Dushyant Varshney · Manmohan Singh
Editors

Lyophilized Biologics and Vaccines

Modality-Based Approaches

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Editors
Dushyant Varshney
Novartis Vaccines and Diagnostics
Holly Springs
North Carolina
USA

AND

Director, Manufacturing Assessment, MS&T
Hospira
Lake Forest
Illinois
USA

Manmohan Singh
Novartis Vaccines and Diagnostics
Holly Springs
North Carolina
USA

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Preface

Lyophilization has become a popular approach for stabilization of the biologics. In the recent years, advances in biotechnology have resulted in various modalities of antibodies, antigens, and cancer drugs being explored in the development of therapeutic proteins, effective drugs, and vaccines. Diverse forms of antibodies (e.g., monoclonal, domain, fused), complex biologics (e.g., antibody drug conjugate, PEGylated proteins), peptides (e.g., cyclic peptides), and vaccines (e.g., combination type, inactivated virus, recombinant protein based) are being stabilized in the lyophilized form. Recent advances in lyophilization equipment, formulation, analytical instrumentation, delivery devices (e.g., cartridges), and manufacturing processes are being explored to overcome challenges posed by differences in the biophysical and chemical stability of each modality. The book "*Lyophilized Biologics and Vaccines—Modality-Based Approaches*" covers advances in lyophilization theories, product and process development approaches, and delivery approaches based on new modalities of biologics and vaccines. Recent advances in alternate drying methods and bulk lyophilization are also discussed in depth. The book is composed of four major sections having a total of 17 chapters, presented by expert and world renowned authors from academia, industry, and regulatory agencies.

Part I—Lyophilization History and Fundamentals is covered in five chapters. First a detail account of the historical development of lyophilization is discussed followed by recent advances in the understanding of heterogeneity of protein environment in the frozen or dried state, new developments in understanding buffer behavior and instrumental analysis of lyophilized biologics or vaccines is described. Special focus is given on recent advances in controlled ice nucleation with a specific discussion on VERISEQ® nucleation technology.

Part II—Lyophilized Biologics and Vaccines—Modality Considerations are discussed in five chapters. First an overview of the challenges and developments in lyophilized formulations for different modalities of biologics or vaccines is presented. Next, recent advances in quality by design (QbD) and process analytical technology (PAT) approaches for process scale-up of therapeutic protein are discussed in depth. The chapter on lyophilized vaccine provides a complete and detailed overview of a typical vaccine product and process development, from scale-up to optimization.

A special highlight on advances in stabilization of plasmid DNA and lipid-based therapeutics as dehydrated formulations is covered.

Part III—Advances in Alternate Drying methods is covered in four chapters. A detailed account of alternate drying methods compared to traditional vial lyophilization is discussed. Some of these include sterile spray drying, sterile powder filling, vacuum drying and drying on a fiber matrix. Chapters on recent advances in the spray drying, bulk freeze drying and crystallization provide an in-depth understanding of technology, challenges, and advantages, with nicely illustrated case studies.

Part IV—Regulatory, Packaging, and Technology Transfer Considerations is discussed in three important chapters, providing the latest regulatory perspective on lyophilized biologics, recent trends in lyophilized delivery devices, and packaging. The chapter on lyophilization technology transfer process provides critical considerations with case studies in detail for successful process scale-up to process validation and launch of lyophilized biologics and vaccines.

Dushyant Varshney
Manmohan Singh

Contents

Contributors

Thomas J. Anchordoquy School of Pharmacy, University of Colorado Denver, Aurora, CO, USA

David Awotwe-Otoo Division of Product Quality Research, Office of Testing and Research, Office of Pharmaceutical Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

Akhilesh Bhambhani Greater Philadelphia Area, USA

Jeffrey T. Blue Telford, USA

Levon A. Bostanian College of Pharmacy, Center for Nanomedicine and Drug Delivery, Xavier University of Louisiana, New Orleans, LA, USA

Joseph Brower IMA Life, Tonawanda, NY, USA

Monica Caldwell Linde Gases, Murray Hill, NJ, USA

Byeong S. Chang Integrity Bio, Inc., Camarillo, CA, USA

Hana Chang Integrity Bio, Inc., Camarillo, CA, USA

Mathew Cherian Hospira, Inc. One 2 One® Research and Development, Lake Forest, IL, USA

Steven Finley Linde Gases, Murray Hill, NJ, USA

Richard A. Graves College of Pharmacy, Center for Nanomedicine and Drug Delivery, Xavier University of Louisiana, New Orleans, LA, USA

Hiten Gutka Thermalin Diabetes LLC, Cleveland, OH, USA

Renaud Janssen Datwyler Pharma Packaging International NV, Alken, Belgium

Madhav Kamat Kamat Pharmatech LLC, North Brunswick, NJ, USA

William J. Kessler Physical Sciences Inc., Andover, MA, USA

Mansoor A. Khan Division of Product Quality Research, Office of Testing and Research, Office of Pharmaceutical Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

Grace A. Ledet College of Pharmacy, Center for Nanomedicine and Drug Delivery, Xavier University of Louisiana, New Orleans, LA, USA

Ron Lee Linde Gases, Murray Hill, NJ, USA

Tarun K. Mandal College of Pharmacy, Center for Nanomedicine and Drug Delivery, Xavier University of Louisiana, New Orleans, LA, USA

Timothy R. McCoy Technical Development, Genzyme Ireland Ltd. IDA Industrial Park, Waterford, Ireland

Marion dC. Molina Independent Pharmaceutical Consultant, Ashland, MA, USA

Mircea Mujat Physical Sciences Inc., Andover, MA, USA

Nicole M. Payton School of Pharmacy, University of Colorado Denver, Aurora, CO, USA

Michael J. Pikal Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT, USA

Krishna Prasad Julphar Pharmaceuticals, Ras Al Khaimah, United Arab Emirates

Michael Reilly Integrity Bio, Inc., Camarillo, CA, USA

Maya Salnikova Novartis Vaccines and Diagnostics, Holly Springs, NC, USA

Jim Searles Hospira, Inc. One 2 One® Research and Development, McPherson, KS, USA

Evgenyi Shalaev Allergan, Irvine, CA, USA

Sheri Shamblin Pfizer, Groton, CT, USA

Puneet Sharma Genentech Inc., South San Francisco, CA, USA

Jessica R. Sinacola Collegeville, USA

Manmohan Singh Novartis Vaccines and Diagnostics, Holly Springs, NC, USA

Peter Studer Linde Gases, Murray Hill, NJ, USA

Dushyant Varshney Novartis Vaccines and Diagnostics, Holly Springs, NC, USA

MS & T Hospira, Inc., Lake Forest, IL, USA

Bingquan (Stuart) Wang Late Stage Process Development, Genzyme, A Sanofi Company, Framingham, MA, USA

Protein Formulation Development, Biogen Idec, Cambridge, MA, USA

Eugene Wexler Linde Gases, Murray Hill, NJ, USA

Cindy Wu Allergan, Irvine, CA, USA

Part I
Lyophilization History and Fundamentals

History of Lyophilization

Dushyant Varshney and Manmohan Singh

Introduction

Lyophilization also known as freeze-drying is a process known from the ancient times, since 1250 BC, for preserving material by dehydrating the sample, which includes first freezing the sample and then drying under a vacuum (or low pressures) at very low temperatures [1–4]. The term lyophilization or lyophilisation literally means "solvent-loving process" or "process for loving dry state." The term has the origin from the ancient Greek root word, *λύω*/leo meaning "to break up, to dissolve," *φιλέω*/phileo meaning "to love, to kiss, to have tenderness for," and *πίλναμαι*/pilnamai meaning "contact, approach" [5]. The term lyophilization, as we know, is mostly attributed to Rey LR's work in 1976 described by taking into account the porous nature of the dried product and its "lyophil" characteristic to rapidly reabsorb the solvent and restore the substance [6]. Lyophilization (noun)/ly·oph·i·li·za·tion/(li-of″ĭ-lĭ-za′shun), transitive verb form is lyophilize, where lyophile or lyophilic in chemistry terms means—noting a colloid the particles of which have a strong affinity for the liquid in which they are dispersed. The suffix *–ize* or *-ise*, meaning—to cause to become [7].

Freeze-drying is in fact a sublimation process where the frozen liquid transforms to gaseous state directly without going through a liquid phase. Lyophilization is a dehydration process to preserve material and make lighter for transportation, a popular freeze-dried ice-cream treat for space astronauts [8]. Interestingly, lyophilization is also defined based on its applications [7]. For example, lyophilization (noun) is a method of drying food or blood plasma or pharmaceuticals or tissue without destroying their physical structure; material is frozen and then warmed in a vacuum so that the ice sublimes. Lyophilization also defined as the process of iso-

D. Varshney (✉)
MS & T Hospira, Inc., 275 N. Field Drive Lake Forest, Lake Forest, IL 60045, USA
e-mail: dushamaya@gmail.com

D. Varshney · M. Singh
Novartis Vaccines and Diagnostics, Holly Springs, NC 27540, USA

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lating a solid substance from solution by freezing the solution and then evaporating the ice under vacuum [7].

In this chapter, we have attempted to cover some of the key historical events that led to the development of modern day freeze-drying or lyophilization, and its widespread applications expanding from basic food needs to biotechnology products. Special consideration is given to lyophilization process, and the history of lyophilized vaccines and biopharmaceuticals.

Historical Events in Development of Lyophilization

Prehistoric Events

The method of freeze-drying, although not claimed as named "freeze-drying," has been utilized from ancient time since 1250 BC. Freeze-drying procedure can be traced back to prehistoric times of Eskimos, who preserved the fish in the cold temperatures of artic by dehydration [1–4]. In 1250–850 BC, ancient Peruvian Incas placed their potatoes and crops above Machu Picchu that caused freezing of their produce. They did not realize that low pressure at the high altitudes vaporized or sublimed the water from the produce and basically freeze-dried it [3, 9]. Although this process was relatively slow, but during drying the quality of the food was preserved due to its final frozen state. Interestingly, South Americans living in the Andes used a primitive freeze-drying method to preserve potatoes. They carried the tubers high into the mountains where temperatures drop below the freezing point of water and atmospheric pressure is low [3]. It is also known that monks living on Koya, the famous Buddhist sacred mountains, were packing tofu in snow on mountain sides that were conducive for drying due to high altitudes and extremely cold winds [3]. Vikings freeze-dried their favorite food, cold fish, by utilizing the local cold and dry conditions [10]. Such dehydration occurs under a vacuum, where the plant or animal product is solidly frozen during the process. The shrinkage of product is minimized or eliminated that result in a good preservation and long lasting.

Realization of Freeze-Drying Process: Early 1900s

In 1890, Altman reported that he was able to obtain dry tissue, at subatmospheric pressures, at a temperature of approximately −20 °C [1, 11]. The report in the literature does not easily reveal who named or first called the equipment as freeze-dryer. In 1905, Benedict and Manning reported the drying of animal tissue at pressures less than 1 atm by means of a chemical pump [11]. Shackell independently rediscovered the technique in 1909 for the preservation of biologicals, and was the first one to realize that the material had to be frozen before commencing the drying process, hence freeze-drying [12]. In 1910, Shackell took the basic design of Bene-

dict and Manning, and to produce the necessary vacuum he utilized an electrically driven vacuum pump instead of displacement of air with ethyl ether [12].

In the 1920s, lyophilization was established as a stabilizing process for heat-liable materials [13]. Interestingly, in 1925 the Dry Ice Corporation of America first trademarked the name Dry Ice [9]. In 1927, the first US patent was issued to Tival which made a reference to the drying of frozen materials under vacuum conditions [13]. The industrial applications of freeze-drying do not appear to have been appreciated prior to patents of Tival in 1927. In 1934, a US patent was issued to Elser who described drying equipment that replaced Shackell's sulfuric acid desiccating system with a cold trap chilled with dry ice.

Development and Popularity of Lyophilization in Food Products

In 1938, the freeze-dried coffee was first manufactured, which led to the development of powdered food product [9]. The 1940s marked great development. The first commercial use of the freeze-drying was reported. Equipment and techniques were developed to supply blood plasma and penicillin to the armed forces during World War II [14]. Greaves was the first to show scientific insight into the drying process by identifying the key operating parameters [15].

From 1950s to 1960s, with increasing popularity of lyophilized food products, further development in freeze-drying process was realized. In 1960, the coining of the term lyophilization is generally attributed to LR Rey who described the porous nature of the dried product and its "lyophil" characteristics to rapidly reabsorb the solvent and restore the substance to its original state [6, 11].

Freeze-drying which requires the use of special equipment, is called a freeze-dryer or lyophilizer. It contains a large chamber for freezing and a vacuum pump for removing moisture or sublimation of ice. Since the 1960s, more than 400 different types of freeze-dried foods have been commercially manufactured. Lettuce and watermelon are considered the two worst candidates for freeze-drying, not surprising, due to its very high water content [9].

Freeze-dried coffee is the best-known freeze-dried product. Nestle company invented freeze-dried coffee, when asked by Brazil to find a solution to their coffee surpluses. Nescafe, first introduced in Switzerland, was Nestlé's own freeze-dried coffee product. After Nescafe, the Taster's Choice Coffee, another famous freeze-dried product is derived from the patent issued to James Mercer. During 1966–1971, Mercer at Hills Brothers Coffee Inc. in San Francisco led the development of a continuous freeze-drying capability and was granted 47 US and foreign patents [9].

It was after the World War II that drying was converted into an industrial method utilizing the tray-type lyophilizer to improve the shelf life for pharmaceuticals. Also, for instant coffee granules, the tray-type freeze-dryer was used for the sublimation of the water.

In 1968, Whirlpool Corporation under contract from NASA for the Apollo missions developed the freeze-dried ice cream, well known as astronaut's ice cream

[8]. Freeze-dried food persists longer than other preserved food and due to very lightweight is ideal for space travel. Astronaut John Glenn became the first American to orbit the Earth more than 20 years ago. Glenn's experience helped designing the space food systems, by the first time experimenting eating food in weightless conditions.

By 1970, freeze-drying was commonly used for taxidermy, food preservation, museum conservation, and for pharmaceutical products [9, 16]. In 1980s, companies learned and applied freeze-drying for making long-lasting freeze-dried flowers [17]. In medicine, Altmann used freeze-drying for the preparation of histological sections as early as 1980.

In 2003, Kellogg Company started freeze-drying of strawberries and blueberries for their innovative cereal, "Fruit Harvest Strawberry Blueberry" [18].

Current Scope of Lyophilization

In modern times, lyophilization is widely used in the biotechnology, pharmaceuticals, and biomedical industries for preserving products such as antibiotics macromolecules, electrolytes, proteins, hormones, viruses, vaccines, bacteria, yeasts, blood serum, liposomes, transplant collagen sponge, active drugs. Freeze-drying is used for preserving biological materials like bacterial cultures. Freeze-drying stabilizes them for a long-term storage while minimizing the damages caused by strictly drying the sample. It is observed that many microorganisms survive well after freeze-drying, easily rehydrated, and can be grown in culture media after the long-term storage [19]. Also, many lyophilized products can be supplied easily to different parts of the globe with relatively less concerns of storage conditions. Compared to traditional freeze-drying methods in vials, in recent years, there has been a growth of alternate methods for drying (e.g., spray drying) and more advanced drug delivery devices (e.g., dual-chambered cartridges) containing dried biopharmaceutical products.

History of Lyophilization of Vaccines

The origin of lyophilization of vaccines for mass immunization has a very interesting history. The initial advent of this technology as it relates to current concepts was based on enhanced stability, storage at ambient conditions, shipping at room temperature, ease of administration, and distribution. The first known documented evidence of freeze-drying of a vaccine relates to that of a small pox vaccine in the early nineteenth century [2, 19–24]. Initial efforts to form a "dry powder" by air drying of vaccine components laid the foundation for subsequent advances in dehydration by "freeze-drying." The first freeze-dried formulation for a small pox vaccine was published in 1909 [2, 19–24]. Subsequently, both the bacillus Calmette–Guérin

(BCG) and the small pox vaccine were formulated as multidose dried powders. "DryVax" small pox vaccine was launched in the 1940s for mass immunization in several countries [25–27]. This was one of the first commercial launches of a sterile dry formulation for parenteral use. The 100-dose single-vial composition also contained phenol as a preservative. DryVax was manufactured by Wyeth Laboratories until the early 1980s and manufactured by Aventis Pasteur (now Sanofi) and is no longer licensed in the USA; it revoked in 2008 [25–27].

Leslie Collier is credited with helping develop a viable vaccine that led to the eradication of smallpox. Collier's major contribution was in perfecting the art of freeze-drying method of producing the vaccine for mass use (Leslie H. Collier, b. 1921). As a graduate student at the Lister Institute of Preventive Medicine, Elstree, Hertfordshire, England, in the early 1950s, Dr. Collier developed the method of freeze-drying of vaccinia virus that was subsequently adapted to large-scale freeze-dried vaccine production in many laboratories throughout the world [28, 29].

This was a very critical step in making the vaccine available for mass distribution, as earlier vaccine required refrigeration. The original method of freeze-drying of a small pox vaccine employed by Camus and others was suboptimal. Phenol was added to the freeze-dried vaccine as an antimicrobial agent. Despite its effectiveness as an antimicrobial agent, phenol also damaged the virus. This opened the door for researchers looking for an alternative excipient to add to the composition for a longer shelf life. Collier added a key component "peptone" to the process of freeze-drying. The powder was reconstituted with a solution of glycerin [28, 29]. This modified freeze-drying process to a large extent was the main driver in leading to global eradication of small pox. Similar efforts in Europe were also demonstrated effectively with a BCG freeze-dried composition. This further led to the acceptance of BCG vaccination in endemic countries very quickly. Most of these freeze-dried vaccines were distributed through governmental agencies around the world. This also enabled the reduction in the cost of goods as some of these were formulated in multidose vials for broader population coverage [30–38].

Despite the success with small pox and BCG, most toxoids and adjuvants used from 1930 onwards did not succeed in being freeze-dried. The primary reason attributed to their failure was adsorption on mineral salts. Aluminum hydroxide and aluminum phosphate, the two primary salts used for adsorption with diphtheria and tetanus toxoid, were not compatible with the dehydration approach as significant aggregation of the mineral salts was seen upon freezing and the loss of potency in vivo of toxoids upon freezing was observed [30–36].

Another vaccine that has made a major impact on providing postexposure protection in endemic countries using a freeze-dried vaccine, is the rabies vaccine. A freeze-dried rabies vaccine was developed in the 1960s that led to a longer shelf life and aided in better utilization in a postexposure setting in developing countries. To this date, in many developing countries, a freeze-dried rabies vaccine is used after reconstitution by the intramuscular route and also in smaller volumes of 100 μl through the intradermal route. Some of the licensed freeze-dried rabies vaccines on the market are RabAvert and Rabipur [34–38].

The large-scale commercial application of a freeze-dried vaccine formulation is credited to this date in eradicating small pox and better utilization of a BCG and rabies vaccine especially in the developing world.

Summary

Lyophilization is now a standard platform with multiple utilities extending from pharmaceuticals to food industry. Its evolution and understanding over the years has increased significantly. In this chapter, we covered the origin, definition, and key historical events that led to the development of modern day freeze-drying, and its widespread applications expanding from basic food needs to biotechnology products. Special consideration was given to the history of lyophilization process, vaccines, and biopharmaceuticals. A detailed account of recent developments in lyophilization of biologics and vaccines, with a focus on different modalities, is discussed in the subsequent chapters of the book.

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Heterogeneity of Protein Environments in Frozen Solutions and in the Dried State

Maya Salnikova, Dushyant Varshney and Evgenyi Shalaev

Introduction

Typical solutions for freeze-drying contain 80–95 wt.% of water and several solutes, including an active ingredient and excipients, such as buffer components, lyoprotector or/and bulking agent, and a stabilizer such as surfactant. Behavior of such systems during freezing and freeze-drying is commonly described with the aid of supplemented phase diagrams, also known as solid–liquid state diagrams and extended phase diagrams. Use of the state diagrams for cryobiology and freeze-drying was pioneered by Luyet, Rasmussen, MacKenzie, and Franks, based on the evaluation of binary water–sucrose system and similar systems in which solutes do not crystallize [1–3]. Solid–liquid state diagrams of aqueous systems containing both crystalline and amorphous solutes were introduced for cryobiology [4] and freeze-drying applications [5]. In particular, the state diagrams allowed a generalized description of the phase behavior of typical aqueous solutions used in freeze-drying [6, 7], as follows. When an aqueous solution is cooled below its equilibrium melting point, a fraction of water molecules is isolated in a separate phase as hexagonal ice, leaving behind amorphous freeze-concentrated solution

E. Shalaev (✉)
Allergan, Irvine, CA 92612, USA
e-mail: Shalaev_Evgenyi@Allergan.com

M. Salnikova
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway,
Holly Springs, NC 27540, USA
e-mail: mayadusha@gmail.com

D. Varshney
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway, Holly Springs, NC 27540, USA
e-mail: dushamaya@gmail.com

MS & T Hospira, Inc., 275 N. Field Drive Lake Forest,
Lake Forest, IL 60045, USA

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consisting of all the solute molecules and residual water. At this point, a two-phase system is formed consisting of hexagonal ice and freeze-concentrated solution. Upon further cooling, a behavior of such a two-phase system follows one of three scenarios below, depending on the solutes, cooling rate, and other variables such as the presence and properties of interfaces (e.g., particles) which can serve as nucleation centers: (i) the freeze-concentrated solution forms a kinetically stable amorphous phase, the so-called maximally freeze-concentrated solution; (ii) the freeze-concentrated solution forms a "doubly unstable" glass (i.e., unstable in both kinetics and thermodynamics sense), in which solute + water crystallization may occur later in the process, during annealing or drying; (iii) a secondary solute + water crystallization may occur during further cooling, resulting in a three-phase system of hexagonal ice, crystalline excipient, and the remaining freeze-concentrated solution.

Overall, the solid–liquid state diagrams have been extensively and successfully used to represent fundamentals of the freeze-drying processes [1, 2, 5–7]. It should be recognized, however, that the solid–liquid state diagrams reflect phase behavior under either equilibrium or metastable conditions. In particular, the assumptions of thermal equilibration across the sample and a sufficiently fast mass transfer between phases as related to the rate of temperature changes apply. In many real systems, however, these conditions are not satisfied and specific details of the freezing process need to be taken into consideration. For example, it was demonstrated using a carefully designed cryo-microscope and a model system (aqueous solution of $NaMnO_4$) that equilibrium conditions as assumed in phase diagrams do not always represent a good approximation. Deviations from equilibrium was more prominent at higher cooling rates where the redistribution of solute in front of the advancing ice–liquid interface was observed [8, 9]. The nonequilibrium features of the freezing process, including events on the ice/solution interface have been reviewed extensively [10–12]. In particular, an existence of the concentration gradients (for both neutral molecules and ions) on the ice/solution interfaces is commonly acknowledged [13–15]. Such concentration gradients could lead to significant inhomogeneity in the environment of an active pharmaceutical ingredient, including variations in the environment of protein molecules. Furthermore, as the protein stability depends on the composition and properties of their immediate environment, the heterogeneity would result in different populations of protein molecules, all having different stability characteristics, leading to a distribution of the degradation rates. As a result of the heterogeneity, shelf life of a pharmaceutical protein formulation would be limited by the most unstable population of protein molecules, which may represent a relatively minor fraction. Identifying this least stable portion of protein molecules and targeting formulation development efforts on this fraction, rather than going after the main (and potentially the most stable) part would allow a formulator to optimize stabilization and formulation development efforts.

An obvious practical challenge in studying heterogeneity in protein systems is that the majority of experimental tools provides an average measure of a property (e.g., structure), and may not have sufficient sensitivity or resolution to detect the

presence of protein molecules in different environments or conformational states. For example, Fourier transform infrared spectroscopy (FTIR), the now-standard formulation tool, would reflect destabilization of secondary structure [16], but only if the majority of molecules are affected. Also, measurements of storage stability include reconstitution of the freeze-dried cake and analysis of the resulting solution using stability-indicating methods (e.g., size-exclusion chromatography). Such tests provide sample-averaged degradation extent but would not allow extracting information about potential heterogeneity. Such challenge in the detection of heterogeneity in protein formulations represents probably the main reason why the subject of heterogeneity has not attracted much of attention in the biotech community until recently, although a few exceptional studies should be noted [17, 18]

In this chapter, we first discuss examples of experimentally determined heterogeneity of protein environment in frozen solutions and freeze-dried preparations, following by a discussion of several mechanisms leading to such heterogeneity. These mechanisms are predominantly related to events during freezing, and include concentration gradients created due to difference in the diffusion coefficients of proteins and other solutes, redistribution of the charged species and electric potential on the ice/solution interface [19, 20], and solution inclusions by ice crystals [21]. It should also be added that heterogeneity is a fundamental property of amorphous systems including both solutions and glasses, as was previously discussed in some details [22]. This chapter is focused on heterogeneity which can be expected within a single container (e.g., vial). Discussion of vial-to-vial variability is outside of the scope of the present study.

Experimental Evidences of Heterogeneity of Protein Environment in Frozen Solutions and Dried Solids

An extreme case of heterogeneity would be a phase separation between a protein and excipients, resulting in two amorphous phases, protein-rich and excipient-rich [23–25]. A potential protein/polymer phase separation in human brain-derived neurotropic factor (BDNF) and BDNF-polyethylene glycol (PEG) co-lyophilized with dextran was suggested based on scanning electron microscopy [26]. Additional evidences of phase separation between proteins and polymers were obtained using Raman mapping, which detected amorphous/amorphous phase separation between a protein and a lyoprotector, e.g., in lactoglobulin–dextran system [27–29]. It was also shown that trehalose had a greater propensity for phase separation from protein than sucrose, with phase separation detected for lysozyme–trehalose and lactoglobulin–trehalose (but not for protein–sucrose) systems. The occurrence of phase separation was correlated to higher instability of proteins.

Raman and FTIR spectroscopy were used to detect the heterogeneity and adsorption of proteins to ice surfaces [30]. It was shown that concentration of albumin in dimethyl sulfoxide (DMSO)/water solutions was high at the ice interface at low temperatures and as much as 20 % of the albumin (for 32–53 mg/mL solutions) can

be adsorbed on the ice or entrapped in the ice phase. In a recent study of the freeze-dried recombinant human growth hormone (rhGH) [31], the amount of protein on the surface of the freeze-dried cake was determined using electron spectroscopy for chemical analysis in formulations with sucrose, trehalose and hydroxyethyl starch (HES). The freeze-dried formulations were prepared at five different freezing conditions that include standard lyophilization cycle with slow freezing, pre-annealing before primary drying, post-annealing after secondary drying, fast freezing by immersion of vials into liquid nitrogen, and fast freezing of droplets by pipetting solution into immersed in liquid nitrogen vial. The surface concentration of rhGH was higher than in the bulk and was related with the rate of freezing and the use of annealing in frozen solids prior to drying, or annealing in glassy solids after secondary drying. Lower fraction of the protein was observed on the surface after slow freezing and annealing. In the same study, the average degradation rate was separated into two contributions, from bulk and surface degradation. It was shown that the degradation of protein molecules on the interface was approximately two orders of magnitude faster than the bulk degradation for chemical processes (deamidation and oxidation), whereas bulk versus surface difference for the aggregation rate was even more pronounced. Similar impact of the heterogeneity on stability was observed in the earlier studies for methionyl human growth hormone formulations prepared by freeze-drying, spray-drying, and film-drying [17].

In another important study, it was shown that protein concentration on the air/solid interface was higher than in the bulk for both spray-drying and lyophilization processes in trehalose/potassium phosphate formulations. [18]. The addition of polysorbate 20 reduced protein surface adsorption and decreased (but did not completely prevent) aggregation.

Appearance of two populations of protein molecules in the frozen state was detected in lysozyme/sorbitol/water system by small-angle neutron scattering (SANS). In that study, two populations of the protein were observed in frozen samples whereas the initial solution consisted of a single population of protein molecules [32], as illustrated in Fig. 1. In one of the populations (with intermolecular center-to-center distance of approximately 3 nm), protein molecules were in close

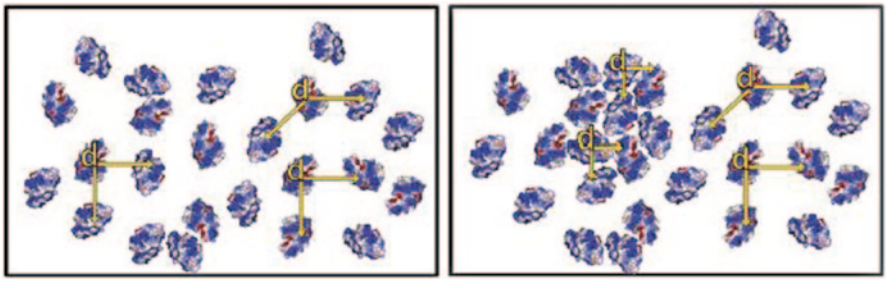

Fig. 1 Schematic drawing of increase in lysozyme crowding from solution (*left*) to freeze-concentrated solution (*right*), showing reduced protein–protein distance (*marked as d*) in one of the two populations of protein molecules, as a precursor for aggregation. The figure is reproduced from [32]

contact and interaction with each other, thus creating favorable spatial conditions for agglomeration and aggregation.

It was noted also that many chemical and physical processes in freeze-died formulations, both proteins and small molecules, do not follow simple kinetics law, and suggested that such observations can serve as an indirect evidence for heterogeneity of the local environments [22]. Indeed, heterogeneity would result in different populations of molecules of the active ingredient with different individual rate constants. As the common experimental methods (e.g., high-performance liquid chromatography) would measure bulk-averaged concentration of the reaction products or the extent of the conversion of a reactant, the apparent rate constant would represent a weight-averaged sum of the individual rate constants. In this case, even if the kinetics of each individual reaction corresponds to a simple reaction order, e.g., first-order, the average kinetic curve would reflect distribution of the individual rate constants, resulting in a more complex kinetic curve.

Mechanisms for the Inhomogeneity

Common mechanisms for the inhomogeneity (heterogeneity) are related to freezing (ice formation), resulting in redistribution of solutes via, e.g., inclusion inside ice crystals. In addition, we note that heterogeneity is a general property of solutions in both liquid and solid state (glasses). One specific case of heterogeneity was reviewed [33], where water clustering in solutions and amorphous solids was discussed as a probable case of heterogeneity on the sub-nanometer to nanometer-length scale. In the following two sections, we consider large-scale heterogeneities which are directly related to ice/solution interfaces.

Protein Sorption on Interfaces

Protein partitioning between bulk solution and interfaces would be an obvious case of heterogeneity, with properties of protein molecules on the interface be different from the bulk phase. Interaction of proteins with ice surface was studied for antifreeze proteins in some details. The propensity of some proteins to interact with ice surfaces is one of the defense mechanisms in nature that prevents ice growth due to increase in the curvature of the ice–water interface and thus resulting in non-colligative local freezing point depression [34–36]. The interaction of the antifreeze proteins with ice is mainly based on hydrogen-bonding mechanisms. Propensity of antifreeze proteins to ice/solution interface was used to purify antifreeze proteins, to separate them from other proteins present in *Escherichia coli* lysate [37]. In this case, non-antifreeze proteins were actually excluded from ice interface. Such exclusion of “common” proteins from ice interface is an important observation, considering that pharmaceutically relevant proteins are obviously not antifreeze proteins and

that the exclusion would not be consistent with a hypothesis of "pharmaceutical" proteins interacting with ice surface.

As evidence to the contrary, i.e., of interaction of non-antifreeze-proteins with ice interface, studies reported in [38, 39] are commonly invoked, and discussed below in some detail. Conformational changes of globular proteins were studied by employing the phosphorescence emission of tryptophan (Trp) residues as a monitor of the conformational changes of six proteins in response to variations in conditions of the medium [38]. Changes in well-structured compact cores of the macromolecules were monitored by the direct correlation between the phosphorescence lifetime τ and the rigidity of the protein matrix surrounding the chromophoric probe. The solidification of water markedly decreased τ and indicated unfolding related changes in conformation of the proteins, which was related primarily to the protein–ice interaction. Additionally, tryptophan florescence was employed to monitor unfolding of azurin mutant C112S from *Pseudomonas aeruginosa*. The thermodynamic stability (ΔG^0) of the macromolecule in frozen aqueous solutions was studied by introducing guanidinium chloride and monitoring tryptophan fluorescence for native and denatured states. The evaluation of the guanidinium chloride-induced unfolding in the frozen state allowed assessment of the thermodynamic stability of proteins in frozen solutions. The results obtained with C112S azurin demonstrated that the stability of the native fold may be significantly perturbed in the frozen solutions depending mainly on the size of the liquid solution pool in equilibrium with the solid phase. It was proposed that the effectiveness of stabilizing additives in preventing protein unfolding in the frozen state will be a combination of two influences: the ability to stabilize the N-state at low temperature and high-solute concentration (a preferential hydration mechanism), plus a specific action of the additive to contrast perturbations deriving from protein–ice interactions. We should note that, while these studies provided convincing evidence that destabilization of protein can be induced by formation of ice, it is not obvious if such destabilization is the results of the direct protein/ice interaction, as other mechanisms can be invoked.

In another study, concentration of the freeze-dried human interferon-γ on the surface of freeze-dried and spray-lyophilized materials was measured by electron spectroscopy for chemical analysis [18]. A higher concentration of the protein was observed on the surface of the dried materials, whereas the use of a surfactant (polysorbate 20) minimized the surface excess. The observations of the high surface concentration of a protein on the surface of freeze-dried cakes were taken as an evidence of protein sorption on solution/ice interface during the freezing step. This would certainly be a logical conclusion, but these studies did not provide a direct proof of protein sorption on the surface of ice crystals.

To reconcile the studies which showed rejection of non-antifreeze proteins from ice [37] with reports of both protein destabilization due to ice formation and the higher concentration of proteins on the surface of dried materials, we note that the formation of ice crystals during freezing usually results in formation of air bubbles [40], thus greatly increasing solution/air interface and potential for protein destabilization. Protein sorption on solution/air interface is well-documented, e.g., [41], and will not be discussed here. Therefore, protein sorption on the air bubbles created as

a result of freezing can be expected, with a corresponding increase in the surface concentration of protein in dried material and protein destabilization. Another possible explanation for the two apparently conflicting observation, that is, exclusion of non-antifreeze proteins from ice interface in the presence of antifreeze proteins versus destabilization of proteins by ice, is that the antifreeze proteins compete with other proteins for the interface, whereas in the absence of antifreeze proteins, other proteins might indeed be sorbed by ice surface. In either case, the use of nonionic surfactants to stabilize proteins, presumably by preventing the binding of the proteins to air/liquid surfaces, and also possibly ice/liquid interfaces, was demonstrated in many systems, e.g., in the example of recombinant human factor XIII [42].

Solute Inclusion Inside Ice Crystals

Solubility of essentially all common freeze-drying solutes in hexagonal ice is negligible, in other words, one can expect that the ice phase consists of 100% water. However, on a macroscopic scale, solution can be entrapped by growing ice crystals under certain conditions. In this section, several examples of such entrapment are discussed.

Entrapment of a solution phase by growing ice crystals depends on the freezing conditions, i.e., geometry of the crystallization front, rate of progression of the ice/solution interface, and macroscopic viscosity of the solution phase. In one study, freezing of small droplets of solutions containing sucrose, pullulan, bovine serum albumin (BSA), antifreeze glycoprotein, polyvinyl alcohol (PVA), and PEG was studied using optical refractometry [43]. Relatively diluted solutions, with the solute concentration of <5 wt.% (for sucrose) and <1 wt.% (for other solutes), were used. A concentration gradient of solute was observed at the ice/solution interface (length scale up to 200 μm) for all solutes but antifreeze glycoprotein, for which the concentration measured at the ice/solution interface, was the same as in the bulk. No incorporation of the solution phase into ice crystals was observed at the growing speed of 2 μm/s, when the ice/solution interface remained approximately planar, whereas at the higher growth speeds dendritic ice morphology was observed with a significant amount of solution trapped between the dendrites as liquid inclusions.

In a recent report, freezing behavior of ternary system water–DMSO–albumin was studied using FTIR and confocal Raman microscopy [30]. Solutions with different albumin/DMSO ratios were equilibrated at various subzero temperatures to create a two-phase (ice + freeze-concentrated solution) system. The albumin/DMSO ratios in the freeze-concentrated solution (FCS) were measured using FTIR and confocal Raman microscopy. In such a two-phase system, one would expect that the ratio would not change from the original single-phase solution, as can be shown using the temperature–composition phase diagram of a ternary system [7]. It was observed, however, that the ratio changed in a complicated manner. In particular, the albumin/DMSO ratio increased at relatively higher temperatures of -4 and -6 °C, which was interpreted as due to trapping DMSO inside ice crystals, whereas the

trend switched at lower temperatures, with FCS containing lower fraction of albumin as related to the initial solution.

An indirect evidence of trapping of protein molecules by ice crystals, and the heterogeneity associated with this was obtained [44] where the bulk concentration of *lactate dehydrogenase* (LDH) in the frozen sample was measured as a function of the distance from the container wall, with resolution of several mm. The concentration of LDH was the same across the sample, whereas there was a noticeable concentration gradient for small molecular weight solutes ($NaMnO_4$ and NaCl). A natural interpretation of these results is that, while small solutes are expelled from the ice crystallization front, larger protein molecules are trapped because of their slower diffusion rate. Therefore, local concentration of salts around protein molecules is likely varies with the position across a frozen sample.

Furthermore, solute inclusion was also invoked to explain the "double T_g" events, which are commonly observed in differential scanning calorimetry (DSC) studies of aqueous solutions. It should be stressed that the physical nature of these two events is still controversial and a subject of a number of publications [1, 21, 45–51]. The lower-temperature event (so-called T_g') has been attributed to a glass transition of the freeze-concentrated solution, whereas the second event (T_g') is proposed to be due to either the onset of ice melting/dissolution in the freeze-concentrated solution, or a glass transition of the freeze-concentrate. In the case of the latter interpretation, i.e., under the assumption that both events are the glass transitions, it was proposed that the two T_gs are due to the existence of two freeze-concentrated solutions with different concentrations of a solute (e.g., sucrose) in the same sample [45].

In order to consider this hypothesis, one would need to answer a question-why would there be two freeze-concentrated solutions with different sucrose concentration in the same sample? For a multicomponent system, heterogeneity in the composition of the freeze-concentrated solution is indeed possible, due to differentiation of the solutes by growing ice crystal because of differences in the diffusion coefficients or/and interaction with the ice surface. However, binary sucrose–water system has only one solute, and a sucrose solution trapped inside an ice crystal would achieve the same sucrose concentration as the solution outside of ice crystal which is also in direct contact (and local equilibrium) with ice, under a reasonable assumption that both temperature and pressure are the same in the trapped versus expelled parts of the solution. As a potential resolution of this problem, we hypothesize that the volume expansion due to water-to-ice transformation may result in differences in local pressures in different parts of the sample. A higher pressure would change the T_g of the freeze-concentrated solution either due to lower solute concentration as a result of the pressure-depression of the ice melting temperature (shift in the water liquidus), or increase in the T_g due to higher pressure. An indirect evidence of elevated local pressures was obtained in a study in which simple aqueous solutions were studied by synchrotron X-ray diffraction [52]. In that study, complex X-ray diffraction (XRD) patterns, with two or more poorly resolved peaks in place of each of the four diagnostic peaks of hexagonal ice, referred to as "splitting," were observed in the majority of cases. Deformation of the lattice of hexagonal ice, probably due to local stress created on the ice/ice or ice/container interface during water-to-ice transformation, was proposed as a possible mechanism for the

observed splitting of XRD peaks. It was also estimated using molecular modeling that the observed shifts in the peak positions are equivalent to applying a hydrostatic pressure of 2–3 kbar.

Furthermore, in a separate and a carefully designed study of water–sucrose system it was shown that, when the trapping (and therefore a probability of creating regions with elevated pressure) was minimized, a single-glass transition event was observed, followed by the onset of ice melting [21]. In that investigation, solution enclosure by ice crystals was prevented by using either scraped-surface freezing process or slow growing of ice crystals from solution containing ice nucleus.

To summarize the discussion on heterogeneity of protein environments due to solution inclusion by ice crystals, we note that the absolute majority of pharmaceutical formulations contain more than one solute. The solutes have different diffusion coefficients and/or interaction with ice surface, and therefore can be expected to develop variable extent of spatial heterogeneity under nonequilibrium freezing conditions. Protein environment in such materials will obviously be different in different parts of the sample, which could lead to distribution of degradation rates. An additional mechanism for the heterogeneity, i.e., due to local pressures as a result of volume expansion during water-to-ice transformation, should also be taken into consideration.

Inhomogeneity as a General Property of Solutions and Glasses

Inhomogeneity on the length scale of sub-nanometer to nanometer is a fundamental property of amorphous liquids (solutions), as summarized earlier [33]. Moreover, even a single-component system such as liquid water is nonhomogeneous on the molecular level, as illustrated by the Frank and Wen's model [53] (Fig. 2).

Many important events in solutions, such as crystal nucleation are expected to be dependent on local heterogeneity, with small clusters of molecules serving as nucleation centers. In addition, larger-scale heterogeneities (on the scale of hundreds of nanometer) were observed in solutions under certain conditions, [55] although the origin of the driving force for such large-scale heterogeneity is obscure.

For amorphous solids (glasses), their intrinsic heterogeneity and its pharmaceutical significance were discussed in [22], and briefly outlined below. The heterogeneous nature of glasses is reflected in non-exponential behavior of the structural relaxation, as expressed in the well-known Kohlrausch–Williams–Watts (KWW) equation:

$$X(t) = \exp\left(-\frac{t}{\tau_{KWW}}\right)^{\beta_{KWW}}$$

where X(t) is a property of the material, τ_{kww} and β_{kww} measure the average relaxation rate and the extent of non-exponentiality and the distribution of relaxation times, respectively. Values of β_{kww} vary from 0 to 1, with 1 representing a single-exponential relaxation process. Typically, many organic amorphous materials have

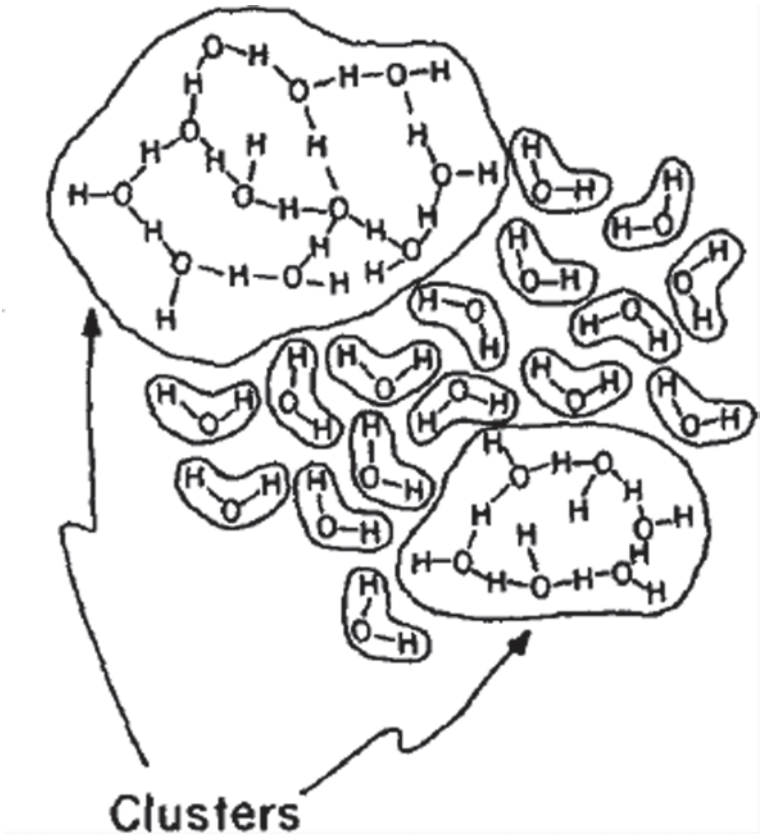

Fig. 2 Schematic representation of the model of liquid water, showing hydrogen-bonded clusters and unclustered water molecules. The molecules in the interior of the clusters are tetracoordinated, but not drawn as such in this two-dimensional diagram. The representation is based on the Frank–Wen model of liquid water (Reproduced with permission from [54]. Copyright 2014, AIP Publishing LLC)

values of β_{kww} from 0.3 to 0.8, indicating a broad distribution of relaxation times and significant inhomogeneity.

Domains with different relaxation times may also have different degradation rates. Note that heterogeneity in freeze-dried amorphous materials can be easily observed by DSC. Indeed, DSC traces of such samples are commonly reveal sub-T_g transition, which can be eliminated by thermal cycling (i.e., heating above the T_g followed by quenching) or annealing below the T_g [56]. In this respect, it is possible that the high-temperature annealing, which was shown to improve stability of both proteins and small molecules, [57–62] reduces population of the least stable molecules thus resulting in the decrease of an average degradation rate.

An experimental observation of large-scale heterogeneities developed after cooling a model system (concentrated sorbitol/water solution) below its T_g was reported

using SANS [63]. In that study, significant increase in the scattering (which is an indication of increased inhomogeneity) was observed during cooling 70% sorbitol solution below its glass transition temperature. Note that the sample retained its amorphous structure (i.e., no sorbitol or water crystallization) during the experiment. It was also observed also that this increase in the scattering was partly reversed during heating, although some hysteresis persisted. A power-law analysis of the SANS data indicated the existence of domains with well-defined interfaces on the submicrometer length scale, probably as a result of the appearance and growth of microscopic voids in the glassy matrix. The SANS results provided an example of long-range inhomogeneity in aqueous glasses, and also suggested an intriguing possibility of a thermal memory retained by the glasses even after heating above the glass transition temperature. The large-scale heterogeneity in glasses was also reported for another system, lysozyme/sorbitol/water mixtures, in which heterogeneity was detected in freeze-concentrated solutions [32]. It was suggested that the interfaces, which were detected by SANS below the T_g, can impose additional stresses on proteins, resulting in destabilization and degradation.

Conclusions

Heterogeneity in protein environment can be created by several mechanisms including sorption of proteins on ice crystals, inclusion of freeze-concentrated solution by ice crystallization front, and amorphous/amorphous phase separation, as well as due to creation of voids and corresponding interfaces in freeze-concentrated solutions. Also, note that crystallization of a solute (such as buffer or lyoprotector) would be an additional source of a heterogeneity, solute crystallization and its relevance to stability of frozen and freeze-dried formulations was discussed elsewhere [5, 10, 11, 64] and not considered here. Difference in the stability of different populations of proteins was convincingly demonstrated for the case of proteins on air–solid interface in freeze-dried cakes, [31] with the degradation rates on the interface exceeding the rate of bulk molecules by at least two orders of magnitude. As a result of such heterogeneity, shelf life of a pharmaceutical protein formulation would be limited by the most unstable population of protein molecules. Identifying the least stable portion of protein molecules and targeting formulation development efforts on elimination or stabilization of this fraction would allow a formulator to optimize formulation development efforts. Use of surfactants is an example of such strategy, allowing minimizing protein sorption on the interface and therefore reducing concentration of the least stable fraction of protein molecules. Phase separation of lyoprotector from protein, either due to crystallization or amorphous/amorphous phase separation represents another commonly accepted mechanism of heterogeneity and creation of a less-stable fraction of protein molecules. Overall, the identification of heterogeneity of protein molecules should be an essential part of a scientific design of freeze-dried and frozen protein formulations.

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Advance Understanding of Buffer Behavior during Lyophilization

Cindy Wu, Sheri Shamblin, Dushyant Varshney and Evgenyi Shalaev

Introduction

Therapeutic proteins are becoming an ever increasing important class of drugs in the pharmaceutical industry. Since the turn of the century, there has been a significant increase in the number of biologics ranked in the top ten blockbuster drugs. In 2012, seven out of the ten blockbusters were categorized as biologics with Humira®, a tumor necrosis factor (TNF) inhibitor leading sales at US $ 9.3 billion [24]. Due to the challenges of maintaining long-term stability in aqueous solution, many proteins are formulated as lyophilized or freeze-dried powder. In fact, roughly half of all commercial biologics are lyophilized products with Remicade, a TNF-α monoclonal antibody, leading sales at US $ 8.2 billion in 2012 [24].

The diverse modalities of biologics (e.g., monoclonal antibody, antibody drug conjugates, fusion proteins, enzymes) and the different routes of administration (e.g., intravenous, subcutaneous, intramuscular) require critical consideration of the type of buffer, stabilizers, and bulking agents used in the formulation. For example, solutions administered by the parenteral route typically formulated at a pH of 4–8 to minimize pain upon injection, which then dictate to some extent the range and type of buffers used. The types of buffers used in some of the best-selling lyophilized biologics are listed in Table 1.

E. Shalaev (✉) · C. Wu
Allergan, Irvine, CA 92612-1531, USA
e-mail: Shalaev_Evgenyi@Allergan.com

C. Wu
e-mail: Wu_Cindy@Allergan.com

S. Shamblin
Pfizer, Groton, CT 06340, USA

D. Varshney
Novartis Vaccines and Diagnostics, Holly Springs, NC 27540, USA

MS & T Hospira, Inc., 275 N. Field Drive Lake Forest, Lake Forest, IL 60045, USA

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Table 1 Buffers in best-selling lyophilized biologics

Product	2012 sales (US $ billions)	Indication	Drug class	Buffer
Remicade (infliximab)	8.2	Arthritis	Monoclonal antibody	Sodium phosphate, pH 7.2
Enbrel (etanercept)	8.0	Arthritis	Fusion protein	Tris (tromethamine), pH 7.4
Herceptin (trastuzamb)	6.0	Breast cancer	Monoclonal antibody	Histidine, pH 6.0
Nutropin, Humatrope, Genotropin, Norditropin, Saizen, Serostime, Omintrope (recombinant growth hormone)	2.9	Growth hormone deficiency	Protein	Phosphate, phosphoric acid, pH 6.5 to 8.5
Avonex (interferon beta-1)	2.3	Multiple sclerosis	Protein	Phosphate, pH 7.3
Advate (antihemophilic factor)	2.2	Hemophilia	Protein	10 mM or 25 mM of each Tris (hydroxymethyl aminoethane) and L-histidine
Betaferon/betaseron	1.6	Multiple sclerosis	Protein	0.54 % NaCl, neutral pH

It is well documented that the selection of buffers, stabilizers, and bulking agents are important for stabilizing the protein during the drying process and subsequently during storage [9]. Therefore, it is critical to have a good understanding of the behavior of these formulation components during the freeze-drying process.

Freeze-drying or lyophilization is widely used to improve the long-term stability of biologics. However, freeze-drying can impose stress that adversely affects the protein if not designed properly. Freeze-drying converts liquid to a solid and generally involves the following steps: freezing, optional annealing, primary drying, and secondary drying. During each of these steps, it is important to consider and understand the stresses, the role of the formulation components and process conditions on the stability of protein and product quality. In the freezing step, the protein can experience supercooling, freeze concentration, crystallization of the formulation excipients, exposure to interfaces and sheer stresses due to formation of ice crystals, and cold denaturation, which may be deleterious to its stability [53]. In the secondary drying step, the protein experiences dehydration as the water is removed from the noncrystalline or amorphous phase.

This chapter begins by providing a general description of the types of buffers and the methods used to characterize buffer behavior during freeze-drying, including pH shifts and crystallization. The behavior of three classes of buffers (e.g., phosphate, carboxylic acids, and amines/amino acids) during freezing and thawing will be discussed next. The chapter ends with a case study that describes the impact of buffers on the stability of a lyophilized powder.

Definition, Functional Role, and Classification

Buffers are commonly used in freeze-dried dosage forms to control pH, in order to improve the long-term stability of protein. Knowing pH of maximum stability is a critical requirement for achieving a stable formulation. During the formulation development, buffering capacity and the potential for buffer catalysis are two main buffer properties to consider. The general rule of thumb is not to exceed the effective buffer capacity by maintaining pH within one unit from its pK_a [39]. For freeze-dried products, special consideration needs to be made for buffer selection including buffer crystallization during freezing, impact on collapse temperature of the freeze-concentrated solution and T_g (glass transition temperature) of the dried formulation, and the volatility of the buffer components under vacuum [40].

Most lyophilized products on the market are formulated in the pH range from 4 to 8 [18]. Common buffers and their pH range are listed in Table 2.

During the selection of buffer system for freeze-dried formulations, it is essential to minimize changes in the pH and apparent pH during freezing and drying. Changes in apparent acidity can lead to acceleration of drug degradation, either directly, via specific acid/base catalysis, or indirectly, by changing ionization state of the protein and thus making it potentially more susceptible to both physical (e.g., aggregation) and chemical (e.g., oxidation) degradation pathways. The propensity for crystallization of buffer components during freezing is the most commonly recognized root cause for pH shifts. It is widely known, for example, that in frozen phosphate buffer solutions, crystallization of disodium hydrogen dodecahydrate ($Na_2HPO_4 \cdot 12\ H_2O$) causes a pronounced acidic pH shift in the freeze-concentrate [20, 37, 48]. Similarly, the pH swing, with pH jumping from 4 to 8 followed by major drop to pH 2, was observed in frozen succinate buffer solutions and attributed to sequential crystallization of the buffer components [47].

Table 2 Common buffers and their pH buffering range

Buffer	Type	Approximate buffering range
Sodium phosphate	Phosphate	6.2–8.2 [15]
Sodium acetate	Carboxylic acid salt	3.8–5.8 [15]
Sodium citrate	Carboxylic acid salt	2.1–6.2 [15]
Glycolate	Carboxylic acid	2.6–4.6 [15]
Malate	Carboxylic acid	2.4–6.1 [15]
Succinate	Carboxylic acid	3.2–6.6 [15]
Glutamic acid	Amino acid	2–5.3 [15]
L-histidine	Amino acid	5.0–7.0 [25]
Glycine	Amino acid	8.8–10.8 [15]
Tris	Amine	7.1–9.1 [15]
TEA (triethanolamine)	Amine	7–9 [15]
Citrate-phosphate	Combination	2.2–8.0

In addition to pH shifts as a result of crystallization of the buffer components, there are two other possible causes for pH changes during freezing, i.e., temperature dependence of pH, and change in an apparent pK_a as a result of decrease in polarity of the amorphous (liquid) phase due to freeze concentration. Buffers vary in their pH temperature dependence. For example, phosphate buffer demonstrates relatively minor temperature trend, with pH increasing by approximately 0.13 units (from 6.86 to 6.99) as temperature decreases from 25 to 0 °C [3]. Tris buffer is an example of much stronger temperature dependence, with a significant pH increase upon cooling, e.g., from 8.1 (25 °C) to 8.8 (0 °C) [54]. Dependence of the ionization constant (pK_a) from the media polarity is another potentially significant contributor to pH changes during freezing. Water crystallization (ice formation) results in significant decrease of water concentration in the solution coexisting with ice crystals, with a corresponding decrease in polarity due to increase in solutes concentration. In sucrose–water system, for example, dielectric constant of the 10 wt.% solution is 76.2 at 25 °C [30] while the maximally freeze-concentrated solution (sucrose concentration approximately 80 wt.%) is estimated to have dielectric constant of approximately 51 at 25 °C (graphical extrapolation using data from Mathlouthi et al. [30]). Such decrease in the dielectric constant may result in a major pK_a change, for example, the apparent pK_a of acetic acid increases significantly with decreased in polarity, from 4.76 in water (dielectric constant 78.3) to 10.32 in ethanol (dielectric constant 24.3). [4] Such media-dependent pK_a changes are highly variable between different functional groups; for example, the pK_a of an amino group ($–NH_3^+/–NH_2$ equilibria) is much less sensitive to changes in the dielectric constant of the solvent than the carboxylic group.

Further decrease in the water content by removing water during secondary drying would result in further decrease in polarity, with corresponding changes expected in the extent of proton transfer and ionization, and therefore, apparent acidity. Indeed, the changes in the apparent acidity as a function of water content were observed in a study of model trehalose–citrate system, in which the Hammett acidity function [23], was measured in amorphous lyophiles as a function of water content [21], as discussed in the next section. The study described in Govindarajan et al. [21] also demonstrated that ionization state is not fixed during the freezing stage, with proton transfer in amorphous solids taken place well below their calorimetric T_g.

The effect of buffer types on freeze-drying is described in more details below, to cover shift in the apparent “pH” during freezing and drying both in systems with buffer crystallization and in cases when buffer crystallization was avoided.

Characterization Methods

Various analytical methods have been utilized to characterize buffers in the lyophilized products to ensure the buffer functions and provide long-term stability of drug products. Mainly these methods have included direct characterization using pH electrodes [19, 20, 48, 49, 50] or indirect characterization, in which pH shifts are monitored based on crystallizing buffer components or use of pH indicators.

Gomez et al. developed an elegant method, utilizing a low-temperature pH electrode, to measure the pH shifts during the freezing stage under far-from-equilibrium conditions [20]. The use of a low-temperature pH electrode indicated major decrease in pH, presumably due to disodium hydrogen phosphate dodecahydrate (DHPD) crystallization, at initial buffer concentration down to 8 mM. Nonetheless, the pH electrode (e.g., Inlab®cool, Mettler Toledo) could only be used at temperatures $\geq -25\,°C$. Another significant limitation of pH electrodes is that they work only in liquid media, and cannot be used to monitor changes in the acid/base relationships during drying. Therefore the utility of the low-temperature pH electrode is limited, as the apparent acidity can change in frozen solutions as they are cooled below $-25\,°C$ as well as during drying.

Indirect methods can be divided into two categories, one is based on detecting crystallization of buffer components (low-temperature X-ray diffractometry (XRD) [8, 17, 38], sub-ambient thermal analysis [10], differential scanning calorimetry (DSC) [32], scanning electron microscopy [33]), and the other monitoring changes in the ionization of ionizable groups (e.g., using pH indicators as probe molecules [34] and measuring the extent of proton transfer either in probe molecules by visible diffuse reflectance spectroscopy or in a buffer itself by Raman spectroscopy [28]).

In the case of succinic acid sequential crystallization and "pH swing" in frozen solutions, for example, low-temperature pH measurement and sub-ambient XRD proved to be excellent complementary tools in the characterization of frozen systems [45–47].

While laboratory-based low-temperature X-ray diffraction method is an essential tool for observing solute crystallization (that can cause pH shifts), it suffers from low sensitivity. For example, based on both XRD and DSC, crystallization of DHPD could not be detected at initial buffer concentrations < 190 mM. The poor sensitivity of the laboratory-based XRD method could be attributed to the low flux of the X-ray source, and the use of a point detector [8, 38]. These limitations could be overcome with the use of highly collimated and brilliant synchrotron radiation, coupled with a high-resolution 2D detector. Synchrotron XRD (SXRD) method, in addition to the increased sensitivity, provides a very rapid data collection (< 1 s) enabling time-resolved studies.

Low-temperature SXRD offers numerous advantages as follows: (a) capability to monitor phase transitions *in situ*, during the entire freeze-drying cycle. (b) Potential for obtaining quantitative information based on the intensity of the analyte peak(s). By collecting the entire diffraction data (the Debye rings), errors in net intensity measurement due to preferred orientation can be minimized. (c) Capability to determine, in real time, the effect of processing. For example, the kinetics of solute crystallization can be studied as a function of annealing time and temperature. (d) Quantification of analyte crystallinity in complex, multicomponent systems.

Varshney et al. [51] demonstrated the sensitivity of low-temperature SXRD for detecting solute crystallization in frozen sodium phosphate buffer and glycine solutions. Crystallization was detected at initial phosphate buffer concentrations down to 1 mM. In addition, the use of a high-resolution 2D detector enabled the visualization of numerous diffraction rings of the crystalline solute. The detection of DHPD was

unambiguous, based on several unique peaks, and was accomplished under far-from equilibrium conditions. The whole range of pH 1–9 at different solute concentrations of frozen glycine solutions was studied by low-temperature SXRD method to quantify complex multi-crystalline phases (with the capability to detect 0.2% crystallinity based on peak integrated intensities). Also the method was utilized for understanding the effect of annealing, primary and secondary drying on buffer crystallization. Phase transition of the phosphate buffer, partial and complete dehydration of the DHPD to amorphous phosphate buffer was shown at commercially relevant buffer concentration and freeze-drying conditions (temperatures and pressure).

Spectrophotometery and the use of pH indicator dye were demonstrated by Li et al. [28]. Authors studied the acid–base characteristics of various citrate buffer systems alone and in the presence of the pH indicator dye, bromophenol blue, in aqueous solution, and amorphous material produced after lyophilization. Fourier transform Raman and solid-state nuclear magnetic resonance spectroscopy were used to monitor the ratio of ionized to unionized citric acid under various conditions, as a function of initial pH in the range of 2.65–4.28. Ultraviolet (UV)–visible spectrophotometry was used to probe the extent of proton transfer of bromophenol blue in the citrate buffer systems in solution and the amorphous solid state. This work was extended to focus on measurement of the apparent acidity using pH indicators, with the acidity expressed as the Hammett acidity function (Equation below), and to establish relationships between the Hammett acidity function and the degradation rate of acid-sensitive molecules in the amorphous freeze-dried formulations [12]. Figure 1 below shows the main principle and example of Hammett acidity function measurement in lyophile. Where the ratio of ionized to unionized species of pH indicator dye, measured by UV–vis diffuse reflectance (DR) spectrophotometer, provides solid state acidity for lyophiles. The relationships between the Hammett acidity function and solid-state degradation were demonstrated for several small molecular weight compounds [2, 12, 17, 22, 29]. Expect that similar relationships between the Hammett acidity function and degradation rate of acid-sensitive protein molecules would be observed for freeze-dried protein formulations as well, although publications in this area are lacking.

$$\mathrm{H}_x = pK_a + log_{10}\frac{C_d}{C_p} = pK_a + log_{10}\left[\frac{F(R)_{\mathrm{d}^{\varepsilon'}\mathrm{p}}}{F(R)_{\mathrm{p}^{\varepsilon'}\mathrm{d}}}\right]$$

where C is the indicator concentration, and the subscripts “p” and “d” refer to the protonated and deprotonated indicator species, respectively. Ka is the ionization constant of the indicator, F(R)d/F(R)p is the ratio of the peak signals of the deprotonated to the protonated indicator forms determined from the DR spectra, and ε′p/ε′d is the ratio of the extinction coefficients of two species in the lyophiles. Subscript x corresponds to the charge of the basic form of the probe molecules; for sulfonephthalein pH indicators in typical pharmaceutical materials, x is equal to either -2 or -.

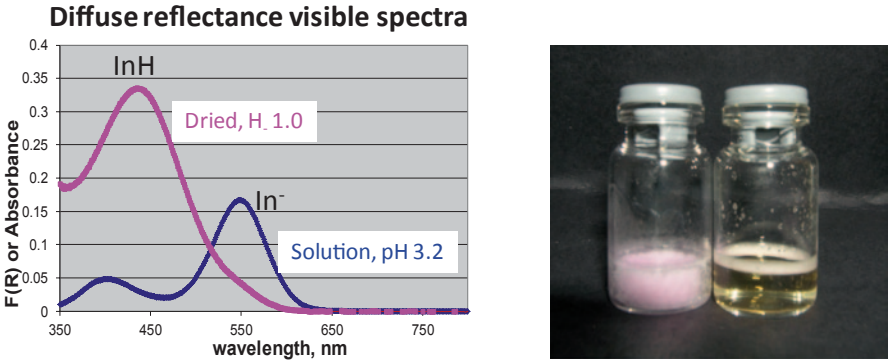

Fig. 1 Change in the apparent acidity from solution to lyophile measured via change of the color of an indicator (photos), quantified by measuring ionization extent of the indicator in solution and lyophile (diffuse reflectance visible spectra), and expressed as the Hammett acidity function, H_x (the numbers in the graph). The change in color from solution to lyophile reflects significant change in the extent of protonation of the indicator and therefore major shift in the apparent acidity during freeze-drying. Visible diffuse reflectance spectra of solution and freeze-dried formulation show a major shift in the protonation of an indicator, from partially protonated in solution (pH 3.2) to completely protonated in the freeze-dried state ($H_- < 1.0$)

Phosphate Buffer

Phosphate buffer is widely used and has been extensively studied in the pharmaceutical field. As early as the 1950s, Van den Berg conducted pioneering work to describe the effect of freezing on phosphate buffer system under equilibrium conditions [49]. Van den Berg showed that the pH and composition of maximally freeze-concentrated solutions can be defined by the eutectic point and is independent of both the initial pH and buffer concentration for sodium phosphate buffer and potassium phosphate buffer. An important finding was that the pH at the eutectic point is very different for sodium and potassium phosphate buffers with pH of 3.6 and 7.5, respectively [50]. This result has significant implication on protein stability during freezing, because independent of initial solution pH, under equilibrium freezing conditions, the unfrozen portion of phosphate buffer may approach pH 3.6 for sodium phosphate. Experimental studies have shown that freezing of 0.1 M sodium phosphate buffer resulted in a significant decrease in pH from 7.4 to 3.4 [27]. The pH behavior becomes even more complex for mixed solutions of sodium/potassium phosphates, where ten eutectic points ranging from 3.3 to 9.3 have been reported to exist. The pH change in this mixed system is influenced by both the sodium and potassium concentration and the ratio of pH of the initial solution, so the pH drop may be closer to 5.3 [27].

Because of the many cases where equilibrium is not achieved during freezing and freeze-drying, it is important to understand what happens under these conditions. It has been shown that under non-equilibrium freezing conditions (e.g., relatively fast cooling rates and small sample size), monosodium phosphate did not typically crystallize, whereas disodium phosphate readily crystallize [33]. It has been more recently shown that conditions that influence crystallization kinetics such as cooling rate, sample size, and initial buffer concentration also play a role in pH changes. For example, it was shown that an initial buffer with a pH 7.4 drops to pH 5.2 for 8 mM buffer as compared to pH 4 for 50 and 100 mM buffers [20].

The pronounced pH shifts in frozen solutions may be minimized by inhibiting the selective crystallization of a buffer component. Studies have shown that the crystallization of sodium phosphate buffer can be inhibited in the frozen state by the incorporation of glycine at a low ratio [52]. However, if the molar ratio of glycine to sodium phosphate is too high, this can lead to the crystallization of glycine. Furthermore, it was shown that at low glycine concentrations ($\leq$50 mM), pH shift in 10 and 100 mM sodium phosphate buffer can be prevented [37]. These findings show the importance in the selection of the buffer and excipient components for a freeze-dried protein formulation.

While it is well understood that pH shifts occur in frozen aqueous phosphate buffer solutions due to disodium phosphate buffer crystallization, there is recent evidence that shows a pH shift upon cooling to −25 °C in the absence of solute crystallization. For example, a pH shift from 6.2 to 4.5 was observed in a buffer system containing sucrose with no evidence of buffer salt crystallization [7]. We speculate that this could be due to preferential inclusion of the basic component (disodium phosphate) by the ice crystals, thus leaving more acidic freeze-concentrated solution in a continuous freeze-concentrated phase, in which pH is measured by low-temperature pH electrode. It should be noted, that a similar explanation of microheterogeneity was proposed in the original study, although specific reasons why heterogeneity would result in acidic shift were not elaborated [7].

Carboxylic Acid Buffers

The risk of pH shifts in frozen solutions for phosphate buffers has played a role in the investigation of other buffer types. Carboxylic buffers are of interest because of its broad buffering capacity ranging from 3 to 7. The crystallization propensity of carboxylic buffers has been studied. Several studies with sodium and potassium citrates have shown that these buffers do not crystallize and have minimal pH change during freezing. Examples include DSC freeze/thaw experiments [44] and a low-temperature pH probe study [27]. In this latter study, it was shown that the pH upon freezing of a citrate buffer increased slightly from 6 to 6.4 [27]. Similar findings were observed with a pH indicator study, which showed no pH change for citric acid/sodium citrate pH 5.5 during freezing [34]. Lastly, a concentration study from 0.1 to 0.6 molal showed no significant change in pH for citrate buffers at pH 6 and 4.4 [5].

Besides citrate buffers, other carboxylic acid buffers such as acetate, glycolate, and malate have shown to have negligible to minimal potential for crystallization and hence stable pH during freezing and thawing. Electrical resistance measurements have shown no crystallization for glycolate buffer at pH 5 during freeze/thaw cycles [26]. A DSC study showed that during freeze/thaw of sodium acetate, no crystallization was observed [10] and similar findings were obtained for sodium malate [44].

Like the other carboxylic acid buffers described above, succinate buffer at pH 5 shows minimal pH changes during freezing [35] and no crystallization during freezing for a 0.25 M solution at pH 4–6 [44]. However, crystallization was observed during heating of the frozen succinate buffer [44]. This is consistent with other studies using electrical resistance measurement [26]. The crystallization can be attributed to the propensity of monosodium succinate to crystallize. It has been shown that neither disodium succinate nor succinic acid crystallized during freeze-thaw experiments.

Recent studies using XRD have shown a "pH swing" in freeze-concentrate of a succinate buffer. This finding is attributed to sequential crystallization of succinic acid, monosodium succinate, and disodium succinate [47]. Specifically, the study showed that the pH of a 200 mM succinate solution increased from 4 to 8 during initial freezing from RT to −25 °C and then decreased to pH 2.2 due to sequential crystallization. A pH swing in the opposite direction was observed when succinate solution pH 6 was cooled. In this case, the basic buffer component crystallized first.

Co-solutes can influence the crystallization behavior and consequently pH shifts in frozen buffer solution [41]. Four commonly used co-solutes (glycine, mannitol, sucrose, and trehalose) in lyophilized formulations have been shown to inhibit crystallization of succinate buffer components [45]. Specifically, it was shown that only when the co-solute retained amorphous was it able to inhibit buffer crystallization. However, when the co-solute crystallized (e.g., trehalose) or degraded to yield a crystalline composition (e.g., sucrose), buffer crystallization was observed.

Moreover, even with buffers which do not usually crystallize such as sodium citrate buffer, a co-solute can also have an impact on the apparent acidity and the rate of acid-catalyzed degradation processes. This effect was demonstrated in studies which used sucrose as a model of acid-sensitive compound which was lyophilized using citrate buffer and several different lyoprotectors [12, 29]. Freeze-dried formulations, containing sucrose and co-solute (lactose, polyvinyl pyrrolidone (PVP), and dextran of different molecular weights) at 1:10 weight ratio, were prepared from solutions of the same pH with citrate buffer, and the rate of acid-catalyzed sucrose inversion was determined during storage of the lyophiles. The apparent acidity of the freeze-dried formulations was determined using sulfonephthalein indicators and expressed as the Hammett acidity function (H_{2-}). Significant difference in the H_{2-} was observed between formulations, with dextran-based formulations determined to be the most acidic while PVP formulations were least acidic. Accordingly, the rate of the acid-catalyzed sucrose inversion was fastest in the dextran formulations followed

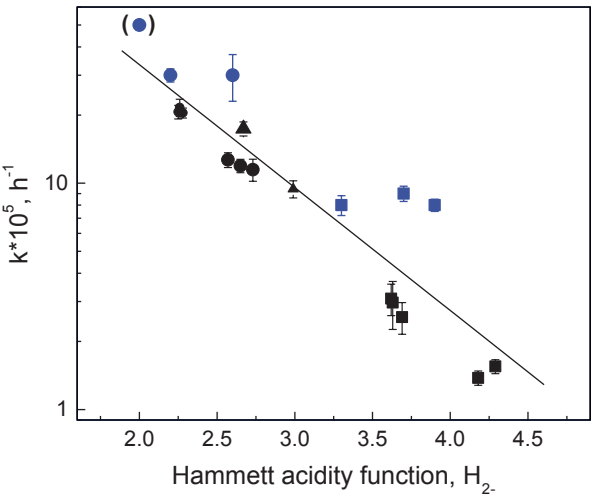

Fig. 2 Pseudo-first-order rate constants of sucrose inversion in PVP (*squares*), lactose (*triangles*), and dextran (*circles*) lyophiles as a function of Hammett acidity function. The line reflects the correlation between the rate constant (logarithmic scale) and the Hammett acidity function (*Blue symbols* are for datapoints from Chatterjee et al. [12] and black symbols from Lu et al. [29])

by lactose- and then PVP-containing formulations. The relationships between sucrose inversion rate and the Hammett acidity function are shown in Fig. 2.

An understanding of the impact of buffer and co-solutes on apparent pH is important during the design of the protein formulations since proteins are sensitive to pH, and the measurement of the Hammett acidity function would provide an essential information for development of freeze-dried proteins

Amines and Amino Acids

Alternative buffers to carboxylic acids which are known to have minor to moderate pH changes (around one pH unit change) during freezing include histidine and Tris [25]. The temperature-pH profile of 20 mM histidine has been studied and shown to have less than one pH unit increase (5.4 to 6.1) upon freezing to −30 °C [25]. The crystallization behavior of histidine during freeze/thaw has been shown to be pH dependent [35]. Specifically, heating of frozen histidine solution with a pH outside the range of 5.5–6.5 results in crystallization of the buffer. Similar to histidine, the pH of Tris–HCl buffer increases by one pH unit from 7.4 to 8.5 upon freezing to −30 °C. However, some pH indicator studies have shown no pH change for 10 mM Tris buffer pH 7.4 up to −40 °C [13] and moderate decrease in pH during freezing of pH 9.8 [14]. Furthermore, by DSC, Tris buffer crystallization was observed during freezing or warming/annealing [10].

Other buffers which show a pH dependence on crystallization include glycine -based systems. The components of glycine buffer (glycine, glycine HCl, and sodium glycinate) have been shown to crystallize during freezing or low-temperature annealing [1, 31]. Specifically, Akers et al. showed a faster crystallization rate at pH 7 as compared to pH 3 and no crystallization at pH 10 [1]. Contrary results have

been reported for glycines depending on the technique and sample tested [27]. Specifically, using a low-temperature pH probe, Larsen showed no pH change at pH 2.8 and moderate increase from 9.3 to 10.2. A possible explanation for the contradictory results may be a consequence of the difference in temperature range studied, in which Akers was working at −30 °C and Larsen was working at −15 °C. Furthermore, crystallization of glycine depends on the cooling rate [17] and can be inhibited by amorphous solutes, such as sucrose, as reviewed by Shalaev and Franks [41]. Presence of sucrose above certain sucrose/glycine wt. ratio, determined to 0.8, results in complete inhibition of glycine crystallization, whereas compositions with higher glycine concentration can crystallize either during cooling (if the cooling rate is less that the critical cooling rate, which also depends on the sucrose/glycine ratio), annealing, or subsequent warming.

In addition to the pH buffering properties, amino acids also may serve as lyo- and cryo-protectors [16]. For example, histidine was shown to stabilize freeze-dried recombinant factor VIII [36]. Note also that histidine forms complexes with divalent cations such as Mg, Ca, Zn, Fe [6], which can have an additional impact on stability and activity of proteins. Indeed, the complex formation between histidine and traces of Fe^{2+} might inhibit oxidative processes, whereas complexation with Zn^{2+} could compromise activity of certain metal-proteins.

Phosphate Versus Citrate Buffer: Case Study

It is commonly accepted that sodium phosphate buffer represents a stability risk for frozen solutions and lyophiles because of its high crystallization propensity and corresponding pH changes. Furthermore, it was shown that inhibition of crystallization can be achieved by using an amorphous solute, such as sucrose or trehalose, if the ratio of amorphous/crystallizable solute is above its critical ratio [41]. In many systems, such critical ratio was determined to be in the range of 1/1–5/1; for example, a formulation with 5% sucrose and 20 mM phosphate buffer is expected to remain amorphous, and the risk of buffer crystallization and corresponding pH changes could be expected to be eliminated. Indeed, it was shown recently that phosphate crystallization was inhibited by sugar [7]. However, it was also shown that the prevention of disodium phosphate crystallization does not necessary avoid significant pH changes during freezing. Therefore, one can hypothesize that even in case when disodium phosphate remains amorphous, use of phosphate buffer may result in significant acidification during freeze-drying and corresponding destabilization of acid-sensitive molecules.

This hypothesis was tested in a case study in which sucrose lyophiles were prepared with either sodium citrate or sodium phosphate buffers adjusted to various pH values ranging from 5.5 to 7.5 before freeze-drying, and the apparent solid-state acidity of lyophilized materials was expressed using Hammett acidity function [11, 42]. The high sucrose/buffer ratio (10 wt.% sucrose/20 mM buffer in the pre-lyo solution) is expected to prevent crystallization of disodium phosphate; also, the lyophiles were confirmed to be X-ray amorphous. However, the phosphate lyophiles

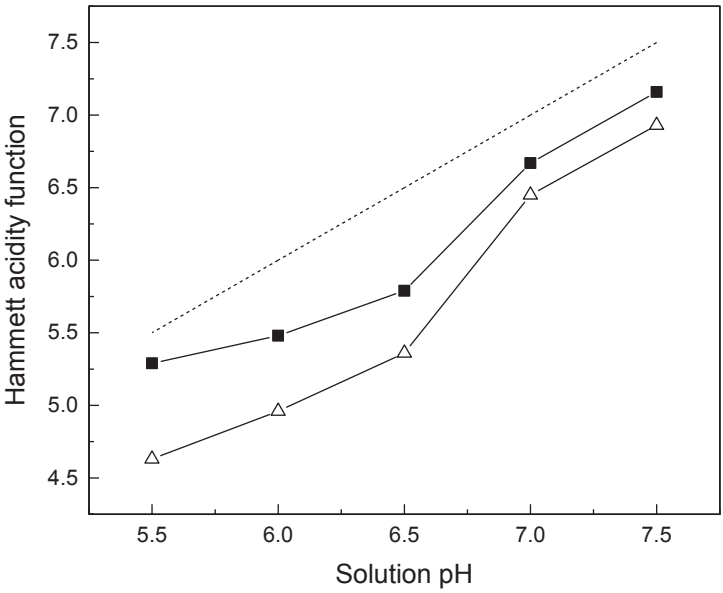

Fig. 3 Hammett acidity functions of lyophilized sucrose/buffer formulations as a function of pre-lyophilization solution pH of citrate (*solid square*) and phosphate (*triangle*) buffered systems. The *dashed line* indicates a hypothetical situation in which the Hammett functions as numerically equal to the prelyophilization solution pH. The other two lines are shown as visual aid (the figure was prepared using data reported by Chatterjee [11])

had lower H_{2-} (higher acidity) than citrate lyophiles, as shown in Fig. 3, indicative of significantly increased apparent acidity of phosphate lyophiles.

This comparison of the two buffers was extended to a system consisting of an amorphous active pharmaceutical ingredient (API) co-lyophilized with either phosphate or citrate buffer. Maximal stability in solution for this particular API was observed at pH 7, with the degradation rate increasing with lowering the solution pH. Three phosphate and four citrate formulations, each containing 200 mg/ml of the API and 20 mM buffer, were lyophilized from solutions at pH 6.6–7.35 (phosphate formulations) and 6.4–6.95 (citrate formulations). The freeze-dried formulations were X-ray amorphous and had water content below 0.5 wt.%. These samples were placed on the accelerated stability, and the concentration of degradation products measured by high-performance liquid chromatography (HPLC) after 8 months at 40 °C. The results are presented in Fig. 4 as a function of pre-lyophilization solution pH.

It can be seen that all formulations with phosphate had higher extent of degradation than citrate formulations, whereas no trend with pre-lyophilization solution pH was observed. To evaluate potential mechanism for such difference in stability, the Hammett acidity function was measured using phenol red as a probe molecule. The Hammett acidity function for phosphate- and citrate lyophiles is presented in Fig. 5 as a function of pre-lyo solution pH.

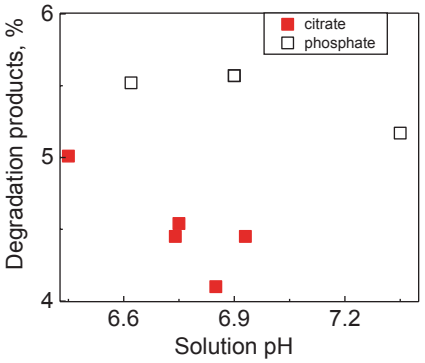

Fig. 4 Total degradation products detected in freeze-dried formulations after storage of lyophilized API with either citrate or phosphate buffer as a function of pre-lyophilization solution pH *API* active pharmaceutical ingredient

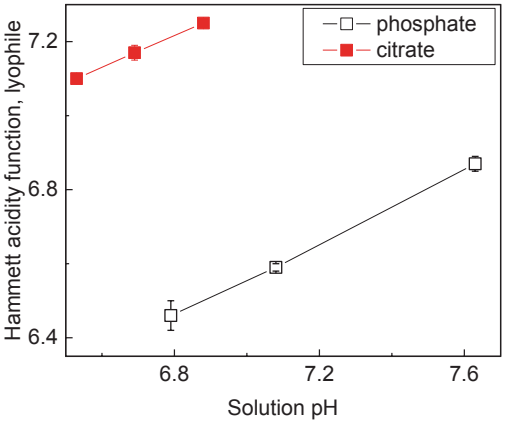

Fig. 5 Hammett acidity function determined in an amorphous API prepared by freeze-drying with either citrate or phosphate buffer from solutions with different pH values *API* active pharmaceutical ingredient

Similar to sucrose lyophiles (Fig. 3), H_{2-} in phosphate formulations were lower than in citrate, i.e., the phosphate lyophiles were more acidic. The stability data from Fig. 4 are presented in Fig. 6 as a function of the Hammett acidity function, which shows a reasonable correlation between the extent of degradation and H_{2-}, with the degradation level higher in formulations with lower H_{2-} (i.e., formulations with higher acidity).

Based on the pH stability profile of this molecule in solution, this molecule is less stable at acidic conditions, with degradation range increasing as pH decreases (data not shown). Therefore, the higher instability of phosphate lyophiles is attributed to their higher acidity.

There are several potential explanations of a higher acidity of phosphate lyophiles, as compared to citrate lyophiles prepared from the solutions at the same solution pH. It is possible that a small amount of disodium phosphate crystalized during freeze-drying, which would be difficult to detect by a conventional XRPD, but probably sufficient to cause an acidic shift in the lyophile. An alternative explanation would involve changes in apparent pK_a of the phosphate buffer, as the media

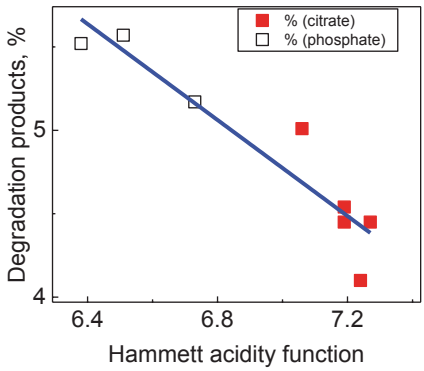

Fig. 6 Total degradation products detected in freeze-dried formulations after storage of lyophilized API with either citrate or phosphate buffer as a function of the Hammett acidity function determined in lyophiles using phenol red *API* active pharmaceutical ingredient

changes from the relatively dilute aqueous solution to an organic (sugar) matrix with low water content and lower polarity. Finally, preferential inclusion of a basic component (disodium phosphate) by ice crystals and resulting acidification of the remaining freeze-concentrate can also be proposed, as discussed above. In either case, the use of pH indicators allowed detection of the difference in the apparent acidity of formulations prepared with citrate versus phosphate buffer. The acidic shift observed with phosphate buffer after freeze-drying correlated the higher degradation rate of the acid-sensitive active ingredient in the phosphate formulations. It means that, in order to achieve a comparable stability with phosphate buffer, phosphate formulations should be lyophilized from a solution with a higher pH than citrate formulations.

Conclusion

Lyophilization of biologics is the most common approach to achieving a robust and stable drug product. The design of a formulated biologic consists of careful selection of excipients and a lyophilization process that maintains biologic activity, promote stability on storage and achieve pharmaceutical elegance and patient acceptability. A critical aspect of this process is knowing how acid–base relationships play a role in the physical and chemical integrity of the therapeutic agent, thus, the selection of a buffer system that maintains optimal pH is paramount. The complex relationships between the extent of proton transfer and temperature, the effect of temperature and dielectric constant on the apparent pK_a and effective ionization states of acids and bases complicate this seemingly simple principle. Furthermore, the potential for crystallization of buffers during cooling and freeze concentration may lead to unexpected changes in the apparent pH leading to irreversible physical or chemical changes that lower therapeutic activity. An understanding of how the therapeutic agent contributes to solution pH, as well as how the addition of stabilizers such as sugars can alter water activity and media polarity is essential to designing buffer system that achieves the desired control of proton transfer and ionization extent. Some buffers, such as phosphate buffers can have a greater propensity to

crystallize, and thus may not always be the best choice. A holistic understanding of how each component in the formulation contributes to the overall apparent pH, polarity, ionic strength, and phase behavior during each step of the lyophilization process and upon subsequent storage is necessary. For example the tendencies for the buffers to crystallize may be altered by the added sugars, and even the biological molecule itself. It was also demonstrated that significant acidification can take place during freeze-drying even if crystallization of a buffer is avoided. In this chapter, what is currently known about the ability of specific buffer systems to achieve and maintain a desired apparent pH have been discussed. In addition, more general considerations for the selection of buffer agents in the design of lyophilized formulations are highlighted and intended as a starting point for the design of lyophilized formulations with the recommendation that each formulation offers unique requirements and challenges.

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Advances in Instrumental Analysis Applied to the Development of Lyophilization Cycles

William J. Kessler, Puneet Sharma and Mircea Mujat

Key Phase Transitions During the Lyophilization Process

The lyophilization process is commonly used to formulate compounds that are unstable in aqueous solution. In order to develop a successful lyophilization process, a comprehensive understanding of the physical and chemical characteristics of formulations in the frozen and freeze-dried form is essential.

The three crucial steps in the lyophilization process are: freezing, primary drying, and secondary drying. The freezing process involves transformation of a liquid formulation to a frozen state. During this transformation, the key transition events are ice nucleation, crystallization of water, and simultaneous increase in solute concentration. Once the formulation is frozen, crystalline components crystallize out. Most disaccharides and proteins tend to remain in an amorphous phase and at the end of the freezing step may contain up to ~20 % of supercooled water. These transitions during the freezing process can be illustrated using a state diagram of a binary solution of sucrose and water (Fig. 1) [1]. Point A shows the initial temperature and composition of the liquid formulation. As the solution is cooled, ice nuclei form in the solution (point B). This event typically occurs below the thermodynamic equilibrium freezing point of the formulation. Ice nucleation is an exothermic event that leads to an increase in the solution temperature (point C). As the solution is further cooled, the solutes (such as buffer salts) may crystallize out (point D) at the eutectic temperature (T_{eu}). For a binary system, W_e represents the weight of crystalline solute at T_e. If the solutes remain amorphous (example sucrose), a glass transition event is observed (point E) at the glass transition temperature of the maximally freeze-con-

W. J. Kessler (✉) · M. Mujat
Physical Sciences Inc., 20 New England Business Center, Andover, MA 01810-1077, USA
e-mail: kessler@psicorp.com

P. Sharma
Genentech Inc., 500 Forbes Boulevard, South San Francisco, CA 94080, USA

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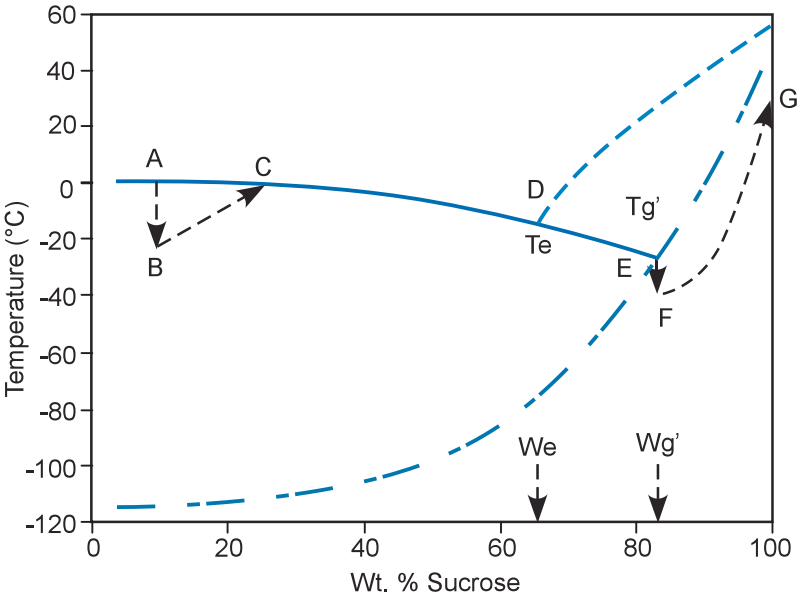

Fig. 1 State diagram of a binary solution of sucrose and water (See text for detailed explanation. Adapted from [1])

centrated solution T_g', which corresponds to ~80% sucrose content (W_g'). Note that there is ~15–20% water (depicted at point W_g') in the sucrose freeze concentrate at T_g'. Below T_g', the solute exists as glass separated from ice crystals. Although the value of T_e is constant, T_g' varies with the measurement technique. A frequent term used while designing a lyophilized formulation is the collapse temperature (T_c) of a formulation. T_c is the temperature above which a frozen concentrated matrix loses its physical structure due to viscous flow as detected using freeze-drying microscopy. In order to produce a stable lyophilized formulation, the temperature of the product (depicted by "*F*" in Fig. 1) during primary drying should be below T_g', T_e, and most importantly T_c. Therefore, estimation of T_g', T_e, and T_c is the first step in designing a lyophilization process. Since primary drying is the longest phase of a lyophilization cycle, optimization of process conditions during this step results in shorter processing times.

The primary and secondary drying processes differ by definition, with the former being related to ice sublimation and the latter to water desorption. These processes influence or facilitate phase transitions. This is primarily due to the presence of high energy states of the compounds or molecules at the very beginning (freezing) of the process, which transform to the lower energy states by the end of the lyophilization process. It is equally important to state that the presence of high- or low-energy solid forms and their interconversion is dependent on the lyophilization process design and also on the complex interaction of coexisting compounds/excipients in the formulation. In an un-optimized formulation, phase transitions from a high-energy to a low-energy form have been shown to occur following lyophilization during storage with adverse impact on the formulation stability [2, 3].

Coupling of Lyophilization Process and Product Attributes: Lyophilization Cycle Design

The estimation of product attributes such as T_g' and/or T_c is essential for successful lyophilization process development. As a general rule, lyophilization cycles should be designed such that the product temperature (T_p) during primary drying remains below the critical temperature. A temperature excursion above the critical temperature during drying could lead to a loss of physical structure of the lyophilized material, high residual moisture content, poor reconstitution times, and compromised long-term stability for the pharmaceutical product. For some formulations, in particular, high-concentration protein formulations, it is possible to lyophilize above the critical temperature (as measured using currently available instrumentation) with no impact on long-term stability [4, 5].

Following the International Conference on Harmonization Q8 R2 guidance and quality-by-design (QbD) approach, generation of a design space for primary drying using heat and mass transfer principles has been discussed in a number of publications [6–9]. Traditionally, three commercial batches at set point lyophilization conditions of time, temperature, and chamber pressure are used to qualify a drug product. However, in the event of a deviation from the set point, extensive investigation, root cause analysis, and additional studies are usually performed to assess the product quality impact. Using the QbD approach for lyophilization, such deviations in the set point conditions can be tolerated, as long as they remain within the design space, justifying completion of the lyophilization process with no impact on product quality. A general scheme of lyophilization cycle development using design space approach is shown in Fig. 2.

During the lyophilization process, T_p of a formulation contained in a vial is a function of heat ($\dot{Q}$) and mass transfer ($\dot{M}$) [10]. The effective $\dot{Q}$ and $\dot{M}$ is controlled by various parameters such as formulation composition, fill volume, vial design, shelf temperature, chamber pressure, design of chamber including shelves, vapor tube (if applicable), and condenser, and design of nitrogen bleed valve and vacuum pump capacity.

The critical temperature of a formulation is most commonly determined using modulated differential scanning calorimetry (mDSC) and freeze-drying microscopy (FDM). As the name implies, mDSC is a calorimetric method wherein thermodynamic (melting point and T_e) and kinetic parameters (T_g') associated with the freezing process are determined. A number of other methods have been used in the literature to characterize the freezing stage such as differential thermal analysis (DTA), thermo electric analysis (TEA), dynamic mechanical thermal analysis (DMA), thermo mechanical analysis (TMA), dielectric analysis (DEA), and thermally stimulated current spectrometry (TSC). Despite the advantages and disadvantages associated with each of these methods, mDSC has remained the method of choice for monitoring thermal transitions and estimation of critical temperature.

Light transmission FDM (LT-FDM) is used to determine the collapse temperature of a formulation. Here, a thin film of a small volume of liquid (1–2 µl) placed on a temperature-controlled stage is freeze-dried. The physical changes in the frozen film during the drying process are visually observed using a microscope as a

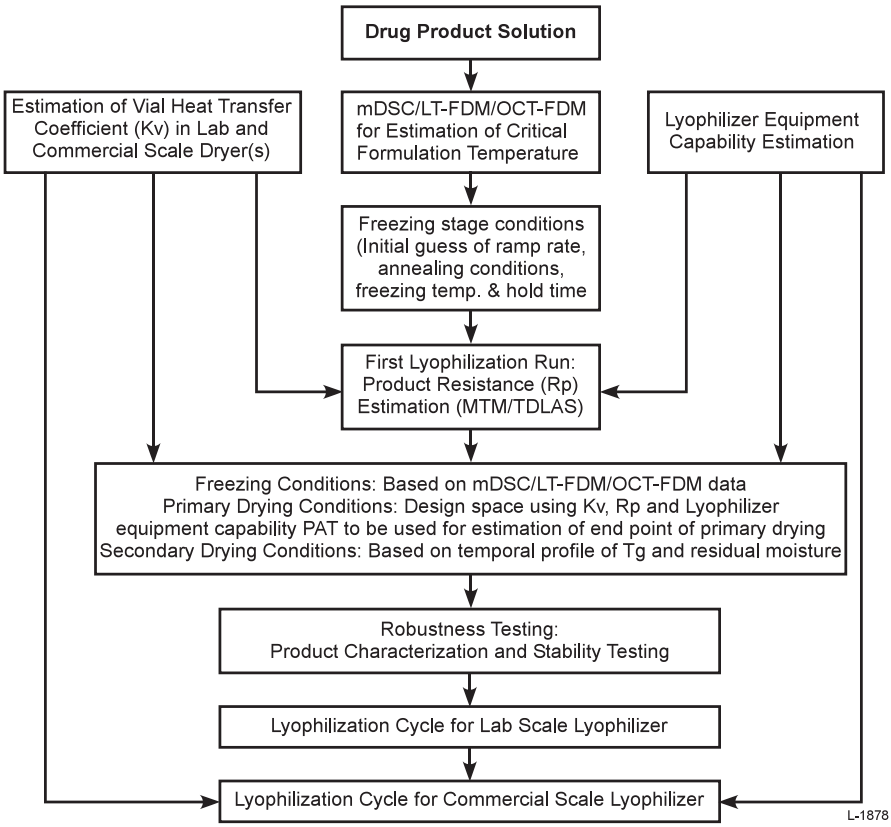

Fig. 2 General scheme for lyophilization cycle development and optimization

function of temperature. The temperature at which physical changes (viscous flow) are observed in the film is defined as the collapse temperature. Evidence in the literature shows that for some formulations differences exist between the collapse temperature of a thin frozen film and a frozen formulation in a vial. For example, Greco et al. have shown that a 25:75 bovine serum albumin (BSA)/sucrose formulation (5% total solids) can be successfully freeze-dried at 5 °C above collapse temperature of −28 °C, determined using LT-FDM [11].

Optical coherence tomography-based FDM (OCT-FDM) has been developed as an alternative to LT-FDM that has the advantage of accurate estimation of collapse temperature of a formulation [11, 12]. The advantage lies in the 3D OCT-FDM measurement approach wherein the collapse temperature of the formulation is estimated in a vial during the freeze-drying process rather than in a thin film. It should be noted that, every 1 °C rise in product temperature during primary drying decreases the primary drying time by ~13% [13] and therefore, cycle time reduction achieved using accurate estimation of the collapse temperature by OCT-FDM should lead to cost reduction and in the overall life cycle of the product. In addition, accurate determination of the collapse temperature using OCT-FDM supports QbD-based development of lyophilization processes that are currently run above the collapse

temperature determined using LT-FDM. Later sections of this chapter provide additional information on OCT-FDM and its application to lyophilization.

Once the target product temperature has been determined, lyophilization cycle conditions are estimated using fundamentals of heat and mass transfer [13, 14]. Central to the application of heat and mass transfer principles are estimation of K_v (heat transfer coefficient) of the vials, $\hat{R}_p$ (area-normalized mass transfer resistance) of the product and lyophilizer equipment capability to support the mass transfer rate. With the advancements in process analytical technologies (PAT), the task to estimate these parameters has been simplified. PAT tools such as manometric temperature measurement (MTM) and tunable diode laser absorption spectroscopy (TDLAS) have been used and demonstrated to be quite useful in estimation of the parameters critical to development of freeze-drying processes [7, 15–19]. Use of MTM requires the presence of a rapidly closing/opening isolation valve between the chamber and condenser [19]. During primary drying, this valve is closed for a 1-min interval and the pressure rise is recorded as a function of time. The MTM equation is fitted to the pressure rise data to enable the determination of parameters such as product temperature at the sublimation interface (T_p) and $\hat{R}_p$. TDLAS is a noncontact spectroscopic technique which, in conjunction with fluid dynamics modeling of the lyophilizer gas flow in the duct connecting the product chamber and condenser during the lyophilization process, can be used to measure the water vapor concentration and gas flow velocity in the spool connecting the product chamber and condenser. These measurements are combined with knowledge of the spool cross-sectional area to calculate the mass flow rate (g/s) of water vapor exiting the product chamber. The mass flow rate is combined with a heat and mass transfer model of vial freeze-drying to calculate the important freeze-drying parameters, such as K_v, $\hat{R}_p$ and T_p. The advantage of TDLAS over MTM is that it does not require the isolation valve between the chamber and the condenser, provides continuous measurements, does not result in a rise in product temperature associated with interruptions in freeze-drying, and can be used to provide accurate determination of parameters throughout both primary and secondary drying. However, a vapor tube of sufficient dimensions is a prerequisite to apply the TDLAS technique to any lyophilizer. Nevertheless, the TDLAS sensor has been shown to be applicable to estimation of K_v [17], $\hat{R}_p$ [20], product temperature at the vial bottom and sublimation interface [17], residual moisture content [18], and choked flow limits of a lyophilizer [7, 15, 21]. The principle of TDLAS and its application in lyophilization are described later in this chapter.

Other than estimation of choked flow limits (the maximum mass flow of water vapor supported by a vapor tube), equipment capability is also dependent on the condenser design and capacity. Un-optimized condenser design leads to condenser overload and a rise in condenser temperature during primary drying which, if it remains uncontrolled, would cause a loss in pressure control in the chamber. Condenser overload can be caused by loading the lyophilizer with water in excess of the condenser capacity. Other factors to consider when reviewing the condenser design are: vapor tube position inside the condenser, location of vacuum pump outlet, and condenser coil design. These factors affect vapor dynamics and the ice distribution pattern [22]. While the condenser coils surface area may be sufficient to condense

a higher load of water vapor, an un-optimized condenser design and nonuniform vapor flow over condenser coil reduce the "effective" surface area for vapor condensation and limits the capability of the equipment.

After thermal characterization of the formulation for T_g', T_c, and other thermal events (crystallization, melting, etc.), target freezing conditions (freezing ramp rates, annealing conditions, and ramp rates) and product temperature are selected. A conservative cycle is designed, that yields a good cake appearance of the product. The primary aim of the conservative cycle is to estimate $\widehat{R}_p$ (using product temperature data, MTM or TDLAS). Note that cycle optimization is not the goal for this first cycle. Conservative freezing conditions (hold times, temperature, and ramp rates) are selected based on prior knowledge and mDSC, FDM characterization studies. Conditions for primary drying are selected based on predetermined K_v values for the vials (using TDLAS or gravimetric measurement), equipment capability, and a hypothetical value of $\widehat{R}_p$, corresponding to higher mass transfer resistance. Data and guidelines described by Tang et al. [13] can also be used for selection of primary drying conditions. The primary drying end point should be estimated using a method that yields the gas composition inside the chamber such as TDLAS and/or a Pirani gauge [23]. Conservative conditions for secondary drying are used (slow ramp rate, extended hold time, temperature based on literature data on T_g of the components and formulation). Lyophilized samples from this initial study should be characterized for T_g, crystallization temperature, and melting temperature. It is also advised to conduct small-scale stability testing of the samples. The stability data from initial time points can serve as an important indicator of the lyophilization process feasibility. The information generated during this first study is used to construct a design space for a particular formulation and lyophilization process.

Lyophilization Design Space for Primary Drying

Steady-state heat and mass transfer equations as described by Pikal [10, 24, 25] can be used for construction of the process design space. Figure 3 shows a graph of the sublimation rate as a function of chamber pressure used to construct the design space for the primary drying stage. The area "choked flow regime" represents the equipment limitation to support the "maximum" mass flow during primary drying [21] and therefore shelf temperature and chamber pressure conditions generating mass flow of water vapor in the "choked flow regime" should be avoided. In cases where the condenser design is the limiting factor in achieving the target mass flow, the choked flow regime is replaced with the "condenser overload regime."

The product temperature (T_p) during primary drying is a function of shelf temperature (T_s) and chamber pressure (P_c). A general representation of steady-state heat and mass flow during vial-based freeze-drying is useful for the selection of shelf temperature and chamber pressure is presented in Eq. 1:

$$\dot{Q} = A_v \cdot K_v \cdot (T_s - T_p) \tag{1}$$

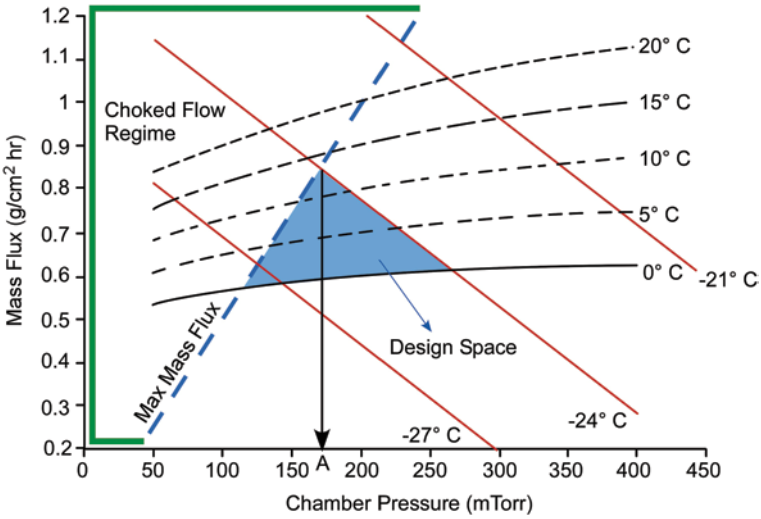

Fig. 3 Design space graph for primary drying stage of lyophilization with design space, control space, and set point

where $\dot{Q}$ is the heat transfer rate (cal/s), A_v is the outer cross-sectional area of the vial (cm^2), K_v is the vial heat transfer coefficient (cal/s.cm^2.K), T_s is the shelf surface temperature (°C), and T_p is the product temperature at the sublimation interface (°C).

Mass transfer of water vapor through the dried product layer (ignoring the small resistance of the stopper) can be represented using [25]:

$$\frac{dm}{dt} = A_p \cdot \frac{p_{ice} - p_c}{\hat{R}p} \tag{2}$$

where dm/dt is the sublimation rate per vial (g/h/vial), A_p is the internal cross-sectional area of the vial (cm^2), P_{ice} is the vapor pressure of ice (Torr), P_c is the chamber pressure (Torr), and $\dot{Q}$ is the area-normalized dry layer resistance (cm^2.Torr.h.g^{-1}) of the product in the vial. P_{ice} (the vapor pressure of water above ice at temperature T_{ice}) can be expressed as [26]:

$$p_{ice} = 2.69 \times 10^{13} \times e^{-6144.96/T_{ice}} \tag{3}$$

where T_{ice} (or T_p) is the temperature of the ice (or product) at the sublimation interface (K) which can be expressed as [26]:

$$T_p = T_b \frac{(\dot{Q}) \cdot L'}{A_v \cdot K} \tag{4}$$

T_b is the product temperature at the bottom of the vial (K), $\dot{Q}$ is calculated using the relation $\Delta H_s * dm/dt$, where ΔH_s is the heat of ice sublimation (cal/g). L' is the thickness of the dry layer (cm), and K is the thermal conductivity of ice (cal/h.cm.K). L' as a function of time (t) can be represented using [26]:

$$L'(t) = \frac{m_o - m(t)}{p_{ice} \cdot A_p \cdot E} \tag{5}$$

where m_o is the initial mass of water (g) per vial, $m(t)$ is the cumulative mass of sublimed ice up to time t (g), p_{ice} is the density of ice (g/cm^3), A_p is internal cross-sectional area of the vial (cm^2), and E is the ice volume fraction.

$\hat{R}_p$ as a function of dry layer thickness $L'(t)$, can be represented as [26]:

$$\hat{R}_p = R_o + \frac{A \cdot L'}{1 + B \cdot L'} \tag{6}$$

The parameter R_0, A, and B are constants and can be evaluated from a fit of the Eq. 6 to data using regression analysis.

Selection of the shelf temperature and chamber pressure for the purpose of defining the design space is dependent upon the target product temperature and primary drying time. The approach for generation of the process design space has been discussed by Mockus et al. [7]. In order to construct the design space, shelf and product temperature isotherms are developed using Eqs. 1–6. Multiple shelf temperature isotherms (relationship between chamber pressure and sublimation rate at constant shelf temperature) are generated by using predetermined values K_v (using TDLAS and sublimation tests) and $\hat{R}_p$ (from the first lyophilization run). Here, at a particular shelf temperature and chamber pressure, Eqs. 1–6 are solved iteratively for a product temperature which is then used to calculate the sublimation rate. This approach is repeated for the same shelf temperature but by varying chamber pressure. Ultimately, a series of sublimation rate values as a function of chamber pressure at constant shelf temperature are obtained which can be plotted to generate the shelf temperature isotherm. Product temperature isotherms (relationship between chamber pressure and sublimation rate at constant product temperature) are generated using Eqs. 2–3. Here, at a particular product temperature, P_{ice} is calculated first using Eq. 3 and then at a particular chamber pressure, the sublimation rate is calculated using Eq. 2. This approach yields a series of sublimation rate values as a function of chamber pressure at a constant product temperature, which are then plotted to generate the product temperature isotherm. Using the equipment capability curve and the shelf and product temperature isotherms, the design space for primary drying is generated (Fig. 3).

Figure 3 shows primary drying design space for a hypothetical product. Assuming a target product temperature of −24 °C, chamber pressure and shelf temperature conditions of 170 mTorr (A) and ~13 °C, respectively, would result in the fastest

sublimation rate that is also supported by the equipment (not in the choked flow region) leading to shortest drying times. A slightly more conservative set of conditions would typically be chosen to operate closer to the center of the design space.

The total heat transfer coefficient K_v of a vial is influenced by three heat transfer mechanisms: (1) radiation heat transfer (K_r) from the chamber walls and shelves above and below of a vial, (2) heat transfer due to gas conduction (K_g) between the top of the shelf and the bottom of the vials, and (3) heat transfer due to the contact between the glass vial and the shelf surface (K_c). Due to the differences in vial locations, vial manufacturing processes which lead to nonuniform vial contours, shelf inter distances, hot and cold spots on the shelf surfaces and shelf surface nonuniformity, the contribution of these three modes of heat transfer to the total K_v varies in a batch undergoing freeze-drying. Moreover, due to differences in ice nucleation temperature of different vials during freezing, $\dot{Q}$ per vial varies in a batch. Due to these batch variations in K_v and $\hat{R}_p$ different vials in a batch have different drying times. This variation in K_v and $\hat{R}_p$ should be considered when using the steady-state heat and mass transfer equations to generate the design space. Using the average values K_v and $\hat{R}_p$ for a batch of vials may lead to a situations where if the cycle is designed to run close to the boundary of the design space, there may be product failure in a subset of vials due to some of vials experiencing higher than average heat transfer (high K_v) leading to collapse or drying too slowly (high $\hat{R}_p$ and low K_v) leading to melt back and high residual moisture.

To check the validity of the mathematical model and design space, robustness studies should be carried out. At least three runs should be conducted at a set of conditions (shelf temperature, chamber pressure, time) that test the high, medium, and low limits of product temperature, primary drying time, and residual moisture. The samples from these studies should be characterized for stability (accelerated and long term). During the transfer of a cycle to a large-scale clinical or commercial dryer, modifications have to be made in the design space to account for the differences in equipment capability (choked flow or condenser overload regime), K_v, and $\hat{R}_p$. Differences in equipment capability are due to differences in equipment design and scale. Differences in K_v arise primarily due to changes in the percentage of vials on the edge of the array (edge vials) which changes the contribution of K_r to the total K_v of all the vials. Due to differences in the number of particulates in the air between a laboratory and class 100 environment, the ice nucleation temperatures and hence $\hat{R}_p$ may also differ b etween a laboratory scale and commercial scale lyophilizer. Typically, compared to a laboratory scale dryer with a plexiglass door, the value of K_v is lower in a commercial scale dryer with a stainless steel door due to reduced K_r and a reduction in the percentage of edge vials in a commercial scale dryer. The average value of $\hat{R}_p$ is expected to be higher in a commercial scale dryer due to a lower ice nucleation temperature as compared to a laboratory scale dryer. The use of a PAT tool such as TDLAS in a commercial scale dryer will enable these parameters to be estimated in a time-efficient manner.

Recent Advancements in Thermal Analysis

As previously described, freeze-drying is an example of a process where material properties dictate the drying dynamics and these properties and their response to thermal input must be considered when developing lyophilization process parameters. Since the driving force for sublimation is the difference between the temperature-dependent vapor pressure of ice at the product-drying interface and the chamber pressure, the duration of primary drying (i.e., the ice sublimation stage) is very sensitive to product temperature. An increase of 5 °C in product temperature decreases the primary drying time by approximately a factor of two, and since primary drying is normally the longest part of the process, significant time savings can be achieved by running the process at the highest possible temperature consistent with producing high-quality product. However, there is an upper temperature limit above which damage to product quality occurs. For a purely crystalline system, this upper temperature limit is the eutectic temperature, T_{eu}. In most cases, however, the solutes are at least partially amorphous, and it is the viscous flow resulting from being above the glass transition temperature of the freeze-concentrated amorphous solutes, T_g', that causes loss of structure and collapse [27]. The structural deformation classified as collapse normally occurs a few degrees above the T_g', since the system is drying at the same time when viscous flow is taking place, and some time is required for sufficient viscous flow to occur to produce significant structural deformation. The loss of structure is referred to as a "eutectic melt" (crystalline systems) or "collapse" (amorphous systems), and is normally unacceptable in a pharmaceutical product from the viewpoints of the customer, regulatory agencies, and the manufacturer. Collapse frequently results in loss of product elegance, high residual moisture in the final product, possible product degradation, prolonged reconstitution times, and extended secondary drying times, all of which are unacceptable for product quality [27]. Freeze-drying below the collapse temperature, T_c, is typically required to manufacture high-quality pharmaceutical products. Thus, there is a need to accurately determine the product formulation collapse temperature and to design lyophilization processes which maintain the product temperature during primary drying close to, but safely below the collapse temperature. In fact, process QbD is not possible without accurate knowledge of T_c.

In most academic and pharmaceutical industry laboratories, the pharmaceutical product formulation collapse temperature is currently measured using LT-FDM or estimated by determining T_g', the glass transition temperature of the maximally concentrated solute in contact with ice, using differential scanning calorimetry (DSC) [27]. The T_g' is normally 1–3 °C lower than the T_c; however, differences of 5–10 °C have been reported [28]. Using T_g' as an estimate for T_c, typically results in a primary drying temperature that is lower than required and therefore results in significantly longer processing times.

The use of microscopy to monitor the freezing behavior of product solutions and the growth of ice crystals was reported by Luyet in 1960 [29]. The use of a freeze drying microscope to monitor product drying was introduced by MacKenzie

[30, 31]. Within the past 10–15 years, advanced commercial instrumentation has been introduced to provide scientists with a robust tool for routinely performing FDM and determining product formulation collapse temperature of thin-film product samples. LT-FDM is a scattering-limited light transmission measurement that is performed using a vacuum cold stage and a high-resolution imaging microscope which typically includes an option to record movies of the images. A thin film (10–100 μm) of solution (1–2 μl) is frozen between microscope coverslips and is subjected to vacuum. The temperature is raised, typically at a ramp rate of ~1 °C/min, and stabilized at a new temperature set point to initiate sublimation. Light transmission images are acquired to monitor the progression of the sublimation front through the frozen, thin-film sample. The process of raising the temperature, stabilization, and imaging is repeated and correlated to thermocouple (TC)-based product temperature measurements until visual changes are observed that are indicative of viscous flow and product collapse. Collapse is indicated by the consolidation and growth in size of dried product pores. Polarized light microscopy is often used to enhance the imaging and the visualization of collapse.

The use of thin-film samples in LT-FDM has a number of limitations for the measurement methodology. Although LT-FDM provides useful information regarding collapse on thin samples, it does not provide information regarding product formulation micro collapse or the onset of collapse in 3D samples, representative of commercial freeze-drying in vials or other container systems. T_c in a product vial may be quite different than in a thin film due to differences in ice nucleation causing differences in dry layer resistance, pressure gradients in the dry layer, and the heat transfer and drying rates [27]. Collapse is a dynamic event which is dependent on viscous flow of the product; therefore, if drying occurs before significant viscous flow occurs, the cake will not collapse even if the temperature is above the glass transition temperature of the solute phase, T_g'. LT-FDM of a thin film may not be representative of these dynamic processes. Both theoretical and experimental evidence suggests that freeze-drying in a vial is sufficiently different than in a 2D sample such that T_c for a product in a vial may be several degrees (or more) higher than measured using conventional LT-FDM, particularly for protein formulations [27]. This will result in the use of lower primary drying shelf temperatures and thus lower than required product temperatures. The reduction in primary drying temperatures will lead to longer drying times and un-optimized lyophilization processes.

Optical Coherence Tomography-Based FDM

Recently, a new collapse temperature determination approach was demonstrated based upon the application of OCT interrogation of a product formulation contained within a standard 10-ml pharmaceutical vial undergoing drying in a single-vial freeze-dryer [11, 12]. OCT is the optical analog of ultrasound [32, 33] providing cross-sectional imaging enabled by the measurement of the magnitude and echo time delay of backscattered light using a Michelson interferometer. The OCT-based

measurements provided a product image resolution of ~5–8 μm over a ~1–2-mm depth and a ~3–4-mm height in a vial during product formulation freeze-drying. The OCT 2D image slices where used to construct 3D images which were combined with TC-based product temperature measurements during freeze-drying to determine the product collapse temperature, T_c, in a container of practical significance, a vial.

Instrument Description

The OCT-FDM system consisted of an optical microscopy system combined with a single-vial freeze-dryer as shown in the Fig. 4 schematic. The experiments focused on developing and demonstrating a product collapse temperature measurement system that enabled determination of T_c in a container of practical importance under drying conditions typically encountered during standard pharmaceutical product lyophilization. The new approach was designed to overcome the limitations of LT-FDM described above. The single-vial freeze-dryer included a temperature-controlled product shelf, a liquid nitrogen-cooled cold-finger-based condenser for cryo-pumping water vapor, a mechanical pump for removing the noncondensable gases, a Pirani gauge and capacitance manometer for monitoring the product drying chamber pressure, and a vacuum isolation valve and a window to enable optical interrogation of the product drying within the vial. A top-view photograph of the single vial dryer loaded with a vial and the imaging coordinate system is shown in Fig. 5. Optical interrogation was performed through the window port on the left side of the product chamber. Vacuum pump out was accomplished through the right side of the chamber.

A time domain OCT (TDOCT) imaging system was used to interrogate the pharmaceutical product contained in the vial during freeze-drying. Figure 6 shows a schematic of the TDOCT setup. The OCT system was driven by a fiber-coupled 1275 ± 50-nm superluminescent diode whose output was split into two optical paths using a 90/10 % fused fiber optic splitter. The 10 % output from the splitter was used to illuminate the reference arm optics through an acousto-optic modulator (AOM)

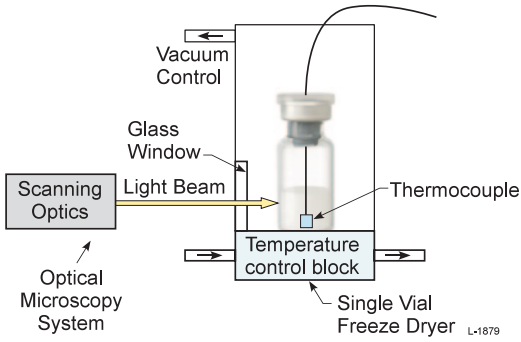

Fig. 4 Schematic diagram of the OCT-FDM imaging system including the single-vial freeze-dryer and the optical microscopy system. *OCT-FDM* optical coherence tomography freeze-drying microscopy

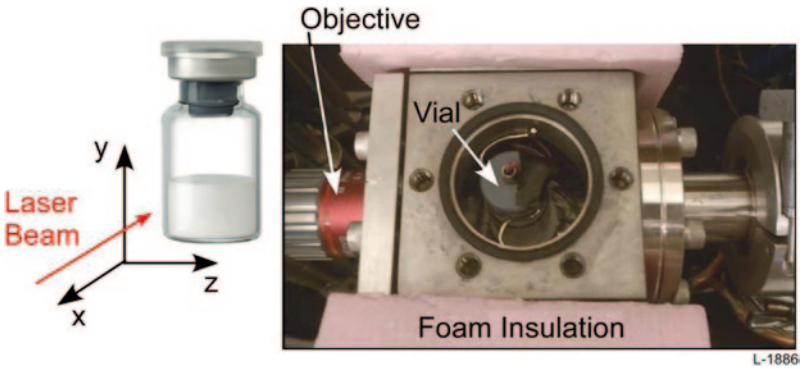

Fig. 5 Photograph of the single-vial freeze-dryer product chamber loaded with a product vial and a schematic of the vial image coordinate system

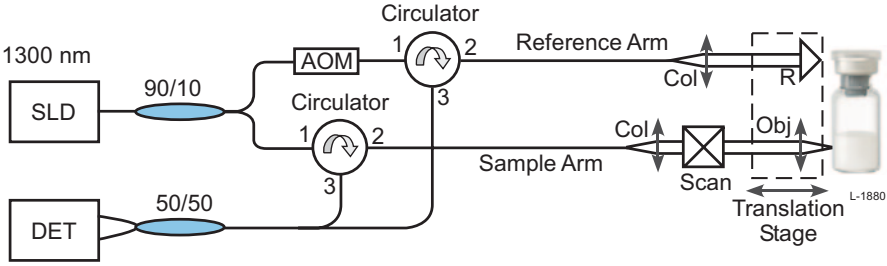

Fig. 6 TDOCT setup. *SLD* 1300 nm superluminescent diode, *DET* balance detector, *AOM* acousto-optic modulator, *Circ* circulator, *Col* collimator, *R* retroreflector, *Scan* scanning optics, *Obj* microscope objective, 90/10 and 50/50 fiber beam splitters. *TDOCT* time domain optical coherence tomography [12]

which phase modulated the output to provide a signal detection carrier frequency. The optical output from the AOM passed through a fiber optic circulator (Circ; 1→2) before transmission to a delay line in the reference arm which consisted of a collimator, a retroreflector, a prism, and two mirrors for folding the optical path in a compact space. The collimator was mounted on a translation stage to allow for adjustment of the reference arm length to overlap the coherence gate with the microscope objective focal plane. The retroreflector was mounted on a motorized translation stage together with the sample arm microscope objective for *z*-scanning and dynamic focusing [34, 35] to maintain optimized imaging resolution at variable depths within the product contained in the vial. The 90 % output from the fiber splitter illuminated the sample arm through a second circulator and scanning optics which consisted of a collimator, a microscope objective (5X), two galvanometers for *x*–*y* scanning, and five mirrors for folding the optical path. Movement of the microscope objective was used to focus the signal beam at known depths within the sample to maintain optimized image resolution. The light coming back from both the reference and the sample arms (and passing through the circulators along the

2→3 route) was recombined with a 50/50 fiber splitter and the interference signal was detected with a balanced detector which rejected the background and the common signal noise.

Image data were collected by optical scanning in the *x*–*y* image plane (en-face) at a selected *z*-axis depth within the sample. Following the capture of a single en-face 2D image the position of the microscope objective was adjusted to a new depth and a subsequent 2D image was acquired. This procedure was repeated over a time period of 282 s to enable the construction of a 3D image cube at a single shelf temperature and thus product temperature setting. The temperature of the shelf was then adjusted to obtain a new product temperature and the process was repeated. The 3D image covered an area of approximately 0.75 mm in width, 4 mm in height, and 1.5 mm in depth (*x*/*y*/*z*). The reported instrument measurement resolution was 6.9 μm/7.5 μm lateral/axial. Additional details regarding the single vial freeze-dryer and the OCT optical imaging systems are described in Reference [12].

Measurement Results

Mujat et al. [12] have provided a detailed description of the OCT-FDM hardware while Greco et al. [11] described its use for determining pharmaceutical product formulation collapse temperature. Demonstration of the new measurement technique focused on comparing OCT-FDM collapse temperature determinations to LT-FDM determinations and DSC measurements of T_g'. The authors reported that in general the collapse temperatures of product formulations drying in vials determined using OCT-FDM were warmer than those determined using LT-FDM of thin-film samples. For example, 5 % sucrose formulations were investigated using both LT-FDM, T_c: −32 °C (Fig. 7), and OCT-FDM, T_c: −28 °C (Fig. 8), measurement tech-

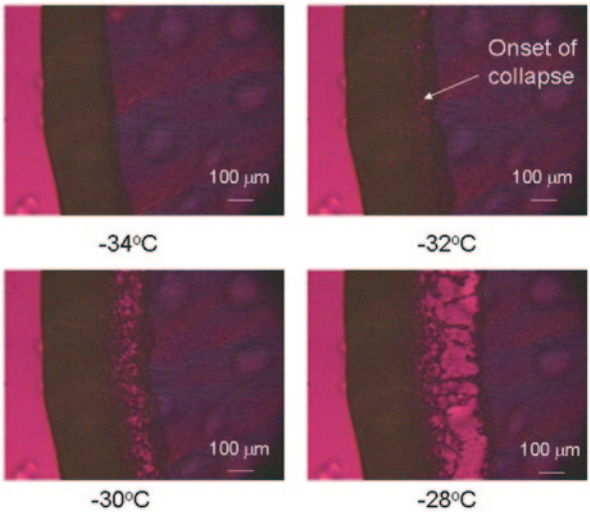

Fig. 7 Light transmission freeze-drying microscopy for a 5 % sucrose solution (Adapted from [11])

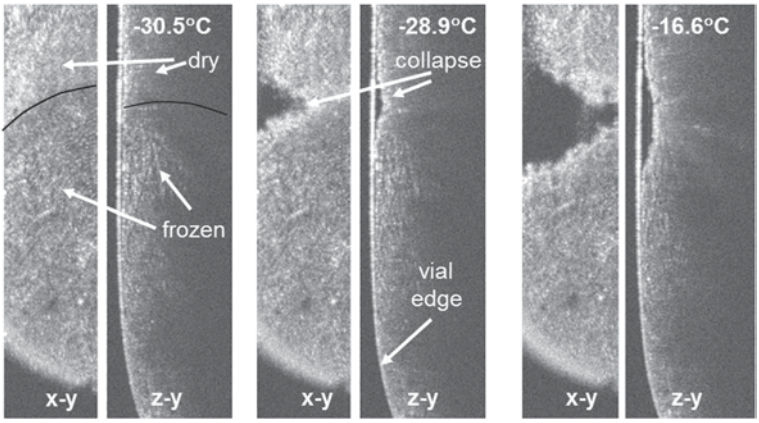

Fig. 8 OCT-FDM data for 0.6 ml of a 5 % sucrose solution. *OCT-FDM* optical coherence tomography freeze-drying microscopy (Adapted from [11])

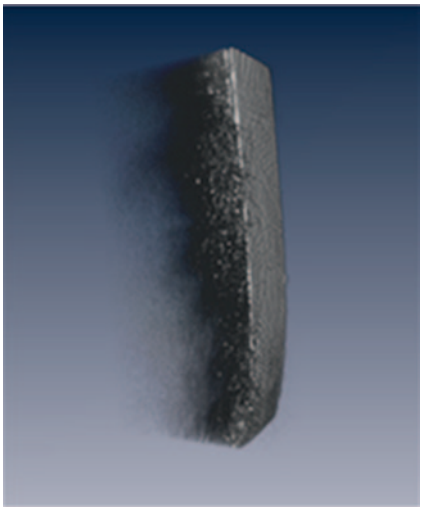

Fig. 9 OCT-FDM 3D image of 5 % sucrose product structure during freeze-drying at −30.9 °C. *OCT-FDM* optical coherence tomography freeze-drying microscopy (Adapted from [12])

niques. A 3D image of the 5 % sucrose product structure at −30.9 °C is shown in Fig. 9. The smooth right side of the image is the product interface at the wall of the vial. Figure 10 shows the same product (with the image counterclockwise rotated) following product shrinkage from the vial wall and internal collapse. These two images indicate the detailed structural information that was provided by the OCT-FDM imaging technique. The T_g' was measured to be −34 °C by DSC.

The OCT-FDM measurement predicted a collapse temperature 3.6 °C higher than LT-FDM. There was concern that the TC measurements made during the vial-based OCT-FDM displayed discontinuities at temperatures equal to or slightly warmer

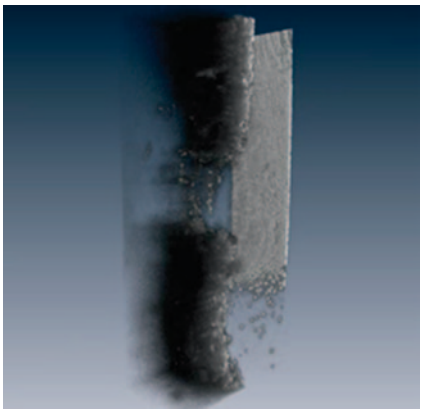

Fig. 10 OCT-FDM 3D image of 5 % sucrose product structure following product collapse. *OCT-FDM* optical coherence tomography freeze-drying microscopy (Adapted from [12])

than the OCT-FDM-determined collapse temperature. This observation raised concern that the TC-based temperature measurements possessed measurement error, possibly due to radiation effects or thermal conductivity down the TC wire. The collapse temperature determination is dependent upon accurate product temperature measurements made by the TCs placed within the vials for comparison to the product images. Measurement errors might result in an incorrect conclusion that the product behaved differently in the vial as compared to the thin film.

To address this concern, Greco et al. performed an experiment drying a 5 % NaCl formulation within a vial. The determination of an amorphous product formulation collapse temperature may be affected by the measurement technique, temperature ramping rate, etc. A NaCl product formulation forms a crystalline matrix which undergoes a eutectic melt, a thermodynamic event that will occur at the same temperature independent of the measurement technique. Determination of the eutectic melt temperature and comparison to published values enabled an assessment of the TC-based temperature measurement accuracy and the combination of the temperature measurements and imaging used to determine collapse or eutectic melt temperatures. These experiments demonstrated good agreement with the published value of −21.1 °C [36]. The LT-FDM determination of melt was −21 °C while the OCT-FDM determination was −21.2 °C. Figure 11 shows a 3D image of the NaCl product structure following eutectic melt. Once again the image clearly indicates the capability of the measurement technique and its potential to detect the onset of melt or collapse and the growth of structural voids due to the consolidation of pores. The NaCl experiment provided evidence of the validity of the TC-based temperature measurements up to the collapse or eutectic melt temperature. The authors hypothesized that the TC measurement discontinuity at the collapse temperature was due to product pulling away from the TC and the reduction of local cooling due to sublimation.

The final set of experiments reported by Greco et al. [11] involved the drying of a disaccharide-based protein formulation, 25 % BSA, 75 % sucrose, and 5 % total solids. T_g' measured using DSC was −28 °C. The collapse temperature as deter-

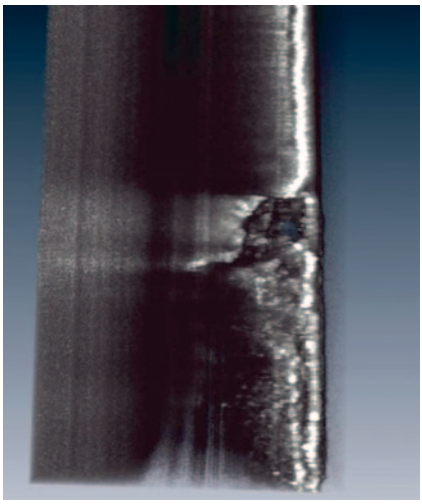

Fig. 11 OCT-FDM 3D image of 5 % NaCl product structure following eutectic melt. *OCT-FDM* optical coherence tomography freeze-drying microscopy

mined by LT-FDM was also −28 °C, with complete collapse occurring by −26 °C. OCT-FDM-based imaging of the BSA/sucrose product drying within a vial did not display internal collapse at any temperature. The authors observed product shrinkage, a form of viscous flow, from the wall of the vial beginning at −22.3 °C. The product continued to shrink from the wall at higher temperatures, but they observed no evidence of significant changes to the internal product structure as had been previously observed for 5 % sucrose and 5 % NaCl product formulations. The LT-FDM and OCT-FDM data are shown in Figs. 12 and 13.

A demonstration of the potential impact of the observed differences in BSA/sucrose collapse temperature determination was undertaken by developing lyophilization cycles based on two collapse temperature determinations. Two drying cycles were performed in a Lyostar II freeze-dryer (SP Scientific) using one full tray of

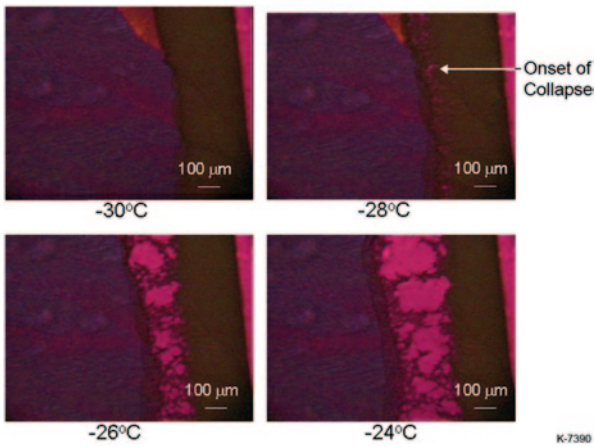

Fig. 12 LT-FDM 2D images of 5 % BSA/sucrose (25 %/75 %) product formulation showing the onset of collapse at −28 °C. *LT-FDM* light transmission freeze-drying microscopy, *BSA* bovine serum albumin (Adapted from [11])

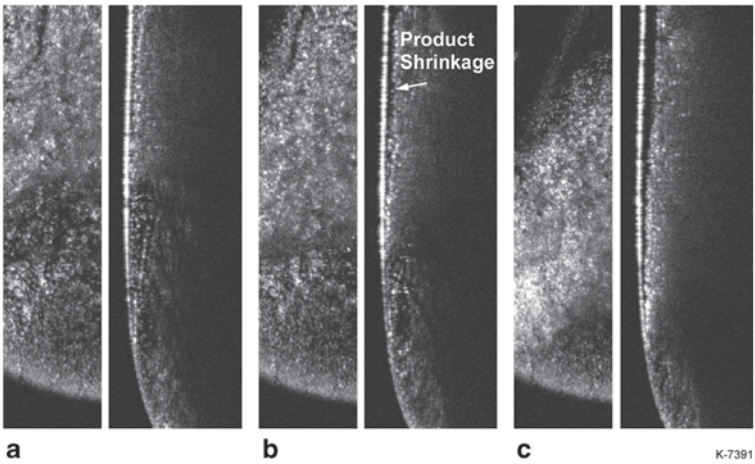

Fig. 13 OCT-FDM 2D images of 5% BSA/sucrose (25%/75%) product formulation showing the onset of shrinkage at −22.3 °C [11]. *OCT-FDM* optical coherence tomography freeze-drying microscopy

vials, filled with 25%/75% (5% total solids) BSA/sucrose formulation. The low-temperature drying cycle based on the LT-FDM collapse temperature determination (−28 °C) was operated at a shelf temperature of −22 °C and 65 mTorr chamber pressure to achieve an average product temperature of −34 °C during primary drying, measured using TCs placed in the bottom center of sample vials. The secondary drying shelf temperature was 40 °C. The primary drying time for this batch was 20 h. The high-temperature drying cycle based on the OCT-FDM shrinkage temperature (−22.3 °C, no internal collapse) was operated at a shelf temperature of 50 °C and 300 mTorr chamber pressure to achieve an average product temperature of −23 °C. The secondary drying shelf temperature was 50 °C. The primary drying time for this aggressive cycle was 4 h, an 80% reduction in time compared to the low-temperature process. The authors reported that macroscopic product collapse was not observed in vials from either cycle, although product shrinkage from the vial wall was observed in vials from both batches, consistent with reports and prior experience drying sucrose-based formulations. Additional product quality assessments were performed on the dried product from each batch to enable comparison of T_g (high temp: 65.6 °C, low temp: 72.2 °C), residual moisture (high temp: 1.7%, low temp: 1.2%), and specific surface area, SSA (high temp: 0.138 m^2/g, low temp (0.760 m^2/g). *Scanning electron microscope* (SEM) analysis was also performed on dry product samples from each batch showing a reduction in the number of pores and an increase in the pore size for the product dried at the higher primary drying temperature based on the OCT-FDM-based collapse temperature determination, consistent with the SSA measurement. Thus, the authors not only observed differences in the dry product characteristics but also demonstrated agreement between the batch freeze-drying data and the OCT-FDM observations. No collapse

was observed when drying at the higher product temperature, consistent with the OCT-FDM collapse temperature determination data.

The publications by Mujat et al. and Greco et al. reported on the use of OCT-FDM to measure T_c and T_{eu} of three formulations freeze-dried in vials. This novel use of OCT provided a new technique for measuring T_c that was predictive of freeze-drying in vials, a container of practical significance. Use of OCT-FDM will be a significant advantage when processing formulations that can be freeze-dried above the LT-FDM-determined T_c without loss of product quality. There have been numerous reports of successful freeze-drying above the LT-FDM determined collapse temperatures with no evidence of macroscopic collapse of freeze-dried cake [5, 37–44], but these investigations were performed on a trial and error basis with no representative data of T_c measurements in a vial to validate the decision to dry at higher temperatures. T_c measured by OCT-FDM provides quantitative justification for freeze-drying above the T_c as measured by LT-FDM, which is in line with QbD principles.

Future application of OCT-FDM for the determination of T_c may provide more accurate determinations for drying within vials and support the development of lyophilization processes that reduce the primary drying time and increase process efficiency. The data reported in the Greco et al. [11] and other researchers suggest that the differences between the LT-FDM and OCT-FDM collapse measurements may be large for high-concentration protein formulations. Additional studies are ongoing with the goal of acquiring additional data to compare the two measurement techniques and to investigate the product formulations that display large differences when dried as thin films and bulk samples in vials. OCT-FDM may also be applicable for monitoring the growth of ice during the freeze-phase of lyophilization. Further studies are required to investigate this potential application.

Tunable Diode Laser Absorption Spectroscopy

The application of optically based process analytical technology sensors has the potential to provide real-time, online process information using nonintrusive measurement techniques. As described above, TDLAS is one such technique that can be applied to monitoring lyophilization to provide direct measurements of the water vapor temperature (K), concentration (molecules/cm^3), and flow velocity (meters/second) within the duct connecting the lyophilizer product chamber and condenser. These measurements can be combined with knowledge of the cross-sectional area of the duct to calculate the instantaneous mass flow rate, dm/dt (g/s). The mass flow rate can be integrated as a function of time to provide a continuous determination of total water removed. The mass flow rate may also be combined with freeze-drying heat and mass transfer models and additional process measurements (e.g., product chamber shelf temperature) and process-specific parameters (e.g., vial cross-sectional area and heat transfer coefficients) to determine the batch average product temperature. Continuous monitoring of product temperature, especially during pri-

mary drying, is a powerful tool for maintaining product quality, as product temperature affects the structure of the dry cake which in turn affects product residual moisture content, reconstitution time, product shelf life and potentially product efficacy. Thus, a TDLAS sensor can provide real-time processing information that can be directly linked to critical product quality attributes.

TDLAS sensors utilize the unique characteristics of diode lasers (narrow spectral linewidth and rapid wavelength tunability) combined with ultrasensitive detection techniques (e.g., frequency modulation, FM, spectroscopy [45–47] or balanced ratiometric detection, BRD [48, 49]) to accurately and sensitively measure gas phase analytes using the Beer–Lambert law shown in Eq. 7:

$$\mathrm{I_v = I_{o,v} \exp[-S(T)g(v - v_o)N\ell]} \tag{7}$$

where $I_{o,v}$ is the initial laser intensity at frequency v, I_v is the intensity recorded after traversing a path length, ℓ, across the measurement volume, $S(T)$ is the temperature dependent absorption line strength, $g(v-v_o)$ is the spectral line shape function, and N is the number density of absorbers (the water concentration). The lineshape function describes the temperature—and pressure-dependent broadening mechanisms of the fundamental line strength. Measurements within the low-pressure (~100 mTorr) conditions present during lyophilization, with limited gas collisions, result in $g(v-v_o)$ described by a Gaussian function. Using the BRD technique [50] and scanning the diode laser frequency across the entire absorption lineshape from baseline to baseline removes any pressure dependency of the lineshape function from the number density measurement, $\int g(v-v_o)\,dv = 1$. Scanning the fully resolved absorption lineshape also reduces the effect of broadband absorbers in the background gas and nonresonant scattering from any aerosols or particulates that may be present in the flow.

The water concentration, [H_2O], in molecules cm^{-3} is calculated using Eq. 8:

$$N = \frac{\int \ln\left[\frac{I(v)}{I_o(v)}\right] dv}{S(T)\ell} \tag{8}$$

where dv is the laser frequency scan rate per data point (cm^{-1}/point).

The gas temperature is determined by analyzing the water vapor absorption lineshape using Eq. 9, where Δv_D is the absorption lineshape full width at half maximum (FWHM) frequency (cm^{-1}), v_0 is the line center frequency (7181 cm^{-1}), T is the gas temperature (K), and M is the molecular weight (g/mole) of the absorbing species (water vapor):

$$\Delta v_D = 7.162 \times 10^{-7} v_o \sqrt{(T/M)} \tag{9}$$

Real-time measurement of the gas temperature is required to calculate the temperature-dependent water absorption line strength and properly determine the water vapor concentration. The isolated, near-infrared (IR), 7181 cm^{-1}, ($3_{03} \leftarrow 2_{02}$ rotational

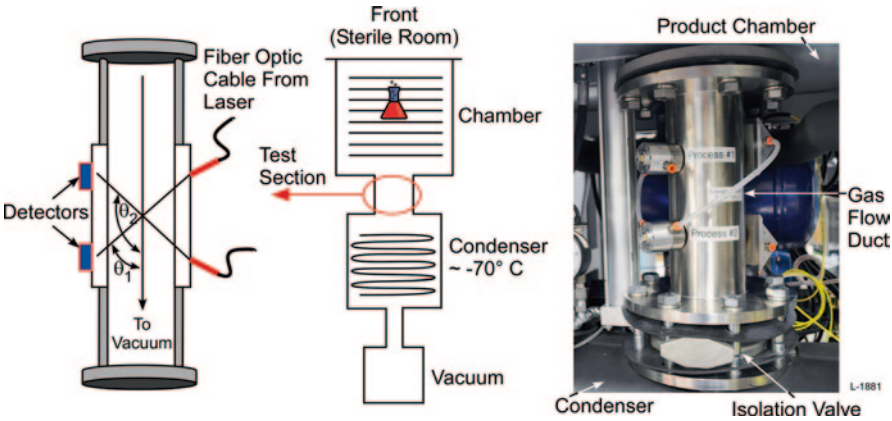

Fig. 14 Schematic diagram of the Doppler-shift absorption spectroscopy velocity measurement concept and a spool outfitted with two optical detection paths installed in a Lyostar 3® lyophilzer

line within the v_3+v_2 vibrational band), water vapor absorption feature is chosen due to its strong line strength, its weak temperature dependence (~2.7% per 10 K gas temperature change under conditions of interest during lyophilization [51]) and to leverage the existence of robust telecommunications grade, fiber-coupled diode lasers.

The measurement of the gas flow velocity within the duct connecting the lyophilizer product drying chamber and the condenser is enabled by orienting the laser beam vectors (**k**) crossing the duct at an angle ($\boldsymbol{\theta}_1$ and $\boldsymbol{\theta}_2$) with respect to the gas flow vector (**u**). This measurement architecture results in a Doppler shift of the absorption lineshape to higher or lower frequencies depending upon the orientation of the laser light propagation with respect to the gas flow. Figure 14 shows a schematic diagram of a two line-of-sight measurement configuration across the flow duct and a photograph of the optical setup mounted within a Lyostar 3 laboratory-scale freeze-dryer. Using two line-of-sight measurements across the duct results in two simultaneously recorded lineshapes shifted to higher and lower frequencies and enables the determination of the gas flow velocity, u, using Eq. 10 [52]:

$$u = \frac{c \cdot \Delta v}{v_o \cdot (\cos\theta_1 - \cos\theta_2)} \tag{10}$$

where c is the speed of light (3×10^{10} cm/s), Δv is the peak absorption shift between the two absorption lineshapes in cm^{-1}, v_o (7181 cm^{-1}) is the absorption peak frequency at zero flow velocity, and θ_1 and θ_2 are the angles formed between the laser propagation vectors across the flow and the gas flow vector. The frequency shift, Δv, is determined by the shift in data points between the two absorption profiles and converted to absolute frequency using the calibrated diode laser frequency scan rate (cm^{-1}/point).

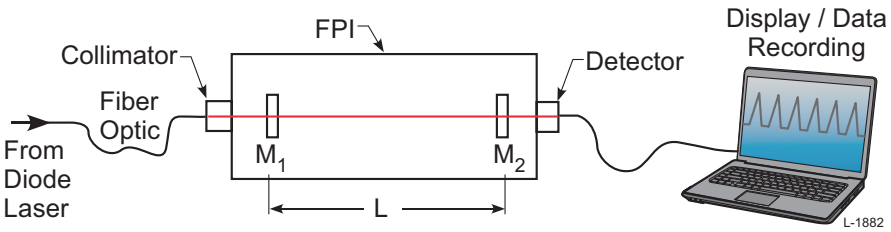

Fig. 15 Schematic diagram of the experimental setup used to calibrate the diode laser frequency scan rate (cm^{-1}/point)

The mass flow rate, dm/dt, (g/s) is calculated using Eq. 11 by the product of the measured number density (N, molecules cm^{-3}), the gas flow velocity (u, cm/s), and the cross-sectional area of the flow duct (A, cm^2; and the appropriate conversion factors).

$$\mathrm{d}m/\mathrm{d}t = N \cdot u \cdot A \cdot (\mathrm{g/s}) \tag{11}$$

The standard measurement angles θ_1 and θ_2 are 45 and 135°. These angles combined with typical laser frequency tuning rates of approximately 1 MHz/data point result in a velocity measurement sensitivity of 1 m/s/data point shift. The application of the BRD-based detection technique and the resulting high signal-to-noise ratios enables peak shift measurements of less than one data point and the measurement of gas flow velocity and mass flow rates throughout both the primary and secondary drying phases of freeze-drying.

All three measured parameters, water vapor temperature, water vapor number density, and gas flow velocity use the same instrument calibration factor, the diode laser frequency scan rate (MHz/point or cm^{-1}/point). The diode laser frequency scan rate (cm^{-1}/point) is determined by launching the fiber-coupled diode laser output into a Fabry–Perot interferometer (FPI) optical cavity. The measurement principle and an experimental schematic of the calibration technique are illustrated in Fig. 15.

The laser light is transmitted through the mirrored cavity only when cavity conditions and the laser wavelength or frequency results in constructive interference of the oscillating light waves. The transmission maxima occur separated in frequency by $\Delta\bar{\nu}$ (free spectral range, FSR) as described by Eq. 12:

$$\frac{1}{2\mathrm{n}L} = \Delta\bar{\nu}(\mathrm{cm}^{-1}) \tag{12}$$

where n is the index of refraction of the medium between the two cavity mirrors (typically air, $n = 1$) and L is the separation between the mirrors M_1 and M_2.

Figure 16 shows a water vapor absorption lineshape and an FPI fringe spectrum simultaneously recorded using a TDLAS monitor. The peak locations of the FPI interference spectrum in combination with the interferometer FSR (2000 MHz) are used to calculate the laser frequency scan rate calibration. Typical laser tuning rates

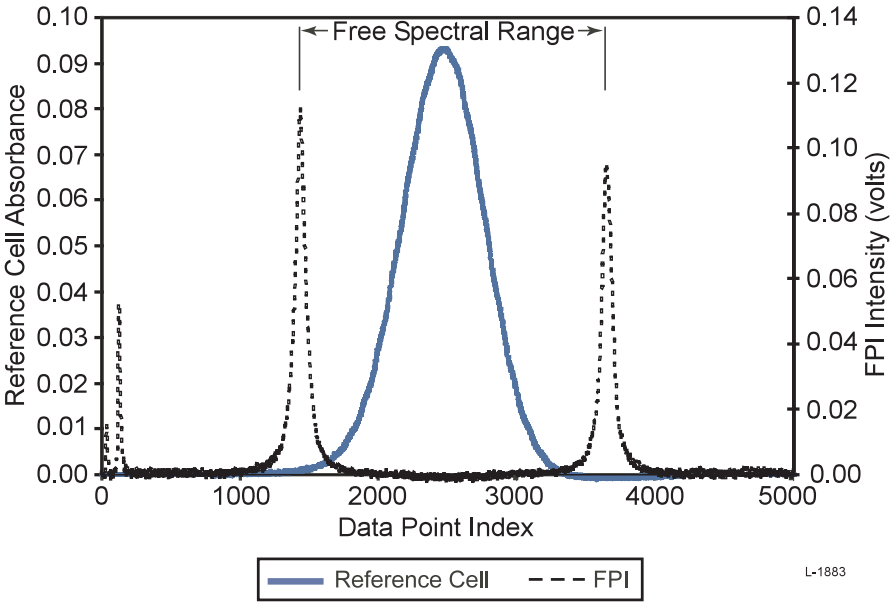

Fig. 16 Simultaneously recorded reference cell water vapor absorption spectrum and Fabry–Perot interferometer (*FPI*) interference intensity spectrum. The FPI free spectral range (*FSR*) = 2000 MHz (6.67×10^{-2} cm^{-1})

are ~ 1 MHz/data point (3.33×10^{-5} cm^{-1}/point) corresponding to a gas flow velocity of ~ 1 m/s/data point.

The diode laser scan rate is dependent upon the laser operating conditions (temperature and injection current) and the sensor is calibrated using a fixed set of operating conditions. The instrument software is configured to prevent user adjustment to these calibration sensitive parameters. Experience has demonstrated that the laser frequency scan rate calibration is typically stable for the life of the diode laser (> 10,000 h of operation) provided the laser is not operated near its current and output power damage threshold limit and in the absence of electrostatic discharge events. A verification of the instrument calibration is typically performed using ice slab sublimation tests and comparing the total amount of water removed as measured using the integrated TDLAS measurements and a gravimetric determination of total water removed [15, 17].

The sensor determines the water vapor mass flow rate by measuring the gas temperature, water concentration and flow velocity through the lyophilizer duct. Line of sight absorption measurements across the lyophilizer duct are influenced by the developing fluid flow within the pipe. While the pressure is expected to be relatively uniform across the measurement path, the gas temperature and flow velocity profiles may not be uniform depending upon the duct geometry and the flow parameters. The developing flow profile may have a significant effect on the measured temperature and velocity determinations. The sensor obtains a Doppler velocity pertaining to the line of sight measurement path. This velocity may be

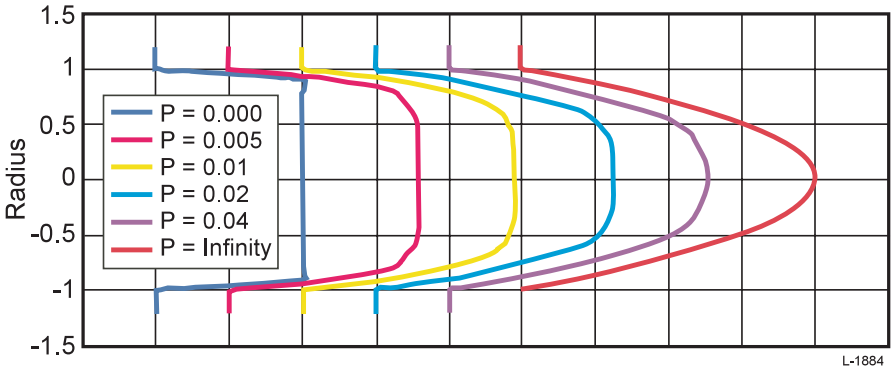

Fig. 17 Velocity profiles in the entrance region of a circular pipe

thought of as a radial segment average velocity along the measurement path, while the desired mass flow involves an integral over the duct cross section. To precisely determine the mass flow, an estimate of the velocity profile to relate the Doppler velocity to the mass flow is required.

In many lyophilizer installations, the gas flow may be approximated as a laminar entrance flow in a straight circular pipe. The entrance effects are governed by a dimensionless flow parameter shown in Eq. 13:

$$\mathrm{P} = \frac{\mathrm{X} \cdot \mathrm{v}}{(2 \cdot \mathrm{a}^2 \cdot \rho \cdot \mathrm{U_o})} \tag{13}$$

Here X is the distance from the pipe entrance to the measurement station, v is the gas viscosity, a is the radius of the pipe, ρ is the gas density, and U_0 is the uniform entrance velocity. A typical value of the flow parameter for a manufacturing scale lyophilizer with a duct diameter of 40 cm, measurement station one diameter from the beginning of the spool piece (X=40 cm), at a pressure of 100 mTorr and with a average flow velocity of 30 m/s is 0.019.

Figure 17 displays a plot of radial, r, velocity profiles for laminar flow in the entrance region of a circular pipe as replotted from Rosenhead 1963. [53].

The figure shows the expected uniform flow ($V = U_0$) at the entrance, ultimately evolving into a parabolic profile in the fully developed limit, $P = \infty$, as described by Eq. 14:

$$\mathrm{V} = 2\mathrm{U_o}\left(\frac{1-\mathrm{r}^2}{\mathrm{a}^2}\right) \tag{14}$$

A uniform velocity core persists at intermediate stations, although the core flow is accelerated above $V = U_0$.

The measured water vapor absorption lineshape data are analyzed to determine the average line of sight temperature, $\bar{T}_{\mathrm{meas}}$ and gas flow velocity, $\bar{U}_{\mathrm{meas}}$ The absorption line shape at any radial position in the flow is a function of both the temperature and velocity at each radial position described by the developing profile. These

latter quantities will have changing values across the flow (with the exception of plug flow) and thus the observed absorption lineshapes are essentially averages over these *U, T* profiles. Thus, for all flows more developed than plug flow, $\bar{U}_{\text{meas}}$ is always larger than $\bar{U}$, with the difference increasing as the temperature deficit between centerline and outer radial positions becomes larger. This is because the velocities near the center line of the flow tend to be higher than $\bar{U}$ and the number densities are also higher than $N(\bar{T})$ Thus, these values are overweighted compared to values nearer the wall. As the flow distribution becomes more parabolic (towards fully developed flow), this effect becomes more pronounced, thus a larger correction is required to determine the line of sight average flow velocity.

As described above, $\bar{T}_{\text{meas}}$ is determined from the measured water absorption linewidth. The predicted behavior of the temperature ratio, $\bar{T}_{\text{meas}} / \bar{T}$ is more complex than that of the velocity ratio because the measured temperature is affected by two competing phenomena. Firstly, the density is highest on the center line where the temperature is lowest, thus the absorption at center radial positions is weighted more than that near the wall, making the measured temperature appear cooler than the line of sight average temperature. Alternatively, the effect of the velocity distribution is to make the absorption line shape appear broader, due to the variation in the frequency position of the peak line shifts. This latter effect makes the measured temperature appear warmer than the average temperature.

In order to accurately correct the measured gas temperatures and velocities to account for the effect of the developing flow profile, absorption lineshapes were calculated as a function of flow parameter, velocity, and gas temperature difference between the duct wall and average gas temperature. The calculated lineshapes were analyzed and compared to the average flow profile (Fig. 17) gas temperature and velocity. The ratios of the calculated lineshape temperature to the average flow profile temperature, $\bar{T}_{\text{meas}} / \bar{T}$, and the corresponding velocity ratios, $\bar{U}_{\text{meas}} / \bar{U}$, are used to develop temperature and velocity scaling factors to provide the instrument user with line of sight average measurements of the water vapor number density, $N(\bar{T})$, temperature $\bar{T}$, and velocity $\bar{U}$.

Following the calculation of the average line of sight water vapor number density, and velocity, the water vapor mass flow rate may be calculated using Eq. 11. The resulting mass flow rate does not account for developing velocity flow profile. The line of sight average mass flow rate overweighs the centerline radial positions, which subtend smaller cross-sectional areas of the duct as compared to outer radial positions. To correct the calculated mass flow rate, a mass scaling factor is determined by integrating the velocity profiles shown in Fig. 17. Figure 18 displays the mass scaling factor as a function of the calculated flow parameter (Eq. 12).

TDLAS Applications

TDLAS applications in lyophilization process monitoring have been discussed in recent publications [15–18, 20, 21]. While the authors acknowledge that some of the most important applications of TDLAS will lie in its use in lyophilization

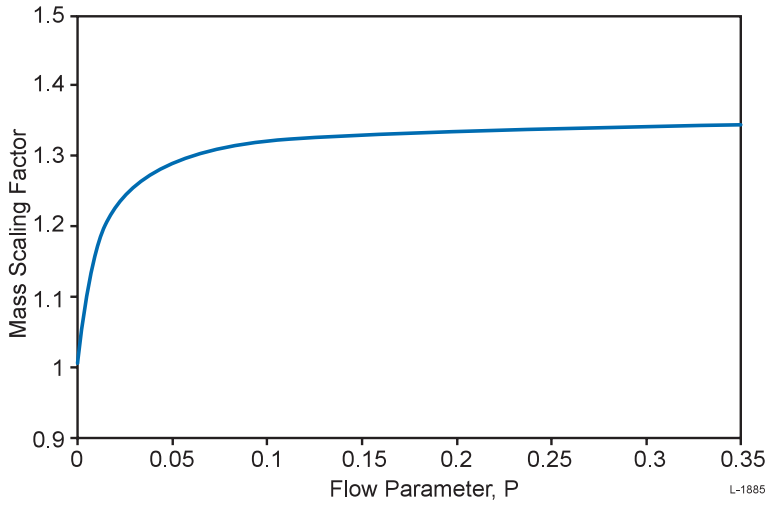

Fig. 18 Mass flow rate scaling factor as a function of the flow parameter

process monitoring during manufacturing operations, here, we make an attempt to evaluate applicability of this technique during freeze-drying cycle development. As has been previously mentioned, generation of primary drying design space for cycle development utilizes the vial heat transfer coefficient (K_v) and product mass transfer resistance (R_p) data. It has been shown by Schneid et al. [17] and Kuu et al. [20], that estimation of these parameters is achievable using TDLAS mass flow rate in conjunction with heat and mass transfer mathematical models.

While one aspect of primary drying, design space generation, focuses on screening shelf temperature and chamber pressure conditions that yield acceptable product temperature, estimation of choked flow conditions for a small-scale lyophilizer is another important aspect of the design space generation. Shelf temperature and pressure conditions that generate sublimation rate causing choked flow in the lyophilizer sets the boundary in the design space [21]. TDLAS-derived sublimation rate measurements can be rapidly applied to estimate the limits of choke flow conditions.

Another application of the TDLAS-derived sublimation rate is an estimation of residual moisture content in the product. Lyophilized formulation stability is sensitive to the presence of residual water left in the formulation after completion of secondary drying. In general, formulations are lyophilized to contain $<1\%$ w/w moisture content. However, for some formulations [54, 55], it has been shown that long-term stability is optimum at 1–3 % w/w moisture content. Schneid et al. [18] devised a technique to use TDLAS-derived sublimation rate to estimate residual moisture in 2 % BSA/5 % sucrose formulation. The technique involved a two-step process. In step 1, an "anchor point" was estimated which was essentially residual moisture content in the vials at the beginning of secondary drying process. In step 2, using this "anchor point," the total amount of water required to get to a predeter-

mined target moisture content in the product was estimated. The TDLAS-derived sublimation rate was then integrated from the start of secondary drying to the time when the predetermined amount of water, as indicated by TDLAS instrument, was removed. At this time, vials were stoppered and the residual moisture content in the product was measured using the Karl Fisher assay. A reasonable degree of accuracy (0.5 %) was obtained, at the anchor point, between the TDLAS and Karl Fisher moisture content values suggesting that the use of TDLAS technique was feasible for predicting product residual moisture at the end of secondary drying. It is worth noting that the instrument used in this study was a first-generation TDLAS instrument with single line of sight water vapor detection capability. The single line of sight measurement is less sensitive for the low-velocity of water vapor (0.5–2 m/s) that is typically experienced during secondary drying. A second-generation TDLAS sensor utilizes two lines of sight detection capability, which significantly enhances the measurement of low water vapor velocity during secondary drying stage (unpublished results).

To gain further understanding of the applications of TDLAS, interested readers are encouraged to refer the chapter "Advances in Process Analytical Technology in Freeze Drying."

Conclusion

The scientific understanding of lyophilization cycle development has evolved tremendously over the past decade. Through this chapter one strategy for lyophilization cycle development was presented that can be used for development of primary drying design space. The use of heat and mass transfer coefficients to build the design space was emphasized for the rationale design of the primary drying stage. Application of heat and mass transfer mathematical modeling together with the use of new spectroscopic tools, such as OCT-FDM and TDLAS, provides a refinement to lyophilization cycle development strategy. While OCT-FDM provides in situ imaging of product structure during the lyophilization process, the cycles developed using the more accurate T_c data from the instrument are much shorter compared to the traditional LT-FDM technique. This is a relatively new technique and work is in progress to explore other areas where the advanced imaging capabilities of the instrument can be suitably employed. Compared to OCT-FDM, a substantial amount of work demonstrating the use of TDLAS in lyophilization has been published in the scientific literature that shows the technique has the potential to be used as a tool during lyophilization cycle development as well as for continuous monitoring during manufacturing operations. The next step in the application of TDLAS will be the implementation of automated process control.

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New Developments in Controlled Nucleation: Commercializing VERISEQ® Nucleation Technology

Joseph Brower, Ron Lee, Eugene Wexler, Steven Finley, Monica Caldwell and Peter Studer

Introduction

Freeze drying, which is also known as lyophilization, plays an important role in drug manufacturing by stabilizing delicate pharmaceutical and biological products. Over the past several decades, significant progress has been made in the design of freeze-drying processes enabling the industry to take full advantage of the recently developed technologies, including those in the field of controlled nucleation.

Freeze-drying was developed commercially in the 1940s to produce a dry product that can be readily reconstituted to its original form by adding solvent (usually, water) when required [1]. Removal of moisture facilitates the slowing down of chemical, microbiological, and physical degradation processes, and therefore, extend the shelf life of the products.

The freeze-drying process is comprised the following three main steps: freezing (solidification), primary drying (ice sublimation), and secondary drying (moisture desorption) and can be quite lengthy (taking up to several days). During the first step, serum vials or other containers filled with a liquid drug formulation are cooled down to low temperatures until the liquid content nucleates and completely solidifies. The temperature is lowered further until the solute portion also solidifies by forming either a crystal or a glass. During the second step, the frozen solvent (primarily, ice) is removed by sublimation under reduced pressure and increased temperature. During the third step, the remaining unfrozen solvent, which is chemically bound to the solid product, is removed by a desorption process.

E. Wexler (✉) · R. Lee · S. Finley · M. Caldwell · P. Studer
Linde Gases, Murray Hill, NJ 07974, USA
e-mail: eugene.wexler@linde.com

J. Brower
IMA Life, Tonawanda, NY 14150, USA

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The overall efficiency and consistency of the entire process, as well as ensuring high quality of the products, largely depends on the nucleation temperature. This temperature directly affects the size of ice crystals and in turn determines the pore size distribution and the pore network of the porous freeze-dried matrix.

The temperature difference between the equilibrium freezing point and the ice nucleation point is known as *supercooling*. A lower nucleation temperature, or a higher degree of supercooling, results in more ice nuclei and smaller ice crystals. On the other hand, higher nucleation temperature, or a lower degree of supercooling, results in fewer ice nuclei and larger ice crystals forming pores and pore networks. Larger pores enable higher sublimation rates, hence shorter drying cycles, as well as reduced reconstitution times and improved finished product attributes. It is also important that all vials nucleate at the same temperature, to ensure consistency of the product morphology, resultant cake structure and appearance, as well as uniformity of the product.

Due to its spontaneous and stochastic nature, uncontrolled nucleation occurs over a wide range of temperatures and a lengthy amount of time, which results in nonuniform ice crystal structure. To overcome such ice nucleation heterogeneity, the process known as annealing is commonly used, during which larger ice crystals are formed at the expense of smaller ones (Ostwald ripening phenomenon) [2]. Annealing can in itself be a time-consuming process; therefore, the potential benefit of reduced drying time may result in little or no overall cycle time reduction.

Therefore, the freezing step is one of the most important steps in the lyophilization process. Handling it in a “controlled” versus “uncontrolled” or “random” fashion, results in a number of benefits to the product manufacturers as well as end users.

Products that could benefit from implementation of controlled nucleation include: biological products like protein and peptide formulations, vaccines, liposome, and small chemical drugs susceptible to physical and chemical degradation, as well as other injectables, which must remain effective from manufacture to patient administration [2]. Due to the rapid growth in biological therapies, the demand for freeze drying has never been higher. This trend is expected to continue for the next decade [3].

Recently, Linde has introduced a novel ice-fog technology, called VERISEQ® Nucleation, which was developed in cooperation with IMA Life North America [4]. This patented cryogenic technology enables controlling the parameters of the nucleation process by using a sterile ice “fog”, produced using liquid nitrogen and water vapor and offers a new degree of control over freeze-drying process as well as the ability to develop more robust lyophilization cycles. VERISEQ® Nucleation technology can be implemented on virtually any freeze-dryer from laboratory lyophilizers to commercial-scale units, as well as aseptic and nonaseptic units, and new as well as retrofits, regardless of pressure rating. The technology is adaptable to both container-based and bulk freeze-drying [5].

Overview of Current Technologies Offering Controlled Nucleation

The challenge of providing a solution to enable controlled nucleation of the liquid preparations in terms of nucleation temperature and time greatly depends on its scalability. A number of proposed techniques that work well in laboratory conditions, however, are difficult or sometimes impossible to implement at a production level [6].

Technologies utilizing ice crystals as nucleation sites are commonly referred to as "ice-fog" technologies. The first ice-fog method was demonstrated in 1990 by introducing a cold gas into a humid freeze-dryer chamber to produce a vapor suspension of small ice particles [7]. This method was further combined with reduced pressure to achieve more rapid and more uniform nucleation [8].

Additional laboratory approaches to controlled nucleation include the following [2]:

- *Vial pretreatment* by producing surface defects or glass particles (for example, by scoring, scratching, or roughening the internal vial walls) to provide nucleation sites and accelerate ice nucleus formation. However, this method does not ensure any control over nucleation temperature and time, and may result in undesirable contamination of pharmaceutical products.
- *Ultrasound treatment* to cause nucleation as a result of ultrasonic vibrations. However, implementation of this method in a commercial-scale freeze-dryer without compromising the ability to clean the equipment is challenging.
- *Utilization of additives*, such as silver iodide, *Pseudomonas syringae* bacteria, and adventitious environmental particulates, to introduce artificial nucleation sites. However, the presence of additives is generally unacceptable and this approach does not provide sufficient control over nucleation parameters.
- *Electro-freezing* by delivering relatively high electric fields (~0.01 V/nm) either continuously or pulsed between narrowly spaced electrodes immersed in the solution to be freeze-dried, in order to induce nucleation in subcooled solutions. However, such method is generally impractical for use in commercial pharmaceutical applications and is sensitive to ionic molecules (for example, NaCl).

The two main techniques currently offering the potential of achieving controlled nucleation in the commercial scale are ice fog (e.g., Millrock's FreezeBooster nucleation technology and Linde's VERISEQ® Nucleation technology) and depressurization (ControLyo™ technology, developed by Praxair).

Millrock's patented FreezeBooster™ controlled nucleation technology, combined with the company's patent pending AccuFlux™ technology works by first cooling the chamber to the desired temperature below the equilibrium freezing point, say −5 °C, reducing the chamber pressure creating a predetermined volume of condensed frost on an outer surface of the coils inside a condenser chamber separate from the product chamber, and then rapidly introducing ice crystals, formed by breaking down the condensed frost due to gas turbulence created as a result of open-

ing the vapor port into the product chamber maintained at a lower pressure than the condenser chamber. According to the developers of this technology, such ice crystal injection enables uniform and rapid nucleation of the product in different areas of the product chamber. Furthermore, the technology allows the crystal structure to be consistently created, monitored, and controlled by producing a consistent and repeatable ice formation, which results in a highly uniform finished product with reduced primary drying times.

The depressurization technique used by Praxair's ControLyo™ nucleation on-demand system, involves pressurization of the freeze-dryer chamber with an inert gas, such as nitrogen or argon to pressures ranging from 18 to 28 psig, followed by reducing the product temperature in all vials to a selected value. When thermal equilibrium between the vials has been achieved, the excess pressure is released rapidly [9]. With this method, nucleation is induced at essentially the same time for all vials in the batch. However, the technique does involve pressurization, which requires the use of pressure-rated freeze-dryers, and could be problematic when working with laboratory units, which are typically not pressure rated. This could also represent a challenge when retrofitting existing units.

Nevertheless, the developers believe that depressurization technology can be successfully used from laboratory to manufacturing scale. The ControLyo™ nucleation on-demand technology developed by Praxair was licensed to SP Scientific exclusively for use on dryers with shelf areas of less than 1.0 m^2. According to reports, the Lyostar™ 3 pilot freeze-dryer with ControLyo™ technology introduced by SP Scientific was the first commercially available freeze-dryer with the control nucleation capability.

VERISEQ® Nucleation Technology: Background, Technical Approach, Scale-Up

The VERISEQ® Nucleation technology, developed by Linde Gases Division of the Linde Group in cooperation with IMA Life North America, offers a commercially viable technique for cryogenically generating a uniform dispersion of microscopic ice crystals (or ice fog). The ice fog is a result of contact between liquid nitrogen (produced from sterile-filtered gaseous nitrogen) and water vapor in a mixing device outside the lyophilization chamber. Upon introduction into precooled vials, containing the product to be freeze dried, these ice-fog crystals serve as nucleation sites. This causes a rapid and uniform nucleation of the product in a vial as well as between vials of the same batch at very low degrees of supercooling [10].

A key challenge for the commercial implementation of VERISEQ® Nucleation technology was to generate a sufficient amount of ice fog to fill the chamber, and to ensure its penetration inside the vials given various lyophilizer volumes, as well as container/closure geometries. Efficient ice-fog generation and distribution were achieved using an ejector assembly, which provides an extremely efficient method of quickly forming the ice fog and circulating it throughout the freeze-dryer chamber.

The VERISEQ® Nucleation system has no moving parts or other complicated mechanisms that would be difficult to steam or otherwise sterilize. Also, no chamber pressurization is required.

The VERISEQ® Nucleation technology is realized by means of an aseptic apparatus for commercial applications requiring steam sterilization, and a nonaseptic stand-alone version for laboratory use and technology evaluation.

The aseptic unit is fully sterilizable. Complete sterilization of its critical components using biological indicators has been demonstrated during the same steam in place (SIP) cycle used to sterilize the freeze-dryer chamber.

The general schematic of the aseptic version of the ice-fog-generating apparatus is presented in Fig. 1.

After passing through a series of sterile (0.2 μm) gas filters, pressurized sterile nitrogen gas condenses inside a smooth coiled tube immersed in a liquid nitrogen bath. Next, the produced sterile nitrogen liquid enters the ejector (also known as a jet pump), where it expands resulting in a two-phase flow, which serves as a motive flow that drives the circulation through a freeze-dryer chamber.

Once in contact with "hot" steam, introduced via steam line, and "warm" recirculating flow, supplied via recycle loop, cold liquid–gas mixture is converted into a

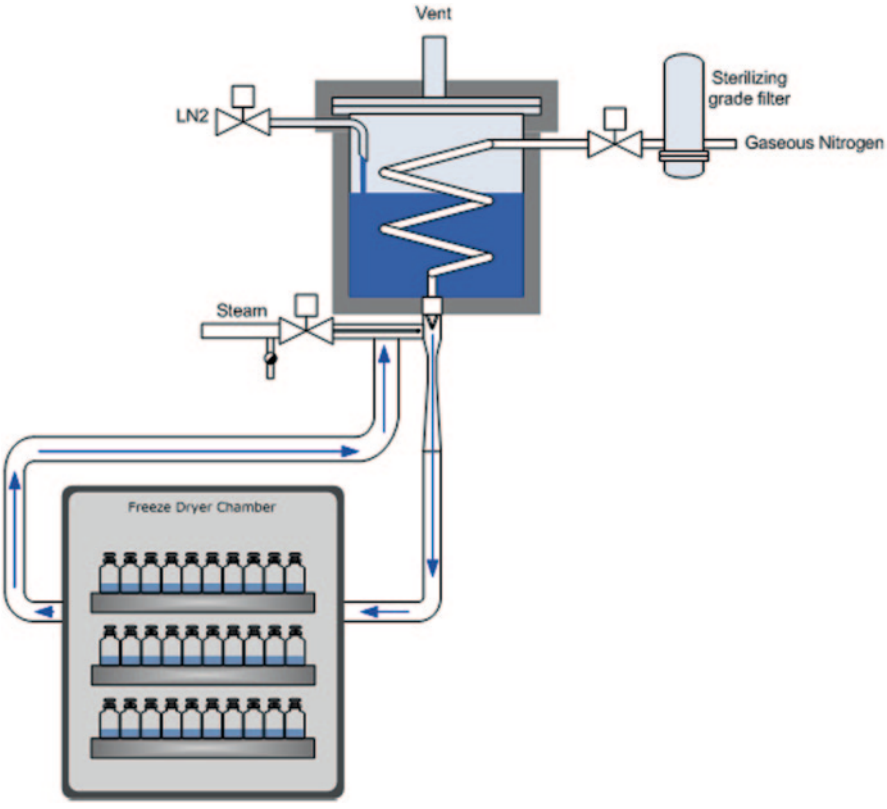

Fig. 1 Schematic of the VERISEQ® Nucleation apparatus (Aseptic version)

cold gas, which freezes the water droplets and forms microscopic ice crystals, thus creating an ice fog—generally, a suspension of ice microcrystals in a stream of gaseous nitrogen. Accordingly, the freeze-dryer chamber is equipped with an inlet port for introducing ice fog and an outlet port for recycling the fog from the chamber.

Ice-fog introduction follows a two-step approach. First, the vials containing the product to be freeze-dried are placed on the cooled shelves inside the freezing chamber and cooled to a selected suitable temperature at or below their freezing point. Once this temperature is achieved, a cryogenic ice fog produced as described above is introduced into the chamber to facilitate nucleation.

A nonaseptic unit is a stand-alone portable device (Fig. 2) powered by 110 V (220 V, optional) of electricity and fed by a liquid nitrogen dewar with pressure control capability. The unit utilizes liquid nitrogen and humidified nitrogen gas to generate ice fog. While liquid nitrogen comes directly from the liquid nitrogen dewar, the humidified gas stream is produced by bubbling dry nitrogen gas from the nitrogen gas tank through a water column. The unit can be attached to a freeze-dryer chamber via two ports to enable ice-fog introduction and circulation as described above.

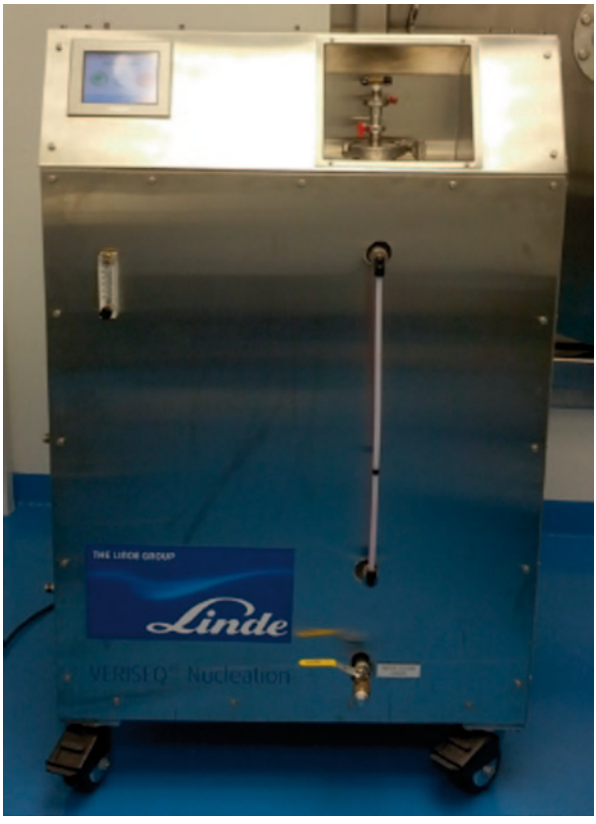

Fig. 2 VERISEQ® Nucleation nonaseptic unit

The unit is isolated from the freeze-dryer before and after the nucleation process and is fully compatible with laboratory freeze-dryers of any size and brand.

The VERISEQ® Nucleation system can be easily sized based on the volume of the freeze-dryer, as opposed to the area, to ensure adequate ice-fog filling and circulation within an acceptable period of time. System design considerations include location of inlet/outlet ports and their size to ensure optimal ice-fog distribution within the chamber, as well as available space limitations.

Results and Discussion

Experimental detection of nucleation inside the vials can be assessed by a combination of direct observation and temperature measurements. Measurements are taken using wired and/or wireless temperature sensors attached to the external surface of selected vials or placed inside the vials. Figure 3 below shows a comparison between temperature profiles during uncontrolled (random) and ice-fog-controlled nucleation using water. Note, that some of the variation between vial temperatures is due to the fact that some temperature probes were placed on the outside of the vials. While accurately reflecting the time of nucleation, this may introduce some uncertainty with regard to temperature.

As can be seen from the provided temperature curves, uncontrolled nucleation of vials takes place over the interval of about 27 min within the temperature range between −8 and −15 °C. In the case of controlled nucleation, all the vials nucleate almost instantaneously at a temperature close to that of the shelf (about −7 °C, based on internal temperature sensors).

To demonstrate ice-fog nucleation utilizing different vial/stopper sizes, tests were performed using the combinations shown in Table 1 (see also Fig. 4):

Consistent ice-fog-induced nucleation was demonstrated in all vials.

In addition, the water intake during ice-fog-induced nucleation was evaluated based on the weight measurements. The results are shown in Table 2.

These results indicate that the water intake is minimal and practically does not affect the duration of primary drying.

Ice-fog-induced nucleation at a lower-degree supercooling results in much larger ice crystals and, hence, pore size, which reduces resistance to mass transfer and decreases the primary drying time.

Case Studies

As with anything related to lyophilization, many of the potential benefits of controlled nucleation vary from product to product, and indeed for the same product, from presentation to presentation. In order to better understand these variables, a number of studies were performed.

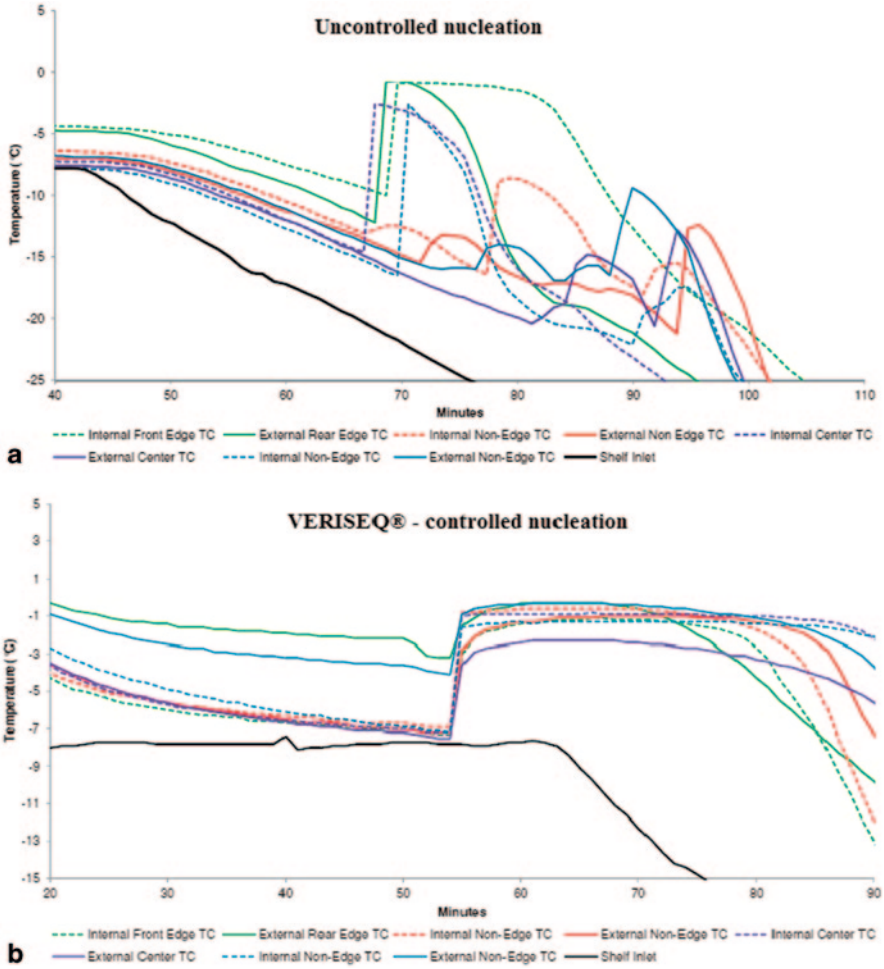

Fig. 3 Temperature profile during uncontrolled **a** and controlled **b** nucleation using VERISEQ® Nucleation technology (Note: *Solid lines* denote probes on exterior of the vials)

Table 1 Vial/stopper size combinations

Vial volume (ml)	Fill volume (ml)	Stopper size (mm)	Number of vents
15	5	20	2
10	4	20	2
6	2	13	1

Mannitol and Sucrose These are very common excipients used in pharmaceutical freeze drying. Mannitol is used as a bulking agent, is crystalline, and its freezing and drying characteristics are well documented in the literature. Sucrose is used as a cryoprotectant, is amorphous, and is likewise well understood. Two lots of each

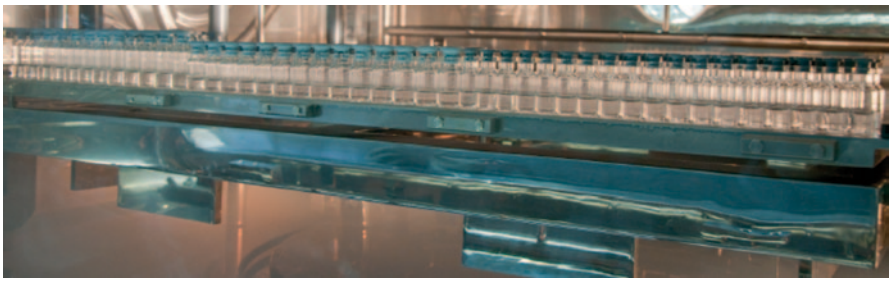

Fig. 4 Testing various vial/stopper combinations

Table 2 Water intake results

Vial volume (ml)	Fill volume (ml)	Stopper size (mm)	Net weight increase (mg)
2	1.0	13	0.1
5	1.0	20	0.2
10	1.0	20	0.2
20	1.0	20	0.4

solution were prepared and filled into 896 vials per batch under class B conditions in order to minimize particulate contamination. Both lots of the same solution were subjected to identical freeze-drying cycles with the exception of controlled nucleation using ice fog on one of the lots. The cycle data and finished product attributes were then compared.

As Fig. 5 demonstrates, the use of ice fog to control nucleation results in homogeneous nucleation of all vials within a narrow temperature range, that is near the equilibrium freeze temperature of the solution.

Figure 6 shows the impact of this effect during drying. The upper two traces are the average product thermocouple temperatures for naturally nucleated vials and those that were nucleated using ice fog. The lower temperature of the ice-fog-nucleated vials implies faster sublimation, and therefore a greater degree of cooling from the sublimation process. The lower two traces show the variation among the product thermocouples. The lower variation in the ice-fog-nucleated vials is a result of more homogeneous behavior in the probed vials.

Figure 7 demonstrates a direct measure of sublimation during the cycles via tunable diode laser absorbance spectroscopy (TDLAS). The chart shows the faster sublimation during primary drying, and in the naturally nucleated lot, a higher peak during the transition to secondary drying. This indicates a higher percentage of moisture remaining after primary drying.

Figure 8 compares the residual moisture remaining after the drying cycle for each lot, and clearly shows not only lower moisture values, but again, better homogeneity in the sample set.

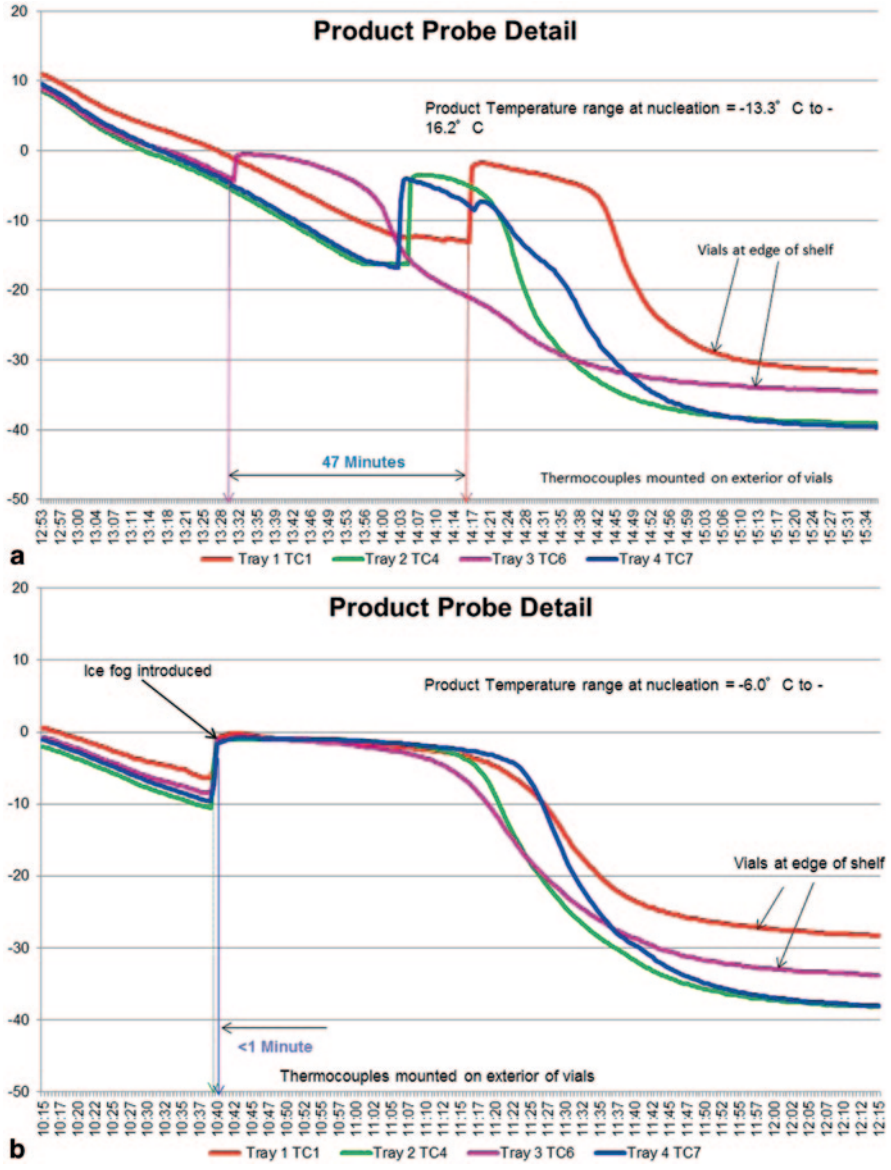

Fig. 5 Nucleation comparison for mannitol first without **a** and then with **b** ice fog

In addition to analytical comparisons, each lot was subjected to physical inspection and reconstitution evaluations. Table 3 summarizes the findings. As can be seen, controlling nucleation results in measurable benefits for chosen product(s).

In addition to the mannitol/sucrose studies, a number of other studies were performed on actual drug products, or surrogates.

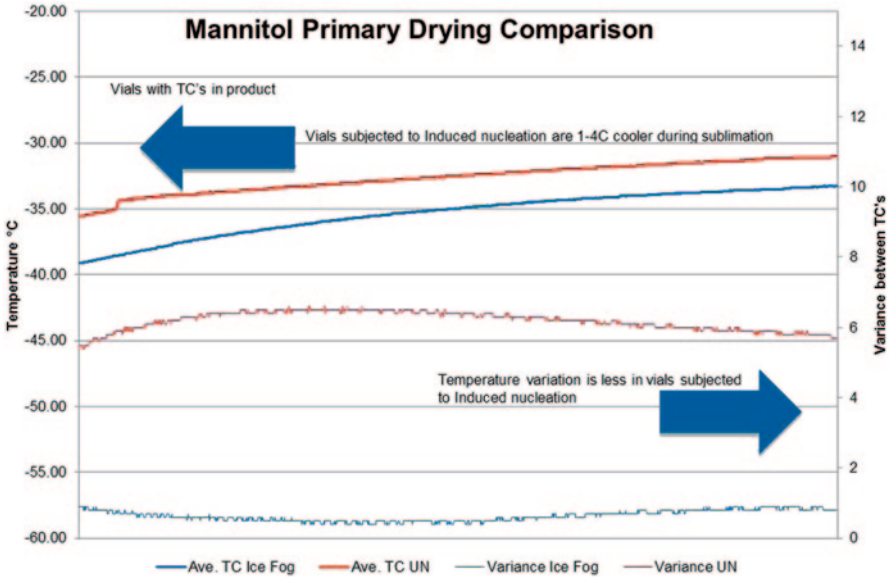

Fig. 6 Product thermocouple comparison

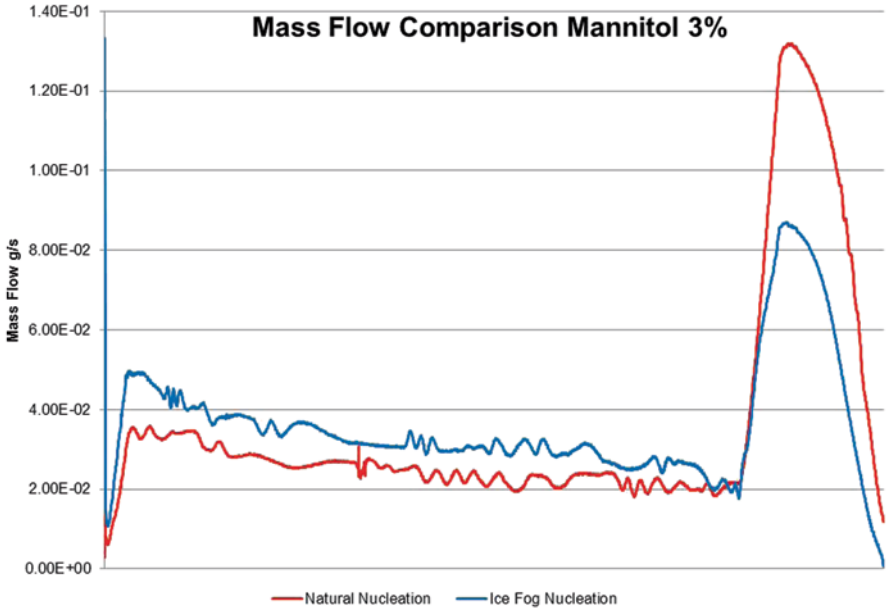

Fig. 7 TDLAS comparison

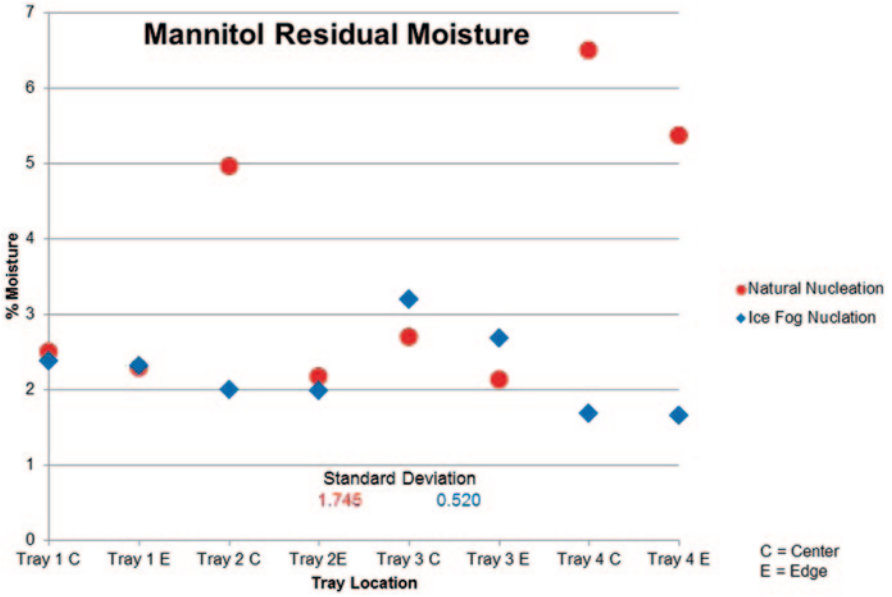

Fig. 8 Residual moisture comparison

Table 3 Physical attribute comparison

Inspection results for 896 vials			
Batch	Broken/cracked	Collapsed/deformed cake	Other
Mannitol natural nucleation	2	0	108[a]
Mannitol ice-fog nucleation	0	0	0
Reconstitution results			
Batch	*Test 1*	*Test 2*	*Test 3*
Mannitol natural nucleation	30.69 s	34.76 s	33.27 s
Mannitol ice-fog nucleation	6.70 s	10.49 s	10.96 s

[a] Vials exhibited small protrusions on cake surface

Vancomycin Hcl This is a very common antibiotic that is made by a number of generic drug manufacturers. In most dosage forms, this product can be freeze-dried quite efficiently due to its very high glass transition temperature (> −7 °C). One particular dosage that defies efficient drying is the 10 g/vial presentation. The vial used by one particular customer is a very thick, molded vial with variable heat transfer characteristics. The fill volume is 65 ml, resulting in a fill height of about 45 mm. The product solution itself contains >15 % solids. Due to these factors, attempted rapid sublimation creates high pressures within the vials, causing the vials to shatter during primary drying. The result is a very conservative and long cycle. A study was

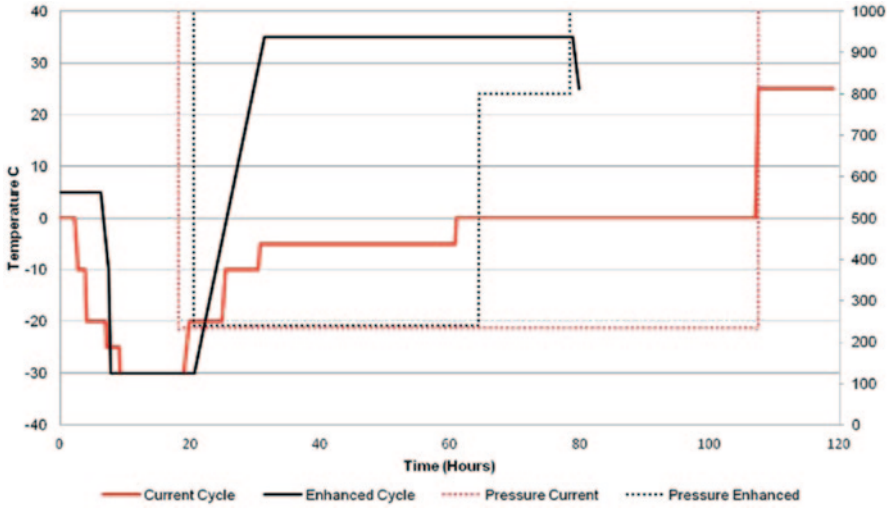

Fig. 9 Vancomycin HCl 10 g/vial cycle comparison

proposed to determine if controlled nucleation could mitigate the factors causing breakage allowing a more aggressive, shorter freeze-drying cycle. Figure 9 outlines the cycle parameters. As in the earlier trials, identical numbers of units were used and filled under the class B conditions. Table 4 summarizes the findings.

Human Serum Albumin (HSA) Used as a surrogate for a protein formulation, a nucleation evaluation was conducted along with an evaluation of tubing versus molded glass vials. This particular product cycle included an annealing step, which is designed to accomplish much the same phenomenon as controlled nucleation, which is to create large ice crystals to allow faster mass flow of water vapor through the product matrix during drying. In this study, both molded and tubing vials were processed in one of two cycles. The first cycle with natural nucleation and annealing, and the second cycle with controlled nucleation and annealing.

Figure 10 displays the average thermocouple traces during drying. The data imply that for tubing vials, annealing combined with controlled nucleation has no perceived benefit from annealing following natural nucleation. Of course, the time spent on the annealing step could be saved by using controlled nucleation only. For the tubing vials, the data indicate that due to more inefficient vial heat transfer, annealing is not as effective, and controlled nucleation provides a significant benefit in cycle time reduction.

Monoclonal Antibody Monoclonal antibodies (mAbs) therapies are a being developed by a large number of firms and appear to be one of the leading growth product segments for years to come [3]. A study was proposed to see if a model mAbs product could benefit from ice-fog nucleation. Factors tested were cycle time reduction, surface area reduction, and reconstitution.

Table 4 Vancomycin HCl 10 g/vial cycle comparison

Inspection results for 315 vials				
Batch	Broken/cracked	Collapsed/deformed cake	Other	Cycle duration
Vancomycin natural nucleation	0	0	0	119 h
Vancomycin enhanced ice-fog nucleation	0	0	0	80 h
Reconstitution results				
Batch	*Test 1*	*Test 2*	*Test 3*	
Vancomycin natural nucleation	145.03 s	208.66 s	130.33 s	
Vancomycin-enhanced ice-fog nucleation	29.72 s	37.31 s	30.61 s	
Moisture results specification ≤5%				
Batch	*Natural nucleation*	*Ice-fog nucleation*		
Percent moisture by KF	3.76	3.49		

KF Karl Fischer

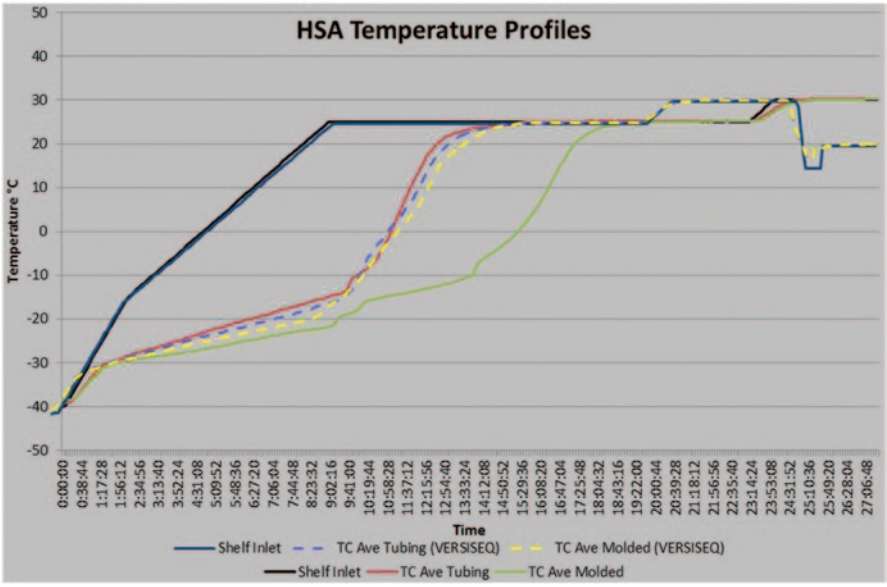

Fig. 10 HSA in molded and tubing vials cycle comparison. *HAS* human serum albumin

Figures 11, 12 and 13 summarize the findings. In addition to these, reconstitution times were reduced by 30%. In these studies, a comparison of pressures indicated by capacitance manometer and pirani gauge was used to identify drying end point. This is a useful and accurate tool, because a pirani gauge will react differently to water vapor than to nitrogen gas. When water vapor pressure is present, the pirani will indicate pressure well above those indicated by a capacitance manometer. Once most of the vapor is removed, the indicated pirani pressure drops to nearly the same value as that measured by the capacitance manometer.

One interesting result of the study was that product probes, that are typically not very useful for end point determination, show very close agreement with the drop in pirani pressure when used in conjunction with controlled nucleation. This is because normally vials with product probes in them tend to nucleate before most of the other vials, and therefore dry at a faster rate. This bias is removed when nucleation is controlled, and the probed vials dry at similar rates to all the rest.

Full-Scale Trial In order to test the adaptability of the VERISEQ® Nucleation system to commercial applications, a trial was performed at a customer site. An IMA Life Lyomax 28 m^2 freeze-dryer was loaded full with 15,000 30-cm^3 vials filled with 17.5 ml of 5% mannitol solution. Thirty-six thermocouples were attached to the outside of the vials throughout the load to indicate whether or not nucleation occurred. A VERISEQ® ice-fog generator was attached to the lyophilizer. The shelf temperature was stabilized at −10 °C, and the product temperatures reached approximately −4 °C. Nucleation was initiated and all vials nucleated as indicated in Fig. 14.

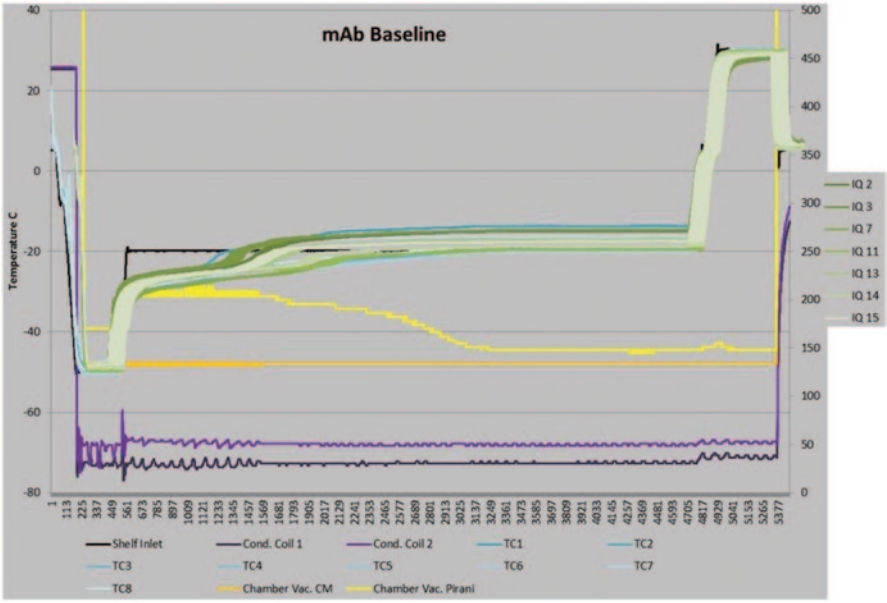

Fig. 11 mAbs cycle with natural nucleation. (Note: vial probes in *green* and *blue* indicate drying end point at approximately 2240 min, but pirani indicates drying end point at approximately 3250 min). *mAbs* monoclonal antibodies

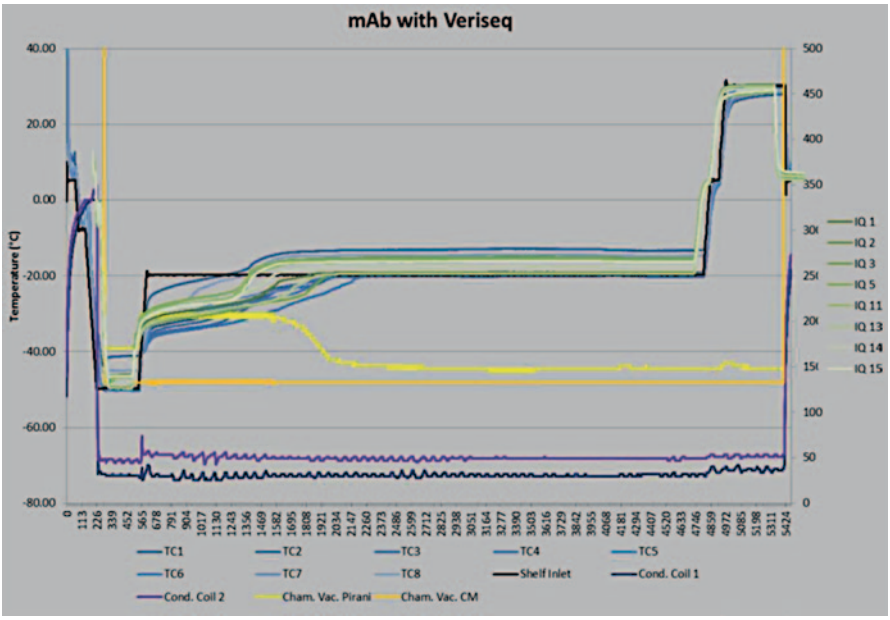

Fig. 12 mAbs cycle with ice-fog nucleation. (Note: vial probes in *green* and *blue* indicate drying end point at approximately 2260 min, and pirani indicates the same time (drying time reduction of ~1000 min)). *mAbs* monoclonal antibodies

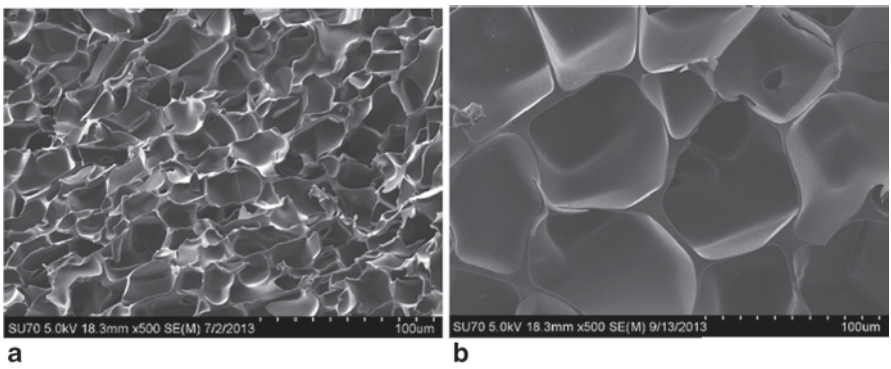

Fig. 13 SEM images of product microstructure obtained as a result of random **a** and ice-fog-induced **b** nucleation. *SEM scanning electron microscope* (Note the reduced surface area in **b**)

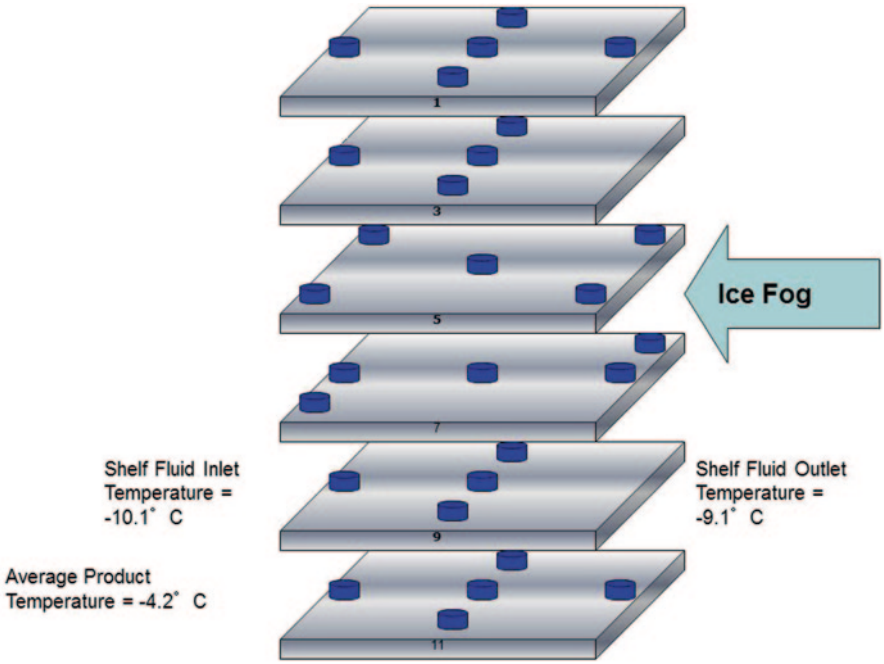

Fig. 14 Thermocouple locations for maximum load study in 28 m^2 freeze-dryer

Conclusions

Thus, it is shown that ice nucleation during freeze drying (lyophilisation) is an important process parameter that needs to be controlled. The described scalable cryogenic ice-fog technology known as VERISEQ® Nucleation can be used in laboratory-, pilot-, and production-scale lyophilizers to induce uniform ice nucleation at

reduced degree of supercooling and eliminate vial-to-vial variability, which in turn, can help mitigate a host of related issues and lead to improved process parameters as well as product quality.

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Part II
Lyophilized Biologics and Vaccines – Modality Considerations

Lyophilized Biologics

Byeong S. Chang, Michael Reilly, and Hana Chang

Lyophilized Formulations

Delicate three-dimensional structures essential for the biological activities of proteins are often susceptible to various modes of denaturation or degradation when they are isolated and prepared for therapeutic purposes. Upon exposure to routine manufacturing processes, storage, and transportation, proteins experience physicochemical changes, including microscopic changes at the level of amino acid side chains (e.g., oxidation, deamidation, or isomerizations) to macroscopic changes, like structural changes, oligomerization, aggregation, or precipitation [2, 3, 8, 9, 16, 22, 24]. Undesirable consequences associated with degradation products include loss of biological activities and undesirable immune responses [17, 20, 25]. Antibodies generated during an immune response can further affect therapeutic potential, as neutralizing antibodies can compromise naturally existing proteins while nonneutralizing antibodies can affect therapeutic protein availability.

For the successful introduction of biopharmaceuticals in the past two to three decades, advancement in stability indicating analytical methods has been instrumental in delivering good quality protein pharmaceuticals with excellent stability profiles [1, 12, 14, 23, 26].

Among the many different and practical solutions for maintaining the integrity of biopharmaceuticals until sufficient expiry, lyophilization has been the most extensively researched [4, 5, 21]. This is primarily due to the high stability provided by a dried powder formulation. Lyophilized formulations generally offer additional advantages, such as a unique way of achieving sufficient expiry for intrinsically labile proteins, potential stability at ambient temperatures, removal of water as a reactant or media for undesirable degradations, and inhibition of autolysis from

B. S. Chang (✉) · M. Reilly · H. Chang
Integrity Bio, Inc., 820 Calle Plano, Camarillo, CA 93012, USA
e-mail: byeong.chang@integritybio.com

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biological activities of proteolytic enzymes. Another advantage of lyophilization is the potential to modify the formulation after manufacturing prior to administration, such as introducing volatile additives, preservatives, or salt; changing pH, viscosity, or volume; initiating reactions, adding adjuvants, or diluting with buffers or non-aqueous diluents for special applications.

Among biopharmaceuticals, different issues have been observed in lyophilizing different modalities, e.g., antibodies, enzymes, hormones, cytokines, peptides, vaccines, viral vectors, and nonviral vectors. Therefore, general approaches in optimizing any specific class of biologics have been different from lyophilizing other modalities. To achieve successfully lyophilized formulations, specific issues were addressed by employing relevant excipients, including stabilization during freezing, dehydration, or storage, and formation of an elegant cake with an efficient cycle.

While lyophilized formulations offer multiple advantages over liquid formulations, they also bear substantial disadvantages, like the inconvenience of reconstitution for end users, additional steps for cycle development and optimization, expensive equipment, and high costs for manufacturing. The choice of implementing lyophilization, therefore, requires an intelligent strategy to maximize the advantages and minimize the disadvantages.

In this chapter, challenges and practical solutions associated with the development and commercialization of lyophilized biopharmaceutical formulations are discussed along with examples of relevant industrial practices and the list of currently commercialized lyophilized protein therapeutics.

Lyophilization Design and Stability Considerations of Various Biologics

As the primary purpose of utilizing lyophilization is enhancing product stability, the best product candidates for lyophilization go beyond considerations of pharmaceutical modality. Generally, the products with the following attributes are good candidates for lyophilization:

- Products with limited stability in a liquid state, even after formulation optimization, are obvious candidates. Extensive research has been conducted to predict the intrinsic stability of biopharmaceuticals based on structural information. However, accurately determining product stability profiles without real-time stability data remains a challenge, as stability relates to complex relationships of many different variables. Although it is difficult to generalize, many nonglycosylated proteins, interferons, hemophilia factors, and vaccines requiring biological function are good candidates for lyophilization.
- Intravenous infusions, among various routes of parenteral delivery, are generally better suited for lyophilization primarily due to the experience of health-care professionals in reconstituting lyophilized formulations.

- Products with a strong intellectual property positions that do not anticipate strong competition in the market are good candidates for lyophilization, as the convenience factor of a liquid formulation may not be necessary.
- Products that are globally distributed, especially to areas where a cold chain is not well established, generally benefit from the added stability of lyophilization.

In order to maximize the benefits of a dry powder formulation, the following topics should be addressed during a general formulation development process.

Stabilization by Lyophilization

For all lyophilized formulations, adequate short-term stability of the product in a liquid state needs to be established during manufacturing, handling, storage, transportation, and administration, to accommodate formulation and filling operations as well as stability after reconstitution. However, the major focus of the development process should be to achieve sufficient stability during long-term lyophilized storage. In order to develop a stable formulation, various formulation factors need to be optimized.

Protein degradation rates are heavily dependent on formulation pH. As different proteins are susceptible to various degradation pathways, it is crucial to identify the optimal pH range where the protein of interest is most stable. A proper buffer with a sufficient buffering capacity should be selected in order to maintain this pH range.

An efficient lyophilization cycle is an ideal attribute of a lyophilized formulation, which is discussed in further detail in Sect. 5.1.2. As part of this design, bulking agents are often introduced to the formulation to achieve an elegant cake. The physical properties of the bulking agents, especially their characteristics in a frozen state, need to be understood for successful optimization [2]. Various classes of bulking agents such as crystalline, amorphous, ionic, nonionic, polymer-based, or protein-friendly sugars can be considered during lyophilized formulation development. As the physical properties of the selected bulking agents would directly affect product stability as well as the lyophilization cycle, careful compatibility studies will need to be conducted. Bulking agents that have a tendency to crystallize during lyophilization, e.g., mannitol or glycine, are commonly used to achieve an elegant cake. However, the large surface areas generated by crystalline structures often enhance undesirable degradations of proteins, so they need to be selected with these considerations. Other commonly used tonicity modifiers with low collapse temperatures like sodium chloride or monosaccharides, e.g., glucose or sorbitol, can be challenging to lyophilize without cake collapse unless they are formulated with other excipients or with a high concentration of protein.

Another inactive ingredient often crucial for successful lyophilized formulation development is a surfactant. Surfactants have been used to maintain the integrity of proteins against surface-induced degradations derived from agitation, filtration, filling, freeze-thawing, etc. [10]. Nonionic surfactants like polysorbates or poloxamers have been successfully used in protein formulations. Some optimization is required

to determine the ideal surfactant and concentration as different proteins are best stabilized by different surfactants.

It has been well reported that proteins undergo structural changes during dehydration processes [4, 18]. The main driving force of this conformational change is the loss of polar water molecules, which make hydrophobic amino acid residues hide inside the folded native protein structure. When polar additives like sugars are added into the formulation, they can replace the water and maintain a polar environment. These "water replacement" sugars have been successfully used to maintain the native structure of proteins during lyophilization. The most effective water-replacing sugars are nonreducing sugars, like sucrose and trehalose. While water-replacing sugars have been used as bulking agents in many commercial formulations, they generally have lower collapse temperatures and can require longer drying times in the absence of crystalline bulking agents. When selecting a water-replacement sugar it is prudent to investigate other relevant aspects of the sugar, including the glass transition temperature in a frozen state and its stability at the targeted pH.

The diluent used for reconstitution of a lyophilized formulation is another factor to optimize during formulation development. The most common diluent is commercially available water for injection (WFI), while other commercially available diluents such as normal saline can be used. Although not ideal due to its reducing nature, 5% dextrose in water (D5W) can also been used as a diluent. Bacteriostatic WFI or bacteriostatic normal saline, both containing 0.9−1% benzyl alcohol as a preservative, have also been used as diluents. These diluents are often used when reconstituted products are susceptible to bacterial contamination. For example, a multidose formulation filled into a cartridge has been effectively used in pen injectors. However, whenever diluents containing preservative(s) are introduced to the formulation, additional compatibility studies are required to ensure protein compatibility. For some products requiring specific configurations, other custom diluents can also be recommended or packaged with the lyophilized vials.

Lyophilization Cycle Development

Once a good lyophilized formulation is developed with optimized excipients, it is important to develop an efficient drying cycle that can achieve a pharmaceutically elegant lyophilized cake with a desired moisture content capable of rapid reconstitution.

The first step to optimizing a lyophilization cycle is to characterize the frozen formulation with thermal analyses, e.g., subambient differential scanning or lyophilization microscopy, to better understand relevant physical changes that may occur in a frozen state. Crucial information can be obtained through these characterizations, such as glass transition temperature of the unfrozen fraction (T_g'), possibility of recrystallization upon annealing, the devitrification temperature, eutectic melting temperature, and freezing point depression. Based on these data, along with infor-

mation about the lyophilizer, the lyophilization cycle can be optimized for process efficiency while product quality is maintained.

A lyophilization cycle generally consists of three distinctive steps: freezing, primary drying, and secondary drying.

During the freezing step, the shelf temperature is decreased to bring the product temperature below its freezing point. As dust-free environments such as good manufacturing practice (GMP) suites can be conducive to supercooling, the shelf temperature is lowered 10–20 °C below the formulation's freezing point to ensure the formulation reaches a solid state. The rate of freezing can be optimized to induce the most ideal ice crystal shape for the efficiency of the cycle and for product integrity.

Once the formulation is frozen, the shelf temperature can be manipulated to modulate the ice crystals. Larger ice crystals can be formed by coaxing smaller ice crystals to melt and join larger ice crystals upon cooling. The crystallization of targeted excipients can also be controlled through the rate of cooling or heating during annealing. Excipients have tendencies to crystallize but can remain amorphous during rapid freezing/concentration processes.

Once an ideal ice structure is formed, the primary drying step is initiated. A vacuum is introduced to the drying chamber and the chamber pressure is decreased to induce sublimation of the ice. Both the shelf temperature and the chamber pressure contribute to the rate of sublimation and the product temperature. Maintaining the product temperature below the formulation's T_g' has been essential for achieving an elegant cake, although the rate of sublimation is typically faster at higher product temperatures. Therefore, the optimization is centered on balancing shelf temperature and chamber pressure. Once the combination of shelf temperature and the chamber pressure is achieved, the primary drying step is continued until all ice is removed from the chamber. Typically, the shelf temperature can be set 10–30 °C above the desired product temperature, e.g., T_g', and then reduced through sublimation heat loss induced by the vacuum.

As the decrease of product temperature caused by the sublimation heat loss stops at the conclusion of primary drying, the product temperature naturally converges with the shelf temperature. This temperature convergence, along with the absence of water vapor in the drying chamber, are good indicators to proceed to the secondary drying step. The secondary drying is carried out at a much higher shelf temperature than the primary drying, e.g., 25–50 °C. During this time, any water molecules that failed to sublimate are removed from the lyophilizing cake through evaporation. Again, the absence of water vapor in the drying chamber will confirm the completion of secondary drying. At this point, the lyophilization process can be completed by replacing the vacuum chamber with an inert dry gas, e.g., nitrogen.

The quality of lyophilized formulations can be determined by various product-specific analyses. Some examples of specific characterizations most relevant to lyophilized formulation include moisture content analysis, protein secondary structure analysis by Fourier transform infrared spectroscopy (FTIR), and thermal analysis. These analyses will not only ensure the quality of the product upon the acute

lyophilization stress but will also be useful to predict the long-term stability of the dry powder formulation.

General Development Approaches

Due to limited time and drug substance availability, it is typically difficult to develop a formulation at an early stage of product development that is feasible for commercialization. For this reason, most biopharmaceutical companies adopt a two-step formulation approach: formulation development for a formulation suitable for preclinical and early clinical trials, followed by formulation development for a commercially feasible formulation before phase III clinical trials. An efficient alternative approach would be to develop a lyophilized formulation with the possibility of reaching the market without further modifications followed by an amendment after licensing approval. General formulation development approaches can include:

- A frozen formulation for the initial formulation followed by a liquid or lyophilized formulation, depending on product stability
- A lyophilized formulation for the initial formulation followed by a liquid formulation, if feasible, for commercialization
- A liquid formulation with limited expiry for the initial formulation followed by an extension of the expiry for commercial purposes

After receiving approval for commercialization, additional formulation optimizations can be performed for competitive purposes and to manage the life cycle of the product.

A rough diagram of various formulations and steps in formulation development that occur during product development and commercialization is shown in Fig. 1.

Strategy for Initial Formulations

The development of an initial formulation can be initiated as early as the process development stage, as the establishment of a good formulation would also benefit upstream purification processes. A pre-formulation characterization can be conducted with minimal drug substance to establish the following basic formulation parameters: pH, ionic strength, and the necessity of a surfactant. In general, pH and ionic strength conditions can be determined by a simple accelerated stability study. An effective surfactant can be identified through an agitation or shear stress study. When these three formulation parameters are optimized, the formulation is often stable for about 6 months in refrigerated storage or frozen state, which may be sufficient to support preclinical studies and early clinical trials. This “initial formulation” will most likely differ from the “commercial formulation,” which is discussed later in this chapter. After the product is introduced to the market, there will be a continued effort to improve the formulation through life-cycle management.

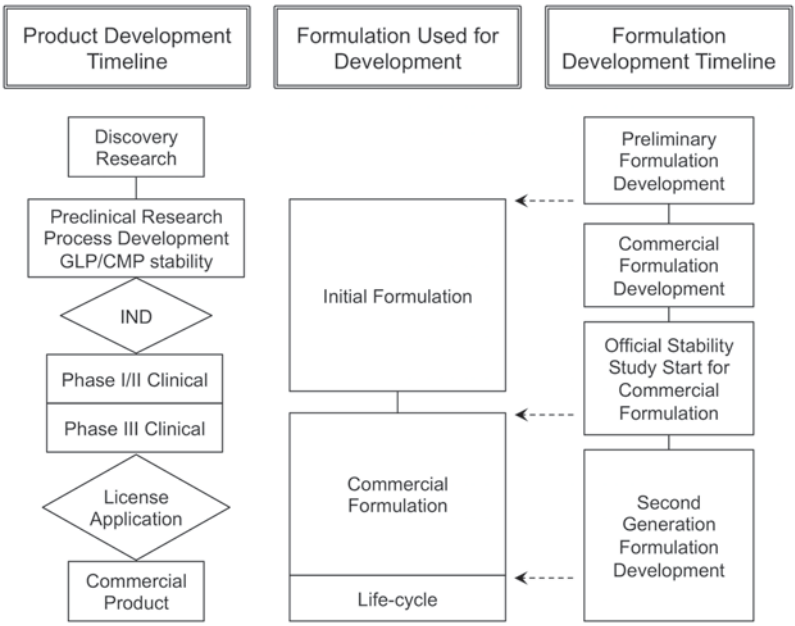

Fig. 1 Formulation strategy for phases of biopharmaceutical development and introduction

Frozen Liquid Formulation

For products with a reasonably well-defined stability in solution and good solubility at the anticipated dose, a frozen formulation can be a cost-effective way to accelerate the timeline and provide a good candidate for an initial preclinical and/or clinical formulation. As the rates of most degradation reactions decrease with decreasing storage temperature, frozen storage is often effective in maintaining product integrity over a short storage duration. Fortunately, freezers with suitable temperature control are available at most research facilities, doctors' offices, and hospitals. Additionally, there are warehouses and transportation options that can accommodate frozen products and reliable temperature control.

Overall, a frozen formulation is considered a relatively safe and effective way to introduce the initial formulation. Once its long-term real-time stability in a liquid state is demonstrated, the same formulation can be commercialized without further changes. However, simply freezing a liquid formulation does not necessarily improve the stability of a formulation. Freezing can accompany various undesirable environmental changes, such as shifts in localized concentration, crystallization of excipients, shifts in pH, phase separation of potential stabilizers, etc. It is important to note that certain excipients are prone to these undesirable changes during

freezing. In formulations including sodium chloride as a tonicity modifier, the salt crystallizes around its eutectic melting temperature of −21 °C. Other excipients such as mannitol, glycine, disodium phosphate, or sorbitol also have a tendency to crystallize in a frozen state, which could generate undesirable stability issues. These crystallizations generally do not occur at early stages of frozen storage, and such stability issues may not be apparent during short-term stability studies. For this reason, unexpected problems such as aggregation and/or precipitation are often not observed until after 6–9 months of frozen storage. Due to these considerations, the decision to use a frozen formulation needs to be made after thorough research and investigation.

Lyophilized Formulation

Many biopharmaceutical companies choose a lyophilized formulation as the initial formulation if proper expertise and resources are available to support rapid development. The primary reason for considering an early development lyophilized formulation is the higher probability of success in achieving a stable formulation. If the company has established a strong intellectual property for the product and no competition is anticipated in the market, the lyophilized formulation could potentially be continued for commercial purposes.

A lyophilized formulation is also recommended if the product has limited stability in a liquid state. As most degradation reactions are facilitated by surrounding water molecules, e.g., hydrolytic reactions, deamidation, and proteolytic activities, the removal of water can be an effective approach to enhancing stability.

As briefly discussed in Sect. 5.1.1, proteins may experience undesirable structural changes during dehydration. Therefore, proper formulation development is required to stabilize proteins in order to achieve suitable lyophilized formulations. In addition to stability, a lyophilized formulation should satisfy other attributes such as an elegant appearance, consistent moisture content, rapid reconstitution, etc., so comprehensive knowledge around specific excipients' compatibility to lyophilization is essential for proper formulation development.

Formulations developed for lyophilization may be stored as a frozen liquid in cases where lyophilization resources or equipment are not readily available. The development of an ideal lyophilization cycle as well as the manufacturing of lyophilized drug product would require several months worth of additional research, additional drug substance, and a properly validated facility. This part of development can be delegated as the commercial formulation development stage.

Liquid Formulation with Limited Expiry

For proteins with demonstrated stability in a liquid state at refrigerated storage with limited expiry can be introduced as an initial formulation. This will require

Table 1 Initial formulations routinely used up until early clinical trials

Initial formulation options	Pros	Cons	Applications
Frozen liquid	Simple, rapid development and implementation, formulation change may not be necessary if stable in liquid state	Stability in frozen state can be difficult to assess, challenging logistics to establish a controlled cold chain	Products with known stability profile, stable in low ionic strength formulations
Lyophilized	Enhanced stability, potential to commercialize, flexible to modifications during development like manufacturing, dose, etc.	Longer development, additional cost of manufacturing	Products with known stability issues, proteases, good IP position
Liquid	Most convenient for patients and health-care professionals	Longer development time (real-time stability data), less flexible to changes in manufacturing, dose, etc.	Products with good stability

a good understanding of a product's stability profiles: susceptible stresses, major degradation products, stability indicating assays, and formulation "sweet spot(s)." If the product maintains stability during a couple of months of storage at 25 °C and against other pharmaceutically relevant stresses such as agitation and transportation, a refrigerated liquid formulation may be a good recommendation for an initial formulation. The expiry may be extended as real-time stability data from long-term studies become available.

One of the major disadvantages of the refrigerated liquid approach is its limited capacity to accommodate common but unexpected excursions. Such excursions could include accidental freezing, exposure to ambient temperature, exposure to ultraviolet (UV) or visible light, excessive agitation during transportation and/or handling, etc. Generating sufficient stability data to cover the full spectrum of potential excursions is often improbable to achieve during early stages of development.

Factors to consider while selecting proper initial formulations are summarized in Table 1.

Strategy for Commercial Formulations

Various drug delivery methods have been pursued to gain a competitive edge in the market. For example, controlled release products for less frequent administrations and noninvasive delivery methods such as oral or pulmonary applications have been considered for competitive purposes. Applications such as these have largely been

limited due to formidable technical challenges. However, the formulation itself can be implemented to gain a competitive advantage. This chapter mostly focuses on parenteral formulations. Different routes of administration, e.g., subcutaneous, intravenous, intramuscular, intraperitoneal, intravitreal, etc., may require varying formulations to be compatible with its specific application.

In general, a minimal expiry of 2 years is required for commercialization. Since real-time stability data are required for setting expiry, preliminary stability data at the target storage condition, i.e., 6 months, are required before recommending the formulation for commercial purposes. When introduced before phase III clinical trial(s), the real-time stability data can be obtained concurrently with the pivotal study.

Frozen Formulations

It is important to acknowledge that frozen formulations are not competitive for commercial purposes. The market will be limited to areas where an adequate cold chain can be established. Maintaining the cold chain itself can become very expensive, especially when specific freezing conditions need to be provided. Also, the time and effort involved in thawing a product before use is inconvenient to the administrator of the product.

Liquid Formulations

In general, liquid formulations, especially when presented in a prefilled syringe, should be considered the best option for commercialization. It is preferred over lyophilized formulation due to the convenience of direct administration without preparation. This attribute is essential when competing products exist in prefilled syringes with convenient devices such as auto-injectors or pen injectors. Even when liquid formulations may not be feasible during the initial entry to market, many protein formulations are switched from lyophilized to liquid formulations during life-cycle management for competitive reasons. Readers are referred to other references for various options for liquid formulation development [8].

Lyophilized Formulations

As many biopharmaceuticals are not sufficiently stable to achieve a 2-year expiry in a liquid state, more than 50% of currently marketed biopharmaceuticals are introduced as lyophilized formulations. Lyophilized formulations offer other advantages over liquid formulation due to their superior stability during transportation and storage. In addition, the enhanced stability of lyophilized drugs often avoids the emergence of stability issues during later stages of development or at the commer-

cial stage. This makes lyophilized formulations an excellent commercial candidate for products with minimum competition and/or a strong intellectual property (IP) position.

Considering that even relatively unstable proteins such as interferon or hemophilia factors were introduced as room temperature stable lyophilized formulations, it is safe to assume that properly developed lyophilized formulations can allow room temperature storage of most protein therapeutics. This would not only make the storage, distribution, and handling of the product more convenient but also allow the distribution of biopharmaceuticals, including vaccines, to developing countries or regions where a cold chain has not or cannot be established.

Furthermore, the possibility of using various types and volumes of diluent allows flexibility in lyophilized formulation applications. Lyophilized formulations can be reconstituted with smaller volumes of diluent to overcome stability issues at higher concentrations or achieve smaller injection volumes. Preservatives can often compromise longer-term stability of proteins in a liquid state, but can be added in diluents to produce shorter-term multidose formulations. For instance, lyophilized formulations can be reconstituted with commercially available bacteriostatic diluents containing benzyl alcohol or custom diluents containing preservatives specifically selected for the product.

Delivery Devices

As previously stated, the main disadvantage of a lyophilized formulation is the inconvenience of reconstitution prior to administration. In order to take advantage of the improved stability of lyophilized formulations without compromising ease of use, various devices have been introduced to the market to aid the reconstitution process. These include vial adapters, vial-to-vial systems, needless transfer systems, or direct connection to vial systems. Other advanced container/closure systems, such as dual chamber syringes, e.g., Lyoject® or Lyotip™, are also available. For multidose formulations, cartridges designed to be reconstituted with bacteriostatic diluents followed by injections with pen injectors are available.

As device technology evolves, it is feasible that a lyophilized formulations could be manufactured as easily and cost-effectively as current container/closure systems, and delivered as conveniently as a prefilled syringe. The most ideal attributes of a product could be achieved with this technology, with the stability of a lyophilized formulation and the convenience of a liquid formulation.

Strategy to Switch Formulations

The best time to switch from an initial formulation to a commercial formulation is before phase III clinical trials. This is the time when all development activities, including manufacturing of drug substance, process to formulate and fill/finish, final container-closure and delivery options, stability study programs, and clinical dosing

Table 2 Studies needed to support changes in formulation during early clinical trials

Research requirements	Supporting results
Purity	Stability, compatibility, structural analyses
Potency	In vitro/in vivo bioassays, preclinical, and/or clinical pharmacokinetic comparability
Safety	Preclinical safety

schedules, are scaled up and poised for pivotal trials. As many other changes relevant to the chemistry, manufacturing, and controls (CMC) package are occurring at this time, it is the most efficient time to incorporate any formulation changes to the final campaign to demonstrate the commercial feasibility of the product.

Since a formulation change can potentially introduce substantial adverse effects on the purity or potency as related to the safety or effectiveness of the product, solid justifications are required for any proposed changes. These justifications must be based on relevant methods and results to evaluate the effect of the change as related to the product's safety or effectiveness (Appendix Regulatory Documents 18–20). Although changes in degradation profiles after formulation modifications are undesirable, if the degradation profile changes qualitatively or quantitatively it is recommended to follow the impurity-related guidelines (Appendix Regulatory Documents 2,3).

Assuming that the formulation change does not affect the route of administration, pharmacokinetic, or pharmacodynamic properties of the product, additional purity, potency, and safety studies (Table 2) should be conducted. Also, results from real-time stability at the recommended storage condition are required to establish an expiry date. However, comparable stability between the old and new formulations can be demonstrated based on results from accelerated stability studies. This will require both good stability data from the old formulation, including both an accelerated stability study and a real-time stability study, as well as a good understanding of how degradations observed from accelerated stability studies can be safely extrapolated to recommended storage conditions.

Switching formulations after reaching the market will require a formal submission and approval of amendments to regulatory agencies. As a part of this process, clinical studies to demonstrate the comparable safety and efficacy of the product will be required, which will involve human pharmacokinetic comparability studies in addition to all the studies listed in Table 2.

Strategy for Different Modality of Biologics

While general approaches in formulation optimization and processes for various biologics should be similar, different characteristics of individual modalities require different formulation attributes. These differences appear to be consistent among commercialized products, so general strategies applied for different modalities can

be derived by comparing the formulations of antibodies, Fc-fusion proteins, antibody drug conjugates (ADCs), peptides, and general proteins including enzymes, cytokines, antihemophilic factors, etc.

A list of commercialized antibodies, Fc-fusion proteins, and ADCs is shown in Table 3. A majority of antibody products are formulated with sucrose or trehalose as a bulking agent or tonicity modifier with surfactants. This particular trend primarily results from general experience with antibodies; most antibodies are reasonably stable in a liquid state and do not require additional stabilization beyond water-replacing sugar(s). These products contain higher concentrations of proteins and, consequently, can form good cakes without the need for additional bulking agents.

This trend changes significantly for Fc-fusion products or ACDs (Table 3). Fc-fusion products have demonstrated inferior stability compared to naturally existing antibodies and thus require additional stabilizers in their lyophilized formulations. The structural compatibility between Tc and Fc regions has been discussed among possible sources of instability [6, 19]. Likewise, the conjugation of antibodies with small molecule pharmaceuticals results in less stable molecules than the original antibodies. Due to this stability issue, more product-specific stabilizers are generally included in these commercial products.

Table 4 lists other general protein therapeutics. As the advantages of antibody products, i.e., high stability and high concentration, do not generally apply for other protein therapeutics, more customized formulations include additional crystalline bulking agents like mannitol or glycine and product-specific stabilizers such as amino acids, salts, etc. As some of the necessary ingredients are relatively difficult to lyophilize, additional formulation components, e.g., crystalline bulking agents or polymeric excipients are added to overcome the low collapse temperatures of such stabilizers. For example, antihemophilic factors generally require high ionic strength as well as specific stabilizers like calcium chloride, both of which have very low collapse temperatures [7] and can be difficult to lyophilize. To address these challenges, additional excipients like sugars or mannitol are commonly used in the formulation. In addition to formulation optimization, the development of an appropriate lyophilization cycle is critical for products containing such additives.

Commercial peptide products and their formulations are outlined in Table 5. Unlike other protein therapeutics discussed above, peptides are formulated with a simple crystalline bulking agent such as mannitol, without the need for other stabilizers like surfactant or sugars. The primary reason behind a simple formulation is that peptide products do not typically possess a complex three-dimensional structure, so the stabilization of a delicate conformation may be not necessary. On the other hand, peptide products are relatively more vulnerable to chemical degradations during storage in liquid state which can be readily eliminated by lyophilizing the product.

Table 6 shows a list of commercial vaccine products which are available in lyophilized forms. Just as there are various modalities even within vaccine preparations, there are complicated and diverse formulations presented to the market. Unique stabilizers like gelatin, HSA, and glutamate commonly appear in vaccines

Table 3 Commercial lyophilized antibody related products

Generic name	Brand name	Molecule source/type	Firm	Route	Bulking agent/stabilizer
Basiliximab	Simulect	Chimeric ab	Novartis	IV	Mannitol, sucrose, NaCl, glycine
Belimumab	Benlysta, LymphoStat-B	Monoclonal ab	BMS	IV	Sucrose, PS 80
Canakinumab	Ilaris	Monoclonal ab	Novartis	SC	Sucrose, PS 80
Certolizumab pegol	Cimzia	PEGylated Fab	UCB	SC	Sucrose, PS
Digoxin immune Fab	Digibind	Polyclonal ab	GSK	IV	Sorbitol, NaCl
Efalizumab	Raptiva	Monoclonal ab	Genentech	SC	Sucrose, PS20
Infliximab	Remicade	Chimeric ab	J&J	IV	Sucrose, PS 80
Omalizumab	Xolair	Monoclonal ab	Novartis/Roche	SC	Sucrose, PS20
Oprelvekin	Neumega	Monoclonal ab	Wyeth	SC	Glycine
Trastuzumab	Herceptin	Monoclonal ab	Genentech	IV	Trehalose, PS20
Etanercept	Enbrel	Fc fusion	Immunex	SC	Mannitol, sucrose
Alefacept	Amevive	Fc fusion	Astellas	IM	Glycine, sucrose
Abatacept	Orencia	Fc fusion	BMS	IV	Maltose, NaCl
Belatacept	Nulojix	Fc fusion	BMS	IV	Sucrose, NaCl
Rilonacept	Arcalyst	Fc fusion	Regeneron	SC	Glycine, sucrose, arginine, PEG3350
Romiplostin	Nplate	Fc fusion	Amgen	SC	Mannitol, sucrose, PS20
Brentuximab vedotin	Adcetris	ADC	Seattle Genetics	IV	Trehalose
Ado-trastuzumab emtansine	Kadcyla	ADC	Genentech		Sucrose, PS20
Gemtuzumab ozogamicin	Mylotarg	ADC	Wyeth	IV	Dextran, sucrose, NaCl

PEG polyethylene glycol, *J&J* Johnson & Johnson, *BMS* Bristol-Myers Squibb, *IV* intravenous, *SC* subcutaneous

Table 4 Commercial lyophilized protein pharmaceuticals

Generic name	Brand name	Firm	Route	Bulking agent/stabilizer
Agalsidase beta	Fabrazyme	Sanofi	IV	Mannitol
Aldesleukin	Peolwukin	Prometheus	IV	Mannitol, SDS
Alglucosidase alfa	Myozyme	Sanofi	IV	Mannitol, PS80
Alpha 1 antitrypsin	Aralast	Baxter	IV	Albumin, PEG, PS80, Zinc
Alpha 1 antitrypsin	Zemaira	CSL	IV	Mannitol, NaCl
Alteplase	Activase	Roche	IV	L-Arginine, PS80
Anistreplase	Eminase	Wulfing	IV	*p*-Amidinophenyl-*p*′-anisate, mannitol, L-lysine, HSA, glycerol, aminocaproic acid
Botulinumtoxin A	Botox	Allergan	SC	HSA, NaCl
C1 esterase human	Cinryze	Viropharma	IV	Sucrose, NaCl, L-valine, L-alanine, L-threonine
C1 esterase inhibitor	Berinert	CSL	IV	Glycine, NaCl
Collagenase	Xiaflex	Auxilium	IM	Sucrose
Drotrecogin alfa	Xigris	Lilly	IV	Sucrose, NaCl
Factor IX	Mononine	CSL	IV	Mannitol, sucrose, PS80, NaCl
Fibrinogen	RiaSTAP	CSL	IV	HSA, arginine, NaCl
Follitropin alfa	Gonal-F	EMD Serono	SC	Sucrose, methionine, PS20
Glucarpidase	Voraxaze	BTG	IV	Lactose, zinc
Imiglucerase	Cerezyme	Genzyme	IV	Mannitol, PS80
Interferon beta-1b	Betaseron	Chiron	SC	HSA, mannitol
Lepirudin	Refludan	Bayer	IV	Mannitol
Lutropin alfa	Luveris	Serono	SC	Sucrose, methionine, PS20
Menotropins	Menopur	Ferring	SC	Lactose, PS20
Menotropins	Repronex	Ferring	IM, SC	Lactose
Palifermin	Kepivance	Amgen	IV	Mannitol, sucrose, PS20

Table 4 (continued)

Generic name	Brand name	Firm	Route	Bulking agent/stabilizer
Peginterferon alfa-2b	PEG-Intron	Merck	SC	Sucrose, PS80
Pegvisomant	Somavert	Pfizer	SC	Glycine, mannitol
Protein C	Ceprotin	Baxter	IV	HSA, NaCl
Rasburicase	Elitek	Sanofi	IV	Mannitol, alanine
Reteplase	Retavase	Centocor	IV	Sucrose, PS80
Rfactor IX	BeneFIX	Pfizer	IV	Glycine, sucrose, NaCl, PS80
Rfactor IX	Rixubis	Baxter	IV	Mannitol, sucrose, NaCl, $CaCl_2$, PS80
Rfactor VII	NovoSeven	Novo Nordisk	IV	mannitol, sucrose, NaCl, $CaCl_2$, PS80, glylglycine, methionine
Rfactor VIII	Advate	Baxter	IV	mannitol, trehalose, NaCl, $CaCl_2$, PS80, glutathione
Rfactor VIII	Helixate	CSL	IV	Sucrose, glycine, $CaCl_2$, PS80
Rfactor VIII	Refacto	Pfizer	IV	Sucrose, NaCl, $CaCl_2$, PS80
Sargramostim	Leukine	Berlex	IV/SC	Mannitol, sucrose
Somatropin	Humatrope	Lilly	SC	Mannitol, glycine
Somatropin	Saizen	EMD Serono	SC	Sucrose
Streptokinase	Streptase	Avantis	IV	Gelatin, glutamate, HSA
Taliglucerase alfa	Elelyso	Pfizer	IV	Mannitol, PS80
Tenecteplase	TNKase	Genentech	IV	Arginine, PS20
Thymalfasin	Zadaxin	SciClone	IV	Mannitol
Thyrotropin alfa	Thyrogen	Genzyme	IM	Mannitol, NaCl
Urofollitropin	Bravelle	Ferring	IM, SC	Lactose, PS20
Velaglucerase alfa	VPRIV	Shire	IV	Sucrose, PS20

IV intravenous, *SC* subcutaneous, *IM* intramuscular, *HSA* human serum albumin

Table 5 Commercial lyophilized peptide pharmaceuticals

Generic name	Brand name	Firm	Route	Bulking agent/stabilizer
Abarelix	Plenaxis	Praecis	IM	CMC
Bivalirudin	Angiomax	MDCO	IV	Mannitol
Cetrorelix	Cetrotide	EMD Serono	SC	Mannitol
Chorionic Gonadotropin	Novarel	Ferring	IM	Mannitol
Corticorelin ovine triflutate	Acthrel	Ferring	IV	Lactose, ascorbic acid
Cosyntropin	Cortrosyn	Amphastar	IV/IM	Mannitol
Daptomycin	Cubicin	Novartis	IV	None
Degarelix	Firmagon	Ferring	SC	Mannitol
Enfuvirtide	Fuzeon	Roche	SC	Mannitol
Glucagon	Glucagen	Novo Nordisk	SC, IM, IV	Lactose
Nesiritide	Natrecor	J&J	IV	Mannitol
Secretin	SecreFlo	Repligen	IV	Mannitol, cycteine
Sermorelin	Sermorelin acetate	Sereno	SC	Mannitol
Teduglutide	Gattex	NPS	SC	Mannitol
Tesamorelin	Egrifta	Theratechnologies	SC	Mannitol

IV intravenous, *SC* subcutaneous, *IM* intramuscular, J&J Johnson and Johnson, MDCO The Medicines Company

Table 6 Commercial lyophilized vaccine products

Generic name	Brand name	Molecule source/type	Firm	Route	Bulking agent/stabilizer
BCG	BCG vaccine	Live bacteria	Merck	SC	Glutamate, reconstituted with NaCl, PS80
BCG	Mycobax	Live bacteria	Sanofi	SC	Glycerin, asparagine, MgSO4, and iron ammonium citrate
BCG	TICE BCG	Live bacteria	Merck	SC	Sucrose, ammonium phosphate, PS80
Diphtheria and tetanus toxoids and acellular pertussis adsorbed, inactivated poliovirus and haemophilus b conjugate vaccine	Pentacel	Inactivated	Sanofi Pasteur Limited	IM	Sucrose, ammonium phosphate, PS80
Haemophilus b conjugate vaccine	ActHIB	Bacterial conjugate	Sanofi	IM	Sucrose, reconstituted with NaCl
Haemophilus b conjugate vaccine	Hiberix	Bacterial conjugate	GSK	IM	Lactose
Japanese encephalitis virus vaccine	JE-Vax	Inactivated	Research foundation for microbial diseases of Osaka University	SC	Gelatin, PS80
Measles virus vaccine	Attenuvax	Live	Merck	SC	Gelatin, HSA, sorbitol, sucrose
Measles, mumps, and rubella virus vaccine	M-M-R II	Live	Merck	SC	Gelatin, HSA, sorbitol, sucrose, NaCl
Measles, mumps, rubella, and varicella virus vaccine	ProQuad	Live	Merck	SC	Gelatin, HSA, sorbitol, sucrose, NaCl, glutamate
Meningococcal (groups A, C, Y, and W-135) vaccine	Menveo	Bacterial polysaccharide + conjugate	Novartis Vaccines and Diagnostics, Inc.	IM	Minimal

Table 6 (continued)

Generic name	Brand name	Molecule source/type	Firm	Route	Bulking agent/stabilizer
Meningococcal polysaccharide vaccine	Menomune-A/C/Y/W-135	Bacterial polysaccharide+conjugate	Sanofi	SC	Lactose
Mumps virus vaccine	Mumpsvax	Live	Merck	SC	Gelatin, HSA, sorbitol, sucrose, NaCl
Rabies vaccine	Imovax	Inactivated	Sanofi	IM	HSA
Rabies vaccine	RabAvert	Inactivated	Novartis	IM	HSA, glutamate
Rotavirus vaccine, oral	Rotarix	Live	GSK	Oral	Sorbitol, sucrose, DMEM
Rotavirus vaccine, oral, pentavalent	RotaTeq	Live	Merck	Oral	Sucrose, PS80
Rubella virus vaccine	Meruvax II	Live	Merck	SC	Gelatin, HSA, sorbitol, sucrose, NaCl
Smallpox (vaccinia) vaccine	ACAM2000	Live	Sanofi	Percutaneous	Mannitol, HSA, NaCl,
Typhoid vaccine live oral Ty21a	Vivotif	Live	Berna Biotech, Ltd.	Oral	Lactose, sucrose, ascorbic acid, amino acids, magnesium stearate
Varicella virus vaccine	Varivax	Live	Merck	SC	Gelatin, sucrose, NaCl, glutamate
Yellow fever vaccine	YF-VAX	Live	Sanofi	SC	Sorbitol, gelatin
Zoster vaccine	Zostavax	Live	Merck	SC	Gelatin, sucrose, NaCl, glutamate

BCG Bacille Calmette–Guerin, *IV* intravenous, *SC* subcutaneous, *IM* intramuscular, *HSA* human serum albumin

derived from viral particles, while more general stabilizers may be employed for vaccines of other modalities.

There are other modalities of biologics on the horizon. Although these products are yet to be approved by the FDA, various DNA-based biologics are currently under development. While most gene therapy products are stable enough to be stored in liquid solution, some show instability, e.g., topological change of plasmid products that would require lyophilization for adequate stability. Lyophilization has been also effectively been applied for delivery vehicles without intrinsic stability issues, e.g., liposomes and nanoparticles for nonviral vectors.

Formulation Role in Business Strategy

The choices of drug formulation and delivery are significant in determining the clinical and commercial profile of a product. Choices made early in formulation development may impact the frequency of administration, drug level, safety, and efficacy of a product. Depending on the indication, medical practice, and competition, these development choices can have positive or negative effects on the success of a product.

As a result, the formulation representative should work as part of a larger drug development team to develop a comprehensive strategy for planning and testing potential attributes of the drug as it evolves through drug development. The target product profile, or TPP, is a document used by many companies to manage and communicate these product attributes.

Target Product Profile

The TPP is a management tool used to communicate product attributes throughout the drug development process. Early in development, targets are based on the characteristics of the molecule, clinical needs, and commercial requirements, as many attributes have yet to be fully characterized through experimentation.

The format of the TPP captures elements of a product insert. Some companies use a marketed competitor's product insert as a template.

Major sections of the TPP that involve the entire development team include:

1. Indication
   a. Launch indication
   b. Additional indications
2. Efficacy
3. Safety
   a. Tolerability
   b. Adverse events

Table 7 Examples of TPPs by functional area with impact on formulation

Drug development	Product development	Manufacturing	Legal/regulatory
Indication	pH	Selling price	Freedom to operate
Route of administration	Tonicity	Cost of goods sold	Approved excipients
Dosage range	Excipients	Equipment required	
Dose frequency	Necessary dilutions or reconstitutions	Product processing time	
Treatment duration	Single or multiuse containment		
Administration date	Packaging type		
Dose volume	Storage conditions		
API concentration	Shelf life		
Pharmacokinetic profile	Shipping requirements		

TPP target product profile, API active pharmaceutical ingredient

4. Administration
   a. Route of administration
   b. Dose range
   c. Dose frequency and duration
   d. Volume and concentration
   e. Delivery device
5. Pricing and Reimbursement

Sub-teams may prepare more detailed TPPs with specific attributes which are of concern to the sub-team, but are of secondary importance to the global project team. Process development, packaging, and formulation groups may find such lists can provide a common organization of joint activities. Specific TPPs for drug, product, legal, or manufacturing components of development (Table 7) would address different attributes of the product.

These attributes can be designated as required elements or "targets." Failure to meet a target may be grounds for stopping or delaying a program.

Target elements are designed to communicate critical goals worth company time and investment. Targets should be scientifically feasible, although they may be challenging objectives for the team.

Companies can also strategically use the TPP to explore alternative investment strategies. For this reason, it is important for the formulation scientist to lay out meaningful options for the entire team. Formulation and delivery device could be one of the earliest experimentally confirmed attributes, allowing a product team to lock in certain competitive advantages early in the development process.

Table 8 Market research factors in evaluating a reconstitution device

Ease of use	Number of parts
Prefilled systems	Waste products
Syringeability	Time
Visibility	Injection safety
Leakage	Injectability
Number of steps	Dosage range

Table 9 Desirability of prefilled syringes compared to vials and importance of syringe attributes [11, 15]

		Physician $N=45$	Nurse $N=70$
Desirability of prefilled syringes compared to vials	Prefilled syringes vs. vials	67%	77%
	Prefilled syringes vs. vials, price being equal	100%	89%
Importance of syringe attributes	Reduced risk of contamination	91%	97%
	Accurate labeling and identification	91%	97%
	Ease of use	93%	94%
	Convenience	89%	93%
	Dosage accuracy	89%	91%
	Needle sharpness	73%	90%

Market Feedback

Product development teams often need to make investment trade-offs, so companies often use market research to understand the potential value of different product profiles. For instance, market research can explore the value of an investment in a room-temperature stable formulation or technology that makes a lyophilized product stable in liquid (Table 8).

Device teams can also explore the additional value of investment in reconstitution systems. For example, it has been shown that the reconstitution device for a lyophilized product can be as important as any difference in efficacy or safety when the patient is choosing between treatment options (Table 9). Market research can be also be used to project potential market shares, which in turn can be used to justify a particular option.

Furthermore, market research is able to test a product with consumers that may not be satisfied with a basic product. Convenient formulation and delivery have become increasingly important for certain chronic diseases immediately at launch. This requires integration of a device early in development so it can be part of the clinical trials and be ready at launch.

Through market research, formulation and product teams can quantify and justify an argument for the value of additional investments.

Issues Specific to Lyophilized Formulations

As nearly 60% of biopharmaceuticals, including antibodies, vaccines, and plasma products, are lyophilized, optimization of lyophilized formulations should concern most biotech professionals. Nevertheless, the value of extra investments in an optimized formulation may differ by indication and geography, so it is important to evaluate the product within different populations.

For example, many orphan diseases require patients to take medication starting at a young age for their entire lives. Suboptimal reconstitution devices can directly and indirectly impact treatment efficacy and patient quality of life [13]. The pediatric population also has other considerations, such as different safety and tolerability issues for certain excipients or device components as adults.

Lyophilized drugs have a great advantage with regard to geography, since a cold chain encompassing storage, distribution, and use may not be necessary for a stable lyophilized formulation. This can be a significant asset in the vaccine market, where vaccine access can be limited to vaccines which are stable at ambient temperatures. Additional investments in stabilized formulations and easy-to-use drug delivery systems could improve the reach and effectiveness of these international efforts.

Business Issues by Development Phase

Discovery

Early in development, a formulation team should prepare a survey of formulation technologies and delivery devices available for consideration. This activity should be updated on a regular basis so these considerations can be shared with members of various product teams. Not all innovations result from in-house activities, so the team should be open to licensing or purchasing access to technology platforms which allow their products to have meaningful differentiation in the market.

Pre-formulation activities may start during this time period, during which the potential for a liquid or a lyophilized formulation may become evident.

Manufacturability issues may also arise at this time. Flagging these issues to process development will allow ample time for preparation for pre-Investigational New Drug (IND) product supply.

Pre-IND

Once a team commences preparations for registration, an ideal formulation becomes an item on the critical path and thoroughness is often at risk of being compromised for development speed. Formulation professionals need to properly advise the team on the trade-offs between formulation risk and overall program risk. If a rational

argument is made to take advantage of an opportunity for additional studies which could lower the future risk of a bad formulation during clinical trials, the team may allocate extra investment and time.

For instance, if a team observes particulates at low concentrations during short-term studies, the team may invest extra time and resources in a more rigorous screening of the formulation. This may avoid future delays during clinical trials due to an unstable or dangerous formulation.

Considerations of device technologies can begin with the improved clarification of whether a product will be in a lyophilized or liquid formulation. If no device exists, device development can also take place at this time. Device development tends to have a long timeline and can run in parallel with drug development. The goal is for the device to be ready in time for phase II or phase III trials, at the latest. If not ready, regulatory issues or even launch delay can occur. This occurred to MannKind when they proposed a late switch in inhaled delivery devices during phase III trials.

Early Clinical

As the product enters clinical trials, the team will acquire valuable information on likely dose, volumes, and schedule. Frequent dose or higher volume products may benefit from a higher concentration formulation to improve patient convenience and competitiveness.

With the improved likelihood of success, the product development team should prepare a commercial formulation that can be brought through late stage clinical trials to the market. Since phase I and IIa trials can be very short, depending on the indication, teams should prepare accordingly.

Late Clinical and Launch

Formulation teams should have a commercial product in the course of a long-term stability study during phase III trials, which can last from 6 months to 5 years depending on the indication.

Reconstitution systems, delivery devices, kits, and other product presentations should ideally be part of any phase III trial, where quality-of-life data can be captured. These data will be used at launch by marketing to demonstrate the ease of use and cost of delivery. Government and private payers have increasingly required such data to set access, reimbursement, and pricing.

Packaging requirements may differ in various geographical locations due to local regulations, differences in volumes due to weight, or local clinical practices. The formulation and packaging team need to anticipate these hurdles in preparation for an effective launch.

Table 10 Examples of life-cycle management

Product	Purpose
Rituxan	Switch from IV to subcutaneous delivery
Hizentra	Highest concentration biologic on the market at 200 mg/ml
Afrezza	Inhaled insulin

IV intravenous

Table 11 Time, cost, and sales of discovery versus optimization (Tufts center for drug development, 2. Pharma life-cycle management planning, June 2005)

	Years to develop	Development costs (US$ MM)	Average peak sales (US$ MM)
NCE/discovery compound	9–12	900	300–500
Optimization/reformulation	4–5	40	100–200

NCE new chemical entity

Post Launch: Life-Cycle Management

Once a product has been successfully launched, companies should look for ways to enhance product competitiveness and expand the product's patent. If any additional intellectual property establishes market relevant advances, it can extend the life cycle of an established product by a couple of years.

This is especially true in markets where efficacy and safety are relatively comparable, such as plasma therapeutics. For this reason, plasma therapeutics companies invest heavily in new formulations, reconstitution systems, and packaging.

Formulation and delivery can be effective ways of blocking generic or biosimilar entrants. Roche's deal with Halozyme focuses on switching products like Herceptin and Avastin from intravenous cancer therapy to subcutaneous therapy via new formulations and delivery devices.

Life-cycle management can also be applied to biosimilar products. Following approval of a biosimilar which is by definition undifferentiated, a firm can quickly differentiate this product through formulation and delivery. A major change, such as a high concentration formulation, can quickly stand out from other competitors, including the originator. Examples of life-cycle management are listed in Table 10.

One potential improvement for a lyophilized drug could be a formulation improvement such as a switch to a liquid or improved storage temperatures or shelf life. Prefilled reconstitution systems such as Lyoject® or Lyotip™ can make the administration of lyophilized products as simple as that of a prefilled liquid syringe.

Delivery devices and formulation technologies have a low technology risk compared with other life-cycle product improvements, such as a new molecular entity or indication. They also tend to be fast to implement and cost relatively less compared to other life-cycle options (Table 11).

Formulation and delivery experts should prioritize those improvements which are valued by the patient or health-care providers who are making the choice between therapies. If they value the change, the company can maintain or gain market share. By addressing life-cycle management before patents expire, companies can transition to new products and retain market share.

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Lyophilization of Therapeutic Proteins in Vials: Process Scale-Up and Advances in Quality by Design

Bingquan (Stuart) Wang, Timothy R. McCoy, Michael J. Pikal and Dushyant Varshney

Introduction

Recent advances in biotechnology have resulted in various modalities of therapeutic proteins, which cover diverse forms of antibodies (e.g., monoclonal, domain, fused), small proteins (e.g., cytokines, hormones, enzymes), and complex biologics (e.g., antibody drug conjugate, peglyated proteins). Each modality of the protein pose unique challenges in stabilization due to differences in the biophysical and chemi-

B. Q. Wang (✉)
Late Stage Process Development, Genzyme, A Sanofi Company, Framingham, MA 01701, USA

Protein Formulation Development, Biogen Idec, 15 Cambridge Center, Cambridge, MA 02142, USA
e-mail: Stuart.wang@biogenidec.com

T. R. McCoy
Technical Development, Genzyme Ireland Ltd, IDA Industrial Park, Old Kilmeaden Road, Waterford, Ireland
e-mail: timothy.mccoy@genzyme.com

M. J. Pikal
Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT, 06269, USA
e-mail: michael.pikal@UCONN.edu

D. Varshney
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway, Holly Springs, NC, 27540, USA

D. Varshney
MS & T Hospira, Inc., 275 N. Field Drive Lake Forest, Lake Forest, IL 60045, USA
e-mail: dushamaya@gmail.com

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cal properties, and due to target dose (e.g., high concentrations) or drug delivery (e.g., subcutaneous). Lyophilization (freeze-drying) has been widely utilized (almost ~50% of marketed proteins products) for stabilization of biological drug products, which are, unstable in the liquid form or preferred over the frozen solutions. During lyophilization process development and scale-up using quality-by-design (QbD) principles, these differences should be carefully considered for a successful process validation and commercialization of each product. In this chapter, we focus on recent advances in lyophilization and scale-up of therapeutic protein products in vials. Also, advances in QbD approaches and a case study will be presented in detail.

Lyophilization Scale-Up

The delicate nature of protein molecules generally results in their poor long-term storage stability in aqueous solution, and thus lyophilization (Lyo) is often used to stabilize protein therapeutics as a solid-state dosage form. The lyophilization process depends heavily on the protein molecule and excipient components used in the formulation [5], and readers can refer to previous chapter for more details about the impact of formulation on lyophilization process design. In addition, the container–closer system also plays an important role in lyophilization process development. Among the many different primary containers available, a glass vial is the most commonly used container in the lyophilization field mainly because of long history of use and the inert nature of glass as well as the effective barrier to moisture relative to plastic containers. At the early stage of a product development, a lyophilization process in a vial container is initially developed in a small laboratory freeze-dryer, and then scaled up to a pilot-scale lyophilizer for early-stage clinical trial manufacturing. At the late-stage clinical phase III and commercial stage, the lyophilization process generally needs to be further scaled up to a large manufacturing-scale lyophilizer to meet the supply requirement.

The lyophilization scale-up process is quite challenging due to multiple differences between the small- and large-scale dryers. There is some guidance to facilitate the development of an efficient lyophilization cycle in the laboratory [50]. However, even an optimized cycle from the laboratory freeze-dryer may not transfer smoothly to manufacturing scale. In addition to the scalability differences, there are several other differences between laboratory- and manufacturing-scale lyophilizers, which may pose a serious challenge for scale-up. These challenges are discussed below.

Ice Nucleation Differences During Freezing

One important goal of freezing is to produce a homogeneous product in terms of ice crystal size so that variation in drying behavior within the batch is minimized and an efficient drying process can be easily developed. However, the random nature

of ice nucleation means variation in the ice nucleation temperature, which in turn means variation in drying behavior as the ice nucleation temperature plays a major role in determining the size of the ice crystals formed. The size of the ice crystals determines the size of the "pores" through which water vapor must pass during primary drying and therefore the "resistance" to mass transfer. The degree of supercooling depends on the solution composition, the shelf temperature–time profile during freezing, and the operating environment. In the laboratory, ice nucleation generally occurs at a higher temperature than in manufacturing, probably due to the higher level of ice-nucleating particulates in the laboratory [48]. This systematic difference in freezing causes the formation of cake with a higher resistance to mass transfer in manufacturing, which causes primary drying to be longer in manufacturing but at a higher product temperature. The freezing difference between small and large manufacturing freeze-dryer is considered to be a major challenge to scale-up of lyophilization cycles.

Heat and Mass Transfer Differences

Generally, there are differences in dryer design and configuration between laboratory and manufacturing scale, and such differences in the chamber, duct, condenser, or refrigeration system could lead to systematic shelf surface temperature differences between laboratory and manufacturing even when using the same set points for shelf temperature control. Gas flow variations may result in "choked flow" in one dryer but not in another, leading to the loss of chamber pressure control in the dryer with choked flow. The loss of pressure control may also result from inadequate condenser design and/or insufficient refrigeration capacity to convert the water vapor back to ice at a low temperature [40]. Therefore, different dryers do not necessarily perform the same way, and some modification in cycle design during scale-up is normally necessary to avoid problems.

Differences in Primary Drying Time

Due to the expected differences in freezing and heat transfer between laboratory and manufacturing, the cycle would be expected to take longer at commercial scale, especially during the primary drying step. As a general rule, one would expect that primary drying needs approximately 20% more time to complete in production scale [40]. However, as with any general rule, there will be significant deviations from the rule, and it will be important to predict the length of the primary drying step for both scales for each specific case, and also important to directly monitor each step using process analytical technology (PAT) tools.

QbD Approach

These differences between laboratory- and manufacturing-scale lyophilizers can pose a serious challenge for scale-up, and a systematic approach is needed to ensure a robust scale-up. Recently, "QbD" concept has become quite popular in guiding the development of protein formulation and lyophilization processes. The term "quality-by-design, QbD" stems from the perspective of building the quality into the product by a rational design, meaning quality is assured, rather than dependence of analytical testing of produced batches to detect individual defects and possibly reject batches. One might argue that the concept of designing the formulation and process in a way that assures quality, such that testing of the batch is superfluous, is hardly a new concept. What is perhaps new in the current QbD focus is the commitment to actually take the time to execute the required scientific and engineering studies that indeed will assure product quality, and to perform these studies with full consideration of the risk to product quality in each aspect of product and process design. This means that aspects that pose little risk to product quality will receive minimal attention, but aspects that may pose serious risk to critical product quality attributes (normally, safety and efficacy) will be systematically and completely studied. Thus, the QbD concept is a scientific and risk-based approach for drug development and the term "design space" is its key element [22].

QbD: Elements and Protein Modality Considerations

In recent years, industries have adopted the US Food and Drug Administration (FDA) International Conference on Harmonization (ICH) Q8, Q9, and Q10 guidelines at different stages of lyophilized product development and commercialization [7–10]. Some of the key elements include: defining a quality target product profile (QTPP), prior knowledge, risk assessment, design of experiment (DoE) studies, defining design space and control strategy utilizing PAT, identifying critical quality attributes (CQA), critical process parameters (CPP), process qualification/validation, and continuous verification of process.

During early stages while defining the QTPP and design space, a special consideration should be made to differences in modality of proteins (e.g., monoclonal, domain, fused antibodies, antibody drug conjugate, or peglyated proteins). Each modality of protein along with differences in dose (e.g., high vs. low concentrations) or drug delivery route (e.g., subcutaneous vs. intravenous) may require quite a different approach towards defining which key quality attributes and what steps in lyophilization are critical for controlling and hence assuring QbD principles [14]. For example, freezing step in case of one modality of protein might be more crucial than controlling primary drying step [15, 37]. The use of statistical methods (univariate statistical process control (SPC)- or multivariate statistical process control (MSPC)-based models) has been used to evaluate the process parameters and develop the design space [1]. In addition, there have

been great advances in the freeze-drying theory and computational methods, which allow modeling the drying process in vials for a design space construction and avoid many trial-and-error experiments [18, 22, 38]. Also, scale-up and scale-down models are used towards freeze-drying process optimization, keeping in mind the manufacturing equipment capabilities, towards successful technology transfer, process validation/qualification batches.

QbD: Quality Risk Management

It is important to identify potential risks and their criticality during early development of product and process, which is an essential part for applying QbD principles. Definition of risk is the combination of the probability of occurrence of harm and the severity of that harm. During the entire product life cycle, quality risk management (QRM) provides a systematic process for assessment, control, communication, and quality risk review of product. Three main pillars of the QRM are risk assessment, risk control, and risk review.

Risk assessment involves risk identification, risk analysis, and risk evaluation, where risk identification utilizes prior knowledge, historical data, flow charts, cause-and-effect diagrams (e.g., Fishbone or Ishikawa), and fault tree analysis. Next step is the risk analysis that involves risk ranking (high–medium–low, quality attributes, and process impact scores); failure modes and effects analysis (FMEA); failure mode, effects, and criticality analysis (FMECA). This leads to a risk evaluation by various tools such as DoE, control charts, Pareto charts, and process capability analysis.

Second major pillar is the risk control that involves risk reduction and risk acceptance, which enables a robust QRM process providing control strategy, improvement, data flow optimization, and achieves overall risk management goals. Finally, a risk review of events is conducted, which provides continuous improvement opportunities. Such an effective QRM approach can provide confidence in the decision-making process and biopharmaceutical company's capabilities to handle potential risks to ultimately provide right quality and regulatory assurance.

The scope of this chapter is to explore the latest development and scale-up of the lyophilization process for protein therapeutics in the vial using a QbD approach. First, the fundamentals of lyophilization process in vials are briefly presented in terms of the heat transfer theory and vial heat transfer coefficient measurement. Then, the applications of QbD concepts to lyophilization processes are discussed for the freezing and drying steps, and the latest developments for designing robustness into the cycle, based on the theoretical modeling specifically for primary drying, are presented. A case study is presented for a protein product using both theoretical modeling and experimental scale-down model approach to obtain a wide design space. Finally, the application of QbD to facilitate the process development and scale-up is summarized.

Lyophilization in a Vial

Vial Heat Transfer Coefficient, K_v

A good understanding of heat transfer during primary drying allows for greater efficiency in process development and minimizes problems encountered during scale-up process [35]. The product temperature during primary drying is directly related to the heat transfer from the heat source (normally, the shelf) to the product itself.

Heat transfer may be defined as the ratio between the area-normalized heat flow and the temperature difference between heat source and heat sink [35]. The vial heat transfer coefficient, K_v, is defined by the following equation for a vial in direct contact with the shelf:

$$\frac{dQ}{dt} = A_v \cdot K_v \left(T_{shelf} - T_{product}\right), \tag{1}$$

where A_v is the external cross-sectional area of the vial, dQ/dt is the heat flow from the shelves to the product in a given vial, T_{shelf} is the temperature of the shelf surface and $T_{product}$ is the temperature of the product at the bottom center of the vial.

The vial heat transfer coefficient (K_v) consists of three mechanisms: (1) heat transfer due to direct conduction (K_c) between the contact points at the vial bottom and shelf surface; (2) radiation heat transfer (K_r) to the bottom, top, and sides of the vial; and (3) heat transfer due to gas conduction (K_g) in the space between the vial bottom and shelf surface. Therefore, the vial heat transfer coefficient (K_v) may be expressed as:

$$K_v = K_c + K_r + K_g. \tag{2}$$

The gas conduction term, K_g, is dependent on the pressure within the chamber during sublimation. This heat transfer is achieved by energy exchange during direct collisions of gas (i.e., H_2O) between vial bottom and shelf surface, and this is conduction through the dilute gas. It has been reported that the dominant mechanism for heat transfer for a center vial during primary drying is gas conduction [12]. The gas conduction mechanism has been expressed as a function of pressure [39]:

$$K_g = \frac{\alpha \cdot \Lambda_0 \cdot P}{1 + l_v(\alpha \cdot \Lambda_0 / \lambda_0)P}, \tag{3}$$

where Λ_0 is the free molecular heat conductivity of the gas at 0 °C, λ_0 is the heat conductivity of the gas at ambient pressure, P is the gas pressure, l_v is the constant "effective" distance characterizing the gap between the shelf and the vial bottom, and α is a term related to the energy accommodation coefficient (a_c) and the absolute temperature, T.

Heat transfer due to radiation, which is affected by the material emissivity, is an important heat transfer mode during lyophilization [11]. In laboratory dryers, the chamber may be fitted with a Plexiglas door which has higher emissivity than stainless steel. Due to emissivity differences, the vials close to the door (front edge vials) dry at a higher rate than the vials placed in the center of the array. These vials also dry faster than the vials on either side (left or right) of the array and back of the array. This phenomenon is known as the edge effect [39]. The percentage of edge vials in a commercial lyophilizer is less than a laboratory-scale lyophilizer. Moreover, presence of a stainless steel door reduces the atypical heat transfer as observed on a laboratory-scale lyophilizer.

The contribution of radiation to the overall heat transfer model has been studied in detail [11]. It has been shown that radiation is an important source of heat transfer under vacuum conditions for a vial in direct contact with the shelf. As expected, the closer to the wall or door, the higher the contribution of radiative heat transfer, which is also represented in the form of weight loss in Fig. 1. It was reported that the contribution of radiation to the overall heat transfer is about of 49% for a vial directly in contact with the shelf at a pressure of 100 mTorr and a shelf temperature of +25 °C. Similar radiation contribution was found at a shelf temperature of −20 °C at the same pressure condition [11].

An uncontrolled vial manufacturing process can lead to differences in the area of actual contact between vial and shelf (i.e., the term K_c) and the gap between the shelf and the bottom of the vial (l_v) for vials manufactured either in the same batch or within batches. Variation in the term "l_v" can lead to differences in the heat transfer through K_g and variation in the vial surface area can affect all modes of heat transfer [35]. Therefore, it is critical to have a good estimate of the differences in the K_v between vials within a batch and also between different batches of vials.

It is important to understand the impact of K_v on product temperature and product quality. It is well documented that a higher K_v results in a higher product temperature profile and in turn a higher rate of sublimation. A higher product temperature may result in an already aggressive primary drying step to become much more aggressive and potentially resulting in cake collapse or even melt, although actual melting is rare. Collapse may result in stability issues for protein formulations. In addition, an increase in the product temperature and subsequent increase in sublimation rate may result in the inability to control pressure due to limitations imposed by dryer capability.

The measurement of K_v can be accomplished through a series of sublimation runs using water at different chamber pressure set points (refer to Fig. 1). Water is filled into the vial to a fill volume of 50% of the vial capacity and subjected to sublimation for a target time at which approximately 25% of water is lost due to sublimation. Thermocouples are placed in selected vials across the shelf to allow measurement of product temperature, and the weight loss (refer to Fig. 1a) is calculated for all vials by weighing each vial before and after the run. As heat transfer is a function of chamber pressure, it is important to measure K_v at several pressure set points close to the actual sublimation conditions. The K_v values measured for each set point are fitted using nonlinear regression analysis, resulting in a model for K_v

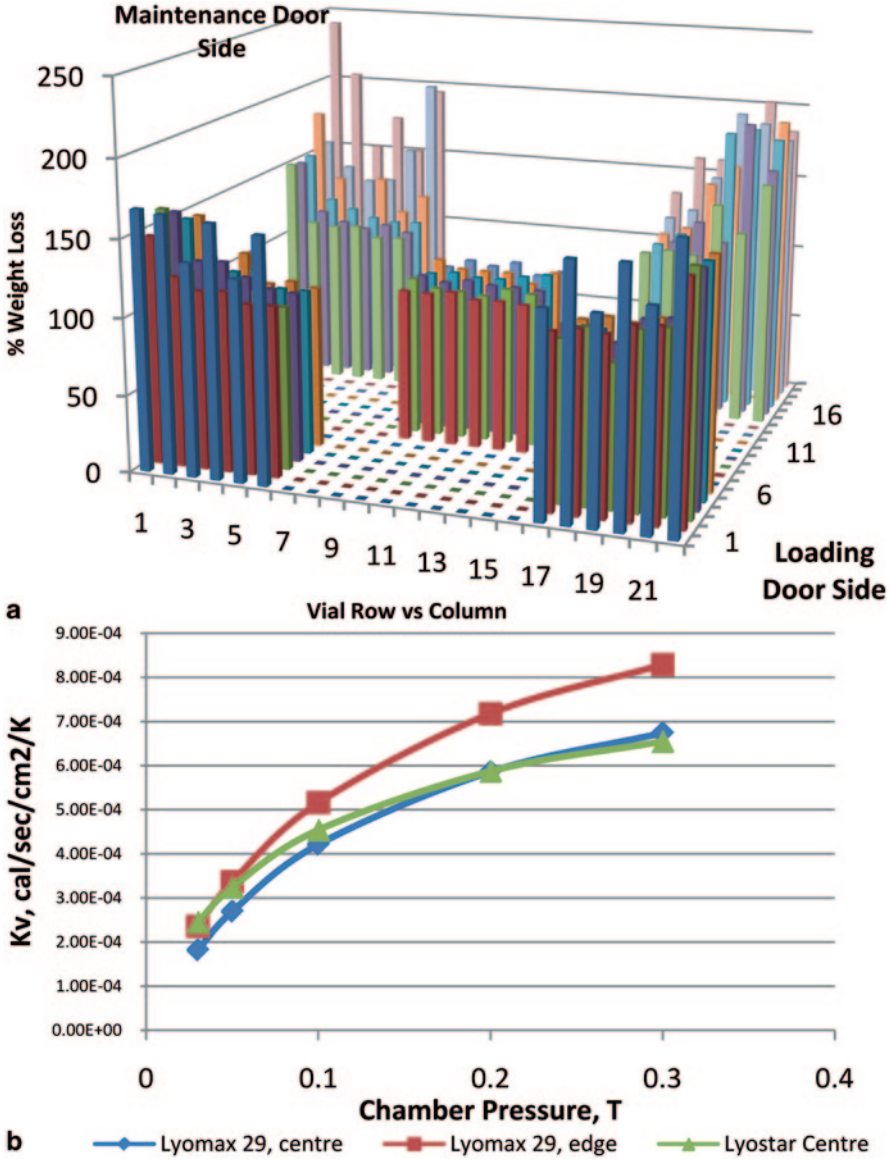

Fig. 1 Vial heat transfer coefficient K_v of a 10-cc tubing vial, measured as a function of chamber pressure and vial location in an IMA Lyomax 29 manufacturing dryer and a LyoStar II pilot freeze-dryer. **a** Weight loss map as a function of shelf location at a chamber pressure of 100 mTorr, measured during a K_v experiment in an IMA Lyomax 29 freeze-dryer. **b** K_v of center and edge vials at different chamber pressure set points as measured in an IMA Lyomax 29 freeze-dryer and a LyoStar II pilot freeze-dryer

that is specific for that vial and the freeze-dryer. Figure 1 shows the K_v of a 10-cc tubing vial as a function of chamber pressure and vial location in an IMA Lyomax 29 manufacturing dryer and a LyoStar II pilot freeze-dryer. The normalized weight loss map as a function of shelf location is given in Fig. 1a, and it shows that there is a clear difference in weight loss between the center and edge vial. In this case, the edge vials result in a maximum weight loss of approximately 2.4 times of that in the center vial at 100 mTorr. The maximum weight loss was recorded at the edge of the shelf on the maintenance door side of the freeze-dryer. The high K_v at this location is expected since the door has a major effect on the K_v of the edge vial due to elevated heat radiation from the door itself. Figure 1b shows that the K_v increases with increasing pressure within the pressure range studied. The data indicate that at lower pressures, the K_v of the center vial is closer to that of the edge vial and therefore the edge effect can be deemed minimal at lower chamber pressures. However, using lower pressures will result in lower product temperatures, lower sublimation rates and therefore, a longer primary drying time. When developing the primary drying segment of the cycle, it is important to balance conditions with time, while obtaining acceptable product stability should always be the major consideration. Secondly, when developing the primary drying step, it is important to consider the edge vial along with the center vial as each vial will dry at different rates due to the edge effect. In addition, the K_v of the same 10-cc tubing vial in the LyoStar II pilot unit was slightly higher than that of the center vial of the Lyomax 29 at low pressures but equalized at higher pressures. Therefore, the data suggest that if the primary drying step was to be developed in the LyoStar II unit (this is the scale where most cycles are developed), differences in K_v must be considered during scale-up.

With a model for K_v developed, primary drying design and scale-up are simplified. Once the product resistance (R_p) is obtained for samples subjected to a representative freezing process, it is possible to predict product temperature performance for conditions within the experimental chamber pressure range at various shelf temperatures, thereby developing a primary drying design space. In terms of scalability, it is recommended to measure K_v at laboratory, pilot, and commercial scale.

Variation in drying behavior and statistical uncertainty in parameter estimation should also be incorporated in building a design space [42]. Using only the mean values of K_v and R_p to build the design space may lead to errors in selection of appropriate primary drying conditions. There is variation of both drying temperature and drying time within a batch, and after all, only a few vials dry at the mean values. Moreover, there is always uncertainty in the evaluated input values of mean K_v and mean R_p. Evaluation of the impact of these "variations" on design space is nontrivial, and requires a combination of modeling calculations and use of statistics to evaluate the distribution of drying times and temperatures arising from variation in K_v, R_p, shelf temperature, fill volume, and chamber pressure. Variation in this context means both intervial variation in a batch and the impact of experimental error on the characterization of the input parameters such as K_v and R_p. Attempts to quantitatively evaluate the impact of variation on process design (and design space) have been outlined in the literature [2, 3, 31]. Alternately, one can impose arbitrary "safety margins" in an attempt to include variation; but even if chosen based on

Table 1 The K_v of different vials at typical chamber pressures. Data are adapted from reference [35] with permission

Vial size	Type	Chamber pressure, mTorr	$10^4 K_v$, cal/(s cm^2 ºC)
10 cc (A_v=4.71 cm^2)	W5800	100	4.89
	W5800	200	6.10
	K5800	100	5.45
	K5800	200	6.78
	W5811	100	5.21
	W5811	200	6.86
20 cc (A_v=6.83 cm^2)	W5816	100	4.22
	W5816	200	5.40
	K5816	100	4.69
	K5816	200	6.14

representative examples from the literature [2, 3, 31], this practice has significant risk of leading to collapse or excessively long duration of primary drying.

Summary of K_v Values in Common Vial Types

The K_v of different vials has been studied extensively in the field, and the vial type, size, dryer design, and chamber pressure, all play an important role in determining the K_v value. Table 1 summarizes the K_v of common vials used at typical chamber pressures, which were measured using water runs in laboratory freeze-dryers [35].

Figure 2 shows the dependence of K_v on the chamber pressure for different types of vials. These data were obtained using the sublimation rate profiles measured with

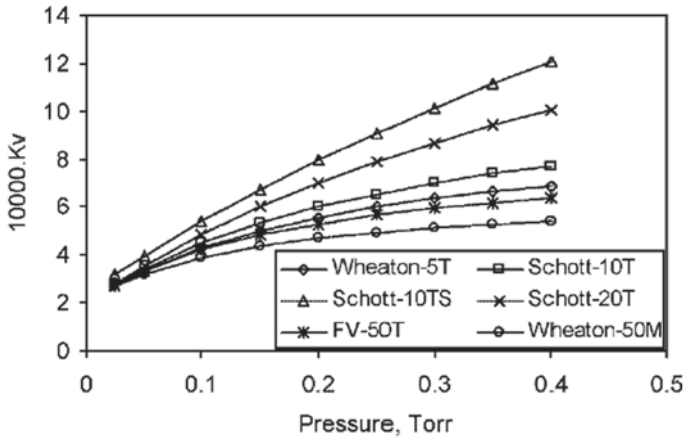

Fig. 2 Computed K_v values at a shelf temperature of – 10 °C (in cal s^{-1} cm^{-2}°C^{-1}) as a function of chamber pressure and vial size. Adapted from reference [20] with permission

a tunable diode laser absorption spectroscopy (TDLAS), and a computer program was used to perform the heat and mass transfer computations with the nonlinear least-squares algorithm. It can be seen that the order of vial heat transfer rate is: Schott-10TS > Schott-20T > Schott-10T > Wheaton-5T > FV-50T > Wheaton-50 at typical chamber pressures [20].

Lyophilization Development and Scale-Up

Due to the significant amount of publications on quantitative analyses of lyophilization in the past two decades, the QbD approach presented in this chapter focuses on the use of a mathematical model based on heat and mass transfer. Estimation of K_v and R_p is essential to the application of this model to obtain a design space [13]. The key element here is to estimate the K_v for a given vial as a function of pressure and to evaluate the R_p of the formulation for an ice nucleation temperature and product temperature range during primary drying that are characteristic of the process one intends to run. This is normally accomplished by running a conservative cycle of the formulation in question, and assuring that drying is performed below the critical product temperature, where R_p is essentially independent of product temperature. Further details are provided in this chapter in relation to the QbD concept and it applications to different stages of lyophilization process.

Liquid Characterization

For a rational development of a stable formulation, complete characterization of the protein solution is necessary. In addition to the structural characterization of protein native structure using conventional spectroscopic methods such as circular dichroism (CD), florescence, or Fourier transform infrared spectroscopy (FTIR), thermal analysis of the frozen state using differential scanning calorimetry (DSC) and freeze-drying microscopy (FDM) is very important to obtain the maximum allowable product temperature to be used in the primary drying process.

During the initial freezing stage, water will be converted into ice and the solute will be concentrated. Unless a critical low temperature is reached, the protein system still exhibits high “mobility” on an experimental timescale and degradation can still occur. This critical temperature during freezing step is called T_g', which is referred as the glass temperature of the maximally concentrated freeze-concentrate [32]. The freezing temperature should be below T_g' to ensure the whole system is completely frozen.

DSC is commonly used to measure the T_g' for a system. The protein solution is first completely frozen (to about $-60\,^{\circ}\mathrm{C}$), and then the temperature is scanned linearly to about $0\,^{\circ}\mathrm{C}$. T_g' is the temperature at which there is a sharp change in the

baseline, suggesting a sudden increase in heat capacity. The operating conditions such as the scanning rate and sample size will depend on the formulation and product properties. For formulations containing crystalline excipients and multiple components or phases, modulated DSC (MDSC) is preferable. MDSC is a development of standard DSC, whereby, a sinusoidal heating modulation is superimposed on the underlying linear heating or cooling signal, thereby facilitating measurement of the experimental glass transition and the heat capacity [45]. The reversing flow signal is determined by multiplying the measured heat capacity by the average heating rate, and it typically clearly reveals the glass transition event.

The critical process design parameter for an amorphous system is the collapse temperature (T_c), which is the temperature above at which the product undergoes structural collapse and loses cake structure [32]. Freeze-drying below T_c is necessary to ensure elegant appearance. Collapse may also lead to higher residual water after secondary drying and can also impact stability in some cases by either negatively impacting or improving stability [25, 46]. FDM is the most common method used for the measurement of collapse temperature. FDM involves monitoring a freeze-drying process at small scale on a temperature-controlled cold stage using a microscope. A small amount of solution is first applied between two glass surfaces and this thin film is then completely frozen. The system is subjected to vacuum to initiate primary drying. The sample temperature is slowly increased at a typical heating rate of 1 °C/min to allow sublimation. As the temperature increases above T_g', viscous flow will result in faster mobility and the structure change of the freeze-dried solid, which ultimately cause the collapse of the dried region. T_c is normally 1–3 °C higher than T_g' measured by DSC; however, differences of 5–10 °C have been reported for high concentration protein system [6].

The thin film used in the traditional FDM freezes differently than product in a vial and drying rates may be quite different than bulk products drying in vials or other commercial containers. Thus, current FDM may not always accurately estimate T_c for freeze-drying in commercial containers. Recently, a new technique based on optical coherence tomography (OCT) was developed to monitor changes in product structure in a vial to estimate T_c during typical freeze-drying. This technique can overcome the limitations associated with FDM, and also provides 3D imaging capability with better resolution to follow the product structure changes. For 5 % sucrose system, the onset of collapse was determined to be −28.9 °C using OCT based on the first-observed gaps in the dried cake, which is about 3 °C higher than measured value using FDM for the same formulation, but with a sucrose-to-BSA formulation, collapse was observed with FDM at about −27 °C, but with OCT no collapse was observed. Moreover, freeze-drying in vials in a laboratory dryer was performed more than 5 °C higher than the FDM collapse temperature with no observable collapse [23]. Therefore, the OCT measurement may provide a more reliable measure of collapse temperature in a commercial container and therefore allow a cycle with a higher shelf temperature to be used to shorten the primary drying stage.

Table 2 Summary of tests associated with a traditional OQ/PQ of a typical freeze-dryer

Test detail
Shelf cooling manual and automatic
Ultimate shelf cooling temperature
Shelf heating manual and automatic
Ultimate shelf temperature
Evacuation time
Ultimate vacuum
Vacuum set point control
Condenser ultimate temperature
Condenser cooling time
Condenser load test
Shelf set point control (various set points)
Shelf uniformity (various set points); with shelf surface thermocouples positioned throughout chamber
CIP coverage testing (generally using Riboflavin)
SIP verification (using biological indicators)

OQ operational qualification, *PQ* performance qualification, *CIP* cleaning in place, *SIP* sterilization in place

Freeze-Dryer Qualification (OQ/PQ)

Table 2 represents a classic testing list associated with operational qualification (OQ) and performance qualification (PQ) of a freeze-dryer, and this list excludes tests associated with installation qualification (IQ). Testing on lyophilizers in relation to heating and cooling has been traditionally performed under no-load conditions. An empty chamber environment is not representative of the reality of batch manufacturing, and executing such protocols under dry conditions (i.e., no sublimation) results in a limited impression of the performance characteristics of the freeze-dryer in question. It was recognized that standard protocols for qualifying freeze-dryers are inadequate, and that it was suggested that appropriate qualification protocols that were representative of actual batch manufacturing conditions needed to be employed [42].

Heat transfer and sublimation studies were performed to evaluate the following [42]:

- Vial heat transfer coefficient, K_v
- Minimum achievable/controllable chamber pressure
- Maximum condenser temperature at a given sublimation rate load
- Heat and mass transfer resistances of the freeze-dryer
- Emissivity of the chamber to evaluate impact of radiation on heat transfer

OQ and PQ testing does not provide enough information to evaluate the performance of the freeze-dryer in detail. Further work must be performed to enable a detailed understanding of performance characteristics and limitations, which are critical to cycle development and scale-up. Combining the understanding of performance design and limitations with the developed K_v model enables the development of a design space for primary drying. It is important to design the primary drying step such that the design limitations of the commercial freeze-dryer in question do not prevent execution of the cycle as designed.

Freezing Step

The overall objective of the freezing step is to separate the solvent from the solutes. The solvent in most cases is water, but may also be organic such as ethanol. Another objective of the freezing step is to crystallize any excipients that may be crystallized under conditions during freezing, for example, mannitol or glycine, in order to maximize the collapse temperature, optimize the primary drying step, and improve cake appearance. Although a certain amount of crystallization would occur under normal freezing conditions, the best way to achieve maximum crystallization would be to perform an annealing step, as discussed later.

Freezing is achieved by cooling the product from the loading temperature (after a certain hold time at the end of the loading stage) to a target freezing temperature below the T_g' with a predetermined shelf cooling rate. It was observed that the degree of supercooling can vary by as much as 10 °C throughout the batch, and thus freezing is sometimes referred to as stochastic freezing. The target freezing temperature would normally be ~5 °C below the T_g' and the vials are held at the target temperature for a number of hours. During freezing, as the heat transfer is a result of conduction at high pressure, perhaps with convection, heat transfer is generally good in a vial, and thus the product temperature and shelf temperature should trend closely.

In order to maximize the crystallization of the amorphous excipients, annealing is generally performed before initiation of primary drying. After freezing is complete, the shelf temperature is increased to a temperature above the T_g' and below the eutectic temperature. The vials are held at this annealing temperature for a few hours. This step has several advantages: First, it allows the ice to restructure to an optimum ice crystal size, which is known to increase the rate of primary drying and to decrease the variation in drying time within the batch and between laboratory and manufacturing batches [47]. Second, it enables crystallization of the bulking agent such as mannitol to a high degree. This crystallization event removes the excipient from the amorphous phase and typically results in a higher T_g'.

Ice Nucleation, Supercooling, and Annealing

Ice nucleation is a stochastic event and the temperature at which ice nucleates in a vial is usually different between the laboratory-scale and commercial-scale dryers. Further, the nucleation temperature is variable from vial to vial within the same batch. The uncontrolled nature of ice nucleation presents complications during the transfer of a lyophilization cycle between laboratory and manufacturing and potentially also between established manufacturing environments. Ice nucleation occurs well below the equilibrium freezing temperature under normal atmospheric pressure conditions. The degree of supercooling is the difference between the thermodynamic (equilibrium) freezing point and the temperature at which ice crystals actually first form in the sample.

Variations in the degree of supercooling reflects the random nature of nucleation and depends on product properties and conditions [32]. Convention wisdom states that a low particulate "class 100" environment leads to a higher degree of supercooling and conversely, a high particulate laboratory environment leads to a lower degree of supercooling. However, while plausible, direct experimental confirmation of this interpretation is limited. Higher degree of supercooling will result in the formation of smaller ice crystals, and smaller pores will be left during sublimation in the dry layer which increases the mass transfer resistance to the flow of water vapor. On the other hand, lower degree of supercooling will lead to lower resistance to the flow of water vapor through the dried layer. Primary drying time is longer for a product with higher cake resistance even using the same conditions of shelf temperature and chamber pressure. Therefore, primary drying time is longer for a cycle running on a commercial freeze-dryer than for a cycle at laboratory scale. This duration difference between laboratory and commercial scale makes it challenging to estimate the end point of primary drying in manufacturing based on typical laboratory data. The traditional approach is to add few hours, or perhaps ≈ 20 % of the laboratory primary drying time, to the duration of primary drying in the manufacturing process. However, this is not an ideal approach and may lead to product defects if the secondary drying is initiated before the end of primary drying. Alternately, using a much longer (arbitrary) duration of primary drying in manufacturing likely will waste time without any positive impact on product quality. Using a PAT tool such as a Pirani gauge is an ideal solution to establish the end point of primary drying [29], and this methodology can be used in dryers of nearly all sizes and designs.

Ice nucleation plays an important role in the secondary drying process, as residual moisture (RM) is a function of specific surface area (SSA) of the lyophilized cake [36]. Large ice crystals result in lower resistance to vapor flow during sublimation but also a lower SSA [48]. This, in turn, results in a reduced efficiency during secondary drying, as the rate of secondary drying is roughly proportional to the SSA [36]. Therefore, a larger SSA (smaller ice crystals) is optimum for an efficient secondary drying process. However, the impact of having smaller ice crystals is generally more severe on primary drying because it is the longest step in the cycle.

Therefore, it is normally more efficient to increase secondary drying temperature and/or time to compensate for the impact of smaller surface area on the desorption characteristics of the product post primary drying.

Nucleation Control: Principles and Methodologies

To deal with the stochastic nature of the onset of ice nucleation, a number of homogeneous nucleation methodologies have been examined [16, 27]. One consists of an ice-fog technique, which was found to result in rapid ice nucleation (< 1 min) due to the ice-fog-seeding nucleation within the container [27]. A second technique, which utilizes rapid depressurization within the chamber, has been shown to achieve instantaneous and homogeneous ice nucleation. The ice nucleation temperature chosen is often relatively high (e.g., − 5 °C), which means that larger ice crystals are formed which then reduces the primary drying time. Other methods such as vacuum-induced freezing [17] were also examined. Overall, controlling nucleation during freezing is of critical importance to improve freeze-drying conditions, resulting in a more scalable and reproducible freezing step. If implemented in the cycle design stage, this could provide more confidence in reproducibility of the process and control of product quality in general. In fact, one could argue that, given the availability of controlled ice nucleation at both laboratory and commercial scale [4, 49], it will soon be a part of current good manufacturing practice (cGMP) for freeze-drying to control ice nucleation just as it is cGMP to control primary drying by control of shelf temperature and chamber pressure.

Achieving homogeneous ice crystal size and morphology is very important from a QbD perspective. Without the solid foundation of the freezing step, no matter how well the primary drying step is modeled, there will always be a degree of variability in the performance of both primary and secondary drying, thereby resulting in batch uniformity issue and variation in product quality attributes, which may not be acceptable. Lastly, without the homogeneity of ice crystal formation, scalability will remain a challenge due to the different environments between the laboratory and manufacturing.

Primary Drying

Primary drying is modeled using a series of heat and mass transfer algorithms with the goal of establishing a design space within the freeze-dryer capacity for chamber pressure (P_{ch}) and shelf temperature (T_{sh}) that results in an acceptable product. These two parameters ultimately control the desired product temperature (T_p) during primary drying. Other factors for establishing a design space include the limitations of the freeze-dryer to control chamber pressure and shelf temperature at the desired set points. All these factors make up the design space for primary drying. The set of operating conditions preserving the product quality defines the design space [13].

Mass Transfer: Sublimation and Cake Resistance (R_p)

During the primary drying stage, ice is sublimed from the vial and the water vapor migrates from the chamber to the condenser and finally condenses on the surface of cold condenser coil or plates. Resistance to the mass transfer of water vapor has contributions from the dried product, stopper, and chamber. The resistance of the stopper and chamber are normally about a factor of 10 less than the resistance of dried product, and thus the cake resistance is the dominant factor for the mass transfer resistance [35]. The area-normalized resistance (independence of product area) of the dried product layer is defined as:

$$R_{PN} = A_{in} \frac{P_0 - P_c}{\left(\frac{dm}{dt}\right)}, \tag{4}$$

where the A_{in} is the internal cross-sectional area of the vial, P_0 is the vapor pressure of ice at the temperature of sublimation surface, P_c is the chamber pressure, and $\left(\frac{dm}{dt}\right)$ is the ice sublimation rate.

The normalized product resistance as a function of the dry layer thickness (ł) can be described by:

$$R_{pN} = R_0 + A_1 ł (1 + A_2 ł), \tag{5}$$

where the constants R_0, A_1, and A_2 are product resistance parameters of water vapor flow through the dry layer. When freeze-drying below the T_g' or eutectic temperature, these parameters are independent of product temperature. This resistance equation is applicable to various types of the resistance profiles, such as the type I (linear), II (linear with a large intercept), III (curve with a small curvature), and IV (curve with a large curvature) models [34].

In order to better understand the lyophilization process and facilitate the process development and scale-up, the resistance of the dried product should be measured experimentally for every formulation because it is heavily dependent on the product formulation and fill height. Several different approaches have been developed for this purpose, and they are briefly listed as follows (in no particular order):

1. The microbalance technique

The microbalance method is the first technology developed for measurement of the cake resistance. The primary experimental technique was based on freeze-drying a cylindrical sample under isothermal conditions, with the sample suspended from one arm of a vacuum microbalance [34]. This method monitors the weight loss of samples at various time intervals, and measures the sublimation rate. This technique has been successfully tested for numerous formulations including crystalline and amorphous systems, and the effects of the potential process variables including freezing rate, sample concentration, and product temperature have been systematically investigated. One of the issues associated with this methodology is that the

sample in the capillary may not freeze with the same degree of supercooling as in a vial.

2. Manometric Temperature Measurement (MTM)

In this technique, the isolation valve connecting the chamber and the condenser is closed very quickly and the chamber pressure is recorded for about 25 s before the valve is reopened. The MTM equation has been used to fit to the recorded pressure rise data as a function of time [51], and the values of dried layer resistance can be obtained as a function of dry layer thickness. It was shown that for 5% glycine, 5% mannitol, and 5% sucrose formulations, the R_p data obtained using MTM method were in good agreement with R_p data calculated with vial thermocouple method when a thermal shield was used in the experiment to remove the influence of a typical radiation. Note that the MTM obtained resistance is no longer accurate once the first row of vial completes primary drying [51, 52]. More details about the MTM application can be found in the PAT chapter.

3. Thermocouple

The product temperature profile alone can normally be used to obtain a useful estimate for the resistance, provided that the temperature can be accurately measured. The product temperature profiles (T_p) during primary drying were recorded and became the input data for the parameter estimation [35]. Based on the steady-state model and least-square minimization algorithm, the sublimation rate and the vapor pressure of ice at the sublimation surface can be calculated from the product temperature, thus the cake resistance can be obtained from Eq. 4. This method has been used for 5% mannitol, 3% lactose, and sucrose, and the results obtained using this modeling approach is similar to the values obtained using the MTM method [21].

4. TDLAS

TDLAS can also be applied to the measurement of R_p. TDLAS is designed for measuring the real-time sublimation rate, and the sublimation rate profile obtained can also be used for cake resistance calculation using the steady-state heat and mass transfer equations. In addition, cake resistance can be easily obtained by combining the TDLAS and the temperature probe profile. Using both the product temperature measured in the vial and the average sublimation rate of the entire batch, the resistance and effective pore radius of the dry layer has been estimated during primary drying [21]. This method does not require solution of the complex heat and mass transfer equations, and has been demonstrated with product runs with mannitol, sucrose, lactose, etc. The resistances obtained with three different approaches were compared, and the resistances obtained at the same dry layer thickness are reasonably close for each system [21].

In order to model the primary drying step accurately, it is essential to use a representative cake resistance measurement. The differences in freezing rate and the degree of supercooling play a significant role in determining cake resistance, and thus the cake resistance measured in the small scale dryer cannot be directly used for modeling in large scale production dryer. This value needs to be either mea-

sured directly in a large scale production dryer or corrected using a correction factor based upon measured or estimated differences in degree of supercooling. In addition, the potential impact of drying temperature on resistance needs to be considered. The effect of drying temperature on resistance was found to be quite dramatic for potassium chloride or a dobutamine hydrochloride–mannitol (weight ratio of 1.12:1) system, and a large increase in shelf temperature resulted in a transition to a type-IV curve with a significant reduction in resistance [34]. At higher temperature close to the eutectic temperature for these crystalline systems, hydrodynamic surface flow of adsorbed water may account for the observed behavior [34]. However, with systems exceed a T_g', changes in pore structure do occur as a result of what is sometimes termed "microcollapse," with the net result of reducing resistance. For example, mass transfer resistances of an antibody (rhuMAb HER2) formulated in trehalose as well as protein-free formulations containing trehalose and sucrose were investigated. Mass transfer resistance of all three formulations was found to decrease significantly with increases in shelf temperature for each system. For 4.5% sucrose formulation, about a sixfold decrease in the cake resistance was noticed at a dryer thickness of 1 cm when the shelf temperature increased from −30 to −20 °C [24]. The microscopic determination of cake structure supported the view that the decreased resistance at elevated temperature may be the result of small-scale product collapse. Thus, when planning to run near or above a T_g' or eutectic temperature, it would be prudent to evaluate the impact of product temperature on the resistance parameters, at least the impact of temperature on the parameter, A2, which was done previously [34].

Primary Drying Design Space Modeling: Designing Robustness Upfront

The goal of this section is to provide a scientific approach for building a design space for evaluating the impact of CPP (shelf temperature, chamber pressure, and primary drying time) on the quality attributes (RM content, reconstitution time, and product physicochemical stability) of the product. A robust and optimized cycle can be designed through the use of mathematical modeling, and a design space can be obtained in which the cycle can produce consistent product quality [42].

In terms of QbD, the emphasis for developing the primary drying step is on using heat and mass transfer simulations along with risk assessment. Mathematical modeling of primary drying has been previously presented [13, 19, 31, 42] and enabled development of a design space as a function of two primary control variables shelf temperature (T_{shelf}) and chamber pressure ($P_{chamber}$) with product temperature and primary drying time being the output.

Once K_v and cake resistance have been determined, the cycle performance can be modeled and optimized. Using the K_v ratio established, cycle performance can be predicted for both edge and center vials over a range of conditions, resulting in a design space similar to the one shown in Fig. 3. Such a plot allows the user to express the $T_{product}$ as a function of the independent variables, T_{shelf}, and $P_{chamber}$. This modeling allows the user to choose cycle conditions such that the primary dry-

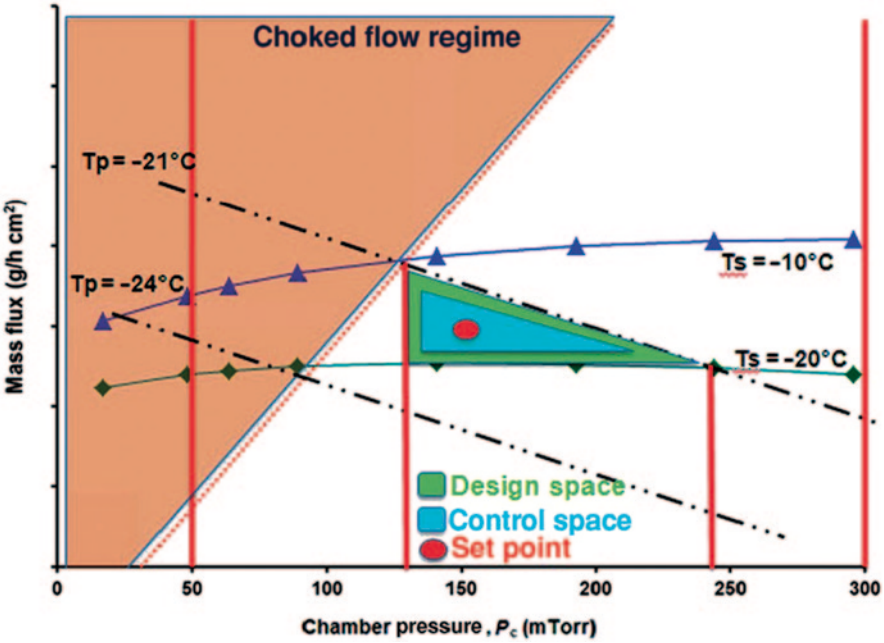

Fig. 3 Primary drying design space for a lyophilized product. Adapted from reference [26]

ing time is optimized, while operating safely below the critical temperature for the formulation and operating within the limits of the freeze-dryer itself. The modeling also provides robustness data around the cycle performance, which can be useful in determining critical parameters and investigating the potential effect of deviations from the specified parameters on product quality.

Figure 3 represents a design space for a lyophilization process [26]. The figure clearly shows the relationship between the primary drying conditions (shelf temperature and chamber pressure), freeze-dryer limitations (mass flux) with product temperature as an output. The green-highlighted area represents the design space. In general, a set of conditions in the center of the graph, which is lower than the critical temperature (collapse or melt), may be chosen as the preferred conditions for primary drying (set point), and the space outside these conditions but within the green-highlighted area represents the primary drying robustness ranges.

Primary Drying End-Point Determination

At the end of primary drying, the cake structure should be retained without any collapse, and it should not "melt back" even after exposure to ambient temperature [33]. This structural retention indicates that essentially all ice and much unfrozen water has been removed by sublimation and the cycle can be progressed into secondary drying to further remove the unfrozen water. Several PAT tools such as Pi-

rani Gauge, capacitance manometer, LyoTrack, manometric temperature measurement (MTM), and TDLAS have been used for the determination of end point of the primary drying stage, and readers can refer to the later PAT chapter for more details. Our view is that the use of the pressure difference between Pirani and differential capacitance manometer is perhaps the best methodology from a perspective of sensitivity, reliability, cost, and preservation of product quality.

Secondary Drying

Once all the ice is removed by sublimation during primary drying, product temperature can be increased to remove the unfrozen water in the product, which is known as desorption drying during the secondary drying stage. A high percentage of RM content in the final product can lower the glass transition temperature (T_g) of the amorphous product below the storage temperature which will lead to collapse and/or perhaps result in crystallization. A high percentage of RM may also have direct effect on chemical stability of the product; normally increased water content, at least above a certain level of ≈3 %, will give an increased rate of chemical degradation. For these reasons, the secondary drying is generally designed to remove most of the unfrozen water to achieve low RM, frequently < 1 % RM content in the final product.

Both the shelf temperature and the shelf temperature ramp rate can affect the finished product quality. The higher shelf temperature ramp rate has been shown to lead to cake shrinkage and therefore, as a general guideline, ramp rate in excess of 0.25 °C/min should be avoided, at least in the first few hours of the ramp from the primary drying temperature to the secondary drying temperature. An increase in shelf temperature leads to an increase in both product temperature and water vapor concentration in the chamber. That is, as product temperature increases sharply with a significant amount of water remaining in the product, there is usually a transient increase in the pressure reading of the Pirani gauge (normally calibrated with Nitrogen). Of course, the increase in product temperature also leads to more rapid drying and decreased RM content. For products that remain amorphous during lyophilization and storage, it is important to keep the shelf temperature during secondary drying below the T_g. Crystalline products carry no real risk of collapse during secondary drying and therefore, higher shelf temperature ramp rates and higher secondary drying shelf temperature may be used. The rate of decrease in moisture content is high during the initial few hours of secondary drying, and typical drying time in excess of 4–6 h is normally not necessary. If at the end of about 6 h the residual water content is still too high, one needs to increase the product temperature. Products having higher solid content (> 10 %) may need extra time for secondary drying. It is understood that the secondary drying desorption rate is not a function of chamber pressure in the range typically used for pharmaceutical systems [36].

For secondary drying, dryer limitations of heat or mass transfer are not a factor, shelf temperature and product temperature are almost the same, and chamber pres-

sure is not a significant variable. Thus, the design space is controlled by the shelf temperature and any constraint on product temperature imposed by any potential product stability issues (upper temperature limit). It is important to note that while the degradation rate will increase with an increase in product temperature, the residence time at a given temperature required to reduce the water content to the desired level will decrease as temperature increases. These effects tend to cancel and the net degradation required to obtain a target water content is relatively insensitive to the exact temperature used. Of course, the time required to reach the target water content decreases sharply as drying temperature increases, so it is common practice to carry out secondary drying at temperatures well above ambient, even as high as 50 °C or greater. In this regard, it should be noted that protein denaturation is never a problem in secondary drying as a relatively dry protein does not denature until temperatures well over 100 °C are reached.

Structural collapse is possible in secondary drying but is rarely a practical problem as the protocol will nearly always call for a hold at the shelf temperature used for primary drying for at least a few hours after all vials are devoid of ice. That is, the ramp begins only after the vapor composition in the drying chamber has decreased below 50 % water vapor, as measured by one of the PAT techniques. Alternately, one delays the ramp several hours past the time when, for whatever reason, the end of primary drying is expected. Moreover, for amorphous systems, a relatively slow shelf temperature ramp (≈ 0.1–0.2 °C/min) is employed, at least up to ambient temperature.

Finally, it must be emphasized that the rate of secondary drying is also sensitive to SSA of the solid, which is controlled in part by the ice nucleation temperature and the primary drying temperature relative to T_g'. That is, drying above T_g' will cause some micro-collapse which will reduce SSA. Therefore, both the freezing and primary drying process will also impact the rate of secondary drying. It would be prudent to optimize the secondary drying stage only after the freezing and primary drying steps have been developed optimally with a well-defined primary drying phase. RM content is the quality attribute that is used to define secondary drying performance.

Lyophilization Cycle Scale-Up

A lyophilization process is first developed to run with defined parameters of shelf temperature and chamber pressure at a laboratory scale freeze-dryer, which is then transferred to a pilot or production scale-dryer. Differences in heat and mass transfer characteristics between a laboratory- and manufacturing-scale freeze-dryer can lead to significant differences in the product temperature profile. One must remember that the objective is to reproduce the *product* temperature history in going from laboratory to manufacturing, not simply reproduce the shelf temperature–time profile.

Utilization of the experimentally determined vial heat transfer coefficient, for the dryer of interest, in calculations that attempt to model drying behavior in man-

ufacturing is the preferred procedure. Likewise, this "scale-up" factor to convert laboratory-measured K_v data to production K_v data can be applied when transferring from one commercial dryer to another to simplify technology transfer. Techniques such as MTM are also useful in primary drying design along with other temperature and vapor pressure monitoring techniques detailed in the PAT chapter. These PAT tools are critical in estimating the end point of primary drying and also important in monitoring the sublimation process to better understand and control the freeze-dryer process.

Other factors can also affect the heat and mass transfer, and thus have an impact on process scalability. These factors are briefly discussed below:

Lyophilizer Design Various design elements of the lyophilizer may affect different modes of heat transfer, e.g., shelf thickness (conduction), shelf-to-shelf and shelf-to-wall inter-distance (radiation), vapor tube dimensions, chamber door, etc. When scaling up or transferring lyophilization cycles, these differences in lyo design must be considered as they may impact the overall performance and product attributes. Heat transfer due to radiation is an important heat transfer mode during lyophilization [39]. Presence of a stainless steel door reduces the atypical heat transfer as observed on a laboratory-scale freeze-dryer with a Plexiglas door. There is a significant reduction in the magnitude of heat transfer via radiation in a commercial dryer relative to the usual laboratory dryer. Highly polished stainless steel as used in commercial dryers has an emissivity of less than 0.3 while the stainless steel used in laboratory dryers typically have wall emissivities of about 0.7, and emissivity of the plastic door of about 0.95. Thus, the primary drying time in manufacturing may increase significantly when compared to a laboratory-scale dryer due simply to the differences in wall and door material, particularly for cycles that employ low chamber pressure and low shelf temperature, as is common for sucrose-based formulations.

Shelf Temperature Homogeneity Presence of hot and cold spots on the surface of shelf will lead to variation in the product temperature in the vials. Normally, shelf temperature homogeneity is determined using surface thermocouples on a clean and empty dryer during OQ. However, running an empty freeze-dryer does not give representative information since there is no load on the heating and cooling system. Compared to running the dryer clean and empty during PQ, the presence of load will escalate any temperature variability between the locations on the shelf. Studies [42] suggest that, at least for the dryers compared, these differences are small. However, the differences are likely to be highly dryer dependent, and the data available are meager.

Ramp Rates in Dryer Compared with the laboratory scale-dryer, commercial freeze-dryers have limited capability to achieve high ramp rates. For example, in systems where freezing rates in excess of 2 °C/min are desired to impede crystallization, a laboratory-scale process cannot translate equivalently in a commercial-scale dryer where maximum ramp rates of only 0.5 °C/min can be achieved.

Dryer Load Compared with a full load, demand for heat would be significantly less for a partially loaded dryer and consequently shelf surface temperature will be higher [30]. This would lead to a shorter primary drying time for a partial loaded dryer. The importance of this effect will be high for systems where high sublimation rates are employed, for example, mannitol-based formulations. However, these effects do not become important until the load is less than ≈ 50 % of full load, assuming the vials are concentrated on a fraction of the total number of shelves to maintain the same hexagonal packing of vials in a given array that is characteristic of a full load. Some other complications can arise because of dryer limitations in heat and mass transfer. High flow rates at low chamber pressure may result in "choked flow" and loss of pressure control [28]. Condenser and/or refrigeration system limitations may also result in loss of pressure control. For a partial loaded dryer, the dryer may be capable of handling the mass transfer and heat transfer demands, but when going to full load, one may experience loss of pressure control.

Solid-State Characterization

Upon obtaining the dried product from a laboratory or large-scale lyophilizer, it will be essential to characterize the dry product to document the important product properties and predict product quality attributes such as stability, and also to gain insights about the freeze-drying process. These characterizations are often overlooked, or misused, even though these solid-state characterization tools can give very useful information about the lyophilization process and product stability.

DSC is generally used to measure the glass transition temperature of a dry protein formulation. All dried pharmaceutical products should be stored below the glass transition temperature, which will help to ensure product elegance and better stability leading to longer shelf life. In addition, the accelerated stability studies need to be performed below T_g in order to remain in the glassy phase and obtain representative behavior. Comparisons of stability in a series of formulations in a test where some formulations are above the T_g and some below is relatively meaningless and likely to be subject to serious misinterpretation. MDSC is most commonly employed because it facilitates the measurement of the glass transition, and normally clearly separates the heat capacity change from the relaxation endotherm and other confounding irreversible thermal events. This technique is extremely useful in formulation screening and development, and also can be used to monitor the manufacturing batch-to-batch consistency.

A Brunauer, Emmett, and Teller (BET) surface area analyzer can give the surface area of the dry powder, which is directly related to the dry layer resistance as well as serving as a surrogate for the ice nucleation temperature, provided drying was carried out below the collapse temperature so the pore size did not change post freezing. That is, at least with an amorphous system, the freeze-dried glassy solid is a template for the ice crystals that formed during freezing but were removed during drying. The BET adsorption method is based on the physical adsorption of an inert

gas onto the surface of a solid, and the BET equation assumes a simple physical adsorption mechanism. The BET experiment is generally performed using inert gases such as nitrogen, argon or krypton, and the experimental temperatures are kept low enough (liquid nitrogen at 77 K) in order to form a sub-monolayer of gas on the sample surface at moderate pressures. The SSA of various freeze-dried 5% mannitol were studied [41]. The SSA of the dried product was found to correlate well with the degree of supercooling and also with the cake resistance of the product, with a higher degree of supercooling resulting in higher SSA and larger resistance. Using this correlation and SSA measurements from both small-scale and large-scale products, one could obtain the cake resistance data from large manufacturing-scale dryer, and further predict scale-up drying differences or modify the freeze-drying process to bring equivalent product temperature profile during scale-up [40].

The formation of crystals in the lyophilized product can be monitored using several techniques, but the "gold standard" is powder X-ray diffractometry (XRD). XRD is an analytical technique used for phase identification of a crystalline material, and it can also provide information on degree of crystallinity in the lyophilized product. Dried sample is loaded into a sample holder, and the scans are generally conducted in the 2θ range between 5° and 60° angle. The identification of the polymorph can be carried out by comparing the diffraction pattern of the sample with reference standard data. Mannitol or glycine is often used as a bulking agent in a protein formulation to obtain a good cake appearance and a robust formulation that resists changes in cake structure during drying, and crystallization of these bulking agents has a large impact on the product appearance and stability. The degree of crystallinity can be estimated by integrating the relative intensities of the crystalline peaks and amorphous halos, and this quantitative analysis can be used to ensure the batch-to-batch consistency and investigate process deviation.

Case Study to Obtain Design Space

Modeling of Primary Drying for a Robust Design Space

A case study is presented to illustrate the use of heat and mass transfer theory modeling in construction of a design space. Primary drying behavior was calculated for a protein product containing sucrose and a bulking agent, filled to 5.3 mL in a 20 cc tubing vial. The protein-active ingredient was of 78 kDa in size, and the collapse temperature was clearly observed at −30 °C with FDM. K_v was measured for the 20 cc vial in a Virtis 35EL laboratory freeze-dryer as per the procedure introduced earlier and is represented by the following equation:

$$K_v = 0.0002 + \frac{0.0025 P_c}{1 + 3.644 P_c}, \tag{6}$$

where P_c is the chamber pressure with the unit of Torr.

Once K_v was measured, cake resistance was measured experimentally. A conservative cycle was performed with a target product temperature of −32 °C, which was below the collapse temperature. Thermocouples were used to obtain the product temperature, which is in turn used to obtain the cake resistance. R_p is represented by the following equation:

$$R_p = 1.15 + \frac{8.58H}{1 + 0.319H}, \tag{7}$$

where H represents the product dry layer.

Once K_v and R_p were measured, a primary drying calculator was then used to predict the primary drying conditions. Figure 4 shows the calculated product temperature profiles during the primary drying stage when different shelf temperatures were employed at 100 mTorr chamber pressure.

The duration of the primary drying step was also estimated based on the time to completely remove the ice. When executing the recommended primary drying conditions, it is very important to verify the end point of primary drying using conventional methods such as a Pirani gauge. In this case, the Pirani gauge was used to verify the end point and this may be examined in Fig. 5. As observed from the predictions in Fig. 4, the −25 °C shelf temperature was expected to result in a product temperature below −33 °C with primary drying duration of approximately 45 h for the primary drying. The −20 °C shelf temperature set point was estimated to have a predicted product temperature of −30 °C and the primary drying length can decrease significantly to about 32 h. At a higher shelf temperature of −18 °C, the

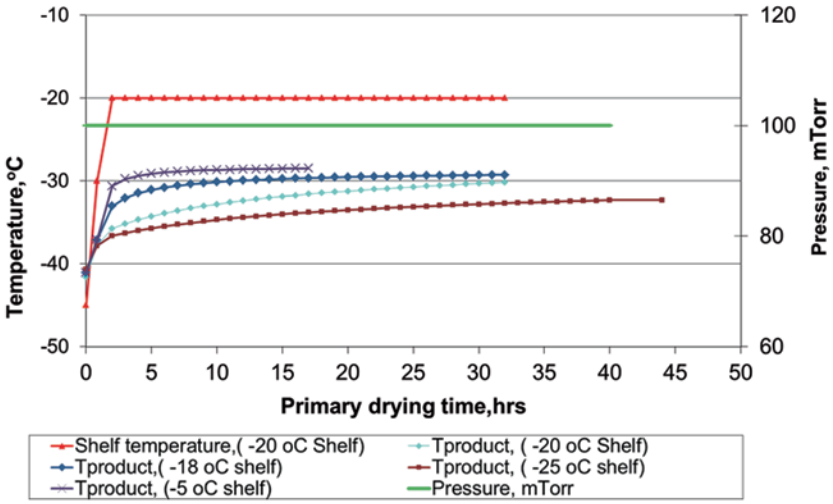

Fig. 4 Prediction of product temperature profiles for various shelf temperatures at a chamber pressure of 100 mTorr

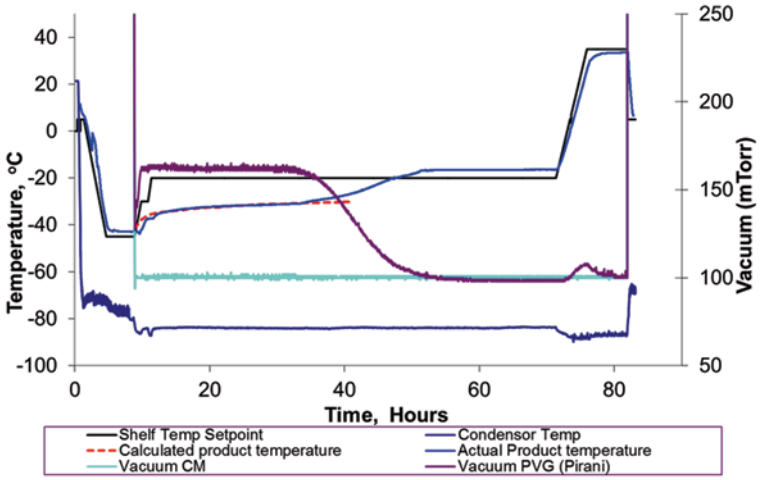

Fig. 5 A lyophilization cycle developed for a model product showing the agreement between the experimental data and the calculated product temperature based on the heat and mass transfer model. Capacitance Manometer (CM), Pirani Vapor Gauge (PVG)

product temperature was estimated to go beyond the collapse temperature (−30 °C), which has the potential to result in poor cake appearance and poor product quality. Therefore, at typical 100 mTorr chamber pressure, the acceptable range of primary drying shelf temperature will be from −25 °C to −20 °C based on this modeling.

Figure 5 gives the comparison of calculated product temperature and the actual thermocouple data during primary drying stage. Good agreement was found between the product temperature measured by thermocouple (blue line) and the predicted product temperature profile (red dashed line). This comparison shows the validity of this K_v and R_p model, which suggests that the developed model allows an accurate prediction of a design space for primary drying at these conditions, in terms of shelf temperature and a set chamber pressure. In addition, the design space was further developed by considering a chamber pressure range along with the shelf temperature to better understand the overall effect on the product temperature profile, relative to collapse. A low limit of 50 mTorr was used as the minimum controllable chamber pressure based on the manufacturing lyo capability, and 200 mTorr was selected as the top pressure limit. A design space considering both shelf temperature and chamber pressure, along with the limitations of the system itself was found to be similar to the one shown in Fig. 3.

Further design of the secondary drying step was conducted, where the shelf temperature was selected with due recognition of the dry state T_g, as measured at the end of primary drying using MDSC. The time was determined by experimental study of the rate of desorption, or residual water content, as a function of time during the secondary drying step. Using a "sample thief," vials were removed for RM analysis by Karl Fisher titration at the end of primary drying, in the middle of the ramp to the secondary drying set point, and after 2, 4, and 6 h of secondary drying

at a shelf temperature of 35 °C. Final moisture of approximately 0.5 % was obtained in the drug product.

Figure 5 represents the final cycle developed for this product. The length of primary drying stage was intentionally increased by approximately 25 % to accommodate for any potential scale-up differences. The impact of variation in K_v due to both position effects and nonuniformity among vials and the impact of variation in R_p due to ice nucleation variation were also considered using the modeling approach, and an extra safety factor in primary drying length was included to ensure the completion of the primary drying step for the worst case scenario. The cycle was further verified experimentally, and the resulting product showed acceptable product quality attributes along with a good product temperature profile, suggesting the suitability of the lyo process. To complete the development and scale-up of this lyophilization cycle, the model was adjusted with the K_v data obtained from the commercial scale lyo scale-up. The operating ranges were then adjusted to maintain the original product temperature profile as determined in the laboratory. Therefore, comparability in the product temperature profile was targeted as opposed to comparability in the shelf temperature and pressure parameters as in the traditional approach.

Expand the Design Space Using a Scale-Down Model

The above modeling approach, which is based on the results of steady-state heat and mass transfer theory, is very useful for cycle development. It allows the prediction of product temperature at different drying conditions, and has been widely used for primary drying robustness studies and design space construction. The process design space as illustrated above is based on process limitations imposed by the product and equipment capability. However, this modeling approach also has some limitations. First, the product temperature limitation for an amorphous system is mainly based on the collapse temperature (T_c) measured by FDM, and the obtained maximum product temperature may not be the "failure point." It has been reported that collapse may not be detrimental to the in-process or long-term stability of freeze-dried proteins [46, 53], and it is possible to perform primary drying at temperatures above the T_c for some formulations such as high concentration protein formulations or formulations containing both crystalline and amorphous components [6]. Systems such as these may be dried at much higher temperature, well above the T_c, without macroscopic product collapse or loss of any perceptible measure of product quality. Secondly, it is generally assumed that the cake resistance measured at a low product temperature can be used to predict primary drying performance at a higher temperature. This assumption could generate large error in sublimation rate calculations for a system where cake resistance is highly dependent on the drying temperature. As discussed earlier, when the shelf temperature was increased from −30 ºC to −20 ºC, the product temperature in a 4.5 % sucrose-based formulation increased above T_c, which resulted in a sixfold decrease in cake resistance [24].

However, obtaining product resistance data at several temperatures in a relevant range is straightforward, but does require at least one additional experiment. One does indeed need to measure the corresponding cake resistance at different drying temperatures when drying close to or exceeding the T_g' in order to get more accurate result using this modeling approach.

The ICH Q8, Q9, and Q10 guidelines stress a continuous process verification strategy, spanning from the process design stage at a development laboratory through commercial production. The guidance aligns well with the QbD concept, and reinforces the importance of performing more upfront development work to have a deep understanding of the relationship between process parameters and product quality. In order to establish the process robustness ranges to support the validation process and potentially expand the design space for this protein product, systematic experimental studies have been performed based on a scale-down model approach. The systematic study employed typical QbD elements including identification of potential CQA and potential CPP, risk assessment based on prior knowledge, establishment of a scale-down model, experimental studies to identify the failure point, etc. Finally, a broader design space is generated based on the process and product understanding. More details are discussed below.

Establishment of a Scale-Down Model

Since performing a useful number of studies at the manufacturing scale is not practical, development of a scale-down model is needed to support process validation and commercial manufacturing activities. In addition, its establishment will enable a better understanding if a change in material (e.g., drug substance source, container, etc.) or lyo process will have any product impact. The objective is to create a laboratory-scale system that is comparable to its large-scale counterpart. The established scale-down model can then be used to achieve reliable process understanding about the relationship between process parameters and final performance [44]. This concept has been widely used in the cell culture and purification field, and has been found to be extremely useful for process characterization and production support [43, 44].

A scale-down model needs to be first developed by taking into account of scalable factors. Similar geometries would be important in that the overall design geometries of each lyo (small vs. large) are close enough to result in insignificant performance differences. For small and large lyophilizers, it is ideal to have the same ratio of shelf area/condenser area, and it is perhaps important that the ratio of shelf area to duct area divided by duct length be the same in both dryers. In addition, one need to consider the dryer-dependent factors such as the position of hot and cold spots on the shelf, duct position/configuration, loading effect, etc. As part of this study, a qualified Virtis laboratory scale Lyophilizer was used. Water sublimation tests were performed, and the sublimation rate normalized by shelf area was found to be comparable between both scale lyophilizers at same drying condition.

A scale-down model should be qualified to demonstrate equivalence between manufacturing process and laboratory- or pilot-scale processes in order to support process validation and commercial manufacturing process deviations. The general approach to scale-down model qualification is to run all process parameters at the center of the operating range of the manufacturing process [44]. However, in terms of lyophilization, it is important to compare the product temperature profiles and adjust the conditions of the laboratory-scale cycle to match the product temperature history characteristic of the commercial scale cycle, if necessary. It is recommended to use representative raw materials or process feed streams, preferably from full-scale manufacturing. Also, analytical methods for assessing process performance should be identical between different scales to minimize the potential differences. A well-defined acceptance criterion needs to be established prior to the scale-down runs performed in the laboratory, which can be based on historical large-scale data from engineering runs, process validation, or previous manufacturing runs. The output parameters and process control sensitivity at both scales should be compared. A minimum number of runs are needed at both scales in order to establish the confidence for the comparability [43].

Based on this guidance, the laboratory lyophilizer has been deemed as a suitable scale-down model for the large-scale manufacturing lyophilizer. Based on the prior knowledge for the product, the most important product CQAs for this product were selected from the release assays list. These CQAs include cake appearance, moisture, reconstitution time, aggregation, activity, concentration, purity, oxidation, etc. In addition, characterization assays such as glass transition temperature, protein secondary and tertiary structure, subvisible and visible particle counting, and SSA were performed on the product to evaluate the lyophilization process impact. The product temperature profile must be assessed and compared at both scales. A risk assessment was also performed for the lyophilization process, and risk factor number is calculated for each process parameter as the product of frequency, severity, and detectability. Through this exercise, several lyophilization process parameters including primary drying temperature, secondary drying temperature, and chamber pressure and freezing rate were identified as “potential” CPPs.

Experimental Studies for Process Characterization

The primary drying temperature was first investigated in order to establish the failure point imposed by the product. Based on the modeling approach, a shelf temperature robustness range was determined (between –25 and –20 °C) as shown in Fig. 4, and a maximum shelf temperature of – 20 °C was obtained. In order to understand the quality attributes of the product for more aggressive condition, shelf temperature of – 15 °C was examined. An acceptable cake structure was observed from the resulting vials, and further analytical tests showed that product quality attributes were well within the acceptable range and aligned with the historical trend. Thus, it is possible to expand the design space for this product. Several shelf temperatures higher than – 15 °C were also tested. At a shelf temperature of – 5 °C, micro-

collapse was suggested based on a drop in the thermocouple product temperature measurement data. However, the resulting product was still acceptable in terms of cake appearance and product quality. However, a further increase in the shelf temperature to 0 °C results in significant increase in product degradation, which is considered as the failure point for the product. The acceptable cake appearance from these cycles is mainly due to the presence of crystalline material in the cake, which was confirmed by the XRD data. Therefore, the incorporation of a crystalline bulking agent into a protein formulation can significantly increase the primary drying temperature without compromising the product quality. The final design space for primary drying was obtained as the combinations of conditions identified from theoretical modeling and the expanded conditions from experimental studies. This much wider design space (shelf temperature ranges from −25 to −5 °C) can build robustness into the process, facilitate a smooth scale-up, and provide a scientific basis for dealing with process deviations which are still within the design space.

The secondary drying temperature impact was also investigated. Temperature ranges from 30 to 40 °C were studied in terms of moisture level and other CQAs. A 30 °C cycle gave a slightly higher moisture level, while 40 °C secondary drying condition produced acceptable product quality even though there is a slight increase in protein degradation. Thus, the secondary drying is robust between temperature of 30 and 40 °C.

The shelf temperature cooling rate during freezing was also examined as it could impact the crystallization behavior of ice and bulking agent and thus affect the final product quality. Different rates between −0.1 and −0.5 °C/min were examined with other lyo parameters fixed. The cake appearance was not impacted; however, a larger increase in protein degradation was observed upon lyophilization with the fast freezing rate. Large number of smaller ice crystals can be formed at a fast freezing rate, which could cause a larger protein–ice interface and result in a higher degree of protein degradation. In addition to this, freezing rates above −0.5 °C may not be achievable at large scale largely due to limitations of the production dryer. In order to make any recommendation of freezing ramp rates, it is very important to use experimental data with nucleation temperature and/or SSA measurements, rather than the lyophilizer limitations alone.

Finally, robustness studies with the combinations of Lyo process temperature and chamber pressure were performed to assess product quality. Aggressive runs above the collapse temperature were used to generate the stability data and cycles with both primary and secondary drying temperature set points ±5 °C and pressures ranging from 50 to 200 mTorr were used to run beyond normal manufacturing conditions. The working cycle with manufacturing set points were used as a control, and all samples from these cycles were tested for long-term stability. It was found that the product quality attributes from these runs were well within the acceptable ranges upon storage, suggesting the process robustness. These stability data along with theoretical modeling are extremely useful to support cycle deviations from manufacturing lyophilizer.

These studies can form the basis for a thorough process and product understanding and support process validation. However, scale-down experiments may not

capture all interactions and also environment factors such as clean room and an isolator usage at large scale. As discussed earlier, ice nucleation may lead to the major differences between the laboratory- and large-scale dryer, and play a significant role in differences in product temperature during primary drying. In order to minimize gaps between the scale-down model and large-scale process performance, extra equipment and process characterization studies in the small scale and large scale may be necessary. In the laboratory-scale studies, all product vials were washed and depyrogenated, and the solution was filtered and filled in a laminar flow hood to minimize the particle introduction. In this case, it was found that the degree of supercooling was comparable to that in the large scale (about 12 °C), and thus the potential impact of nucleation is relatively small in this case. In addition, continuous process monitoring from commercial batches can build more process knowledge at large scale. By combining the scale-down studies, large-scale studies, comparability evaluation, and stability monitoring, a robust process validation at manufacturing scale is expected. This QbD approach can be used to accelerate the scale-up process and facilitate a smooth technology transfer.

Summary

Rational development of a freeze-drying cycle requires a deep understanding of the heat and mass transfer process. The vial configuration is most commonly used in the lyophilization field, and the most important parameter associated with the vial is the K_v value as a function of pressure. This K_v value will enable measurement of R_p and with these data, will allow the impact of the freeze-dryer design on the product temperature history during primary drying to be quickly and accurately calculated. Understanding the variability in K_v from vial to vial as a function of location is very important. Edge vials, especially at the door of the freeze-dryer, will complete primary drying at a faster rate because they normally display higher product temperatures as a result of the increase in K_v due to high radiation heat transfer during primary drying. This product temperature must be carefully monitored so that it will not result in product quality issues during development and scale-up. Ice nucleation also plays an important role in freeze-drying cycle performance. Due to the random nature of ice nucleation, control of this event is very important if one is to control and standardize the freezing step and minimize vial-to-vial variability. In general, larger ice crystals result in lower cake resistance and shorter primary drying times.

Scaling-up of a freeze-drying cycle is challenging due to the many differences between the laboratory- and manufacturing-scale lyo. Various design elements of the lyophilizer may affect heat transfer, e.g., shelf thickness, shelf-to-shelf and shelf-to-wall inter-distance, vapor tube dimensions, chamber door, etc. Different tools should be used to understand the performance of each freeze-dryer. IQ/OQ can provide very useful information about the equipment capability, especially under the load conditions. Extra studies to measure the K_v value are critical to characterize each dryer, and the vial-to-vial variability needs to be addressed. Dryer loading also

plays an important role in the primary drying time due in large part to the difference in the fraction of edge vials as load changes. The vapor tube dimension is also an important factor to be considered in terms of the potential for choked flow leading to loss of pressure control at high sublimation rate.

QbD application in lyophilization development is becoming popular due to the potential flexibility in regulatory filing. A key feature of this approach is that it combines prior knowledge, a formal risk assessment process, and well-designed experiments. Prior knowledge can come from published literature, preliminary experiments, and past experience. The well-established theory in lyophilization makes cycle development much more efficient. Mathematical modeling of the primary drying step is extremely critical in the QbD paradigm. Blind usage of factorial DoEs in freeze-drying development should be avoided, as long as the modeling produces accurate prediction of product temperature profiles as a function of cycle conditions, a condition normally met and easily proven with a few key experiments. Establishment of a design space for primary drying is straightforward when applying this modeling strategy, where simulations of various shelf temperature and chamber pressure conditions may be conducted using a simple excel-based computer program. As demonstrated in the case study, some experiments are needed to evaluate K_v and R_p and to verify accuracy of the simulation results. For cases where the model gives a narrow range based on the collapse temperature, a scale-down model approach may be needed to expand the primary drying design space, and further establish the robustness of other process parameters such as secondary drying, freezing rate, etc. In addition, the combination of mathematical simulation and this scale-down model can be used to facilitate the continuous lyo process validation, and also support investigations to understand if a change in material (e.g., drug substance source, container, etc.) or lyo-process will have any product impact.

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Advances in Process Analytical Technology in Freeze-Drying

Bingquan (Stuart) Wang and Timothy R. McCoy

Introduction

Rational design and utilization of freeze-drying processes are essential to minimize their impact on drug product quality and assure consistent clinical performance. Development of the formulation and lyophilization process should focus on the product quality attributes. The International Conference on Harmonization (ICH) guidance Q8 (R2) suggests that the product quality cannot be tested in a product by using limited off-line measurements, instead it should be built in by design of the formulation and manufacturing process. This guidance lays the foundation for a quality-by-design (QbD) paradigm, which stresses the scientific understanding of formulation and processing factors on product quality and also the ability to assure product consistency [25, 26]. Process analytical technology (PAT) is a vital part of the implementation of QbD. Based on the in-line real-time measurement of critical process parameters (CPPs), the manufacturing process can be monitored and further controlled with appropriate feedback mechanisms. PAT can also facilitate the trending of the process operations to support continuous improvement efforts. Even though multiple off-line analytical techniques, such as chromatography and

B. Q. Wang (✉)
Late Stage Process Development, Genzyme, A Sanofi Company, 45 New York Ave, Framingham, MA 01701, USA

Protein Formulation Development, Biogen Idec, 15 Cambridge Center, Cambridge, MA 02142, USA
e-mail: Stuart.wang@biogenidec.com

T. R. McCoy
Technical Development, Genzyme Ireland Ltd. IDA Industrial Park, Old Kilmeaden Road, Waterford, Ireland
e-mail: timothy.mccoy@genzyme.com

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spectroscopy, can give useful information about the quality of the final product, these technologies do not directly provide real-time process information. Here, we mainly focus on the online monitoring devices used in the freeze-drying process.

QbD design of a robust freeze-drying process relies on the thorough understanding of the impact of the formulation and process parameters on product quality. The critical formulation factors include the properties and amount of the drug and each excipient used and their interactions [31]. The CPPs include, but are not limited to drying shelf temperature, chamber pressure, freezing/heating ramp rate, etc. Process optimization depends on understanding each freeze-drying stage, monitoring CPPs and controlling them whenever necessary.

Due to the extreme conditions used in the freeze-drying process (low temperature, high vacuum, and high temperature for sterilization), only limited PAT devices are compatible with the harsh environment. During the early development history in freeze-drying, there was not much understanding about the required technology and visual inspection was mainly used to guide process development. The Pirani gauge was later employed to monitor or control the pressure during the drying process, and it is still being explored for regular use in good manufacturing practice (GMP) environment. Also the thermocouple was implemented to monitor product temperature, which has greatly accelerated the development of lyophilization field. Lately, more advanced tools such as manometric temperature measurement (MTM), tunable diode laser adsorption spectroscopy (TDLAS), and near-infrared (NIR) are being developed to monitor the product temperature, moisture level, and sublimation rate during lyophilization. These new technologies have a great potential in monitoring and controlling the freeze-drying process.

Based on their design applications, PAT tools for lyophilization can be categorized to the following four types:

1. Product temperature monitoring

The product needs to be fully frozen and should be dried below a critical temperature (collapse temperature for amorphous system or melt temperature for crystalline system) to ensure an elegant cake appearance. In addition, the product quality is heavily influenced by the thermal history which the product has experienced during the sample preparation process [35]. Therefore, it is critical to monitor and control the product temperature during the freeze-drying process. Both wired product temperature probes such as thermocouples and resistance temperature detectors (RTDs), and wireless probes such as the temperature remote interrogation system (TEMPRIS) have been developed for this purpose.

2. Primary drying endpoint

The endpoint of primary drying is the point at which sublimation rate is low enough to suggest that primary drying is almost complete. At the end of primary drying, the cake structure should be retained without any collapse, and it should not melt back even after exposure to ambient temperature [23]. This structural retention indicates that most frozen water has been removed by sublimation and the cycle can be progressed into secondary drying to further remove the “bound” water. Pirani gauge,

MTM, TDLAS, and plasma emission spectroscopy (Lyotrack) can be used to detect the primary drying point.

3. Moisture content

Since the freeze-drying process is essentially a water removal process, it is of great importance to constantly monitor residual moisture. NIR-based technology has been developed for this purpose, and can be used to provide a complete inspection of all the vials from the same batch. In addition, TDLAS has been developed to monitor the sublimation rate, which can also be used to track the amount of water removed and monitor the moisture content.

4. Changes in molecular structure

Recently, spectroscopic methods such as Raman and NIR were developed for monitoring of water–ice phase transition and mannitol polymorphism transition. In addition, protein conformational changes and the protein–excipient interactions during the freeze-drying process can also be studied. These techniques can give more insights into protein conformational stability and the lyoprotectant–protein hydrogen bonding interaction in real-time during the dehydration process.

This chapter intends to provide a comprehensive review of latest PAT tools for freeze-drying, with emphasis on suitability for a large-scale manufacturing process where PAT implementation and sterility concerns become critical. First, each of the latest process monitoring devices is reviewed in terms of the major applications and limitations. Second, all these techniques are summarized based on their capabilities, practical advantages, and scalability to a large-scale freeze dryer. Finally, the current most commonly used PAT tools are discussed, and future implementation of promising PAT tools is presented.

Process Analytical Technology

Product Temperature Probe

It is well understood that product temperature is the critical parameter of interest for an effective freeze-drying process [15]. In general, during the primary drying stage, the product temperature should be maintained below the maximal allowable critical temperature [19]. Frequently, thermocouples are used to monitor this parameter during the freeze-drying cycle at the laboratory or pilot scale. At a large scale, thermocouples or RTDs may also be used, the latter of which is preferred due to its mechanical robustness and effective sterilization. Lastly, thermocouples are used to determine the endpoint of primary drying [18–20].

During the primary drying stage, there is a difference between the shelf and product temperature, which is due to the heat absorbed by the sublimation process. The endpoint is the point at which the product temperature equilibrates with the shelf temperature. In the example shown in Fig. 1, the product temperature remains quite

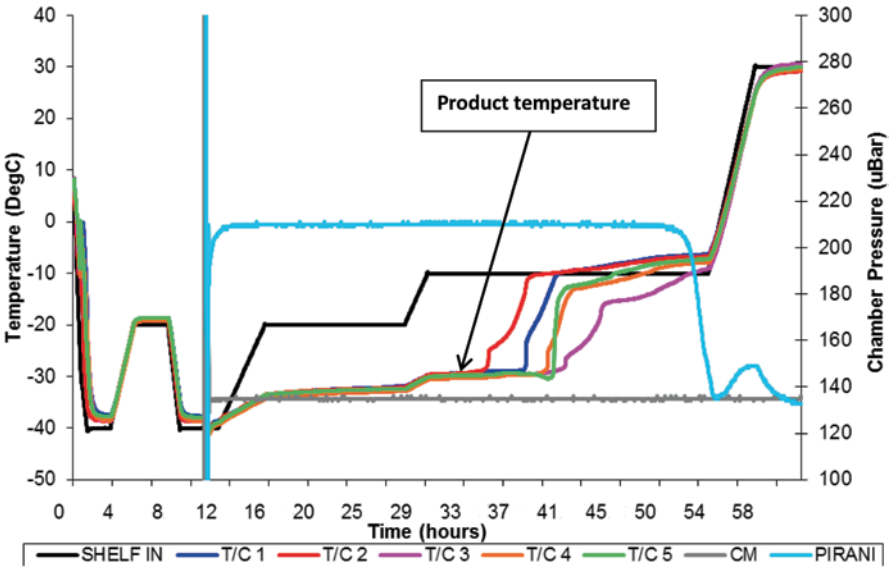

Fig. 1 Typical freeze-drying cycle showing thermocouple trending and endpoint during the primary drying stage of the cycle

low around −30 °C during the early stage of primary drying, and then it quickly merges with the shelf set point at about 40–52 h, depending on the product location. It can be concluded that primary drying has completed at this point as sublimation process has ended.

Even though thermocouples are a useful tool to determine the primary drying endpoint, caution must be taken in the validity of the data. It is well described in the literature that product vials with thermocouples placed within are not representative of the rest of the batch [20]. This is mainly due to the invasive nature of thermocouple placement, as they are inserted directly within the product in order to obtain best results.

The main challenges associated with using a thermocouple to monitor product temperature are:

1. Placement within the product vial
2. Impact on ice nucleation and cake resistance

The ideal placement of the thermocouple should be center-bottom of the product touching the bottom of the vial [28]. Thermocouples placed too high will lead to an early indication of the completion of primary drying step, as the thermocouple tip will lose contact with ice at an earlier stage, indicating that all ice has been removed, even though there would still be ice remaining in the vial. In order to compensate for this, the use of a Pirani gauge to determine endpoint would be an option [20] or, as it has been suggested, a conservative approach would be to add between 10 and 30 % of time to the primary drying step [31].

The most important impact of thermocouple presence is on ice nucleation and in turn cake resistance. Simply stated, invasive product temperature measurement techniques impact the freezing of the product. This is the main reason for the vial with the thermocouple not to be considered as being representative with the rest of the batch [9, 20]. The presence of thermocouples in the product interferes with the ice nucleation process in that the thermocouple itself acts as a nucleation site. The nucleation temperatures of the product in the thermocouple containing vials are often much warmer than that of the rest of the batch, resulting in a lower degree of supercooling, larger ice crystals and, in turn, a lower resistance to vapor flow during primary drying as compared to its non-thermocouple containing neighbor.

Lastly, other challenges include placement of thermocouples during scale-up in a commercial freeze dryer, which poses issues of mechanical and sterility nature. Thermocouples are difficult and impractical to use in a manufacturing environment as they are often incompatible with stoppering and auto-loading systems. Wireless technology may be an option to deal with this challenge.

Wireless Product Temperature Probe

In order to address the pitfalls of the thermocouple use at the commercial scale, the TEMPRIS was developed. The TEMPRIS (IQ Mobil Solutions GmbH) consists of eight sensors, an interrogation unit, and a computer system with CarLog software [28].

TEMPRIS is an invasive technique, where the probe is placed within the product to monitor product temperature, similar to a traditional thermocouple. The major concern associated with this system is the size of the probes themselves, as it has been suggested that the probes should be smaller in size [19]. A maximum product fill depth of $\leq$0.4 cm was required when using the TEMPRIS probes [28]. It was reported that the correct placement of the TEMPRIS probes as shown in Fig. 2 was crucial in order to obtain reliable temperature profiles and endpoint monitoring, and a larger tip resulted in a more sensitive measurement of ice sublimation [28].

Studies have been performed to examine the performance of the TEMPRIS monitoring system and to compare to a traditional 36-gauge thin wire thermocouple. Using a 25-mg/mL sucrose solution and a LyoStar II lyophilizer equipped with MTM, it was found that the product temperature profile gave a similar trend as the traditional thermocouple measurement, which was also used during the same cycle. The study showed that the product temperature data generated from a center vial using the TEMPRIS monitoring system were in good agreement with both thermocouple data and predicted data using MTM. The TEMPRIS data also provided a more representative bias between edge and center vials as compared to that of a thermocouple [28]. Similarly, agreement was noted between TEMPRIS and thermocouples for a system consisting of 50 mg/mL mannitol/sucrose solution (10:1). In addition, the TEMPRIS probes indicated a more representative primary drying endpoint in that they all displayed a delayed increase in temperature during the end of primary drying in comparison to traditional thermocouples [28].

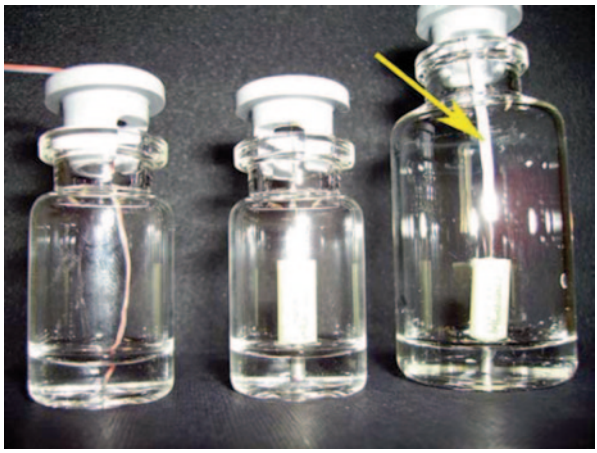

Fig. 2 Correct placement (*bottom center*) of a thermocouple (*left*) and two TEMPRIS sensors (5 and 20 mL vial, *right*) (Adapted from [28])

Another benefit of this approach includes the adaptability at scale, where due to the wireless technology, the TEMPRIS may be used with an automatic loading system. Second, the fact that the probe may be placed anywhere on the shelf enables understanding variation in product temperature across the shelf and chamber [19].

Disadvantages are the same as for thermocouples and RTDs, where product vials with probes are not representative of the entire batch [19]. The product temperature data generated with TEMPRIS would not be representative due to its influence on ice nucleation. In addition, the industry is moving towards noninvasive methodologies of measuring product temperature during freeze-drying process.

Pirani Gauge Versus Barometric Endpoint Determination

A Pirani gauge is commonly used to monitor the primary drying process. It consists of a metal wire (often platinum) open to the applied pressure. The wire is heated by a passing current and cooled by the surrounding gas, and the gauge wire temperature is dependent on the rate of heat loss to the surrounding gas. The rate of heat loss is directly proportional to pressure, and thus this measurement is based on the thermal conductivity of the gas present in the chamber [18]. The gauge can give accurate result from 10 to 10^{-3} Torr, but only for the gas against which it is calibrated (normally nitrogen). Since the Pirani gauge is sensitive to the chemical composition of the gases being measured, its use could result in inaccurate pressure measurements in the chamber due to the gas composition changes (i.e., from water vapor to nitrogen) during drying. However, as described later, a Pirani gauge is commonly used with a capacitance manometer for the detection of the primary drying endpoint.

The Pirani gauge is an inexpensive and small tool that can withstand steam sterilization without loss of performance [20]. In addition, it can be easily installed in a large-scale lyophilizer, which makes it popular for process monitoring. However,

with prolonged usage, heat transfer via radiation can become significant due to the increasing emissivity of the filament; some periodic maintenance/replacement may be needed to ensure accuracy.

A capacitance manometer (CM, also called MKS Baratron) is commonly put in the drying chamber to measure and control the chamber pressure during lyophilization [18]. The CM device is made of a metal diaphragm placed between two fixed electrodes, with one side being evacuated to high vacuum to serve as a zero reference pressure, and the other side being exposed to the chamber pressure. Since a change in device output voltage is directly proportional to the applied pressure, the CM is able to measure the absolute pressure independent of the gas composition, which is different from the Pirani gauge [19]. In addition, CM can give a stable output within a very wide pressure range (0–760 Torr), and can withstand steam sterilization. Therefore, the CM is commonly used for chamber pressure measurement in the freeze-drying process.

The joint use of a Pirani gauge with a CM has been employed to determine the endpoint of primary drying for the entire batch. At the early stage of primary drying, essentially all of the gas in the drying chamber is water vapor, and thus the Pirani gauge gives about 60% higher result than CM reading because thermal conductivity of water vapor is about 60% higher than that of nitrogen [22]. At the late stage of primary drying, the gas composition in the drying chamber changes from mostly water vapor to mostly nitrogen, and thus the Pirani gauge will show a sharp drop in pressure and finally merge with CM readout as shown in Fig. 1. This sharp transition and the small pressure differential (ΔP) between Pirani and CM is an indication of the completion of primary drying. Once the primary drying endpoint has been reached, ΔP will be close to zero and remains constant. The $\Delta P = 0$ criteria could be used as a reliable detection of endpoint of primary drying, and this ΔP between Pirani and CM can be used as a control tool if a feedback loop is programmed to allow the automatic progression to the next drying step.

The drying process can also be monitored by taking vials out of chamber at different stages of primary drying using a "sample thief." A systematic study was performed for both 5% sucrose and mannitol systems, and residual water was measured either by gravimetric or Karl Fischer method [20]. Figure 3 shows the Pirani and CM pressure trends and percent residual water profile during primary drying for 5% sucrose (a typical amorphous system). At the onset, midpoint, and offset points of pressure drop in the Pirani gauge, the residual water content is about 25, 9, and 5%, respectively. When the sample vial is warmed to ambient temperature, the midpoint vial cake (taken out at about 48 h) showed collapse behavior, suggesting that residual moisture present is high enough to depress T_g to be below 25 °C. However, the offset point gives acceptable cake structure due to sufficiently low moisture. For the 5% mannitol system, the onset, midpoint, and offset points of pressure drop from the Pirani gauge give about 9, 5, and 4% residual water, respectively. Also, all three points give good cake structure for this crystalline system [20].

It is often helpful to define the endpoint of primary drying based on the Pirani transition alone. After primary drying is complete, most "unfrozen" water has been removed. However, the product still contains a relatively high level of residual water

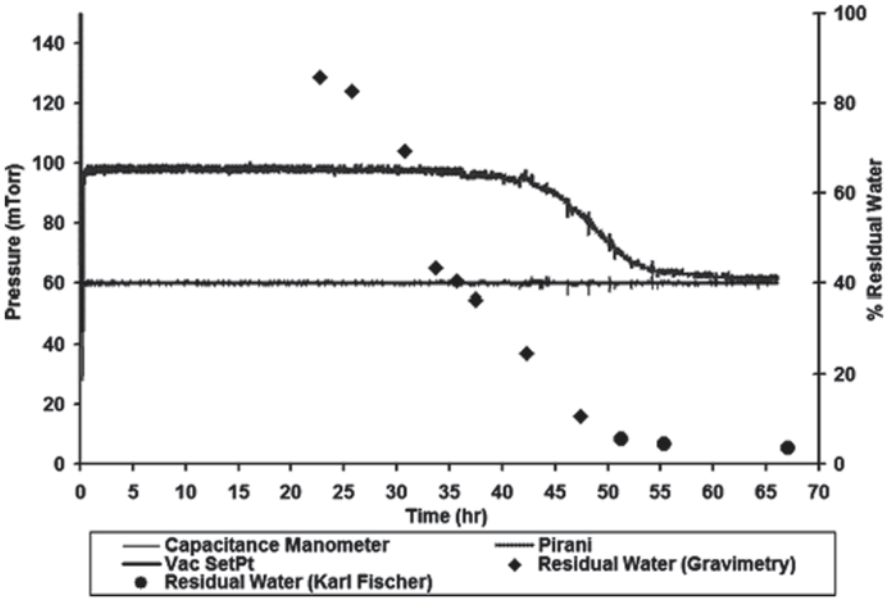

Fig. 3 Pirani versus differential capacitance manometer and percent residual water profile during primary drying for 5% sucrose (Adapted from [20])

(about 5–20% on a dried solid basis). Since the remaining "bound" water will be further removed during the secondary drying stage, a criterion of < 10% residual water may be considered to define the endpoint of primary drying [20]. As shown in Fig. 3, the midpoint of Pirani pressure drop gives low residual water < 10% for both sucrose and mannitol systems, thus the midpoint of the Pirani transition can be considered a good indicator of the endpoint of primary drying, at least for sucrose and mannitol [20]. Of course, the offset point of Pirani drop with longer primary drying time can also give acceptable result.

Manometric Temperature Measurement

It has been suggested that MTM may be used as an alternative method to monitor product temperature during primary drying [17]. The product temperature can be predicted by closing the isolation valve between the chamber and condenser for approximately 25 s to allow the pressure rise to be recorded over this time period. Due to the nature of the process, MTM is more likely employed at laboratory and pilot scale [19, 33]. When compared to invasive methods such as thermocouples, RTDs, or TEMPRIS, MTM offers a noninvasive product temperature measurement, which would be more representative as all the product remains intact and untouched during the freeze-drying process. As discussed in earlier sections, the use of sensors in the product results in nonrepresentative lyophilization behavior, in comparison to vials not containing any sensors [27, 33].

The principle involves fitting the MTM equation (Eq. 1) to the pressure rise data, generated from opening and closing the isolation valve, by nonlinear regression analysis [33].

$$P(t) = P_{\text{ice}} - (P_{\text{ice}} - P_0)\exp\left[-\left(\frac{3.461\text{NAp}T_s}{V(\hat{R}_p + \hat{R}_s)}\right)t\right] + 0.0465P_{\text{ice}}\Delta T\left[1 - 0.811\exp\left(-\frac{0.114}{L_{\text{ice}}}t\right)\right] + X_t \quad (1)$$

where P_{ice} is the vapor pressure of ice (Torr) at the sublimation interface; P_0 is the chamber pressure (Torr); N is the total number of filled vials; A_p is the inner cross-sectional area of the vial (cm^2); T_s is the shelf temperature (K); V is the chamber volume (L); $\widehat{R_p} + \widehat{R_s}$ is the total area normalized product and stopper resistance (determined by the fit); L_{ice} is the ice thickness (cm); ΔT is the temperature difference between ice sublimation interface and bottom of vials; and X is a constant (Torr/second).

Fitting Eq. 1 to the pressure rise data yields a vapor pressure of ice, P_{ice}, and the $\widehat{R_p} + \widehat{R_s}$ total resistance of product and stoppers. The MTM product temperature can be calculated from the vapor pressure of ice (P_{ice}) by Eq. 2.

$$\ln(P_{\text{ice}}) = \frac{-6144.96}{T_p} + 24.01849 \quad (2)$$

where T_p is the product temperature (K) and P_{ice} is the MTM fitted vapor pressure of ice.

When considering the application of MTM to collect product temperature data during the primary drying stage of the cycle, it should be noted that the product temperature is measured from pressure rise data, calculated using a CM and applying Eq. 1. As the pressure rise measurement is a function of the full chamber over a time period of 25 s, it does not discriminate between vials at specific locations within the freeze dryer. The calculated product temperature using the MTM equation is therefore an average of the entire batch [24].

Studies were performed to understand the comparability between product temperature measurement using thermocouples and MTM [34]. Sample of 5% sucrose was used with a target product temperature of −40 °C, which is well below the sucrose collapse temperature of −32 °C. It was found that the temperature gradient was negligible during the drying step, the comparative thermocouple and MTM measurements were in good agreement. A second experiment using 5% glycine suggested that the average product temperature was heavily skewed towards the coldest vials (interior or center vials as opposed to edge vials). Interior vials form the majority of vials when considering the overall array on a given shelf. As the edge vials display higher temperatures due to the influence of radiative heat transfer, this should be taken into account from thermocouple placement.

MTM limitations include the fact that the product temperature is an average temperature with bias towards the coldest vials and a minimum ice sublimation area is required for accurate results, estimated by a Q value of ≥ 0.23. For a laboratory freeze dryer with a 50-L chamber, the minimum area would be 150 cm^2 [34]. MTM is only useful for 2/3 of the primary drying step due to the high degree of drying heterogeneity towards the end of primary drying [19].

Benefits of using MTM are clear in that it provides a product temperature profile by noninvasive means, arguably resulting in a more representative profile. This principle could prove to be a powerful PAT tool. It has been observed that MTM is effective at product temperatures as low as −45 °C, which covers the majority of freeze-dried formulations. MTM has also been used to measure dry layer resistance (Rp), where it was shown that for 5 % glycine, 5 % mannitol, and 5 % sucrose formulations, R_p data were in good agreement with actual data (vial thermocouple method) when using a thermal shield to remove the influence of atypical radiation [34]. When measuring without a thermal shield, it was noted that the R_p values were consistently lower than actual data.

MTM was also examined as a method to measure the vial heat transfer coefficient (K_v) and sublimation rates in different conditions with and without thermal shields. Using an FTS Dura-Stop/Dura-Top freeze dryer, it was found that K_v was in good agreement with the traditional gravimetric method for 5 % sucrose when using a thermal shield such as aluminum foil. Conversely, without the thermal shield, the K_v measurement was consistently higher than for the gravimetric method, which was supported by data generated for multiple concentrations of mannitol, sucrose, and glycine [33].

SMART™ freeze dryer was developed using MTM [32]. The system was designed to enable the scientists to arrive at an optimized cycle in one run, once certain parameters were entered into the software prior to initiating the cycle (maximum allowable temperature, amorphous or crystalline, concentration, fill volume, vial geometry, vial type, number of vials, etc.). In short, the cycle is adjusted in real time (i.e., shelf temperature and chamber pressure), while maintaining the product temperature below the maximum allowable temperature.

Lastly, it has been reported that MTM has applications in the secondary drying step, where it has been shown to be able to predict average moisture content across the batch and the secondary drying endpoint [19, 32]. Residual moisture measured experimentally using Karl Fischer was measured for vials of freeze-dried sucrose (5 %) and glycine (5 %), where the samples were taken using a sample thief. In another experiment, the residual moisture was predicted by using a series of pressure rise steps (MTM method), where the rate of desorption was estimated using the ideal gas law [19, 32]. Good agreement was found between the residual moisture data generated from both methods.

Overall, MTM is a useful tool for the design and monitoring of the primary drying step, even with a number of limitations that need to be considered. It was also found that MTM was useful in measuring desorption rates and therefore residual moisture during the secondary drying step. However, implementation at commercial scale may be difficult due to the nature and requirements of the tool.

Tunable Diode Laser Adsorption Spectroscopy

TDLAS can be used to continuously measure trace concentrations of selected gases for various applications, and has recently matured into the lyophilization field as a process-monitoring tool. The TDLAS control unit provides two coupled outputs from the same laser, which are connected to two fiber optic collimators mounted on the duct connecting the drying chamber and the condenser. The diode laser light is transmitted through the gas mixture containing water vapor, and the beam's wavelength is adjusted to water vapor absorption lines to accurately measure the absorption [5]. The concentration of water vapor was measured based on the Beer–Lambert's law, and the gas flow velocity can be obtained from the Doppler-shifted water vapor absorption spectrum.

TDLAS was used to measure sublimation rate during the lyophilization process on both a laboratory and a pilot scale freeze dryer [5]. The time integrated sublimation rate obtained from TDLAS was compared to a gravimetric determination of the total weight of water removed based on the mass difference before and after the sublimation. The ratio of ''gravimetric/TDLAS'' measurements of water sublimed was 1.02 ± 0.06, suggesting that this in-line tool can be used for accurate measurements of total amount of water removed. In addition, the application of TDLAS for endpoint detection has been studied for 5% mannitol runs in both laboratory and pilot scale freeze dryers. As shown in Fig. 4, the sublimation rate was about 0.5 g/s during the early primary drying stage, and then gradually decreased to almost zero at the end of primary drying. During secondary drying, a bump in the baseline mass flux was observed with a maximum value of 1.0×10^{-2} g/s, and then dropped to zero at the end of secondary drying. Therefore, the TDLAS can also be used to detect the endpoints of primary and secondary drying for the product run.

TDLAS can be used for a rapid determination of vial heat transfer coefficient based on the sublimation rate profiles. Traditionally, several cycles are needed to be performed at different pressures in order to obtain the pressure-dependent vial heat transfer parameters. Since the sublimation rates can be frequently measured by TDLAS, the pressure effect can be incorporated into one cycle by using step-changes in chamber pressure, which makes it much more efficient to measure vial heat transfer parameters. Kuu et al. showed that both the contact parameter K_{cs} and the separation distance ℓ_v can be rapidly determined using the sublimation rate continuously measured by TDLAS within a short cycle. The determined K_{cs} and ℓ_v values closely fit both the sublimation rate and product temperature profiles, suggesting the validity of the approach employed [11]. Note that the TDLAS measured sublimation rate is the average of all vials in the whole batch, and thus the measured vial heat transfer coefficient using TDLAS is also the average value.

In order to evaluate the drying heterogeneity of vials, position-dependent vial heat transfer coefficients (K_v) were studied using TDLAS during sublimation tests with pure water [29]. The K_v data obtained from TDLAS were found to be comparable with K_v data obtained by the traditional gravimetric procedure. Edge vials were found to run at higher temperatures, which results in that K_v of edge vials was

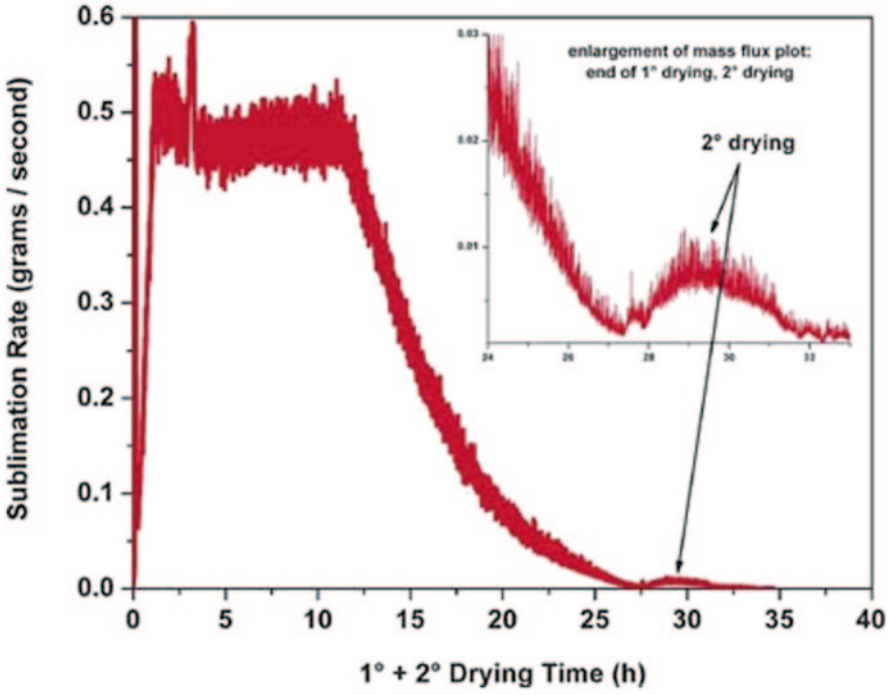

Fig. 4 Mass flow temporal profile over time for a 5% mannitol formulation in a pilot scale freeze dryer (Adapted with permission from [5])

about 20–30% higher than that of center vials. The combination of TDLAS mass flow rate measurements and the heat and mass transfer model based on a weighted K_v were employed for nonintrusive, real-time product temperature determinations. It was demonstrated that product temperatures calculated from TDLAS mass flow data were in excellent agreement with thermocouple data in the center vials during product runs with 5, 7.5, or 10% (w/w) sucrose, mannitol, and glycine, respect tively. TDLAS product temperatures for all freeze-drying runs were within 1–2 °C of "center vial" steady-state thermocouple data [29].

TDLAS has also been applied to the measurement of dry layer mass transfer resistance. By combining the TDLAS and the pore diffusion model, the effective pore radius of the dry layer can be estimated from the sublimation rate and product temperature profiles measured during primary drying. This method does not require solution of the complex heat and mass transfer equations, and has been demonstrated with product runs with 5% mannitol, sucrose, lactose, 3% mannitol plus 2% sucrose, etc. [12].

In addition to the process monitoring, effort has been made to achieve automatic product temperature control to reduce the primary drying time. Kuu et al. developed computer programs to determine the optimal shelf temperature and chamber pressure while ensuring the product temperature profile is below the target temperature

[10]. TDLAS was used to continuously measure the sublimation rate during 5% mannitol run, and the program was able to quickly perform the calculations based on heat and mass transfer equation. It was demonstrated that maximum product temperatures were controlled slightly below the target temperature, and a cascading temperature-ramping cycle is the most efficient cycle design [10].

Unlike thermocouple and other spectroscopic methods mentioned below, TDLAS gives the behavior for the whole batch. Overall, TDLAS shows a great potential to be used as an online monitoring tool for freeze-drying process, especially during manufacturing runs where the application of thermocouples is not possible. However, TDLAS also has some constraints. First, the integration of the technique to freeze dryer is not straightforward. The retrofitting of an old freeze dryer may be quite challenging, and the technique can only be applied to a freeze dryer with a long duct to allow absorption measurement at an angle (typically 45°) to the gas flow velocity vector. Second, the technology requires sophisticated calculations based on the gas concentration and gas flow velocity profile in the duct from the raw data. Therefore, depending on the duct size and configuration, the gas flow dynamics may be quite different between dryers. Spray balls and clean-in-place (CIP) nozzles in vapor duct at large scale may also impact calculations. In order to better understand these factors and facilitate process scale-up, more studies on large-scale manufacturing dryers are required.

Plasma Emission Spectroscopy (LyoTrack)

Lyotrack is a new online device developed to measure the humidity, and thus can be used to determine the endpoint of primary drying for the full batch. Lyotrack relies on inductively coupled plasma/optical emission spectroscopy (ICP/OES). This technology is adapted to the freeze-drying environment to measure water vapor concentration during the drying process [19]. The device is composed of two parts: the ICP plasma generator and the optical spectrometer. The ICP part creates a high power radio frequency wave, and the gas present in the freeze/dryer chamber forms cold plasma. The electrons from the gas atoms and molecules in the chamber are excited to a discrete higher energy state by absorbing this radio-frequency energy. The unstable excited atom will relax to its initial energy level and emit light. The optical spectrometer can thus identify the water vapor in the chamber based on the characteristic wavelengths of the emitted light.

Mayeresse et al. have systematically studied the Lyotrack using different formulations. The results indicate that Lyotrack can be used to determine the endpoint of primary drying with high sensitivity and good reproducibility [16]. The impact of probe location on the signal sensitivity was also studied, and it was found that the Lyotrack was sensitive to gas composition when it was placed in the chamber and in the duct connecting the chamber and the condenser but not in the condenser. The gas composition profile with drying time was similar when two lyophilizers of different scales were used for the same product, suggesting that the device can be used as a characteristic tool in process scale-up.

Hottot et al. compared five different types of sensors for the detection of the sublimation endpoint in syringe configuration [6]. Results showed that the Lyotrack and Pirani sensors provided similar results, with clear and precise sublimation endpoint determination. The Lyotrack sensor gave better signal-to-noise ratio than the Pirani gauge, which is possibly due to the signal disruption to Pirani caused by air injection for pressure regulation.

De Beer et al. simultaneously implemented four newly introduced PAT tools to monitor freeze-drying processes [2]. It was found that a combination of Raman and Lyotrack allows the monitoring of nearly all critical process aspects. Raman spectroscopy can give insight into the product behavior during freezing stage and also the bound water removal dynamics during secondary drying, whereas plasma emission spectroscopy gives information about the drying process.

The Lyotrack is simple to integrate into a lyophilizer via a tri-clamp flange due to its portable nature. Also, it is steam-sterilizable thanks to its durable construction. However, it also has some limitations. The humidity signal from the Lyotrack ranges between 0 (no water vapor) and 1 (saturated with water vapor), and it is difficult to transform this qualitative value into a measure of water quantity. In addition, Lyotrack involves ionization of the gas present in the chamber and the formation of free radical, and there is a potential risk to the stability of the freeze-dried product. In a recent study, oxidation of human growth hormone (HGH) was found to be significantly increased (about 12%) upon using Lyotrack in the drying chamber to freeze-dry HGH and HGH/sucrose formulations [20]. Thus, this technology could compromise the product quality of a molecule which is sensitive to oxidation. Moreover, Lyotrack is relatively expensive as compared to a Pirani gauge, and both sensors give very similar gas composition profiles, therefore, there is no strong advantage of using Lyotrack instead of the Pirani gauge for the primary drying endpoint determination.

Near-Infrared Spectroscopy

NIR is an effective tool for the understanding of the lyophilization process and evaluation of lyophilized pharmaceuticals. The NIR spectrum has a frequency range from 4000 to 12,500 cm^{-1} (800–2500 nm), and is highly sensitive to vibrational motions of the hydrogen atom in different molecular environments. Due to its ability to penetrate glass and plastic containers, NIR spectroscopy can also be used as a fast and noninvasive method to monitor the lyophilized product [1]. NIR has been not only used to determine the residual moisture in lyophilized samples but also evaluated for in-line process monitoring during freezing and drying stages. In addition, it offers many unique advantages such as the monitoring of protein conformational change and excipient morphology conversion during the drying process. NIR can therefore be a valuable tool for speeding the development of formulations and lyophilization process.

Off-Line Determination of Residual Water in Freeze-Dried Solids

Water exhibits strong absorption in the NIR region around 970, 1190, 1450, and 1940 nm [36]. Many efforts have been made to evaluate NIR spectroscopy as a fast nondestructive approach to determine the residual moisture content in the lyophilized products. Residual water was determined in freeze-dried sucrose samples using NIR diffuse reflectance spectrometry by scanning through the bottom of the glass vial [7]. A good correlation ($r^2 = 0.97$) was observed between residual water determined by traditional Karl Fischer method and NIR. The impacts of cake porosity, buffer concentration, and surfactant on NIR signal were also studied for a lyophilized monoclonal antibody system [14]. These factors do not have significant effect on the residual moisture when the cake thickness and diameter was much larger than the NIR penetration depth. However, it was found that the disaccharide concentration has a large impact on the NIR determined moisture content, which results from the strong NIR absorbance of disaccharide at the same wavelength as residual water. Therefore, the NIR method for moisture quantification is highly sensitive to the drug formulation, and a calibration curve needs to be developed against the standard Karl Fischer method. It was reported that the calibration curve developed for one formulation cannot be used for another drug concentration [13]. Even for a given formulation, samples from different batches could give differences in the correlation between NIR and Karl Fischer. Therefore, it is critical to develop robust calibration curves incorporating all relevant factors and manufacturing process variables.

In-Line Process Monitoring for Protein Conformation and Protein–Excipient Interactions

NIR has also been used as in-line PAT tool to monitor the lyophilization process since water and ice give strong signals in NIR spectra. De Beer et al. have studied the performance of a NIR probe directing to the sidewall of a vial for mannitol formulation [4]. Chemometric tools were used to extract useful information from the large raw data sets, and it was found that mannitol starts to crystallize when ice crystallization is complete. Since sublimation finishes at the bottom center of the vial, the drying endpoint can also be measured by the NIR tool. However, NIR may underestimate the drying endpoint as compared to the endpoint measured by Lyotrap method.

Another application of NIR spectroscopy is the differentiation of mannitol hydrate and surface water. The different crystalline mannitol polymorphs can be distinguished via NIR in the 4330–4450 cm^{-1} spectral range, and a nondestructive method was developed to determine the amount of metastable mannitol hydrate and surface water in lyophilized products [3]. This study indicated that NIR could be employed to monitor the formation of mannitol hydrate during lyophilization process.

NIR was recently used as an in-line process analyzer for monitoring protein unfolding and protein–lyoprotectant interactions during drying [21]. The amide A/II band near 4850 cm^{-1} was monitored along with the water absorbance band near 5160 cm^{-1}. This amide A/II frequency was found to correlate well with the water absorbance intensity during protein dehydration in the absence of protein unfolding, whereas deviation from the linear relationship was found to be related to protein unfolding. For sucrose formulations, the amide A/II frequencies decrease immediately after sublimation, suggesting an increase in protein–sucrose hydrogen bond interaction. This approach could provide more insights about protein conformational stability and the lyoprotectant interaction with protein at real time during the dehydration process.

Even though NIR has demonstrated some utility for in-line process monitoring, the technique has several limitations. First, NIR is based on the detection of a single vial in the edge, and only small part of the cake is measured by the NIR probe. Therefore, the result measured is not representative of the entire batch. Second, this technique requires an unobstructed view of the NIR probe to the contents of the vial and the insertion of a fiber-optic cable into the chamber, which makes it difficult to integrate into a manufacturing scale lyophilizer. While NIR has low potential as an in-line PAT tool for freeze-drying, it could find off-line applications as a nondestructive method for high throughput residual water measurements.

Raman Spectroscopy

Since water and ice are weak Raman scatterers, Raman spectroscopy is becoming popular for monitoring formulation characteristics during the freeze-drying process. De Beer et al. have studied Raman as the PAT to monitor the endpoint of primary drying. The Raman probe was placed above the product vial in the drying chamber. The Raman technique gave a significantly lower endpoint of primary drying, which was due to the inappropriate placement of Raman probe [3]. However, Raman spectroscopy was able to provide information about the endpoint of freezing and the mannitol solid-state conversion throughout the entire process. It was reported that a combination of Raman and Lyotrack allows the monitoring of nearly all process aspects. Raman spectroscopy can provide insights into the product behavior during freezing stage and also the dynamics of bound water removal during secondary drying. Conversely, Lyotrack gives information about the primary drying endpoint [4].

Recently, Raman spectroscopy was used for in-line monitoring of a microscale freeze-drying process [8]. The effect of cooling rate and annealing step on the solid-state formation of mannitol was studied. Principal component analysis was used to qualitatively analyze the solid-state forms of mannitol, while classical least-squares regression analysis was used to estimate the solid-state form ratios of each polymorphism. The results showed that mannitol hemihydrate emerged and subsequently transformed to more stable forms during the secondary drying step. Similar to NIR, the Raman measurement is based on one vial, and it is not representative of

the whole batch. In addition, since it requires a special arrangement of the Raman probe, it will be difficult for an in-line application in an industrial lyophilizer.

Impedance Spectroscopy

During this literature review, a potential new technology to measure product temperature was identified. An impedance spectroscopy method was introduced as a minimally invasive method of measuring product temperature during the freeze-drying process [30]. This approach was shown to be useful to monitor the freezing step, but at this point in the early development of the technology, it cannot monitor the product temperature during primary drying. The experimental setup consists of placing planar electrodes on the external vial wall, and these electrodes are then coupled to a high impedance analyzer. The impedance measurement is converted to temperature using an algorithm [30]. The placement on the external vial wall does not influence the nucleation event of the freezing process. If this technology evolves to monitor the product temperature during primary drying, it will significantly improve its value to lyophilization cycle monitoring. However, similar to the thermocouple approach, this system must be applied to the vial manually, which means that it would be a challenge to apply to commercial process monitoring.

Summary

Table 1 summarizes the major advantages and disadvantages of each PAT tool discussed above. It gives an overview of each PAT tool's capabilities and its practical application and scalability to large-scale freeze dryer. The major limitations associated with each tool are also listed in the Table.

Conclusions and Future State

Based on the QbD requirements, an ideal PAT tool for freeze-drying should be able to meet the following needs: (1) monitor the product temperature during *all* steps of freeze-drying process including freezing, primary drying, and secondary drying, and the temperature measured should be representative of the whole batch; (2) determine the endpoint of major steps including primary drying and secondary drying; (3) monitor the sublimation and determine the residual moisture during primary drying and secondary drying; (4) provide real-time product quality information such as the protein structure and excipient phase transition; and (5) integrate to different types of lyophilizer from laboratory scale to GMP manufacturing scale.

Table 1 Summary table for assessed online PATs

Criteria	Thermo-couple	TEM-PRIS	Pirani versus CM	MTM	TDLAS	Lyotrack	NIR	Raman	Impedance spectroscopy
Sensitive to placement positions	+	+	−	−	−	−	+	+	+
Measure product temperature	+	+	−	+	+	−	−	−	−
Detect endpoint of primary drying	+	+	+	+	+	+	−	−	−
Monitor moisture level	−	−	−	−	+	−	+	−	−
Monitor molecular structural change	−	−	−	−	−	−	+	+	−
Representative to the whole batch	−	−	+	+	+	+	−	−	−
Noninvasive (no direct product contact)	−	−	+	+	+	+	+	+	+
Simplicity of integration and use	+	+	+	+	−	+	−	−	+
Application to large-scale dryer	+	+	+	−	+	+	−	−	−

(+) indicates the ability to monitor the considered process aspect, while (−) means the non-applicability of the tool
TEMPRIS temperature remote interrogation system, *MTM* manometric temperature measurement, *TDLAS* tunable diode laser adsorption spectroscopy, *NIR* near-infrared *CM* capacitance manometer

Upon review of PAT technologies available to date, we found that not a single PAT tool currently exists to cover these broad requirements, possibly due to the harsh conditions of the freeze-drying process (deep vacuum, low temperature during freezing, high temperature during sterilization, and sterility requirement at GMP environment). Most PAT tools only meet one or two criteria listed above, and we currently have to rely on the combination of several PAT tools in order to achieve this ideal state. In addition, while more options are available as PAT tools for application in a small-scale lyophilizer, there are fewer PAT tools which can be used in GMP commercial manufacturing scale lyophilizer. Therefore, further development of these existing and new PAT tools to cover these gaps is strongly needed.

Product temperature monitoring with thermocouples will still be used to develop a safe lyophilization cycle. Traditionally, thermocouples or RTDs have been used to monitor product temperature in real time during technology transfer and scale-up. Over the years, thermocouples have even been used to control freeze-drying cycles during commercial manufacturing. However, thermocouples are less than ideal due to their effect on the nucleation process and potential sterility challenges when used in a GMP environment. Other approaches such as TEMPRIS or the new impedance spectroscopy system may be a more advanced means to measure product temperature, but they are not yet ready to replace thermocouples due to their limitations. The invasive TEMPRIS yielded data not representative to the other vials in the chamber, and the impedance system is limited at this point to the freezing step. MTM can measure average product temperature for the whole batch and it is very useful for development; however, MTM is limited to laboratory and pilot freeze dryer and is not scalable to a large-scale manufacturing dryer.

From a primary drying endpoint perspective, the Pirani gauge remains the best option for reasons outlined earlier in the chapter. Lyotrack can be a good option when used with a stable system which is not susceptible to oxidation degradation. A Pirani gauge is less expensive than Lyotrack, while it can provide very similar gas composition profiles. In addition, the Pirani gauge may also be used as a PAT control tool if a feedback loop was programmed to continuously measure the ΔP between the Pirani and the CM and allow the automatic progression to next step when $\Delta P = 0$ is reached at the endpoint of the primary drying.

Several new PAT tools such as TDLAS, NIR, and Raman have been developed in the past decades, and they have provided insights about the water removal and process transition. Among them, TDLAS is the most promising PAT tool as it can meet several criteria for lyophilization. First, it can measure sublimation rate during the primary drying and secondary drying process, and has the potential to monitor the residual moisture in the cake during secondary drying. Second, it can be used to determine the endpoint of both primary drying and secondary drying based on the change in sublimation rate. Third, based on the steady-state heat and mass transfer model, the signal measured can be used to obtain the average product temperature during primary drying, which will give the behavior for the whole batch. In addition, it can provide information about the vial heat transfer coefficient and dry layer mass transfer resistance. Research is currently ongoing to assess TDLAS for

other applications and thus far, even with certain limitations, this system looks very promising.

In summary, the end of thermocouple as an application to lyophilization does not appear to be anytime soon. There are other systems available to monitor the product temperature profile, which is ultimately the critical parameter during lyophilization. In terms of primary drying endpoint, tools such as a Pirani gauge and MTM are excellent for pilot scale applications; a Pirani gauge can also be used in commercial manufacturing. To date, it appears that TDLAS is the most promising PAT tool used during the freeze-drying process. TDLAS is applicable to more than one step of the freeze-drying process. It can be used to monitor product temperature during primary drying and determine primary drying endpoint, and also has the potential to control the secondary drying step by monitoring the residual moisture in the cake in real time. Development of new PAT tools for lyophilization is still needed in order to meet the multiple QbD requirements. The most applicable PAT tools will be able to monitor and control multiple steps of the cycle and also assure the product quality attributes in real time.

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Process Scale-Up and Optimization of Lyophilized Vaccine Products

Jeffrey T. Blue, Jessica R. Sinacola and Akhilesh Bhambhani

Introduction

Development of the formulation processes for vaccines relative to therapeutic proteins (TPs) and small molecules is challenging and results in increased regulatory oversight. The increased oversight is mainly due to the population of patients that are receiving the treatment. Unlike TPs and small molecules, vaccines are administered to a healthy population where the disease state is not present. Thus, ensuring safety and efficacy are important and increase the regulatory review of the products. The complexity of developing vaccines is compounded by multiple factors including: lack of structure and function with immunogenicity, the inability to have a true biomarker as a correlate for protection or effectiveness, and not having a good in vitro/in vivo model. The aforementioned issues with development of vaccines lead to large clinical studies in multiple patient populations to show efficacy and safety.

Due to the large clinical trial sizes, the clinical development timelines are longer than both TPs and small molecules. One example would be the licensing of RotaTeq®, where the efficacy study consisted of approximate 5700 patients and the phase III trial consisted of ~71,000 patients [1, 2]. The overall timeline for the phase III clinical trial (REST study) took more than 4 years to complete. From the initial proof of concept in 1993, the final licensure of the vaccine occurred in 2006.

When developing a vaccine, the formulation scientist must consider not only the formulation conditions to ensure stability but also the delivery mechanism, tar-

J. T. Blue (✉)
Telford, USA
e-mail: jeff_blue@merck.com

J. R. Sinacola
Collegeville, USA

A. Bhambhani
Greater Philadelphia Area, USA

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get population (e.g., pediatric), and the necessity for a potential adjuvant to ensure the vaccine is immunogenic [3, 4]. This information will be captured early in the global target product profile (GTPP) and will be an evolving document as the program moves through product development. During the generation of the GTPP, it is important to ensure not only that the stability profile aligns with the requirements but also that the proper packaging of the product will suffice within the respective markets.

Early in development, different primary images including single- and multidose vials, prefilled syringes, liquid, and lyophilized products will be explored. The decision to move forward with a respective primary image will be based on the necessary stability profile required to meet the specific market demands and be customer driven. Beyond exploring the primary image, the formulation scientist must also consider the secondary package (1x vs. 10x, kits vs. single vials) and the tertiary package to ensure the cold chain can be kept under control (i.e., gel packs, dry ice pack-outs, and nano-cooling technology). Aligning the formulation scientist to this GTPP will ensure the customer is provided with a vaccine that meets the market demands, is safe, efficacious, and has been developed with customer centricity in mind.

Complexity of vaccine formulation development is often further enhanced by combination vaccines. An example currently on the market is the ProQuad® and RotaTeq® vaccines each consisting of four or five different live viruses, respectively [3, 5]. Another example of a multicomponent vaccine is Pnuemonvax®, which consists of 23 different polysaccharides [6]. Selecting a formulation that can stabilize these multicomponent vaccines and achieve the desired GTPP is a significant hurdle for the formulation scientist. The formulation must be compatible with all components within the vaccine as well as the manufacturing process. This may result in some compromises with regard to stability for one entity and is a balancing act for the combined vaccine.

Vaccines serve an important medical need in preventing disease and lessen the overall health care burden. As a result of their health care benefits, there has been increased pressure on vaccine manufacturers to increase their global footprint and reach areas of the world that were not previously covered [7, 8]. As the markets for vaccines continue to expand into the developing world and emerging markets, the need to ensure that vaccine and formulation can withstand increased thermal stress becomes paramount. This is due to the developing and emerging markets not having proper command of the cold chain and thus, the vaccine product being exposed to increased temperature and excursions during routine shipping and administration. By ensuring that the vaccine stability profile can withstand increased thermal stress, the impacts associated with temperature excursions can be minimized and ensure that a safe and efficacious vaccine is delivered to the patient. It is important to not only deliver a robust formulation that can withstand thermal stress but also have a formulation and filling process that can be manufactured robustly. The robust manufacturing process is essential since there are specific regulatory guidances requiring local manufacturing of the vaccines, thus, requiring technology transfer and well defined critical quality attributes (CQAs) [9] to allow manufacturing to be successful.

Drivers for Vaccines and Lyophilization of Product

The driving force for choosing between a liquid image and a lyophilized image is mainly linked to the stability and storage requirements within the GTPP. Lyophilization has the advantage over liquid images in terms of stability, storage, and ease of shipment for clinical development and marketing, especially with the desire to move into new emerging and developing world markets where increased thermal stability is highly desired [10].

Another advantage and driver for products to move to a lyophilized form is that lyophilization normally meets the product sterility assurance without terminal sterilization due to the process being a trusted aseptic operation [10]. For biopharmaceuticals, lyophilization is the industry benchmark for attaining a dried product, and any deviation or innovative approach to obtain dried product will have the burden of overcoming the technology barrier while maintaining comparable (if not superior) quality assurance as the lyophilized product [11].

Lyophilized products, especially vaccines and biologics, add an additional benefit when a multidose image may be required. Most vaccine products are incompatible with preservatives, and thus, achieving the required stability profile in the liquid state is unobtainable when in a preservative-containing formulation. By lyophilizing the vaccine without a preservative, the negative stability effects associated with the preservative, can be removed form the product. This is achieved by having the preservative be part of the reconstitution diluent and minimizing the exposure of the vaccine to the preservative. Thus, long-term stability of the vaccine is not impacted.

In addition to improving the success for a preservative-containing vaccine, there are instances where vaccine antigens may need to be detoxified. One common approach for detoxification is the use of formaldehyde. However, long-term liquid stability with a formaldehyde product may not be feasible due to the potential for reversion. To overcome this, lyophilization can be utilized to mitigate reversion to toxicity [12].

Finally, lyophilization of live virus vaccines (LVV) is usually necessary to achieve the desired long-term stability profile. Unlike protein-based vaccines, LVV (enveloped and non-enveloped) are significantly challenging in their intrinsic thermal stability [13]. Liquid degradation rates for LVV can be as high as 10% per hour in the liquid state at refrigerated temperatures. Thus, achieving a stable liquid formulation for an LVV is usually not obtainable, and thus, necessitates the use of lyophilization to achieve a vaccine that is safe and efficacious over the desired shelf life of the product [13].

Although there are some potential benefits associated with a lyophilized vaccine product, the freeze-drying process does have some disadvantages [10, 14–16]. These include the additional unit operation of lyophilization, higher cost with manufacturing, large capital investment in equipment, the potential impact of freezing and drying stresses, inability to lyophilize an aluminum-based vaccine, and differences in equipment across laboratory, pilot, and commercial scale when developing a robust process. As the formulation scientist initiates vaccine development, the scientist must assess the GTPP and determine how best to achieve the desired

product. During the assessment to proceed with liquid or lyophilization, the formulation scientist will utilize past experience with similar vaccines and likely complete the risk assessments to help shape the formulation strategy moving forward. Both liquid and lyophilized vaccines are feasible and it is the job of the formulation scientist to ensure a proper image is defined and able to support the product long term commercially.

Vaccine Drug Product Development Stages

In our opinion, prior to any drug product development, it is essential that the GTPP has been defined and aligned across the development team. The GTPP outlines the target population, desired shelf life of the product, dosing schedule, and route of administration. It is expected that as the program advances in development that the GTPP will evolve. However, to ensure that the formulation scientist does have an initial target, the GTPP is utilized. An example of a GTPP is outlined in Table 1 below.

After establishing the GTPP, drug product development can be initiated. It is our opinion that vaccine drug product development can be broken down into three main stages. The first stage of development consists of the early development space where preformulation activities occur. The main goal is to identify the proper antigen to bring forward as a preclinical candidate. Identifying the main degradation pathways for the antigen is critical and using that knowledge will help shape the initial formulation. Early screening of wide ranges of excipients is usually completed here and may involve design of experiment (DOE) approaches to find factors that impact product stability. Once the preclinical candidate has been identified, additional early formulation screening occurs that leads to a formulation that can

Table 1 Global target product profile

Attribute	Minimally acceptable profile
Vaccine serotypes	Vaccine consists of 5 serotypes and covers at least 75 % of invasive disease
Target population	Vaccine to be administered to children < 5 years of age and particularly effective in infants < 2 years of age
Safety, reactogenicity	Safety and reactogenicity should be similar to other marketed vaccines. No significant AEs
Dosing schedule	A two-dose regimen is required
Route of administration	Intramuscular, intradermal, or subcutaneous
Product images	Single-dose vial, syringe, and multidose vials desired
Product formulation	Liquid formulation preferred
Product stability	Refrigerated product desired for minimum of 24 months, desire a minimum of 6 months of RT stability, request vaccine vial monitoring 14 (VVM14)

be brought forward into safety assessment/toxicology studies. Normally, the formulation is not optimized at this point, but contains excipients that are likely to be utilized in the clinical formulation for phase I and beyond.

As the program proceeds through the development and enters into phase I, the second stage of development is initiated where additional formulation changes may be examined, as more knowledge of the degradation mechanisms become known and the ability to characterize the vaccine increases. It is likely that the analytical methods and stability-indicating assays have been improved and can significantly improve the success of the vaccine candidate by better teasing out differences in product stability. This stage of development is focused on delivering a robust manufacturing process and an optimized formulation for clinical development. Use of QbD and PAT becomes more common in this stage of development and is used to shape the CQAs and CPPs associated with the product while de-risking the investment [17].

Although QbD is common within this stage of development, it is likely that the application of QbD is limited. Unlike TPs and small molecules, the variability and complexity associated with vaccines make it difficult to apply to all areas of product and formulation development. Two operations where QbD can be applied and often is applied in vaccine development include lyophilization and adjuvant production. Application of QbD to the vaccine drug product development should be evaluated on a case-by-case basis.

The final stage of vaccine drug product development consists of working toward technology transfer into the final commercial manufacturing facility and the potential issues with scaling the process. Here, it is essential to have a deep understanding of the regulatory environment within the expected markets, identify the potential differences between development and manufacturing facility (e.g., glycol cooling systems), equipment comparability between development and manufacturing (e.g., shelf to condenser ratio, internal vs. external condensers), and respective process compatibility and process map (e.g., tank design and formulation suite capability). This information is necessary to ensure a robust technology transfer and successful phase III campaign can occur, thus ultimately leading to licensure of the new vaccine.

Introduction to Formulation Development

During early drug product development, the formulation scientist is challenged due to the lack of representative bulk drug substance that can be utilized for formulation screening. As a result, formulation scientists must try and utilize high throughput screening technologies to examine formulations and complete the studies at accelerated conditions (i.e., 25, 37, and 45 °C) to try and identify conditions that will stabilize the product [18–24]. One caveat of utilizing accelerated conditions is that it does not always mimic the degradation mechanisms observed under marketed shelf-life storage conditions. Thus, it is important for the formulation scientist to

understand the most probable degradation pathways associated with the candidate, and assess the appropriate accelerated conditions to utilize during screening. Real-time stability, although preferred, is time consuming early in programs to utilize for screening.

Due to the route of administration associated with vaccines, mainly parenteral, the formulation scientist is also challenged with a limited range of excipients that are available for use [25–27]. Although generally regarded as safe (GRAS) excipients are examined across TPs, pharmaceuticals, and vaccines, the list is significantly constrained for injectable products (Table 2). Additionally, as the global markets for vaccines expand, animal-derived raw materials and excipients must be removed to satisfy regulatory requirements in many markets such as China and Japan.

The range of vaccines and the stability issues associated with them are quite variable. Vaccines can be as diverse as live-attenuated or inactivated viruses, polysaccharides or polysaccharide conjugates, subunit/peptide vaccines consisting of native or recombinant proteins, and unadjuvanted or adjuvanted vaccines [28]. This adds additional layers of complexity to the formulation scientist since there are not "platform" formulations or technologies that can be utilized to support the different vaccine candidates. In addition, the assays required to monitor the stability and degradation pathways vary significantly from vaccine product to product.

Since the variability in vaccine candidates is vast, the formulation scientist relies substantially on their analytical colleagues to establish stability-indicating assays that can shape the final drug product process and formulation. From an analytical perspective, vaccines can be significantly challenging. The methods being developed in many cases must be able to distinguish differences for each individual antigen associated with the multicomponent vaccines. The large size of vaccines (>10,000-fold larger than a pharmaceutical) and low concentrations of the active

Table 2 Common excipients in vaccine drug product development and expected impact in product

Excipient	Common examples	Impact in formulation
Salts	Ammonium sulfate, calcium chloride, sodium chloride, magnesium chloride, potassium chloride	Tonicity modifier
Buffers	Succinate, sodium phosphate, potassium phosphate, histidine, hepes, tris	pH
Sugars and polyols	Cyclodextrin, sucrose, sorbitol, trehalose, lactose, glycerol, mannitol	Stabilizing effect
Amino acids	Arginine, proline, glycine, glutamic acid, aspartic acid	Stabilizing effect, aggregation modifiers, bulking agents
Surfactants	Poloxamer 188/407, polysorbate 20/80, sodium lauryl sulfate	Air surface interfaces, mitigation of surface adsorption
Antioxidants	Ascorbic acid, glutathione, methionine	Prevention of oxidation
Polymers	Dextran, polyethylene glycol	Bulking agents, freeze-point depressors
Preservatives	M-cresol, phenol, 2-phenoxyethanol, chlorobutanol, methylparabens	Antimicrobials

product (mcg/mL) levels limit the analytical tool kits for vaccines and can challenge the limit of detection increasing the challenge in establishing the necessary stability profile required to achieve the QTPP requirements. To emphasize the challenges with analytical development, two different examples are presented below.

The first example where characterization of the degradation mechanisms is truly challenging is associated with live-attenuated vaccines. These vaccines usually consist of crude cellular extracts that have multiple proteins and components. This can often lead to significant matrix effects in the characterization and stability assays. Furthermore, in live-attenuated combination vaccines (e.g., ProQuad®), stability assays must be developed carefully to ensure there is no interference across vaccine components [13]. In many cases, it may be necessary to inactivate with anti-sera to get a true measure of one virus' stability profile [13].

Another example where analytics can be challenged is in the case of Prevnar®13. Here, there are 13 different serotype-specific conjugates combined into the vaccine. The dose level associated with 12 of the serotypes is at 2.2 mcg while the 13th is 4.4 mcg [29]. Having the analytics to tease each serotype stability independent of the others is difficult and ensuring that the sensitivity within the assays is met places a significant burden on the analytical method. Additionally, with the low-dose levels, there can be a potential for surface adsorption of the antigens during routine testing. As a result, the analytical scientist may need to add surfactants into their assay buffers to mitigate the loss of antigen in the actual assays. This could impact the assays that can be utilized for determining stability indication and need to be carefully considered.

Each stage of the formulation development is further described in greater detail below. This is usually developed in a staged approach and may include: preformulation, early-stage formulation development, drug product optimization, establishment of design space, and late-stage formulation development. For clarity and alignment with this book regarding lyophilization development, the discussion around vaccine development and the use of lyophilization have been specifically mentioned in a stand-alone format. Additionally, guidelines to achieve these stages and relevant case-studies are presented below.

Stage 1

Preformulation

One of the main goals during preformulation development is for the formulation scientist to initiate understanding and characterization of the respective antigen [19, 22–24]. Preformulation allows the formulation scientist an ability to overcome any inherent physical or chemical instabilities associated with the respective antigen and improve the antigen prior to moving into full preclinical development. At this stage of development, the availability of robust stability-indicating assays are lim-

ited and usually in the development stages in the analytical laboratories. To aid in developing stability-indicating assays, the formulation scientist usually will stress samples and provide to the analytical areas for generation and evaluation of monoclonal and polyclonal antibody reagents to shape the in vitro potency assays as the project continues through development.

Both biophysical and biochemical characterization is completed and multiple preclinical animal studies need to be conducted to try and identify the proper antigen/adjuvant combination and impact of minor process and formulation changes on immunogenicity [30]. This work is essential so that as the program continues to advance within the pipeline, a fundamental understanding of the degradation pathways for the product are known, enabling formulation development to focus on ways to minimize degradation and enhance the stability of the vaccine target.

Often due to limited thermal stability of vaccines, especially LVV and their expected global markets, examining lyophilized formulations for LVVs is preferred in the preformulation space to align end-to-end with commercial process. However, due to lack of drug substance (DS) availability in preformulation and capital and time-consuming lyophilization development, early stage preformulation may focus on identifying a stable frozen formulation to combat stability challenges associated with LVVs and phase I clinical development. Such knowledge gained during preformulation will help shape the design of future experiments and can be utilized in building the QbD approaches and steps to examine later in formulation development.

In preformulation experimental studies, the vaccine candidates are usually examined in both liquid and frozen conditions rather than in lyophilized forms. Liquid formulations allow a quicker read on the degradation pathways associated with the vaccine and expedite product development. In addition, liquid formulations are more convenient when completing early preclinical animal studies. Field-mixing of the antigens and potential adjuvants is easier since compatibilities may not be known and stability profiles are still being explored.

Thus, in summary, the main goal during the preformulation activities is to help the discovery areas identify the proper antigen(s) to bring forward as a preclinical candidate. Additionally, the formulation scientist is working to characterize the antigen as best they can so that as the program progresses, the main degradation mechanism has been outlined. After successfully completing preformulation development, it is likely the program has advanced into preclinical development and the main focus for the formulation scientist will be on completing initial screens to identify a formulation that will lead to a safety assessment/toxicology formulation.

Identifying an Initial Formulation for Safety Assessment and Toxicology Studies

After entering preclinical development, the formulation scientist is charged with identifying a formulation that will be utilized in safety assessment/toxicology studies. The main screening in this initial stage will likely examine the common ex-

cipients and factors that need to be outlined to ensure an acceptable formulation is achieved. These include formulation screening as a function of pH, excipients, buffers, adjuvant, and their interactions, and therefore it is recommended that the formulation scientist should begin exploring ranges around them to build in robustness to the product. Utilization of DoEs allows multiple combinations of the formulation to be explored and optimized efficiently.

Design of experiments within vaccines is, at a minimum, broken down into two stages. The first stage will be a low-resolution study where a broad range of conditions for each input is explored (e.g., for pH a range from 4.0 to 8.0). Once the initial factors/interactions are identified that impact the product significantly, a more detailed exploration can be accomplished in stage 2 of DoE to establish the design space for the vaccine product. In addition, simulation and modeling efforts, where possible, can aid in reducing the number of experiments required in establishing the design space. Common factors explored within DoE for a vaccine formulation are outlined below (Table 3).

After a better understanding of the degradation mechanisms and the factors within the formulation that impact product stability is established, the formulation scientists can now focus on developing a stable product that will meet the necessary GTPP. It is however recognized that timeline/resource constraints might limit the detailed exploration of degradation mechanism and a business need might drive the product development forward with a suboptimal formulation. It is our recommendation that CQAs must be identified, investigated, and the formulation variables to ensure the stability is enhanced are explored. Here, the proper delivery method is explored, dose levels for the product through animal studies and early device development is determined. Once accomplished, a formulation is moved forward into safety assessment/toxicology studies and into phase I. The formulation may not be fully optimized, but there is a strong understanding of what will be required to meet the necessary GTPP and a formulation process, albeit not optimized for routine manufacture is in place so that clinical manufacturing at the pilot-scale can be achieved.

Table 3 Common factors explored in vaccine formulation development DoE

Common factors	Ranges investigated
Buffer species	Phosphate, histidine, succinate, citrate, acetate, tris
Buffer concentrations	5–50 mM
pH	4.0–8.0
Salt concentrations	0–300 mM
Sugar (sucrose, trehalose, lactose, etc.)	1–10%
Surfactant concentration (PS80, PS20, P188)	0–0.3%
Adjuvant	Aluminum phosphate, aluminum hydroxide, (proprietary adjuvants like MF59, AS03, AS04, etc.)
Antigen concentration or potency target	1–5 mcg/mL

DoE design of experiment

Early Stage Formulation Development (Identification of Safety Assessment/Toxicology Formulation):

Based on initial efforts in preformulation, the formulation scientist can begin to determine whether a liquid formulation can be achieved. However, since preformulation efforts are minimal, it is necessary for the formulation scientist to assess both real time and accelerated stability within the formulation development space and assess the ability to generate a liquid product that will meet the necessary GTPP characteristics [19]. In the event that the liquid stability data will not align with the desired GTPP, the development of a lyophilized or frozen product will be initiated. Data generated during the liquid formulation screening early in formulation development can be then utilized to aid in identifying a good lyophilization formulation for the vaccine product (if required).

Liquid formulations are preferred when compared to lyophilized formulations [10]. This is driven by many factors including convenience of delivery to the patient, minimizing the operations within manufacturing, and not requiring a reconstitution time in the patient setting. Although preferred, developing a liquid formulation that will align with the desired GTPP can be challenging, especially with the desired 2–3 years shelf life expected for vaccines to enhance drug product supply.

One specific example where the likelihood for a liquid vaccine product is low is when developing an LVV. From experience, liquid degradation rates can be as high as 10% per hour even under refrigerated conditions [13]. Additionally, it has been observed that during freeze-thaw of frozen liquid material, losses of greater than 30% can be observed [13]. As a result of these issues, developing a liquid formulation is not feasible and lyophilizing the product becomes a viable, and in most cases the only option.

Similar to preformulation efforts, an in vivo animal model will be utilized to help shape the final formulation, as well as the formulation process for both DS and DP. This ensures that any changes during DS processing and DP formulation and filling do not impact the immunogenicity of the product. It should be noted, that although in vivo models will help the formulation scientist during development, there are limitations in correlating animal study results with human immunogenicity. The preclinical animal models can be utilized to infer differences, but usually can be considered disaster checks for gross changes in the process or final drug product and the impact to immunogenicity. Identifying a definitive in vitro immune marker to build a correlation of protection with human immunogenicity is usually lacking and can impact the development timelines. As a result, making changes within the formulation or formulation process after initiating clinical development is challenging and usually met with significant resistance once entering the pivotal clinical studies for efficacy (phase II). If changes are necessary following phase II, it is likely that additional clinical bridging studies will be required to demonstrate comparability, this in turn increases the overall development time for the product as well as development cost. Thus, it is important that most of the formulation development and optimization has been completed before entering phase II.

Developing good quality control for vaccines has been a challenging task. One of the main reasons around this is the ability to define the CQAs associated with

a vaccine, especially for LVV. The formulation and formulation process relies not only on the lot release assays, but the consistency in which the manufacturing process is completed and is a large part of establishing vaccine quality. This usually results in the formulation and formulation process defining the product.

Stage II

Vaccine Drug Product Optimization (Exploration of the pH, Excipients, Stabilizers, and Process Conditions)

After developing a formulation for safety assessment/toxicology and the initial phase I clinical development, the formulation scientist must begin to "optimize" the formulation for long-term commercialization.

Usually, experiments are completed to assess various stress conditions including thermal stress, agitation, impact of freeze-thaw, exposure to light, and interactions with formulation process conditions (i.e., mixing, pumping, tubing, filtration). These different stress conditions help determine the proper conditions to generate a robust formulation so that the product can be scaled and commercialized [19]. This is often achieved by further evaluating and optimizing various buffers, pH ranges, excipients, and ionic strengths to try and identify the ideal conditions for the vaccine target and achieve the stability profile necessary to support the GTPP.

As discussed earlier, vaccines usually consist of multiple antigens and the formulation scientist should examine both the combined final drug product as well as separate the multicomponent vaccine into monovalent vaccines during formulation screening and development. Since analytical methods are usually still often in development for the multicomponent vaccines, examination in a monovalent format will allow the formulation scientist the opportunity to better characterize the product and identify degradation mechanisms for each component found within the vaccine. By better understanding monovalent stability, the formulator can balance the stability profiles effectively to achieve the final multicomponent drug product. At times, there will be compromises on the stability of one or more of the multicomponents to ensure that a final drug product can support the GTPP. Additional CQAs may be required for conjugated or subunit vaccines where an adjuvant may be included and will be considered on a case-by-case basis.

The formulation scientist must also consider that any changes to scale, the formulation itself, or the formulation process requires ways to ensure comparability before and after that change. This comparability will help to ensure that there is no change to the safety and efficacy associated with the vaccine and is required by regulatory authorities to justify the changes. Comparability protocols and extensive analytical characterization must be put in place to justify the changes. Even when comparability has been established, regulatory agencies may still require additional clinical trials to show equivalency between the new and old vaccine.

Currently, regulatory agencies in both Europe and the USA have guidance in place for ensuring vaccine quality. One example of this is the Center for Biologics Evaluation and Research (CBER), the regulatory body responsible for vaccines within the USA, is responsible for release of all commercial lots of vaccines and can conduct testing of manufactured vaccines on a routine basis.

Late-Stage Formulation/Drug Product Development

In this stage, the end-to-end process (drug substance to final drug product) is explored, defined, validated, and utilized for manufacturing of the vaccine product. The formulation scientist along with process engineers works to ensure that a robust process has been defined with range finding around the formulation and process. Failure modes are identified and the control strategy is established to ensure successful commercialization of the product. Scale-up and tech transfer are often the most challenging and essential aspect of commercializing the product. The rationale and available tools, critical issues during vaccine development, along with case studies in tech-transfer principles in the context of PAT and QbD approaches are discussed below. Guideline for vaccine lyophilization development will be outlined and a recommended approach for ensuring a robust process for tech transfer will be discussed.

Application of QbD to Vaccine Formulation Development

Defining the Global Target Product Profile

Similar to TPs and small molecule products, establishing a well-defined GTPP is critical to the success of the vaccine program. The GTPP consists of required stability profile to meet the global markets, route of delivery (e.g., subcutaneous), dosage regimen, reconstitution time (if dried vaccine), and primary package.

As stated in the ICH Q8 R2 document: “The quality target product profile forms the basis of design for the development of the product. Consideration for the quality target product profile could include:

- Intended use in clinical setting, route of administration, dosage form, delivery systems;
- Dosage strength(s);
- Container closure system;
- Therapeutic moiety release or delivery and attributes affecting pharmacokinetic characteristics (e.g., dissolution, aerodynamic performance) appropriate to the drug product dosage form being developed);
- Drug product quality criteria (e.g., sterility, purity, stability, and drug release) appropriate for the intended marketed product.”

An example of a GTPP for a new vaccine is shown in Table 1.

The GTPP allows the formulation scientist to define the potential CQAs for the product. ICH Q8 (R2) defines a CQA as "A physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality."

Input from preformulation and formulation development as well as prior knowledge from similar vaccines are essential in establishing the initial CQAs associated with the vaccine candidate. During formulation development, the CQAs are further explored and optimized. It is commonly accepted that risk assessments are essential to understand which processes within the vaccine product are considered critical.

Quality by design (QbD) within the vaccine development space, as well as other modalities, has been evolving. QbD approach requires that the quality is built into the process and not into the final drug product. This requires the formulation scientist to understand variability associated with raw materials, equipment changes between scale, and correlation of CQAs between product and process [31]. As mentioned above, QbD approaches are not applied holistically to vaccines due to their complexity and inherent variability, especially LVV. As a result, a combination/hybrid approach will be the norm in regulatory filings for vaccine products. For example, lyophilization is one unit operation where QbD can be applied for vaccines. It should be noted that although QbD is being applied more routinely to vaccine product, regulatory agencies do not expect to give the manufacturers regulatory relief when applied. It is assumed that this is building better quality into the product, but does not ensure comparability when minor changes within the design space occur.

End-to-End Development for Vaccine Lyophilized Products

Early in preclinical development, the availability of bulk (DS) is limited for formulation, analytical, and DP process development. Lack of well-established stability-indicating assays, limited knowledge on factors impacting product quality (CQAs), the inability to utilize platform formulation approaches for vaccines, especially LVV, and the ability to establish a LOS (line of sight) to clinical and manufacturing facility limits the DP scientist in how to optimize the formulation and process while documenting the corresponding risks.

Due to the poor stability profiles, vaccines suffer from substantial losses during the formulation and filling processes. These include bulk thaw yields, time out of refrigeration losses, liquid degradation, and lyophilization yields. This results in an overall process yield from the DS to the final DP. Additionally, the DP scientist must account for losses during routine shipping and storage following release of the product. Given the complexity of LVV, the use of accelerated stability to determine final DP characteristics likely will lead the scientist astray. Real-time statistically powered stability is key to choosing the optimal formulation for a vaccine product. This is due to the lack of following Arrhenius kinetics and the high variability associated with the analytical methods to determine stability.

Lyophilized formulations are common within the biopharmaceutical area, especially when associated with LVV. Selecting the optimized formulation either through DoE or empirically, as described above, needs to meet the GTPP and the necessary CQAs related to a lyophilized product (i.e., reconstitution time, moisture level, cake appearance, etc.) [32–34]. Additionally, a technology transfer protocol and a robust manufacturing process will need to be completed to ensure success of the program. The fundamentals and guidelines associated with lyophilization formulation development are described in greater detail below.

Lyophilized Formulation Development

When starting lyophilization development, the critical properties to understand include but are not limited to the glass transition temperature (T_g, T_g), collapse temperature (T_c), the phase behavior of the formulation (amorphous vs. crystalline), liquid degradation rates of the product, and overall yield and stability requirements [33, 34].

Given the fact that the lyophilization process generates a variety of stress on the vaccine product, the formulator must balance these to achieve an optimized product. Some of the common stresses include pH shifts during freezing, freezing method, and type of ice crystals formed during the freezing process, potential phase separation, and in certain instances cold denaturation of enveloped viruses in the freeze concentrated state [14, 16, 36, 37]. In addition to identifying the proper formulation to mitigate product stresses, the formulation scientist should also examine the actual lyophilization conditions to improve product success. Both the formulation and lyophilization cycle are inter-connected [32, 38–41]. Factors impacting formulation selection have been detailed above, while factors impacting lyophilization are described below. In general, lyophilization consists of three stages: freezing, primary drying, and secondary drying [42]. Each stage generates its own stress on the vaccine product and should be explored independently and in conjunction with the others. The reader is reminded that basic lyophilization consists of a drying chamber with a heat transfer fluid circulated through shelves, a condenser that is connected to the drying chamber through the spool piece, and a refrigeration/heating/vacuum system to control the temperature and pressure of the cabinet. During a scale-up process, environmental conditions associated with the formulation process, shelf temperature, radiation effect, chamber pressure, process time, and process monitoring capabilities could account for scale-up differences between laboratory-, pilot-, and commercial freeze-dryers [43]. Furthermore, it is recommended that the significance of these aspects must be studied early in program development to avoid delays in scale-up and technology transfer.

Furthermore, the highly labile nature of LVV renders freeze-drying a challenging task. An example is the respiratory syncytial virus (RSV) which has been shown to be highly thermolabile even when stored with excipients at refrigerated and frozen conditions. As a result, the lyophilization cycle will result in poor drying yields and stability profile with minimal process changes resulting in significant potency losses. Similarly, Zostavax® a live enveloped virus manufactured by Merck® is stored

either frozen or refrigerated following lyophilization due to the poor thermal stability of the dried product [44]. Thus, the significance of each step in optimizing the product quality cannot be overlooked. Each stage of the lyophilization process is outlined below in greater detail.

Stage 1: Freezing

Destabilizing stresses associated with stage 1 of the lyophilization process may include degradation at the ice–water interface, increase in ionic strength or shifts in pH during the freeze-concentrate state, and potential cold denaturation and aggregation of the product during freezing.

The function of freezing is to convert water into ice and is usually carried below the T_g' or T_{eu} (eutectic temperature), or T_c (collapse temperature) associated with the formulation [33, 35]. The T_g' and Teu can be obtained using differential scanning calorimeter (DSC) while T_c measurements are obtained using a freeze-drying microscope. Ice crystal formation is impacted by super-cooling nucleation and the rate of freezing. Slower freezing rates result in larger ice crystal formation, while LN2 (liquid nitrogen) blast freezing will result in microcrystals being formed. Although larger ice crystal structures are generally easier to dry during primary drying due to their large pores, LVVs are usually blast frozen to minimize time in solution due to high liquid degradation rates. This results in the microcrystal structures and increased mass resistance during drying, in the end leading to more conservative and lengthy cycles to minimize cake collapse.

For subunit vaccines, an annealing step may add value to the process. Annealing allows the crystallization of a bulking agent (i.e., glycine, mannitol, etc.) and redistribution of ice crystals into a more homogenous cake morphology. Annealing may also help minimize the freezing inhomogeneities that could occur between a laboratory-, pilot-, and commercial-scale lyophilizer due to differences in particulates present since GMP manufacturing occurs in a low-particulate environment (Class 100) [45].

The reader is reminded that unwanted crystallization during storage could severely impact the stability of the product due to increased moisture content and corresponding lower T_g. Formulation selection, when utilizing a crystalline component, must be achieved in a way that allows successful crystallization during annealing [46]. This can be achieved by maintaining a ~2:1 ratio of crystalline to amorphous material [47]. Similarly, surfactants may be added to the formulations [40] due to the potential of the vaccine to degrade ice–water interfaces and also to prevent adsorption to glass surfaces, especially for subunit vaccines.

Stage 2: Primary Drying

The frozen water is sublimed in the primary drying step (i.e., lowering the chamber pressure and raising the shelf temperature). Primary drying optimization is often considered a challenging task due to that fact that the product temperature is indi-

rectly controlled using shelf temperature and chamber pressure and is dependent on formulation, primary container, and the corresponding apparent heat transfer coefficient. Primary drying, however, can have significant economic impact as it is the longest step in drying. In general, it is recommended that a conservative cycle is obtained by maintaining product temperature several degrees (~2–5 °C) below collapse temperature which in turn is a few degrees below glass transition temperature [48]. Operating at higher (scorch) temperature, however, may result in uniform distribution of bound water and temperature and a balance between cycle time and product safety must be established [49]. It must be noted that high viscosity of the formulation matrix in the frozen state can severely limit the reaction kinetics (Stokes–Einstein relationship) and it might be feasible to obtain a quality dried product with drying temperature above T_g' [50]. In certain cases, crystalline bulking agents such as glycine and mannitol (Teu ~ −3 °C) might be added to increase the collapse temperature of the product and to achieve faster primary drying. In general, approximately 5 °C increase in shelf temperature increases the product temperature by approximately 1 °C and every 1 °C change in product temperature may expedite the primary drying by approximately 13 % [16, 42]. However, in such cases, an annealing step must be added to ensure complete crystallinity and the ratio of crystalline to amorphous excipient must be > 67 %. Amorphous bulking agents such as sucrose and trehalose are also added to the low concentration solids (< 1 %) containing products to prevent "blowout" [10]. Radiative heat from the freeze-dryer walls and door can induce heterogeneity through edge effects. Additionally, ultra-low pressure may further add to inter-batch heterogeneity and even cause product contamination (pump oil, stopper components, etc.) [51, 52]. Thus, it is recommended that optimal chamber pressure that allows for fastest sublimation and homogenous heat transfer must be chosen. End of primary drying can be monitored using multiple methods such as convergence of shelf temperature and thermocouple, convergence of pirani and capacitance measurement, tunable diode laser adsorption spectroscopy (TDLAS), mass spectrometry, dew-point sensor and/or MTM/pressure rise method [10, 41]. If a thermocouple is used to monitor end of primary drying, an additional 20 % soak time must be allowed for nonthermocouple vials to finish primary drying.

Stage 3: Secondary Drying

Post completion of primary drying, the shelf temperature is ramped slowly (approximately 0.1–0.5 °C/min) to avoid product collapse while removing water by desorption during this stage. It is recommended that secondary drying must be carried out for a shorter period of time at higher temperature as the desorption rate of water decreases substantially with time. Furthermore, shorter secondary drying results in faster cabinet turnaround during manufacturing. It must be noted that the rate of desorption is dependent on product-specific surface area (i.e., freezing and formulation specific), shelf temperature, and container heat transfer coefficient. In our experience, lower moisture is not always better for stability and an optimal

moisture level is often desired especially for LVV. Such optimal residual moisture and the corresponding glass transition for each product must be empirically quantified along with their drying yield, molecular mobility, and stability to ensure successful long-term shelf stability [53].

PAT Development

In August 2002, the FDA announced an initiative, "Pharmaceutical Current Good Manufacturing Practices (cGMPs) for the 21st Century—A Risk-Based Approach," to improve the overall understanding of the product and process better in the pharmaceutical industry [54]. The process would be for the addition of process analytical testing (PAT) to occur during development and in manufacturing.

PAT allows for quality to be a part of the process and product, rather than relying on testing during release. The definition of PAT is "a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process material and processes, with the goal of ensuring final product quality" [55]. PAT allows one to examine the process and determine the process performance by using risk-management tools as well as online sensors to monitor, control, and design the process [56].

Thus, PAT should improve the desired state of the manufacturing process and result in the following:

Manufacturing processes are designed efficiently and effectively, and thus improve the quality of the product and an improvement in the performance of the manufacturing process.

- A better mechanistic understanding of the formulation and process which results in improving the product performance and sets the product and process specifications based on knowledge learned during development.
- Continuous feedback during the formulation process by online monitoring improving the product quality. "Real-time" feedback loops.
- Based on the current level of scientific knowledge, regulatory policies and procedures are tailored to the product and process understanding.
- Using PAT allows a better understanding of the formulation and manufacturing process that impact product quality and consistency, as a result PAT allows for better risk-based regulatory approaches to be considered.
- PAT allows for better process control strategies to be implemented and improve the overall performance of the product and improve product quality.

PAT's main goal is to improve the formulation process and product to ensure final product quality. This is accomplished through designing a system that analyzes samples in real-time and can control the manufacturing process. The PAT should not only consider the process but also understand the key inputs into the system including raw materials and in-process intermediates.

Regulatory agencies believe that quality should not and cannot be tested into the product, but should be built into the process [55, 57]. Utilizing PAT allows the development team and manufacturer a thorough understanding of the product and allows for better knowledge of the process. This includes utilizing the GTPP to understand the desired target, patient population, and route of administration. Additionally, it ensures that the design of the product and product image are amenable to the GTPP and achieve the desired chemical and physical stability necessary to enter the desired markets without impacting product quality. Finally, to ensure that a reproducible and robust manufacturing process has been developed, leading to a stable final product, the design and implementation of a manufacturing process utilizes principles of engineering, material science, and quality assurance.

Process analytical tools' main goal is to aid in the design and development of a sound fundamental understanding of the product and processes to ensure a high-quality product is produced. Ensuring a fundamental understanding of the process is a basic tenet of QbD and could potentially reduce risks to the product during manufacturing and lead to a more robust product. The long-term benefit of PAT will vary depending on the product and process where employed. The potential gains in efficiency and product robustness can include [55, 58]:

- Reduction in overall cycle time and increasing capacity within the manufacturing facility.
  - Utilization of online monitoring allows for efficient product development and control.
  - Improved efficiency in the process and increasing capacity within the facility leads to reduction in energy consumption.
- Minimizing rejected material by implementing more automation within the process, leading to reductions in human errors and improving product quality.
  - Results in improved yields for the manufacturer by lessening rejects, scrap, and need to reprocess.
- Real-time release of the final product and facilitating continuous processing to manage variability within the manufacturing facility.

A well-characterized process should be defined as follows:

- Areas of variability have been identified and explained.
- The process can manage the variability associated with the product.
- The product quality attributes can be predicted over the entire design space including the process conditions, raw material inputs, environment, and other conditions.

Although PAT within the small-molecule space is common, implementation into the vaccine and biotherapeutic area has been slower and more challenging. Some of the reasons for the slower adoption in the space are listed below:

- Proteins and vaccines are significantly larger in size, and can be very heterogeneous in nature. Additionally, vaccines can be multicomponent making it difficult to track the individual components with current PAT instrumentation.

- The formulation and filling processes associated with vaccines are more complex. This results in multiple batch records, increased product quality and consistency tests, and the number of critical process steps are increased [59].
- The sensitivity of a vaccine to the final manufacturing process is extreme. Thus, there can be substantial lot-to-lot variability when manufactured using the same process and that can impact product quality. In addition, raw materials utilized within the process can be complex and vary from manufacturer to manufacturer. This variability in the raw materials can impact product quality as well and should be carefully monitored [60, 61].
- Building a strong link between product quality attributes and the clinical safety and efficacy for vaccines is difficult to ascertain [59, 60]. This results in differences in how PAT is employed with vaccines and where within the process, if at all, it is utilized to its full potential [58, 62].

Applying PAT in Drug Product Operations

PAT tools that have been utilized in traditional small molecule development and approach could be employed in the vaccine space as well. These technologies, while not fully discussed here, include Raman spectroscopy with wide area illumination (WAI) [63], laser-induced breakdown spectroscopy (LIBS) [64], and nuclear magnetic resonance (NMR) [65].

For example, as described previously, in vaccine development and especially in the case of an LVV, lyophilization is a common unit operation. Here, water is removed for the product by first freezing and then sublimation of water occurs in both primary and secondary drying. Lyophilization is a long unit operation in manufacturing and delivering an efficient cycle is critical to minimize production costs and ensure product quality is achieved. Because of the criticality of this unit operation, it is a desired place for implementation of PAT. Real-time monitoring of residual water during lyophilization can be achieved by utilizing TDLAS. This technology can measure the water vapor pressure between the lyophilization cabinet and the condenser and can predict the end point of sublimation and secondary drying [66]. Understanding the end points of both primary and secondary drying, a more efficient and cost-effective unit operation can be achieved.

Similarly, as a follow-up to ensure that what has been determined through TDLAS as an efficient lyophilization cycle, near infrared technology (NIR) can be used to examine the final moisture content of all vials following lyophilization [67, 68]. Moisture is a CQA associated with vaccines and can significantly impact stability. Historically, moisture analysis was done on a subset of vials within a lyophilization cabinet run and was a destructive test. With the implementation of NIR, 100% of lyophilized vaccines can be analyzed for moisture in a nondestructive way. By now, monitoring the moisture associated with all vials, improved product quality can be achieved. Vials where moisture levels in the past may have been missed due to a small sampling analyzed, now can be fully monitored and discarded when

outside the quality specifications. This should result in better safety and efficacy of the product in the patient's hands.

PAT can be implemented in the vaccine space and will begin to become more common within the vaccine drug product development arena moving forward. Although beneficial, understanding where to implement is still being evaluated. Drug product operation that appear to fit well with PAT (i.e., lyophilization, moisture analysis, mixing times) across modalities will likely see more implementation in the future. Utilization of PAT will continue within the vaccine space and allow the formulation and process scientist a better understanding of the product and process.

Relevant PAT tools and principles, as described below can enable process optimization and control while improving efficiency and addressing the limitation of time-defined end points. PAT tools can be characterized into:

- Multivariate tools for experimental process design and ability to acquire data and analysis.
- Process analyzers for collecting process data relevant for QA and regulatory decisions.
- Control tools to aid in better understanding the link between the process design and CQA.
- Continuous improvement through data analysis and knowledge management for establishing understanding of multifactorial relationships.

Additionally, risk based, integrated systems and real-time release allow evaluation and builds quality in-process and final product.

For additional information, the reader is referred to the Guidance for Industry: PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance [54, 55, 57].

Scale-Up to Clinical Development/Early Launch Commercialization

A critical step in vaccine development is scale-up of the process from the laboratory-scale operations often used for preclinical studies to pilot and ultimately commercial scale operations required for the production of clinical supplies and marketed product. The scale-up and technology transfer from a developmental laboratory environment to a production environment increases capacity, assures compliance with GMP requirements, and preferably improves the consistency and overall robustness of the process. The primary objective at each stage of scale-up is to preserve the CQAs that assure the safety and efficacy of the product. Secondary objectives often exist to maximize facility throughput and minimize the opportunities for manufacturing deviations. Thus, the scale-up process includes careful consideration of both the technical aspects of each manufacturing unit operation as well as the GMP, quality, and operations systems within each manufacturing environment.

Numerous sources are available that describe considerations for transfer of a lyophilization cycle developed at laboratory scale to a pilot- or commercial-scale aseptic processing environment [51, 69–71]. A brief summary is provided here, but the reader is directed to the references for greater depth. Lyophilization is a controlled heat and mass transfer process, primarily leveraging chamber pressure and shelf temperature to drive the sublimation process and bound water desorption. Equipment and environmental differences between the laboratory and production suite result in significant differences in product microstructure, equipment capability, effective heat transfer rates, and ultimately, drying results when the same drying cycle parameters are used. Choices made in the design and installation of the production freeze-dryer can significantly affect the driving forces for sublimation, leading to potential differences in the speed and consistency of the drying process within and between runs. A successful scale-up program will evaluate the ability to maintain control of heat and mass transfer throughout the process and across the intended range of batch sizes for each freeze-dryer.

Environmental and Equipment Considerations

One of the first differences to manifest in scale-up is a result of the increasing environmental controls that are required in the production environment. Process operations from vial filling to loading of the lyophilizer are completed in a class 100 environment for parenteral products. As a result of this very clean environment, few particles are present to serve as ice nucleation sites. A greater degree of product supercooling prior to freezing occurs in the production suite than in the laboratory [72]. This can lead to extended primary drying times of up to 10% and a 1 °C increase in primary drying temperature. A useful generalization is that primary drying times increase by ~2% for each 1 °C decrease in the ice nucleation temperature. Thus, cycle modifications are often desirable even before consideration of equipment differences between laboratory and production scale lyophilizers simply due to cascading consequences from the cleaner production environment.

Ice nucleation is a stochastic process, and a contributor to the vial-to-vial variability observed in both laboratory and production processes. One approach that is used to overcome the vial-to-vial variability in ice nucleation and the high drying resistances associated with more significant supercooling is frozen product annealing above the glass transition temperature.

An important step in scale-up or transfer of a lyophilization process to a new freeze-dryer is characterization of the heat transfer rate by position within the freeze-dryer. This should be established for both the laboratory unit used in development as well as the destination production unit, as no two units (even those of identical model) are truly identical. Understanding the differences in positional heat transfer rates, caused by a variety of equipment design and use parameters, is important from several perspectives. First, from a product quality standpoint, this will result in vials having different drying rates. Care must be taken to ensure that primary drying

time is sufficiently long to allow all vials to complete this step. If the time to accommodate the lagging vials is not appropriately factored into the cycle, it is possible to cause product collapse when ramping to secondary drying. For particularly labile products such as LVV, vials with the fastest heat transfer coefficients during secondary drying may be exposed to warmer temperatures for an extended period while the slower vials continue to come up to temperature, which may have negative product quality consequences. Large differences in the vial heat transfer coefficients may limit the robustness of the cycle to unanticipated manufacturing deviations. Second, from an operational perspective, significant heterogeneity in heat transfer coefficients leads to longer overall cycles and thus limits facility throughput.

Typical approaches for establishing the effective heat transfer coefficient by vial position include partial sublimation studies under representative load conditions. In these studies, a lyophilization run is simulated using pre-weighed water filled vials. The run is stopped prior to completion of primary drying and the mass sublimed for each vial is used to estimate the effective heat transfer coefficient at that selected location. Significant differences in the effective heat transfer coefficient often exist based on shelf as well as location within a shelf, with differences of 15% not uncommon (Table 4). Many factors contribute to these observed differences, including (1) nonuniform shelf surface temperatures, (2) position-dependent radiation, (3) sizing and design of the condenser, spool piece, port, and refrigeration system, and (4) vial loading approach.

Nonuniform shelf surface temperatures can be caused by design, installation, and operational factors. Heat transfer fluid should be uniformly distributed to all processing shelves with well-designed serpentine flow or baffling systems. Shelf to shelf imbalances in the flow of heat transfer fluid, as well as blocked passages within a shelf, can be detected by a variety of field tests. Multi-shelf temperature mapping studies that include unsteady state conditions, such as transition to new shelf temperature set points, can be particularly useful for start-up of new lyophilizers to confirm proper installation and operation.

Radiative heat transfer effects, which often manifest as edge effects, can be significant contributors to the total heat flow to the product under conditions typically used for primary drying and should be evaluated as part of the scale-up process. Studies have shown that vials in proximity to chamber walls and doors can have considerably different effective heat transfer rates, and hence drying times, than

Table 4 Variations in heat transfer coefficients across laboratories and manufacturing scale lyophilization cabinets

Lyophilization cabinet	H_{center} (W/m^2 *K)	H_{corner} (W/m^2 *K)	Inhomogeneity ratio
Lab-scale unit 1	5.1	16.4	3.21
Lab-scale unit 2	4.5	13.1	2.90
Lab-scale unit 3	4.7	13.7	2.89
Commercial unit 1 site A	5.6	13.5	2.41
Commercial unit 2 site A	3.7	12.8	3.41
Commercial unit 3 site A	3.7	13.2	3.48
Commercial unit 1 site B	7.7	22.3	2.81

interior vials, due to the difference in emissivity values for chamber walls, shelves, view ports, and door. Various design choices at the laboratory scale as well as production scale can influence the extent of radiative heat transfer contributions. For example, laboratory freeze-dryers are typically equipped with a transparent door that has been shown to induce drying rates up to twofold higher for edge vials than interior vials. For such units, metal door shields (even as simple as aluminum foil) can be helpful to minimize extreme edge effects. Production units often have features to minimize the extent of radiative heat transfer, such as temperature controlled walls, stainless steel doors, and low emissivity electropolished surfaces (although this may change with equipment age and the repeated exposure to cleaning and sterilization cycles).

Understanding the degree of radiative heat transfer in the system used for development studies, as well as the destination lyophilizer, is critical when thermocouples are used to assess the progress of the drying cycle. Best practices for aseptic manufacturing require that operators not reach over the vials to place thermocouples, therefore when thermocouples are used they are often only placed in front edge vial locations, which can have different drying rates than the interior vials that comprise the majority of the batch.

The sizing and design of the condenser and refrigeration systems influence the maximum allowable sublimation rate that can be supported without loss of pressure control. Additionally, these systems can influence the minimum operating pressure of the freeze-dryer. As part of scale-up, it is critical to design studies that span the range of intended pressures and load conditions. Partial load studies, which do not address the high load, high sublimation operating conditions, are necessary but not sufficient. In addition to partial load studies, it should be confirmed that the intended cycle can run without loss of pressure control at maximum load. Extreme minimum load studies are helpful to evaluate the impact on drying times for pressure controlled cycles using an inert gas such as nitrogen. Under these conditions, the low level of water vapor relative to nitrogen and the difference in thermal conductivities of these gases can result in a significant reduction in heat transfer coefficients, leading to extended drying times.

Choke flow tends to be a greater concern with laboratory lyophilizers than production units due to port and pathway design differences [73]. The exit of vapor into the condenser relative to the condenser coils or plates can also be critical. Under some sublimation conditions, it is possible to have ice buildup immediately at the entry into the condenser that, over the course of the run, effectively constricts the pathway and impedes controlled operation. Visual observations of the condenser ice buildup should be made to determine if an extension tube on the pathway exit into the condenser is needed to prevent choke flow.

The most common approach for loading vials into the lyophilizer is the use of bottomless trays, in which the vials sit directly in contact with the lyophilizer shelf. Alternate systems in use include perforated aluminum trays and stainless steel trays. The use of such alternate systems introduces additional sources of variability, particularly as trays are subjected to repeated cleaning and sterilization cycles that can lead to slight warping and suboptimal contact with the lyophilization shelf. If not properly controlled, this can lead to high rates of collapsed product.

Systems Considerations

There are several other elements of the process and equipment design that, when looked at as an end-to-end process, have the potential to significantly impact the quality and consistency of transferred process. One example is the choice of lyophilizer door design, often related to the decision to accommodate automated vial loading. Commercial lyophilizers are in use with standard, side-hinged, full doors that enable access to the entire chamber from top to bottom as well as slot-style "pizza" doors that only expose a few shelves at a time and are most amenable to automated loading. Differences in the loading and shelf indexing approach used in these systems should be considered from a standpoint of operational ease and efficiency as well as compatibility with intended product types. For example, the product warming associated with multiple openings of a standard lyophilizer door and multiple shelf indexing steps was found to result in unintended and variable product annealing for a labile live virus product transferred to the lyophilizer in prefrozen vials [74]. While not a standard industry process, this example highlights how small operational choices, such as the lyophilizer loading and indexing procedure, can influence final product quality outcomes.

The extent of process automation to pursue should balance total system cost, consistency, and flexibility. While manual and automated systems can both deliver high quality products, there are some considerations to keep in mind both for the transition from laboratory to production scale as well for routine production-scale operation where automation is concerned. First, many activities that were performed manually during laboratory development will be automated in a standard production line. One example of this is the automated partial stoppering of vials that occurs on filling lines. Drying cycles should be designed to be robust to the typical line setup variability and resulting small differences in stopper position.

Second, the implementation choices for automating a process can have unexpected consequences for supply chain management and process validation. For example, a sample that was easy to obtain for a manually loaded and unloaded process at laboratory or production scale may be very difficult to obtain for an automated process. If nonstandard supplemental samples are expected to be required as part of process validation activities or potential manufacturing deviation resolution, forethought should be given to the process for obtaining such samples, including alignment with existing media challenge interventions. The cause for such sample challenges can range from lack of human operators in the processing area where nonstandard samples would need to be collected to handling and subsequent automated transfer concerns caused by disruption of tray or shelf pack integrity. One example of unintended supply chain consequences is from an automation choice in a new facility that leveraged automated transfer carts for movement of vials in and out of the lyophilizer on a shelf by shelf basis. Although this system greatly streamlined certain operational steps and improved sterility assurance, it could only accommodate transfer of full shelves, equivalent to thousands of vials. Therefore, it was highly desirable to plan batches that minimized partial shelves, as partial shelves would have to be backfilled with empty glass and represented lost lyophilization capacity. This represented a new planning constraint that needed to be managed for efficient operation. An example is shown below.

System considerations

- Manual versus automated systems
  - Partial stoppering by hand versus machine
  - Impact that this has on sampling
  - Impact on final product inspection
  - Pizza door versus full door, shelf indexing
- Facility and media differences
  - Formulation, order of addition, accuracy of small transfers
  - Data on more parameters may be needed—if more
  - Media source—site-to-site differences, hold times, containers/materials.
  - Operational constraints—need to fit cycle with shift coverage or fit standard rhythm.
- Overall time to perform operations—longer in production than in laboratory
  - TIS versus batch size, effect of extended mixing times
  - Product settling before freezing
  - Need for end to end
- Process monitoring
  - Laboratory versus production facility capabilities
  - Thermocouples—maybe yes maybe no—how to control?

Case Study 1: Impact of Lyophilization Cabinet Loading Process on Product Appearance

Case study I presented here is based on author's personal experience and is documented in detail in Wallen et al. [74]. Impact of loading a flash-frozen complex biological product altered the cake appearance leading to cake collapse was observed and further investigation attributed the findings to unwanted annealing. It should be noted that freezing was optimized for a liquid nitrogen freezer, a process not commonly utilized in the vaccine field. Most production scale lyophilization processes rely on shelf freezing; however, due to the sensitivity of the active ingredient within the vaccine, it required a rapid freezing process to maintain product yields. The method utilized to load the product impacted the rate of product collapse during the lyophilization process. As mentioned above, the reader is referred to the article by Wallen et al. for a detail description; only a brief relevant description is provided below.

During product development in the production setting (engineering batches) a set of lyophilization cabinets were utilized with expanded shelf loading (all shelves fully indexed). The results during those batches indicated the ability to utilize the full cabinet with minimum product impact. In an attempt to optimize the loading

Table 5 Impact of lyophilization cabinet loading process on product rejection rates

Shelves in cabinet	Collapsed loading rejection rate (%)	Expanded loading rejection rate (%)
1–4	Not utilized	Not utilized
5–6	20.0	0.2
7–8	13.2	0.4
9–11	0.3	0.2
12–13	0.3	0.4
14–15	0.2	0.3
16–18	–	0.2

process and transition to new cabinet expansion with the manufacturing setting, a new method of loading the shelves where the majority of the shelves were collapsed during loading and shelves were indexed up during loading. This resulted in an increased product collapse on the top shelves of the cabinet and unacceptable rejects (Table 5). Through root cause investigations in the manufacturing facility, it was determined that the main cause was associated with unwanted annealing during loading. This annealing step resulted in ice structure changes as well as amorphous/crystalline state and ultimately leading to product collapse. Remediation of the cake appearance impact was to discontinue the use of the top few shelves of the cabinet, thereby causing underutilization of the full cabinet for long-term production.

This study clearly demonstrates the necessity to understand the process transfer between cabinets and ensure the formulation and process scientist understands the impact of annealing on product quality. It is highly recommended that proper temperature mapping studies and the corresponding correlation between the equipment and the facilities is documented to ensure proper technology transfer can occur in an efficient manner.

Case Study 2: Equipment and Facility Design Considerations

The freeze-dryer equipment design, facility, and their operation protocol (e.g., single shift vs. continuous operation, bottomless vs. perforated trays, etc.) must all be considered to achieve success during transfer and scale-up process. An illustrative example characterizing equipment and operational considerations is presented below in Table 6.

Choke flow measurements were quantified between the two industrial dryers. For example, the choke flow measurement in the industrial dryer using ice sublimation study revealed a value (1 kg h^{-1} m^{-2} at the target cycle pressure) much greater than the value obtained for pilot scale lyophilizer (0.34 kg h^{-1} m^{-2} at the target cycle pressure) suggesting that choke flow is not a concern during the scale-up. Besides

Table 6 Differences in lyophilization cabinets between pilot- and commercial scale

	Characteristics	Pilot plant	Commercial manufacturing
Equipment differences	Shelf area	1 m^2	40 m^2
	Condenser	External	External
	Monitoring end of Primary Drying	Mass Spectrometer	Thermocouple
	Pressure gauge	Capacitance	Capacitance
Operational differences	Batch size	4000 vials	120,000 vials
	Trays	Bottomless	None-automatic loading system (ALS)
	Shifts	One 8-h shift/day	Three 8-h shift/day

choke flow measurement, the apparent heat transfer coefficient (K_v in W m^{-2} K^{-1}) of the various vials types at various location on the shelf, as obtained using gravimetric measurements, were used to correlate the drying in the two lyophilizers and correspondingly the shelf temperature and cycle times were adjusted. For example, an increased K_v for a vial type in moving the product from pilot (approximately 25 W m^{-2} K^{-1}) to commercial scale (approximately 30 W m^{-2} K^{-1}) suggested a drop of shelf temperature by approx. 3 °C. Additionally, the experimental findings were supplemented with a quasi-steady state mono-dimensional model [75], to simulate optimal drying conditions and build the appropriate design space [76]. The model and the corresponding design space were evaluated by correlating the difference in the critical process parameters and its corresponding impact of product. The findings, as measured by calculating the difference between the theoretical and the calculated value, on the pilot scale are presented below in Table 7:

Prior to active runs for process qualification and validation, engineering runs were performed on commercial scale to verify the suitability of the scale-up protocol. In the absence of active runs, appropriate surrogates (as measured by correlating, T_g', T_c, and dry layer resistance using MTM pressure rise method) were used to correlate the two processes (pilot and commercial). Table 8 summarizes various analytical tools to characterize product quality during various stages of the formulation and filling process. Primary drying time, moisture (center vs. edge vials) and cake appearance (i.e., reject rates) were used as the success criteria for engineering batches. Success of the engineering batches (similar moisture, slightly longer PD but within safety margin and acceptable vial reject rates) served as the basis to continue process validation.

Table 7 Illustrative examples depicting differential between theoretical and calculated values

Critical process parameters	Technique	Differential
$T_{product}$	Averaging 5 T-type thermocouple	0.7 °C
	Pressure rise test	0.9 °C
$\Delta T_{end\ of\ sublimation\ time}$	MKS/Pirani gauge	30 min
Δ(maximum rate of sublimation)	TDLAS	0.05 kg h^{-1} m^{-2}
Rejection rate	Cake appearance	0.2%

Table 8 Summary describing various steps of form/fill and inspection along with suitable analytical tools to characterize the product quality

S. No.	Steps	Analytical tools	Characterize
1	Freezing	DSC, FDMS, XrD, optical tomography, etc.	T_g, T_c, nucleation temperature, crystallization, etc.
2	Primary drying	Thermocouple, Pirani gauge, MTM, mass spectrometry, modeling, TDLAS, and other PAT tools, etc.	Sublimation rate, product temperature, cycle time, vapor flow resistance, heat transfer coefficient, etc.
3	Secondary drying	DSC, DVS, TDLAS and other PAT tools, etc	Moisture, product temperature, drying time, etc.
4	Post-drying	Visual inspection, karl fisher, DSC, MicroCT Imaging, XrD, SEM, BET, etc.	Cake appearance, moisture, T_g, reconstitution time, crystallinity, specific surface area, etc.

DSC differential Scanning Calorimetry, *FDMS* freeze-drying microscopy, *XrD* X-ray diffraction, *TDLAS* tunable diode laser absorption microscopy, *DVS* dynamic vapor sorption, *SEM* scanning electron microscopy, *BET* brauner-emmett-teller

Summary

In contrast to other modalities, vaccine drug product development is more challenging and requires a thorough and systematic approach to achieve success. Lyophilization adds to the inherent complexity associated with vaccine development. Additional freeze-drying steps involved in formulation, filling, and inspection of dried products (Table 8) adds additional failure mode in the system. Thus, a GTPP is recommended to be discussed and agreed upon by the team early so that a quality product and efficient processes are established with clean line of sight to commercialization. To ensure the clean line of sight to the commercial process, the early development scientists and the process engineers should be communicating early and often at the respective manufacturing sites to properly align product development. Risk associated (worse case recommended) with scale-up and unit operations known to be critical to the product must be determined on a case-by-case basis and corresponding mitigation strategies should be documented.

Unlike other modalities, in general “the product is the process” for vaccines and minor changes to the process may result in extensive analytical comparability plans to be established, and in certain instances, additional comparability clinical trials to show the changes did not impact product efficacy when analytical comparability cannot be established.

Preformulation, early- and late-stage formulation development and product optimization efforts must all funnel into the desired GTPP, and any technical or strategic failure modes should be identified, documented, and communicated. During this effort, the CPPs must be identified and their impact on CQAs must be documented. Proper documentation and communication between early development scientists and the process engineers will help ensure that a robust manufacturing process has been established (form/fill, inspection, release, and distribution) and the vaccine reaches the targeted population.

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Stabilization of Plasmid DNA and Lipid-Based Therapeutics as Dehydrated Formulations

Marion dC. Molina, Nicole M. Payton and Thomas J. Anchordoquy

Introduction

Over the last decade, gene therapy has regained more attention as a promising strategy in providing opportunities for the development of novel nucleic acid-based therapeutics (e.g., DNA) for disorders that need to correct or replace the responsible defective gene(s). Indeed, the discovery of the RNA interference (RNAi) mechanism in mammalian cells revolutionized even more the field of gene therapy [1]. Since the first clinical gene-therapy trial was reported by Rosenberg and collaborators in 1990 [2], over 1900 protocols have been approved worldwide as therapeutic interventions against a wide variety of diseases [3, 4]. Diseases such as cancer, infectious diseases, muscular dystrophy, hemophilia, cystic fibrosis, and many others have been the focus of broad research and clinical activity [4–9]. In fact, private- and public-sector resources have been widely dedicated to these efforts [10–12]. There is a consensus, however, that the success of gene therapies will highly depend on the efficacy and safety of the delivery vector, of either viral or nonviral origin [13]. Significant gains in this field have principally resulted from studies involving viral vectors which are highly efficient at transferring genes (see cited reviews, [14–16]). Although the majority of clinical protocols to date involve viral vectors (~67%, [3, 4]) as major gene therapy vehicles, their use still poses significant safety concerns as a result of previous adverse events due to potent immunogenicity [17] and insertional mutagenesis [18, 19]. Based on these outcomes, viral-based gene therapy has recently seen a clear decline. For instance, the use of retroviral vectors in clinical protocols has decreased from 28% in 2004 [20] to 19.2% in 2014 [3]. The aforementioned disadvantages have prompted the emergence of nonviral alternatives. Nonviral vectors are indeed considered simpler, potentially safer for clinical use, and more amenable to large-

M. dC. Molina (✉)
Independent Pharmaceutical Consultant, 36 Cutler Drive, Ashland, 01721 MA, USA
e-mail: mdc.molina@gmail.com

N. M. Payton · T. J. Anchordoquy
School of Pharmacy, University of Colorado Denver, Aurora, 80045 CO, USA

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scale manufacture [13, 21–23]. Nearly 26% of the total approaches used in clinical trials have been based on some kind of nonviral system [3]. It is clear, however, that despite the great potential of synthetic vectors, gene transfer is modest as compared to its viral counterparts [24, 25]. Of the developed nonviral gene-based therapeutics, the simplest approach involves vaccination using plasmid DNA (usually termed "naked DNA or pDNA") by subcutaneous or intramuscular injection [26–29]. It is important to point out that this approach is technically still considered within the nonviral gene-delivered approaches even though DNA is not bound to any compacting chemical carrier (e.g., cationic agents such as lipids or polymers). This technology is growing fast, and as of January 2014, around 782 completed or active phase I, II, and III studies were registered with the National Institute of Health (NIH, http://www.nih.gov, accessed 4 January 2014) for applications of a variety of diseases. Furthermore, as per gene therapy clinical trials using pDNA, ~17.8% (355 protocols) are currently under investigation worldwide [3]. Most of these studies include infectious and cancer-related diseases [30, 31]. Although the use of naked DNA has been associated with acceptable transfection and potent cellular and humoral immune responses as DNA vaccines [32–34], additional efforts have been recently dedicated to the optimization of specific viral vector-based DNA vaccines [28, 35] or in combination with cytokine adjuvants (e.g., granulocyte-macrophage colony-stimulating factor (GM-SCF) or interleukin-2 (IL-2) [30, 31]). Yet, no human vaccine has been approved by the Food and Drug Administration (FDA) or other regulatory agencies, the licensure of three distinct DNA vaccines for veterinary use (prophylactic West Nile virus vaccine for horses [36], prophylactic hematopoietic virus vaccine in salmonid fish [37], and a therapeutic DNA vaccine for melanoma in dogs [38]) has increased the enthusiasm in developing DNA vaccine technology as human therapeutic interventions. Due to physiological barriers, however, the naked DNA approach is not applicable for systemic gene delivery *in vivo* with the exception of hydrodynamic injections [39]. Hence, significant efforts have been directed toward developing efficient synthetic vectors for target cell delivery of nucleic acids (pDNA; small interfering RNA, siRNA; oligonucleotides; synthetic antisense molecules; among others) in animals and humans [40]. Cationic liposomes have been among the most studied and developed nonviral vectors since the pioneering publication of Felgner and collaborators showed that these positively charged drug vehicles are capable of mediating binding and delivery of DNA molecules to cultured cells [41]. Since this initial study, a considerable interest in exploiting liposomes as carriers of nucleic acids has led to the development of a number of cationic lipids (e.g., 1,2-dioleoyl-3-trimethylammonium-propane, DOTAP; 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol, DMRIE-C; 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane, DODMA; 1,2-dilinoleyloxy-3-dimethylaminopropane, DLinDMA; among others) for both *in vitro* and *in vivo* gene transfer [40, 42–47]. Cationic lipids are considered the most efficient systems for nonviral gene delivery. Their relatively higher efficiency has been the result of the continuous development of new generations of cationic lipids that have been designed via chemical modifications to headgroup, hydrophobic domain and linker, and the development of better lipid compositions (i.e., lipid mixture or formulation). To date, approximately 5.5%

(113 protocols) of all gene transfer clinical trials worldwide have used some kind of lipid/DNA complexes [3, 4]. For instance, an adjuvant cationic liposome–DNA complex (CLDC) platform (JVRX-100, formulated with noncoding plasmid DNA [48, 49]) has been developed by Juvaris Biotherapeutics Inc., a Pleasanton, CA-based company, and is gaining momentum in cancer gene therapy [50]. Currently, a clinical study is recruiting for a phase I trial for patients with relapse or refractory leukemia [51].

While much attention in the field of gene therapy has been mainly focused on the development of more promising gene-based therapeutics in terms of highly efficient delivery vectors with reduced toxicity and increased stability *in vivo* [22, 23, 52, 53], surprisingly little attention has been paid to their physicochemical stability on a pharmaceutically relevant time scale (i.e., 18–24 months). Clearly, stable formulations must be developed as nucleic acid-based therapeutics moves from clinical trials to market approval. Indeed, a prominent challenge of preparing naked DNA and a suspension of lipid/DNA complexes as aqueous formulations is that they generally face physical and chemical instabilities during processing and storage [54–60]. It is acknowledged that prolonged storage of DNA and lipoplexes in aqueous formulations is difficult to achieve considering the high sensitivity of both DNA [61–64] and lipids [65–67] to hydrolytic and oxidative degradation. Furthermore, aqueous suspensions of nonviral vectors are known to aggregate over time [46, 68]. For instance, the high tendency of lipid/DNA complexes to aggregate in aqueous suspensions has prompted the use of these liposomal formulations within a short period of time after preparation [69, 70], and aggregation is further exacerbated in the highly concentrated suspensions prepared for clinical studies [71]. Additional stresses such as agitation and freeze-thawing can also contribute to lipid/DNA complex aggregation [72]. Accordingly, long-term storage of vector systems in suspensions presents crucial difficulties [55, 69, 72]. By analogy, it is well accepted that chemical degradation of DNA is enhanced in aqueous formulations (e.g., depurination [73], deamination [74], depyrimidination [75], and hydrolytic cleavage [76]), and researches in an attempt to inhibit acid-catalyzed degradation mechanisms during prolonged storage have formulated DNA at alkaline conditions (pH 8.5, [61]). However, oxidative damage (i.e., free-radical oxidation-induced chemical degradation) becomes a serious problem not only for DNA formulations (i.e., loss of supercoil (SC) content and base modification [61–63, 77, 78]) but also for those preparations containing a lipid component that is known to degrade rapidly under these conditions (i.e., lipid peroxidation, [65, 66, 79]). Alternatively, frozen formulations have been developed in order to prevent the aforementioned problems (reviewed by Anchordoquy et al., [80]). While recovery of biological activity and DNA integrity has been demonstrated for frozen formulations [60, 71, 72, 81], the maintenance of stable liquid and/or frozen nonviral formulations require formulation and cold storage conditions that limit their large-scale production, storage, as well as their distribution to some regions of the world [55, 69, 82]. Therefore, the development of dehydrated formulations represents an effective approach for stabilization of large standardized batches of DNA or lipid/DNA complexes that would be resistant to shipping stresses and offer the potential for prolonged storage at room temperature [55, 83, 84]. In fact, there has been

much interest in preparing nonviral vectors as lyophilized formulations [83, 85–90]. The lyophilization process subjects preparations to two distinct stresses, freezing and drying, which can alter DNA structure [91] and promote aggregation of nonviral vectors [69, 83]. Like other biomolecules (e.g., proteins [92–94]), previous work has demonstrated that sugars, especially disaccharides, have the ability to preserve lipid/DNA complexes [83, 86, 95], DNA [91, 96] as well as liposome formulations [97] when present above a critical mass ratio of stabilizer to nonviral particle during lyophilization. Particle size is considered a critical pharmaceutical quality attribute of the product that must be maintained during processing regardless of its effect on gene transfer [80, 98]. Although both freezing and drying can promote aggregation, earlier studies indicate that particle size increase is typically manifested during the freezing step of the lyophilization process [72, 83, 88, 99]. Even though the precise mechanism by which sugars stabilize nonviral vectors during freezing and drying remains unclear, it has been suggested that two mechanisms could potentially contribute to the observed particle stabilization during freezing: glass formation [100, 101] and particle isolation [102]; for further discussion read our previous review on the topic [80]. Furthermore, Allison and collaborators have demonstrated that an excipient to DNA (weight-to-weight ratio, *w/w*) ratio of around 1000 is sufficient to prevent aggregation of nonviral vectors during freezing [102], which is in good agreement with later studies on the stabilization of different types of lipid/DNA complexes [103]. In a further study, Armstrong and Anchordoquy [104] showed that particle aggregation in the frozen state is time and temperature dependent. More recently, a research study based on a theoretical modeling had suggested that both the initial sample viscosity and the residence time of the particle in the low-viscosity fluid state are predominant factors in the retention of particle size during freezing. This model has been applied only to polyplexes (DNA complexed with a cationic polymer [105]), and further studies are needed to better understand the relevant mechanism of stabilization of other systems that are known to aggregate during freezing (e.g., liposomes, proteins). More than two decades ago, Crowe and collaborators proposed the so-called water replacement hypothesis to explain how sugars preserve the integrity of liposomes during drying [106, 107]. This theory states that sugars function by replacing water that is bound to the lipid headgroup. To date, this hypothesis has also been implicated in the preservation of nonviral vectors in the dried state [86, 95, 102, 108]. It has recently been suggested that this theory may not explain the ability of sugars to protect lipoplexes (e.g., with polyethylene glycol (PEG)ylation-based components into the particle) for (e.g., with polyethylene glycol (PEG)-modified nonviral vector, PEGylation-based) that adopt a nonbilayer structure [98, 109]. Indeed, more studies are needed in order to investigate further the mechanism(s) involved in the protection of nonviral vectors during freezing and drying.

As mentioned above, it would be advantageous to develop dehydrated formulations that could be shipped and stored at ambient conditions, thus limiting the need for a dependable cold chain. Despite the growing body of research on nonviral vector dehydration, to date, lyophilization studies have mostly focused on the stability of vectors (e.g., naked DNA [91, 110–114], lipoplexes [83, 86, 115–117], polyplexes [85, 88, 99, 109, 118, 119], and lipopolyplexes [95, 120]) during acute

freeze-drying stress. Considering the complexity involved in the stabilization of individual vectors (e.g., naked DNA, lipid) as dehydrated formulations, this chapter addresses these challenges separately. Mechanisms of lipid degradation as liquid and dried formulations are considered. Information highlighting progress to date on the development of dehydrated formulations are reviewed, with particular emphasis on the instability events occurring in the storage of lyophilized naked DNA, unsaturated lipids, and lipid/DNA complexes, and their implications on the development of stable dried pharmaceutical preparations.

Preservation of DNA

The interest in preserving DNA as dried preparations has increased vastly in response to the growing demand from a variety of fields, including pharmaceutical sciences [56, 82, 91, 113, 121, 122], forensics [123], molecular diagnostics [124], and biorepository management [125]) for storing DNA samples or tumor specimen banks [114, 126] for prolonged periods of time, sometimes in large numbers, in regions where sophisticated storage equipment may not be available. Although the requirements are quite different in each of these fields, the need for strategies that provide highly stable storage conditions (e.g., room temperature storage) seems to be a common factor. From a pharmaceutical point of view, while clinical trials are advancing and consequently large amounts of pharmaceutical-grade DNA will be required at the industrial scale, the necessity of developing stable dehydrated DNA formulations, with diminished chemical and/or physical degradation, has gained considerable attention in the recent years. Among the several existing approaches to dehydrate DNA (spray freeze-drying, spray drying, air drying, freeze-drying, or lyophilization), freeze-drying is an established process that can offer a practical alternative to remove water and reduce molecular mobility [127], and, consequently, diminish hydrolytic reactions that are known to damage DNA in solution [76, 128]. Among these technologies, lyophilization has been the preferred approach to remove water due to the general perception that uncomplexed plasmids as well as oligonucleotides may undergo shear-induced damage [129–132] during the process of spray drying. However, recent studies on the mechanisms of nucleic acid shear-induced damage have concluded that smaller plasmids (~5 kb) are not as sensitive to shear stress as larger plasmids (~9.8 and 37 kb) [133, 134] which is consistent with previous findings by Levy and collaborators where the level of damage during shear stress was found to be dependent essentially on plasmid size and ionic strength [135]. These findings are important when considering the practical challenges of producing large amounts of plasmid DNA for therapeutic use in humans, for both clinical trials, and, in the end, full-scale production. In this respect, it is recognized that SC plasmid DNA is frequently subjected to fluid stress during primary isolation processes, and, thus, shear forces involved in upscaling must be controlled [136, 137].

The general understanding of nucleic acid chemistry is mostly dominated by the view of molecular biologists, and, in this respect, the chemistry of DNA degradation in solution is well understood [61, 62, 73, 138, 139]. However, mechanistic studies describing DNA storage stability in the dried state are scarce. Indeed, storing DNA-based formulations in a solid state may be highly desirable not only to reduce the requirement for costly cold storage (e.g., −80 °C, −20 °C [60, 140]) but most importantly to preserve product quality for prolonged storage at ambient conditions. From a historical perspective, the interest of lyophilizing naked DNA began a long time back. Sixty years ago, in April of 1953, Watson and Crick made history describing the double-helix structure of DNA [141]. Although a year prior to this significant achievement, investigators had already isolated and handled dried DNA for research purposes [142]. However, early results in the literature on lyophilized DNA are conflicting in terms of the effect of dehydration on the stability of DNA. For instance, some initial dehydration studies reported a stabilizing effect [142, 143], whereas other investigators found that DNA was readily damaged during lyophilization [96, 144, 145]. Of note, DNA purity was uncertain during those early years of nucleic acid lyophilization, and its effect on stability may explain these contradictory findings. In fact, 60 years ago, scientists did not possess the sophisticated technology that is available nowadays in terms of DNA isolation at high purity [59, 137, 146–149]. Furthermore, it has been recently shown that impurities have a detrimental effect on plasmid quality and gene delivery performance [150].

The lyophilization process has been shown to alter the hydration shells around DNA molecules and, ultimately, influence its conformational stability [91]. Indeed, DNA structure is intrinsically dynamic and plasmid DNA under certain conditions (e.g., humidity driven) can assume conformational changes. Dehydration (e.g., lyophilization) has a remarkable influence on the B- (most commonly found DNA conformation under normal physiological conditions) to A-DNA (found under low-hydration conditions) conformational transition [151, 152]. Typically, A- and B-DNA vary in the slope of base pairs to the helical axis, major- and minor-groove proportions, crystal packing, and deoxyribose ring conformation [153–155]. Studies have reported that during lyophilization, DNA loses its native helical structure [91]. An observation that appears to be different for RNA molecules which preserve an A-like structure independently of their local water activity [156]. Although this well-defined DNA B-to-A transition promoted by removal of water is likely reversible after rehydration [80], recent studies have demonstrated that sugars (e.g., trehalose) when employed completely stabilized DNA formulations during the lyophilization process [82, 91, 96]. Furthermore, our recent unpublished studies monitoring the phosphate antisymmetric stretching vibration (i.e., wave number redshift from 1240 to 1223 cm^{-1}) of dried DNA formulations in trehalose through Fourier transform infrared (FTIR) analysis demonstrate that the right-handed B form could be maintained during lyophilization in the presence of sugars. For instance, when DNA is lyophilized in the presence of sufficient amounts of trehalose (i.e., 5 % trehalose to DNA ratio, w/w), we clearly have observed that dry DNA persists in the B-conformation after acute lyophilization, and it is comparable to the hydrated state (Fig. 1, unpublished observations). This finding is consistent with a previous work

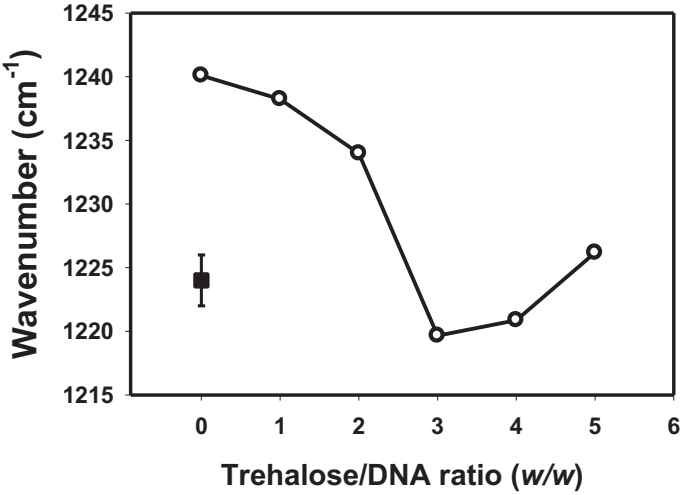


Fig. 1 Sugar maintains dry plasmid DNA (5.9 kb) in its native B-conformation: A gradual antisymmetric phosphate (PO_2^-) stretching redshift from 1240 to 1227 cm^{-1} is observed upon an increase of trehalose in the formulation from 0 to 5 trehalose to DNA ratio (*w/w*). Hydrated (*closed square*), dry DNA-containing trehalose (*open circle*) formulation absorptions obtained by Fourier transform infrared (*FTIR*) spectroscopy. The results are expressed as mean values ± 1 SE of measurements of triplicate samples. *w/w*, weight to weight ratio, *SE,* standard error

by Zhu and collaborators [157], who reported that trehalose has a stabilizing effect on the structure of calf-thymus NaDNA (CT-DNA) in the dry state. When lyophilizing nucleic acid-based pharmaceuticals, scientists most likely formulate DNA, as seen in Table 1, in the presence of sugars (e.g., disaccharides) under conditions of low ionic strength (e.g., 2–10 mM salt concentration) and in the presence or absence of a chelator (e.g., diethylene triamine pentaacetic acid, DTPA; or ethylenediaminetetraacetic acid, EDTA) and, generally, dry DNA will be probably in its native B form [157, 158]. However, further studies are needed to conclusively elucidate if the maintenance of the B-like structure in dried DNA formulations is beneficial for pharmaceutical applications (e.g., prolonged storage).

Formulation of Plasmid DNA

Development of a stable and efficacious plasmid DNA formulation (drug product) involves careful consideration of factors (e.g., DNA purity, optimum pH, identification/addition of stabilizers including antioxidants and/or metal ion chelators) that can affect its safety, potency, as well as its chemical and physical stability. Production of high quality of plasmid DNA (i.e., current good manufacturing practice (cGMP)-grade SC of > 3000 base pairs) is an essential prerequisite to ensure that the product specifications are met (purity, potency, identity, efficacy, and sterility). In addition, bulk plasmid purity specifications must meet minimum release values of

Table 1 Storage studies of lyophilized (LF) plasmid DNA-based therapeutics

Vehicle	Initial buffer	Excipients (w/w)	Protocol	Duration	Recovery	Reference
DNA (4–4.8 kb)	10 mM Tris-HCl 1 mM EDTA pH 7.4	Gluc, Suc, Lac sugar/DNA=0.1, 1, 2, 4 & 4000	LF, stored at 75 °C	3 wks	Biological activity retained	[91]
DNA (5.9 kb)	2.5 mM Tris-HCl pH 8.5	Treh sugar/DNA=1000	LF, stored at 60°C	8 wks	SC≥80 % up to 8 weeks	[179]
DNA (4 kb)	2 mM NaPhosphate pH 8.5	HES, Suc, Treh sugar/DNA=1100 DTPA=200 μM	LF, stored at 50 °C, 60 °C	9 wks	Treh: SC maintained up to 9 weeks Suc: SC reduced after 4 weeks (~80 %) HES: marked reduction in SC (~30 %) after 4 weeks of storage	[110]
DNA (5.9 kb)	2.5 mM Tris-HCl pH 8.5	Treh sugar/DNA=1000	LF, stored at −20 °C, RT, 40 °C	6 M	−20 °C: SC≥ 80 % up to 12 weeks progressive strand breakage at all storage conditions	[82]
DNA (3.8 kb)	None (WFI)	Suc, Treh, Man, PVP sugar/DNA=10, 20	LF, stored at −20 °C 2–8 °C stored at 25 °C/60 %RH stored at 40 °C/75 %RH	6 M 1 M, 2 wks* 1 M, 2 wks*	Suc : SC≥ 90 % up to 6 M Treh, Man: SC≥ 90 %; PVP*: SC <90 % Treh, Man, PVP*: SC<90 %	[122]
DNA (3.8 kb)	None	Lac, Mal, Suc, Treh sugar/DNA=10, 20	LF stored at 25 °C/60 %RH stored at 40 °C/75 %RH	6 M 6 M 6 M	NC: Treh Lac; PC: Mal; TC: Suc Lac, Mal, Suc, Treh: SC≥90 % Lac, Mal, Suc, Treh: SC <90 %	[121]
DNA (3.8 kb)	None (WFI)	Suc sugar/DNA=20	LF stored at −20 °C stored at 2–8 °C stored at 25 °C/60 %RH	66 M 24 M 6 M	SC maintained up to 66 M SC maintained up to 24 M SC maintained up to 6 M	[112]

DTPA diethylene triamine pentaacetic acid, EDTA ethylenediaminetetraacetic acid *Gluc* glucose, *kb* kilobase, *Lac* lactose, *Mal* maltose, *M* months, *NC* non collapse, *PC* partial collapse, PVP polyvinylpyrrolidone *RH* relative humidity, *SC* supercoiled, *Suc* sucrose, *Treh* trehalose, *TC* total collapse, *WFI* water for injection, *wks* weeks, *w/w* weight to weight ratio, *DTPA* diethylene triamine pentaacetic acid

* Storage study of 2 wks when using PVP as excipient

key contaminants: *Escherichia coli* genomic DNA, RNA, and protein (<1%), and endotoxin levels should not exceed 40 EU/mg plasmid [159, 160]. Briefly, to date, manufacturing processes (i.e., upstream, fermentation, and downstream processing steps; for in-depth discussion, the reader is referred to articles that offer an extensive and up-to-date review of the processes [136, 161–163]) involved in the production of cGMP-grade plasmid DNA (drug substance) for human clinical trials have been designed in order to be cost-effective, efficient, and capable of producing large-scale quantities of SC pharmaceutical-grade plasmid DNA (e.g., ≥200 mg for pilot and early clinical trials or gram-to-kilogram quantities at industrial scale [122, 140, 164–168]). Accordingly, these higher capacities allow the preparation of DNA gene therapy or DNA vaccine formulations for clinical trials at the higher concentrations required (≥0.5 mg/mL) [30, 169–171].

Furthermore, it is important to mention that plasmid DNA homogeneity (i.e., most plasmid is in its SC form) is a major concern to pharmaceutical manufacturers since efficacy and stability depend, in part, on the topology of DNA (i.e., comparative topology in the relative amounts of SC; open circular, OC; and linear forms) [110, 140, 172–174]. It is generally recognized that via single-strand break, the SC form (used as a standard measure of DNA quality [160]) can convert to OC form, and further degradation can produce undesirable amounts of the linear form that has been associated with lower levels of expression once transfected [174, 175]. Typically, the different isoforms of plasmid DNA (i.e., relative amount of each of the forms—SC, OC, and linear—in a given sample) are determined by agarose gel electrophoresis [176]. This method has been routinely applied in the industry to analyze clinical-grade plasmid DNA. Unfortunately, the undesirable complications associated with gel staining (e.g., ethidium bromide) such as background variability, nonlinearity in staining, and the requirement of corrections for more accurate determination of SC content due to the distinct intercalating affinities of ethidium bromide for the different species [176] have encouraged the development of more accurate chromatographic methods: ultra performed liquid chromatography (UPLC; [110]) and anion-exchange high-pressure liquid chromatography (AE-HPLC; [173]). For instance, UPLC has proven to be a valuable tool to monitor the distribution of plasmid isoforms in dried samples subjected to extreme conditions such as metal contamination and storage under accelerated conditions [110]. Certainly, maintenance of plasmid DNA integrity (i.e., ≥80% SC as recommended by FDA, [160]) is an important structural characteristic that will have an impact not only on bulk production but also long-term stability (e.g., in the dried state), efficacy, and eventually clinical approval.

As indicated above, the plasmid DNA-containing formulation must be sufficiently stable (e.g., 18–24 months) against physical and chemical pathways of degradation. To this end, the formulation scientist must consider a series of relevant approaches (e.g., raising pH, addition of chelators and antioxidants, alone or in combination) to improve the stability of clinical-grade plasmid DNA during storage either in solution to minimize acid-catalyzed degradation pathways and oxidation (aside from nuclease-related contamination) [61, 64, 177] or in a dehydrated form to inhibit mainly free radical-mediated oxidative damage [82, 178], as described below. Plasmid DNA is typically manufactured in low-salt conditions (2–10 mM),

with sugar excipients such as sucrose or trehalose added not only to stabilize the formulation during the freeze-drying process but also to achieve isotonicity after rehydration (e.g., 10% sucrose). While current pharmaceutical preparations for clinical use are more concentrated (e.g., ≥0.5 mg plasmid DNA/mL), formulate at high excipient to DNA ratios will make it more challenging. It is also recognized that high concentrations of sugars lengthen the lyophilization cycle, resulting in considerable production costs [56, 102]. Therefore, the development of more stable compositions at lower excipient to DNA ratios is highly desirable. For example, the group of Quaak and collaborators [122] has been able to manufacture a GMP-lyophilized dosage form at a low sugar to DNA ratio of 20% (*w/w*).

Stability of Dried Plasmid DNA

Despite the wide interest for the need of preserving DNA in the dried state, there are limited studies that provide stability data during and/or after lyophilization of naked DNA as pharmaceuticals. With regard to the stability of naked DNA during the lyophilization process, it has been reported that naked DNA undergoes double-strand denaturation in the absence of lyoprotectants [91], an observation that is consistent with previous findings where DNA does not retain its native structure (i.e., double helix) and is more vulnerable to oxidative damage when stored under extremely dry conditions (e.g., in the presence of phosphorus pentoxide) [138]. However, DNA must resist the freezing and drying stresses encountered during processing before stability can be addressed. In this respect, it has been demonstrated that the addition of disaccharides, such as sucrose and trehalose, are beneficial to prevent loss of SC DNA during lyophilization [55, 56, 82, 91, 96, 113, 115, 121, 178, 179].

Prolonged stability of plasmid DNA as dried formulations is crucial for their development as viable pharmaceutical products. The effects of prolonged storage (18–24 months) of dried DNA were first reported by Kolobov and Vainberg almost 40 years ago [180]. Their initial findings demonstrated that dry CT-DNA samples, in the absence of lyoprotectants and stored under refrigerated conditions, experienced a progressive strand breakage (~35% loss in molecular weight) over a period of 14 months. However, biological activity was not measured. More than 20 years later, a preliminary accelerated study by Cherng and collaborators have suggested that dried naked plasmid DNA containing high amounts of sucrose (~2500 sugar to DNA weight ratio) could be stored at room temperature and slightly higher temperatures (40 °C) for up to 10 months [181]. Yet, despite the increasing interest in preparing dry plasmid DNA formulations for pharmaceutical purposes, only a limited number of studies have reported the stability of plasmid DNA during storage (as shown in Table 1). One report, for example, showed that biological activity of lyophilized sugar-containing naked DNA preparations could be fully maintained for up to 3 weeks at high temperatures (i.e., 75 °C). However, formulations were stored at very high sugar to DNA ratios (i.e., 4000, *w/w*) [91]. In contrast, a later study has reported that purified plasmid DNA prepared at a ratio of sugar to DNA much lower (i.e., 34.2 *w/w*) and dehydrated under extreme conditions (on the surface of phosphorus pentoxide powder, P_2O_{50}) at room temperature underwent oxidative damage after 56 days even in

the presence of a nonreducing disaccharide (i.e., trehalose) [182]. Furthermore, storage studies conducted for longer periods of time in trehalose have been performed in our laboratory and similarly, we observed a progressive strand breakage (i.e., loss of SC content) over a period of 6 months in purified plasmid DNA formulations [82]. Particularly surprising was the observation that chemical damage took place at a very-low-storage temperature (e.g., −20 °C; $T_g - T_{storage} > 50$ °C) despite samples being lyophilized in the presence of highly purified trehalose (at a higher ratio of sugar to DNA, 1000 *w/w*) and optimal glassy-state conditions (high T_g, 113 °C and low moisture levels of ~ 0.5 %, [82] and Molina M.—unpublished data]. We also observed significant degradation (lost of SC) at room temperature, and, not surprisingly, at higher temperatures. The observation that nonreducing sugars fail to stabilize naked DNA during storage strongly suggests that other degradation mechanisms such as oxidative damage (formation of reactive oxygen species; ROS) are active in the dried cake. Considering that biomolecules are immobilized in a glassy-excipient phase where reactions requiring positional specificity would be considerably inhibited in the dried state [127], our recent findings show that degradation can still occur at significant rates despite maintenance of a glassy phase (e.g., in trehalose). In fact, our validated fluorescent-based method [115] has been used consistently to detect the formation of ROS in lyophilized naked DNA, lipid alone, as well as lipid/DNA complexes, and demonstrated that ROS are still active in the solid state [115, 178, 179, 183]. Furthermore, our findings suggest that trace metal contamination (Cu^{2+}, Fe^{2+}, and Fe^{3+}) is a prominent factor affecting the stability of these biomolecules, particularly when an unsaturated lipid is present in the formulation composition (e.g., dried lipid/DNA complexes, as described in corresponding section of this chapter) [179, 183]. It should also be noted that even very small quantities (parts per billion, ppb) of transition metals (e.g., ferrous iron $-Fe^{2+}$) can mediate generation of ROS in the dried state [115]. Furthermore, trace amounts of metal contaminants are present even in the cleanest materials available commercially (e.g., clinical-grade DNA and GMP-grade sugars) [103, 115, 183]. It is generally recognized that free radical oxidation is a major chemical degradation pathway for DNA-based pharmaceuticals [61, 62, 64, 184] and that it is substantially stimulated by molecular oxygen [185, 186]. In an attempt to prevent oxidative damage and extend the shelf life of lyophilized DNA-based formulations in the dried state, our laboratory has evaluated various strategies including metal removal from reagents (demetalation, [61, 77, 187]), air displacement in the vial headspace [188], and fortification with chelator/antioxidant agents [62, 184]. We found that demetalation (Chelex 100 resin) and headspace oxygen displacement were insufficient to completely inhibit the formation of ROS in dried formulations [178]. Although removal of low levels of oxygen contamination from the gas stream in the vial headspace is a general practice in pharmaceutical manufacturing processes; however, it has been suggested that the low efficiency of a demetalation step to both eliminate metal contamination and prevent the formation of ROS in dried formulations is impracticable for large-scale purification and production [56]. Yet, undetectable metal levels (below detection limits, < 5 ppb) may induce damage during prolonged storage [61, 115, 183]. An effective strategy is to incorporate chelating agents into DNA preparations [82]. Indeed, our recent investigation has shown the great effectiveness of the ion-chelating agent DTPA to counteract the damaging effect of ROS (e.g., lost in SC) in lyophilized DNA-based formulations

during acute lyophilization [115] and during storage [110, 178]. DTPA-prevented ROS levels increase in the presence of Fe^{2+} (400 μM), which is consistent with the fact that its affinity for iron (log K_a of Fe^{2+} = 16, [189]) is larger than the affinity of iron for double-stranded DNA (log K_a of Fe^{2+} = 4.73, [190]). Similar to that discussed below for lipid oxidation (see "Stability of Lipid/DNA Complexes" section).

Although vitrification is not required for vector stability during acute freezing or dehydration [83, 102], minimizing biomolecule (e.g., DNA) mobility via storage well below the glass transition (T_g) of the dried product might be necessary for long-term stability. Recently, our group has dried DNA in the presence of sugar combinations (Fig. 2a, [110]), showing that some formulations are better than others during acute lyophilization. We observed that 5 % trehalose and 2.5 % sucrose/2.5 % hydroxyethyl starch (HES)-based formulations seem to maintain most of the initial SC content in the presence of DTPA. Compared to fresh samples, formulations containing higher amounts of HES did not work very well and dropped down to approximately 80 % (e.g., pure HES, 4 %HES/1 % trehalose or 4 % HES/1 % sucrose). It is important to point out that this loss in SC content during acute lyophilization is unacceptably high for a pharmaceutical product. Furthermore, we have observed that trehalose-containing formulations maintain DNA integrity for 9 weeks at higher temperatures (e.g., 40, 50, and 60 °C) in the presence of DTPA, and also resulted in better preservation of the initial biological activity (Figs. 2b, 3a, b, [110]). In our hands, however, pure sucrose-containing formulations did not protect naked plasmid DNA in terms of biological activity and DNA integrity even at 40 °C (Figs. 2b, 3d). Although the addition of HES into sucrose-containing formulations (e.g., 2.5 % HES/2.5 % sucrose) increased biological activity during storage, it is clear that adding more HES to the formulation diminishes the protective effect (compare Fig. 2d–f). Similarly, when plasmid DNA was freeze-dried in HES alone and stored under accelerated conditions, our results showed that after 4 weeks of storage the biological activity of formulations is mostly lost (data not shown). Although these data are not sufficiently extensive to permit firm conclusions concerning the role of T_g in prolonging stability of plasmid DNA in the dried state, the observed DNA degradation even in formulations with maximized T_g values (e.g., HES) suggests that glass formation does not correlate with DNA stability which is consistent with our previous reports [110, 183, 185]. Supplementary strategies need to be developed in addition to chelator(s) incorporation in dried DNA formulations in order to reduce product degradation during storage.

Moisture Effect on DNA Preservation

Considering that nucleic acids are highly susceptible to hydrolysis and oxidation in solution [62, 63, 191], one might conclude that the removal of water would increase DNA stability. Although residual moisture (RM) is considered a contributing factor to the degradation of dehydrated protein formulations [192], the role of RM in naked plasmid DNA formulations has yet to be investigated. Research, however, has shown that stability of dried viral vectors is not always maximized at the lowest

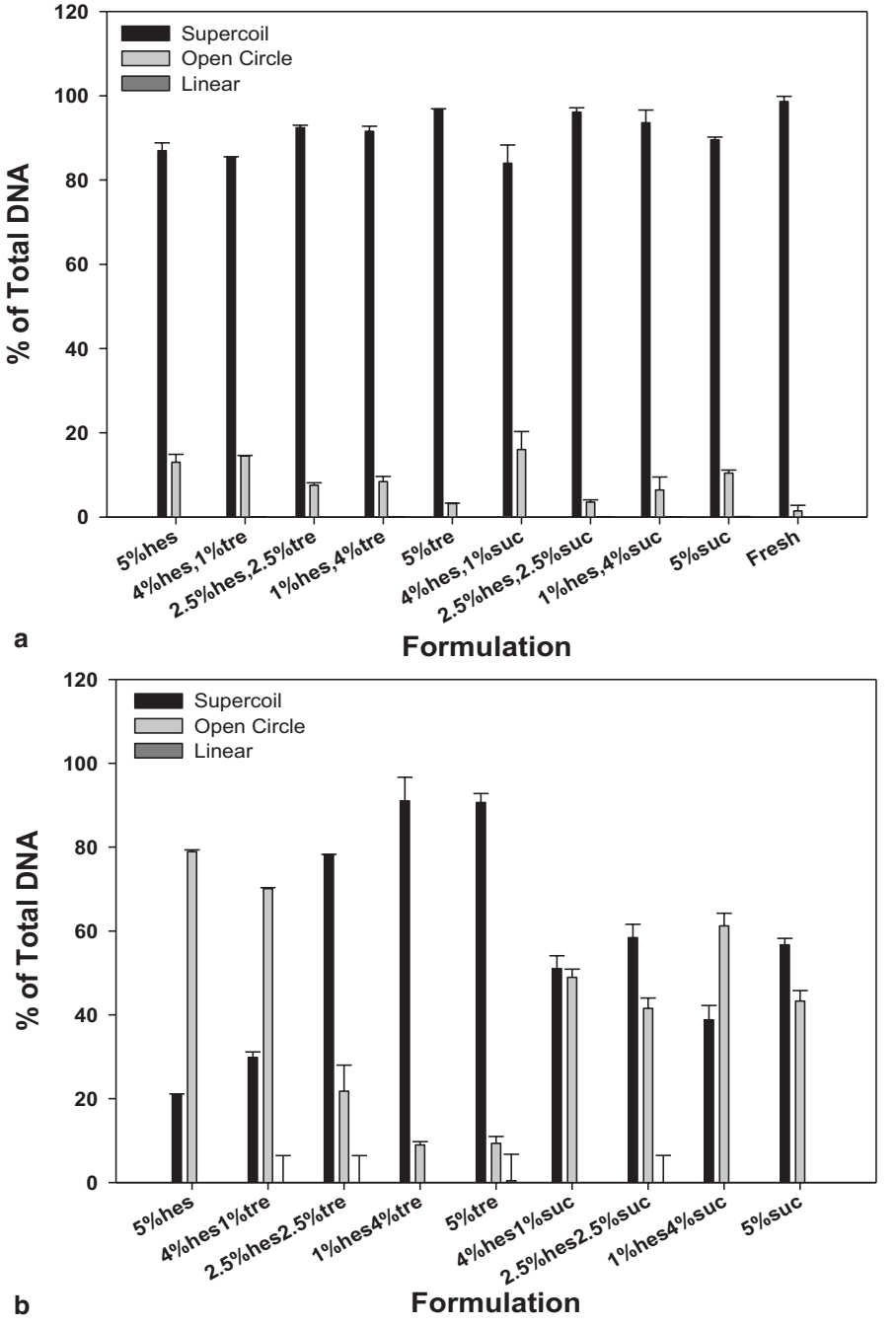


Fig. 2 DNA integrity of lyophilized DNA formulated in pure sugars (trehalose, sucrose and HES) and in sugar to HES combination ratios at **a** initial and **b** after 9 weeks of storage at 60 °C. Reported values are represented as mean values ± 1 SE of measurements of triplicate samples. *HES* hydroxyethyl starch, *SE* standard error

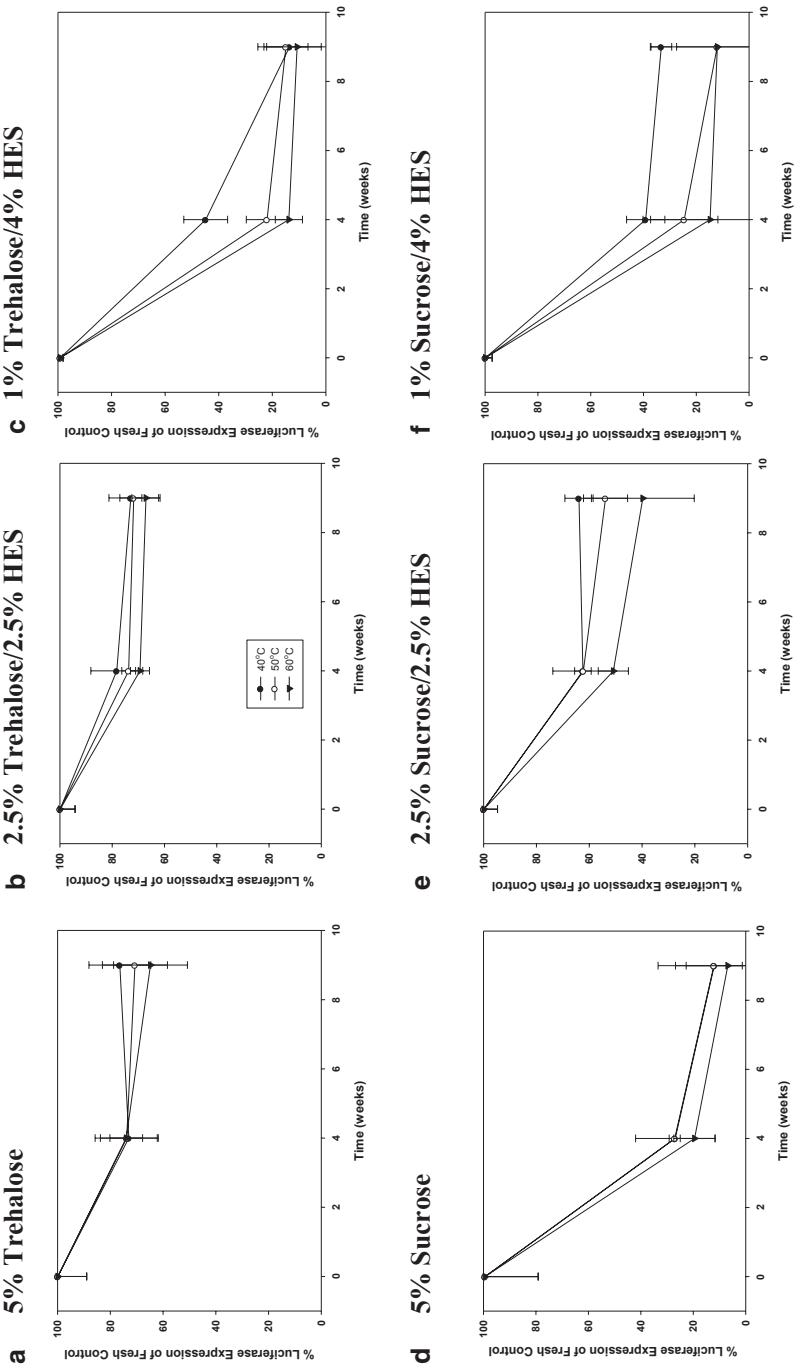


Fig. 3 Biological activity of lyophilized DNA stored during 9 weeks at distinct sugar to HES ratios and high temperatures: (**a**–**c**) trehalose/HES formulations and, (**d**–**f**) sucrose/HES formulations stored both at 40 °C (*closed circle*), 50 °C (*open circle*), and 60 °C (*closed triangle*). Symbols represent the mean values ± 1 SE of measurements of triplicate samples. *HES* hydroxyethyl starch, *SE* standard error

water contents (< 1 % water content) [193], which is the condition normally used for the preparation of dried nonviral lipid/DNA formulations [102, 178, 179, 185]. Our preliminary attempt on the effect of low (Initial %, *w/w*: ~ 0.41 ± 0.06) and moderate (Initial %, *w/w*: ~ 1.13 ± 0.15) RM on the stability of dried plasmid DNA-based formulations containing trehalose and chelator (DTPA) are shown in Fig. 4a, b. Our findings show that very dry DNA (< 0.5 %, *w/w*) was less prone to oxidative damage than moderate moisture-containing (1.51 %, *w/w*) formulations within 8 weeks under storage conditions of forced degradation (e.g., 40 °C; see Figs. 4a, b). Furthermore, DNA recovery (SC content, ROS levels) as well as biological (transfection, data not shown) was not affected by higher levels of moisture when formulations were stored at room temperature (Fig. 4c, d). Although DNA purity (≥ 90 % SC) for up to 6 months at 25 °C/60 % RH has been documented in lyophilized cGMP-DNA preparations with relatively high RM (2.41 %), these increases in time (from initial 0.7 %) were due to rubber stoppers [194] that exceeded water-content specifications [112]. Although these results are not ample to provide definitive conclusions regarding the role of water in preserving DNA during storage, our data clearly show that DNA degradation (higher levels of ROS and loss in SC content) was exacerbated in formulations with higher levels of RM at temperatures approaching the glass transition temperature (Fig. 4a–d). The fact that ROS are still active in the dried cake [115, 178, 183, 185] and considering that water [191] and traces of transition metal contaminants are a potential source of ROS together with the role of molecular oxygen in the propagation step of the oxidative damage in DNA samples [111, 191, 195, 196], we suggest that levels of RM need to be optimized (and maximize T_g) in combination with the aforementioned strategies to minimize formation of ROS (molecular oxygen displacement, including chelators and/or antioxidants, using metal-free materials as possible) during prolonged storage in order to prevent free radical-mediated damage to DNA-based pharmaceuticals.

Lipids

Liposomes are bilayer vesicles that are composed of cationic, anionic, and/or neutral lipids. Liposomes are often used as delivery vehicles; currently 12 liposomal products are being marketed which deliver small molecule therapeutics [197]. The commercially available products, which employ lipid-based delivery vehicles, are typically composed of saturated, hydrogenated, or singly unsaturated lipids. These lipids are primarily used due to their greater chemical stability. Although this chapter examines the chemical stability of lipids with a focus on pharmaceutically relevant work, other studies have investigated the stability of lipids in relation to food and beverage products [198–204].

The main degradation pathways that are of concern for liposomal formulations are peroxidation and hydrolysis. Peroxidation, which is a complex free radical-mediated process, consists of three distinct steps: initiation, propagation, and termination [205, 206]. Peroxidation initiation involves the abstraction of a hydrogen atom from the fatty acid tail of a lipid resulting in the formation of lipid radicals. The ease with which the hydrogen can be abstracted is related to the number of unsaturated sites

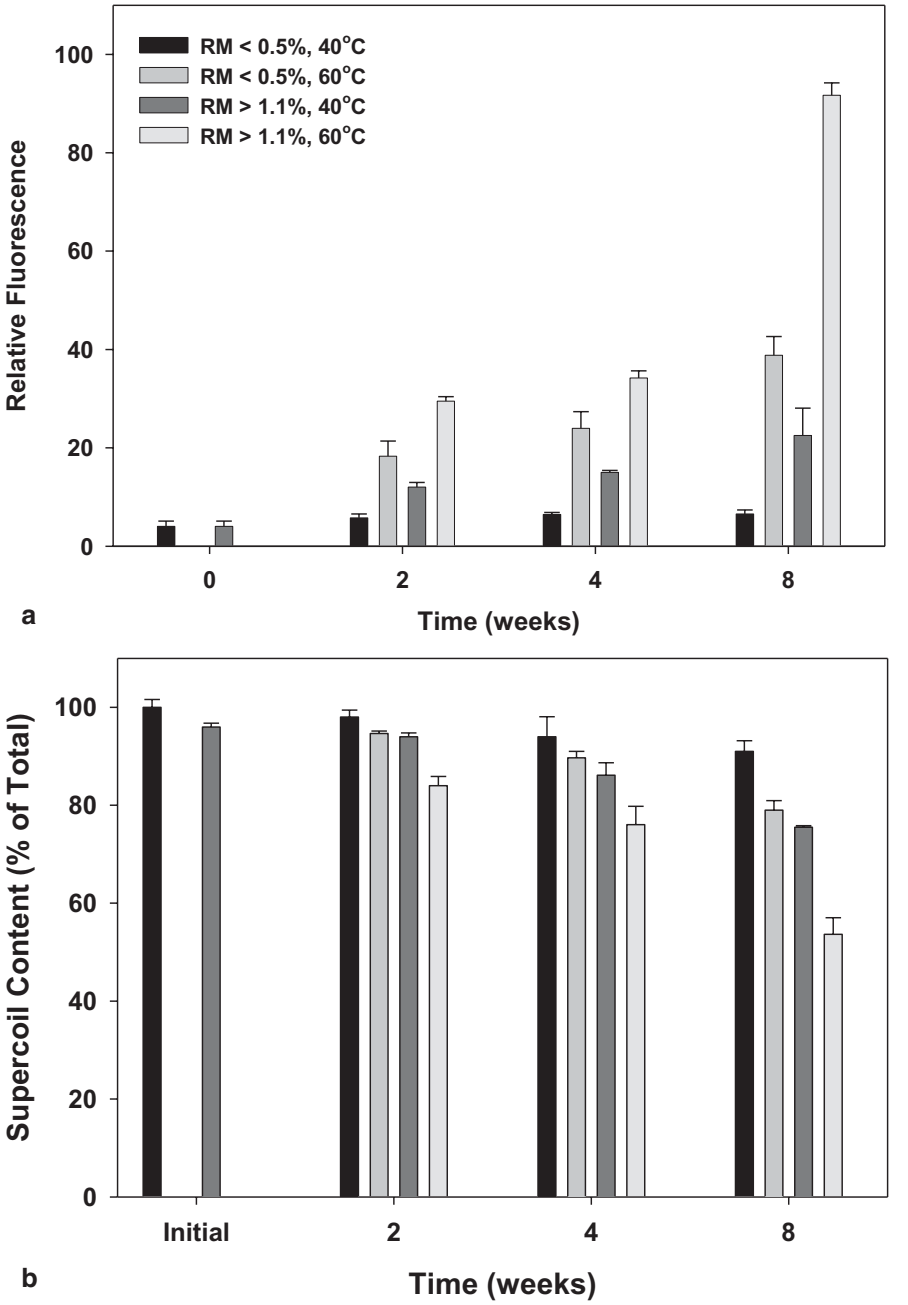
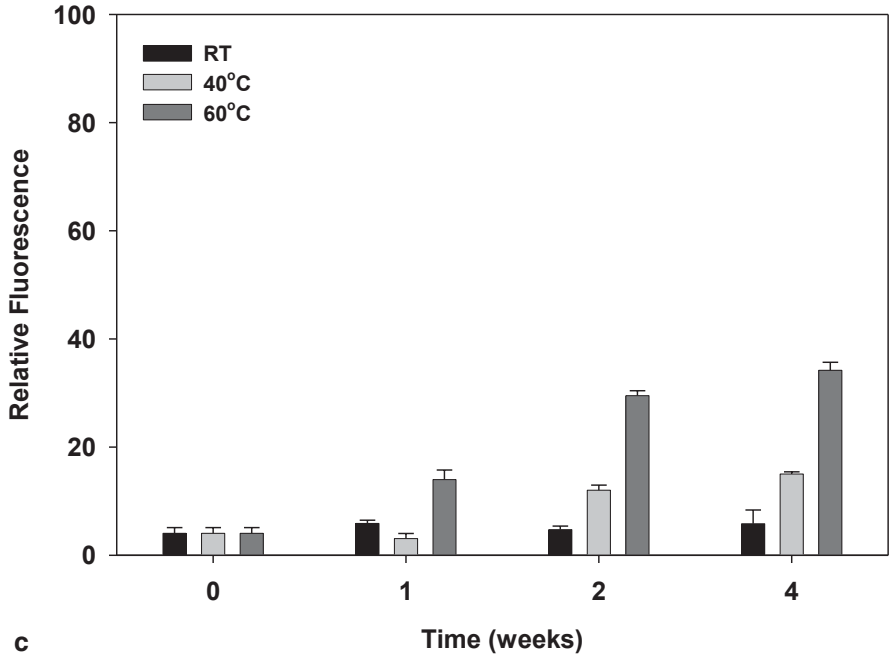


Fig. 4 Effect of residual moisture (*RM*) on the physicochemical stability of lyophilized DNA formulations during storage: Comparison low and moderate RM: **a** ROS and **b** DNA integrity at 40 °C, 60 °C; and moderate RM: **c** ROS and **d** DNA integrity at all storage conditions (RT, 40 °C, 60 °C). Percent of DNA is shown as supercoil content (*SC*) as total remaining in samples. ROS formation assessed by using a fluorogenic spin trap probe, proxyl fluorescamine, that was added to the formulations prior to freeze-drying as earlier described [115]. Moisture contents and glass transition temperatures, low RM (Initial %, *w/w*: ~0.41 ± 0.06/T_g onset: 95 °C ± 1.8; after 2 months of storage at 40 °C: 0.46 ± 0.02/T_g onset: 87 °C ± 3.3, and 60 °C: 0.46 ± 0.07/T_g onset: 88 °C ± 2.7),



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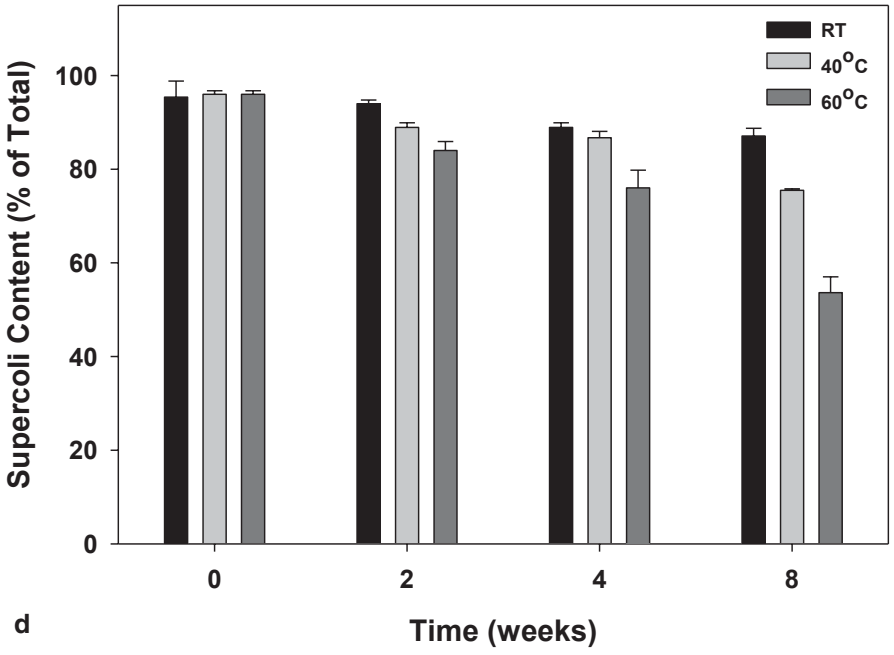

d

and moderate RM (Initial %, *w/w*: ~ 1.13 ± 0.15/T_g onset: 54 °C ± 2; after 2 months of storage at RT: 1.34 ± 0.38/T_g onset: 57 °C ± 4, 40 °C: 1.51 ± 0.15/T_g onset: 54 °C ± 2, and 60°: 1.44 ± 0.15/T_g onset: 57 °C ± 1). All formulations: 1000 excipient to DNA ratio, *w/w* + 2.5 mM Tris-HCl + 200 ∝M DTPA in metal-free material as previously reported (178). Symbols represent the mean values ± 1 SE of measurements of triplicate samples. *RT* room temperature, *SE* standard error, *ROS* reactive oxygen species, *w/w* weight to weight, *DTPA* diethylene triamine pentaacetic acid

in the fatty acid tail of the lipid. The carbon–hydrogen bond energy is decreased for allylic (88 kcal/mol) and bisallyic (75–80 kcal/mol) carbon–hydrogen bonds relative to alkyl carbon–hydrogen bonds (101 kcal/mol; Fig. 1) [207]. The decreased bond energy as well as the resonance stabilization of the resulting lipid radical underlies the greater propensity of lipids containing two or more unsaturated sites to undergo peroxidation [206, 208]. During propagation, the reaction of molecular oxygen with a lipid radical results in the formation of peroxyl radicals [208]. Once formed, the peroxyl radical can then abstract a hydrogen atom from another lipid molecule leading to the formation of hydroperoxides and lipid radicals [208]. In addition to these reactions, a number of other reactions such as fragmentation, rearrangement, and cyclization can also occur [208]. Termination, the final step of lipid peroxidation, occurs when nonradical products are formed as a result of the coupling of two radicals. It has been demonstrated that peroxidation can affect the physical properties (i.e., permeability and fluidity) of liposome bilayers [209–211]. Besides peroxidation, hydrolytic degradation of lipids is also of concern. The ester functionality which connects the glycerol backbone with the fatty acid tails is susceptible to hydrolysis and results in the formation of free fatty acids, lysophospholipids, and glycerophospho compounds [212]. Hydrolytic degradation can impact liposome size, rigidity, and drug entrapment [213]. Due to the limited stability of aqueous liposomal formulations, these formulations are often lyophilized. Little is known about the chemical stability of lyophilized liposomal formulations (i.e., lipids in the dried state). Predominately, the studies which have investigated lipid stability have focused on aqueous liposomal formulations. However, liposomal leakage and the retention of small molecules have been studied in lyophilized and aqueous formulations [107, 214, 215]. To better understand how to maximize the stability of lipids in the dried state, it is necessary to review the studies which have investigated the chemical stability of lipids in aqueous formulations. The continued development of lipid-based therapeutics necessitates the further investigation of the stability of lyophilized liposomal formulations.

Oxidation

In the studies which have examined the stability of liposomal formulations, typically, transition metal (Fe^{2+}/Fe^{3+}) catalysts or azo compounds are utilized to facilitate the initiation of lipid peroxidation. The various ways that transition metals can affect lipid peroxidation has been studied extensively, and several possibilities have been proposed. The reaction of iron with oxygen and/or hydrogen peroxide via the Haber–Weiss and/or the Fenton reactions results in hydroxyl radical formation which can then serve as the initiator free radical [216, 217]. It is well known that the direct reaction of oxygen with lipid molecules is spin forbidden due to the electronic configuration of these molecules [218]. Iron has also been suggested to mitigate the spin restriction of oxygen and increase the rates of oxidation of biological molecules (e.g., lipids) by serving as a bridge [219]. Another route by which iron can affect lipid peroxidation is through direct initiation. Direct initiation of lipid

peroxidation by higher valence state metals, such as Fe^{3+}, via one electron transfers can lead to the formation of lipid radicals [220]. The use of azo compounds is another strategy which has been employed to investigate lipid peroxidation. Azo compounds, often referred to as azo initiators, are molecules which decompose, leading to the generation of radicals. Azo compounds vary in terms of the efficiency with which they generate radicals and their propensity to reside in the lipid bilayer or in aqueous solution [221]. Because of the difficulties that have been encountered when using traditional azo compounds (e.g., 2,2-azobis-(2-methyl-propanimidamide) dihydrochloride, AAPH; 2,2′-azobis (2,4-dimethylvaleronitrile), AMVN) to investigate lipid peroxidation, significant research has been conducted to synthesize and characterize azo compounds that reside and generate radicals in the lipid bilayer efficiently [221, 222]. Although many studies have endeavored to determine the factors that play a role in the oxidative degradation of aqueous liposomal formulations, this chapter focuses on reports which have investigated the effect of lipid saturation, inclusion of cationic or anionic lipids, and the use of antioxidants.

Aqueous Liposomal Formulations: Effect of Lipid Saturation

The effect of the particular lipids incorporated into the liposomes has been examined as well as the effect of the addition of cholesterol. Mowri et al. [223] investigated the peroxidation of liposomes composed of either 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (POPC; 16:0, 18:1) or 1-hexadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine, PAPC (16:0, 20:4) in the presence of iron (II) and ascorbic acid. POPC was expected to be insensitive to peroxidation. Conversely, PAPC was expected to undergo peroxidation due to the presence of a polyunsaturated fatty acid, arachidonic acid, at the sn-2 position. The thiobarbituric acid-reactive substances (TBARS) assay, which detects secondary products such as aldehydes, was utilized along with gas chromatography (GC) to assess changes in the fatty acid content. Analysis of the PAPC samples showed an increase in peroxidation products with a concomitant decrease in arachidonic acid. In contrast, the formation of peroxidation products was not detected in POPC liposomes, and no detectable change in the fatty acid content was observed [223]. The effect of mixing PAPC with different peroxidation-insensitive lipids, POPC (16:0, 18:1) or 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC; 18:0) was also assessed. For the PAPC:POPC formulation (1:9 mol ratio) the concentration of peroxidation products detected was negligible; however, a significant concentration of peroxidation products was detected for the PAPC:DSPC (1:9 mole ratio) formulation. Analysis of the arachidonic acid fatty acid content revealed no detectable change in the arachidonic acid content for the PAPC:POPC (1:9) liposomes; however, for the PAPC:DSPC (1:9) liposomes, a significant decrease in the arachidonic acid was detected. This study was conducted at 37 °C, which is above the liquid crystalline-to-gel transition temperature for both POPC and PAPC. Mowri et al. [223] suggested that POPC and PAPC were homogeneously distributed in the bilay-

er, and the distance between the PAPC molecules decreased peroxidation. Further Mowri et al. [223] suggested that PAPC and DSPC would not be homogeneously distributed in the bilayer. Because the liquid crystalline-to-gel transition temperature of DSPC is greater than the temperature at which this study was conducted, DSPC would be expected to be in the gel state resulting in the clustering of PAPC. Thus, the increased peroxidation of PAPC was attributed to the greater density of PAPC molecules when formulated with a gel-phase lipid-like DSPC.

Studying the effects of lipid saturation, Vossen et al. [224] investigated the copper (II)/H_2O_2 catalyzed peroxidation of liposomes containing increasing levels of unsaturation by measuring the accumulation of conjugated diene formation. This study utilized liposomes composed of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLinPC; 16:0, 18:2), 1-palmitoyl-2-linolenoylsn-glycero-3-phosphocholine (PLPC; 16:0, 18:3) or PAPC (16:0, 20:4). Since palmitic acid (16:0) is insensitive to peroxidation, the formation of conjugated dienes resulting from peroxidation was attributed to either linoleic acid (18:2), linolenic acid (18:3), or arachidonic acid (20:4). The experimental system in this study used only phosphatidylcholine (PC) lipids eliminating any variation that would be caused from using lipids with different headgroups and is free of other molecules that would obscure the results (e.g., proteins). The formation of conjugated dienes was much faster in PLPC samples relative to PLinPC samples. No conjugated diene formation was detected for 16:0, 16:1, or 18:0, 18:1 liposome formulations. Surprisingly, the conjugated diene formation in PAPC samples was slower than either of the other liposome formulations that were tested [224]; however, the underlying cause of this surprising result was not discussed.

Cholesterol is often included in liposomal formulations to increase the rigidity of the bilayer and increase transfection efficiency of lipid–nucleic acid complexes [213, 225–227]. The effect of the addition of cholesterol on the oxidative degradation of liposomal formulation is not consistent and appears to depend on the composition of the liposomal formulation and the temperature at which the study was conducted [228]. The effect of cholesterol on PAPC (16:0, 20:4) liposomes was examined. In the absence of cholesterol, significant peroxidation was observed. However, in the formulations which contained cholesterol (1:2 and 1:1 cholesterol:PAPC), a progressive decrease in oxidative degradation was observed [223]. It was proposed that the effect of cholesterol on the fluidity of the lipid bilayer caused the reduced peroxidation which was observed. Samuni and Barenholz [229] examined the stability of egg PC (EPC) liposomes that were formulated with and without cholesterol and stored at room temperature for up to 10 months. Decreased peroxidation was observed in EPC–cholesterol (10:1) liposomes relative to pure EPC liposomes. The decreased oxidative degradation of EPC–cholesterol liposomes was attributed to a more tightly packed bilayer, hindrance of the free radical-mediated propagation reactions, and decreased bilayer hydration. Another study examining the effect of the incorporation of cholesterol on the stability of EPC liposomes reached somewhat different conclusions [230]. In this latter study, the hydrophilic azo intiator, AAPH, was utilized and the cholesterol content was varied from 0 to 45 % (mol %). Overall, the presence of cholesterol did not alter the oxidation of egg PC liposomes except for the formulation which contained 10 % cholesterol. The oxidation detected in this formulation was actually greater than any of the formulations containing higher

cholesterol contents [230]. The authors suggested that increased peroxidation at 10 mol% cholesterol could be attributed to the coexistence of liquid disordered (LD) and liquid ordered (LO) phases.

Incorporation of Charged Lipids

The interest in the effect of the inclusion of cationic or anionic lipids in a liposomal formulation is often driven by use of cationic lipids such as 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) in lipid-based nucleic acid delivery vehicles or the desire to better understand the peroxidation of biomembranes which are inherently negatively charged. A significant amount of research has been conducted to assess the effect of the inclusion of cationic or anionic lipids in liposomal formulations on iron-catalyzed lipid peroxidation. Some studies have concluded that the inclusion of anionic lipids, in particular 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS), inhibits lipid peroxidation due to binding of the cationic iron ions by the negatively charged headgroup, thereby preventing the iron-catalyzed decomposition of lipid hydroperoxides [231, 232]. Conversely, Kunimoto et al. [210] found that anionic liposomes readily underwent peroxidation, whereas neutral and cationic liposomes were not as prone to oxidative degradation. The increased peroxidation of anionic liposomes was attributed to increased binding with iron or the close proximity of the ferrous ions and the surface of the bilayer. In many studies, there was either a slight or a negligible effect of liposome charge on liposomal peroxidation initiated by azo compounds [231–233].

Incorporation of Antioxidants

The addition of antioxidants to liposomal formulations is a common strategy which is used to slow or, in some cases, inhibit the oxidative degradation of susceptible liposome components (e.g., polyunsaturated lipids). Antioxidants are typically characterized as either hydrophilic and water soluble (e.g., ascorbic acid, uric acid) or lipophilic (e.g., α-tocopherol, 2,2,5,7,8-pentamethyl-6-chromanol, PMC). Noguchi et al. [222] examined the peroxidation of both methyl linoleate micelles and PC liposomes in the presence of AAPH (water soluble) or 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile (Meo_AMVN, lipophilic). Methyl linoleate micelle oxidation was followed by measuring the formation of hydroperoxides and the consumption of oxygen. The oxidation of methyl linoleate in the presence of MeO–AMVN was inhibited by PMC but not by ascorbic acid. In the presence of AAPH, methyl linoleate oxidation was inhibited by ascorbic acid. Not surprisingly, methyl linoleate oxidation was inhibited effectively when the antioxidant and the source of the radicals (i.e., the azo initiator) were together in either the aqueous solution or the lipid bilayer. When the MeO–AMVN (lipophilic) and ascorbic acid (water soluble) were added to methyl linoleate samples, oxidation was only inhibited when a cationic surfactant, tetradecyltrimethylammonium bromide (TTAB), was also present. It was proposed

that the electrostatic attraction between the cationic TTAB and the anionic ascorbic acid (at pH 7.4) brought the ascorbic acid in close contact with the surface of the micelle [222]. Similar experiments were carried out to examine the peroxidation of PC liposomes in the presence of antioxidants [222]. The peroxidation of PC liposomes was initiated by the addition of MeO–AMVN, and the ability of several antioxidants to inhibit peroxidation was tested. PC liposome peroxidation was monitored by measuring the formation of lipid hydroperoxides. PC liposome peroxidation was decreased in the presence of antioxidants as indicated by the decreased hydroperoxide formation. Antioxidants, such as PMC, that were able to efficiently partition into the bilayer were able to suppress PC oxidation most efficiently [222]. We have observed related phenomena when using fluorescent probes to monitor lipid oxidation. In our previous studies on lipid oxidation, we observed that water-soluble probes were not able to accurately monitor oxidation occurring in the suspended lipid particles [115].

Lyophilized Lipid Formulations

Particularly in the 1960s and 1970s, an increased interest in the stability of freeze-dried food products arose. A number of studies were conducted that focused on the factors that would deleteriously impact the stability of lyophilized food products. These studies focused on lipid peroxidation in lyophilized foods and a variety of experimental systems were used to investigate the effect of moisture content, transitional metals, and antioxidants. Interested readers are referred to several studies [198–204] which utilized lyophilized salmon and dehydrated milk as well as an experimental system comprised by methyl linoleate, cellulose, and glycerol to study lipid oxidation. However, because of the significant differences and complexity in these experimental systems, this work will not be discussed. Years later, a unique study conducted by Mouradian et al. [234] investigated the chemical stability of lipid-based vesicles over the course of 49 days. Sarcoplasmic reticulum (SR) vesicles prepared from lobster tail muscle were isolated and lyophilized. These vesicles were not a pure lipid system and contained proteins inherent to the SR. The oxidative degradation of these lipid-based vesicles was assessed by measuring the accumulation of lipid hydroperoxides and secondary oxidation products (i.e., aldehydes) with the TBARS assay. Oxidation was expected to be a major degradation pathway due to the presence of polyunsaturated lipids in the SR membrane. The effect of varying the concentration of trehalose (0–2 g trehalose/gram membrane) was tested, and it was observed that the trehalose content of SR vesicle samples had no effect on oxidation. However, the moisture content, which was dictated by the relative humidity samples were exposed during storage (0 and 35%), and exposure to light had significant effects on the stability of the SR vesicles. As might be expected, exposure to light correlated with increased hydroperoxide accumulation. Surprisingly, a greater concentration of secondary oxidation products was detected in the samples with lower moisture content (exposed to 0% relative humidity). Mouradian et al. [234] proposed that the secondary oxidation products were less stable chemically in samples with a higher moisture content preventing their accumulation and subsequent detection.

Recently, in our laboratory, we have studied the chemical stability of a triply unsaturated lipid, 1,2-dilinoenoyl-sn-glycero-3-phosphocholine (DLPC; 18:3), in a lyophilized formulation. This work specifically focused on the oxidative degradation of DLPC during lyophilization [235]. Iron (II) was added to the DLPC–sucrose samples to mimic the trace transition metal contaminants that are known to be present in the pharmaceutical-grade sugars that are used as lyoprotectants. During lyophilization, DLPC peroxidation occurred during the freezing step and the DLPC peroxidation increased as the iron (II) concentration was increased. It was determined that the DLPC peroxidation occurred during the freezing process, and did not continue, while the DLPC samples were maintained in the frozen state. The concentration of sucrose in DLPC samples as well as the buffer ionic strength also affected DLPC stability. Increasing the sucrose concentration caused a decrease in the buffer ionic strength in the freeze concentrate which increased DLPC peroxidation. The effect of the buffer ionic strength on DLPC stability was confirmed by conducting an experiment in samples which were stored at 4 °C overnight, and the buffer ionic strength was increased in these samples to mimic the freeze-concentrated state encountered during the freezing step of lyophilization. Considering that iron ions are known to interact with lipid bilayers (i.e., liposomes and microsomes) through electrostatic interactions with the negatively charged phosphate in the lipid headgroup [236], the increased concentration of ions (in our case, ionized Tris molecules and chloride ions) may inhibit interactions that promote lipid degradation. Alternatively, the reduced DLPC degradation may be due to the formation of iron–chloride complexes which decreased the iron–lipid interactions; previous studies have shown that chloride ions can reduce iron-catalyzed lipid oxidation [237–240]. Another potential explanation involves the Tris salts playing a more direct role in decreasing DLPC peroxidation. In this regard, it is important to note that Tris has been reported to react with hydroxyl radicals formed during lipid peroxidation to a greater extent than fatty acids, such as linoleate [241]. It was also observed that the addition of a reducing agent to DLPC–sucrose samples when exogenous iron was not added significantly increased the DLPC oxidative degradation. These results are consistent with the suggestion that the sugar used in the formulation possesses trace quantities of iron (III) contaminants such that addition of sodium sulfite (Na_2SO_3) undergoes redox with the sucrose–Fe^{+3} complex to produce iron (II) and/or displaces the iron (III) species which then undergo in situ redox reactions to produce the more reactive iron (II) ion which ultimately catalyzes DLPC degradation [235]. Our laboratory has continued the work with DLPC and extended our focus to examine the long-term storage stability of DLPC (18:3), DLinPC (18:2), and DOPC (18:1; NM Payton, manuscript in preparation). DLPC, DLinPC, and DOPC were lyophilized in a trehalose formulation and stored at 4 °C, room temperature, 37, 50, and 60 °C. DOPC degradation was not observed at any of the temperatures which were studied. However, significant degradation of DLinPC and DLPC samples was observed over the course of this study (i.e., 8–11 months), and DLPC samples exhibited the greatest degradation. The increased rate of degradation with the increased degree of lipid unsaturation was anticipated due to studies conducted using aqueous liposomal formulations [207, 224, 242, 243]. In a related experiment, DLPC was formulated with trehalose, sucrose, and sorbitol, and, after lyophilization, these samples were stored

at 60 °C. This experiment was designed to evaluate the effect of utilizing different lyoprotectants (i.e., sugars) on the storage stability of DLPC (18:3) samples. It was hypothesized that the rate of degradation for the DLPC–sugar samples would correlate with the glass transition temperature of the sugars, and that the samples lyophilized in trehalose (113 °C), sucrose (72 °C), and sorbitol (−3 °C) would exhibit slow, intermediate, and fast degradation rates, respectively [183, 244]. In contrast, the rate of degradation for DLPC formulated with trehalose, sucrose, and sorbitol was 0.069 day^{-1}, 0.024, and 0.021 day^{-1} which is not consistent with a predominant role of glass transition temperature on lipid degradation. One possible explanation for this surprising result involves the structure of the lyophilized cakes. At 60 °C, the DLPC–trehalose cakes maintained their structure with no visible change in the cake. Conversely, the DLPC–sucrose cakes collapsed over time and the DLPC–sorbitol cakes collapsed during the lyophilization process itself. It is possible that the porosity of the noncollapsed trehalose cakes allowed for the greater permeation of the headspace gases, which promoted DLPC degradation. It was also observed that the degradation of DLPC plateaued, that is, the rate of degradation slowed considerably for the DLPC–sugar samples. Perhaps the change in the rate of degradation reflects the rates of oxidation for the lipid on the surface versus the interior of the cake. The lipid on the surface of the lyophilized cake is more exposed to the headspace gases, (e.g., oxygen) in the lyophilization vial and would be expected to be more susceptible to peroxidation than the lipid embedded in the cake interior. Alternatively, the decomposition of preexisting lipid hydroperoxides could have been playing a role. Both iron (II) and iron (III) are known to catalyze the decomposition of hydroperoxides leading to the formation of alkoxyl and peroxyl lipid radicals which can further propagate the reactions leading to oxidative degradation [220]. Thus, the change in the rate of DLPC degradation could be the result of depletion of the preexisting hydroperoxides (i.e., the source of alkoxyl and peroxyl radicals). Further studies are being conducted to investigate these hypotheses.

Hydrolysis

The hydrolysis of lipids leads to the formation of lysophospholipids and fatty acids [245]. The carboxy esters which form the connection between the glycerol backbone and the fatty acid tails are most susceptible to hydrolytic degradation. Lysophospholipids can also be further hydrolyzed leading to the formation of glycerophospho compounds and fatty acids [245]. Hydrolysis can be acid or base catalyzed. Although several studies have examined the factors that affect the hydrolysis of lipids in aqueous formulations, it is difficult to compare the results due to the use of different liposomal systems and conditions. Temperature, pH, and the use of anionic/cationic lipids are the factors which have been shown to have the largest effect on the rate of hydrolysis [246].

Effect of pH

Several studies have looked at the effect of pH on the hydrolysis of aqueous liposomal formulations. The hydrolysis of DPPC–DOPE (16:0, 18:1) and DOTAP–DOPE (18:0, 18:0) liposomes was evaluated as the pH was increased from 4 to 9. A distinct trend was observed indicating that the rate of hydrolysis was minimized between pH 5.6 and 6.7 for all of the formulations which were investigated [246]. Another study also examined the effect of pH on the rate of hydrolysis using partially hydrogenated egg phosphatidylcholine (PHEPC) liposomes. Consistent with the results reported by Vernooij et al. [246], the rate of hydrolysis was slowest for PHEPC liposomes at approximate pH of 6.5 [212]. Somewhat different results were reported by Ho et al. [247] in their investigation of the hydrolysis of POPC (16:0/18:1) at pH 1, 4, 7, and 10. Contrary to results reported by Vernooij et al. and Grit et al., where increased hydrolysis at pH 4 was observed, Ho et al. [247] observed that the hydrolysis of POPC only occurred at an appreciable rate at pH 1. The underlying reasoning regarding the occurrence of hydrolysis only at pH 1 was not discussed. Instead of varying the pH and examining the effect on the rate of hydrolysis, Kensil and Dennis [248] focused on base-catalyzed hydrolysis of egg PC liposomes at pH 12.7. The ability of hydroxide ions to access the ester bond, which connects the glycerol backbone to the fatty acids, was examined by measuring the rate of hydrolysis when the EPC bilayer was perturbed. A nonionic surfactant (Triton X-100) was spiked into EPC samples to perturb the bilayer. As the Triton X-100:EPC molar ratio and the resulting bilayer perturbation was increased, small increases in the rate of hydrolysis were observed. The effect of increasing the fatty acid chain length was investigated by measuring the rate of hydrolysis for DLPC (12:0), DMPC (14:0), and DPPC (16:0) Triton X-100-PC liposomes (8:1). It was observed that the fatty acid chain length did not have a significant effect on the rate of hydrolysis. The effect of the varying lipid headgroup was also investigated. To eliminate any variation caused by a difference in the fatty acid tails, phosphatidylethanolamine (PE) lipids were prepared from EPC via transesterification. PC liposomes were hydrolyzed three times faster than PE liposomes, which were attributed, in part, to the overall neutral and anionic charge of the PC and PE liposomes, respectively, at pH 12.7. The electrostatic repulsion between the anionic PE liposomes and the hydroxide ions was thought to contribute to the lower hydrolysis rate, although the differences in packing of the PC and PE molecules in the bilayer and the penetration of the bilayer by solvent molecules may also have contributed to the different hydrolysis rates. The authors also investigated the positional specificity of hydrolysis using ^{14}C radiolabeled DPPC (16:0) liposomes, and the hydrolysis of the palmitic acid tails was evaluated below and above the liquid-to-crystalline gel transition temperature (40 °C). Hydrolysis of the fatty acids at the sn-1 and the sn-2 positions were not significantly different and the physical state of the DPPC bilayer (liquid crystalline or gel) did not preferentially increase the hydrolysis at sn-1 or sn-2 [248].

Inclusion of Cationic and Anionic Lipids

DOTAP is a cationic lipid that is often utilized in liposome formulations, especially those which are intended to deliver nucleic acid therapeutics. The effect of the incorporation of DOTAP into a liposomal formulation on the rate of hydrolysis was evaluated by comparing DOTAP–DOPE (18:0, 18:0) liposomes with liposomes composed of the zwitterionic/neutral lipids, DPPC–DOPE (16:0, 18:1), at pH 7.0 [246]. An increased rate of hydrolysis was observed for both lipids in the DOTAP–DOPE formulation. The Increased rate of hydrolysis for liposomes containing DOTAP was attributed to increased accessibility of ester linkage between the glycerol backbone and the fatty acid tails, thereby promoting hydroxyl-catalyzed hydrolysis [246]. The effect of the incorporation of an anionic lipid, egg phosphatidylglycerol (EPG), into a PHEPC liposomal formulation has also been investigated. An increased rate of hydrolysis was observed for EPG–PHEPC (10:4) liposomes relative to pure PHEPC liposomes, particularly in the pH range of 4–6.5 [65]. The increased hydrolysis rate for EPG–PHEPC liposomes was attributed to an accumulation of protons at the lipid bilayer–water interface leading to increased acid-catalyzed hydrolysis [65]. To our knowledge, there have been no studies which have examined the hydrolytic degradation of lyophilized liposomal formulations. Admittedly, such a study would be very difficult to perform at the low-water contents (~1 %) typical of lyophilized formulations.

Nonviral Vectors as Dehydrated Medicines: Lipid/DNA Complexes

A wide variety of nonviral gene delivery (lipid-based) systems has been developed and continually being improved for *in vitro* and *in vivo* gene delivery (e.g., DNA, siRNA) as a viable alternative to viruses [41, 43, 249–252]. However, it is recognized that these therapeutic systems have been hampered by critical pharmaceutical issues, such as physical and chemical instability, that need to be addressed before nonviral vectors can become a pharmaceutical reality. Due to their rapid deterioration (i.e., aggregation) the prolonged storage of lipoplexes in aqueous formulation is difficult to achieve considering the high sensitivity of DNA to hydrolytic and oxidative degradation [61–63]. Furthermore, as mentioned earlier, agitation is a stress that can occur during shipping and presents crucial difficulties [55, 69, 72]. Since lipoplex-based pharmaceuticals could benefit from ambient storage, lyophilization is a feasible approach to prepare dehydrated formulations of nonviral gene vectors. To date, various additives including trehalose and other sugars have been investigated in an effort to stabilize lipid/DNA complexes during lyophilization, but room temperature stability for pharmaceutically relevant timescales (i.e., 2 years) has yet to be demonstrated. Knowing the high tendency of lipid-based particles to aggregate, appropriate quantities of virtually any excipient should offer protection during freeze-drying [72, 83, 88]. In fact, it has been suggested that the volume

of the unfrozen fraction at a critical excipient to DNA ratio is sufficient to isolate lipid/DNA complexes in a viscous sugar matrix, thus preventing aggregation during freeze-drying. An excipient to DNA weight ratio of approximately 1000 was sufficient to achieve complete protection by either sucrose or glucose [83], which is in good agreement with later studies on the stabilization of different types of lipid/DNA complexes [103]. Indeed, a prominent drawback of these potential medicines in a solid phase is their tendency to undergo physical and chemical damage during storage [179, 183], which in turn constitutes a critical pharmaceutical problem for the development of lipoplexes as marketable therapeutic products [56, 58]. These stability challenges are discussed below.

Formulation of Lipid/DNA Complexes

A well-designed liposomal delivery composition will be capable not only of increasing potency of gene-based drugs and decrease the risk associated with toxicity, but also of maintaining its physicochemical characteristics during storage. Certainly, a nucleic acid-containing particle should have a small size (less than 100 nm) and better *in vivo* stability in order to be suitable for systemic gene delivery [253, 254]. Furthermore, formulation factors (i.e., number of components, cation to nucleic acid phosphate charge ratio (+/−), concentration of components, order and mixing rate of the components, ionic strength, as well as temperature of assembly and/or preparation) required to prepare stable particles requires appropriate vector design and a robust method of manufacturing. Considering that most delivery systems incorporate cationic and unsaturated agents (as mentioned above) that interact electrostatically with nucleic acids to facilitate both encapsulation and intracellular delivery [255], various encapsulation technologies have been developed to generate more advanced lipid delivery systems (i.e., multiple lipid component compositions that employ a mixture of cationic lipid, neutral lipid, fusogenic helper lipid, and PEG lipid) needed for therapeutic application. For example, Tekmira Pharmaceuticals (British Columbia, Canada), in partnership with Alnylam Pharmaceuticals (Massachusetts, USA) has developed specialized liposome nanoparticles termed a stable nucleic acid lipid particle (SNALP) that represents the most advanced systemic siRNA delivery system [256–259]. Furthermore, Alnylam Pharmaceuticals has recently reported their positive initial data of phase II open-label extension (OLE) study with patisiran (ALN-TTR02), an RNAi therapeutic targeting transthyretin (TTR) that is currently under development for the treatment of TTR-mediated amyloidosis (http://www.alnylam.com/product-pipeline/ttr-amyloidosis-fap/; [260]). Indeed, lipid-based formulations manufactured at large scale for clinical applications will need to employ pharmaceutically robust methods that are easy to scale-up, cost-effective, and meet regulatory requirements. As a matter of fact, within the past two decades, several methods have been developed to enhance nucleic acid encapsulation in multicomponent liposomal systems including passive (lipid film) technology [261, 262], ethanol dialysis [257, 263], reverse-phase evaporation [264], detergent dialysis [265–267], and, recently, hydration of a freeze-dried matrix [268, 269].

Recent reports have shown that some advanced formulations prepared by these alternative methods have been applied with significant success for *in vivo* studies of therapeutic siRNA [249, 259, 270]. Despite this great progress, prolonged storage of lipoplexes in aqueous formulation yet is difficult to achieve considering the high sensitivity of both DNA and lipid components to hydrolytic and oxidative degradation [61, 66, 67, 79, 271–273]. Furthermore, it has been reported that DNA functionality and structure may be damaged directly by co-oxidation with lipids [274–276]. In the last decade, there was a growing consensus that lyophilization technology is a suitable approach to stabilize batches of nonviral gene delivery systems as dehydrated formulations. However, lyophilization studies have mostly focused on the stability of nonviral vectors during acute freeze-drying stress [83, 85, 89, 90, 95, 99, 116, 117, 120, 185, 277–280]. Previous work has demonstrated that sugars, especially disaccharides, have the ability to preserve lipid-based nucleic acid formulations during acute freeze-drying [83, 86, 90, 95]. In fact, these authors have suggested that a critical sugar to DNA ratio (e.g., 1000, *w/w*) should be sufficient to preserve particle aggregation and vector physicochemical features in a viscous sugar-based matrix [83, 103].

Stability of Lipid/DNA Complexes

Before lipid-based therapeutics develop into a market reality, preparations will need to be physically and chemically stable when stored as dried solids at pharmaceuticals timescales (i.e., 18–24 months). Despite the benefits of storing lipoplexes as dehydrated formulations, only a limited number of studies have assessed their stability in the dried state during short-term and/or prolonged storage (Table 2). As a number of studies have implicated maintenance of particle size as a critical factor for the recovery of transfection activity [83, 90, 91, 95, 99, 103, 120, 181, 193], our recent results have demonstrated a relationship between retention of particle size and T_g of the glassy excipient phase during prolonged storage [183]. This is clearly illustrated by the fact that lyophilized vectors stored at room temperature in trehalose (high T_g; [178, 183, 276, 279]) show less tendency to aggregate as compared to glucose formulations (lower T_gs; [183]). These findings are consistent with the idea that vectors possessing more restricted mobility are less prone to aggregation [127]. It is important to realize that the viscosity of these lyophilized formulations is too high (even slightly above T_g) to allow aggregation in the dried solid. Therefore, we think that the observed particle size increases in our studies were most likely due to physicochemical changes in lipoplexes that promote aggregation upon rehydration [183]. However, despite the maintenance of particle size in many formulations (see Table 2), our previous reports have shown that progressive vector degradation occurred in spite of the high T_g values and low moisture contents, indicating that mechanisms other than aggregation are responsible for the loss of biological activity during storage [178, 179, 183, 276]. It is important to mention that under the experimental conditions of prolonged storage (e.g., 24 months), dried lipoplexes for-

Table 2 Storage studies of lyophilized (*LF*) lipid-based therapeutics

Vehicle	Initial buffer	Excipients (*w/w*)	Protocol	Duration	Recovery	Reference
Lipid/ protamine/DNA	None Water	Sucrose (10%)	LF, stored at 42 °C, 25 °C, 37 °C	2 M	Size, transfection retained (4 °C, 25 °C)	[95]
Lipid/DNA	2.5 mM Tris-HCl pH 8.5	Trehalose (sugar/ DNA=1000)	LF, stored at 60 °C	2 M	SC up to 2 weeks (~80%) ROS, TBARS & size increased after 2 weeks Transfection reduced after 2 weeks	[179]
Lipid/DNA	2.5 mM Tris-HCl pH 8.5	Trehalose sugar/DNA=1000 DTPA=200 μM α-ocopherol=50 μM L-Methionine=10 mM	LF, stored at 25 °C, 40 °C, 60 °C	2 M	Treh alone: progressive degradation +DTPA: SC, transfection, size retained +α-Toc: SC, transfection, size retained +L-Met: progressive degradation	[178]
Lipid/DNA	20 mM HEPES pH 7.8	Trehalose sugar/DNA=1000 DTPA=200 μM+ α-Toc=2.5 μM	LF, stored at 25 °C, 40 °C, 60 °C	3 M	Size retained at all storage conditions SC~80% up to 3 months (40 °C) SC~75% up to 3 months (60 °C) TBARS retained at 25 °C	[276]
Surfactant/lipid/ DNA	None ultra pure water	Trehalose (10%), sucrose (9.5%)	LF, stored at 25 °C	3 M	Size retained (in suc, treh formulations) Transfection up to 3 M: Suc; Treh ~70%	[279]
Lipid/ protamine/ siRNA	None DEPC-treated water	Trehalose sugar/liposome=11.6	LF, stored at 4 °C	3 M	Size: progressive decrease, initial (>600 nm) Biological potency up to 1 month	[281]
Lipid/ protamine/DNA	None Sterile water	Lactose sugar/DNA=900	LF, or spray-dried stored at 25 °C/ vacuum	3 M	Transfection retained up to 84 days	[120]
Lipid/DNA	None Water	Trehalose (10%)	LF, stored at 25 °C, 30 °C, 40 °C	12 M	Transfection up to 9 months (25 °C) Large size after LF (>300 nm)	[287]
Lipid/DNA	2.5 mM Tris-HCl pH 8.5	Sucrose, trehalose, glucose sugar/DNA=1000	LF, stored at −20 °C, 4 °C, 25 °C, 40 °C, 60 °C	24 M	Size retained, 24 months (−20 °C) Transfection, 3 months (−20 °C)	[183]

DTPA diethylenetriaminepentaacetic acid, *M* months, *TBARS* thiobarbituric acid reactive substances, *w/w* weight to weight ratio, *DEPC* Diethylpyrocarbonate

mulated with glucose also exhibited severe physical degradation (i.e., brown cake) indicative of nonenzymatic glycosylation via the Maillard reaction [183]. However, even nonreducing sugars (sucrose and trehalose) failed to stabilize vector formulations during storage, suggesting that other degradation mechanisms such as oxidation (generation of ROS) are active in the dried cake [178, 179, 183]. Similarly, Kundu and collaborators [281] recently have suggested that their nanosome degradation may be the result of ROS formation-related siRNA oxidation. As a matter of fact, fundamental issues associated with the role of ROS in the chemical stability of lipid/DNA complexes in the dried state have been recently elucidated by our group, and data consistently showed that ROS are generated in the dried cake. [115, 179, 183, 185]. We have speculated that trace amounts of transition metals in excipients can catalyze the generation of ROS, and that the high free volume of glasses might facilitate their diffusion [115, 183]. Furthermore, we have determined that the lipid component contributes substantially to the observed oxidative damage in dried cationic lipid/DNA complexes[179]. We have also suggested that the close proximity of DNA to the lipid component in lipoplex formulations facilitates the interaction of DNA with oxidized lipids (e.g., peroxyl radicals) or with other by-products of lipid peroxidation, thereby compromising stability. In fact, our group has observed that TBARS: lipid peroxidation end products or aldehydes [282, 283]) are formed and accumulated in lyophilized cakes during storage [179]. Considering that unsaturated lipids constitute the cornerstone of lipid-based therapeutics [45, 284] and noting that oxidation of lipids via a free radical mechanism is exacerbated in lipids possessing higher degree of unsaturation [235, 285], our group also explored different strategies to minimize oxidative damage in the dried solid during storage [178, 179]. We found that in spite of the inclusion of demetalation and headspace oxygen displacement steps, formation of ROS was still observed in dried formulations, which suggests that these approaches are not sufficient to protect lipoplexes. However, the inclusion of a metal ion chelator (DTPA, 200 μM) or a lipid-soluble antioxidant (α-tocopherol, 50 μM) to our formulations prior to lyophilization enhanced the stability of dried lipoplexes during storage [178]; formulation strategies that have been applied in subsequent lipid-related storage studies [276].

Effect of Moisture on Lipid/DNA Complex Stability

The effect of moisture content on the chemical and biological stability of lipid/DNA complexes during storage has been recently explored by our group and others [178, 276, 279]. Considering that water is a potential source of ROS [191, 286] and that it can participate in hydrolytic reactions that can chemically degrade biomolecules during storage [138, 229], it is typically assumed that lower water contents might enhance the stability of lipoplexes in the dried state. To this respect, recently, Molina and Anchordoquy [178] showed that oxidative damage was considerably enhanced in lyophilized preparations possessing low T_gs (~56 °C) and relatively higher moisture content (~2%) after 2 months of storage at higher temperatures (i.e., 40 °C, 60 °C), consistent with previous studies that suggest that maximal sta-

bility of glassy solids can be achieved when T_g is 50 °C above the storage temperature [127]. In contrast, subsequent studies by Yu and Anchordoquy [276] showed that formulations possessing comparable moisture contents (~1.9%) maintained lower levels of TBARS and higher SC content than those containing lower levels of moisture (~0.4%). Despite these initial efforts, no correlation has been found between the changes in RM with time and the physicochemical and biological activity upon storage in lipid/DNA-based pharmaceuticals [276, 279]. Therefore, future studies should explore the effect of moisture contents in different lipoplex compositions (alone or in combination with the aforementioned strategies) in order to better define the optimal moisture content range for storage.

Conclusions

In conclusion, lyophilization is considered a suitable tool to preserve nonviral vectors in the dried state. Nucleic acid- and lipid-based formulations have been shown to undergo oxidative damage (generation of ROS, loss of SC) in the dried solid, and these species have been associated with the instability of formulations during storage. Studies have demonstrated that nucleic acid-based formulations can be preserved in trehalose as dehydrated preparations when chelators (DTPA) and/or antioxidants (α-tocopherol) are included in the formulation. In addition, our studies have clearly shown that naked DNA is relatively stable, and that the lipid component is more prone to oxidation during storage. Considering the growing interest in the development of more efficient and stable nonviral vector systems, studies focusing on the stability of more advanced lipid/nucleic acid (DNA; oligodeoxynucleotides, ODNs; siRNA) compositions are needed to achieve shelf lives similar to other biopharmaceuticals (24 months).

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Part III
Advances in Alternate Drying

Alternatives to Vial Lyophilization

Jim Searles and Mathew Cherian

Introduction

While lyophilization possesses many advantages, there are also drawbacks to its use. At its best, successful formulation and lyophilization yields a sterile, active, stable product that is easily reconstituted, and is sealed within its final container. However, it could not be accurately described as highly efficient, and in some cases a vial as a final container may not be optimal. For some products, reconstitution is neither fast nor easy, and sometimes a free-flowing bulk powder is more useful than a rigid cake encased in a vial.

One should always consider the liquid form as the primary alternative to lyophilization. During product development, it is standard to attempt discovery of a liquid formulation, with the lyophilized form as the main alternative. The choice of lyophilization is usually driven by the failure of liquid formulations to sustain sufficient stability over shelf life.

Examples of how some of the alternatives to lyophilization ameliorate some of the disadvantages inherent to lyophilization:

- Lyophilization has very high-energy requirements. Temperatures and pressures far from ambient must be sustained over long periods of time while supplying the energy for sublimation plus the energy for condensation. The economics and scale ability of spray-drying are such that a single spray-dryer can produce five to ten times the product that a lyophilizer of similar size and cost can produce.

J. Searles (✉)
Hospira, Inc. One 2 One® Research and Development, McPherson, KS, USA
e-mail: James.Searles@hospira.com

M. Cherian
Hospira, Inc, Early Stage Research & Development
Lake Forest, IL 60045, USA

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- Loading and unloading of lyophilizers requires either direct human intervention for manual loading, or complex, high-precision automatic loading and unloading equipment. The presence of humans in such close proximity of open sterile product is a concern for sterility assurance, and has high costs associated with training, gowning, airlocks, room pressure differentials, and microbial monitoring support.
- Reconstitution of some lyophilized products such as antivenoms [12] and highly concentrated therapeutic proteins [4] can take an hour or more. There is some evidence in the literature that spray-drying can yield powders that reconstitute faster than the lyophilized version [7].
- Free-flowing powders produced by some alternatives to lyophilization are more versatile than lyophilized cakes because they can be filled into a wide variety of containers for innovative reconstitution/injection technologies [9].

This chapter covers the following alternative drying methods with current or emerging capability to serve as alternatives to lyophilization: sterile spray-drying, bulk tank freeze-drying, aseptic crystallization and drying, vacuum-drying, and drying on a fiber matrix. Most of these generate flowable powders, so we have included information on aseptic powder filling into vials as well as innovative reconstitution-injection technology under development. Finally, we offer some insight into reformulation as a liquid.

Sterile Spray-Drying

Spray-drying is a manufacturing technology that has a long history in the production of dry powder forms of foods, chemicals, pharmaceutical excipients, and active pharmaceutical ingredients (APIs). A comprehensive review of biopharmaceutical spray-drying can be found in [19] and [20].

Sterile spray-drying is an emerging technology, in that it is close to commercialization in the pharmaceutical industry. It offers the potential to generate sterile powders that can be filled into vials as a direct replacement for lyophilization, with the promise of higher throughput/lower cost [10, 11].

Several vendors are having success in delivering integrated production equipment over a range of sizes, and the authors understand that some pharmaceutical companies are developing sterile spray-dried products. And while there are contract manufacturers that offer sterile spray-drying, we know of no sterile injectable drugs or biologics produced by sterile spray-drying that have been approved by the regulatory body of a large market. The coming decade will certainly see regulatory approvals of such products, and a rapid proliferation of equipment and process parameters that have been "proven" as fully GMP.

Spray-drying is a mechanistically simple, continuous process, in which two streams are pumped simultaneously into a drying chamber: a fine spray of the solution to be dried (usually aqueous), and a drying gas. What exits the other end of the

chamber is a single stream containing dried particles suspended in the gas. In the ideal case, each droplet of the solution has been dried to form a single particle, as it has been carried downward through the chamber by the surrounding gas and the force of gravity. The particles are removed from the gas stream with a cyclone or a filter.

There is a common belief that the high temperatures usually used for the inlet drying gas would lead directly to product degradation. However, the drying droplet's temperature is much closer to the wet-bulb temperature of water due to evaporative cooling, and the residence time of a given droplet from the time it is sprayed to the time that it has completed drying and is in a cooler collection vessel is on the order of seconds. In addition, it is possible to use very low drying gas temperatures if one is willing to accept lower throughput. As discussed in the recent reviews cited above, many have found that spray-drying can be used for thermolabile products, including vaccines [5, 15, 18].

Development of a sterile spray-dryer requires addition of full sterilization-in-place (SIP) capability, sterilizing vent filters on the inlet gas line, and fully aseptic powder harvesting. In addition, one must have the ability to perform sterile powder filling into vials or other suitable containers. Fortunately, sterile powder-filling technology has been in use for many years. One challenge associated with filter sterilization of the inlet gas is that relatively large filter banks are required, and these must be tested for integrity. Integrity testing of very large filter banks can be a challenge.

While formulation and process development for lyophilization can be reliably carried out on a small scale, spray-drying presents special challenges in this regard. Benchtop spray-dryers are known to lead investigators to "false negative" conclusions of the feasibility of using spray-drying. The principal challenges with benchtop spray-drying are that it is very easy to "overheat" the sample during drying, and the percent recovery of powder can easily be $<50\%$ due to deposition on the walls of the drying chamber and powder collection cyclone. Both of these problems are known to diminish with increasing spray-dryer size.

A recent paper addresses these challenges, showing that for multiple recombinant monoclonal antibodies, a "laboratory scale" dryer (chamber diameter 30 cm) significantly outperformed three benchtop scale units (chamber diameters 11–17 cm) with respect to product recovery, which was $>90\%$ [3]. They also observed that higher ratios of antibody to trehalose resulted in higher recoveries, because the trehalose imparts additional tackyness or stickyness to the formulation, increasing the deposition of partially dried droplets on the walls. Reconstitution times were <3 min. After 3 months of stability testing at 40 °C, the decrease of monomer content was greater (worse) for formulations with less trehalose. This observation brings about the need to optimize the formulation composition in concert with process development and scale-up, for while higher trehalose concentrations improved stability, they negatively impacted product recovery. This suggests the need for an additional excipient such as maltodextrin to be used as a "drying aid" to reduce tackyness and improve product recovery [21].

With full commercialization of sterile spray-drying, the full advantages that the technology offers over lyophilization will be fully realized. One of those advantages is discussed in a section below on innovative, lower cost packaging, and delivery solutions that require a flowable powder or liquid product.

Bulk Tank Freeze-Drying

This section describes new technologies for producing sterile powders by freeze-drying in tanks. The processes used by each are similar to drying on lyophilizer shelves, except that freeze-drying is carried out in tanks that agitate the product as it is progressed through the freezing, primary drying, and secondary drying steps. With some systems it is possible to attach a separate spray-freezing chamber. Such systems offer the prospect of greatly reduced drying times compared to conventional tray freeze-drying with product pre-filled into vials.

The "Active Freeze-Dryer" from Hosokawa Micron Powder Systems uses a conical tank with a mixer/wall scraper to mix the material, as well as to remove and refresh it from contact with the wall. The drying tank is jacketed for heat transfer. Figure 1 shows a schematic of the system components, which includes a separate freezing tank [14]. Below is a quote from the Hosokawa literature on how the process works:

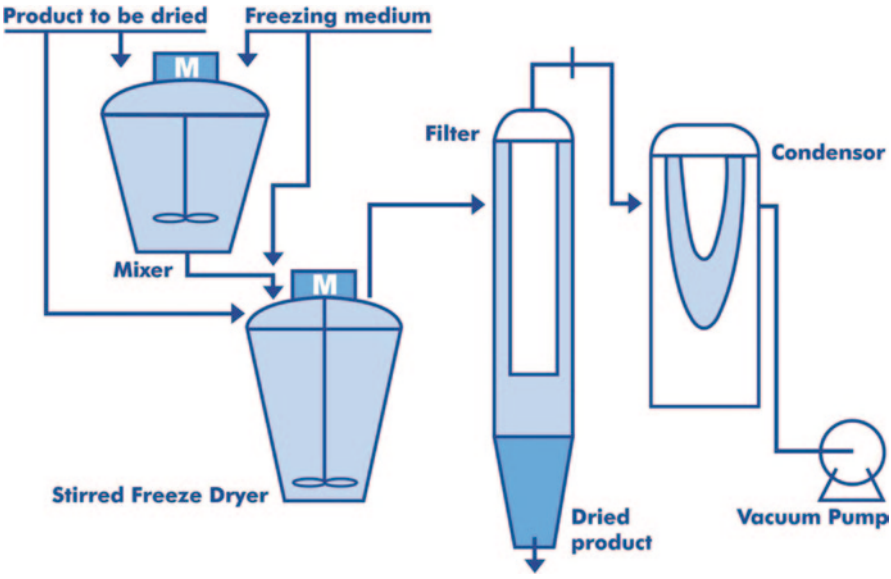

Fig. 1 Schematic of Hosokawa "Active Freeze Dryer" system (Image courtesy of Hosokawa Micron Powder Systems)

In a dedicated designed stirred drying chamber the material to be dried is frozen. Due to stirring motion the material, being a liquid, paste or solid, will be transformed into solid granules. The granules can have sizes and shapes which are controlled by the mixing characteristics of the machine.
Once the freezing step is completed the drying chamber is closed and vacuum is applied. After evacuation of the freezing agent the sublimation process will start. From this stage the product temperature is dictated by the vacuum level. During sublimation the heat is supplied through the jacket and efficiently distributed throughout the product by the stirrer. The initially coarse granules will gradually reduce in size due to the sublimation of the connecting ice structure in between the frozen material. The released dried particles will make up a loose powder.
Towards the end of the drying process when most of the frozen solvent is sublimated the product temperature will start to rise. Finally the product temperature will equalize the wall temperature, indicating that the drying process is finished. By then all material is transformed into a fine and loose powder, after breaking the vacuum the dryer can be discharged easily from the dryer vessel, assisted by the transporting characteristics of the mixing element.

Figure 2 shows images of freeze-dried material from the Hosokawa Active Freeze Dryer and a standard tray freeze-dryer, providing evidence that the Active Freeze Dryer can achieve similar particle sizes of dry product. Per the manufacturer, the minimum pressure that can be currently achieved is approximately 400 mTorr, and they have not yet developed a sterile version.

Meridion Technologies GmbH promotes a bulk freeze-dryer, LyoMotion, that tumbles its contents in a rotating cylinder that is mounted horizontally, as shown in Fig. 3 (Meridion Technologies n.d.). Heat transfer is by thermal radiation and heated product-contact surfaces. They also offer spray-freezing technology (*Spray-Con*) that can be connected to their *LyoMotion* dryer.

IMA Life North America has applied for patents for using spray-freezing to create frozen droplets, followed by freeze-drying in an agitated tank, as shown in Fig. 4 [8]. The spray-freezing concept includes separate product and liquid nitrogen spray nozzles at the top of the freezing tank.

A concern with all of these concepts is that one must be mindful of product loss by deposition on the internal surfaces of the equipment; however, this disadvantage is also a concern for spray-drying.

Aseptic Crystallization and Drying

Aseptic crystallization/precipitation and drying is a current technology that is commonly used for nonsterile as well as sterile APIs. The process stream entering the crystallization step of the API manufacturing is sterile filtered, using 0.2 μm filters, into pre-cleaned and sterilized tanks, and the product, after crystallization, is filtered and dried in aseptic fluid drying step. This last stage of the API manufacturing process is carried out under International Organization for Standardization (ISO) 5, aseptic conditions. This part of the process will need to be validated for asepticity by media runs simulating the process.

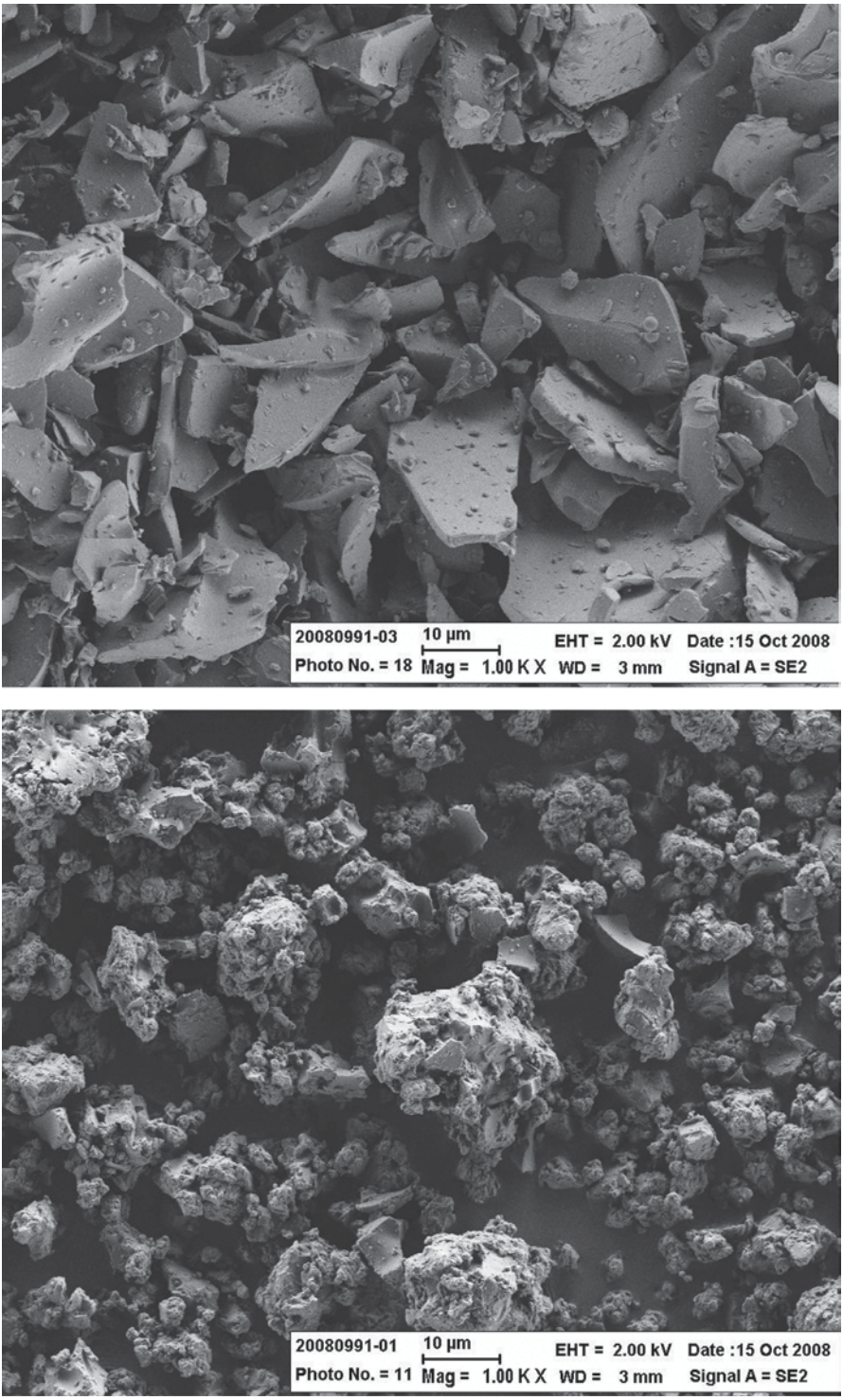

Fig. 2 Scanning electron micrographs of product freeze-dried on a standard tray dryer (*top*) and the Hosokawa "Active Freeze Dryer" (*bottom*). Scale bar is 10 µm (Image courtesy of Hosokawa Micron Powder Systems)

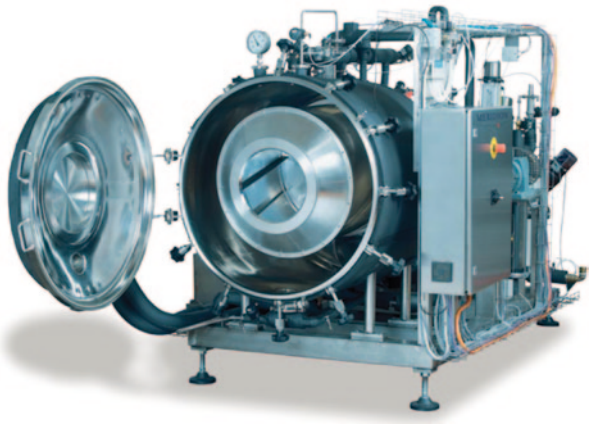

Fig. 3 Meridion Technologies GmbH pilot scale LyoMotion dynamic bulk freeze-dryer. The company also offers SprayCon spray freezing technology that can be connected directly to LyoMotion (Image courtesy of Meridion Technologies GmbH)

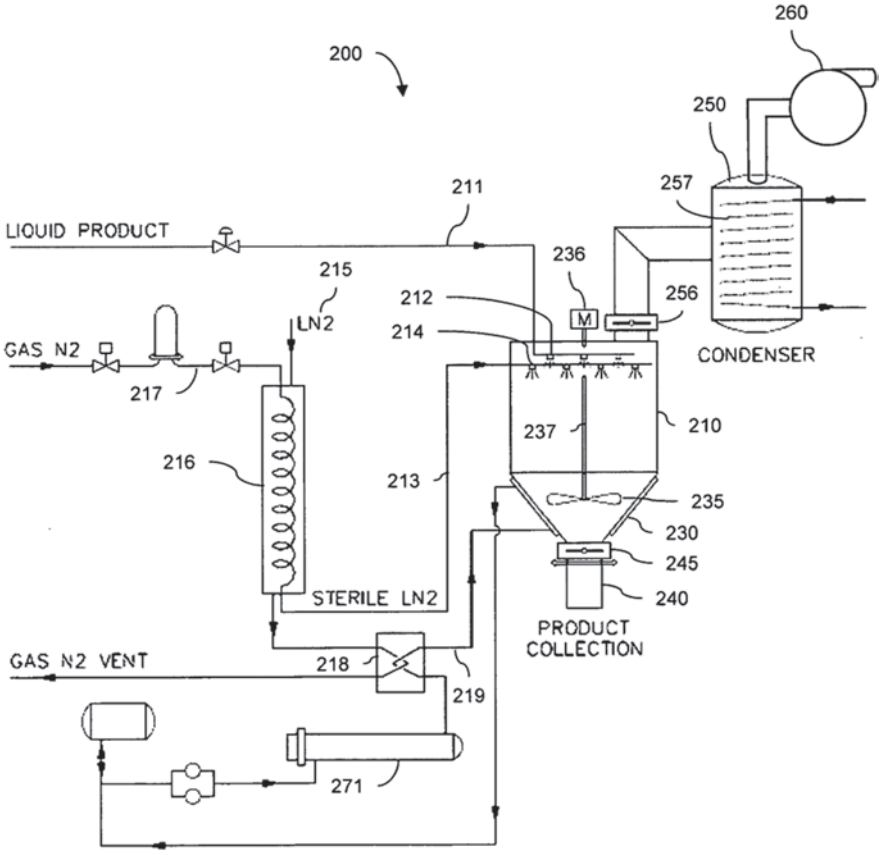

Fig. 4 Schematic from patent application titled "Bulk freeze drying using spray freezing and stirred drying" from Patent Application# WO 2012018320 A1 by Francis W. Demarco and Ernesto Renzi, Filed 04 August 2010, assignee IMA Life North America Inc. (Reprinted by permission from IMA Life North America, Inc.)

Special care is taken during process development to ensure that the crystalline form, size range, moisture content, etc. are optimal to help the powder flow freely during the powder-filling step. Unimpeded powder flow is essential to do aseptic powder fill operation. The sterile powder is collected in aluminum containers, and ported to sterile powder-filling machines, and filled in de-pyrogenated/presterilized vials to obtain the finished product, described further below.

Vacuum-Drying

Although less often used than freeze-drying, vacuum-drying has found its use in making pharmaceutical products. As the name suggests, the process does not entail freezing, but application of vacuum only, at low temperatures. Typically the drug is dissolved in ethanol, loaded on to freeze-dryers, the shelf temperature brought down to −25 to −35 °C, and a vacuum is applied. Care is taken to ensure that the contents of the vial does not "boil" at the low pressure, as this will likely lead to the contents being expelled from the vial. At the end of the cycle, the vials will be left with the active drug in the form of a film. Often the film is barely visible—depending upon the quantity of active used. Sometimes, an excipient soluble in ethanol is added to the formulation, so that the finished product does appear to be a visible film, amenable to easier visual inspection.

In cases in which a lower vapor pressure solvent is required to achieve dissolution of the API, a cosolvent approach can be taken to increase the vapor pressure to make the formulation amenable to vacuum-drying [6]. Examples of such solvents are dimethyl acetamide and dimethyl sulfoxide.

Vacuum-drying cycles are easier to develop than standard freeze-drying cycles. There is no need to determine a collapse temperature or eutectic point. Formulation development is significantly reduced, as the only requirement to be met is that the active be soluble in ethanol and that there is no alcohol—drug chemical reaction. The one critical parameter to observe is the potential for the contents of the vial to boil over. Typically, vacuum-drying cycles are shorter when compared with freeze-drying. In view of the flammability of ethanol, explosion-proof equipment will be necessary. Typically, this can add significant cost for capital equipment, and may explain why vacuum-drying is not more widely used.

The process for manufacturing vacuum-dried products is not significantly different from that used for regular freeze-drying. The active is dissolved in ethanol (sometimes an excipient is added for enhancing visual appearance), sterile filtered into pre-cleaned and de-pyrogenated vials, and loaded on to freeze-dryers. Shelf temperature is lowered to −25 to −35 °C, and vacuum is applied. Prior laboratory work will have been done to select the optimal vacuum and shelf temperature, in order to ensure that the contents do not boil over.

Commercially available pharmaceutical products which avail vacuum-drying technology include the cytotoxic drugs carmustine and lomustine.

Drying on a Fiber Matrix

In another example in which the physical form of the dried product enables value-added packaging, reconstitution, and delivery technology was demonstrated by Alcock in a 2010 publication that product solutions can be dried while suspended within a filter-like matrix of inert fibers [2]. This "coupon" of dried product + fiber matrix can then be packaged in a housing that is installed between a syringe containing diluent and a needle, and the product can be reconstituted as the diluent is pushed through the matrix, as the reconstituted product flows out into the needle and then the patient. Figure 5 shows a scanning electron micrograph of dried product on the fiber matrix as well as a photo of the assembled reconstitution/injection device.

The formulation of the product is of course very important as always, to provide stabilization. In the published case, live modified vaccinia virus Ankara (MVA)

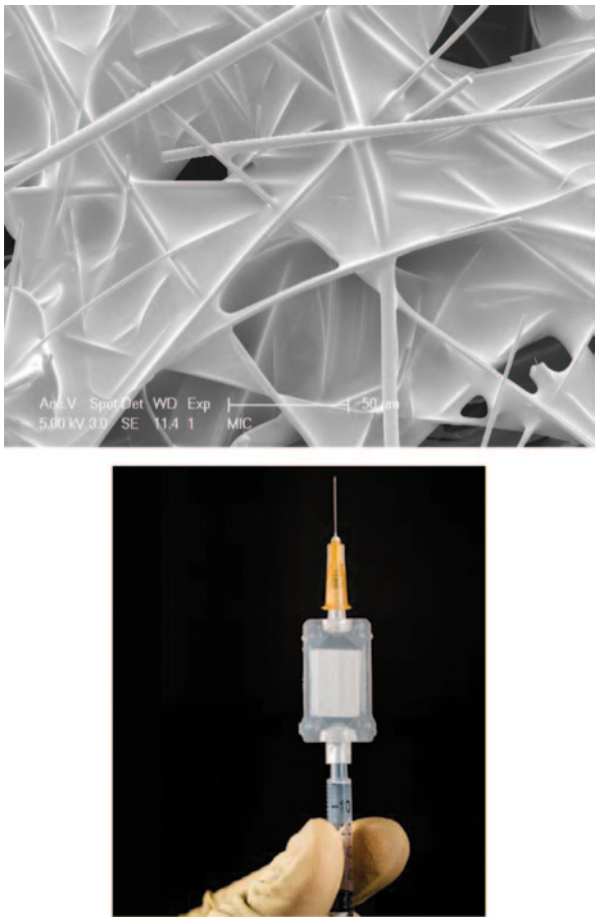

Fig. 5 HydRis® by Nova Laboratories. *Top*: scanning electron micrograph of product dried on fiber matrix. *Bottom*: HydRis® system (Image courtesy of Nova Laboratories Ltd.)

and E1/E3-deleted human adenovirus type 5 (AdHu5), which are currently in use as vectors for several vaccines, were successfully stabilized by formulating with sucrose and trehalose together. They achieved complete recovery of viral titer and immunogenicity after storage at up to 45 °C for 6 months. The prototype product, HydRis®, is offered by Nova Laboratories Ltd. [17].

Successful commercialization of this technology will of course require development and scale-up of robust manufacturing processes and equipment for cleaning and sterilizing the fiber matrix, aseptically applying then drying the product solution, storing and testing the individual doses, and aseptically packaging and sealing the doses into the housings. Nova Laboratories is continuing development of this platform.

Aseptic Powder Filling

Sterile powders can be filled into pre-sterilized vials by commercially available equipment, and this practice has been in commercial use for decades [1, 13]. An example of a commercial sterile powder-filling line is shown in Fig. 6. The use of sterile powder filling enables conversion of a given product to an alternative to lyophilization without changing the primary packaging. However, the ability to manufacture sterile powdered drug product offers the advantage that novel, value-added primary packaging options can be considered. One can imagine powders being sterile-filled into empty single- or dual-chamber syringes.

Fig. 6 Sterile powder-filling machine by IMA Pharma (www.ima-pharma.com) (Image courtesy of IMA Life North America, Inc.)

Sterile powder fill operation obviates the use of expensive and time intensive lyophilization. This is accomplished by preparing the active drug as a sterile powder in the last steps of API manufacturing.

The following pre-formulation studies are typically conducted for sterile powder fill products.

Pre-formulation study	Properties influenced	Analytical technique
Crystalline vs. amorphous, crystal structure, polymorphism	Solubility, chemical stability, hygroscopicity	XRD, DSC, IR spectroscopy, polarized light microscopy, hot stage microscopy
Particle size, crystal habit, particle shape	Flow properties	Optical microscopy, SEM
Particle size distribution	Flow properties, reconstitution time, blend uniformity (for mixed APIs)	Sieve analysis, light obscuration counters, laser diffraction instruments
Bulk density, compactability	Flow properties, compaction in filling machines using vacuum	Angle of repose, Carr Index, Hausner ratio
Water content	Storage stability, flow properties	Karl Fischer
Hygroscopicity	Stability, environmental conditions during filling	Karl Fischer, loss on drying

XRD x-ray diffraction, *DSC* differential scanning calorimetry, *IR* infrared, *SEM* scanning electron microscope

Many antibiotics today are manufactured by sterile powder fill operation. These include cefazolin, ceftriaxone, penems, etc. Interestingly, even cytotoxic drugs can be manufactured in this manner. Most notable among them are cyclophosphamide, bleomycin, and mitomycin. High-level isolation is necessary for sterile powder filling of cyctotoxic to eliminate exposure of operators.

Sometimes, two or more powder species have to be mixed to get the desired product. One such example is piperacillin and tazobactam—two antibiotics. While it is possible to formulate these two very different antibiotics as a single formulation and then freeze-dry it, the challenges would be significant due to potential stability issues. Powder filling the two sterile powders makes this a relatively simple operation. There are also cases where functional excipients are co-filled in a powder fill operation. These functional excipients may be solubilizers, or buffers. Some examples are given below.

Drug	Excipient	Function of excipient
Imipenem/Cilastatin (Primaxin)	Sodium bicarbonate	Buffer
Penicillin G potassium (Pfizerpen)	Sodium citrate/citric acid	Buffer
Aztreonam (Azactam)	Arginine	Solubilizer
Ceftazidime (Fortaz)	Sodium carbonate	Solubilizer
Cephalothin (Keflin)	Sodium bicarbonate	Buffer
Cefepime (Maxipime)	Arginine	Buffer

Powder fill machines use either augers or vacuums to withdraw powder from the aluminum container and deliver to the vials. They are located inside isolators, under ISO 5 conditions. It is possible to control the humidity within these isolators in order to obtain the optimal humidity content for the final product. The current machines are highly automated, with no human interventions necessary. The incoming de-pyrogenated vials are weighed (tare), and weighed again after fill to determine the exact weight of powder filled. If there is an out of limit vial, it is routed to a recollection stream, where the contents can be retrieved without compromising sterility. These machines have speeds up to 400 vials per minute, and have shown very high accuracy for fills.

Powder fill operations are less expensive than freeze-drying, as capital and infrastructure expenditures are less and time to process is significantly reduced. Savings per vial can be as much as US$ 2–4.

Aseptic Reconstitution Cartridge Hybrid

A further drive to efficiency and high-throughput, low-cost manufacturing may someday be manifested as what is shown in Fig. 7, the Aseptic Reconstitution Cartridge Hybrid (ARCH) [9]. It consists of two compartments: one with powder and one with the reconstitution diluent. The high barrier properties of the foil laminate protect the diluent and powder alike from water loss and gain, respectively. The user reconstitutes the powder with the diluent by depressing the diluent blister to rupture the seal between the two compartments, and the product can then be withdrawn

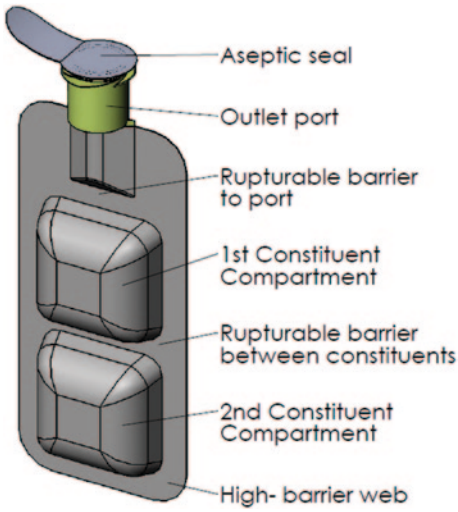

Fig. 7 Aseptic Reconstitution Cartridge Hybrid (*ARCH*) (Image courtesy of AktiVax LLC)

from the collapsible blister by an attached Luer port. Alternate configurations include the needle as well.

This and related technologies can be found in the patent literature. Designs are under development, and are currently undergoing testing by major pharmaceutical companies.

Reformulation as Liquid

The basic rationale for freeze-drying pharmaceutical dosage forms is to protect the active moiety from hydrolytic degradation when in solution. Early in the drug development cycle, the amount of drug substance available to pharmaceutical development teams is typically very small—often less than a gram. The first priority for the pharmaceutical development team is to provide a formulation for the toxicologist. If the active principle is susceptible to hydrolysis, extra efforts at developing a liquid formulation are often deferred for a quick freeze-dried product in a vial. Soon the project transitions to phase I clinical trials, and as often happens the switch to a potential liquid formulation gets low priority for resources and funding. Liquid products are obviously less expensive, and find greater acceptance in the clinic because of ease of use.

A number of freeze-dried products have transitioned to liquid solution products post approval. These include gentamicin, human growth hormone, docetaxel, gemcitabine, bendamustine, doxorubicin, and epirubicin. The conversion of these freeze-dried products to solution was primarily based on finding optimal solution parameters like pH, osmolality, ionic strength, protection from oxygen, and sometimes choice of more suitable excipients, etc. A multifactorial study designed to find the optimum conditions for stability can be pursued to this end.

Yet another approach to liquid formulations has evolved with the advent of encapsulation technologies. These technologies include liposomes, lipid complexes, and emulsions. As is well known, liposomes and lipid complexes are made of phospholipid bilayers, with the tails of the phospholipid molecules aligning to form a hydrophobic region. If the active drug is hydrophobic (or using the base of the active drug instead of the salt) the drug can be "trapped" in the hydrophobic region. Often this is adequate to protect the drug from hydrolytic degradation—even though the hydrophobic region of the bilayers is not completely "walled off" from the aqueous milieu.

One of the best examples of this approach is Abelcet, a lipid complex formulation of amphotericin B. This product is a liquid formulation, refrigerated. Considering that the original amphotericin B formulation (Fungizone) is a freeze-dried product, which needs refrigerated storage conditions for stability, the Abelcet lipid complex liquid formulation is a huge advantage. It is important to remember that reformulating a freeze-dried product in a lipid matrix changes its pharmacokinetics, pharmacodynamics, bio-distribution, and safety and efficacy profiles. Thus, this approach invariably demands complete preclinical and clinical studies.

The above principles can also be as well applied to making emulsified products. If the active drug is soluble in oils (if the active is a salt, often the base is oil soluble), an emulsion will help stabilize the drug.

Conclusions

In conclusion, there are several promising alternatives to lyophilization either currently in use for sterile products (aseptic crystallization/precipitation and drying, vacuum-drying), while sterile spray-drying is very near to commercialization, and leverages mature powder-filling technology. Early-phase concepts include bulk tank agitated freeze-drying, drying on a fiber matrix, and fully contained powder reconstitution and injection devices. Overall, there are strong reasons for optimism that within a decade, higher efficiency alternatives to lyophilization will be available as established technology.

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Spray-Drying of Biopharmaceuticals

Grace A. Ledet, Richard A. Graves, Levon A. Bostanian and Tarun K. Mandal

Introduction

Spray-drying has been utilized in the pharmaceutical industry for more than five decades. Spray-dried granules possess excellent flow properties and, when used for tablet or capsule formulations, result in excellent batch-to-batch dose uniformity. A typical spray-dryer atomizes a stream of sprayed liquid into a fine, uniform dry powder. Sprayed liquids range from simple solutions of drugs to emulsions and suspensions. Even though the principles of spray-drying were established decades ago, spray-drying has gained popularity in recent years, especially for the production of biopharmaceutical formulations. While exposure to high temperature during the drying process is always a concern for spray-dried biopharmaceuticals, spray-freeze-drying, a recent permutation of the conventional spray-drying process, does not require high drying temperatures and has expanded the range of biopharmaceuticals suitable for formulation. This chapter reviews the basics of spray-drying from equipment design to optimization, highlights the advantages and challenges of spray-drying biopharmaceuticals, and explores especially rich areas of research within spray-dried biopharmaceuticals, specifically, pulmonary delivery for vaccines and inhaled insulin.

T. K. Mandal (✉) · G. A. Ledet · R. A. Graves · L. A. Bostanian
College of Pharmacy, Center for Nanomedicine and Drug Delivery, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA
e-mail: tmandal@xula.edu

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Basics of Spray-Drying

The spray-drying process has existed since the latter part of the nineteenth century, but its first commercial use was in the 1920s for the production of powdered milk [1]. Today, not only has spray-drying remained an integral part of food production but also has extensively been used in the pharmaceutical industry—particularly for the formulation of biopharmaceutical preparations. Moreover, the unique environmental restrictions of thermolabile biopharmaceuticals have furthered the design of spray-dryers with the development of nonconventional spray-drying methods. The conventional spray-drying process involves four stages: (1) atomization of the liquid into a spray, (2) spray liquid droplets and air contact, (3) evaporation and drying of the liquid droplets, and (4) separation and collection of the dry particles. The design and operating conditions of spray-drying have been extensively reviewed elsewhere [1–3].

Conventional Spray-Drying

A conventional spray-dryer consists of three main components—an atomizing device or nozzle, a drying chamber, and a collection chamber (Fig. 1). The atomizer reduces the feed stream into tiny droplets ranging in size from submicron size to several hundred microns depending on the method used to "atomize" the sample. The reduction in size results in a tremendous increase in surface area [4]. In the drying

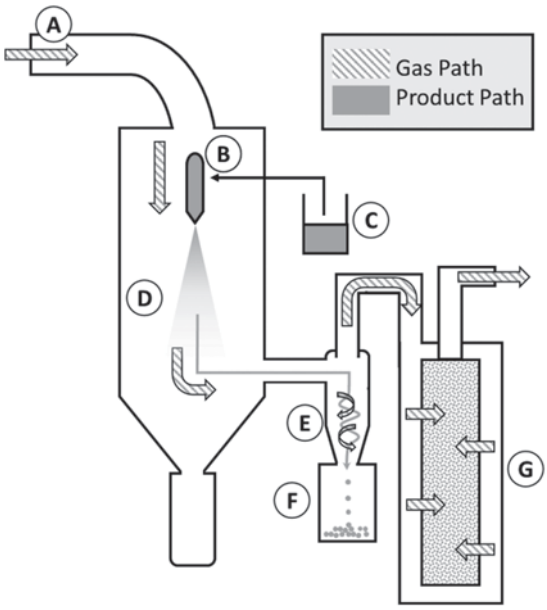

Fig. 1 Typical spray-drying configuration with *A* heated, filtered gas inlet; *B* spray nozzle; *C* liquid feed; *D* drying chamber; *E* cyclone; *F* collection chamber for dry product; and *G* air outlet filter

chamber, the solvent component of the feed stream, which can include water, alcohol, or other organic solvents, is rapidly removed by the continuous flow of a heated gas (usually air or nitrogen). The dried, solid residual particles are collected in a collection device. Spray-drying is well suited to the preparation of biopharmaceuticals despite the heat of the drying gas. Because of the rapid conversion of the solvent component of the feed stream to the gaseous state, the temperature experienced by the remaining constituents of the feed stream is much lower than the inlet temperature of the device, extending the range of tolerable processing temperatures available for sensitive biologics [5]. The process is rapid, energy efficient, and does not require the pretreatment of the feed stream prior to spraying, in contrast with, for example, lyophilization that requires freezing the sample prior to drying.

Several types of atomizers are used in the spray-drying industry including spinning disk atomizers, one-fluid spray nozzles (also referred to as pressure or hydraulic nozzles), two-fluid spray nozzles, ultrasonic nozzles, and piezoelectric nozzles. Each type of atomizer has been utilized in spray-drying of pharmaceutical products, each coupled with their own advantages and disadvantages. The core of a spinning disk atomizer, as its name suggests, is a rotating disk which, when rotating at high speeds, causes the liquid feed stream to impinge on the disk surface and atomize into small droplets. Changing the speed of the disk, the number of grooves, and the shape of the grooves on the disk affects the pattern and size of the atomized spray. The process may be used for fairly viscous samples, and produces a relatively uniform particle size. However, since the droplets are expelled in a horizontal direction, a wide drying chamber is needed for this atomizer. A one-fluid spray nozzle utilizes the shear of the fluid passing through the nozzle's small orifice to atomize the sample, accommodating low-viscosity samples and producing fairly uniform droplet sizes. However, this type of nozzle cannot be used for high-viscosity feed streams, has a tendency to clog, and is subject to variations in the atomization of the feed because the shear is directly dependent on the passage of the fluid through the nozzle. A two-fluid nozzle attempts to alleviate some of these problems by using a stream of gas to shear the feed stream at the tip of the nozzle. The added shear imposed by the gas stream enables these nozzles to spray higher viscosity fluids than those permissible with one-fluid nozzles. The two-fluid nozzle produces a more uniform spray pattern but with some variability in droplet size [6]. Ultrasonic nozzles apply ultrasonic vibration to the nozzle tip to atomize the feed stream. These nozzles produce more uniform droplets, are less likely to clog, have less overspray, and can produce a smaller overall droplet size than conventional spray nozzles. The feed rate tends to be lower for ultrasonic nozzles than for the other nozzle types.

Many different modifications of these basic types of nozzles exist [7, 8]. For instance, researchers have developed three- and four-fluid nozzles that have multiple feed streams in addition to the gas stream [9, 10]. Büchi Labortechnik (Flawil, Switzerland) has developed a piezoelectric spray nozzle that vibrates a perforated steel disk to atomize the feed stream [11]. While the feed rate is relatively low compared with other nozzles, this atomizer can produce submicron particles.

The design of the drying chamber depends on the choice of atomizer and the desired drying characteristics and can be divided into two major types: tall/narrow

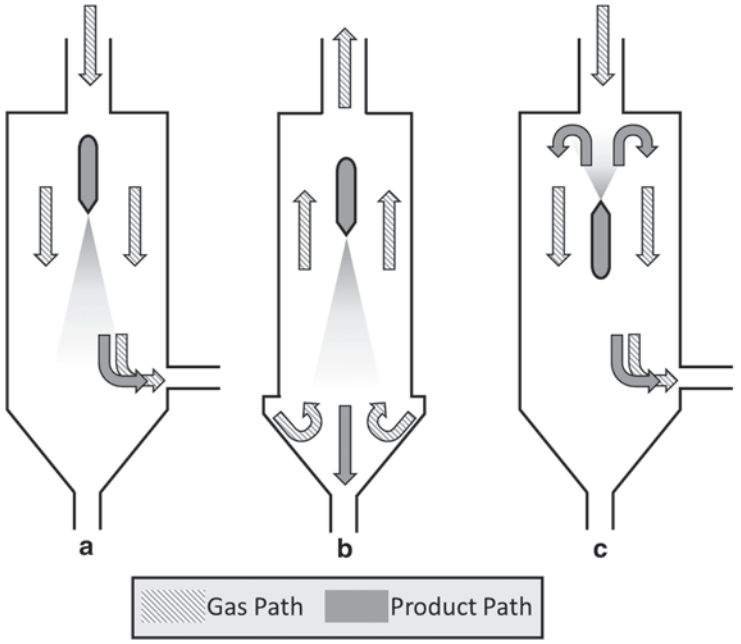

Fig. 2 Different dryer configurations with **a** cocurrent airflow, **b** countercurrent airflow, and **c** mixed airflow

chambers (5:1 height-to-diameter) and wide chambers with a conical bottom [12]. The most important role of the drying chamber is to ensure that droplets are given sufficient time to dry before collection. In the drying chamber, the direction of airflow relative to the atomizer liquid spray can vary depending on the type of air-droplet contact system employed (Fig. 2) [1]. The collection chamber usually involves the use of a cyclone to collect the final dried particles (Fig. 3). Various types of high-efficiency cyclones have been developed specifically for very small particles [13]. For their Nano Spray Dryer B-90, Büchi has developed an electrostatic collector which can collect particles of submicron size [11]. The design of the drying chamber and collection chamber largely depends on the scale of production. Büchi manufactures laboratory scale spray-drying equipment including the Nano Spray Dryer B-90 and Mini Spray Dryer B-290, and they offer accessories for their equipment including the Inert Loop B-295 for preparing samples in an inert atmosphere and the Dehumidifier B-296 for use in conjunction with the inert loop to remove residual moisture. GEA Niro (Soeborg, Denmark) manufactures spray-dryers ranging from the laboratory scale (SD-MICRO™) to pharmaceutical production-scale equipment (PHARMSD™). Other manufacturers include Fujisaki Electric (Tokushima, Japan), Shachi Engineering Pvt. Ltd. (Maharashtra, India), Nanjing SF Machinery Co. (Nanjing, China), and Spray Drying Systems, Inc. (Eldersburg, MD, USA), to name a few—all with unique designs for their atomizers, drying chambers, and collection chambers.

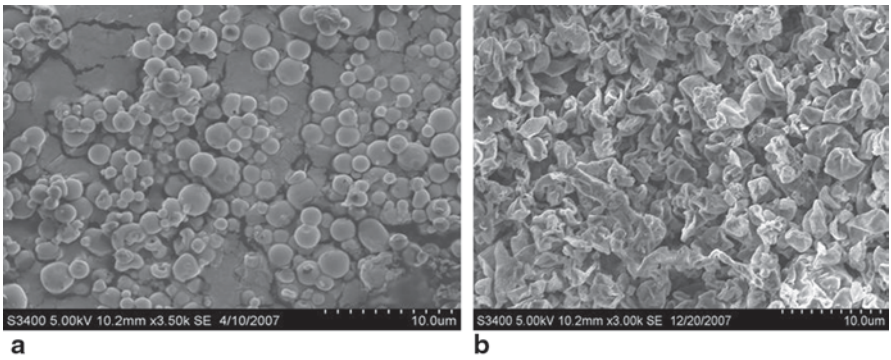

Fig. 3 Representative scanning electron microscope images of spray-dried particles. **a** PLGA/propranolol/trehalose particles sprayed at an inlet temperature of 55 °C, outlet temperature of 38–40 °C, airflow rate of 800 L/h, and pump rate of 3 %, and **b** blank PLGA particles sprayed at an inlet temperature of 55 °C, outlet temperature of 41–43 °C, airflow rate of 600 L/h, and pump rate of 6 %. PLGA poly(lactic-co-glycolic acid)

Alternative Spray-Drying Methods

Conventional spray-drying removes a solvent from a feed stream using heated gas leaving a dry particulate product, but spray-drying has evolved to include other drying processes which are applicable to the pharmaceutical industry. A solvent is not the only component of a feed stream which can be removed; heat can also be removed. Removing heat from the feed stream of a melted material by spraying into a stream of cooled gas is called spray-congealing or spray-cooling and also results in a solidified product [14–16]. Generally, disk and two-fluid atomizers are utilized for spray-cooling/-congealing, but the liquid feed, atomizer, and spray gas must be maintained at the melt temperature of the feed stream, requiring auxiliary equipment to maintain the feed material at the melt temperature. In the drying chamber, a stream of cooled gas is delivered to remove heat from the sample, and a traditional cyclone collector is used. This process is used in the pharmaceutical industry to form solid lipid composites and other hot-melt materials. Spray-cooling has limited use in the biopharmaceutical field to date because the constituents of the feed stream must be stable at the melt temperature of the matrix compound. For those peptides and proteins that are thermally stable, this technique has found some application [17–19]. Another option for spray-cooling biopharmaceuticals is to use a low melting point lipid as was done with milk thistle (*Silybum marianum*) to increase its oral bioavailability [20].

Another alternative spray-drying methodology, which is particularly popular in the preparation of biopharmaceuticals, is spray-freeze-drying [21–23]. Spray-freeze-drying is similar to traditional spray-drying in that the feed stream, which includes a solute dissolved in water, is sprayed with one of the standard atomizers

producing small aqueous droplets. However, instead of removing the water with heated gas, heat alone is removed from the droplets leaving the solute in a matrix of ice. Subsequently, the water is removed by lyophilization. Two major approaches are used to remove the heat from the droplets. In the first, the feed is sprayed directly into liquid nitrogen, sometimes called spray-freezing into liquid (Fig. 4a). The nitrogen is then removed, and the frozen product is freeze-dried [24, 25]. In the second approach, referred to as atmospheric spray-freeze-drying, the sample is sprayed into a chilled chamber (sometimes as low as −90 °C), collected on a filter, and then dried with a stream of cold, desiccated gas (below the eutectic temperature of the target material; Fig. 4b). This approach to spray-freeze-drying was patented in 2008 [26] and has been employed for the development of pharmaceutical formulations [27, 28]. While the atomization is similar to spray-drying, spray-freeze-drying has the additional necessity of liquid nitrogen or other super cooling methods which require specialized drying chambers. Because the feed is sprayed into liquid nitrogen or onto a filter, spray-freeze-drying does not require a collection cyclone. The sample, however, must be either transferred to a freeze-dryer or freeze-dried in place. While much of the spray-freeze-drying research is conducted with in-laboratory produced equipment, there are a few manufacturers of laboratory and production scale equipment. One such company is PowderPro AB (Goteborg,

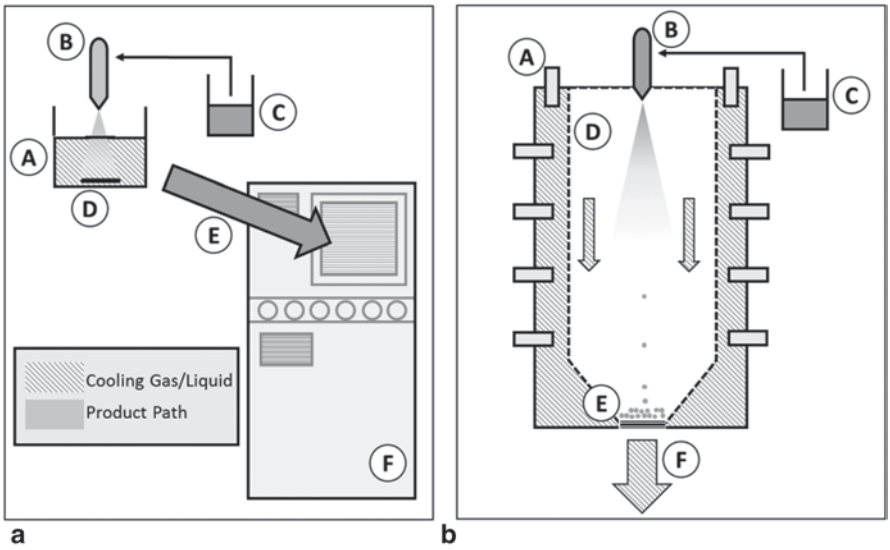

Fig. 4 Different types of spray-freeze-drying. **a** Diagram of spray-freeze-drying into liquid with (*A*) liquid nitrogen; (*B*) spray nozzle; (*C*) liquid feed; (*D*) stirrer; (*E*) transfer of frozen droplets to freeze-dryer after sublimation of liquid nitrogen; and (F) freeze-dryer. **b** Diagram of atmospheric spray-drying with (*A*) nozzles circulating coolant in outer chamber; (*B*) spray nozzle; (*C*) liquid feed; (*D*) drying chamber with porous walls; (*E*) collection filter for dry product; and (*F*) gas outlet to refrigerated condenser. This atmospheric spray-freeze-drying diagram is adapted from one configuration described by [26], but other cooling gas configurations also exist

Sweden) that produces laboratory (LS-2), pilot (LS-6), and production scale (PS-20) spray-freeze-drying equipment [29].

Spray-Drying Process Optimization for Biopharmaceuticals

Whether conventional spray-drying, spray-congealing, or spray-freeze-drying, optimization of a spray-drying technique for a biopharmaceutical formulation depends on many factors. Some of the factors affecting the final spray-dried formulation properties are determined by the material to be sprayed, by the spraying conditions, by the type of equipment, and by the specific spray-drying technique itself. Optimization is determined by the specific properties that are prioritized by the researcher. For biopharmaceuticals, these properties typically include yield, morphology (i.e., shape, size, and porosity), flow characteristics, and product condition (i.e., chemical degradation, protein denaturation, and other physicochemical properties). The prioritization of one or more of these properties is specific to the biologic and its application—what is desirable for one product may be undesirable or irrelevant for another. For instance, spherical, dense particles are usually desired for most spray-dried pharmaceutics; however, for spray-dried, inhalable insulin the target morphology is a light, nanoporous particle [30]. While size, morphology, and flow properties may vary depending on the product and its application, other properties, such as yield and final product condition, are always maximized. For a conventional spray-dryer, the factors that affect the quality of the final product are excipients in the formulation, instrument design, feed solids concentration, inlet temperature, outlet temperature, liquid feed rate, and airflow rate. All of these parameters are interrelated, and adjustment of one parameter may require an adjustment of another. Because the parameters are interrelated and because of the sheer number of parameters, the spray-drying process is usually viewed as an empirical process requiring many trial runs to obtain the desired product [31]. Researchers have attempted to develop models to describe the spray-drying process [32]. All of these approaches are goal-oriented and have a particular formulation target in mind when optimizing the spray-drying process. In addition, because of the interrelation between the parameters, a strict model describing the overall process is difficult at best. However, modeling the spray-dry process is useful in making some generalizations about the overall process.

Two main factors affect the yield in conventional spray-drying—loss of material due to instrument design and loss due to material properties. Loss of material due to instrument design may be minimized by selecting an appropriate spray-dryer design for maximal recovery such as a wide drying chamber for a spinning disk atomizer, a high efficiency cyclone for fine particles, or an electrostatic collector for submicron particles. Other loss due to instrumentation is generally finite and independent of batch size and, therefore, can be minimized by increasing the overall batch size when possible. Suboptimal material properties are the other major hindrances to maximum product yield. When a spray-dried material is “sticky,” it will tend to collect on the walls of the spray-dryer [33]. This problem may be minimized by decreasing the outlet temperature below the glass transition point (T_g) of the

formulation excipients or by the addition of crystalline materials to the formulation. Reducing the inlet temperature may have the opposite effect since the retention of solvent due to incomplete drying will plasticize the formulation, increasing the "stickiness" of the material. In addition to temperature, minimal residual moisture, which translates to better yield efficiency, may be achieved by optimal atomization, lower airflow, and higher feed concentrations. Ståhl et al. (2002) reported yields as low as 8% and as high as 61% for spray-dried insulin simply by adjusting the concentration of the feed stream [34]. Adjustment of the solids concentration in the feed stream produced particles of differing sizes, and some were differentially retained by the recovery cyclone. Likewise, Haj-ahmad et al. (2013) demonstrated the relationship between particle size and yield—with different shell materials resulting in different particle sizes, larger particles resulted in higher yields, and smaller particles correlated with lower yields. This was attributed to the ability of the cyclone to capture large particles [35].

The processing equipment, feed composition, and spray conditions also have a tremendous effect upon the morphology of the spray-dried particles. As will be discussed in the section "Applications of Spray-Dried Biopharmaceuticals," particle size and morphology are some of the highest priorities for inhaled biopharmaceutical products, second only to preservation of biological activity. The morphology of spray-dried particles can be quite diverse—from solid to hollow and from spherical to wrinkled. Several studies have attempted to classify the morphology of spray-dried particles. Prinn et al. (2002) defined four categories of spray-dried particles: Type I, smooth spheres; Type II, collapsed or dimpled particles; Type III, wrinked or "raisin-like" particles; and Type IV which are highly crumpled or folded particles [36]. Paluch et al. (2012) took classification a step further, defining classes of particles based on their surface and interior features as well as shape (spherical vs. irregular, smooth vs. crumpled) [37]. Representative illustrations of these different types of particles are shown in Fig. 5. The diversity of particle types results from

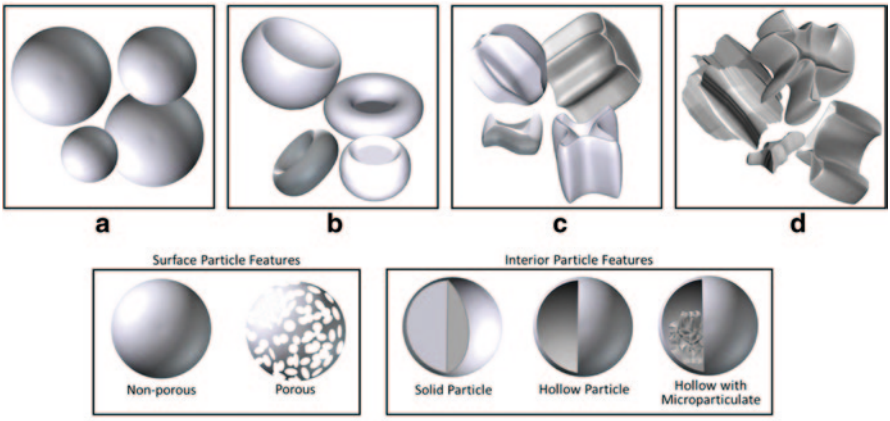

Fig. 5 Spray-drying can result in many different types of particles: **a** smooth spheres, **b** collapsed or dimpled particles, **c** wrinkled or "raisin-like" particles, and **d** highly crumpled or folded particles. Particles can also be classified by their interior and surface features

the evaporation of the solvent from the surface of the droplet, the crystallization of the solute material in the droplet, and the diffusion of the solvent from the interior to the surface of the particle. This chapter covers the general principles which apply to most formulations, but Nandiyanto and Okuyama (2011) review the latest research regarding the correlations between spray-drying parameters and particle morphology [38]. As with yield efficiency, the interplay between the processing parameters, the different types of spray-drying equipment, and the diversity of biopharmaceuticals make the optimization of spray-dried particle morphology very specific to the biologic and its intended application. Regarding particle size and morphology, the type of atomizer, feed composition, and feed concentration are the most influential processing parameters [39]. The drying rate may be considered a significant factor only because a slow drying rate may lead to agglomeration of the particles. For atomization, smaller nozzles and higher shear lead to smaller droplets, translating into smaller dried particles. As the concentration of solids in the feed stream decreases, so will the size of the dried particles. The effect of feed composition is more complicated because both the solvent and solutes can affect the particle size but often affect the morphology of the particles as well. Specifically, the solvent evaporation rate affects the porosity of the particles, and feed composition and temperature mainly impact the morphology of the particles. If the evaporation rate is high (e.g., high drying temperatures for a volatile solvent), surface enrichment is enhanced leading to hollow shells and complex structures. Multiple solvents with different evaporation rates enhance the possibility of obtaining particles with complex interior pores with discrete domains. In addition, the use of solute materials with vastly different solubilities, mass transport properties, and heat transport properties enhance the likelihood of obtaining complex particles [40].

Finally, product stability is always an important consideration for biopharmaceuticals. The main factors that influence the stability and activity of biopharmaceuticals during spray-drying are inlet temperature, oxygen environment, and inclusion of stabilizing excipients. Vaccines and proteins can be successfully spray-dried with little physical or chemical damage [37, 41, 42] and will be discussed further in section “Challenges Specific to Spray-Dried Biopharmaceuticals.” Excipients such as amino acids, sugars, and polymers may be incorporated in the formulation to stabilize biopharmaceuticals during the spray-drying process and for long-term storage following spray-drying [43, 44]. Most spray-dryers are equipped to operate within a nitrogen atmosphere, and, therefore, they can be used for spray-drying biopharmaceuticals that are oxygen-sensitive and require processing in an inert atmosphere. Once again, only broad generalizations can be drawn since the type of stabilizing excipient and the most likely source of degradation is highly dependent on the biopharmaceutical in question.

The optimization discussion to this point has focused on the optimization of conventional spray-drying, but spray-congealing and spray-freeze-drying have additional operational parameters which impact yield, particle morphology, and biological stability. Generally, the yield from spray-congealing is high [45] but, like conventional spray-drying, is affected by adherence of particles to the walls of the drying chamber. This loss can be minimized by lowering the temperature of the

cooling gas to increase the cooling rate of the particles and by spraying smaller droplets. The final size of the lipid particles following spray-congealing is a direct reflection of the atomizer's ability to shear the feed stream into small droplets, and the particle size can be reduced by heating the feed stream to lower its viscosity and by increasing the force of aspiration. The most important difference in particle properties for spray-drying versus spray-freeze-drying is the size and porosity. Spray-freeze-drying results in large, highly porous, fragile particles [46]. The temperature at which the droplets are dried can also change the morphology of the particles. If the drying temperature is above the T_g of the solutes, the particles may not maintain the spherical shape of the original droplets (i.e., becoming collapsed or shrunken), which will affect other properties such as the density of the particles [47].

Lyophilization Versus Spray-Drying

Freeze-drying, or lyophilization, is a well-established pharmaceutical manufacturing procedure, encompassing approximately 50% of currently marketed biopharmaceutical products [48]. The predominant role of freeze-drying is to improve the long-term stability of labile biopharmaceuticals, by inhibiting or sufficiently decelerating chemical and physical degradation [49]. Additionally, solid state, lyophilized formulations are easier to handle, ship, and store [50]. Formulation by spray-drying seeks these same characteristics. Choosing between these two drying methods is ultimately dependent on the final desired storage conditions and formulation characteristics, as well as consideration of the specific tolerable stresses (physical and/or chemical) of the biopharmaceutical in question. However, several overarching advantages and disadvantages remain true regardless of the nature of the biopharmaceutical. First, specific to the manufacturing process, spray-drying is more easily scaled to the industrial production level and has lower initial investment costs [3]. The processing time is much shorter for spray-drying, which is on the order of hours, than for freeze-drying which can take days [51]. Spray-drying is a one-step process, whereas freeze-drying may require another milling procedure to form particles. However, spray-drying requires a secondary bottling or packaging step for the final product, whereas freeze-drying can dry and cap samples aseptically in one step if milling is not required. Freeze-drying is limited to a batch process as opposed to continuous processing with spray-drying. Both drying methods can achieve low residual moisture levels [7, 52], which is important because high residual moisture content negatively affects chemical and physical stability [53]. Spray-drying offers the flexibility to tailor particle properties to suit varied purposes (e.g., controlled release, optimal size and morphology, etc.) as outlined in section "Basics of Spray Drying," while freeze-drying offers few options for particle property manipulation. However, spray-drying subjects the biologic to added shear and temperature stresses that are not present in freeze-drying. Biopharmaceuticals sensitive to high temperatures may not be suited for spray-drying, but those biologics particularly vulnerable to freezing temperatures are not suitable for freeze-drying.

Finally, the arguably most significant disadvantage of spray-drying compared to freeze-drying is the process efficiency. Freeze-drying results in very little loss during drying, whereas yield is a persistent drawback with spray-drying, particularly with small particles, which may not efficiently deposit in the cyclone [37]. The choice of the optimal drying method is largely determined by the tolerable operating temperature for a specific biopharmaceutical and the final dosage form.

Challenges Specific to Spray-Dried Biopharmaceuticals

The benefits of spray-drying for biopharmaceuticals are the same as the overall benefits of spray-dried formulations in general, namely, it is a one-step process, scalable to the industrial level, less time consuming than other drying methods, and offers tight control of particle properties. Formulating proteins and other biologics remains a challenge in pharmaceutical research due to the range of possible chemical and physical degradations to overcome—deamidation, oxidation, hydrolysis, disulfide bond shuffling, conformational changes, adsorption to surfaces, denaturation, precipitation, and aggregation [54]. Because of these sensitivities, formulating biopharmaceuticals is accompanied with limitations on tolerable operating temperatures, pressures, and environments. The ability to tightly control the morphology of the resultant particles, the ability to tune the size of the particles, the ease of incorporation of excipients for the controlled release of compounds, and the final product in a dried state which extends the storage life of the product all contribute to the extensive use of spray-drying in the formulation of biopharmaceuticals.

However, there are several sources of stress in the spray-drying process which are particularly important due to their possible negative effects on the stability of biopharmaceuticals—high pressure, high shear, atomization pressures, temperature, and dehydration. Without the use of stabilizing excipients such as sugars, amino acids, and surfactants, spray-drying can cause thermal degradation, conformational changes, and aggregation. Understanding the interactions between excipients and biomolecules and the excipient(s)' role in stabilization helps to determine optimal excipient concentrations to maximize biomolecule activity. Specifically focusing on protein stabilization, different excipients protect a protein from different sources of destabilization. Several stabilization mechanisms have been put forward to explain the effects of stabilizing excipients and the causes of destabilization in the spray-drying process. Specifically, protein aggregation, and subsequent inactivation and structural changes, have been linked to (1) dehydration during the drying process and (2) interaction with the air/liquid interface during atomization.

Proteins can tolerate shear and shear rates as high as 10^7 and 10^5 s^{-1}, respectively, in liquid medium without protein aggregation and activity loss, though they may still be subject to conformational changes as assessed in recombinant human growth hormone (rhGH) and recombinant human deoxyribonuclease (rhDNase) at high shear rates [55]. Amphiphilic proteins will aggregate at the surface of interfaces, such as air–liquid interfaces, exposing their hydrophobic regions towards

the nonaqueous surface, which leads to denaturation and precipitation. Thus, while proteins can tolerate high shear in a continuous liquid medium, their stability is compromised when introduced to an interface in the presence of even moderate shear forces [56]:

$$\text{amphiphilic protein} + \text{air / liquid interface} \xrightarrow{\text{shear}} \text{aggregation}.$$

The larger the surface area of the interface, the greater the possibility of aggregation and denaturation, which means that smaller droplets are subject to higher rates of aggregation because of the larger size of the interface between air and liquid. Surfactants [57] and amino acids [58] are used to lower the concentration of proteins at the air–liquid interface during spray-drying by competing to occupy the interface, thus lessening the corresponding aggregation and precipitation. Another method of reducing aggregation at the air/liquid interface is the utilization of spray-freeze-drying. Fortunately, diffusion to the liquid/ice interface during freezing is negligible as demonstrated by Webb et al. (2002), who observed that aggregation of recombinant human interferon-γ (rhIFN-γ) after spray-freezing was not significantly different from aggregation during atomization [59]. Yu et al. (2002) suggest spray-freeze-drying *directly* into liquid nitrogen through an insulated nozzle rather than spray-freeze-drying above the liquid surface, which can reduce aggregation by increasing the freezing rate of the process [60]. Yu et al. showed minimal loss of monomer bovine serum albumin (BSA; < 1 %) with rapid-freezing spraying compared to Costantino et al. (2000) who reported 2 % monomer BSA loss in their best case scenario with "traditional" spray-freeze-drying [61]. By reducing the time until freezing, the proteins do not have sufficient time to migrate to the interface and aggregate, thus reducing damage to the protein.

Another explanation for protein denaturation due to spray-drying is denaturation during the dehydration stage of processing. When water is removed from the formulation, a protein can undergo conformational changes when hydrogen bonds with water are broken as the water is removed from the system. Sugars, such as sucrose or trehalose, stabilize the proteins by forming hydrogen bonds with the proteins as the water is removed from the system, preventing the unfolding of the protein in the absence of water. The water substitution/replacement hypothesis also explains why sugars fail to prevent irreversible destabilizations when present in high concentrations [62, 63]. When high concentrations of excipients are present, excipient–excipient bonding may compete with excipient–protein hydrogen bonding and partition into sugar-rich regions and protein-rich regions, thus reducing their stabilizing capacity. Additionally, sugars and other stabilizing excipients form an amorphous glass matrix (preferably with a high T_g) which trap the protein, protecting its structure [64].

In the end, and as with all pharmaceutical formulations, different excipients will have differing effects on biopharmaceutical stability during spray-drying. Understanding the types of instability which are most likely to affect the biopharmaceutical under investigation will aid the scientist in determining the optimal type of stabilizing excipient to use for their biomolecule. As a point of comparison on

the variability of processing procedure and protein stability, Forbes et al. (2007) determined that spray-drying causes perturbations in the secondary structure of trypsin [65], and French et al. (2004) found that spray-drying of recombinant human granulocyte colony-stimulating factor (rhG-CSF) and recombinant consensus interferon-α (rConIFN) with trehalose stabilized their α-helical structures which were also compromised by the spray-drying processing in the absence of trehalose [66]. While these studies and others [67] report changes in the secondary structure of proteins or peptides due to spray-drying, Schule et al. (2007) reported no detectable changes to humanized chimeric monoclonal antibody (IgG1) which could be attributed to spray-drying [67]. While they demonstrated antibody aggregation in the presence of high sugar contents (60–80 % mannitol) and secondary structure changes resulting from thermal stress (80–90 °C), no change in secondary structure associated with spray-dried mannitol-containing formulations could be detected. Even though generalizations may be drawn among similar biomolecules, these examples demonstrate that the type of denaturation and/or aggregation resulting from spray-drying is protein-specific and that stabilizing excipients must respond to the specific sensitivities of the biomolecule under investigation.

Applications of Spray-Dried Biopharmaceuticals

While it is outside the scope of this chapter to fully catalog the range of biopharmaceuticals currently under investigation as spray-dried formulations, we hope to illustrate the flexibility of the spray-drying process in formulating sensitive compounds by highlighting particularly strong lines of research within the realm of spray-dried biomolecules—namely, pulmonary delivery, vaccine research, and diabetes research. The overlap among these fields of research will hopefully give the reader a greater understanding of the interplay between spray-drying processing parameters and the effectiveness of the final product. While the predominant application of spray-dying in the field of biopharmaceutical development is the use of spray-drying to formulate aerosolized therapeutics for pulmonary delivery, it is not the only application of spray-drying for biopharmaceuticals, and several reviews of spray-dried biomolecules exist [68].

Spray-Dried Biopharmaceuticals for Pulmonary Delivery

Most biopharmaceuticals rely on parenteral delivery, but pulmonary delivery affords several advantages, including reduced discomfort to the patient, fewer health-care professionals needed for delivery in low-income countries, improved patient compliance, avoidance of first-pass metabolism, and rapid onset of action [69, 70]. The pulmonary delivery route also exhibits a large absorptive surface area (> 100 m^2), high vascularization, and alveolar epithelium as thin as 0.1 μm [71, 72]. The utility

of spray-drying for pulmonary delivery is immediately apparent when taken with the fact that particle size, density, and shape are the most important design variables for aerosol formulations [73]—all properties which can be precisely controlled by spray-drying. For efficient deep lung penetration, particles must have an aerodynamic diameter between 1 and 5 μm [74]. Thus, aerodynamic particle size is the key determiner of aerosol performance for an inhaled formulation [72, 74]. The aerodynamic particle diameter takes into account the size, density, and shape of the particle and is usually expressed as the mass median aerodynamic diameter (MMAD). To fit within the 1–5-μm range, the aerodynamic diameter of a particle can be decreased three ways: decreasing the particle size, reducing the particle density, or increasing the dynamic shape factor of the particle (i.e., the ratio of the actual drag force experienced by the particle to the resistance force experienced by a sphere of the same volume) [73, 75]. For particles larger than 5 μm, they must have a low-density, nonspherical geometric shape, or surface features which compensate for the larger particle size. For instance, Chew et al. (2005) found that moderate increases in surface corrugation or roughness of spray-dried protein particles improved aerosol performance, as evidenced by an increase in fine particle fraction (FPF) from only 26% for smooth particles to 40% for slightly wrinkled ones [76]. In addition to the particle size requirements, an aerosol product should ideally have a high FPF, high dose, good dose consistency and uniformity, narrow particle size distribution, suitable properties for chosen inhaler device, contain excipients safe for lung tissue, and retain the biological activity of the active compound [75, 77].

Many studies have demonstrated the feasibility of spray-drying for the production of inhaled biopharmaceutical formulations with the desired aerodynamic properties. Inhaled formulations prepared by spray-drying have already been attempted with many proteins, peptides, and other biopharmaceuticals, including insulin which will be discussed further in the section "Applications of Spray-Dried Biopharmaceuticals." Spray-dried particles containing plasmid DNA (pDNA) have been produced with a particle size of 3–5 μm and 44–60% yield, and spray-drying did not compromise the transfection efficiency of the pDNA [78]. Spray-dried rhGH particles with dimethyl-β-cyclodextrin (DMβCD) as a pulmonary absorption enhancer and stabilizer resulted in particles with an FPF as high as 53% [79]. Spray-dried small interfering RNA/poly(lactic-co-glycolic acid) (siRNA/PLGA) particles with different stabilizers, mannitol, trehalose, and lactose produced particles with an MMAD between 2 and 5 μm, yields ranging from 20 to 60%, and preservation of siRNA biological activity [80]. Compared to lyophilized powders, spray-drying of liposomal DNA produced smooth, spherical, 4 μm particles with better gene transfer in cell culture than either fresh solution or lyophilized formulations [81]. An inhaled human parathyroid hormone (PTH) formulation (4.5 μm particle diameter, MMAD 3.9–5.9 μm, 90% emitted dose, and 61% FPF) had 186% bioavailability relative to subcutaneous administration in rats and resulted in no acute lung inflammation after 48 h [82].

Spray-freeze-drying is emerging as an exciting option for inhaled formulation because it produces porous particles with high FPF which may have better aerosol performance than those produced by conventional spray-drying. Spray-dried powders

of two different proteins (deoxyribonuclease (rhDNase) and anti-immunoglobulin E (IgE) monoclonal antibody) had smaller particle sizes than those produced by spray-freeze-drying (3 vs. 7–8 μm) but had poorer FPFs (30–50 vs. 50–70%) [83]. Additionally, the spray-freeze-dried formulations had significantly better dispersibility of particles less than 3 μm (53 vs. 15% for rhDNase and 20 vs. 11% for anti-IgE antibody) [83]. Even with a tenfold difference in geometric particle diameters, spray-dried (1–2 μm) and spray-freeze-dried (approximately 10 μm) DNA/peptide/mannitol particles had similar FPFs (about 50%); however, the spray-dried particles had a significantly higher cell transfection efficiency [46]. Comparing the two methods, spray-freeze drying is more appropriate for temperature-sensitive biopharmaceuticals but is less easily scaled to the industrial level and is more expensive. Spray-drying has poor yield (typically 20–50% but may exceed 70% with a Büchi high-performance cyclone [33]), but spray-freeze-drying can achieve much higher efficiencies, typically >80% yield. Mohri et al. (2010) achieved 83–85% yield for spray-freeze-dried pDNA for gene therapy [84]. However, spray-freeze-dried particles are very fragile and may be adversely affected by handling and shipping. Another challenge for both spray-dried and spray-freeze-dried inhaled formulations is the high excipient content. For example, of the studies referenced above, several have stabilizer concentrations in excess of 95% of the formulation [46, 78, 84]. Special attention must be paid to the pulmonary safety of the excipients. Spray-drying and spray-freeze-drying are the most common production methods for dry powder aerosols of biological macromolecules [46, 73, 83], and the list of biological macromolecules developed for pulmonary delivery is extensive [70]. We discuss two indications of biopharmaceuticals which can greatly benefit from pulmonary delivery—disease prevention through vaccination and the treatment of diabetes mellitus.

Spray-Drying for Vaccine Stability and Delivery

Spray-drying research with vaccine candidates covers the gamut of possible types of biopharmaceuticals which can benefit from preparation as a spray-dried powder—live, attenuated vaccines which are whole, live pathogens; inactivated/killed vaccines which are dead pathogens; subunit vaccines which are generally pathogenic proteins; toxoid vaccines which are inactivated toxins from bacteria; conjugate vaccines which combine subunit or toxoid vaccines with the polysaccharide outer coat from bacteria; DNA or RNA vaccines; and recombinant vector vaccines which combine microbial DNA with a virus or bacterium as a carrier [85]. Indeed, many lyophilized vaccine products are commercially available in the USA, including M-M-R®II (measles, mumps, and rubella virus vaccine live, Merck & Co., Inc.), Hiberix® (haemophilus b conjugate vaccine (tetanus toxoid conjugate vaccine), GlaxoSmithKline), Varivax® (varicella virus vaccine live (chickenpox), Merck & Co., Inc.), and Rotarix® (rotavirus vaccine, live, oral, GlaxoSmithKline), to name a few. Researchers seek spray-dried vaccine formulations for the same reasons

lyophilized formulations were developed—improved storage stability. McAdams et al. (2012) review the stabilization of vaccines via spray-drying [86]. One or both of these drying methods, spray-drying and freeze-drying, may hold the key to the next step for solid storage forms for vaccines, namely, eliminating the need for cold chain storage. "Cold chain" storage refers to the process used to maintain optimal temperature conditions during the transport, storage, and handling of vaccines, starting at the manufacturer and ending with the administration of the vaccine to patients. Most vaccines require storage at 2–8 °C without exposure to freezing temperatures. 17–37 % of vaccine providers expose vaccines to improper storage conditions, usually keeping vaccines too cold [87, 88]. Additionally, cold chain storage requirements hamper efforts to vaccinate in low-income nations and regions where resources for maintaining cold chain storage are thin and can cause the vaccine to be (1) discarded because of temperature exposure or (2) administered without the proper antigenic potency to confer protection [89]. Additionally, emergency immunization programs would benefit from loosening cold chain storage requirements and lengthening vaccine storage time [90]. We review some of the current research into spray-drying for the formulation of stable vaccine storage forms, focusing on processing parameters and storage implications.

Año et al. (2011) evaluated spray-drying as a methodology for the preparation of a candidate cholera vaccine (*Vibrio cholerae*), which may allow mucosal delivery and ease delivery to children and infants, the most susceptible population in endemic areas [91]. Using a Büchi 191 Mini Spray Dryer, they combined their inactivated bacterial strain with polymers (Eudragit®L 30 D-55 or Eudragit®FS 30D) at a 1:10 vaccine-to-polymer ratio. The processing parameters were as follows: a liquid feed rate of 5 L/min, air feed rate of 600 L/h with compressed air, a 0.7-mm nozzle, and three different inlet air temperatures (60, 80, and 100 °C). For both polymers and the three different inlet temperatures, the yield was generally 70–80 % with a particle size around 3 μm. The yield was significantly lower at their lowest air inlet temperature (60 °C) compared to 80 and 100 °C. However, the lower inlet temperature and Eudragit®L 30 D-55 also resulted in more uniformly spherical and smooth microparticles than Eudragit®FS 30D and the other inlet temperatures. Also, the highest inlet temperature (100 °C) and Eudragit®L 30 D-55 resulted in statistically different antigenicity (only 88 % compared to 98 % at 60 and 80 °C). Also, the addition of alginate as a mucoadhesive agent could further enhance the vaccine efficacy for oral administration [92]. Thus, depending on a researcher's prioritization of characteristics, such as morphology, yield, or antigenicity, the optimal parameters and excipients may be different.

Saluja et al. (2010) compared spray-drying and spray-freeze-drying for the formation of an influenza subunit vaccine for inhalation [93]. In addition to comparing spray-drying and spray-freeze-drying, they considered two different aqueous buffers for the vaccine—phosphate-buffered saline (PBS) and Hepes-buffered saline (HBS). They made 1:200 vaccine-to-inulin (their chosen stabilizer) in each buffer and spray-dried these solutions with a Büchi 190 Mini Spray Dryer with a two-fluid, 0.5-mm nozzle with a liquid feed rate of 5 mL/min, an air feed rate of 800 L/h, an inlet air temperature of 130 °C, and an outlet air temperature of 70 °C. For their

spray-freeze-dried formulations, the same two solutions were sprayed with the same nozzle into liquid nitrogen from approximately 5 cm, and the samples were then freeze-dried over 32 h increasing the temperature from −40 to 5 °C. The spray-dried formulation in HBS and spray-freeze-dried formulation in PBS resulted in reduced antigenic potency compared to their counterparts (89 and 82.5% potency, respectively, compared to 100% potency for spray-dried in PBS and spray-freeze-dried in HBS). As expected, the spray-dried particles were smaller, solid particles (median size around 2.6 μm, FPF of 37%), while the spray-freeze-dried particles were highly porous, larger particles (median size around 10 μm, FPF of 23%) with a much larger specific surface area. Pulmonary delivery of both the spray-dried in PBS formulation and spray-freeze-dried in HBS formulation resulted in higher IgG titers than intramuscular delivery in mice and were stable at 20 °C/10% relative humidity (RH) for 3 years.

Ohtake et al. (2010) evaluated spray-drying to make a powdered live, attenuated measles virus vaccine [42]. Using a Büchi B-191 Mini Spray Dryer and a Sonicair sonic nozzle (IVEK, North Springfield, VT, USA), they chose to use a modest atomization pressure (15 psi) and air outlet temperature (40 °C) to minimize heat and pressure stresses on the vaccine. Spray-drying, compared to freeze-drying and foam-drying, resulted in the least virus titer loss and lowest process-associated loss at 37 °C. They identified several stabilizing excipients which contributed to virus stability at 37 °C: sucrose-trehalose (17% w/v) to stabilize against dehydration stresses as discussed in the section "Challenges Specific to Spray-Dried Biopharmaceuticals"; L-arginine (4% w/v) to reduce protein-to-protein interactions and thereby aggregation; human serum albumin (4% w/v) to stabilize the virus by a combination of enhanced T_g, increased viscosity, and surfactant-like effect to protect the virus; glycerol (1.25 wt%) as a plasticizer to increase interactions between the glassy matrix of the sugars and the virus; potassium phosphate buffer (50 mM) which reduced process loss; and divalent cations ($CaCl_2$ and $ZnCl_2$) at pH 6.0 which helped maintain the virus structure during processing. This formulation achieved stability for 8 weeks of storage at 37 °C, exceeding the World Health Organization's stability requirement. Excipient choice is critically important to storage stability of vaccines. In addition to sugars or surfactants as cyroprotectants, amino acids can also be used as stabilizing excipients during spray-drying of biologics. Assessing different amino acid for their stabilizing effects for spray-dried proteins, Ajmera et al. (2014) found that a combination of arginine and glycine was optimal for protection of proteins during spray-drying, demonstrating their protective efficacy with an influenza antigen [94]. Murugappan et al. (2013) evaluated different stabilizing excipients for spray-freeze-drying of a whole inactivated virus vaccine [21]. They studied inulin, dextran, and a mixture of both, and all yielded a stable product for 3 months at storage temperatures up to 30 °C. Tonnis et al. (2014) utilized spray-freeze-drying to increase the storage life of a hepatitis B vaccine antigen [23]. When the antigen was co-sprayed with either inulin or a mixture of dextran and trehalose, the resulting powder could be stored at room temperature for 3 months without loss of immunogenicity or conformational changes. Interestingly, Tonnis et al. found that the sugar excipients may have acted as adjuvants for the vaccine resulting in

higher IgG titers and a more balanced type 1 T helper cells/type 2 T helper cells (Th1/Th2) immune response than the liquid antigen.

In addition to pulmonary delivery, another alternative route of administration is through the skin but as a needle-free ballistic administration rather than an intramuscular injection. For example, Maa et al. (2004) formulated two influenza vaccines (split-virion vaccine and subunit vaccine) by spray-freeze-drying [95]. The excipients considered were dextran, mannitol, trehalose, arginine glutamate, and Pluronic F-68P. Spray-freeze-drying produced yields ranging from 82 to 89% (higher yields than from most spray-drying processes) and large particles with a median size range of 35–56 μm. A final optimized formulation consisted of trehalose, mannitol, and dextran at a 3:3:4 ratio with antigen purity identified as the most critical factor for a successful formulation and with a stability of 1 year at room temperature. Chen et al. (2010) also evaluated the formulation of two different types of vaccine antigens—a subunit vaccine with an aluminum-salt for hepatitis B virus and a polysaccharide–protein conjugate vaccine for meningitis A [96]. In contrast to spray-freeze-drying with Maa et al., Chen et al. studied the production of conventional spray-dried formulations. They used a Mobile Minor™ pilot plant (GEA Niro) to manufacture these preliminary formulation and had similar results with 2+ years of stability at 37 °C and with 90%+ yield for their HepB vaccine. Choice of type of spray-drying is not defined by the type of vaccine but rather by the processing parameters and excipients therein.

Spray-Drying for the Formulation of Inhaled Insulin

Insulin is a good candidate for pulmonary delivery, and the challenges faced by researchers in pursuit of an inhalable form of insulin accurately represent the spray-drying formulation process and the critical need for methodologies which allow fine-tuning of particle properties. Insulin is known to have a high permeability through the alveolar membrane and is a good candidate specifically for spray-drying because it can tolerate a large range of temperatures and maintain its bioactivity following spray-drying. Patel et al. (2001) confirmed that insulin can retain its activity following spray-drying with air inlet temperatures ranging from 110 to 170 °C, only losing activity at 200 °C [97]. Insulin's robust range of tolerable temperatures means that co-sprayed stabilizers can be selected from a larger pool of excipients, although its indication, diabetes mellitus, limits the use of some sugars as stabilizers.

Spray-drying insulin is a popular line of research within the spray-dried pharmaceutical industry, spurred forward by the approval of Exubera® (Pfizer) by the Food and Drug Administration (FDA) in early 2006, and making insulin the first and only approved systemic protein administered through the lungs [98, 99]. In clinical trials, patients receiving Exubera® reached peak insulin concentrations faster than those injected with insulin [100]. However, the product was discontinued in October of 2007 with Pfizer citing poor sales as the reason for discontinuation. Exubera® con-

sisted of recombinant insulin (60%), sodium citrate, mannitol, glycine, and nominal amounts of sodium hydroxide (used to adjust the pH to 7.2–7.4) and was manufactured on a commercial scale spray-dryer with an inlet temperature of 181 °C and resulting outlet temperature of 87 °C [99]. With a final moisture content of < 2% and the formation of a high-T_g amorphous solid, Exubera® was stable for more than 2 years at room temperature [101]. The following section reviews examples of spray-dried insulin formulations reported in the literature with particular focus on those publications which assess the effects of formulation and processing parameters on the final product characteristics.

Balducci et al. (2014) developed a spray-dried insulin formulation without excipients to maximize the insulin content and safety of the final formulation [41]. Their formulation was manufactured with a Büchi Mini Spray Dryer B-191 with an inlet temperature of 120 °C, airflow rate of 600 L/h, feed rate of 3.5 mL/min, and outlet temperature of 40–60 °C. They sprayed an acidic solution (acetic acid solution adjusted to pH 3.6 with ammonium hydroxide) of insulin, achieving an insulin content of 95%, a median particle diameter of 4 μm, and an MMAD of 2 μm. The obtained particles were "shriveled and raisin-like," and they contained an 89.5% respirable fraction of the delivered dose, indicating that the shape and density of the particles contributed to the respirability of their formulation. Additionally, their formulation stayed within the European Pharmacopoeia requirements (< 2.0% molecular masses greater than that of insulin (high molecular weight protein (HMWP)), < 5% A21 desamido insulin (A21), and < 6% for other related proteins (ORP)) for 3 months at 25 °C/60% RH. Following 18 months of storage, they had no content modification at 5 °C (i.e., within the European Pharmacopoeia requirements) and were just outside of specifications at room temperature with approximately 3% HMWP, 5.9% A21, and 4.3% ORP.

Bi et al. (2008) chose to use spray-freeze-drying to manufacture an aerosol formulation of insulin under the supposition that the spray-freeze-drying process produces large particles with small MMADs which would improve aerosolization and dissolution [102]. Their optimal formulation was a spray-dried liposomal formulation containing soya lecithin and cholesterol with sucrose as a lyoprotectant. Using a Büchi Mini Spray Dryer B-191, they sprayed their formulation with a two-fluid nozzle positioned 10 cm above the liquid nitrogen. Drug retention in the formulation was used to select the best excipient with results as follows: sucrose > lactose > glucose > mannitol. Also, in evaluating the spray-freeze-drying procedure, compressed air pressure (optimal = 0.4 MPa) was determined to be the most critical parameter for optimizing the particle size, followed by liquid flow rate (optimal = 5 mL/min) and concentration (dilution of 6% (w/v)). They found that a higher air pressure resulted in finer powders (higher FPF). Bi et al. reported that the spray-freeze-dried liposomal insulin formulations produced a prolonged hypoglycemic effect in rats after pulmonary administration.

Depreter et al. (2010) manufactured a spray-dried solid lipid formulation intended for pulmonary delivery of insulin with cholesterol/phospholipon (75/25) as the lipids [103]. After spray-drying, their best formulation achieved an FPF of 63%, an MMAD of approximately 3 μm, and a yield of 60%. Al-qadu et al. (2012) used chi-

tosan to make carrier particles for an inhaled insulin formulation [104]. With mannitol as a stabilizer during spray-drying with a Büchi Mini Spray Dryer B-290, their processing conditions were as follows: 0.7 mm two-fluid nozzle, liquid feed rate of 2.5 mL/min, inlet temperature of 160 °C, resulting outlet temperature of 40 °C, and airflow rate of 400 L/h—resulting in approximately 73 % yield. The yields of these studies are typical of the yield values expected from optimal spray-drying of any biopharmaceutical. As discussed in sections "Basics of Spray Drying" and "Lyophilization Versus Spray Drying," this loss of product remains one of the drawbacks of any conventional spray-drying process.

Maltesen et al. (2008) examined several processing parameters including nozzle gas flow rate (7.3–17.5 L min), feed flow rate (1.8–5.25 mL/min), inlet air temperature (75–220 °C), and insulin concentration (5–60 mg/ml), using a Büchi B-290 Spray Dryer equipped with a two-fluid nozzle (7 mm) [31]. They found that the insulin concentration influenced the MMAD, mass median diameter (MMD), yield, tap density, droplet size, HMWP content, and morphology—all of these parameters were positively correlated with insulin concentration except HMWP content which decreased with increasing insulin content. The inlet drying air temperature mainly correlated with the outlet air temperature and the final moisture content; as inlet air temperature increased, outlet air temperature increased and moisture content decreased. The particle density was equally influenced by the insulin concentration and inlet air temperature. Maltesen et al. found that the least important processing parameters were the aspirator rate and feed flow rate, with no significant relationship to any of the parameters studied. As observed by Maltesen et al., particle size properties (such as the MMAD and MMD) are the most difficult powder characteristics to predict due to the numerous influential processing parameters. While insulin concentration had the greatest influence on the MMAD and MMD, at high insulin concentrations its influence on the MMAD plateaued, and drying conditions became the controller of particle aerodynamics. This study is a reminder that formulation choices, such as drug solubility, concentration, and excipient choices, should carry equal (and often more weight) than drying parameters with formulating spray-dried biopharmaceuticals.

Concluding Remarks

In recent years, spray-drying has become popular in the preparation of powdered biopharmaceuticals. The root of its popularity lies in the diversity of equipment, excipients, and process conditions available to fine-tune particle characteristics. The introduction of new spray nozzles, alternative spray-drying methods, and more efficient particle collection devices has broadened the range of biopharmaceuticals amenable to the spray-drying process. However, because of the diversity of the research, a more systematic approach of processing parameters is warranted, particularly for the selection of appropriate solvents and excipients. In contrast, freeze-drying offers the advantage of a longer, proven history of extending the storage life

of biopharmaceuticals. Both methodologies offer advantages and disadvantages, and while neither is a silver bullet to biopharmaceutical preservation, both can significantly extend the storage life of a biomolecule, ease handling and shipping, and allow the exploration of alternative drug delivery routes.

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Current Trends and Advances in Bulk Crystallization and Freeze-Drying of Biopharmaceuticals

Hiten Gutka and Krishna Prasad

Introduction

Drug discovery, design, and development process in the post genomics era has changed significantly with an increasing number of approvals for recombinant therapeutics. Such recombinant therapeutics include varied modalities such as enzymes, replacement hormones (e.g., growth hormone, insulin), growth factors, cytokines, fusion proteins, monoclonal antibodies (mAbs), vaccines, nucleic acid-based therapeutics, and antibody–drug conjugates [6, 14]. The pharmaceutical development of these varied modalities has further enhanced our understanding in several platform development processes and manufacturing techniques. The aforementioned biopharmaceutical modalities are marketed in different forms such as liquid products, solid lyophilized formulations, and several device combinations. Protein stability is a principle factor governing the ultimate form and configuration for these commercial biopharmaceuticals [30, 31, 56]. Designing stable liquid prototype formulations is generally the first step towards development of a novel biopharmaceutical; moreover, this approach has been adequately reviewed and routinely applied in the biopharmaceutical industry [54]. Design of a stable lyophilized formulation is an alternative (and often parallel or overlapping) approach taken during product development [7, 55]. Lyophilization, to date, remains a popular means of converting proteins into solid form to achieve reasonable shelf life as a commercial pharmaceutical product. The broader concept of drug product lyophilization and related aspects such as excipient selection and buffer composition, container closure systems, and

H. Gutka (✉)
Thermalin Diabetes LLC, 10000 Cedar Ave, Cleveland, OH 44106, USA
e-mail: hiten1980@gmail.com

K. Prasad
Julphar Pharmaceuticals, Ras Al Khaimah, UAE
e-mail: krishna.prasad@julphar.net

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process development challenges have been discussed in sufficient detail in other chapters of this book.

Figure 1 describes the general steps involved in a typical biopharmaceutical manufacture process. Step 4 in this process, wherein the purified drug substance is obtained represents a critical hold and transfer step in the manufacturing process. The purified biologic may be stored as a frozen liquid, crystallized solid, freeze-dried solid, or a formulated liquid (ready to be filled). Moreover, the subsequent steps may take place in a fill-finish operations facility which can be distantly located from the drug substance manufacture site; in such instances, the hold and transfer step becomes very critical from a logistics and transportation perspective. The fill-finish operations involved in biotherapeutics development have been reviewed [45] wherein the drug substance is typically described as a frozen bulk solution. Freeze–thaw processing of protein solutions, a widely practiced approach in biopharmaceutical manufacture has been reviewed in the recent past. Singh et al. present a practitioners perspective on large-scale freezing of biologics [50, 51] wherein the authors discuss details on the available frozen storage options including jacketed cryovessel and Celsius(R) Pak large-scale bag system and provide practical advice on rational process and formulation development. While bulk freeze–thaw

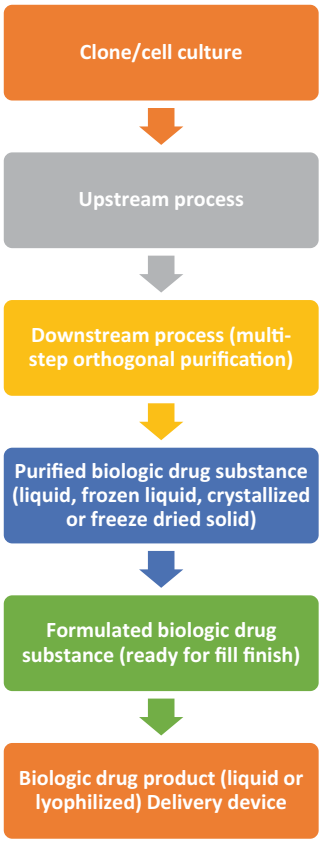

Fig. 1 Process flow diagram describing the manufacture of a typical biopharmaceutical. The purified biologic drug substance is typically held as a liquid or frozen liquid and occasionally also crystallized and processed as a freeze-dried solid

is an attractive option in biopharmaceutical processing, it requires elaborate studies and data-driven development approaches including but not limited to scale-down models, transportation validation, experimental verification of controlled and uncontrolled freeze-thawing processes [27–29]. Moreover, since the process involves volumes of frozen liquid, it presents a potential logistics and business limitation. Recently, there has been a focus on several drying technologies (including lyophilization) for protein therapeutics; each of these drying methods can have a significant impact on product quality and should be rationally selected [1]. Spray-drying and supercritical fluid-drying are two such emerging techniques besides freeze-drying with specific applications in protein biopharmaceutical development [21–23, 33, 48]. Bowen et al. describe a bulk drug substance storage approach based on spray-drying of mAbs [5]. The findings suggested that the physical stability of the spray-dried mAbs was comparable or greater than that of corresponding freeze-dried samples. Finally, extensive characterization of the spray-dried mAbs with regards to reconstitution time and reconstituted solution properties suggested that the said process was feasible for powder-based biologic bulk storage applications of mAbs.

Bulk freeze-drying has been successfully adapted for pharmaceutical processing of small molecules particularly cytotoxic anticancer drugs and antibiotics [4]. Insulin is a classic biopharmaceutical wherein the drug substance is processed as a freeze-dried solid. This chapter discusses the potential of bulk crystallization and bulk freeze-drying applied in succession as final polishing steps in typical biopharmaceutical manufacturing processes.

Protein Crystallography: Principle, History and Applications in Drug Development

Principle underlying protein crystallography can be understood by carefully evaluating the protein phase diagram. Figure 2 describes the schematic illustration of a typical protein crystallization phase diagram formed as a result of varying protein concentration and varying adjustable parameters typically precipitant or additive concentration, pH, and temperature [9]. Protein crystals are formed in the metastable zone which can be achieved by varying both the protein concentration and one or more of the adjustable parameters. The underlying principle of macromolecular crystallization and approaches to protein crystallization have been reviewed by McPherson [35, 36, 37].

The successful application of crystallography in structural elucidation of proteins dates back in to the late 1950s with the first-reported structure of sperm whale myoglobin by Sir John Cowdery Kendrew and Max Perutz, for which they shared the Nobel Prize in Chemistry in 1962 [26]. In the early 1990s, the National Institute of Health, through the Protein Structure Initiative (PSI), funded several centers for high-throughput structure determination of proteins [40]. Large-scale protein purification, crystallization and X-ray crystallography formed the core of such a collaborative effort which provided novel structural information on proteins of

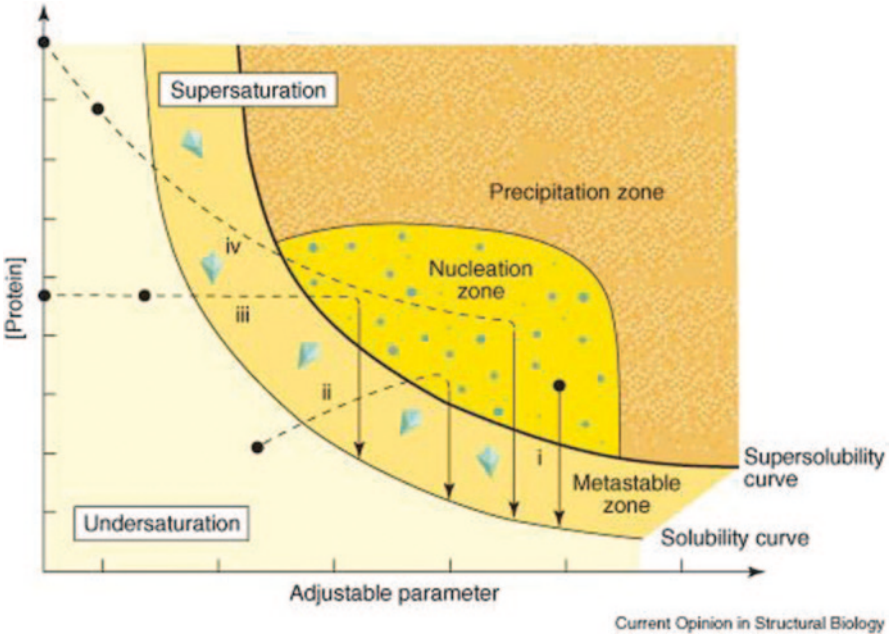

Fig. 2 Schematic illustration of a protein crystallization phase diagram. Adjustable parameters include precipitant or additive concentration, pH and temperature. The four major crystallization methods are represented: (i) microbatch, (ii) vapor diffusion, (iii) dialysis, and (iv) free interface diffusion (*FID*). Each involves a different route to reach the nucleation and metastable zones, assuming the adjustable parameter is precipitant concentration. The *filled black circles* represent the starting conditions. Two alternative starting points are shown for FID and dialysis because the undersaturated protein solution can contain either protein alone or protein mixed with a low concentration of the precipitating agents. The solubility is defined as the concentration of protein in the solute that is in equilibrium with crystals. The supersolubility curve is defined as the line separating conditions under which spontaneous nucleation (or phase separation or precipitation) occurs from those under which the crystallization solution remains clear if left undisturbed (Adapted with permissions from [8])

biological significance [12, 20, 44, 47, 57]. Such consortium-based collaborative efforts utilizing structural genomics have provided fundamental knowledge and understanding for drug discovery, particularly in the area of infectious diseases and other neglected diseases of developing nations [3]. Tuberculosis Structural Genomics Consortium (TBSGC), formed as a part of the PSI is one such consortium dedicated towards solving structures of proteins from the pathogen *Mycobacterium tuberculosis* (*Mtb*) the causative agent of tuberculosis (TB). Novel purification and crystallization means and crystal structures for *Mtb* proteins, as reported by consortium members and nonmembers have provided a meaningful insight into drug discovery and design efforts towards countering TB [2, 10, 11, 15, 16, 32, 38]. Figure 3 describes a typical process flow of structural elucidation for proteins by X-ray crystallography [44]. In the recent past, protein crystallography finds extensive application in drug discovery and design of small molecule inhibitors.

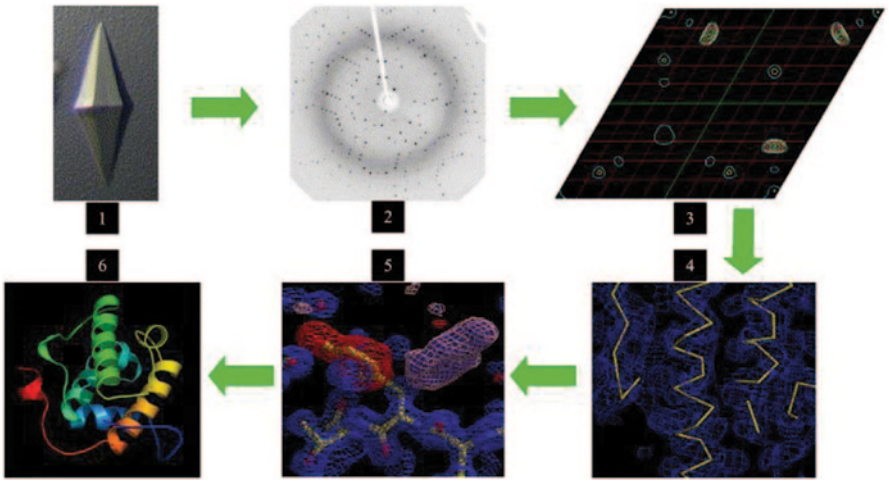

Fig. 3 Process of protein structure determination by single crystal diffraction and exploitation of the anomalous scattering phenomenon. A set of oscillation images (*2*) is obtained by exposing single crystals (*1*) of the protein under investigation to a beam of X-rays. The processed data are then used to "solve" the structure. Shown here is a Patterson map (*3*) (generated with the program XPREP) as used in the determination of a heavy atom substructure. Once the substructure is known, phases are calculated and refined. Phases and diffraction data permit the calculation of electron density maps and the generation of an initial trace of the model (*4*) (screenshot from XFIT). Multiple cycles of validation, model refitting and refinement against the diffraction data (*5*) (XFIT), and geometric restraints produce a model of the protein (*6*) (cartoon generated with PYMOL) for release to the structural biology community (Adapted with permission from [44])

Bulk Protein Crystallization (Process Development, Advantages, and Significance in Biopharmaceutical Development Process)

As discussed in the previous section, protein crystallography as a technique has made a significant contribution to drug discovery, design, and development. However, most of the applications have been in protein crystallography for structural biology and small molecule drug discovery and not in bulk crystallization for purification or polishing of proteins. While bulk crystallization is a popular approach applied in separation and purification of small molecule drugs and fine chemicals, its applications with proteins are limited to a handful of biopharmaceuticals such as insulin, lyzosyme, aprotinin, certain commercial enzymes, and some monoclonal antibodies. Peters et al. describe important criteria that distinguish bulk protein crystallization (a purification and polishing step for proteins) from traditional protein crystallization used for structure determination (Table 1) [42]. Among the several listed criteria, the precipitant or excipients used in crystallization are limited in bulk biopharmaceutical processes, since they have to be pharmaceutical grade, nontoxic, generally regarded as safe (GRAS), easily sourced, and inexpensive. Some aspects such as scalability, process compatibility, redissolution, and crystallization

Table 1 Demands on crystals for structure determination and purification. (Adapted with permission from [42])

Criterion	Crystals for structure determination	Crystals for purification/formulation
Excipients	Free choice	Only pharmaceutically acceptable compounds; no hazardous chemicals
Excipient costs	No issue	Important
Process compatibility	Not important	Very important
Crystal size	As large as possible	As large as possible
Packing quality (unit cells)	High resolution	Not important
Crystallization yield	Not important	Very important
Growth kinetics	Often very slow (several days to month)	Fast (hours to days)
Re-dissolution	Not necessary	Necessary
Scalability of conditions	Not important	Very important
Protein consumption during screening phase	Restrictive (in most cases)	Not restrictive

yield are important in bulk biopharmaceutical crystallization processes which are not so significant for structural biology-related applications. Crystal size and packing quality (structural resolution) are important aspects in crystallization for structural elucidation.

The presence of impurities and related proteins and their influence on crystallization rate is an important consideration in bulk crystallization process development. Judge et al. describe the effect of protein impurities on lysozyme crystallization [25]. The effect of the protein impurities, avidin, ovalbumin, and conalbumin at concentrations up to 50%, on the solubility, crystal face growth rates, and crystal purity for lysozyme was investigated. The findings suggested that the presence of impurities had negligible effect on solubility and crystal purity (>99.99% pure crystals). However, the effect of the impurities on the face growth rates varied from none to a significant face-specific effect leading to growth cessation. These findings suggest the possibility of employing bulk crystallization as a unit operation (separation, polishing, and purification) in downstream processing of therapeutic proteins.

Process design, scale-up, and the design of industrial-scale protein crystallizer are important aspects for consideration in bulk protein crystallization. While industrial crystallizers for chemicals and small molecule drugs can employ methods including evaporation, cooling, precipitation, supercritical fluid-based crystallization, the choice becomes limited for proteins due to the macromolecular (and rather complex) nature, degradation, and potential stability issues with proteins. Hence, the choice of crystallization equipment and process design become extremely critical for bulk protein crystallization.

Gutka et al. describe the generation of larger hexagonal crystals of *Mtb* fructose-1,6-bisphosphatase enzyme by the traditional vapor diffusion method, by increasing the hanging drop volume from 2 µl to 4 and 6 µl (Fig. 4) [16]. It is important to note here that while larger crystals were easily obtained, the edges and surface properties of larger crystals were ill-defined (Fig. 4c).

While protein crystals for structural elucidation are traditionally obtained using vapor diffusion methodology, screening for large-scale protein crystallization development is often performed in batch crystallization mode, because of its inherent advantages. In fact, batch crystallization is now being widely accepted as a robust protein crystallization tool in the structural biology community as well [46]. While batch crystallization has its process advantages, successful transfer of an existing vapor diffusion-based crystallization method to microbatch and then its scale-up to process level remains a challenging task. Hekmat and coworkers describe the successful transfer of vapor diffusion-based crystallization to agitated batch crystallization at milliliter scale using lysozyme as a model protein [19]. The same research group also describes a stirred-tank crystallization-based approach [53] towards successful scale-up of batch crystallization conditions using lysozyme, lipase [18], and therapeutic mAbs [17, 52] as model proteins. Smejkal et al. describe a maximum local energy dissipation-based scale-up approach for stirred crystallization using lysozyme and Canakinumab Fab-fragment as model proteins. Faster onset of crystallization was observed for both lysozyme (ten times faster) and Cankinumab Fab-fragment (four times faster) under stirred crystallization conditions at 6 ml and 1 l

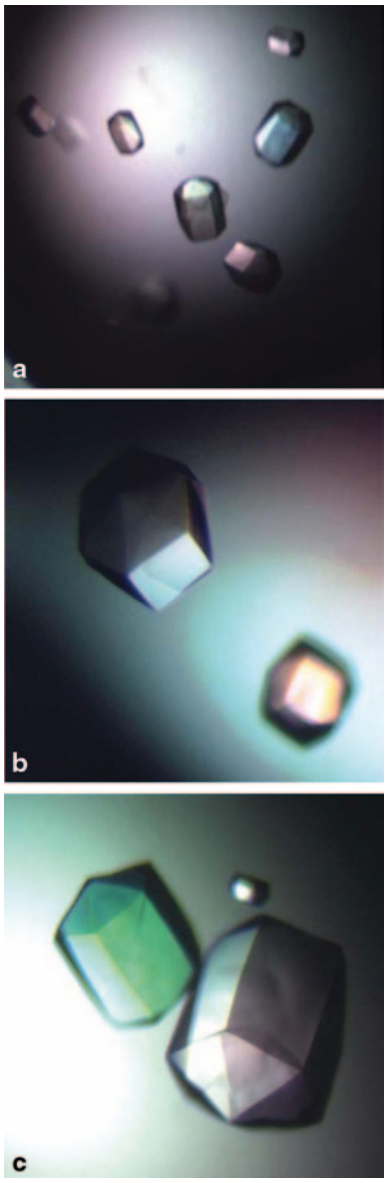

Fig. 4 **a** Hexagonal crystals of *Mycobacterium tuberculosis* FBPase obtained at pH 7.0. **b** and **c** Larger crystals of *M. tuberculosis* FBPase obtained under similar conditions with increasing drop volumes. *FBP* fructose-1,6-bisphosphatase (Adapted with permission from [16]. Copyright© International Union of Crystallography 2011)

scale, respectively, in comparison to the nonagitated 10 µl control. The otherwise helpful scale-up parameter such as stirrer speed or impeller tip speed was not a suitably indicative parameter for crystallization scale-up. Successful and comparable crystallization scale-up was achieved when the maximum local energy dissipation, a parameter estimated by measuring the drop size distribution of an oil/surfactant/water emulsion was kept constant across the different stirred-tank reactors (6 ml, 100 ml, and 1 l scale) used in this study. Figure 5 describes the geometrical design

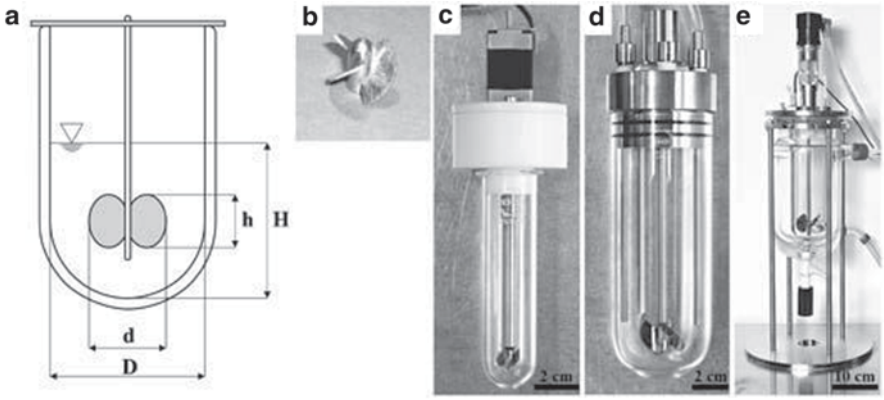

Fig. 5 Geometrical design of the crystallization systems. **a** Schematic drawing, **b** three-bladed segment impeller ($d = 11.3$ mm), **c** 6-ml vessel, **d** 100-ml vessel, and **e** 1-l vessel (Adapted with permission from [53])

of crystallization vessels and the three-bladed impeller used for scale-up development [53].

While there are several considerations and limitations with bulk protein crystallization, it offers the following distinct advantages:

1. It is a general observation that several protein instability reactions proceed slowly in the solid rather than the liquid state, hence crystallization can serve as an ideal polishing step for final processing of biologics wherein water is eliminated or minimized from the system and material is converted into a more stable solid state. In case of insulin, bulk crystallization followed by freeze-drying is commercially adopted to ensure stability of the material for pharmaceutical distribution and long-term storage before formulation.
2. Bulk lyophilization of protein solutions is a rather energy consuming step and can often be deleterious to sensitive proteins (aggregation or protein degradation). In such instances, it becomes practical to crystallize/precipitate the protein and then remove the residual water from the suspension by bulk freeze-drying. The combined application of crystallization and freeze-drying as subsequent unit operations becomes an economically viable option for large-scale biopharmaceutical manufacture.
3. Bulk crystallization, a relatively inexpensive technique, can replace cost-intensive standard biopharmaceutical unit operations like packed-bed and membrane chromatography. Przybycien et al. present a comprehensive review of bioseparation operations wherein they compare alternative bioprocess techniques to standard packed-bed chromatography (Fig. 6) [43]. Crystallization happens to be a relatively mature industrial processing technique with several applications. Several studies describe successful purification and polishing of proteins such as aprotinin [42], mAbs [17, 52], and lysozyme [19, 24, 25] by bulk crystallography approach.

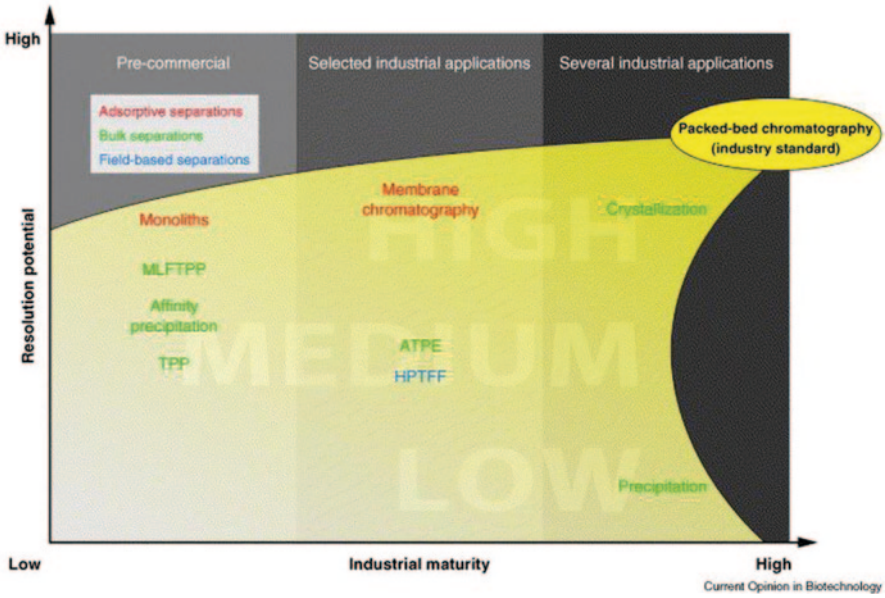

Fig. 6 A parsing of chromatography alternatives in terms of relative resolution potential and industrial maturity. Packed-bed chromatography is arguably the gold standard. *ATPE* aqueous two-phase extraction, *HPTFF* high-performance tangential flow filtration, *MLFTPP* macroaffinity ligand-facilitated three-phase partitioning, *TPP* three-phase partitioning (Adapted with permission from [43])

4. Crystalline protein suspensions offer a novel means to produce high-concentration formulations for biotherapeutics. High-concentration protein therapeutics especially mAbs, remain a formulation challenge due to high viscosity, instability due to charge interactions, and specific chemical degradation at high concentrations [39, 49, 58, 59]. Crystallization offers a formulation advantage; further, such crystalline suspensions can also be designed as sustained or controlled release formulations.

Bulk Freeze-Drying: Practical Considerations in Process Development and Scale-Up

With reference to pharmaceutical development, it becomes imperative to define the term bulk freeze-drying particularly in the context of its application and material properties. There are several possible scenarios that can be referred to as bulk freeze-drying including: (i) crystalline slurry/suspension of protein (with no lyophilization additives) being freeze-dried in trays, e.g., insulins, freeze-dried enzymes, lysozyme etc; (ii) formulated protein solutions being freeze-dried in bulk, i.e., trays;

and (iii) protein solutions (with no formulation additives) being freeze-dried in bulk. For the purpose of this chapter and ease of readers understanding, the term bulk freeze-drying shall be referred to as the first scenario only for practical reasons. Formulated protein solutions are rarely freeze-dried in bulk containers as compared to individual dosage units like vials/syringes/dual chamber cartridges; also, bulk freeze-drying of protein solutions without any additives is not feasible due to energy considerations in process development and protein stability limitations. Moreover, it would be appropriate to conclusively define bulk freeze-drying as an industrial process to bring down water/moisture limit to pharmaceutically acceptable levels, thereby permitting the long-term storage of active pharmaceutical ingredient (drug substance) until it is finally processed as a pharmaceutical product in an appropriate package/delivery device for commercial distribution.

Centrifugation in batch or continuous mode followed by repeated washings of protein crystals/precipitate to eliminate residual impurities and trace contaminants (buffers, salts, residual solvents noncrystalline protein impurities) is performed as a preceding step to bulk freeze-drying. Such a processing step provides uniform quality material for easy handling and transfer during a bulk processing step. Residual water content, total solids content in the slurry, slurry depth, and the uniform distribution/spreading of the slurry in a lyophilization tray are some of the important considerations for optimization and scale-up of bulk freeze-drying process. Slurry depth/fill is an important scale-up parameter necessary to be controlled for optimal drying and process performance. The otherwise important processing parameters like heat transfer, shelf temperature variation in a typical lyophilization process are equally important for bulk freeze-drying optimization. A major limitation of stainless steel freeze-drying trays is that they get warped over repeated use and hence have to be periodically replaced. Patel and Pikal have reviewed the development and scale-up issues with freeze-drying with a particular focus on the effect of load on process design and tray properties and considerations for freeze-drying [41].

Loading and unloading of a bulk tray lyophilizer typically take place under classified area and are important standard operating procedures in a typical manufacturing process. Recently, there has been considerable development towards finding an effective methodology for holding the product on the shelves for optimal freeze-drying. There are two possible ways to hold material.

Framed Structures This is a refined conventional approach for holding the product inside the freezer-dryer. In this approach, a low-density polyethylene (LDPE) sheet is sandwiched between two rectangular stainless steel frames. The LDPE sheet (appropriate pore size and thickness) then serves as the base for holding the crystal slurry. Once the product with optimal slurry concentration is spread uniformly on the sheet, a Tyvec sheet (appropriate pore size and thickness) is covered on top of this and fixed by means of the top frame. The Tyvec sheet avoids any product spilling before and after lyophilization.

Preframed Structures These structures typically are like the GORE® LYOGUARD® trays, their use in bulk freeze-drying has been discussed in sufficient details in subsequent parts of this chapter.

Fig. 7 Contained transfer of freeze-dried powder product to the LDPE bag by means of a hopper. *LDPE* low-density polyethylene (Image courtesy: ATMI Life Sciences)

Once the drying cycle is completed, the freeze-dried material from each tray is carefully transferred to a "pre-sterile" bag by means of a sterile polypropylene/stainless steel hopper (Fig. 7). The transfer operation has to be done as quickly as possible under optimal humidity conditions due to the possible hygroscopic nature of the product.

The product in the bag is then mixed uniformly and samples withdrawn for quality control. The bags offer the flexibility for a short-term storage of bulk product for considerable periods thereby minimizing the storage space. Studies evaluating the suitability of the bag material for a particular product, storage stability studies, and stress testing, need to be performed before selecting such bags as an interim storage system for bulk freeze-dried material. Eventually, the bulk product can be stored in USP type I glass bottles for long term under suitable temperature and humidity conditions, protected from light. For safe and easy transfer of freeze-dried powders, ATMI Life Sciences developed contained powder transfer bags (Fig. 8a, b) with tri-

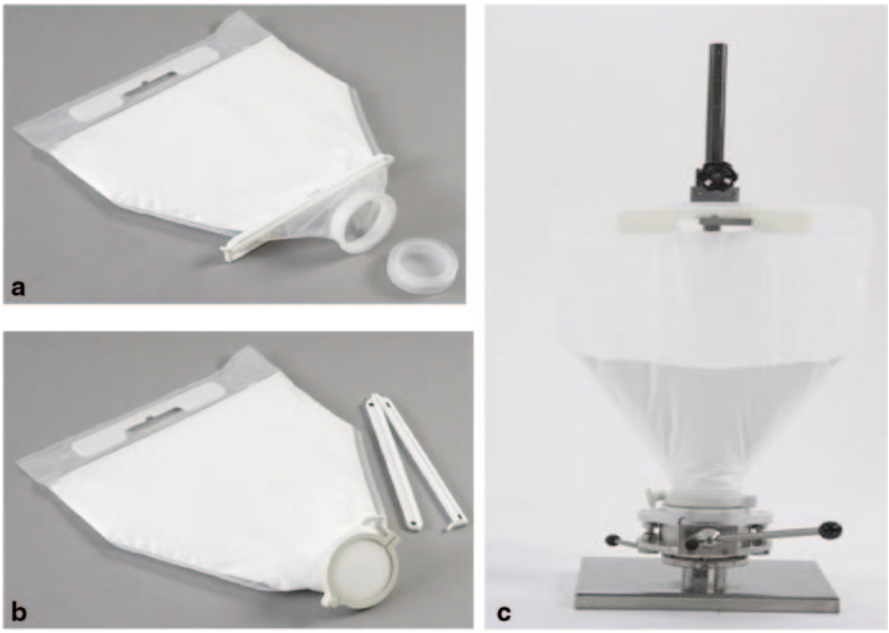

Fig. 8 **a** and **b** Newsafe contained powder transfer bags and accessories. **c** Transfer bag attached to tri-clamp connector (Image courtesy: ATMI Life Sciences)

clamp connectors (Fig. 8c) sealed onto ultra clean LDPE film. Newsafe contained powder transfer bags have antistatic properties, which prevent the buildup of electrostatic charge, thereby permitting safe and efficient handling of hygroscopic and fluffy freeze-dried powders.

Industrial scale-up of bulk freeze-drying process is optimized and performed on similar lines as that for other lyophilization-based processes. Production scale freeze-driers are typically available as single chamber or double chamber types. Cleaning and sterilization (cleaning in place, CIP/sterilization in place, SIP), which may not be essential considerations in laboratory freeze-dryers, are integral aspects of production scale freeze-dryers.

Freeze-drying validation involves validation of three aspects of the operation:

CIP Validation During the product loading and unloading steps and also during drying step, (particularly secondary drying when high vacuum is applied), it is possible that traces of product material builds up on the surface of the condenser and the surface of the drying chamber. The objective of CIP validation is to demonstrate the effectiveness of the cleaning step to remove product traces from the condenser and the drying chamber. Typically, CIP step involves a recipe with low-concentration acid wash or solvent wash (in which the product is soluble) followed by several rinses with water for injection.

SIP Validation In order to ensure the integrity of the machine for aseptic drying, it is imperative to ensure the effectiveness of steam sterilization cycle, which ensures sterilization of the probable product exposure components such as the condenser, the vacuum/nitrogen inlet, and the chamber. Typically, chemical and biological indicators are used to validate the steam sterilization cycle.

Drying Recipe Validation Freeze-drying recipe validation is an integral part of the overall manufacturing process. At the end of drying cycle, samples are withdrawn from key locations in the freeze-dryer and evaluated for critical quality attributes of the product including moisture content (loss on drying), residual solvents if any, particle size distribution, and other material properties (purity or critical impurity levels, assay, protein impurities with high molecular weight, aggregates). The general sampling methodology involves taking aliquot from a minimum of five locations in each tray, one in each corner and in the center of the tray. The pooled sample from these five locations represents a sample from a particular tray. Samples are taken from each tray in all the shelves. Such an elaborate sampling strategy is relatively easy for material being lyophilized in container closure systems such as vials and relatively complicated for bulk drying; however, such a strategy provides assurance on the uniformity of the product quality. It is a common practice to use several sampling locations and generate extensive processing data as a part of product drying validation.

GORE® LYOGUARD® Freeze-Drying Trays: Biopharmaceutical Application

GORE® LYOGUARD® (W. L. Gore & Associates, Inc., Elkton, MD, USA) freeze-drying trays (Fig. 9) represent a significantly improved methodology addressing some of the inherent limitations of the bulk freeze-drying process. In contrast to the routinely used open stainless steel 316 L trays, the GORE® LYOGUARD® trays

Fig. 9 GORE® LYOGUARD® trays. (Courtesy: W. L. Gore & Associates, Inc., Elkton, MD, USA; GORE, LYOGUARD and designs are trademarks of W.L. Gore & Associates)

Fig. 10 GORE® LYOGUARD® trays as loaded for a freeze-drying cycle. (Courtesy W. L. Gore & Associates, Inc., Elkton, MD, USA; GORE, LYOGUARD and designs are trademarks of W.L. Gore & Associates)

can be effectively used for product containment and prevent product loss post the drying cycle. Gassler and Rey describe some of the unique design features of the GORE® LYOGUARD® trays, present a detailed performance evaluation of the GORE® LYOGUARD® trays and also highlight the advantages of using GORE® LYOGUARD® trays in bulk freeze-drying processes [13].

The GORE® LYOGUARD® trays (Figs. 9 and 10) consist of a polypropylene frame, the bottom of the tray is a thin, flexible film with the product contact layer (inner side) made of polypropylene. The top surface of the tray comprises a GORE-TEX® expanded polytetrafluoroethylene (ePTFE) membrane permeable to water vapor. The tray is also provided with a filling port which can be secured by a screw cap. This filling port permits easy filling and unloading of the tray.

Gassler and Rey describe the evaluation of GORE® LYOGUARD® trays with regards to three aspects: (i) freezing and heating temperature profiles of the tray (heat transfer); (ii) mass transfer resistance for the upper membrane barrier (effect on primary drying); and (ii.) effectiveness of the upper membrane barrier [13]. The findings suggest that GORE® LYOGUARD® trays permit better heat transfer compared to the stainless steel open trays. Furthermore, the GORE-TEX® membrane exerted negligible resistance for drying of 5% mannitol solution. Their findings also suggested that the primary drying time for a typical product could be reduced when using GORE® LYOGUARD® tray compared to stainless steel trays. Finally, the authors recommend a thorough evaluation of the product-specific heat and mass transfer characteristics of the GORE® LYOGUARD® tray before any process modifications or transfers.

Mayeresse et al. describe the successful transfer of a bulk freeze-drying process from a glass container to a GORE® LYOGUARD® tray utilizing a robust process validation approach. The investigators designed two optimized lyophilization cycles (namely A and B) using the GORE® LYOGUARD® trays. The transferred process was deemed feasible and profitable with regards to freeze-drying capacity; however, detailed evaluation of container compatibility and estimation of the heat and mass transfer properties for both container types (glass vial and GORE®

LYOGUARD® trays) was suggested to be an integral aspect of the freeze-drying process transfer [34].

While the GORE® LYOGUARD® trays may significantly improve the bulk freeze-drying process depending upon individual product or material properties, some of the definite advantages of GORE® LYOGUARD® trays are product containment post drying, ease of unloading the lyophilizate (which at times may be light and fluffy with significant electrostatic charge built up), and the option to seal the trays in preformed pouches (for shipping to different sites).

Conclusion

Based on the several aspects discussed in this chapter, we can conclude that bulk crystallization followed by freeze-drying of a biopharmaceutical can certainly be a preferred processing strategy. However, the successful implementation of both these processing techniques depends upon several factors including crystallization potential of a protein therapeutic (ability to form crystals), stability of the said protein during such operations, sensitivity to processing conditions, scalability of the processing technique, and overall regulatory compliance of the operations. In a globally expanding biopharmaceutical industry, where logistics and transportation are important considerations, such bulk processing–finishing techniques will certainly find widespread applications. It would be inappropriate to summarize both bulk crystallization and freeze-drying as general biopharmaceutical processing techniques without describing the specific applications of both these techniques by means of relevant case studies or examples. The next chapter discusses specific examples of biotherapeutic modalities wherein both (or either one of) these techniques have been applied as unit processing steps.

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Case Studies and Examples of Biopharmaceutical Modalities Processed by Bulk Crystallization or Bulk Freeze-Drying

Hiten Gutka and Krishna Prasad

Introduction

While the previous chapter describes the basic principle, process development, and scale-up aspects of both bulk crystallization and freeze-drying in general, this chapter describes unique examples of biopharmaceuticals processed by these techniques. A section of this chapter describes in detail the processing approach for human insulin—the world's first approved recombinant biotherapeutic. In the case of insulin, the purified biologic is generally bulk crystallized using certain crystallization aids and finally freeze-dried to ultimately minimize the residual moisture content. This approach has also been extended to certain modifications of insulin and insulin analogs. The next class of biotherapeutics discussed in this chapter are monoclonal antibodies (mAbs) and antibody fragments. Bulk crystallization has been used for several aspects of antibody therapeutic development, including an efficient capture and purification strategy, long-term storage, and high concentration formulation development strategy. The third group of modalities discussed in this chapter are examples like aprotinin and L-methionine γ-lyase which have been crystallized for process and drug development purposes. Bulk freeze-drying has recently also been applied in plasma and plasma fraction product development.

H. Gutka (✉)
Thermalin Diabetes LLC, 10000 Cedar Ave, Cleveland, OH 44106, USA
e-mail: hiten1980@gmail.com

K. Prasad
Julphar Pharmaceuticals, Ras Al Khaimah, United Arab Emirates
e-mail: krishna.prasad@julphar.net

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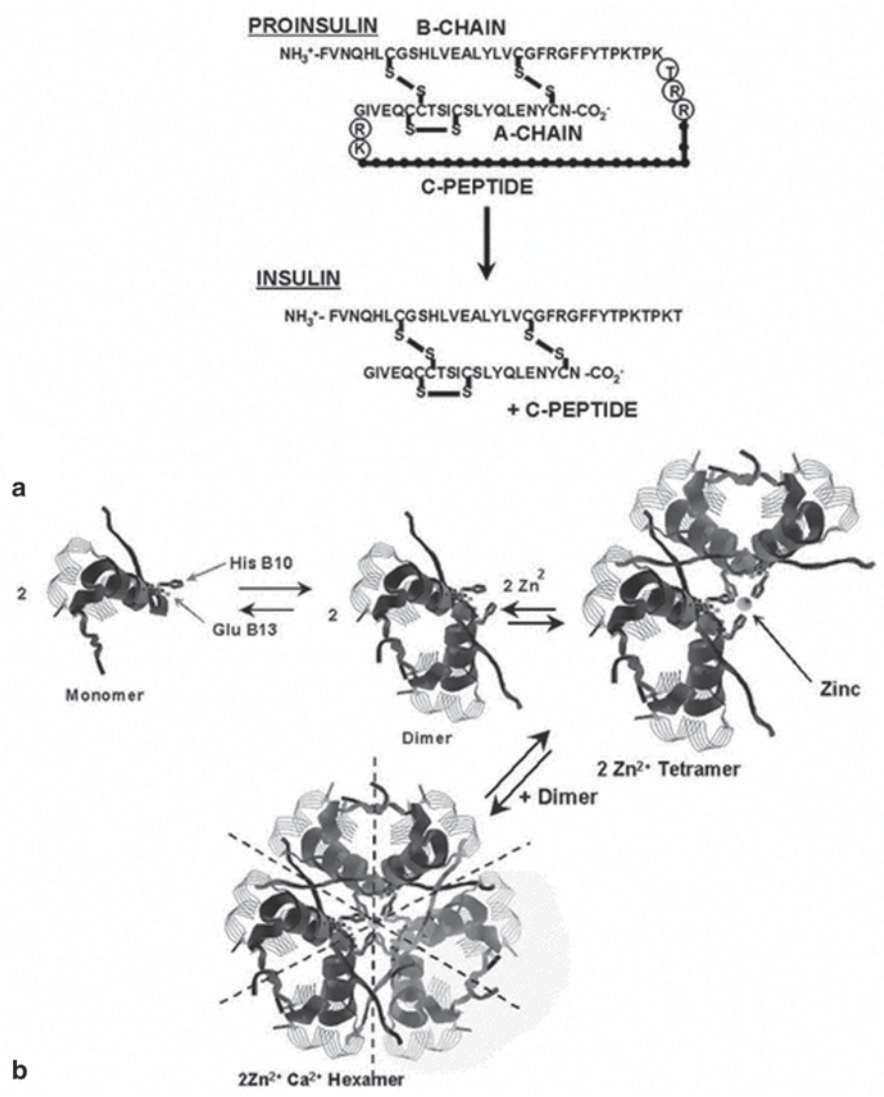

Fig. 1 **a** Scheme depicting the conversion of proinsulin to insulin and the C-peptide through the action of the trypsin-like and carobxypeptidase-like enzymes of the secretory vesicle. **b** Pathway for the assembly of the T_6 insulin hexamer. Structures are viewed along the hexamer threefold symmetry axis. The three pseudo twofold symmetry axes of the hexamer are shown as *dashed lines* (Structures created from 41NS.PDB [2]. Figure adapted with permission from [18])

Insulin

Insulin Structure: Zinc-Based Crystallization Primary sequence of human insulin contains 51 amino acids in two chains, chain A comprising 21 residues and chain B consisting of 30 residues, respectively (Fig. 1a). As indicated, there are three

disulphide linkages—one intrachain between cysteine (Cys) A6 and A11 and two interchain linkages between Cys A7 and B7 and Cys A20 and B19, respectively. The secondary structure of human insulin comprises two α helices in the A chain (A2–A8 and A13–A20) and a single α-helix (B9–B19) and a β strand (B21–B30). While the A chain and the B9–B19 helix form a rigid structural unit, the B25–B30 and the B1–B8 regions are flexible. Endogenous insulin is synthesized after tryptic cleavage of the C-peptide from proinsulin (Fig. 1a). Individual synthesized monomers of insulin, combine to form a dimer, and two such dimers with two zinc ions form a tetramer which is eventually assembled and stored as hexameric complex with two zinc ions and a calcium ion. (Fig. 1b) [12, 38]. The hexameric assembly of insulin–zinc complex and its allosteric regulation is key to biophysical properties of insulin in liquid pharmaceutical preparations. It is well understood now that insulin undergoes phenolic ligand- and zinc-mediated conformational change between the T_6–T_3R_3 and the R_6 states, wherein the T and R conformations, respectively, represent the tense and relaxed structure of the monomers in a hexameric assembly [32, 33, 38, 41–43]. Liquid pharmaceutical preparations of insulin normally containing phenol and/or metacresol as preservatives are effectively stabilized such that the insulin hexamer exists in the R_6 conformation and has optimal chemical stability (Insulin's chemical stability and the underlying instability mechanisms are discussed in subsequent section). It is the underlying knowledge on the physiological hexameric assembly of insulin in complex with zinc that forms the basis for using zinc-based bulk crystallization as a final recovery and polishing step in industrial insulin manufacture process.

Potential Routes of Degradation: Stabilization Approaches Insulin undergoes chemical modifications primarily at the Asn A21 and Asn B3 sites with the respective desamido species (Asp) formed under different pH conditions. The Asp A21 species is formed under acidic conditions, whereas the Asp B3 species is formed under neutral conditions [6–8]. Besides the deamidation pathway, insulin also undergoes covalent dimerization. Following a common pathway as the deamidation reaction, covalent dimerization primarily occurs via covalent linkage of the cyclic intermediate at A21 position with the A or B chain N-terminal from another insulin molecule as described in the scheme in Fig. 2 [45].

Factors influencing the formation of desamido species and covalent dimers at low pH (typically in the range of pH 2–5), and also during lyophilization (particularly at low pH), have been well characterized [14, 15, 44, 45]. Pikal and Rigsbee evaluated the stability of amorphous insulin prepared by freeze-drying of the supernate from a zinc–insulin crystal suspension, the pH of which was previously adjusted to 7.1. Their comprehensive storage stability evaluation (at high temperatures) using size-exclusion chromatography and reverse-phase chromatography suggested that the amorphous form of insulin was far more stable than the corresponding crystals of insulin. The degradation pathways for insulins as evaluated by chromatographic assays suggested that the A21 site was involved in instability mechanisms. The secondary structures of both types of insulins were further evaluated by differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR); the findings suggested that both forms of insulin retained a higher-order structure.

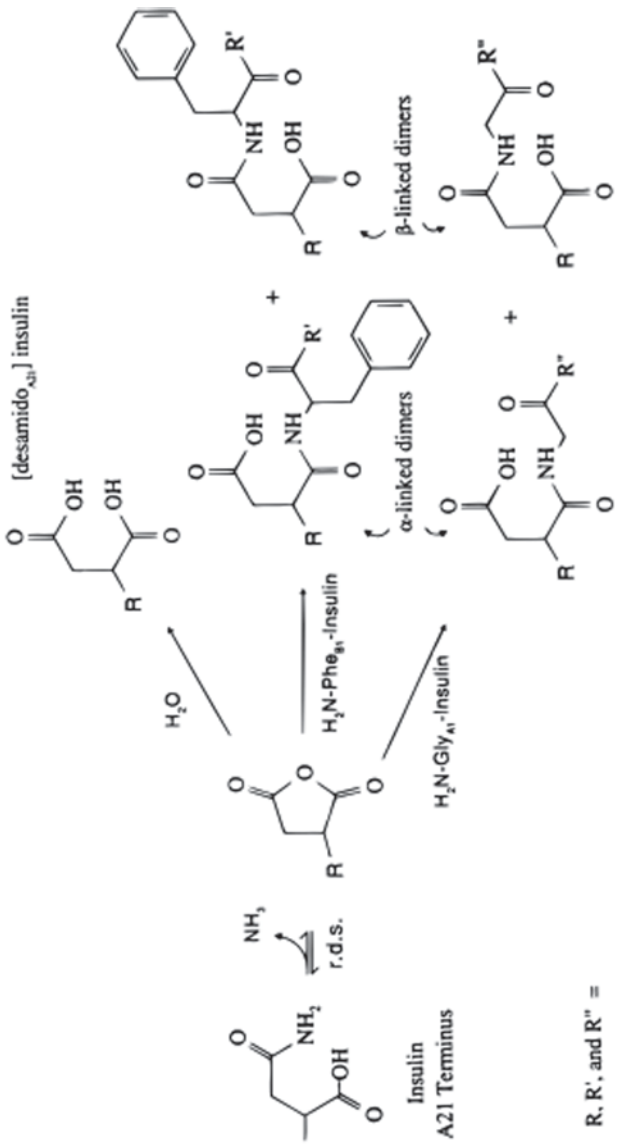

Fig. 2 Schematic pathway describing the formation of desamido insulin and covalent insulin dimmers (Adapted with permission from [45])

However, the secondary structure was significantly perturbed for both forms of insulin compared to the native solution structure of insulin [37]

Constantino et al. investigated the moisture-induced aggregation behavior of lyophilized insulin. Their findings suggested that when exposed to elevated temperature and moisture, lyophilized insulin undergoes both covalent and noncovalent aggregation. The covalent aggregation mechanism involves intermolecular thiol-catalyzed disulfide interchange as a result of β-elimination of an intact disulfide bond in insulin. The investigators also reported that such aggregation could be prevented by Cu^{2++} which is a free thiol scavenger [13]. The above-described instability mechanisms (particularly deamidation, covalent, and noncovalent aggregation) should be considered in designing the crystallization and subsequent freeze-drying steps for insulin manufacture.

Insulin Manufacture Process: Bulk Crystallization and Freeze-Drying an Integral Part of the Manufacturing Process Biosynthetic human insulin, the world's first successful recombinant biotherapeutic, was approved for the treatment of diabetes in the year 1982 [11]. Insulin manufacture process is a typical bioprocess, involving an upstream step and several downstream steps performed in a continuous manner to ultimately yield pure insulin crystals. As described in Fig. 3, bulk crystallization, centrifugation, and bulk freeze-drying are employed as polishing and finishing steps in insulin manufacture process.

A careful review of the literature on insulin preparation processes, suggests that the application of crystallization techniques dates back to the late 1920s. Abel crystallized insulin in its isoelectric region from a solution containing brucine, pyridine, and ammonium acetate [1]

US patent 3,719,655 describes a process for preparing ammonium and alkali metal salts of insulin [26]. In this process, insulin obtained from pork pancreas is dissolved under acidic conditions using acetic acid. The pH of insulin solution is adjusted to 8.2 with sodium hydroxide solution. Insulin begins to crystallize at pH 8.2 within about 15 min, and the crystallization is complete after the solution is stirred for about 18 h at 22 °C. The process described in this patent is popularly referred to as "8.2 process" since the insulin yield is maximum at pH 8.2.

US patent 5,597,893 describes the crystallization of LysB28ProB29 human insulin analogue [3]. LysB28ProB29 human insulin is a structural modification of human insulin, wherein the native sequence of ProB28LysB29 is reversed thereby preventing the dimerization of insulin which makes LysB28ProB29-human insulin a rapid-acting insulin. Unfortunately, the 8.2 process of crystallization for insulin does not work for this analogue. Based on the US patent 5,597,893, LysB28ProB29 insulin is soubilized with glacial acetic acid and sodium chloride solution. Liquefied phenol is added to this solution and the pH is subsequently raised to approximately 9.0 with sodium hydroxide solution. The resulting solution is held at 5 °C with gentle agitation for about 24 h to obtain well-defined crystals of LysB28ProB29-human insulin. The patent also describes various modifications of this crystallization approach using other phenolic preservatives such as metacresol, resorcinol, and methyl paraben.

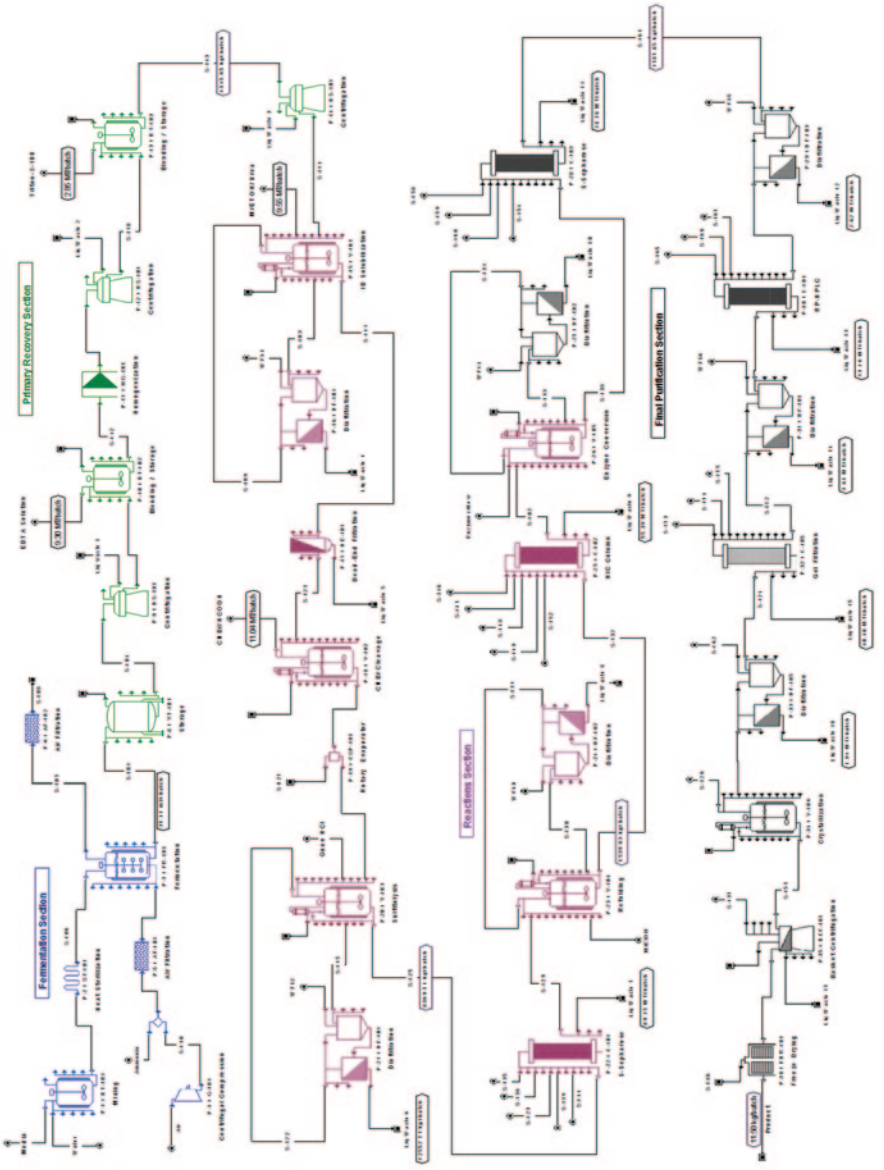

Fig. 3 Insulin production flow sheet (Adapted with permission from Demitri Petrides (personal communication), for details readers can refer to Bioprocess Design and Economics, In: Bioseparations Science and Engineering; Ed: Roger Harrison, Paul Todd, Scott Rudge and Demetri Petrides (http://intelligen.com/literature.html). Copyright © 2012, by Demetri Petrides)

European patent EP 1627642A2 describes a method to obtain zinc-free insulin crystals for pulmonary use [18]. One of the examples in this invention describes a method to generate zinc-free insulin crystals using sodium acetate. Based on this method, purified human insulin is dissolved in TRIS buffer, pH 8.0, and ethanol mixture. The addition of sodium acetate to this solution, forms a precipitate immediately. The crystals formed after about 2 days are examined microscopically and reported to have a diameter between 0.5 and 1 μm. The crystals are washed with ice-cold ethanol in water, isolated by centrifugation, and dried by lyophilization.

Dave et al. describe a process for manufacture of a modified insulin intended for oral delivery [15]. The modified insulin is synthesized by covalent conjugation of native insulin, purified by successive reverse-phase chromatography and cation-exchange chromatography steps. The purified insulin analogue was crystallized by adding phenol and zinc chloride at a pH of about 4.8. The crystals were washed with cold, purified water to remove excess zinc and phenol and lyophilized into dry powder.

In all the above-described processes the residual moisture in the insulin crystals is removed by vacuum drying or bulk freeze-drying. Processing of Insulin crystals after crystallization typically involves a washing step which controls some important quality attributes of the freeze-dried Insulin bulk. Successful washing step ensures that the zinc content, inorganic salts and buffer residues, and residual organic solvent are controlled such that the final freeze-dried Insulin bulk has ideal quality attributes/properties (some desired properties are described in Table 1).

Crystal washing can be performed in either batch mode or continuous mode using an industrial-scale centrifuge for separation of crystals. After the crystals settle, the resulting supernatant is decanted and the crystal slurry washed several times with water for injection. The supernatant is decanted, and the crystal slurry is taken for lyophilization. The suspension and washing steps can be repeated multiple times depending on the process to achieve desired product quality.

Insulin Freeze-Dried Drug Substance a Process, Logistics, Commercial, Formulation Advantage

a. *Process advantage:* Unlike several other biopharmaceuticals which are stored as frozen liquid solutions, Human Insulin drug substance (DS) is a solid [25, 48] and has traces/residual zinc (typically less than 1% w/w). Insulin, unlike other biopharmaceuticals, is a large volume, high-value molecule with typical manufacturing yields in kilograms. The typical polishing steps in insulin manufacture are chromatography steps (reverse-phase or size-exclusion chromatography), wherein the final elution volumes can be as high as 50 L. Converting the liquid samples into a stable solid form is a definite process advantage.
b. *Logistics and commercial advantage:* A crystalline/freeze-dried solid at the end of a lengthy manufacture process is not just a process advantage but also a storage handling and logistics advantage with less volumes to store and handle at the end of the process. Solid DS is a fill–finish advantage as well, wherein cold chain transportation (and the validation of such transportation) of small volumes of freeze-dried solids becomes practically feasible. This is a practical scenario

Table 1 Typical quality attributes of freeze-dried insulin bulk that is directly affected by the crystallization and subsequent freeze-drying steps in the manufacture process.

Quality attribute(s)	Typical properties of freeze-dried insulin crystals
Appearance	White crystals or powder
Solubility	Soluble in solutions of dilute acids and alkalies
High-molecular-weight proteins (HMWP) as determined by size-exclusion chromatography	Proteins with molecular weight higher than that of insulin is typically controlled below 1%[a]
A21 desamido Insulin as determined by reverse-phase chromatography	Desamido insulin (A21 position) is typically controlled below 2%[a]
Related proteins other than Insulin and A21 desamido Insulin as determined by reverse-phase chromatography	Proteins other than Insulin and A21 desamido Insulin are controlled below 2%[a]
Zinc content	Freeze-dried Insulins typically contain about 1% weight/weight (w/w; zinc)[a], which is an important formulation (drug product) consideration
Moisture content/Loss on drying (LOD)	Sufficiently dried insulin bulk generally contain less than 10% moisture content as determined by a suitable method[a]
Residual organic solvents	Residual solvents such as methanol, ethanol, acetonitrile, isopropyl alcohol, and acetone as used in the downstream purification of Insulin should be within approved regulatory limits

[a] Limits/typical properties for each of the individual quality attributes reported herein are general and not compendial in nature. Moreover, such limits may be different based on the expectations of different regulatory agencies around the world

in global corporations where fill–finish sites are located across different places, away from the central DS manufacture facility.

c. *Formulation advantage:* Insulin formulations can be classified as rapid acting, short acting, intermediate acting, and long acting depending upon their pharmacokinetic profiles and formulation composition. While insulin Lispro, Aspart, and Glargine are analogues derived after modifications of the native insulin structure, commercial insulin formulations are subtle formulation variations of native Insulin (change in zinc concentration, addition of protamine sulphate, etc.) which result in an altered pharmacokinetic profile [17]. Present-day insulin formulations are typically formulated as liquid solutions with Insulin strength at 100 U and occasionally 500 U. Typical compositions contain glycerol one or more phenolic preservatives, zinc added as a stabilizer and a buffer system. Neutral protamine Hagedorn (NPH) insulin is a unique type of long-acting insulin suspension, wherein insulin is complexed with protamine. Mixed-acting insulins are designed by combining the solution and NPH suspension formulations in a predetermined ratio—typically 70/30 or 50/50. The availability of the DS in a freeze-dried solid form presents a unique advantage, wherein a DS batch can be used for manufacture of all possible formulation variations (Regular insulin,

NPH, Premixed 70/30 or 50/50) in a fill–finish facility which typically runs manufacture process in campaign mode with appropriate change over controls.

Aprotinin

Aprotinin is a polypeptide that inhibits the action of trypsin, chymotrypsin, and plasmin and is used as a hemostatic in several surgical procedures [34, 35]. Peters et al. describe the successful bulk crystallization of aprotinin variant by employing a statistical design of experiments (DOE)-based approach and a conventional screening approach which was followed by DOE. Figure 4 describes the two crystal forms of aprotinin variant. Monoclinic (plate-shaped) crystals (Fig. 4a) are formed with sodium chloride, whereas clusters of tiny plate-like crystals (Fig. 4b) grew with ethanol and magnesium sulphate.

Both the described crystallization conditions were successfully applied at scale in manufacturing process. The crystals obtained were bulk lyophilized after successive water washings. Furthermore, the crystals could easily be reconstituted in buffer at pH 7.8. Both of these crystallization steps could be employed either as a hold step in the process or the final storage of purified bulk drug. Such an approach is very similar to that taken for insulin, reiterating the possibility of employing freeze-drying/lyophilization as a final storage or hold step for crystallized bulk biopharmaceuticals.

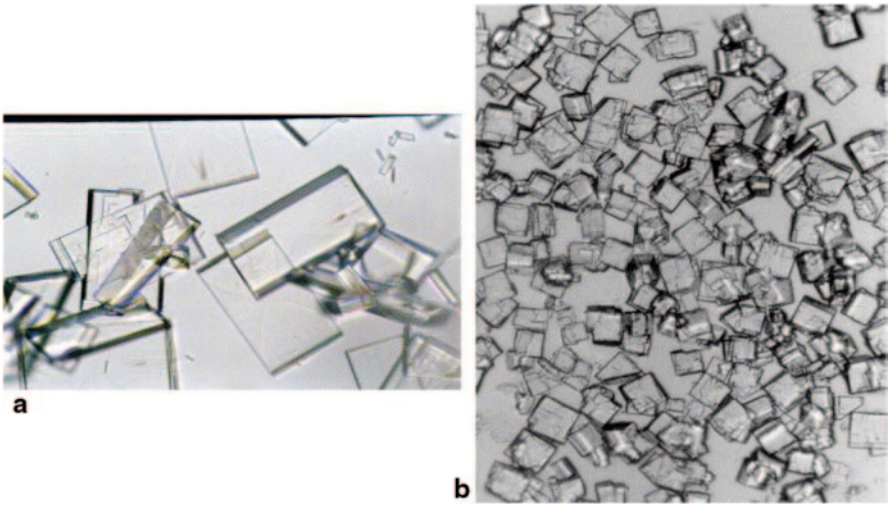

Fig. 4 Photograph of crystals of the aprotinin variant. **a** Conditions: 5 mg/mL protein, pH 4.9, 50 mM NaCl, *T*: 20 °C, 300 µL microbatch. Approximate size of the average crystal: 10–20 µm length, 7–14 µm width. **b** Conditions: 2.5 mg/mL protein, pH 5.0, 13 % ethanol, and 5 mM magnesium sulfate, *T*: 20 °C, 300 µL microbatch. Approximate size of the average crystal: 3–5 µm length, 2–5 µm width (Adapted with permission from [36])

Ovalbumin

Ovalbumin, a *protein* found in *egg white*, has been studied extensively for its structural properties [24]. Judge et al. demonstrate the feasibility of using bulk crystallization for the recovery and purification of ovalbumin. Ovalbumin crystals were obtained in a batch crystallization mode using ammonium sulfate. Factors influencing crystallization such as protein concentration (supersaturation, metastable region) and pH were extensively studied and evaluated. Crystallization was also performed in the presence of related proteins conalbumin (80,000 Da) and lysozyme (14, 600 Da), the obtained crystals were further washed, and protein purity determined to be >99%. These findings suggested that bulk crystallization could be applied as a purification step for bulk processing of ovalbumin; additionally the presence of impurities (related proteins) did not adversely affect crystallization as a purification step [28, 31]. Carbone et al. describe the use of a desupersaturation curve-based method to understand seeded isothermal batch crystallization of ovalbumin. Since the method utilized inexpensive instrumentation, it offers a simple and cost-effective approach for analysis and large-scale protein crystallization [10].

Lysozyme

Similar to ovalbumin, bulk crystallization has also been successfully attempted for lysozyme. As described earlier, Judge et al. discuss the effect of protein impurities on lysozyme crystal growth [29]. The findings suggest that presence of related protein impurities (avidin, ovalbumin, and conalbumin) at concentrations as high as 50% had little effect on solubility and crystal purity but the effect on face growth rates varied and was impurity specific. The effect of process parameters such as temperature and solution pH on the nucleation of lysozyme crystals has also been evaluated [30]. Hekmat et al. describe a systematic approach for the scale-up of crystallization of lysozyme from vapor diffusion-based experiments (20-µl sitting-drop volume) to batch crystallization in agitated milliliter scale vessels [22]. Quantitative phase diagrams were generated for both the 20-µl sitting-drop vapor diffusion condition and the 200-µl microtiter plates. Phase diagrams suggested that rising agitation rates, resulted in a significant reduction of the area of nucleation zone. Batch crystallization of lysozyme was performed under different conditions including 0.2–2 ml Eppendorf tubes in a laboratory rotator, an unbaffled shake flask (5-ml volume) and stirred baffled and unbaffled vessels (4-ml volume). The size of the crystals obtained in the rotated Eppendorf tubes and the stirred vessels was smaller. Large crystal aggregates were formed in the shake flask experiments, which were otherwise unsuitable for scale-up and robust process development. The use of ethanolammonium formate, a biocompatible ionic liquid acting as a crystallization aid, resulted in larger crystals in unbaffled stirred tanks [23]. Based on the crystal properties, it was concluded that ml-scale batch crystallization of lysozyme in stirred

vessels was fast, scalable, and reproducible. Hebel et al. describe the scale-up of protein crystallization in stirred crystallizers from a 5-ml scale onto geometrically similar stirred crystallizers at 100 mL and 1 L scale (stirred crystallizers as depicted in Fig. 4), using lysozyme and lipase as model proteins [21].

L-Methionine γ-Lyase

Takakura et al. describe industrial scale purification and bulk scale crystallization of L-methionine γ-lyase (METase) a pyridoxal 5′-phosphate-dependent enzyme with selective antitumor activity [46]. Bulk crystallization was employed as an initial capture and purification step for processing the crude enzyme preparation. Rod-shaped crystals (with a crystallization yield of 87%) were obtained in the presence of 9.0% polyethylene glycol 6000, 3.6% ammonium sulfate, and 0.18 M sodium chloride using a 100-L crystallizer (Fig. 5a). Repeated second and third crystallizations in the presence of polyethylene glycol 6000 and sodium chloride resulted in octahedral crystals (Fig. 5b).

The individual effects of PEG 6000, ammonium sulfate, and sodium chloride concentrations on the crystal purity and crystallization yield were evaluated on a small scale prior to bulk crystallization (Fig. 6). The crystal purity was assessed by measuring specific activity (U/mg) for the enzyme. As described in Fig. 6, each of the three components in crystallization affected the crystal purity and yield, the final composition of the crystallization recipe was optimized based on these results from

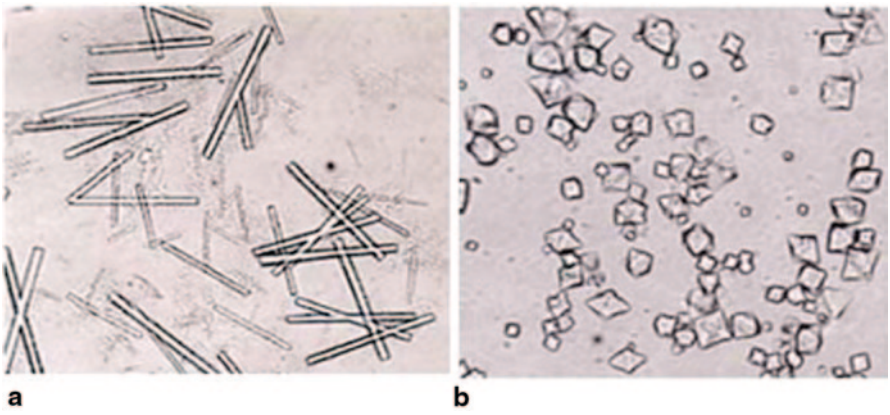

Fig. 5 Morphology of rod-shaped crystals (**a**) and octahedral-shaped crystals (**b**) of recombinant METase. **a** Rod-shaped crystals were obtained from the first crystallization in the presence of poly ethylene glycol (PEG) 6000, ammonium sulfate, and sodium chloride as precipitants. **b** Octahedral-shaped crystals were obtained from the second and third crystallizations in the presence of PEG 6000 and sodium chloride as precipitants. The bar shows 50 μm (Adapted with permission from [46])

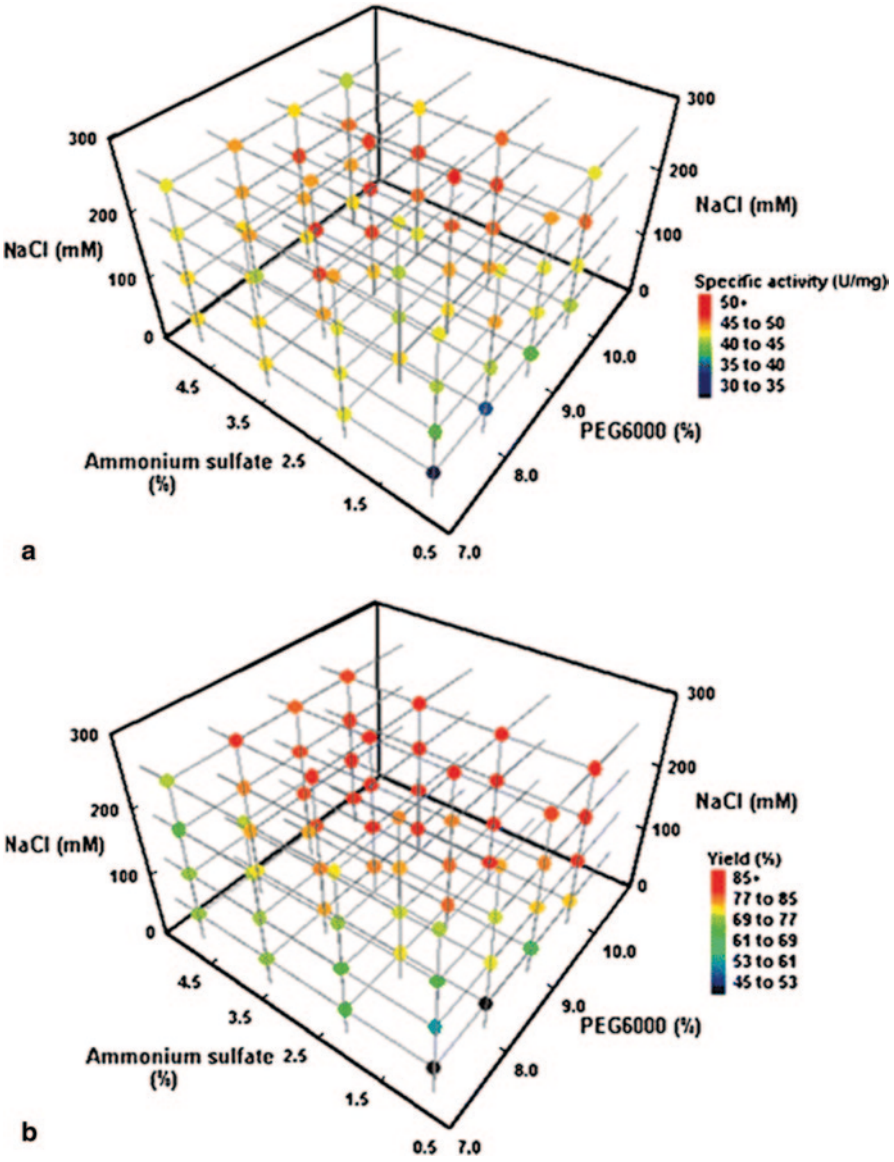

Fig. 6 Effect of PEG 6000, ammonium sulfate, and sodium chloride concentrations on crystal purity (**a**) and crystallization yield (**b**). Purity was represented as specific activity, and yield was calculated from the recovery of enzyme activity after crystallization. Crystallization was performed at concentrations of 7.0–10.0% PEG 6000, 1.0–5.0% ammonium sulfate, and 50–250 mM sodium chloride, respectively. *NaCl* sodium chloride (Adapted with permission from [46])

Table 2 Purification of recombinant METase from *Escherichia coli* JM109 harboring the plasmid pMGL-Trc03. (Adapted with permission from [46])

Purification step	Number of batch	Total activity (mU)	Total protein (g)	Specific activity (U/mg)	Yield (%)	Endotoxin (EU/mg)
Cultivation	2	83.3	3440	24	100	ND
Debris removal	2	81.2	2680	30	97	2×10^6
First crystallization	2	70.8	1320	54	85	6×10^4
Second crystallization	2	64.2	1170	55	77	1×10^4
Third crystallization	2	61.4	1100	56	74	8×10^3
DEAE-Sepharose FF	1	40.8	720	57	49	2
Sephacryl S-200 HR	3	34.1	600	57	41	8×10^{-1}

ND not determined, *DEAE* diethylaminoethanol, *FF* fast flow, *EU* endotoxin unit

small-scale experiments. Finally, the enzyme was purified with anion-exchange chromatography and polished with a gel filtration step to achieve sufficiently high-purity enzyme with significantly reduced endotoxin content for clinical applications (Table 2).

Crystalline Monoclonal Antibodies

Crystallization of mAbs or crystalline mAbs find applications particularly in three areas of structural biology, purification and process development, and high concentration or sustained-release mAb formulations.

Drug Discovery and Structural Biology Fab fragments and nanobodies have been used as chaperons in biology efforts to characterize difficult-to-crystallize targets such as membrane proteins. Bukowska et al. review new concepts and aids to facilitate protein crystallization, wherein the application of mAbs, mAb fragments, and camelid single-chain nanobodies as crystallization aids has been described [9].

mAb Crystallization: Purification Process Development Crystallization of proteins from clarified fermentation broth has been explored for enzymes such as lipase [27]. Such an approach has also been considered for complex biomolecules such as mAbs. Zang et al. describe the utilization of protein crystallization as a downstream processing step for a therapeutic antibody namely immunoglobulin G4 (IgG4) [50]. The purity of the crystalline mAb was found to be about 90%, though the crystallization yields were unsatisfactory. Bean and Matthews describe a similar crystallization-based approach for therapeutic proteins and antibodies for purification and

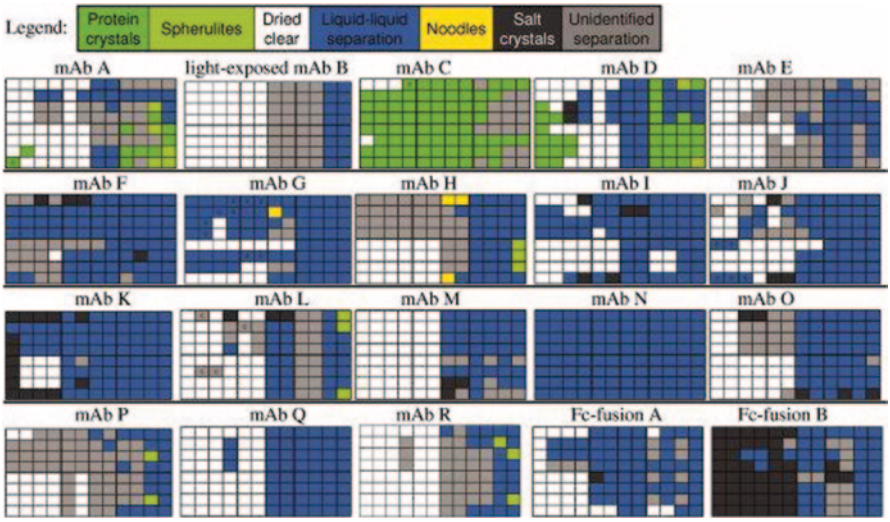

Fig. 7 Evaporative screen results using conditions in the study. Letter C in some wells stands for "clear" and indicates that, although there was phase separation at some point during drying, only a single phase was observed toward the end. *mAb* monoclonal antibodies (Refer to the study for details on screening conditions. Adapted with permission from [47])

bulk storage applications [5]. Crystallization parameters such as crystal morphology, crystal size distribution, and yield were optimized at laboratory scale by varying the salt concentration, pH, temperature and anti-solvents. Finally, the developed crystallization method was implemented into the manufacturing process.

Trilisky et al. examined the crystallization and liquid–liquid phase separation (LLPS) behavior of a set of 20 monoclonal antibodies and 2 Fc-fusion proteins, employing both vapor diffusion and evaporative screening-based methods [47]. Screening was performed using a set of about 100 conditions using reagents that are generally regarded as safe (GRAS), thus allowing the possibility of employing the crystallization condition as a purification step in process development and also as a protein formulation tool. While a small fraction of the tested proteins, could be crystallized in the preliminary screening, four of the IgG2s produced diffraction-quality crystals and three of these IgG2s could be crystallized with inexpensive GRAS reagents. In general, all the tested proteins exhibited LLPS which can be implemented as a concentration or purification step. Figure 7 describes the results of evaporative screen for the 20 tested proteins, depicting different morphologies (crystals, LLPS, spherulites, etc.) formed under different crystallization conditions.

Hebel et al. describe a stirred batch crystallization-based approach for obtaining crystals of a therapeutic antibody fragment FabC225 [20]. Vapor diffusion-based crystallization conditions already identified during the structure determination of FabC225 served as a starting point for development and optimization of the microbatch crystallization process at 10 µl scale. Figure 8 describes the crystals for FabC225 under microbatch conditions with varying concentrations of protein and ammonium sulfate. Figure 9 describes a phase diagram obtained for FabC225 from

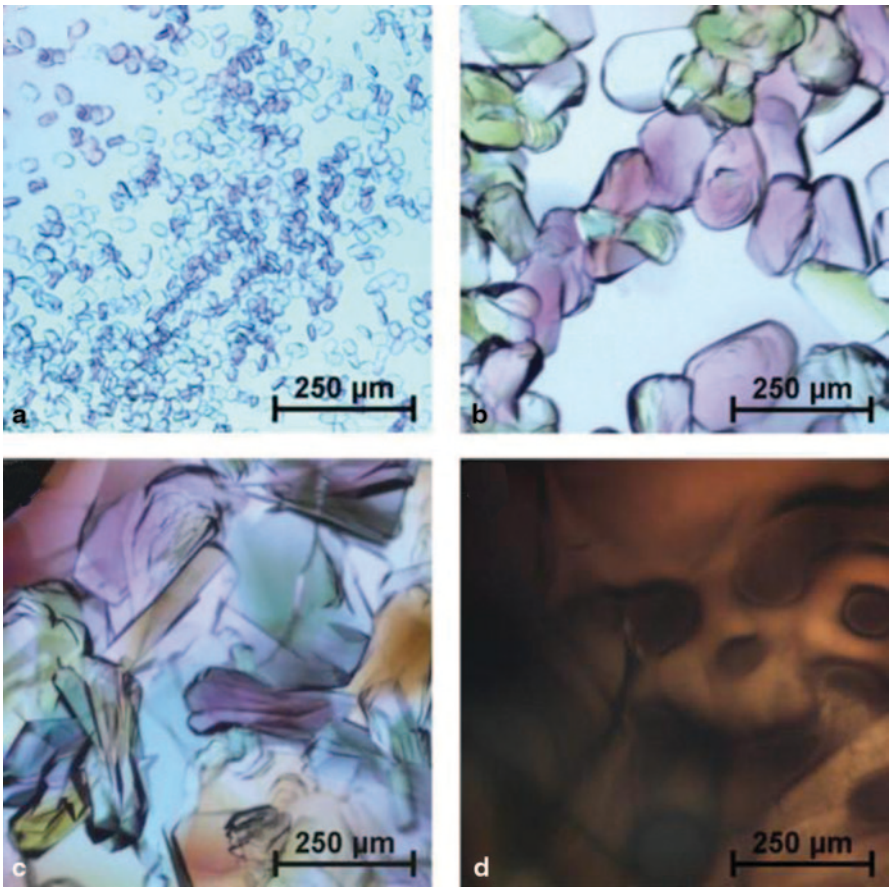

Fig. 8 10 µL-scale microbatch crystallization of FabC225 at 20 °C in 40 mM sodium citrate buffer at pH 6.25. **b** 73.4 g L^{-1} protein, 0.8 M $(NH_4)_2SO_4$, **b** 18.3 g L^{-1} protein, 1.4 M $(NH_4)_2SO_4$, **c** 27.5 g L^{-1} protein, 1.2 M $(NH_4)_2SO_4$, and **d** 91.8 g L^{-1} protein, 1.7 M $(NH_4)_2SO_4$ (Adapted with permission from [19])

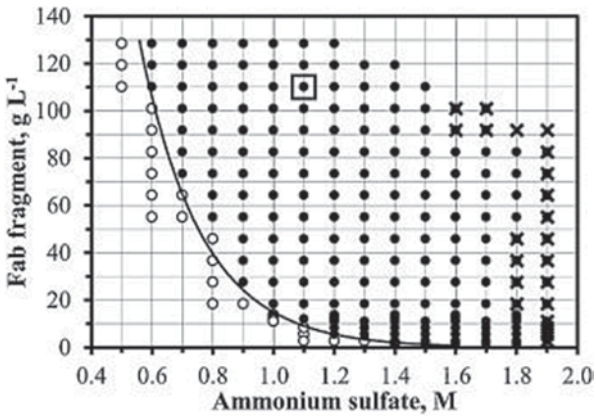

Fig. 9 Phase diagram for FabC225 from microbatch experiments at 20 °C with 40 mM sodium citrate buffer at pH 6.25. *Solid circles*: crystal growth; *crosses*: precipitate formation; *open circles*: no crystallization or precipitation. The *square* denotes the starting condition for both of the subsequent 5- and 100-mL experiments. *Solid line*: measured solubility data of FabC225 (Adapted with permission from [20])

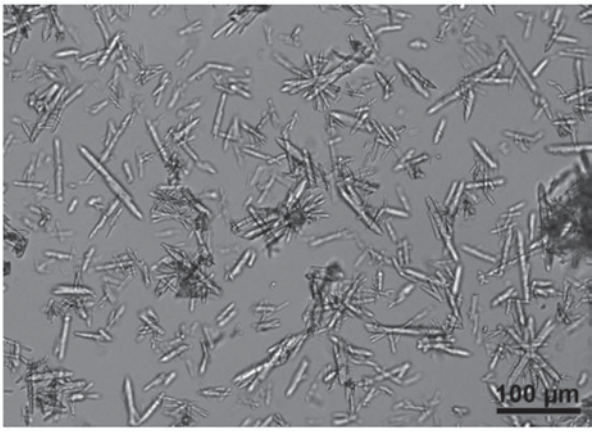

Fig. 10 Stirred-batch crystallization of mAb01 from pretreated harvest on the 1 L-scale (Experiment A). 2.2 g L^{-1} mAb01 in pretreated harvest; 2% w/v PEG 10000 added; TRIS base added to adjust the pH to 6.8. Microphotograph was taken after crystallization overnight at 10 °C. Robust rod-like crystals (the dark spots represent crystal agglomerates containing native antibody). *mAb* monoclonal antibodies, *w/v* weight/volume (Adapted with permission from [40])

similar microbatch experiments. The microbatch crystallization process was successfully scaled to 5 ml and ultimately to 100 ml in a stirred tank crystallizer (as described in previous chapter). Scale-up resulted in reproducible crystal morphologies, similar crystallization kinetics, and comparable crystallization yields.

Smejkal et al. describe a similar fast and scalable crystallization-based purification for a therapeutic IgG1 antibody [40]. While the purified IgG1 could be crystallized easily in microbatch experiments, the crystallization of IgG1 from clarified Chinese hamster ovary (CHO) cell culture harvest required a simple pretreatment. Stirred-batch crystallization of both the purified mAb01 and the pretreated harvest was scaled at 6-mL scale and 1-L scale, using stirred-batch crystallizers (as described in previous chapter). Figure 10 describes crystallization of mAb01 from pretreated harvest culture in stirred-batch crystallizer at 1 L scale.

The developed crystallization method resulted in high crystallization yield, high purity, and significant reduction in related impurities such as host cell protein and host cell DNA. The crystals were easily solubilized and recrystallizable without significantly affecting the biological activity of the therapeutic antibody. Based on the findings, the authors suggested that batch rystallization method could potentially replace protein A chromatography, a generally employed capture step in antibody purification.

Crystalline mAbs: High Concentration and Sustained Release Formulations As described previously, development of high-concentration mAb formulation remains a significantly challenging task due to high viscosity and related colloidal instability. Crystalline antibody suspensions are proposed as a drug-delivery alternative to high-viscosity solutions [4]. Besides reducing the viscosity, crystalline suspensions also offer the unique advantage of sustained release drug delivery. Yang et al. describe a successful application of small-volume, high-concentration crystalline antibody suspensions for subcutaneous delivery employing three commercial therapeutic antibodies rituximab, trastuzumab, and infliximab as examples [49]. The

crystallization conditions resulted in high yields, no detectable modifications in the protein properties, and complete retention of biological activity. The crystalline suspensions had significantly reduced viscosity, compared to similar concentration solutions. The crystalline suspension also demonstrated longer pharmacokinetic profile (sustained release) during in vivo evaluation. Using glucose oxidase and lipase as model proteins, Shenoy et al. describe the preparation of crystalline formulations [39]. Analytical characterization (using FTIR spectroscopy and size-exclusion chromatography) of the crystalline suspension suggested that such an approach generates more stable protein formulations.

Conclusions and Future Trends

Based on the examples discussed herein, undoubtedly crystallization and freeze-drying find applications in different stages of biopharmaceutical development such as, (i) capture or purification (e.g., enzymes, mAb, antibody fragments), (ii) polishing and finishing step for purified biologic (e.g., insulin, aprotinin), and (iii) high-concentration formulation development (e.g., high-concentration antibody formulation). Some of the interesting techniques such as developing high-concentration crystalline mAb formulations and the capture (purification) of antibodies by crystallization instead of a packed bed chromatography step, will definitely have widespread applications in the near future.

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Part IV
Regulatory, Packaging and Technology Transfer Considerations

Lyophilization of Biologics: An FDA Perspective

David Awotwe-Otoo and Mansoor A. Khan

The views expressed in this chapter are those of the authors and do not necessarily represent the position of the Agency.

Introduction

In an effort to facilitate the adoption of modern quality techniques to ensure a coordinated operation of the regulatory review, compliance, and inspection policies, the Food and Drug Administration (FDA) introduced and published "Pharmaceutical cGMP for the 21st Century: A Risk-based Approach" and a pertaining "Guidance for Industry: Process Analytical Technology—A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance," in 2004 [1]. The introduction of this white paper signaled a paradigm shift from the empirical, data-driven submission to a more knowledge-based submission, where the focus is on sound science and engineering principles for assessing and mitigating risks of poor product and process quality.

Publications from the Center for Drug Evaluation and Research (CDER), FDA, indicate that the ultimate goal of this paradigm shift is to reach a desired state which is envisioned as "a maximally efficient, agile, flexible pharmaceutical manufacturing sector that reliably produces high-quality drug products without extensive oversight" [2]. These current good manufacturing practice (cGMP) initiatives have resulted in the "roll out" of three harmonized guidance, namely, Q8, Q9, and Q10,

M. A. Khan (✉) · D. Awotwe-Otoo
Division of Product Quality Research, Office of Testing and Research, Office of Pharmaceutical Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993, USA
e-mail: Mansoor.Khan@fda.hhs.gov

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which fully integrate the principles of quality risk management (QRM), process analytical technology (PAT), and quality systems (QS) [3–5], and also provide a model for drug product submissions, while assisting both industry and the FDA in a move toward a more scientific, risk-based, holistic, and proactive approach to pharmaceutical development [6].

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" [3]. The ultimate goal of QbD is that product and process characteristics should be scientifically designed to meet specific objectives and not empirically derived from performance of test batches, and good product quality should represent an acceptably low risk of failing to achieve the desired clinical attributes [7]. This requires several key elements such as quality target product profile (QTPP), prior scientific knowledge, product/process development, product/process design space and control strategy to be defined and incorporated into the studies and included in the dossier [3–5].

Implementation of QbD has started in all three review programs in the Office of Pharmaceutical Sciences (OPS), namely; Office of Generic Drugs (OGD), Office of New Drug Quality Assessment (ONDQA), and the Office of Biotechnology Products (OBP), at different paces due to the differences in the complexity of products and review practices.

ONDQA has been the forerunner in implementing QbD with a Chemistry, Manufacturing, and Controls (CMC) pilot program which emphasized the use of QbD concepts in the evaluation of critical aspects of pharmaceutical quality, with a strong focus on manufacturing science, integration of review and inspection functions, and use of modern statistical methodologies [8]. OGD started its implementation with a question-based review (QbR) for quality evaluation of generic-drug applications. QbR involves standardized scientific and regulatory review questions whose answers by sponsors helped the reviewer determine the quality of the product and level of risk associated with the manufacture and design of the particular product. This has enhanced the quality of CMC reviews by significantly reducing CMC supplements and subsequently the review time [8, 9]. Full implementation of QbD by OGD started in January 2013 [10].

In 2008, the OBP launched a pilot program entitled "Notice of Pilot Program for Submission of Quality Information for Biotechnology Products in the Office of Biotechnology Products." The goals of this pilot program include (i) obtaining more information to facilitate agency review of QbD risk-based approaches for the manufacture of complex molecules, (ii) define clinically relevant attributes for complex products and linking them to the manufacturing process, and (iii) determine if QbD is appropriate for an entire original application as well as unit operations in supplements [11]. The pilot program has provided an opportunity for the biopharmaceutical industry and the FDA to evaluate and identify best practices for key QbD elements of target product profiles, critical quality attributes (CQA), risk assessment, process characterization for design–space definition, CQA-focused control strategies, and expanded change protocols. Many of the elements of QbD are already embedded in OBP's regulatory review process, with full implementa-

tion slowed by the functional and structural complexity of biotechnology products, their manufacturing processes, as well as difficulty in evaluating the impact of each process parameter on each quality attribute and impact of every quality attribute on safety and efficacy.

Lyophilization process spans other FDA centers too, besides CDER. As an example, some vaccines are also lyophilized for stability considerations, where the sponsors submit applications to the Center for Biologics Evaluation and Research (CBER).

General Steps for Implementing QbD in the Lyophilization of Biologics

The process of lyophilization has become an important part of the biopharmaceutical industry as more biological entities are being explored as therapeutic agents. For such biologics, whose formulation and storage in aqueous solution result in instability, lyophilization, or freeze-drying, represents the most common and important formulation strategy to improving their long-term stability. Currently, roughly 50% of marketed therapeutic protein products are lyophilized [12]. While simple in concept, lyophilization is a complicated process that can result in changes in the structure of the biologics due to several compositional and process variables. A typical lyophilization process is a complex, multistep process which starts with the freezing step, where the aqueous formulation in vials is cooled below its thermodynamic freezing temperature, and the solutes are crystallized or transformed into a solid amorphous system. This is followed by the primary drying step, where the ice crystals are removed by sublimation under vacuum and increased shelf temperature and the secondary drying step, where most of the unfrozen water still absorbed in the interstitial region is removed by desorption at elevated shelf temperatures and low-chamber pressure to allow the desired low moisture content to be achieved [13, 14].

While the scope and philosophy of QbD are well established for small-molecule pharmaceuticals, its interpretation and application in the lyophilization of biologics involves some nuances and complexities due to the unique, sensitive, and higher-level structures of biologics such as proteins, as well as challenges associated with their development and manufacturing [15]. For example, whereas in most cases for small molecules, the term “quality” is defined as “the suitability of either a drug substance or drug product for its intended use, including attributes such as identity, strength and purity” [3], the term “quality” for lyophilized proteins, is, in the first instance, attributed to other physical characteristics such as cake elegance and residual moisture content, rather than the stability of the active pharmaceutical ingredient (API). As a result, lyophilized vials with collapsed cakes would be routinely rejected from a batch even though its API may be stable from a pharmaceutical standpoint. Other factors such as scaling-up, transferring technologies and results from laboratory to major production units, and ensuring homogeneity and compliance with key regulations require a sound understanding of both product-

and process-related attributes to ensure effective QbD implementation. By implementing QbD in lyophilized biologics, the goal is to be able to identify and control possible interactions that may arise between formulation and process parameters that may have significant effects on the quality attributes of the final products [16].

The following sections describe the various key steps in implementing QbD for lyophilized biologics. While the implementation of QbD would generally vary with product class and complexity, it nonetheless follows common, iterative processes, as outlined in International Conference on Harmonisation (ICH) guidance: Q8, Q9, and Q10. We also include information on a case study involving the formulation and lyophilization of a model monoclonal antibody (mAb) to illustrate the application of QbD.

Identifying the Quality Target Product Profile

The quality target product profile (QTPP) forms the basis of design for the development of the final product. It is defined as "a prospective summary of the quality characteristics of a drug product that will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product" [3]. Generally, a drug's QTPP is usually established as soon as the drug is identified as a viable candidate for commercialization, and this serves as a template where the sponsor lists drug-labeling concepts and documents the intended studies to support those concepts. It comprises several key sections such as indications and usage, dosage and administration, contraindications, warnings and precautions, adverse reactions, drug interactions, clinical pharmacology, use in specific populations, nonclinical toxicology, clinical studies, and use in specific populations [18]. For a lyophilized biologic which belongs to the class of mAb, the QTPP should generally include the dosage form, appearance, mode of administration, concentration or content per vial, reconstitution media, reconstitution volume, mode of reconstitution, reconstitution time, post-constitution storage and stability, and drug product quality criteria (e.g., sterility and purity) [19] (Table 1).

Identification of the QTTP provides the necessary information for a constructive communication between the sponsor and the FDA reviewer since it outlines the risks involved with the proposed labeling and also potentially minimizes the risk of late-stage development failures, ensures the safety and efficacy data availability in a timely manner, and improves labeling content and consequently reduces the total time involved with drug development [7, 18].

Identifying Critical Quality Attributes

Once QTTP has been identified, the next step is to identify the relevant CQAs which ensure the desired final product quality. CQA is defined as "a physical, chemical, biological or microbiological property or characteristic that should be within an

Table 1 QTPP for a lyophilized model monoclonal antibody formulation

Dosage form	White to off-white pharmaceutically elegant lyophilized cake that can be reconstituted for IV infusion
Administration	IV infusion (slow)
Infusion volume (deliverable)	5 mL in 100 mL of normal saline
Strength	100 mg/vial
Concentration after reconstitution	20 mg/mL
Reconstitution time	≤5 min
Reconstitution	Reconstitute with SWFI to provide clear solution free from foreign particulate matter
Identity	Positive for protein assay
Isotonicity	Isotonic with plasma
Aggregation	Within acceptable levels (<2%)
Stability	High thermal transition temperature (>100 °C)
Container/closure	20 mL type 1 tubing glass vial with 20 mm closure and crimp seal
Process performance	Efficient process with consistent yield

QTPP quality target product profile, SWFI sterile water for injection, *IV* intravenous

appropriate limit, range or distribution to ensure the desired product quality" [3]. Identification of CQAs is usually done through risk assessment, governed by ICH Q9 [4]. Prior product knowledge through past experience with similar biological molecules and accumulated preliminary laboratory experimental data could be used to create a list of CQAs that relates to or affect the formulation of QTPPs [7]. For lyophilized proteins, which belong to the class of monoclonal antibodies, a typical list of CQAs would comprise the freeze-drying properties such as collapse temperature (T_g' for an amorphous product or T_{eu} for a crystalline product), final cake appearance (color, density, uniformity, absence of melt back, shrinkage, and/or collapse), solution appearance upon reconstitution (completeness, clarity, color), reconstitution time, residual moisture content, protein purity (chemical changes, presence of aggregates, visible and subvisible particles), and potency [19]. These attributes could be further updated and refined in the course of product development based on new findings and/or results obtained during product characterization. A similar list of parameters consisting of formulation components such as excipients, buffer components, surfactant, protein concentration, pH, ionic strength, vial configuration, stoppers and fill volumes; freeze-drying process operating parameters such as freezing ramp rate, hold time, primary drying conditions of shelf temperature, chamber pressure and duration, secondary drying conditions (shelf temperature, pressure, and duration), and equipment conditions may include the batch size/load and scale effects, is created, and the effects of these parameters on CQAs are studied.

Performing Risk Assessment: The Role of QRM in the Lyophilization of Biological

One of the key elements of QbD is the identification of potential risks during early stages of product and process development. Risk is defined as the combination of the probability of occurrence of harm and the severity of that harm. Risk assessment is a valuable science-based process which is used in QRM. QRM is a systematic process for the assessment, control, communication, and review of risks to the quality of the drug product across the product life cycle [4]. It consists of three major steps namely risk assessment, risk control, and risk review (Fig. 1)

Risk assessment provides a mechanism by which process parameters (inputs) are studied against quality and process attributes (outputs) in order to prioritize parameters for experimental studies. It provides an understanding of relationships that exist among formulation parameters, process inputs/material attributes, and product quality attributes and enables the identification of robust process conditions and their acceptable limits. Effective QRM can facilitate better and more informed

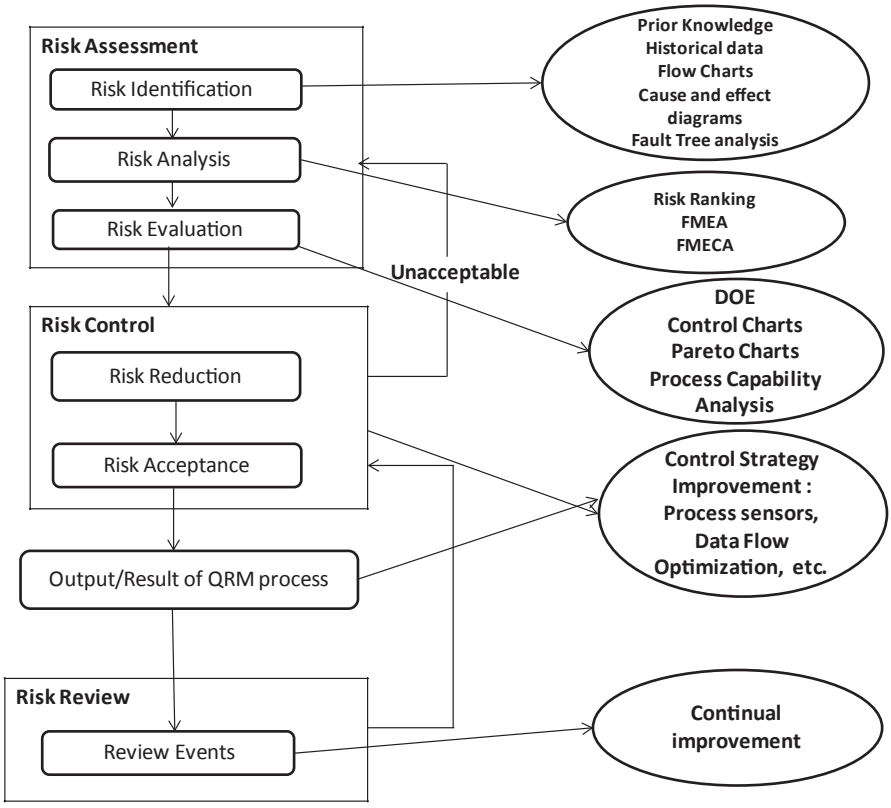

Fig. 1 Quality risk management model for the lyophilization of biologics (Modified from [4])

decisions, provide regulators with greater assurance of a company's ability to deal with potential risks and consequently affect the extent and level of direct regulatory oversight.

Risk assessment begins with the systematic use of information such as prior knowledge, historical data, theoretical analyses, and information available in literature to create a list of product CQAs that affect the QTTP [4, 7, 8]. Once these factors are listed, various methods are applied to analyze and understand the relationships between the process parameters/material attributes and product quality and process attributes in order to prioritize parameters for further experimental studies. Due to resource constraints, it is practically impossible to study the effect of all the listed parameters on CQAs using a systematic set of experiments. Practical ways of analyzing these factors include the use of risk analysis tools such as cause and effect (Ishikawa or Fishbone) diagrams, cause and effect matrices, failure mode effects analysis (FMEA), failure mode effects and criticality analysis (FMECA), fault tree analysis (FTA), and risk ranking analysis [4].

In cause and effect matrix, each process parameter is assigned an "impact" score based on the strength of its relationship with the CQA, while each CQA is also assigned a "weight" score based on its potential impact on safety and efficacy. The purpose of this ranking analysis is to establish a functional relationship between the process parameter (input) and the quality attributes (output) using a scoring exercise to identify and rank each process parameter based on its potential impact on quality attributes or process performance attributes. Table 2 shows an example of a cause and effect matrix ranking and weight scores for process parameters and quality attributes identified for the lyophilization of a model mAb formulation.

The objective is to prioritize potentially high-risk parameters for further process characterization. Once each process parameter is assigned a score based on its effect on the quality attribute of the product, the cumulative score is then calculated using the formula;

Table 2 A cause and effect matrix ranking showing the use of weight scores for quality attributes and impact scores for process parameters

Quality attributes			Process parameters
Weight score	Ranking criteria	Impact score	Ranking criteria
10	Expect a direct or very strong impact on safety and efficacy based on prior knowledge or available data	*10*	Expect a strong relationship based on experience, prior knowledge, or available data
7	Expect a moderate or indirect impact on safety and/or efficacy	*7*	Expect a moderate relationship based on experience, prior knowledge, or available data
5	Expect a low on product safety and efficacy	*5*	Expect a low or moderate relationship
1	Expect no impact on product safety and efficacy	*1*	Known to have no relationship based on experience or available data

$$\text{Cumulative Score} = \Sigma(\text{Impact score of process parameter} \times \text{Weight score of quality attribute})$$

The cumulative score for a process parameter gives an indication of the strength of its potential risk to product quality and/or process performance. Table 3 shows an example of a simple cause and effect matrix analysis with cumulative scores for the optimization of the lyophilization of a model mAb after its formulation has been optimized. Parameters with high cumulative scores are selected for further studies using statistical design of experiments (DoE) to determine the degree of impact each parameter has on the CQA.

The first step in the use of DoE is to screen the selected parameters to determine the main effects of such parameters on the CQA. Screening experiments such as Plackett–Burman, fractional factorial, and full-factorial experimental designs could be used to determine the extent of main effect of such parameters on CQAs [19]. This evaluation could be based on statistical significance in the experiments or Pareto ranking analysis and process parameters that are observed to significantly impact CQAs will be categorized as critical process parameters (CPPs), while the other parameters that are not statistically significant but are important for consistency of process performance are categorized as key process parameters (KPP). CPPs are further studied using DoE involving multivariate combinations and interactions with other parameters to define the optimum operating boundaries. A response surface experimental design, such as Box–Behnken design or central composite design could be used to study main and interaction effects of process parameters on CQAs, depending on the number and levels of the factors selected.

After multivariate experimental study, data obtained are analyzed to determine the acceptable ranges for the KPPs and CPPs within which quality is assured. The validity of the operating boundaries of model can then be confirmed with verification experiments at full scale. This helps to identify robust process conditions and their acceptable limits. Risk assessment is a continuous process and is performed at various unit operations during development. As additional process information is obtained over the life cycle of the product, it is possible for the criticality of some attributes or parameters to change. In such instances, there is the need to revisit risk assessment since the changes in the prioritization must be demonstrated by data.

Defining the Design Space

Design space is defined as the "multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality" [3]. The concept of design space is linked to risk assessment and describes the multivariate functional relationship between CQA and CPPs that impact them. Such relationships are arrived at by the iterative application of risk assessment and experimental design as well as mathematical modeling. It is the combination of these procedures that lead to an understanding of the linkage and

Table 3 Cause and effect matrix for the lyophilization process for a model monoclonal antibody formulation

Rank (5, 7,10)	10	10	10	10	7	7	7	7	7	5	
Process parameters	Cake appearance	Aggregation (insoluble)	Aggregation (soluble)	Potency	Clarity	Residual moisture content	Protein conc	Osmo-lality	pH	Recon time	Score
Chamber pressure (PD)	10	10	10	7	10	10	1	1	1	10	581
Shelf temp (PD)	10	10	10	5	10	10	1	5	1	10	589
Freezing ramp rate	1	1	1	1	1	1	1	1	1	1	100
Hold time at freezing temp	1	1	1	1	1	1	1	1	1	1	80
Ramp rate to PD Shelf temp	1	7	5	5	5	5	1	5	1	5	324
PD time	10	7	5	5	10	10	5	1	10	10	572
Shelf temp (SD)	5	10	10	10	7	10	5	5	1	7	581
Nucleation temp	10	7	10	5	5	10	1	1	5	10	524
Ramp rate to SD temp	7	5	7	5	7	7	5	7	5	7	492
Unloading temp	1	1	1	1	1	1	1	1	1	1	80
Ramp rate to unloading temp	1	1	1	1	1	1	1	1	1	1	80

PD primary drying, *SD* secondary drying

effect of process parameters and material attributes on product CQAs and also help identify boundaries within which consistently acceptable products are produced.

The design space defines the acceptable processing conditions that have been shown to provide repeatable, consistent, and provable assurance of product quality (with underlying assumption of patient safety and efficacy) [8]. For lyophilized biologics, the establishment of the design space involves a thorough understanding of the criticality of the components of the formulation as well as the selection of the appropriate ranges of the process parameters for lyophilization [7]. During the lyophilization of biologics, product temperature at the sublimation interface is the most important factor which affects final product quality attributes such as cake elegance and residual moisture content. However, the product temperature at the sublimation interface cannot be directly controlled and is influenced by other processing factors such as shelf temperature and the chamber pressure [20]. Keeping the product temperature below the "critical" temperature of the formulation (the collapse temperature (T_c) or glass transition temperature (T_g') for amorphous and eutectic temperature (T_{eu}) for crystalline products) would avoid macroscopic collapse of the lyophilized product [14].

For a typical lyophilization cycle, it has been shown that the chamber pressure and shelf temperature have a complex effect on product temperature and sublimation rate of the product. Ideally, increasing the chamber pressure increases the rate of heat transfer to support the sublimation rate by increasing the thermal conductance of gas between the shelf surface and the product vial. However, this has the tendency of causing an increase in the product temperature above the critical collapse temperature of the product, resulting in macroscopic collapse. Macroscopic collapse is a characteristic of predominantly amorphous formulations, and it is undesirable and could lead to rejection of an entire batch. Therefore, there is the need to establish limits of chamber pressure, shelf temperature, and product temperature to ensure that a pharmaceutically elegant product is produced. A very efficient lyophilization process is one which operates within the design space at the highest allowable shelf temperature and the lowest chamber pressure that would still maintain the target product temperature during primary drying [21].

Once the design space has been initially established, there is the need to expand the primary design space beyond the initially tested experimental conditions. This is particularly important during the development phase when the optimized lyophilization recipe has to be transferred to pilot and production scale freeze-dryers. Numerous factors such as freeze-dryer design (such as shelf area and spool piece construction) and environmental factors such as particulate content could influence product performance during method transfer. These factors can cause the process to undergo deviation outside the design space such as a spike in chamber pressure leading to choked flow. Equipment limitations may also result in deviations from the established primary design space. This condition may occur when there are differences in refrigeration capacity as well as heat transfer system due to differences in the lyophilizer size [21, 22]. Under such circumstances, a combination of mechanistic and mathematical modeling represents a powerful guiding tool for scaling up or method transfer from laboratory scale through pilot scale to manufacturing scale [23].

The design space is proposed by an applicant and is subject to regulatory assessment and approval [3]. It allows more effective dialogue between industry and the regulatory agency during the application review process, in that it provides structured information about product and process development that is well aligned with risk- and science-based approach. From a regulatory standpoint, working within the design space is not considered a reportable change. However, working out of the design space is considered a change which requires a regulatory post-approval change process.

Defining Process Control Strategy

The FDA considers a process as well understood when all critical sources of variability are identified, explained, managed, and product quality attribute can be accurately and reliably predicted within the design space [1]. Once a sufficient level of process understanding has been achieved, a control strategy should be developed to ensure that the process remains in control within the normal variation in material attributes and process operating ranges. Control strategy is defined as "a planned set of controls, derived from current product and process understanding that assures process performance and product quality" [5]. Process control strategy is a cornerstone of a modern pharmaceutical quality system and can be a combination of parametric- and attribute-based controls. It is established via risk assessment and includes material controls (qualification and specifications of raw materials, excipients, drug active, packaging material, etc.), procedural controls (equipment, facility, quality system, etc.), process monitoring and controls (CPP and KPP), in-process controls, lot release testing, characterization testing, comparability testing, and stability testing [7, 8]. Generally, real-time monitoring and control of the process is preferred over relying on end-product testing. Real-time monitoring and control of CPPs integrate a broad spectrum of analytical technologies or tools that are capable of providing in-line and at-line measurements. Such analytical tools are usually interfaced to production plant control networks and assimilated into standard procedures to ensure consistency to the process through improved quality, efficiency through reduction of cycle, and prevent batch product rejection.

Application of PAT in the Lyophilization of Biologics

Since lyophilization is a complex, time-consuming, and expensive multistep process during which the starting material (solution) undergoes several transformations to form a solid amorphous product (dry cake) using principles of heat and mass transfer, it is imperative that the steps involved are effectively monitored and controlled so that any sources of variability are identified and controlled to improve process efficiency and guarantee final product quality [23]. The application of pro-

cess analytical technology (PAT) in the lyophilization of biologics ensures a noninvasive, risk-based approach to continuous monitoring and control of critical steps of the process, through timely in-line and real-time measurements of CPPs to improve manufacturing efficiency and ensure predefined product quality. PAT is defined as a system for designing, analyzing, and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality. PAT tools specified by the FDA include multivariate data acquisition and analysis tools such as statistical DoE and chemometric tools used to identify and address interactions of product and process variables; process analyzers capable of in-line, at-line, and on-line measurement and control of CPPs; process and end-point monitoring and control tools to monitor the end point of all intermediate process steps before the next process step is initiated; and continuous improvement and management tools [1].

PAT Tools for Characterization of the Bulk Solution

While the characterization of the bulk solution is not considered as part of the freeze-drying process, it is, however, mandatory since it gives very important information that are critical to the design of an optimum lyophilization cycle for a biologic. In the design of a lyophilization cycle, it is important to know the critical properties of the formulation such as the macroscopic T_c, the stability of the drug, and the properties of the excipients used. The T_c is the most critical lyophilization parameter and is the temperature above which the freeze-dried product loses macroscopic structure and collapses during lyophilization. Generally, freeze-drying below T_c is necessary to ensure elegant appearance, low residual moisture content, good storage stability, and reconstitution characteristics. Therefore, accurate measure of T_c is critical to lyophilization process development [14]. Traditional lyophilization cycle design has been driven by the determination of the T_g' of the maximally freeze-concentrated amorphous solution or the T_{eu} if solutes are crystallized in the frozen solution using differential scanning calorimetry. The T_g' is usually 2 °C below the T_c and is used as a guideline for setting primary drying temperatures so that the product temperature at the sublimation interface is close to but not above the T_c of the formulation. However, differences of up to 10 °C have been reported [25]. As such, using T_g' to estimate T_c would result in significantly lower primary drying temperatures and unnecessarily longer freeze-drying times.

A novel, efficient, and scalable freeze-drying microscopy technique based on time–domain optical coherence tomography (OCT-FDM) allows for real-time, 3D imaging of the formulation in a vial during lyophilization. This instrument monitors the structural changes in the product and allows full exploration of the product response to temperature changes in the sample vial during the lyophilization. Apart from accurately measuring the collapse temperature of the formulation, it also allows the observation of other features of the lyophilization process such as the ice nucleation and freezing process. This technology is more efficient and has

a higher resolution and accuracy than the light transmission freeze-dry microscopy (LT-FDM) and differential scanning calorimetry (DSC) tools that were originally used [24]. The results of such measurement of the T_g' or T_{eu} determine the allowable upper range for the product temperature at the sublimation interface during the lyophilization cycle and such information is expected to be included in a biologics license application (BLA) or new drug application (NDA) by the FDA.

Freezing Step: Monitoring and Control of the Degree of Supercooling

Originally, monitoring and control of the lyophilization process was primarily focused on the primary drying step, since it is the longest step of the entire process. However, with improved process understanding, the freezing step has also been identified as a particularly important step which must also be monitored and controlled. The degree of supercooling during the freezing step is known to dictate the ice crystal morphology, which in turn affects both the primary drying and secondary drying performance and critical process parameters and overall final product quality such as the physical state of the sample, residual moisture content, and reconstitution time [13–28]. During freezing, the onset of nucleation is a random process which usually occurs below the thermodynamic freezing temperature of the solution. Generally, high degree of supercooling results in the formation of smaller ice crystals, and this impacts the primary drying step by increasing the product resistance to ice sublimation. However, when the ice nucleation is controlled, all the vials nucleate uniformly and larger ice crystals are formed, which also impact the primary drying time as well as the final cake structure [26, 29]. Various techniques have been proposed in the literature to control the ice nucleation of the freezing step such as the ice-fog method, ultrasound-controlled nucleation, and electro-freezing method [12]. However, these methods are invasive and are not applicable at the commercial scale due to sterility issues. PAT tools that allow for noninvasive control of the freezing step include a scalable method, called ContrLyo™ Technology, which works by rapidly pressurizing and depressurizing the chamber using an inert gas such as argon to uniformly trigger the onset of ice nucleation during freezing [27, 32]. This causes the product in the chamber to nucleate simultaneously at a lower degree of supercooling (Fig. 2), leading to the formation of larger ice crystals and larger pores during drying. This results in a reduction in the resistance to mass transfer and decreases the primary drying time significantly. Studies have demonstrated that for every 1 °C increase in the nucleation temperature, primary during can be reduced by as much as 3–4 %. This technology has proved to be successful in pilot operations as well as production freezers [22, 30, 31].

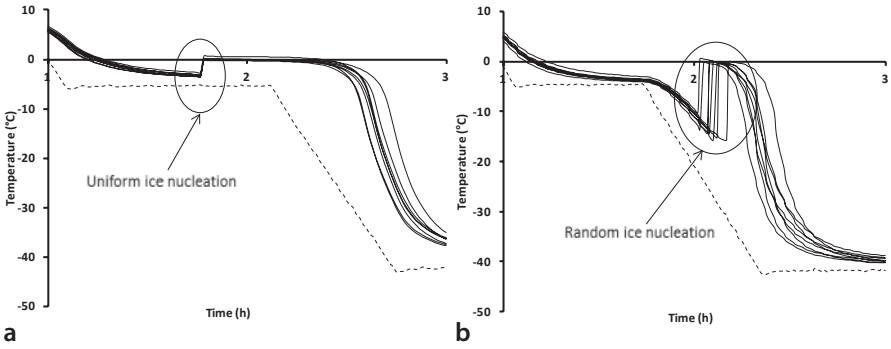

Fig. 2 Product temperature profiles for a laboratory scale lyophilization of a model monoclonal antibody formulation showing the nucleation behavior for **a**. controlled ice nucleation and **b**. uncontrolled ice nucleation. Controlled ice nucleation resulted in uniform nucleation of the formulations, while uncontrolled nucleation resulted in random nucleation

Primary Drying Step: Monitoring and Control of CPPs and End Point of Primary Drying

During primary drying, the accurate measurement of CPPs such as sublimation rate (dm/dt), the product mass transfer resistance, and product temperature at the sublimation interface are critical process analytical needs that must be monitored and controlled since they impact the overall lyophilization process and the final product quality [20]. Traditionally, the mass flow or sublimation rate has been estimated manually using sample thief or the microbalance technique. Various monitoring techniques have been proposed to monitor the product temperature, such as the use of thermocouple sensors and electrical resistance temperature detectors (RTD), which are placed directly inside sample vials. However, such monitors only determine the product temperature at the bottom of the vials and not at the sublimation interface, which is more critically related to product macrocollapse. Moreover, the placement of thermocouples is invasive and the information obtained is not representative of the whole batch. It also compromises the sterility of the product and causes bias in both freezing and drying behaviors relative to vials that do not contain thermocouple. RTD sensors are also not compatible with automatic loading systems during manufacturing scale freeze-drying [4, 32]. Given the sensitivity of process economics and final product quality history with product temperature, there is the need for the real-time monitoring and control of the primary drying step. The introduction of noninvasive methods of monitoring and controlling the primary drying stage include manometric temperature measurements (MTM) and spectroscopic methods such as tunable diode laser absorption spectroscopy (TDLAS) and near-infrared (NIR) and Raman probes.

MTM is a procedure by which the product temperature at the sublimation interface may be measured noninvasively by closing the isolation valve between the

chamber and the condenser for a short time and measuring the pressure versus time data. The MTM equation is then fitted to the pressure rise data, and the analysis provides real-time information about important process parameters such as the product resistance, the cake thickness, and sublimation rate. The data obtained provide information to the lyophilization control software, called SMART™, which makes adjustments to shelf temperature in a closed-feedback loop. By monitoring and controlling product temperature during processing, critically harmful temperatures can be avoided and scientifically derived drying times can be determined. The information obtained is representative of the entire batch and gives valuable insight into the cake morphology and final product quality attributes [30, 31].

TDLAS is also a noninvasive in-line technology that is capable of instantaneous determination of the water-vapor concentration and sublimation rate in the freeze-dryer spool connecting the chamber and the condenser. It works on the spectroscopic principle of measuring the absorption of radiation by water vapor in real time using a laser beam attached to the spool. This information is also used to calculate important processing parameters such as the batch average product temperature, the product resistance, as well as the heat-transfer coefficient. Real-time measurement of sublimation rate by TDLAS can also be used for operational qualification (OQ) testing of lyophilizers, thereby facilitating cycle transfer or scale-up [31, 35].

Real-time measurement of CPPs during primary drying could also by achieved noninvasively using NIR and Raman probes. The location of NIR and Raman probes depends on the type of data needed from such sensors. Raman spectroscopy probes, when placed above vials, offer a noninvasive monitoring of critical process and product aspects during freeze-drying such as water-to-ice conversion, product crystallization, kinetics of polymorphic transitions, solid-state characterization of intermediate and end products [33]. Since water and ice produce strong absorption signals in the NIR region, NIR probes could also be used for in-line and real-time monitoring of the sublimation process during primary drying. The acquired spectra from both Raman and NIR are then subjected to chemometric analysis to build a robust prediction model which can be used to optimize the lyophilization process or determine the residual moisture content in the lyophilized product [34].

Both MTM and TDLAS can also be used to determine the end point of primary. MTM uses a comparative pressure measurement tool which consists of a Pirani gauge and a capacitance manometer. The Pirani gauge works on the principle of measuring the thermal conductivity of the gas in the drying chamber and reads about 60% higher than the capacitance manometer. During primary drying, when all the gas in the chamber is essentially water vapor, the Pirani gauge reads about 1.6% higher than the capacitance manometer (because the thermal conductivity of water vapor is ~1.6 times more than nitrogen gas. At the end of primary drying, when the gas composition in the chamber is essentially nitrogen, the Pirani pressure decreases toward the capacitance manometer and that signals sublimation is essentially completed [32]. TDLAS could also be used to predict the end of primary drying using the water-vapor concentration profile during primary drying. The point where the water vapor starts to sharply decrease indicates that the gas composition is changing and hence sublimation is essentially complete (Fig. 3).

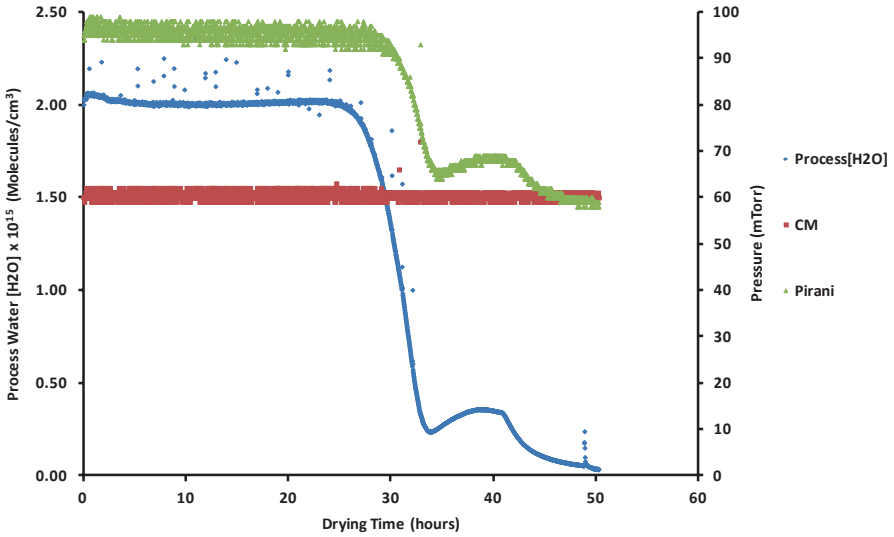

Fig. 3 Process water concentration from TDLAS and Pirani versus Capacitance manometer can be used to determine the end point of primary drying of biologics. In both instances, a sharp decrease in the concentration of water vapor (*depicted by a sharp decrease in the curve*) denotes that sublimation is almost complete. TDLAS tunable diode laser absorption spectroscopy, CM capacitance manometer (Unpublished data)

Secondary Drying Step: Monitoring and Control of the Residual Moisture Content

For an optimized lyophilization cycle, it is very crucial to ensure that the end point of all intermediate process steps is reached before the next process step is initiated since this also affects the final product quality. For example, it is important that the end point of primary drying is accurately determined, when the amount of unfrozen water in the amorphous matrix is typically less than 20% before secondary drying starts. Premature progression into secondary drying could result in product collapse or melt back and ultimately batch rejection due to lack of cake elegance [32, 34]. Moreover, the residual moisture in the final product at the end of secondary drying should be accurately measured since product stability often correlates with residual moisture content. Both MTM and TDLAS have proved vital in characterizing the secondary drying stage of the freeze-drying process. TDLAS has been used for in situ monitoring of residual moisture content in real time during secondary drying on a laboratory scale [36]. Raman and NIR spectroscopy have also been used as rapid and noninvasive techniques to determine the residual moisture content of lyophilized samples during secondary drying.

Characterization of the Lyophilized Cake

While there are not many tools for the real-time characterization of the lyophilized products, many off-line tools allow for both quantitative and qualitative characterization of the lyophilized cakes to ensure that product quality is consistent with the set target. For example, visual inspection for cake elegance, determination of reconstitution time, and off-line determination of residual moisture content using Karl Fischer analyses are standard release tests that are required by the FDA. Other required information that are expected to be included in BLA or NDA include physicochemical characterization information such as the T_g of the dried cake by DSC, which is important in predicting optimum storage conditions, information about the crystallinity of the final product by powder X-ray diffraction (PXRD), the specific surface area of the lyophilized cake by nitrogen or krypton gas adsorption experiments, and secondary structure of the lyophilized biologic by Fourier transform infrared (FT-IR) spectroscopy or circular dichroism (CD) spectroscopy.

The desired improvements from application of PAT are gains in quality, safety and efficiency due to reduced production cycle time, increased automation of the process, and prevention of product rejection due to improved process consistency and understanding [7]. Another major advantage is the possibility for real-time release, which means that the product quality can be ensured by data generated during production, and the batch can be released for sale directly after completion of production without extensive additional final testing procedures.

Summary

One of the key elements of QbD is the development of in-depth process and product understanding and the establishment of a robust manufacturing process. QbD is an umbrella that encompasses overlapping concepts and approaches including QTTP, CQA, risk assessments, design space, PAT, and continuous improvements in the lyophilization of biologics. The application of risk assessment principles in the lyophilization process is very beneficial to guide effective characterization studies needed for lyophilization process optimization. With this approach, it is possible to obtain a lyophilization design space that is relevant for manufacturing.

PAT is an enabling component of QbD with particular emphasis on the creation of a robust control strategy. The continuous real-time monitoring and control of a process would result in more consistent product quality and efficient use of manufacturing capacity. The application of PAT in lyophilization of biologics is likely to facilitate technology or process transfer from laboratory to production and even from one site to another or one manufacturer to another since the process becomes less equipment and recipe dependent and more a function of process outputs.

The application of principles of QbD in the lyophilization of biologics would be mutually beneficial to both regulators and industry since it eventually leads to con-

tinuous process improvements. Further, it enhances in-process controls, provides consistent product quality, higher operational efficiency, and a potential reduction in the volume of data submitted since empirical data is replaced by knowledge-based submissions.

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Recent Trends in Lyophilized Delivery Devices and Packaging

Renaud Janssen

Packaging systems for lyophilized products typically consist of a glass vial and an elastomeric closure as primary packaging components, and an aluminum/plastic flip cap as secondary packaging component. Alternatively, the lyophilization can take place in dual-chamber cartridges where the freeze-dried product is available in one chamber of the cartridge and the diluent in the second chamber.

The first part in this chapter discusses advances in the area of these packaging components and systems.

Elastomeric Closures

Closure Geometry

Lyophilization closures have to be compatible with the freeze-drying process. The first step in the process is the filling of vials with drug solution, followed by partial stoppering of the vials, meaning that the lyophilization closures are only partially inserted in the vial neck (Fig. 1). A major part of the stopper plug is still protruding above the vial neck opening. The partially stoppered vials are then transported into a freeze-drying chamber. There, the aqueous drug solution is first frozen and then the water is removed from the frozen state by sublimation at low temperature and pressure (primary drying). After that the temperature of the vials is increased, leading to the removal of the last parts of water at low pressure and increased temperature, higher than but still fairly close to what is commonly called "room temperature" (secondary drying). Only thereafter, the vials are fully stoppered by the action of

R. Janssen (✉)
Datwyler Pharma Packaging International NV, 3570 Alken, Belgium
e-mail: renaud.janssen@datwyler.com

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Fig. 1 Half-stoppered vial with drug solution (*right*) and totally stoppered with freeze-drying cake (*left*). In front of the vials: freeze-drying closures with their vent openings facing forward

the shelves in the freeze-dryer that come down and push the stoppers on the shelf below entirely into the vial neck. In most cases, the stoppering takes place with a vacuum still being present in the chamber. After bringing it again to atmospheric pressure, the freeze-drying chamber is then unloaded. What comes out are vials under a certain vacuum containing the drug product in the form of a freeze-dried cake and closed with stoppers that are not secured yet with a crimp cap.

During the primary drying phase, when the water in the vial is converted from the frozen state (ice) into the gaseous state (water vapor), the gases are evacuated from the vial via the vent opening of the lyophilization stopper. Since the pressure is so low, the volumes of gas are appreciable, meaning that for a good functioning the vent opening has to be of a certain size. Studies have shown however that the size of the vent opening is not rate determining for the freeze-drying process [1, 2]. The size of the vent opening is far less important than the resistance formed by the already dried product through which additional gases have to make their way to be evacuated from the vial.

As a result of the freeze-drying process and the transport stages of the vials from the filling station to the freeze-dryer in half-stoppered condition and from the freeze-dryer to the capping station in stoppered but still uncapped condition, the design and the dimensioning of lyophilization stoppers have a number of particularities that are not seen with stoppers that are used for a liquid or a dry powder fill.

Flange thickness

The flange of the lyophilization stopper is that part of the stopper that is above the rim of the vial mouth, when the stopper has been firmly brought into its final seating position. The closure manufacturer has to keep flange thickness well under control

for the reason of vial capping. At the time of package development, an aluminum/plastic cap has been chosen where the inner height of the skirt of the cap is capable of gripping the flange height of the stopper plus the height of the vial neck collar, and at the same time being well folded under the collar of the vial. If stopper flange thickness would be excessively high, crimping the cap under the collar could become difficult, while with too low flange thickness there could be an excess of folded aluminum which results in a cosmetically imperfect crimp, while in both cases, worst case, bad container/closure integrity (CCI) results.

Flange markings

The functionality of flange markings (see Fig. 2) for all elastomeric closures can be described in terms of prevention of stopper clumping during storage, steam sterilization, and machining. This functionality is also valid for lyophilization closures; however, there is an additional functionality that is related to the final stoppering of the vials inside the lyophilization chamber at the end of the freeze-drying cycle. As explained above, the stoppers are pressed down by the shelf that is located above them. During this stage, a significant pressure is exerted on the stopper at the time it hits the vial neck. The pressure is transferred to the stopper flange that is in contact with the underside of the shelf, in other words, the stoppers are firmly pressed with the top of their flanges against a stainless steel plate that for reasons of good cleanability has only a low degree of surface roughness. These conditions are ideal to make the stoppers stick to the underside of the shelf. After pressing the stoppers down, the shelves are separated again. At this stage, it is absolutely undesired that the stoppers keep sticking to the shelf. If they do, then the shelf may pull the stopper slightly out of its seated position and thereby impair the integrity of the seal that at that time and until capping is between the plug of the stopper and the inner diameter of the vial neck. If the stopper is firmly stuck to the shelf and at the same time makes a good fit with the vial, then it may also come to the situation that the entire vial at first is lifted, and then, under the influence of gravity, falls down

Fig. 2 Lyo stoppers with one, two, and three vent openings, and with different flange markings

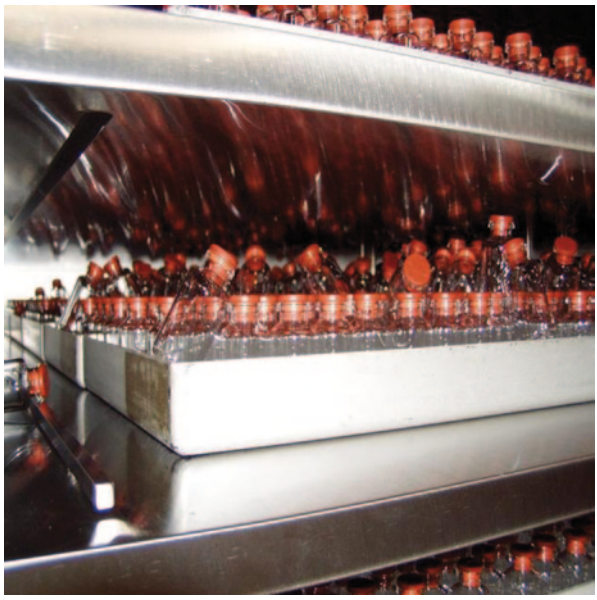

Fig. 3 A shelf of a pilot lyophilization chamber after unsuccessful insertion of the stoppers. Stoppers got stuck to the shelf that pressed them down

again (Fig. 3). This phenomenon has highly undesired consequences that reach from product loss and outside contamination of neighboring vials to the inability to automatically unload the shelves. In all of this, the flange marking of the stoppers, next to other stopper properties and shelf properties plays an important preventive role. If the flange markings are well designed, the probability of shelf sticking can be substantially reduced. Of the flange markings that are depicted below, the one at the right-hand side is the worst in terms of sticking to shelves because the uninterrupted circle on the flange can act as a suction cup on the underside of the shelf that presses the stopper down. With the flange design in the middle, that is composed of two interrupted circles of different diameters, this is extremely less likely. The design on the left-hand side in this respect shows an intermediate behavior.

Penetration thickness

The penetration thickness of an elastomeric closure is the thickness of the stopper in the area where it is penetrated with a needle.

This thickness is an important dimension since it plays a significant role in determining the coring (fragmentation) behavior, the resealing behavior, and the penetration behavior of the stopper. All other factors like needle quality, rubber compound, etc., remaining equal, a higher penetration thickness definitely leads to a higher penetration force, and to a higher likelihood that during penetration rubber fragments are "scraped off" by the needle (named fragmentation or coring). At the same time, at least in theory, a higher penetration thickness leads to a higher probability of adequate resealing upon withdrawal of the needle.

Additionally, after capping of the vial, when it has been ascertained that the closure/vial interface is tightly sealed so that no gas can come in via that route, penetration thickness of the closure determines the permeability to gases of the stopper/vial/cap combination. Given a certain rubber material, higher penetration thicknesses leads to higher resistance to permeation of air and moisture into the vial and thus into the drug. This aspect of the membrane in the center of the stopper acting as a gas barrier after crimping of the vial is especially significant for freeze-drying stoppers. The background of this is two-fold. First of all, many lyophilization vials are stoppered with the lyophilization chamber under underpressure. This vacuum is installed in the vials, mainly to make their reconstitution right before use easier. As a result of the vacuum in the vial, the reconstitution liquid which is in a syringe at atmospheric pressure will be very easily transferred into the freeze-drying vial. Obviously, it is the intention that this vacuum is preserved over the lifetime of the drug product. Therefore, after capping of freeze-drying vials, the role of the penetration area of the stopper as a gas barrier is of very high importance. Second, the reason for freeze-drying a product is that it is not stable in an aqueous environment. The purpose of the primary and secondary drying phases is to remove as much as possible water from the freeze-dried cake in order to ensure drug stability. Consequently, again after capping, the penetration area of the stopper also plays a role as a moisture barrier. (Of course, atmospheric moisture is also a gas!). In order to throw up a higher barrier for the ingress of air and moisture into the freeze-dried vial, the penetration thickness of a lyophilization closure therefore typically is higher than for a stopper that is used for a liquid fill. This is particularly true for 13-mm lyophilization closures.

Plug design

Also the role of the plug of a freeze-drying closure is more critical from a functional point of view than that of a closure used for a liquid or dry powder fill. The plug design for the lyo stopper is more intricate in order to address the different phases of transport and of freeze-drying. The different functionalities of the plug are illustrated using the stopper drawing here below. The discussion below holds for this particular design, but may be transferable only in part to lyophilization stoppers of a different design.

A first feature that is typical of a lyophilization stopper is that the plug wall does not form a full cylinder over an angle of 360°. It is interrupted for the creation of a vent opening that during freeze-drying allows the evacuation of the sublimated ice. In this example, the plug wall is interrupted twice. Thereby, two “legs” are created.

Next, it is easily recognizable that the stopper has a plug with different diameters at various positions along the height of the plug. Diameter D_1 that before capping ensures closure/vial seal integrity is slightly larger than diameter D_2. This D_2 is the diameter that, when positioning the stopper after filling of the freeze-dry solution, is the first to enter in the vial neck. For that purpose, it is advantageous to have a smaller diameter. D_2 on the other hand, must not be too small either. It must be

larger than the internal neck diameter of the vial since this oversizing will contribute to the stability of the stopper in halfway down position. Of course, during all of the transport stages on the filling line itself, on the way to the lyophilizer and during loading of the vials into the freeze-dryer, the stopper must firmly stay on the vial without tilting or falling down because this would jeopardize subsequent steps in the process. Stability of the stopper in halfway down position, however, is not only dependent on the oversizing of D_2 versus the internal vial neck diameter. There is another diameter that plays a role, namely D_3. D_3 is larger than D_2 and also larger than internal neck diameters. The transition between the two diameters D2 and D3 is made by a chamfer, so that a rib is formed that runs over the circumference of the two legs of the stopper. In halfway down position this rib is resting on the rim of the vial. Thereby, it forms an obstacle against tilting and tumbling of the stopper in a plane that is "parallel" to the two vent openings. Equally of interest is a second, this time partial rib that is located a little bit lower on the plug. The rib that is described above, together with this partial rib, over a limited angle form an annular space that can accommodate the blowback rim of the vial on which the stopper is placed. Also this increases the stability of the stopper in halfway down position since a second obstacle against tilting and tumbling is formed. This statement is valid provided that the stopper and the vial are designed to match with each other. If this is not the case, the partial rib will not exert its functionality, but on the other hand is not likely to negatively influence the stopper stability.

After complete insertion of the stopper and before capping, closure/vial seal integrity is assured by the diameter D_1 that is oversized versus the internal vial neck diameter. The seal however, will only stretch over the height H_1 underneath the stopper flange and above the vent openings. This sealing height is a very important dimension for a lyophilization stopper. If the stopper after full insertion, e.g., as a result of flange/shelf sticking or of mechanical agitation during transport to the capping station would creep out of the vial neck, then part of the sealing height is lost and the quality of the seal before capping is affected, in part or in full. Raised stoppers may cause loss of the vacuum that was present before stopper insertion. This is seen as a critical quality attribute for a finished lyophilization vial. Illustration and discussion of this phenomenon can be found in publications like [3]. Additional provisions in the stopper plug design can be made to control and prevent stopper pop-up after full seating, e.g., by providing a blowback below the flange and/or an annular ring that is located on the plug body below the flange and that grips under the blowback of the vial. These provisions are not present in the stopper that is illustrated above. They also will only have their effect if the stopper and the vial are designed to perfectly match with each other. If this is not the case, then seal integrity before capping worst case may even be harmed. Interesting illustrations of exemplary and non-exemplary stopper/vial matches can be found in [4].

There are International Organization for Standardization (ISO) standards for lyophilization closures.[1] These standards are not exhaustive when it comes to stopper

[1] ISO = International Organization for Standardization. The standards in question are ISO 8362-5, "Injection containers and accessories—Part 5: Freeze drying closures for injection vials" and ISO 8536-6, "Infusion equipment for medical use—Part 6: Freeze drying closures for infusion bottles"

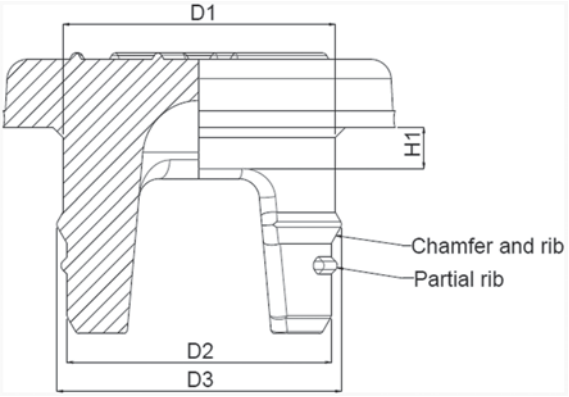

Fig. 4 A particular design of freeze-drying closure

dimensioning. Only a minimum number of dimensions are standardized. This is a consequence of so many lyophilization stopper geometries having been developed and being made available to the market over time. One feature that leads to diversity is the number of vent openings. In Fig. 4, there are two vent openings that are symmetrically located over the plug circumference ("2-leg-lyo-stopper"). Other designs have three vent openings ("3-leg lyo stopper"). A frequently encountered stopper type has only one, but then a larger vent opening ("igloo stopper"). All of these stoppers are illustrated in Fig. 2.

Splitting the vent area over multiple openings also has other consequences that become apparent at the time of lyo cake reconstitution and preparation of the injection into the patient. The single large vent opening of the igloo-type stopper on the left-hand side in Fig. 2 allows to follow the introduction of the needle through the stopper penetration area into the vial, at least if the turbidity of the reconstituted solution and the size[2] of the stopper both permit this. The needle is attached to a syringe in which the drug solution is collected before injection into the patient. With the igloo type of stopper it therefore is possible to visually follow how much of the vial drug solution is transferred into the syringe and eventually into the patient. There thus is a contribution of the stopper design to minimization of the residual volume of the vial. With a 2-leg stopper, this is already not easy anymore. With a 3-leg stopper, it is not possible at all anymore, since the cannula tip will always be hidden, if not by the crimp cap, then by the legs of the rubber stopper. Moreover, with this type of stopper, there is the additional disadvantage that when the stopper is in fully seated position, the legs are forced to come together. Thereby, capillaries are formed between the legs in which reconstituted drug solution is held up. Such 3-leg designs therefore increase the residual volume in the vial. A last disadvantage of 3-leg designs is that during packaging at the closure manufacturer or during transport to the user of the closures, the legs get entangled, resulting in "twin pairs" of stoppers that do not separate again and that disturb good machineability behavior on filling lines.

[2] It depends on the actual igloo design whether this is possible or not. With some designs it is, with others it is not.

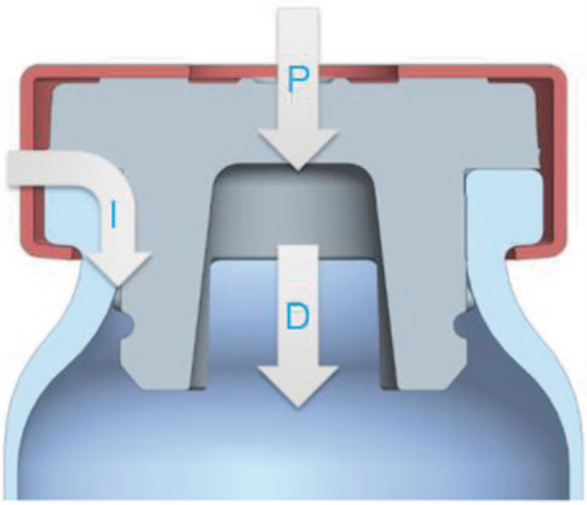

Fig. 5 Moisture making its way into freeze-dried vials

Moisture Absorption/Desorption and Permeability Behavior

Lyophilization is a process that is applied if the drug product on the longer run is not stable in an aqueous medium, and where identity, strength, quality, and purity can only be guaranteed if the product is brought in the form of a lyophilized cake. Upon storage, it is therefore also of importance to keep moisture away from the freeze-dried product. In general, the lower the active dose is, the more important it is to shield the product from moisture. This shielding can be done via appropriate choices of packaging components and crimping conditions.

For a lyophilized drug product there are three principal routes by which, after packing, it can be "contaminated" with water: ingress of water via the closure/vial interface ((I) in the Fig. 5), desorption of water from the elastomeric closure (D), and permeation of water through the closure (P) (Fig. 5).

The ingress route will be given attention under the section CCI of this chapter. The desorption and permeation routes will be discussed here.

Elastomeric closures for parenteral use, at the end of their manufacturing process, are always subjected to a washing and drying process[3]. The purpose of this process is to bring the closures in a controlled state of microbiological and particulate cleanliness, and to satisfy regulatory requirements that are imposed by various regulations [5–6]. During the washing and rinsing phases of this process, the closures are for a certain period exposed to water, while also in the step before washing the closures for a certain time remain in wet condition. This means that right before and during the washing and rinsing there is time for the closures to absorb water. The absorption of the water takes place at the surface of the stopper and will not reach into the bulk of it. Most of the water will be dried off during the drying

[3] Most of the time this washing and drying process is combined with a siliconization process of the closures.

phase that follows washing and rinsing, but part of it will remain in the stopper. The percentage of moisture that is left in the stoppers will depend on parameters such as stopper compound, duration of the exposure to water before washing, duration of the drying cycle after washing, temperature at drying, storage conditions after drying, etc. Typical numbers for moisture content of elastomeric stoppers as delivered by closure manufacturers and as measured by loss on drying are in the order of magnitude of 0.1–0.4% [7–8]. Before aseptic filling, elastomeric closures are however subjected to sterilization. In most of the cases, this is done by steam sterilization of the closures, followed by drying. During the first part of this process, closures are brought in a saturated steam environment, typically for 30 min at 121 °C. The objective of this process obviously is to bring stoppers in a sterile state, ready to be used for filling. However, the side effect of it is that stoppers again will absorb water (steam!) and that the moisture level of the stoppers increases to levels that are four to five times the initial level before sterilization. At longer sterilization times, the effect is even more pronounced [9–10]. In order to reduce this level again, stoppers are dried. The moisture level after drying can be influenced by varying temperature and pressure during drying and by the length of the drying cycle [9–13].

In recent years, a number of "low residual moisture" rubber formulations have been brought to the market. They are characterized by a considerably lower moisture pick up during steam sterilization in comparison with other traditional rubber formulations that are used for lyophilization stoppers. This feature of those formulations can be attributed to their composition in that they do not or only contain little rubber ingredients that easily absorb moisture. Furthermore, they are also characterized by drying rates that are comparable to those of other more moisture absorbing formulations. The moisture absorption and drying behavior of such rubber formulations is illustrated in Fig. 6.

The advantage of this type of rubber formulation is that the stoppers at the beginning of the drug life cycle contain less moisture. Since the moisture in the stopper is in the immediate vicinity of the lyophilized drug and is therefore, by desorption from the stopper, easily accessible, the use of low residual moisture stoppers is

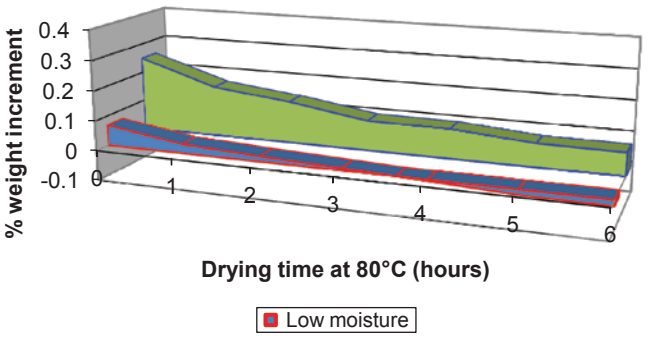

Fig. 6 Moisture absorption/desorption of a low moisture absorption rubber compound in comparison with a typical compound (both bromobutyl)

beneficial for drug stability. For this reasoning to be valid, there are however a number of conditions to be met. The first of those conditions is that the stoppers that are sterilized and dried do not pick up moisture again between the time of drying and the time of being placed on vials. Under normal practical conditions, this is not an issue, since the duration of the storage and the storage conditions of the stoppers after drying are not excessive, and since stoppers that pick up less moisture during steam sterilization display the same behavior upon exposure to atmospheric moisture. A second condition however is that the moisture desorption mechanism from the stopper is not outscored by a second mechanism of moisture transfer to the drug product, namely permeation through the stopper. Desorption of moisture from the stopper and permeation of moisture through the stopper are two phenomena that run in parallel. However, whereas moisture absorption/desorption ("D" in Fig. 5) is determined by stopper composition and the presence of materials with a 'hydrophilic' character, stopper permeation ("P" in the figure) is related to stopper permeability, which is a different physical principle. The moisture that is most readily transferred to a lyophilization cake is the moisture that is left in the stopper after steam sterilization and drying. This water has the shortest way to the cake as it does not need to permeate through the stopper. Desorption of water from the stopper is a phenomenon that starts right away after complete stoppering of the vials. Permeation of water through the stopper is an effect that plays a role on the longer term, since the water that reaches the cake in this way first has to permeate through the entire thickness of the rubber stopper in its penetration zone. The ideal lyophilization stopper shall therefore have both a low residual moisture level at stoppering and a low moisture permeability, or more in general a low gas permeability. Stopper permeability equally depends from stopper composition, but not in the same way as absorption/desorption does. Stopper permeability is primarily dictated by the permeability of the elastomer that is used as base polymer in the rubber, and in second place by the type of fillers that are used in the rubber. Low gas permeability is always achieved by the use of halobutyl elastomer (bromobutyl or chlorobutyl) as elastomer base in lyophilization closures. Fillers that are used are always silicates, but depending on the type a lower or higher permeability will be reached. Blending of bromobutyl or chlorobutyl with elastomers that have a higher permeability such as polyisoprene or styrene butadiene in rubber formulations for lyophilization closures from a perspective of stopper permeability is not indicated. Such blending sometimes is undertaken in rubber formulations for vial stoppers for liquid-fill applications or for plungers for prefilled syringes, in order to improve the mechanical performance of the material, e.g., by increase of its elasticity. A discussion of the role of water absorption and of water permeability can be found in [13–14].

Fluoropolymer Coatings

Whereas the base polymer used for rubber compounds for lyophilization closures is always halobutyl, more and more lyophilization closures, especially for biologicals, are closures that at their surface are covered with a fluoropolymer coating. If

a coating of this nature is applied, then it will always be present in the area of the stopper that is facing the lyophilization cake ("drug contacting area"), and it may or may not be present on the top surface of the stopper flange. The primary reason for applying such a type of coating in the drug contacting area is to achieve a better compatibility of the stopper with the freeze-dried cake. Improving compatibility in fact comes down to further reducing the probability of interaction between the closure and the freeze-dried drug product. Reduction of interaction is achieved by the barrier effect of the coating, meaning that a fluoropolymer coating further reduces the extractability of chemical compounds from the stopper. The extractables 'Omit' level from a coated stopper therefore is lower than the level from a stopper in the same rubber compound but without fluoropolymer coating. The coating in this respect acts as a barrier between the stopper and the drug product. Choosing stoppers with a fluoropolymer coating in the drug contacting area gives the advantage of achieving substantially higher success rates in drug stability studies and in shortening time to market for the drug product.

The reason for applying a fluoropolymer coating on the stopper flange is different. Obviously, the barrier effect of the coating on this part of the stopper does not serve a purpose. The fluoropolymer coating however, very much unlike the halobutyl substrate for the coating, has a totally different tackiness. The fluoropolymer coating makes the stopper completely nonsticky, also without silicone being applied to it. Therefore, stoppers that are fluoropolymer coated on the top of their flanges will not stick to the underside of lyophilizer shelves when they are pressed down. Application of a fluoropolymer coating in the flange area is the most powerful, however also the most expensive measure to avoid stoppers sticking to shelves. A further benefit of a closure that is fluoropolymer coated over its entire surface, not only in the drug contacting area and in the flange area but also present on the sidewall and on the underside of the flange, is that such a closure does not need any surface siliconization at the end of its manufacturing process and yet will not clump together. The absence of surface silicone is beneficial again from a compatibility point of view for silicone sensitive drugs, such as certain biologicals.

Whereas fluoropolymer-coated stoppers offer many advantages, there are also some considerations to make. The first one is that the fluoropolymer coating does not act as a water vapor barrier. The absorption/desorption behavior of a fluoropolymer-coated stopper is not governed by the coating but by the behavior of the rubber compound on which the coating is placed. An illustration of the absence of impact of the fluoropolymer coating on moisture absorption/desorption behavior of a totally coated stopper in steam sterilization and drying can be found in [10]. As a result there is also no effect on headspace moisture levels in vials that are stoppered with uncoated and with fluoropolymer-coated stoppers of this type. A second consideration is that the fluoropolymer coating in the drug contacting area is not limited to just this area, but that the coating extends to the outer surface of the igloo or of the legs that form the plug of the stopper. The latter surface comes in contact with the vial neck when the stopper is in halfway down position before lyophilization and when it is fully down after complete stoppering of the vial in the freeze-drier. The fluoropolymer layer behaves differently in comparison with a typically siliconized

surface of an uncoated stopper. Fluoropolymer-coated stoppers are more slippery, and, all other things such as the vial remaining the same, are more sensitive to displacement in halfway down position and also are more seen to more easily lead to rising of stoppers after full stopper insertion and before capping. This in turn can lead to a transient CCI failure that ends when the stoppered vial is crimped, but in the meantime may have led to effects on the composition of the vial headspace and potentially to lack of sterility assurance. Altogether, the behavior of the stopper/vial combination in transport phases right after filling, during loading and unloading of the freeze-drier, and between unloading and vial crimping shall be closely observed.

Vials

In the area of lyophilization vials, a lot of attention has been paid in research and literature to the role of the vial in mass and heat transfer and its impact on sublimation rates, e.g., [1] and [15] and the references cited there. Noteworthy is that some new types of vials are offered to the market that specifically target a use as lyophilization vial. An example of such a vial is a tubular vial that is subjected to a plasma impulse chemical vapor deposition (PICVD) process [16]. In this process, a transparent Si-O-C-H layer is deposited on the inside of the vial. The coating has a hydrophobic character. Claimed advantages are better cake appearance and prevention of cake collapse, as well as a better emptying of the vial leaving less residue of the reconstituted product, opening the way for reduced overfill. The vial bottom geometry is claimed to be optimized allowing reductions in freeze-dry process times. A second example is a reduced weight molded vial that thanks to its flat bottom is equally claimed to have an optimized heat transfer and a better cake appearance. A comparison of both vial types with a polymer molded vial in cyclic olefin copolymer (COC) is presented in [18]. Through sublimation tests with pure water at varying low pressures, it was shown that the above-cited molded vials have a very similar performance in comparison with the PICVD-coated tubular vials. Both vials clearly outperformed the polymer vials that were used in the study.

Secondary Packaging Components: “Integrated Rubber/Plastic Caps”

In the elastomeric closures section of this chapter, it was pointed out that the closure/vial seal area is not in the same location before and after crimping of the vial with an aluminum cap. Before crimping of the cap, the seal area is between the plug of the stopper and the “vertical” portion of the inside of the vial neck. After crimping, the seal area relocates to the position between the underside of the flange of the stopper and the “horizontal” rim of the vial neck. After lyophilization, vials thus leave the freeze-dryer with a seal between vial and stopper that lacks robustness.

Until the moment the vials are crimped, underpressure in the vials may get partially or completely lost and care is to be taken for preservation of product sterility until crimping has been performed. For many years, it has been a goal of pharmaceutical packaging component development to design a closure system that is able to put in place a robust seal before the vials leave the freeze-dryer. Such closure system could be in the form of an "integrated closure" where an elastomeric part as primary packaging material is combined in some form with a plastic cap as secondary packaging material, the plastic cap taking over the role of the aluminum crimp cap in the traditional way of working. The idea is that this integrated product is placed on the vial after filling, instead of the rubber stopper only. The integrated product just as a traditional lyophilization stopper has a halfway down position allowing primary and secondary drying. At the end of the freeze-drying cycle, the integrated product is then pushed down until a specially designed feature on the plastic part grips under the collar of the vial, thereby at the same time pushing the elastomeric part into its sealing position on the top rim of the vial. Since it is now a hard plastic part that is in contact with the shelves and not an elastomeric stopper, stickiness to shelves is avoided. The vials then are unloaded from the freeze-dryer already in capped condition, avoiding any risks during further transport. Although the idea looks straightforward, its realization was found to be far from trivial. A number of issues were encountered during development of this type of seals. The first was that the design of the plastic part had to cope with all of the stacked height tolerances of the collar of the vial and of the flange of the stopper. Whereas with traditional flip caps (aluminum/plastic caps) this must be a point of attention when the cap is chosen in the design stage of the packaging, usually there is enough choice in different heights of the aluminum part of the cap so that picking an appropriate cap is not problematic. In the case of the integrated product however, there is only the plastic part to deal with stacked tolerances, and there is little or no flexibility to vary the height of the part once the design is frozen and a mold for the part has been constructed. A second issue was that over a longer time plastic, when fixed on the vial, is subject to stresses that, unlike aluminum, can lead to dimensional changes of the cap ("creep"). Such changes in the worst case lead to an elongation of the cap that leaves so little residual force of the elastomeric part on the vial that the closure/vial seal integrity might be endangered. Eventually however, commercial versions of such plastic caps are offered to the market now [19, 20].

Dual-Chamber Systems

By far, the majority of lyophilized products are freeze-dried in vials. The vial then typically comes with a second vial or with a prefilled syringe that contains the diluent. In a dual-chamber system, both the freeze-dried cake and the diluent are present in the two chambers of the same container. The container can be either a syringe or a cartridge. An illustration of both systems can be found in [21]. During the storage life of the system, the two chambers are separated by a rubber plunger that is

sitting in the middle of the system ("middle plunger"). The liquid drug formulation is first filled in the chamber that starts on top of the middle plunger and that ends at the needle end of the system. Then it is freeze-dried from that chamber. What is left at the end of the process is the freeze-dried cake. The diluent is contained in the second chamber that is confined between the middle plunger that separates the chambers and a second rubber plunger that seals the syringe ("end plunger"). Right before drug administration, the diluent is transferred into the same chamber as where the lyophilized cake is stored. This is done by activation of the end plunger. Exertion of force will not only move the end plunger but by transmission of pressure over the diluent also the middle plunger. This goes on until the chamber with the diluent reaches a bypass in the glass barrel of the system. While the end plunger, always under application of force, keeps on moving forward in the syringe, the diluent in the second chamber will be displaced into the first chamber and the freeze-dried cake will be redissolved. The reconstituted drug is then ready for injection.

Dual-chamber systems are not new. They are in the market since at least 25 years but, in spite of offering distinct advantages, have never generally become first choice administration systems. Nevertheless, efforts are being spent on improving dual-chamber systems. A system that is worth noting in this respect is illustrated in [22]. The claimed advantage of this novel system is that in contrast with existing systems, the diluent is filled first and can be sterilized by autoclaving prior to filling the solution that is going to be freeze-dried in a second step. A further claimed advantage is that after filling the solution, the syringe is fully closed by a specially designed rubber closure, that both has vent openings to allow freeze-drying and also has ribs like a syringe or a cartridge plunger. Between filling and positioning in the freeze-dryer, the ribs serve as sealing elements to close the container. Once inside the freeze-dryer, ingenious use of underpressure in the freeze-dryer and relative overpressure in the chamber with the drug solution allow the closure to move as a plunger over a small distance until the vent openings come free and the pressure in the lyophilizer and in the drug solution chamber of the system equalizes. After this self-opening in the freeze-dryer, the system is ready for lyophilization. At the end of the freeze-drying cycle, the rubber closures will be pushed down again into the syringes by the shelves that are coming down, just as is the case with freeze-dried vials and standard lyophilization stoppers. During the cycle, the diluent that is present between the middle and the end plunger will first freeze and then thaw again. A more extensive discussion of the system is available in [23].

Container/Closure Integrity

It has been emphasized several times before that closure/vial seal integrity for lyophilization vials is not highly robust between unloading of the freeze-dryer and crimping of the cap. This fact has been extensively brought under the attention when in the EU the Annex 1, "Manufacture of Sterile Medicinal Products" to Good Manufacturing Practice Guidelines was in a revision process. The process ended

in 2008 with the publication of an updated document [24]. The wording in [24] is as follows: “The container closure system for aseptically filled vials is not fully integral until the aluminum cap has been crimped into place on the stoppered vial. Crimping of the cap should therefore be performed as soon as possible after stopper insertion.” And also “Partially stoppered freeze drying vials should be maintained under Grade A conditions at all times until the stopper is fully inserted.” “Containers closed by fusion, e.g. glass or plastic ampoules should be subject to 100 % integrity testing. Samples of other containers should be checked for integrity according to appropriate procedures” and “Containers sealed under vacuum should be tested for maintenance of that vacuum after an appropriate, pre-determined period.”

This ruling, as well as the 2004 Food and drug Administration (FDA) Guidance on Aseptic Processing [25] that contains statements pointing in the same direction, have spurred many pharmaceutical companies to look more critically at the condition of their freeze-dried products between stoppering and crimping, e.g., [26] that illustrates a 100 % camera control of stopper placement before capping and the rejection of vials with stoppers that have excessively raised, based on results of helium leak rate testing.

The increased attention for CCI for a part has coincided with the advent and acceptance of novel nondestructive inspection techniques. Among the latter, laser-based headspace analysis is in a very prominent position for freeze-dried vials. It is a technique based on laser absorption spectroscopy that is suitable for measuring gas concentrations and vacuum levels in the headspace of vials in a nondestructive manner. When the same vials are measured over time, changes in gas concentrations, also if they are temporary, can be detected on the finished vial after crimping. The technique is extensively discussed in [27–28]. It is meanwhile offered by various companies. References of the most cited providers can be found in [29, 30]. The fact that CCI for lyophilized vials is not a given thing, and that it may vary from batch to batch, is illustrated in [31], where the investigation of in total nearly 14.6 million lyophilization vials revealed an average CCI failure rate of 0.67 % with serious outliers in particular batches.

A factor that may even put more focus on CCI is the revision process for United States Pharmacopeia (USP) <1207>, “Sterile Product Packaging—Integrity Evaluation” [32] that is going on at the time of writing this chapter. So far, in terms of CCI evaluation for sterile products, mostly USP <1207> and Parenteral Drug Association (PDA) Technical Report (TR) 27, “Parenteral Packaging Integrity” [33], were the two most cited documents. In future that will probably remain the case, however, as a result of the revision process in which both documents are in, their relative significance is going to change. At present USP <1207> [32] describes integrity testing as a pharmaceutical product package life cycle testing activity, starting at product package development and later on covering the stages of routine manufacturing testing and marketed product stability testing. It mentions physical tests and microbial challenge tests, but it does not go into details. An extensive listing of testing methods as well as more details on them can be found in PDA TR 27 [33]. The revision process of both documents has not been concluded yet, but from what has been publicly presented so far, it may be inferred that some significant changes are

going to come up [34]. USP <1207> in its revised version[4] will take into account that leak testing methods, notably physical testing methods, have made a lot of progress over the past decades and also it will acknowledge that test method validation requirements are much stricter than they were at the time of publishing the current version of USP <1207>. USP <1207> will stay with the product package life cycle testing approach. More emphasis will be on validation requirements. Ample attention will also be given to the selection of leak test methods. Very significant in this respect is that USP <1207> prefers wherever possible the use of deterministic leak test methods, and not, as is presently often the case, the use of probabilistic methods. Deterministic methods are physical methods where leakage is dictated by predictable fluid flow. Among these methods are the above discussed laser-based gas headspace analysis, pressure and vacuum decay leak tests, and high-voltage leak detection. Probabilistic methods on the other hand are methods such as tracer liquid leak testing ("dye testing") and microbial challenge testing. Such methods are not at par with deterministic methods if it comes to sensitivity, reproducibility, and validatability and therefore are more prone to error. The revised USP <1207> will also eliminate its current requirement of comparison of physical and microbial test method. Since USP <1207> after revision will contain documentation of each leak test method, deterministic and probabilistic, this documentation can be taken out of PDA TR 27. PDA TR 27 in its revised form therefore is planned to focus on discussion of new and emerging leak test methods that have not yet acquired the status of "peer-reviewed" methods.

Extractables and Leachables

Interest in extractables and leachables from primary packaging components may not have started with the FDA Container/Closure Guidance [35], but it definitely spurred the thinking process. Generally accepted definitions are that extractables are compounds that can be extracted from packaging components under model conditions in model solvents, while leachables are compounds that originate from packaging components that are found in drug products during or at the end of shelf life. Leachables can be either extractables itself or may be derived from extractables by reaction with the drug product or its excipients.

Lyophilized drug products are in contact with the glass from the vial and are or may be in contact with the elastomeric closure. Because of their composition, extractables from glass typically are of inorganic nature in the form of ions. This is different for extractables from rubber. The main constituents in rubber are the base polymer (elastomer) and the filler. For the reason of its excellent permeability properties, the base polymer in the case of lyophilization closures is bromobutyl or

[4] Publication is aspired to for 2016.

halobutyl polymer[5]. This is a polymer that results from the polymerization of isobutylene with a small fraction of isoprene, followed by halogenation of the resulting polymeric material. The reaction process takes place in an organic solvent while the precipitation of the finished halogenated polymer requires addition of some stabilizers. The halobutyl polymer contains a number of reactive sites that are used in a cross-linking process. Before going into this process, the polymer however is first mixed according to a fixed recipe with at least cross-linking agents, a filler, and a pigment. Other material constituents might be additional antioxidant, plasticizer, or other materials to tune the rubber compound to the desired property profile. In the cross-linking process, also termed vulcanization or curing, bonds between polymer molecules are created by the cross-linking system by chemical reaction at polymer reactive sites. The physical effect of the cross-linking reaction is the creation of elasticity in the material. This elasticity is desired because it is required to obtain the sealing and resealing capacities of the elastomeric closures that are formed. An undesired effect is that in parallel with the cross-linking process, also side reactions and breakdown reactions are taking place. With this polymer manufacturing process and this mixing and conversion process as background, it can be explained that the extractables profile of a rubber material is quite complex. There are some inorganic extractables, primarily from the filler and the pigment, and chloride and bromide ions from the base polymer, but the majority of the extractables are organic in nature. There might be a residual monomer or residuals from the reaction solvent, but also oligomers that are formed during the polymerization and halogenation process and that are carried through the cross-linking process. Next, there is an antioxidant that was used to stabilize the polymer, mostly hindered phenol-type compounds, and there are the stabilizers used in polymer production (calcium stearate, epoxidized soybean oil) and the extractables related to additional rubber compound ingredients. Of course, they may be an unreacted leftover of the cross-linking reaction plus reaction side products.

For lyophilization products, transfer of leachables into the drug formulation via the headspace of the vial must be considered. Volatile components can affect product stability and can give rise to haze formation in reconstituted solutions. An example of this is the haze that is attributed to butylated hydroxytoluene (BHT) that is used as an antioxidant in rubber closures [36], be it that the case that is described there does not concern a lyophilized product.

Since the publication of the FDA Container/Closure Guidance [35], knowledge and science about extractables and leachables has been given a boost thanks to the work that was done in several collaborative projects. Among these, the projects of Project Quality Research Institute (PQRI) take a very prominent place. The first project of this consortium was in the field of extractables and leachables for orally inhaled and nasal drug products (OINDP). Partners in this project were pharmaceutical companies, academia but also regulators from FDA and component manufac-

[5] Nonhalogenated butyl is another possibility, but is rarely used anymore.

turers. The project ended in 2006 with the publication of an extensive report [37]. The success of this work on OINDP was carried on since 2008 in a second project, this time on parenteral and ophthalmic drug products (PODP). The approach in the PODP project is similar to the OINDP work; however, it gives consideration to specifics for parenterals and for ophthalmic products. The result is expected to be available from 2015 onwards, equally in the form of considerations and recommendations for safety thresholds and best practices.

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Lyophilization Process Technology Transfer Towards Product Launch

Madhav Kamat and Dushyant Varshney

Introduction

In the past two decades, there has been a rapid increase in the number of biological (e.g., therapeutic proteins, biosimilars, biobetters) and novel vaccine (e.g., multivalent) products developed by many small and large biotech companies. Development of such biologics is quite expensive and many companies lack in-house setup and capability to develop biologics from discovery to commercialization. In contrast, large multinational biopharmaceutical companies, engaged in core or noncore business, have realized cost saving by utilizing contract manufacturing organizations (CMOs) and improved productivity trends, as compared to investing in setting up and maintaining own facilities with required expert staff and regular updates [1]. In this diverse and changing industry, technology transfer (TT) of active pharmaceutical ingredients, analytical methods, and drug products from development to market phase is becoming increasingly common and important to deliver safe and quality products in the most cost-efficient way.

A successful TT ensures the quality of product during the entire life cycle of manufacture and validation, in accordance with current good manufacturing practices (cGMPs), providing predictable and consistent operation of the processes. It is

M. Kamat (✉)
Kamat Pharmatech LLC, North Brunswick, NJ 08902, USA
e-mail: madhav.kamat@gmail.com

D. Varshney (✉)
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway,
Holly Springs, NC 27540, USA
e-mail: dushamaya@gmail.com

MS & T Hospira, Inc., 275 N. Field Drive Lake Forest, Lake Forest, IL 60045, USA

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based on the principles of Food and Drug Administration (FDA)/ International Conference on Harmonization (ICH) Q8, Q9, Q10, Q11 guidelines, involves knowledge transfer, science and risk-based approaches which ensure a comprehensive and systematic transfer of information and documentation between transferring site and receiving site [2–6].

Most of the biopharmaceutical products are delivered through parenteral route and a large number of such products are manufactured utilizing lyophilization process to ensure long-term stability of biologic drug substance and drug product [7–9]. The lyophilization process as such is complex, time-consuming, expensive, and requires a robust and reproducible process transfer from development laboratory to commercial manufacturing site, or from one manufacturing site to other manufacturing site within same company or to a CMO.

In this chapter, focus is on the TT of the lyophilized products wherein an overview of general TT activities of sterile lyophilized product including specific case studies is discussed.

Technology Transfer

As described in the FDA ICH Q10, the goal of TT activities is to transfer product and process knowledge between development and manufacturing, and within or between manufacturing sites to achieve product realization [4]. This knowledge forms the basis for the manufacturing process, control strategy, process validation approach, and ongoing continual improvement [2–5].

An overview of different types of TT of lyophilized product is shown in Fig. 1. In general, TTs can be categorized in two types:

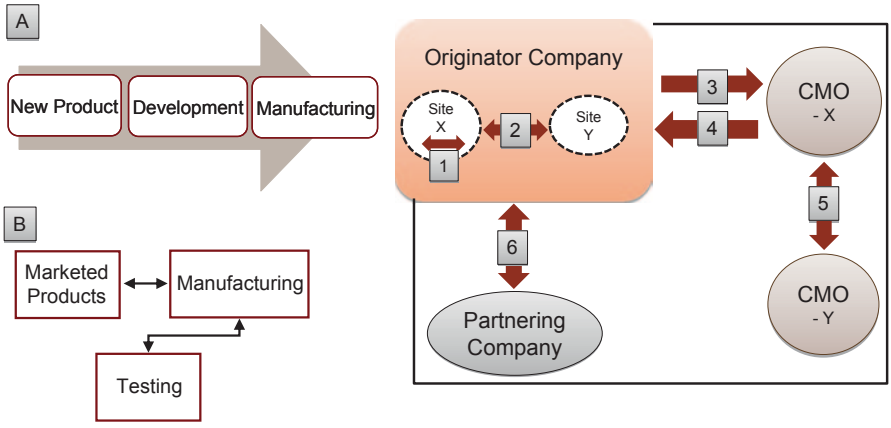

Fig. 1 Types of technology transfer. **a** New product transfer; **b** Marketed product transfer. *1*: Intra-site; *2*: Inter-site; *3*: Outsourcing—CMO; *4*: Insourcing—CMO; *5*: Inter-CMO; *6*: Intercompany (licensing). *CMO* contract manufacturing organization

1. New product transfers during development through manufacturing.
2. Transfers within or between manufacturing and testing sites for marketed products.

Other more descriptive types of TT involve the following types:

1. Intra-site transfer, process scale-up, or transfer with same or larger equipment.
2. Intracompany transfer, from sending (originator) site to receiving site.
3. Outsourcing-CMO transfer, from originator site to contract manufacturing site.
4. Insourcing-CMO transfer, from contract manufacturing to originator site.
5. Inter-CMO transfers, from sending CMO site to other receiving CMO site.
6. Intercompany transfers from innovator company site to licensed partner receiving company site.

Manufacturing of sterile lyophilized product essentially involves two distinct processes. First, manufacture of sterile solution in vials, which involves all the unit processes in the sterile product manufacture for an aseptically produced product, such as compounding, sterile filtration, and filling into sterile containers such as vials. This step is immediately followed by the lyophilization process in which the filled vials are subjected to the lyophilization process inside a freeze dryer to get a final dry product.

Technology Transfer Execution

General Activities

Some of the key general activities involved in the TT process are listed below:

1. Start TT activities at the manufacturing (receiving) site.
2. Establish TT key performance indicators (KPIs).
3. Procure and assemble a complete technical data package from technology originator (or sending) site/R&D/company/CMO and verify completeness of the data.
4. Start site TT activities in preparation for experimental and/or process justification trials.
5. Perform process design for the site based on originator site process information.
6. Initiate and write a technology transfer plan.
7. Initiate and write a validation master plan document.
8. Perform an aseptic processing validation evaluation.
9. Develop the initial manufacturing instructions for experimental, stability, and/or clinical trials.
10. Train operators in the manufacturing process and the master batch records (MBRs).

11. Set initial process limits based on originator site previous experience.
12. Determine optimal batch size based on equipment capacities and/or operational constraints and product forecast.
13. Obtain packaging specifications with originator site primary packaging components.
14. Perform multiple activities required to achieve equipment readiness.
15. Ensure completeness of the qualification activities for the washing, sterilization, and depyrogenation processes for the primary packaging components.
16. Obtain a filter validation package from originator site.
17. Perform cleaning verification and/or validation activities.
18. Develop experimental study plans and/or process justification protocols.
19. Develop validation protocols to show consistency/comparability at originator and receiving sites and ensure that the transfer offers documented evidence that process operated at receiving site can consistently manufacture, meet product specifications and other key attributes, demonstrate comparability to reference standards.
20. Address other site and product specific issues.

Specific TT Activities Related to the Lyophilized Product

In addition to the above general activities, there are numerous technical tasks that must be performed to address completeness of manufacture-related TT elements, which can be specific to the particular lyophilized product. These can be categorized as: (1) formulation-related issues, (2) container-closure-related issues, (3) manufacture of sterile solution for lyophilization, and (4) lyophilization of the sterile solution in vials.

Formulation-Related Issues

1. Formulation raw materials' characterization studies: A full physical characterization profile for each raw material ingredient should be performed since these may affect the quality of the dried product.
2. Raw materials particle size, particle size distribution, particle inner and outer porosity, and particle form and morphology are some typical examples of critical information that should be determined. As part of this characterization effort, a solubility profile for each material and its intrinsic dissolution rate should be developed.
3. Bulk formulation thermal analysis: Lyophilization cycle development for a freeze-dried product is designed based on bulk formulation solution thermal properties. Bulk solution structures' formation during the freezing stage (amorphous or crystalline) must be critically characterized in order to define appropriate drying parameters of shelf temperatures, soak and ramps, chamber pressures,

and duration of all the phases. To achieve this, following information should be obtained from the sending site: freezing temperature, glass transition temperatures (T'_g and T_g), and the eutectic/collapse temperatures.

4. Preliminary operational parameters must be established during experimental phase throughout specific unit process operations used for monitoring, control, and measurement of the manufacturing process. Process specificity parameters should be based on specific manufacturing equipment capabilities. Mixing speed range, mixing shafts placement/inclination, system temperature and pressure range and tolerances, flow rates, filling speed, etc., are some examples of process specific parameters.
5. Product compatibility: The primary packaging components for a parenteral product play a major role during selection in order to assure appropriate stability and sterility state of the finished product throughout its shelf life. The chemical and physical interactions within the stopper/product or syringe system should be evaluated. A stopper is considered product compatible when the fundamental physical and chemical attributes of both elements are maintained after a predetermined contact exposure time and conditions.
6. Photostability studies: Gather light exposure restrictions from the transferring site as part of the initial knowledge transference process, particularly for highly sensitive products. Evaluate the manufacturing infrastructure to determine if existing facility lighting offers the adequate light intensity control. Additional light stability study may be required in order to confirm receiving site light exposure controls are sufficient throughout the manufacturing stages.
7. Determination and characterization of stopper leachable/extractable levels, even if the product is a dry solid, are required to be determined in order to comply with both quality and regulatory requirements.

Container-Closure Issues

1. Container-closure system integrity: A container-closure system integrity studies ensure no intrusion of contaminant into the product occurs. This study should be representative of the receiving site sealing machine capability and established operational parameters.
2. Stoppers coring test: Stoppers must work as a barrier to protect the product. Also, it is important to determine the compatible needle/puncture system and procedure to get the product out of the container in a safe way. The stopper ability to maintain a barrier and its integrity after one or successive punctures along with the associated cores detected in the product should be determined.
3. Glass container issues: Special attention should be given to the issues of delamination of glass surfaces during the storage of the product. Moreover, glass breakage during the cycle and during handling is a problem that is becoming increasingly important in lyophilized vials. Glass particle contamination in vials is a critical defect and must be addressed throughout the process—from procurement, incoming inspection, and through the manufacturing steps.

4. Stopper sealability: Machine-sealing parameters must be identified to ensure proper sealing of the vial with the stopper and thereby providing adequate container closure integrity to maintain sterility of the product. This is typically achieved by establishing the required machine mechanical pressure conditions to achieve a cosmetic and well-seated cap. The pressure operational parameters should be confirmed by integrity test results.

Manufacture of Sterile Solution for Lyophilization

1. Process/equipment machineability studies: Evaluation of the required change parts for the proposed process equipment throughout specific manufacturing stages should be performed. The recommended mechanical operational parameters for each piece of equipment should be defined during the execution of this study.
2. Sterilized stoppers staging/holding time: Sterilized stoppers must be packed in suitable bags and/or containers and transferred into the aseptic staging or marshaling area. Normally, it is convenient for the operation to have them stored temporarily in the aseptic clean room until its final use. Integrity of stopper bag during the holding time must be demonstrated through microbiology studies to monitor the sterility state of stoppers throughout this time.
3. Cleaning/sanitization/fogging agents' interference studies: Clean rooms and other manufacturing areas must be cleaned and sanitized using qualified agents in order to maintain proper environmental conditions. The manufacturing isolators are sterilized using sterilants such as vaporized hydrogen peroxide (VHP). It is common to use a fogging agent for control of spores. Chemical and physical effect of the residual agents on the quality of the product should be assessed to define the tolerable levels and or develop appropriate decontamination procedures during specific manufacturing process.
4. Processing times and bulk solution holding times: The performance of the bulk formulation solution process is normally defined and monitored by establishing time requirements for materials' addition into the formulation vessel/reactor. Once a solution with predetermined quality attributes is obtained, bulk solution holding times are established. The following processing time categories are normally defined:

    a. Excipient ingredients' mixing time: The specific mixing time for total dissolution of each excipient ingredient is monitored and established. A mixing time range should be established.
    b. Active pharmaceutical ingredient (API) mixing time: The specific mixing time needed for total dissolution of the active pharmaceutical ingredient is monitored and established.
    c. Formulation time: This is the elapsed time since the addition of the first ingredient into the formulation vessel until the formulation process is completed (normally end of mixing after final batch volume is made up).

   d. Filtration time: This is the total time required for transfer of bulk solution from the formulation room into the aseptic area through sterilizing filter at predetermined operational parameter conditions.
   e. Bulk holding time: This is the maximum permitted time that the bulk solution can be stored in the formulation tank (from the time active was added into the formulation vessel until the start of freeze-drying cycle for lyophilized products).
   f. Sterile holding time: This applies to situations when the formulation is filtered to a holding tank inside the aseptic area and the filtered solution in the tank is held for further filling operation. For lyophilized products, this is the maximum permitted time the sterile bulk solution can be maintained in the aseptic area (since the end of the sterile filtration process) prior to the time the freezing stage in the freeze-dryer unit is initiated.

5. Sterilizing filters surface area optimization: Determination of optimum size of filter(s) should be done to ensure that problems related to low filter performance issues such as membrane clogging and integrity test failures are not encountered. The filter should be large enough to ensure the passage of the entire batch, but should not be excessively redundant to avoid losses of precious product in the holdup elements as well as to minimize operational costs in a long-term basis.
6. Moisture pickup studies: The lyophilized products are normally highly hydroscopic in nature due to very high porosity and amorphous nature of the dried product. At the end of a lyophilization cycle, the freeze-dried product is fully stoppered within the chamber and the vial trays are unloaded and stored on transfer carts. The seal generated by the stopper placed over the vial mouth maintains dried product cake integrity until it is finally sealed. Studies are performed to demonstrate that the residual moisture content achieved during the lyophilization process is maintained throughout the elapsed time that the last vial is sealed/capped. This study should recommend a maximum freeze-dried product staging holding time in the aseptic area or inside the lyophilizer chamber.
7. Vial headspace studies: Specific product vial headspace composition should be known and is established based on product stability and related degradation kinetics. It is critical to demonstrate that the receiving site manufacturing process is capable to reproduce the headspace composition base line provided by the transferring site in order to ensure proper product storage throughout its shelf life.
8. Storage/shipping studies: Proper storage conditions are critical for assurance of product stability throughout its shelf life. Acceptable ranges for temperature and relative humidity during the storage as well as limits on exposure to light intensity must be established. Proper storage room temperature distribution and humidity control should be demonstrated through a formal qualification effort. Product storage requirements should also be maintained throughout batch shipment to the distribution centers or final destiny.

Lyophilization of Sterile Solution in Vials

Lyophilization process consists of three major stages: freezing (may involve additional annealing step), primary drying, and secondary drying, each of which must be completed before proceeding to subsequent stage for a successful cycle. Also, different mechanical and control systems of the lyophilizer equipment must maintain the process conditions within established limits to produce uniform dryness and quality for the complete batch [10–17].

Some specific considerations in lyophilization scale-up and transfer include:

1. Differences in lyophilizer units
2. Differences in cycle profiles from small to large lyophilizers
3. Determination of the end of various cycle stages at commercial scale
4. Consideration of nonuniformity of rate of drying in the chamber
5. Effect of stopper vent type and size during transfer of lyophilized products
6. Effect of load size on the duration of the lyophilization cycle

1. Differences in lyophilizer units

It is important to note that no two lyophilizer units are identical. There do exist some differences in supposedly identical units such as design, architecture, hardware, and controls, which may result into differences in the drying cycles. Table 1 shows the overview of some relevant technical characteristics of the lyophilizers. The list includes only those equipment parameters that are generally regarded as having the greatest influence on the course of a lyophilization cycle. It should be noted that the parameters that are most critical in maintaining a desired lyophilization profile are the shelf temperature, the condenser temperature, the chamber pressure, and the duration of various stages as well as the control/monitoring systems. As seen from this table, all of the critical hardware and the process control/monitor mechanisms between two units (transferring and receiving lyophilizer units) must be comparable. If such similarity exists, then it is fairly safe to assume that the independent programmable cycle parameters (shelf temperature, chamber pressure, and duration of various steps) will be executed identically in these units and the resultant lyophilization cycles will be equivalent as long as the process conditions do not overburden the system capabilities.

2. Differences in cycle profiles from small to large lyophilizers

Often, the lyophilization cycles are developed in R&D setup where the size of the lyophilizer unit is very small (shelf area of 2–10 sq. ft.). Additionally, these units are not insulated adequately and are located in a general laboratory environment. These conditions result in unintentional heat supply from the surrounding environment to the frozen cakes undergoing sublimation phase inside the relatively small chamber and the dynamics of drying becomes skewed. The drying vials receive heat not only from the shelf of the unit at the bottom surface of the vial but also additional heat from the door as well as walls of the chamber by radiation. In such case, the cycles are usually shorter than calculated or anticipated. If such cycle parameters are trans-

Table 1 Lyophilizer unit considerations

System	Subsystems	Difference type	Considerations
Chamber	Chamber volume	Information	Impact on batch size
	Shelves area	Information	Impact on batch size
	Construction metal/polish	Critical	Heat transfer from the shelf fluid to the bottom of the vial may get affected
	Shelf flatness		
	Dimension of pass-through valve	Critical	Mass transfer flux may choke the flow in inadequately configured pass-through valve and affect the cycle performance
Condenser	Configuration	Critical	Internal or external condenser will affect the cycle
	Minimum condenser temperature	Critical	Must be maintained at $\leq -55\,^{\circ}C$ for all the loads and at all the rate of ice deposition
	Maximum ice capacity in volume	Critical	Must exceed maximum batch size
Refrigeration	Number of compressors	Information	Must be capable to provide necessary heating and cooling power throughout the cycle
	Type of compressors	Information	
	Refrigerants	Information	
Shelf cooling and heating	Thermoregulation range	Critical	Range must match or exceed process requirements
Vacuum system	Vacuum regulation range	Critical	Range must match or exceed process requirements
	Method of vacuum regulation	Critical	Must remain equivalent during transfer (N_2 or air)
	Type/number of pumps	Information	Redundancy for failure mode
Measurements	Temperature probes	Critical	Must be equivalent. Thermocouple or RTD types
	Pressure probes	Critical	Must be equivalent. Pirani or capacitance types do exhibit differences in the temperatures in the presence of moisture
Control and automation	Control of the programmable recipe and data acquisition systems	Critical	Must be capable of executing required process steps

Table 1 (continued)

System	Subsystems	Difference type	Considerations
Temperature uniformity	Temperature mapping: within the shelves	Critical	Typically, ± 2.0 °C. Ensures uniformity of drying among vials in the load
	Temperature mapping: across the shelves	Critical	
	dT—Maximum deviation from set point	Critical	± 3.0 °C. Shelf surface temperature experienced by the vial bottom is the key. Not the set temperature

RTD resistance temperature detectors

ferred to the much larger unit where this radiation artifact is not encountered, then the cycle times are much slower than the ones observed in the laboratory. These differences in the dynamics of drying between the small laboratory units and commercial freeze dryers are the reason why many cycles fail in their initial attempts of technology transfer.

3. Determination of the end of various cycle stages at a commercial scale

A number of techniques are used to determine the end of primary and secondary drying stages of the lyophilization cycles. Some of these are listed below:

a. Measurement of product temperature
b. Pressure rise measurement
c. Differential pressure gauge responses
d. Remote sensing of product temperature
e. Others: dew point measurement (electronic moisture sensor), H_2O concentration from tunable diode laser absorption spectroscopy (TDLAS), manometric temperature measurement (MTM), sample vial extractor, residual gas analysis, near-infrared spectroscopy (NIR), etc.

All of the above are shown to be very effective by many research groups; however, these may not be always available at both the R&D site (technology transfer sending unit) and the commercial manufacturing site (technology transfer receiving unit). It is important that in a validated routine manufacturing process, end of drying should be independent of any variable. Ideally, it should be based on time duration parameter of the process alone. We discuss some of these more practical methods below.

a. Measurement of product temperature

The product temperature agreement with the shelf temperature is very commonly practiced in the industry using thermocouples and/or resistance temperature detectors (RTDs). As the end of the sublimation drying approaches, the product tempera-

ture approaches that of the shelf. No more frozen ice is remaining in the cake and the incoming heat is now used for raising the temperature and not for phase change alone. However, accuracy of this test depends upon the validity of the placement, position in the cake, and location in the chamber. There are also concerns of potential breach of sterility due to manual handling of the probes in sterile vials. Moreover, the newer freeze-drying plants use automated loading and unloading systems and hence placement of the temperature probes becomes difficult, if not impossible.

b. Pressure rise measurement

This technique is a kind of leak rate test in chamber loaded with the drying vials. It is a volumetric test and indicates the drying kinetics of the whole lot as a unit, and not for a particular vial. Based upon the knowledge of intrinsic leak rate of the empty dry unit, one can set or determine allowable higher leak rate of the loaded unit (allowing for residual moisture levels in all the vials) which then can be correlated to the known point of end of drying.

c. Differential pressure gauge response

There are mainly two types of pressure (vacuum) gauges used in most lyophilization units—Pirani and capacitance gauge. Pirani gauge readings vary based upon the moisture composition of the gas (due to change in the thermal conductivity of the measurement filament), whereas the capacitance gauge is of absolute type and is independent of the gas composition. In the early part of drying, when the moisture is a major mole fraction of the gas in the chamber, the Pirani gauge shows higher offset reading. As the moisture level in the surrounding gas goes down upon further drying, this reading is lowered. As the moisture is completely depleted in the surrounding toward the end of drying, the Pirani gauge shows almost same reading as the capacitance gauge, indicating dryness of the environment and in a way, dryness of the vials. In general, the agreement between the two types of pressure gauges (Pirani vs. capacitance) is very indicative of the end of drying of the whole batch. This method is also the most practical from operation point of view. It is most compatible with the: (a) automatic vial loading and unloading systems, (b) clean-in-place (CIP) and stoppering devices, (c) aseptic handling procedures, and (d) steam sterilization. Moreover, these probes are integral to the system (already installed), easy for implementation and calibration, as well as relatively cost-effective.

d. Remote sensing of product temperatures

As described earlier, disadvantages of temperature probes include: (1) they cannot be used with isolators or automated vial loading systems, (2) only the very front few rows of vials can be probed, and (3) the positioning of the thermocouple wires is hard to control as they tend to knock over vials and stoppers in the chamber. Wireless monitoring of the product temperature using quartz-based resonance circuit devices such as commercially available temperature remote interrogation system (TEMPRIS) is a newer technique that has been successfully used in both the R&D and commercial setup to measure the product temperatures and effectively monitor progress of the lyophilization cycle.

4. Consideration of nonuniformity of rate of drying in the chamber

It is well known that there exists a considerable variability in the rate of drying in the chamber due to the variability of product temperatures. This is due to the fact that vials may receive variable heat supply from radiative and other mechanisms depending upon the location of the vials. This also differs from one lyophilizer unit to another and generally results in variable drying rates—mainly during the primary drying phase where the shelf temperatures tend to be at subzero temperatures. The rate is largest for the vials located in the front of the chamber followed by those located on the sides and corners followed by the vials located in the middle of the chamber. The nonuniformity of product temperatures in a large lyophilizer may also occur due to one or more following reasons:

a. Location of vials
b. Position of shelf
c. Uneven freezing due to silicon fluid circulation
d. Dimensional differences in vials
e. Freezing/supercooling
f. Improper stopper placemen
g. Freezing time differences (initial and the last vial)

Figure 2 shows a time/temperature profile of a typical lyophilization cycle in which product temperatures from vials located at various places were monitored. There exists a large variability within the chamber during the early phase of drying as

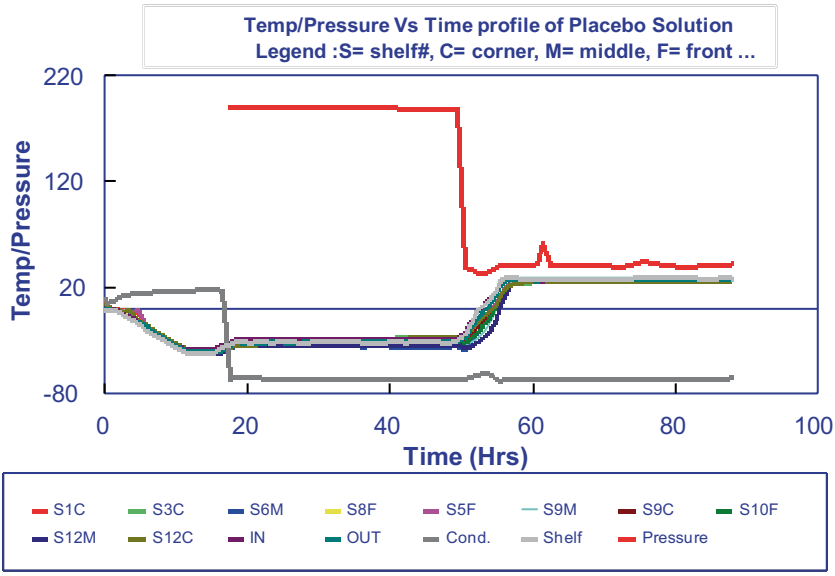

Fig. 2 Time-temperature profile of a typical lyophilization cycle in which product temperatures from vials located at various places were monitored

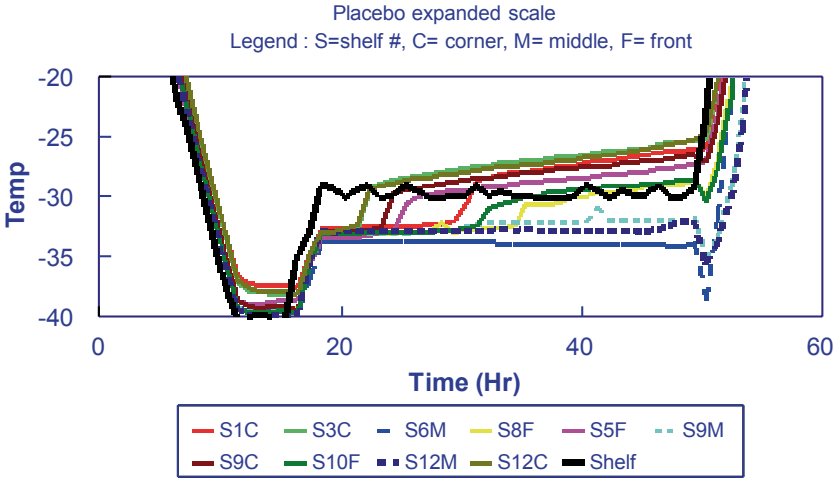

Fig. 3 Expanded view of time–temperature profile of a typical lyophilization cycle. Product temperatures from vials located at various places were monitored. Nonuniformity of product temperature in large lyophilizer is evident

seen from the expanded view in the Fig. 3. During scale-up and transfers of the lyophilization cycles, considerations must be given to the slowest drying vials and cycles should be designed to ensure completion of primary drying step of these slow drying vials before moving on to the next stage of secondary drying where the shelf temperatures are sharply raised.

5. Effect of stopper vent type and size during transfer of lyophilized products

Many a times, it becomes necessary to transfer an existing lyophilization cycle using a different kind of stopper—in type or in the number of vents due to many operational reasons. It becomes a question, if the existing cycle will have to be changed due to such change. A recent paper has described the effect of vent size on the drying rates during lyophilization [17]. Using precisely governed vent area, the authors concluded that as the water vapor transfer across the vents diminishes as the vent area is decreased and the drying rate profile follows choked and non-choked regimes. They further showed that as long as the vent areas were greater than the critical mass transfer restriction threshold, there should not be any difference in the overall drying behavior, and substitution of stoppers within the range should yield similar drying cycles. In very aggressive cycles, where the water vapor flux across the stopper vent is very high, then the dimension of the vent may become rate limiting and impede mass transfer. This may result in accumulation of water vapor inside the vial and become choked flow across the vent resulting in rise in the product temperature and adverse effect on the cake quality.

6. Effect of load size on the duration of the lyophilization cycle

It is often asked if the total duration of a cycle depends upon the load size in the chamber or if the cycle will be longer for a fully loaded chamber than a partially

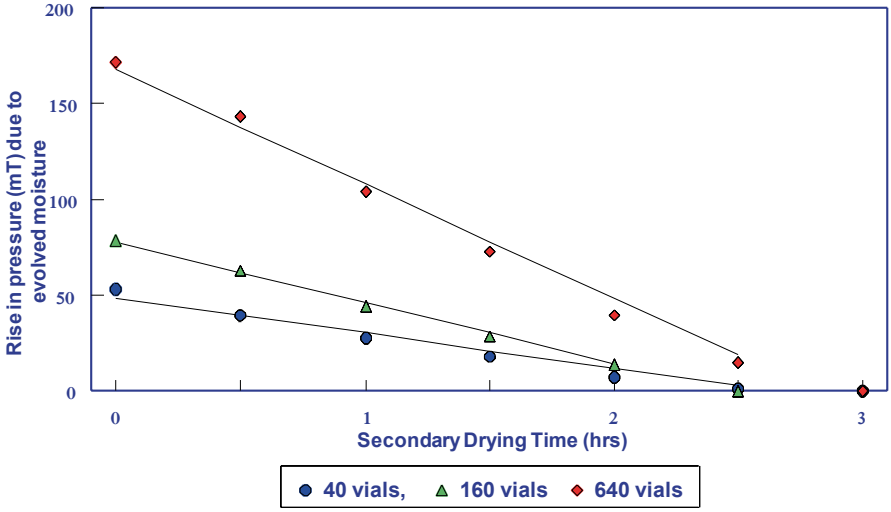

Fig. 4 End of drying as measured by pressure rise measurement method in various loads (40, 160, and 640 vials)

loaded chamber. One must keep in mind, that each vial dries independently of others and hence the cycle should be the same for one vial or thousands, barring minor interdependencies. In one study, chamber bleed method in which the valve between the chamber and the condenser is briefly closed to assess the rise in the chamber pressure was used. As the time passed, the subsequent rise in pressure decreased and ultimately there was no appreciable pressure rise in the system—an indication of the dryness of the load. As seen from the Fig. 4, the 40-vials load, the 160-vials load, and the 640-vials load showed end of drying at approximately the same time independent of the number of vials. Thus, as long as the drying process does not result in exceeding the capacity of the vacuum/condenser system (e.g., efficiency ratio, drying factor) or the rate of drying is not too high which might overwhelm the vacuum/condenser system (e.g., high shelf temperature, high ramps), the duration of cycle is independent of the load in the chamber.

Technology Transfer Report

At the end of the TT process, a TT summary report should be generated to discuss and summarize the TT results (including process robustness/capability and validation results), compare yields, product and process quality for the transferring and receiving sites, list the manufactured process justification lots and rationale, provide completion date for each of the key activities (milestones) in the TT process. This report includes all the experience and knowledge learnt during the TT process and becomes part of a very important aspect of the TT process—knowledge transfer. Such a report will typically include the following documentation:

1. Technical data package from sending site.
2. Chemistry and manufacturing control (CMC) sections.
3. List of monographs for raw materials, active product ingredients, and finish product.
4. Drug substance/API and excipient physical characterizations (solubility, morphology), specifications, and manufacturers.
5. Drug substance/API and finish product stability data, special requirements (sensitivity to light, relative humidity), compatibility studies, storage shipping conditions.
6. Equipment list (model, size, special features or accessories) for the sending site.
7. Process description and flow chart including operating parameters (critical).
8. Formula manufacturing documents (FMD) or master batch records (MBR).
9. List of in-process test procedures.
10. List of validated test methods, analytical cleaning methods (including recovery on different surfaces), and cleaning procedures.
11. Development reports including critical processing parameters, batch size, results, process robustness study results, if available.
12. Regulatory commitments before and after approval, if appropriate.
13. Process justification and validation reports.
14. Quality (deviations, incidents, investigations, out of specification results, rejections) history of the product.
15. Process capability, trends, and actual yield results.
16. Process optimization/productivity changes.
17. Environmental health and safety (EHS) review: waste disposal, toxicity, fire/explosives, industrial hygiene and safety protection assessments, material safety data sheets (MSDS), subject exposure limit (minimum daily dose).

Lyophilization Cycle Transfer: Case Study-Evaluation of Functional Equivalency Among Lyophilizers

Currently, there is no common industry practice or regulatory guidance available that can be used to technically evaluate suitability of different lyophilizer units for successful transfer of lyophilization cycles. Recently, a report was published that explored development of comprehensive methodology for establishing functional equivalence between (different) lyophilizers, which can then be used to predict suitability of cycle transfers [17]. This report described the comparative and operational qualification methods that were used, which included: (a) evaluation of comparability of relevant technical characteristics of the lyophilizer units, (b) comparison of sublimation rates using a model compound, and (c) evaluation of parametric equivalence during the cycles.

Three large-scale production lyophilizer units (one 220 sq. ft. and two 420 sq. ft.) were evaluated by this procedure. The results showed that the proposed meth-

odology provides the necessary basis to establish functional equivalence between the tested units. After establishing functional equivalence, it is expected that testing requirements and process validation protocols can be optimized, resulting in considerable savings of time, resources, and capital. Such a procedure is extremely desirable in large manufacturing operations, where multiple lyophilizer units are employed for processing of a common lyophilized product.

Conclusions

An overview of types of TTs from development to validation of the lyophilized product is discussed. A detailed consideration of various activities that must be performed for successful TT of lyophilized product is also described. Evaluation of functional equivalency among lyophilizers as a tool in transfer of lyophilization cycles is reviewed and described. Such results provide the basis to establish functional equivalence among different lyophilizers. Once established, functional equivalence can facilitate successful implementation of scale-up and transfer of lyophilization cycles. Such equivalency procedures are desirable in large manufacturing operations, where multiple lyophilizers are used for processing a common lyophilized product, and reduced validation campaigns based upon a matrix approach can save considerable resources and time.

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