dryer overnight.	Size Number/ concentration Morphology Proteomic analysis Transcriptomics Cell internalization	[128]
Cerebrospinal fluid	No excipients	Uncontrolled freezing in -80 °C. Drying by benchtop freeze-dryer 24 h.	Number/ concentration Morphology RNA amount miRNA copy number	[126]
HEK293T recombinant EVs	5% trehalose	Uncontrolled freezing in -45 °C. Drying with clear primary and secondary drying steps.	Size Number/ concentration Morphology Density distribution Fluorescence intensity	[127]

There are no reports, to the best of our knowledge, on the use of directional freezing in the freeze-drying of EVs. In most studies, the freezing step was uncontrolled, suggesting a wide variation of local ice front velocities during freezing and an increased risk of damage. In conclusion, the effects of freezing on changes in the chemical, physical, and mechanical environment were not considered in any of the studies. We believe that the field of EVs conservation (both in the frozen state and freeze-dried) could strongly benefit from a more controlled freezing step.

When EVs isolated from cerebrospinal fluid were freeze-dried without any additives, high, approximately 40%, reduction in the number of EVs was observed [126]. However, this is contrary to the results of Frank et al. (2018) who reported only minor changes in size and concentration of MSC and HUVEC derived EVs under comparable freeze-drying conditions [68]. These results highlight that EVs from different origins resist differently to the damage caused by freeze-drying. Although, applied techniques may impact the results [130].

EVs were mostly freeze-dried without controlling the shelf temperatures with benchtop freeze-driers overnight or 24 h [68,126,128,129]. Geeurickx et al. (2019) reported a freeze-drying cycle with clear freezing step, primary drying, and secondary drying. Surprisingly, there were no reports about the detection of Tg' or collapsing temperatures in any paper, even though those temperatures are essential parameters when designing the freeze-drying cycle. However, no differences in properties of freeze-dried EVs resulting from the different freeze-drying cycles can be concluded.

In our study, EVs were freeze-dried with excipients and a hydrogel scaffold (Fig. 10**).** We used a hydrogel scaffold in which the EVs were enveloped in the combination of nature origin excipients, such as saccharides. The idea of hydrogel scaffold is to prevent aggregation and fusion of EVs while the saccharides prevent damage caused by ice crystals and stabilize the lipid bilayer in a dry state similarly as with liposomes [131]. According to our results, the highest concentration was obtained when we addressed the chemical, physical, and mechanical environments accordingly. However, further optimization and evaluation for example with biophotonic equipment is required.

All freeze-dried EVs discussed in this review, except murine MSC derived EVs, were reconstituted by direct addition of distilled [126] or Milli-Q water [68,127]. Murine MSC derived EVs were reconstituted by incubating them in the water at 4 °C for 1 h [128]. As with the freeze-drying cycle, no differences in the results originating from the different rehydration methods can be concluded. Residual water content plays a major role in the shelf-life and preservation of biological samples. Thus, based on our opinion, it is worrying that the community has failed to report residual water contents of freeze-dried EV samples.

Shelf-life studies of freeze-dried EVs varied from 7 days to 28 days and they were performed by subjecting the freeze-dried and stored EVs for similar experiments as before freeze-drying such as determining the protein and RNA contents and measuring the concentration of EVs [68,126,129]. Long-term stability experiments according to the general guidelines in the pharmaceutical industry are highly important and required to further evaluate the success and usefulness of freeze-drying for the preservation of EVs. This issue was also recognized by the groups who have been freeze-drying EVs [129].

5.3. *Freeze-drying mammalian cells and biomaterial cell systems*

"Adult" desiccation-tolerant eukaryotic species such as *Saccharomyces cerevisiae* yeast or tardigrades are rare [132]. Consequently, freeze-drying of viable eukaryotic cells is far more challenging than freeze-drying of biomacromolecules, such as proteins, or liposomes, and EVs. Different aspects should be considered considering the planned usage of the freeze-dried cells: are the cells planned to be used as whole cells or just some parts of cells, such as DNA. In the case of freeze-drying and preserving whole cells, for example, the cell membrane must be protected, intracellular structures must be protected, and DNA must be protected. These can be addressed by adjusting the formulation with excipients to modulate the chemical environment, by controlling the freezing process and the physical environment, and by considering changes in the mechanical environment.

Different kinds of cells have been freeze-dried and they are summarized in Table 3. In addition, there are several reports about freeze-drying of mammalian spermatozoa and preserving the reproduction capacity of gametes [133,134], but those are left out of this review since we aim to focus on and review preservation of the more complicated systems.

Freeze-drying of platelets and red blood cells typically aims to increase the shelf-life of blood components essential for blood transfusion [141,142]. Considering the application, it is essential to formulate the freeze-drying formulation with biocompatible and non-toxic excipients such as sugars. Not surprisingly, trehalose has been the most used excipient in the freeze-drying of blood cells **(**Table 3**).** Platelets have been freeze-dried and stored for up to 22 months and their number, morphology, and aggregation capacity i.e. functionality, have been well preserved [142]. Trehalose and human serum albumin were the most important excipients. The importance of trehalose in the freeze-drying of platelets has been reported also by Zhou and colleagues [137,138], who reported that the highest recovery of human platelets was obtained when the platelets were freeze-dried with bovine serum albumin and trehalose. Furthermore, they state that also other sugars such as glucose,

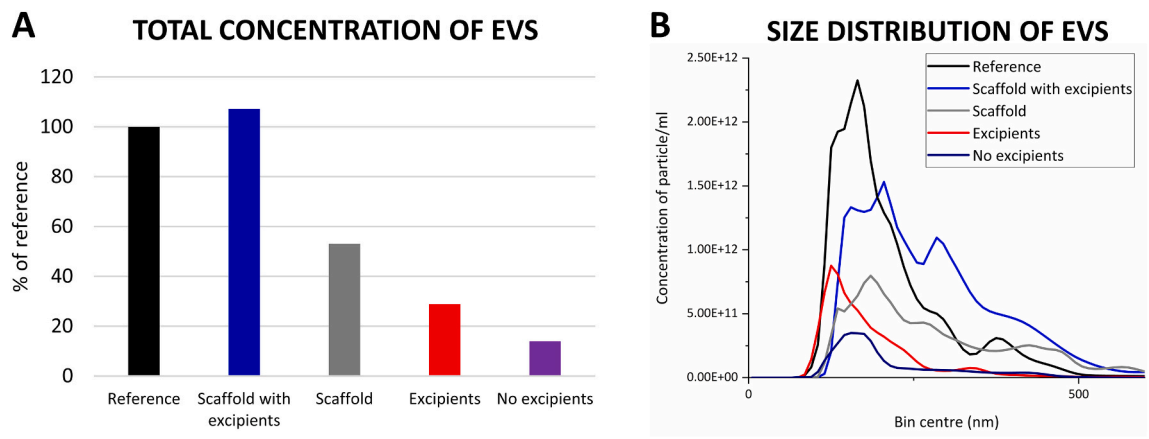


Fig. 10. EVs were freeze-dried with different formulations consisting of a lyoprotective hydrogel scaffold or excipients, or a combination of those, and reconstituted with water. **A)** When EVs were freeze-dried in a lyoprotective scaffold with excipients, the total concentration of EVs was higher compared to the scaffold or excipients alone. The scaffold was removed before measurements. **B)** The size distribution indicates that the EVs tend to aggregate with the scaffold, but they are destroyed without the scaffold. The scaffold was removed before measurements. This is an example of the need for precise formulating with careful consideration of the chemical, physical, and mechanical environments when freeze-drying natural biomaterials such as EVs. EVs were analyzed with Nanoparticle tracking analysis. Biophotonic analysis methods such as Raman or FTIR spectroscopies would provide valuable information about freeze-dried EVs. Unpublished data.

lactose, maltose, and sucrose serve as protecting excipients for platelets during freeze-drying. Hydrogen bonds provided by trehalose and sucrose stabilize the cell structures while lactose and maltose, commonly used bulking agents, support the freeze-dried cells by providing extracellular mechanical support.

Red blood cells are needed in high numbers for blood transfusions. Especially in crisis areas where cold storage is impossible, increasing the shelf-life of red blood cells by freeze-drying, would increase their usability, if the freeze-dried red blood cells can be used for the treatment by only adding water. Again, trehalose was the most used excipient in the freeze-drying for red blood cells as it was for the platelets. Extracellular trehalose showed no protective effect in the preservation of hemoglobin of freeze-dried red blood cells [67]. However, when trehalose was loaded inside the cells by electroporation, trehalose showed beneficial effects on the preservation of red blood cells during freeze-drying [139]. Intracellular and extracellular presence of excipients is probably required to preserve the intracellular structures as well as lipid bilayer during freeze-drying. Furthermore, Arav and Natan (2012) showed the benefit of directional freezing in the freeze-drying of red blood cells [65]. However, the authors reported only up to 65% of water loss which makes it unclear what is the role of excipients, is long-term storage possible and can the samples be considered dry.

Mononuclear cells, and hematopoietic progenitor and stem cells, both isolated from umbilical cord blood, have been freeze-dried [32,140]. Mononuclear cells' capacity to form colonies and viability of the cells were preserved when they were freeze-dried with antioxidant EGCG and frozen by directional freezing [32]. EGCG is reported to stabilize the lipid bilayer and this excipient alone was enough to protect the mononuclear cells during the freeze-drying when the freezing step was performed by directional freezing and controlling the freezing velocity. The authors believe that the combination of correct excipients, number of cells, and directional freezing are all required for the preservation of the cells during and after freeze-drying. Hematopoietic progenitor cells were successfully freeze-dried with trehalose, human serum albumin, and hydroxyethyl starch [140]. Trehalose was loaded intracellularly by permeating the cell membrane via P2Z receptor with adenosine-5-triphosphate (ATP) and benzoyl ATP, to protect the cells from freeze-drying damage and to stabilize the cell membrane. The importance of intracellular and extracellular trehalose has been discussed also earlier with platelets and red blood cells [135,137,139].

In all freeze-drying studies performed with the mammalian cells, trehalose was the most used excipient. Some groups reported that they had loaded trehalose inside the cells to increase the protecting effects of that sugar [66,135–137,139,140]. Albumin, either human or bovine serum originated, was the second most used excipient in the freeze-drying of different cell types. Positive results were obtained when albumin was used in the freeze-drying of blood cells [32,135–138]. However, albumin failed to preserve the viability and DNA integrity of mouse fibroblasts [64] but increases the Tg of dry formulations which is beneficial for storage at room temperature as there is less mobility in the sample when stored under Tg and the freeze-drying cake does not collapse [42,143]. Using albumin in combination with trehalose is one method to benefit from the properties of both excipients even though the exact mechanism, how albumin provides stabilization, is still unknown.

Directional freezing was reported to be beneficial in the freeze-drying of red blood cells and mononuclear cells [32,65,141]. Ice front velocities were 0.2 mm/s and 1 mm/s for red blood cells and 0.02 mm/s, 0.2 mm/s and 2 mm/s for mononuclear cells. Faster ice front velocity was beneficial in the freeze-drying of red blood cells while with mononuclear cells, ice front velocity of 0.2 mm/s resulted in higher preservation of proliferation capacities. Taken together, the optimal cooling rate or ice front velocity appears to be cell line dependent [44]. When optimizing freeze-drying in general, or freezing rates and ice front velocities of biomaterials and cells, both cell line specific requirements and properties of the formulation must be carefully considered.

How dry samples are after freeze-drying has a major impact on viability. This was studied ingeniously by Buchanan et al. (2010) in their study where they studied freeze-drying of hematopoietic progenitor cells and analyzed samples from different points of freeze-drying cycles. They showed that the proliferation and differentiation capacity of cells decreased as a function of the decreasing residual water content of cells. Their results are in line with previous reports concerning the importance of the role of the residual water content to cell viability [40]. Nevertheless, high residual water content does not automatically result in viable cells, which can be concluded from the experiments performed with freeze-drying of mouse fibroblasts [64].

Our group has freeze-dried encapsulated human retinal pigment epithelial cells ARPE 19 cells and 3D cultured human hepatocellular carcinoma (HepG2) cells [66,70]. The cells were loaded with trehalose and mechanically supported by biomaterial encapsulation and hydrogel scaffold, respectively. Encapsulated ARPE 19 cells and ARPE 19-Seap cells were freeze-dried with different saccharides (trehalose, dextran, sucrose) and glycerol [66]. The cells showed viability after rehydration; however, their oxygen consumption was highly reduced. For 3D HepG2 cell spheroids, trehalose and glycerol were used as excipients and nanofibrillated cellulose hydrogel as a scaffold to provide mechanical

Table 3
Summary of freeze-dried cells, most important excipients, and main results. Recovery: Subjective feature that was characterized such as morphology, metabolic activity, or proliferation rate.

Cells	Excipients	Freeze-drying method	Residual water (% w/w)	Recovery (%)	Ref
Platelets	12 mM TRE[a] + 30 mM TRE, 1% HSA	Cooling rate 2–5 °C/min to −40 °C, then frozen at −80 °C, dried in 20 mBar without temperature control for 24 h.	5	85	[135]
Platelets	Loaded with 40 mM TRE, FD with 150 mM TRE, 5% HSA	Cooling rate 2–5 °C/min, then frozen at −80 °C, dried in 20 mBar without temperature control for 24 h.	N/A	85[b]	[136]
Platelets	13.2 mM TRE[a] + 1% BSA, 1% TRE	Freezing: 10 °C/min to −40 °C Primary drying: 18 h, − 38 °C, 2 Pa Secondary drying: heating 0.2 °C/min to 20 °C, 15 h at 20 °C	2	90.7	[137]
Platelets	20% TRE, 1% BSA	Freezing: 0.5 °C/min or 10 °C/min to −50 °C or liquid nitrogen freezing Primary drying: 18 h, − 38 °C, 2 Pa Secondary drying: heating 0.2 °C/min to 20 °C, 15 h at 20 °C	2	93	[138]
Red blood cells	15% TRE	Freezing: 50–60 °C/min to −80 °C Primary drying: -35 °C, 200 mBar for 24 h	4.3	50	[67]
Red blood cells	63.7 mM TRE[a]	Freezing: For two hours at −60 °C Primary drying: -45 °C, 3 Pa, 15 h Secondary drying: 15 °C, 10 h	2	70.9	[139]
Red blood cells	300 mM TRE, 10% HSA	Freezing: Directional freezing 0.2 mm/s or 1 mm/s Primary drying: -55 °C, 5 mTorr, 24 h	1.9	40	[140]
Hematopoietic progenitor cells	Loaded with 200 mM TRE, FD with 6.8% TRE, 2% HES, 5% HSA	Freezing: 2.5 °C/min to −45 °C and 3 h. Primary drying: -35 °C, 80 mTorr, 36 h. Secondary drying: 0.2 °C/min to 5, 10, 15 and 20 °C, 6 h	4.89	91	[32]
Mononuclear cells	100 mM TRE, 0.945 mg/ml EGCG	Freezing: directional freezing 0.2 mm/s Primary drying: Under vacuum, without temperature or vacuum control.	6	0	[64]
Fibroblasts	250 mM TRE	Freezing: -40 °C/min to −80 °C. Primary drying: -30 °C, 6.7 h, 60 mTorr. Secondary drying: 0.1 °C/min to 40 °C, subsequently decreasing temperature to 20 °C.	N/A	10	[66]
Retinal pigment epithelial cells	0.58% TRE, 20 mM dextran, 0.68% sucrose, 0.5% glycerol	Freezing: 1 °C/min to – 40 °C, and increase to −35 °C. Primary drying: −35 °C, 18 h, 150 mTorr Secondary drying: 5 °C/h to 25 °C, 4 h at 25 °C, 100 mTorr.	N/A	18	[70]
Hepatocellular carcinoma cells	50 mM TRE, 1% glycerol, 0.4% NFC	Freezing: Rapid freezing with LN. Primary drying: Under vacuum, without temperature or vacuum control.			

TRE = Trehalose; HSA = Human serum albumin; BSA = Bovine serum albumin; HES = Hydroxyethyl starch; NFC = Nanofibrillated cellulose; EGCG = Epigallocatechin gallate. If different formulations were studied, the formulation with the highest recovery is reported here.

[a] Intracellular trehalose concentration.

[b] Continuing Wolkers et al. (2001) work, partly the same group.

support in the dry state [70]. We successfully preserved the 3D structure of HepG2 spheroids and partly enzymatic activity of the cell spheroids, but we failed to recover fully viable cell spheroids with intact cell membranes. Here again, the importance of controlling all the environments, chemical, physical, and mechanical, is of utmost importance. When we had no control over the physical parameters such as freezing rate and sublimation rate, the cytoskeleton of cells was destroyed. Contrary, when controlling the chemical, physical, and mechanical environments and considering the change of paradigm we are suggesting, the cytoskeleton of freeze-dried and reconstituted cell spheroids was preserved, and the dry aerogel scaffold appeared regular (Fig. 11).

5.4. Future prospects of freeze-dried biomaterials, extracellular vesicles and cells

As discussed in chapter 5.1. freeze-dried biomaterials are already in the market as hemostats and bone fillers. We believe that hybrid materials and biomaterials combined with active substances will be increasingly needed in clinical applications and the market. Freeze-drying is needed to manufacture the biomaterials and to preserve the active substances.

The freeze-dried EVs are commercially offered as standard and reference materials, yet, no freeze-dried EVs are marketed for clinical usage, for example for drug delivery applications. Nevertheless, as the clinical applications of EVs develop further, we believe that also the understanding of freeze-drying must improve.

With the cells, we have to first consider what the intended application of the freeze-dried product is. If we consider pharmaceutical applications, such as drug metabolism experiments, potentially the preservation of the metabolizing enzymes can be already enough (compare to microsomes in drug metabolism studies). However, for more relevant in vitro experiments, the freeze-dried cells should be viable and functional. Another application of cells which we discussed, freeze-dried red blood cells for blood transfusion, would be highly beneficial in the catastrophe areas where cryopreservation is impossible and the need for blood transfusion is high. In that scenario, freeze-dried red blood cells stored at room temperature would outperform frozen blood products, but the production scale should be improved.

One interesting method to preserve and encapsulate EVs and cells with increased drying efficiency would be spray freeze-drying. Spray freeze-drying has been utilized for preservation and encapsulation of a wide variety of products, such as small molecules, for oral and lung delivery, proteins for dermal delivery, and probiotic cells, as reviewed by Vishali et al. (2019) [144]. Spray freeze-drying provides a higher drying efficiency than freeze-drying and for example, it has enhanced the preservation and delivery of probiotic cells. To the best of our knowledge, spray freeze-drying has not been utilized with EVs or mammalian cells.

The freeze-drying technology must develop in a more economic direction, for freeze-dried cell products to become the standard way of preserving cells. Utilizing QbD ideology to carefully develop the process and carefully considering each environment in the freeze-drying, are the first steps to improve the process. In the future, as we are moving to ever greener technology and pharmacy, all the improvements in sustainability will be of utmost importance.

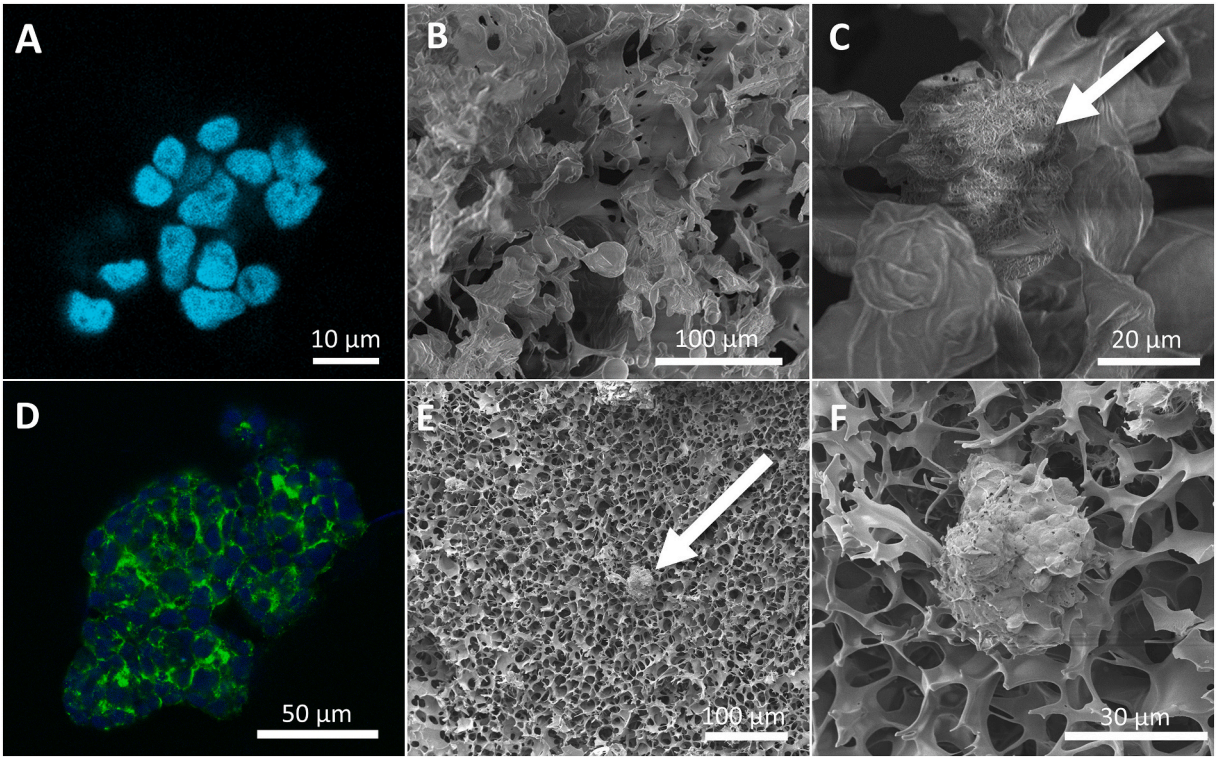


Fig. 11. Freeze-drying of cells and 3D cell spheroids is extremely challenging. A)-C) 3D cell spheroids freeze-dried with a hydrogel scaffold, and excipients, but without controlling the freezing rate or freeze-drying cycle. D)- F) 3D cell spheroids freeze-dried with the optimized method and considering the chemical, physical, and mechanical environments during the process. A) The cytoskeleton of the freeze-dried and reconstituted cell spheroid is destroyed. (Green: actin, Phalloidin Alexa 488; Blue: nuclei, Hoechst 33342. B) The porous aerogel scaffold is irregular and damaged. C) Zoomed in SEM micrograph of a 3D cell spheroid after freeze-drying. D) The cytoskeleton of the 3D cell spheroid was preserved when the freeze-drying process was optimized and the different environments considered (Green: actin, Phalloidin Alexa 488; Blue: nuclei, Dapi). E) A 3D cell spheroid protected by the dry scaffold. The scaffold is porous with a regular structure. F) A zoomed-in SEM micrograph of freeze-dried 3D cell spheroid with intact 3D structure. A)-C): Auvinen et al. (2018) [70] D)-F): Unpublished data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6. Conclusions

Freeze-drying is a potential method to preserve the natural biomaterials and cells in a dry state and to permit their storage preferably at room temperature, but to improve the results a change of paradigm is required. This change of paradigm involves the implementation of Quality-by-Design ideology and careful consideration of the chemical, physical, and mechanical environments before, during, and after freeze-drying. In particular, learnings that are emerging from other disciplines are important to consider. Furthermore, process analytical technology tools, such as biophotonic tools, are needed to monitor the samples during the process. We believe that by this change of paradigm the natural biomaterials and cells can be freeze-dried with even higher yields and freeze-drying applied into even more complex systems.

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