

Lyophilized vaccine development

Nicole M. Payton, Rushit N. Lodaya, Adora M. Padilla

DRUG PRODUCT R&D U.S., GSK, ROCKVILLE, MD, UNITED STATES

Introduction

Vaccine stability has long been a challenge when developing new products, particularly when considering the diverse nature of vaccines. In addition, the distribution and storage of the product in countries that may be limited in the ability to maintain the cold chain storage presents a challenge when developing these often temperature-sensitive antigens. As a result, years of efforts in product development have looked to eliminate or minimize the need for low-temperature storage, i.e., temperatures below 2–8°C. By far, the most widely used method of producing stabilized vaccines is through lyophilization. For vaccines, lyophilization offers many benefits: immobilization of antigens in a glassy matrix through freezing and drying, thereby minimizing time in the solution state; longer shelf life for final products; possible elevated storage temperatures (higher than –20°C); flexibility of dosing different target populations (i.e., pediatric, maternal, and older adult); and reduced impact of temperature excursions on product quality. Besides, lyophilization has the added benefit of enabling multivalent vaccine development by minimizing the potential for antigen–antigen interaction over time. Due to the complexity of vaccines by nature, such as type of antigen(s), susceptibility for degradation, the potential for adjuvant, and multivalence, lyophilization offers an attractive and often faster approach for stabilization during development and progression to commercial manufacturing [1–4].

During early clinical development, the dosing strategy for an indication is under evaluation. Lyophilization of the antigen or antigens may allow for more rapid development, easy dosing, and mixing strategies for early clinical assessment. For example, an evaluation of more than one antigen can be performed by lyophilizing each antigen separately and reconstituting one and then another such that the evaluation of the stability of a multivalent vaccine only needs to be assessed during the short hold time leading up to administration. This evaluation also aids in minimizing the analytical activities needed for multivalent formulations. Additionally, there is flexibility in the reconstituting agent allowing for the use of different adjuvants and diluents or both that may be under evaluation for a target population, i.e., pediatric, adolescent, maternal, or

older adults. This flexibility in dosing options presents an opportunity to address unmet medical needs more quickly with early evaluation.

In some cases, antigens may be valuable to more than one population, such as maternal versus older adults, and thus might require two different reconstitution schemes and perhaps two different doses. Manufacturing capacity may be optimized for both indications by lyophilizing one vial for both purposes: adjuvanted and non-adjuvanted. Lyophilization may even circumvent the need for a two (or more)-vial presentation by allowing for adjuvant and antigen(s) to be lyophilized in a single vial presentation.

Of great concern, and often highlighted as a risk, is the cold chain needed to store vaccine products. A review by Kumru et al. [1] discusses in detail the strategies by which vaccine formulation has looked to address the cold chain concern. Of note are live-attenuated viral and bacterial vaccines, which are often susceptible to elevated temperatures during storage and distribution. These vaccines often require lyophilization for stabilization but still need a 2–8°C storage. Thus, the cold chain is always a potential concern, and sometimes even short-term deviation from the intended storage condition can result in a loss in potency of the vaccine. The reality is that there is not always a strong understanding of what drives this potency loss, and thus, formulation strategies may be insufficient in protecting the antigen(s) from temperature-induced effects.

Cost and time to develop vaccines can vary quite significantly. These attributes can be primarily due to the type of antigen, and from which starting materials, it is produced, e.g., human cell line, CHO cell, synthetically produced RNA, etc. It may also be due to sensitivities of the antigen(s) and the potential for multivalent vaccines requiring more complex processes, analytical assays, and presentation of stability challenges. A recent review from 2017 [5] highlighted the complexities associated with vaccine development and the potentially huge costs associated with this development. Product development alone was estimated to be upward of 500M USD and could take many years to complete. Also, a company will need to invest up to 600M+ USD in just facilities costs to accommodate the capacity required to supply the intended population. Labor costs and overhead also factor into the overall vaccine cost [5]. The Coalition for Epidemic Preparedness (CEPI) performed and published a study in 2018, in which they estimate the total cost of vaccine development from 36M USD (on the low end) to over 2B USD (on the high end) [6]. These numbers highlight just how substantial the investment is, but this certainly pales in comparison with the impact on human life, as vaccines are estimated to have averted millions of deaths from diseases such as measles, mumps, rubella, polio, and more [2].

This chapter aims to review the application of lyophilization in the development of vaccines, from both a historical perspective and a forward-thinking perspective. We aim to outline the benefits and some of the challenges of the employment of lyophilization to vaccines targeting large populations across the globe and therefore being critical on supply. Besides, we outline a variety of vaccine antigens that have been developed or are currently being developed with a focus on challenges and advantages by employing

lyophilization in the product final process. We refer the reader to [Table 11.1](#) as a reference for current FDA-approved vaccines that require lyophilization [7].

Overview of lyophilization formulation and process

The lyophilization of pharmaceutical products, such as vaccines, is often carried out to improve stability during shipment and storage. However, the lyophilization process also exposes active pharmaceutical ingredient (APIs), such as protein or nucleic acid-based antigens, to stresses during each of the three main steps of the lyophilization process: freezing, primary drying, and secondary drying. Upon loading filled vials into the lyophilizer, the shelf is cooled (e.g., -40°C), resulting in the formation of ice but also decreasing the product temperature sufficiently to achieve an immobilizing glassy matrix (i.e., the temperature of the product is below the product T_g'). The glass transition of the freeze concentration or T_g' depends on the characteristics of a particular amorphous excipient such as the molecular weight and shape, the water content, and the presence of other excipient components, such as salts, plasticizers, or small molecule stabilizers [8]. After freezing is complete, the shelf temperature is increased (e.g., -25°C), and the pressure within the chamber is reduced (e.g., 50–150 mTorr), thereby initiating primary drying [9]. As the temperature is increased and sublimation of the ice begins, the product temperature is maintained below the T_g' to prevent undesirable morphological changes, such as collapse. Although collapse may not result in increased degradation, unwanted appearance, longer reconstitution time, longer drying times, and potentially excipient crystallization may occur as a result of cake collapse [10,11]. The endpoint of primary drying can be assessed using several different approaches, from classical methods such as measuring product temperature to more recently developed, robust process analytical technology (PAT) tools such as tunable diode laser absorption spectroscopy (TDLAS). At the end of primary drying, the frozen concentrate contains approximately 20% water [8,11]. Upon completion of the primary drying step, the shelf temperature is further increased (e.g., $+40^{\circ}\text{C}$) to enable secondary drying, which removes bound or associated water molecules.

Each of the steps of the lyophilization process imparts stresses on vaccine antigens, which can result in physical or chemical degradation, depending on the antigen's specific molecular characteristics. Furthermore, due to the complex nature and the diversity of vaccine antigens (proteins, nucleic acids, lipid-based vectors), it is often necessary to evaluate and develop a lyophilization process for each specific antigen, keeping in mind the resulting stresses and the interplay between the formulation and the process. In the subsequent sections, the stress associated with each of the steps of the lyophilization process will be discussed with an emphasis on the impact of these stresses on stability and how formulation can be used as a tool to minimize the effect of these stresses.

Table 11.1 Lyophilized Vaccines approved in the United States.

Vaccine	Trade name	Company/Sponsor	Year approved	Mode of administration	Antigen description (protein/conjugate/polysaccharide etc.)	Storage temperature	Reconstitution medium	Final product excipients
Meningococcal polysaccharide vaccine, groups A, C, Y, and W-135	Menomune-A/C/Y/W-135	Sanofi Pasteur, Inc	1981	subQ	Conjugate	2–8°C	Sterile water for injection (multidose contains thimerosal)	59 ug/mL mercury, 5–10 mg/mL lactose as stabilizer [0.5 mL dose]
Combined Varicella virus vaccine live	Varivax	Merck & Co, Inc	1995	subQ	Live-attenuated virus	2–8°C	Sterile water for injection	36 mg/mL of sucrose, 17.8 mg/mL hydrolyzed gelatin, 7.2 mg/mL of urea, 4.6 mg/mL of sodium chloride, 0.72 mg/mL of monosodium L-glutamate, 0.66 mg/mL of sodium phosphate dibasic, 114 ug/mL of potassium phosphate monobasic, and 114 ug/mL of potassium chloride [0.5 mL dose]
Hemophilus b conjugate vaccine (tetanus toxoid conjugate)	ActHIB	Sanofi Pasteur, SA	1996	IM	Conjugate	2–8°C	0.4% chloride	8.5% sucrose, 0.4% NaCl [0.5 mL dose].
Rabies vaccine	RabAvert	GlaxoSmithKline Biologicals (formerly Novartis Vaccines and Diagnostics)	1997	IM	Inactivated virus	2–8°C	Sterile water for injection	1 mg/mL potassium glutamate, 0.3 mg/mL sodium EDTA [1 mL dose]
Measles, mumps, rubella, and varicella virus vaccine live	ProQuad	Merck & Co, Inc	2005	subQ	Live-attenuated virus	2–8°C	Sterile water for injection	40 mg/mL of sucrose, 22 mg/mL of hydrolyzed gelatin, 5.0 mg/mL of urea, 4.6 mg/mL of sodium chloride,

32 mg/mL of sorbitol, 0.76 mg/mL of monosodium L-glutamate, 0.28 mg/mL of sodium phosphate, 0.50 mg/mL of human albumin, 0.26 mg/mL of sodium bicarbonate, 188 ug/mL of potassium phosphate, 116 ug/mL of potassium chloride; [0.5 mL dose]	63.1 mg/mL of sucrose, 31.5 mg/mL of hydrolyzed porcine gelatin, 13.2 mg/mL of urea, 8.1 mg/mL of sodium chloride, 1.26 mg/mL of monosodium L-glutamate, 1.1 mg/mL of sodium phosphate dibasic, 0.20 mg/mL of potassium phosphate monobasic, 0.20 mg/mL of potassium chloride [0.65 mL dose]	Lyophilized component: 6–8 mM HEPES (pH 6.5–7.5), 20 mg/mL human serum albumin USP, 5–7 mg/mL sodium chloride USP, 50 mg/mL mannitol USP. Diluent vial: 50% (w/v), glycerin USP, 0.25% (v/v), phenol USP in water for injection USP [1 dose = 2.5 uL = 15 jabs]	Sterile water for injection	2–8°C	50% (w/v) glycerin USP, 0.25% (w/v) phenol USP in water for injection USP							
Zoster vaccine, live, (OKa/ Merck)	Zostavax	ACAM2000	Merck & Co., Inc.	2006	2007	subQ	Live-attenuated virus	Percutaneous	2–8°C	Live-attenuated virus	Smallpox (vaccinia) vaccine, live	Emergent Product Development

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Table 11.1 Lyophilized Vaccines approved in the United States. —cont'd

Vaccine	Trade name	Company/Sponsor	Year approved	Mode of administration	Antigen description (protein/conjugate/polysaccharide etc.)	Storage temperature	Reconstitution medium	Final product excipients
Diphtheria and tetanus toxoids and pertussis adsorbed, inactivated poliovirus and hemophilus b conjugate (tetanus toxoid conjugate)	Pentacel	Sanofi Pasteur Limited	2008	IM	Inactivate + Conjugate	2–8°C	Aluminum phosphate suspension vial 1 (adsorbed diphth, Tet and pert Ags + IPV)	3 mg/mL aluminum phosphate, 85 mg/mL sucrose, 0.6% v/v phenoxyethanol (not a preservative) [0.5 mL dose]
Measles, mumps, and rubella virus vaccine, live	M-M-R II	Merck & Co, Inc	2008	subQ	Live-attenuated virus	2–8°C	Sterile water for injection	29 mg/mL sorbitol, sodium phosphate, 3.8 mg/mL sucrose, sodium chloride, 29 mg/mL hydrolyzed gelatin [0.5 mL dose: supplied as single dose vials of diluent and single dose vials of vaccine]
Rotavirus vaccine, live, oral	ROTARIX	GlaxoSmithKline Biologicals	2008	Oral	Live-attenuated virus	2–8°C	Calcium carbonate, sterile water, and xanthan	Amino acids, dextran, Dulbecco's Modified Eagle Medium (DMEM), sorbitol, and sucrose, calcium carbonate (antacid to protect vaccine during passage through stomach), sterile water, and xanthan. [1 mL dose: supplied as single-dose vials of lyophilized vaccine + a prefilled oral applicator of liquid diluent]

Yellow fever vaccine	YF-Yax	Sanofi Pasteur, Inc	2008	subQ	Live-attenuated virus	2–8°C	0.9% Chloride	Sorbitol and gelatin (stabilizers) and NaCl [0.5 mL dose]
Hemophilus b Conjugate vaccine (tetanus toxoid conjugate)	Hiberix	GlaxoSmithKline Biologicals, S.A.	2009	IM	Conjugate	2–8°C	0.9% Chloride	25.2 mg/mL lactose, 0.9% NaCl [0.5 mL dose]
Meningococcal (groups A, C, Y, and W-135) oligosaccharide diphteria CRM197 conjugate vaccine	Menveo	GlaxoSmithKline Biologicals (Novartis Vaccines and Diagnostics)	2010	IM	Conjugate	2–8°C	MenCWY	No excipients listed [0.5 mL dose]
BCG live	BCG vaccine	Organon Teknika Corp LLC	2011	Percutaneous	Live-attenuated virus	2–8°C	Sterile water for injection	Glycerin/asparagine/citric acid/potassium phosphate/magnesium sulfate/iron ammonium citrate/lactose [1.0 mL single dose].
Rabies vaccine	Imovax	Sanofi Pasteur, SA	2011	IM	Inactivated virus	2–8°C	Sterile water for injection	No excipients given [1 dose = 1 mL]
Zoster vaccine, recombinant adjuvanted	Shingrix	GlaxoSmithKline Biologicals	2017	IM	Recombinant subunit protein	2–8°C	AS01 adjuvant	20 mg of sucrose (as stabilizer), 4.385 mg of sodium chloride, 1 mg of DOPC, 0.54 mg of potassium dihydrogen phosphate, 0.25 mg of cholesterol, 0.160 mg of sodium dihydrogen phosphate dihydrate, 0.15 mg of disodium phosphate anhydrous, 0.116 mg of dipotassium phosphate, and 0.08 mg of polysorbate 80

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Table 11.1 Lyophilized Vaccines approved in the United States. —cont'd

Vaccine	Trade name	Company/Sponsor	Year approved	Mode of administration	Antigen description (protein/conjugate/polysaccharide etc.)	Storage temperature	Reconstitution medium	Final product excipients
Dengue vaccine	DENGVAZIA	Sanofi Pasteur Inc.	2019	subQ	Live-attenuated virus	2–8°C	0.4% Chloride	2 mg sodium chloride (from saline diluent) and the following ingredients as stabilizers: 0.56 mg essential amino acids (including L-phenylalanine), 0.2 mg nonessential amino acids, 2.5 mg L-arginine hydrochloride, 18.75 mg sucrose, 13.75 mg D-trehalose dihydrate, 9.38 mg D-sorbitol, 0.18 mg trometamol, and 0.63 mg urea

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Freezing

Initially, a thermal equilibration step in which the shelf temperature is decreased to 5 or -5°C , for example, may be utilized to increase product temperature homogeneity. Then, the shelf temperature is reduced resulting in ice nucleation, the spontaneous formation of molecular water aggregates capable of growing into ice crystals [8]. Typically, nucleation and ice formation occurs stochastically and is due to the presence of particulate impurities [8]. However, with the use of approaches such as ice fog or Praxis controlled nucleation, ice nucleation can occur more homogeneously across all the vials on a sample chamber shelf. When ice forms, the solutes in the remaining liquid, such as the antigen, buffer salts, and sugar stabilizers, become concentrated by as much as 20 to 50 times their initial concentration [12]. Besides resulting in the potential for increased molecular interactions, the increased concentration can dramatically increase the rate of degradation reactions even at temperatures below the equilibrium freezing point of water [8,12]. Freeze concentration and the resulting exposure of the API to the ice–water interface are two of the primary sources of stress encountered during the lyophilization process. The temperature at which ice nucleation occurs and the freezing rate correlate with ice crystal size and the surface area of the ice crystal. For example, a higher freezing rate and lower nucleation temperature result in smaller ice crystals with a larger surface area. Consequentially, more extensive ice–water interfaces and higher levels of surface-inducing protein destabilization [13]. Depending upon the nature and characteristics of an antigen molecule, freeze concentration or exposure to ice–water interfaces may lead to physical degradation (i.e., aggregation) or chemical degradation (i.e., oxidation) [14,15].

Besides a linear decrease in shelf temperature to the final freezing temperature, annealing can be included as a part of the freezing step. Annealing involves increasing the shelf temperature such that the product temperature exceeds the T_g' often by $10\text{--}20^{\circ}\text{C}$ for several hours [9,13] with the shelf temperature subsequently decreased back to the initial freezing temperature. Annealing can be utilized to promote the formation of larger ice crystals, thereby reducing or eliminating the dependence of ice crystal size on nucleation temperature and increasing interval homogeneity [13]. Larger ice crystals form when smaller ice crystals, which have a higher likelihood of melting due to their greater free energy, melt and subsequently recrystallize with the larger ice crystals already present acting as nucleation sites [13]. The use of annealing to create larger ice crystals, which ultimately leads to larger pores within the lyophilized cake, has been shown to impact subsequent steps of the lyophilization process, namely primary drying [16]. Besides affecting ice crystal size, annealing can also be utilized to promote the crystallization of excipients such as mannitol. Because amorphous mannitol has a low T_g (approximately 13°C) which would effectively lower the T_g of an amorphous mixture as observed by Kim et al. [17] and the crystallization of mannitol during storage can result in the release of water into the amorphous matrix [18], achieving complete crystallization of mannitol during lyophilization is critical. An additional aspect to consider

when utilizing mannitol as a crystalline stabilizer is the formation of the hemihydrate polymorph, which can convert to an anhydrous polymorph (i.e., mannitol hemihydrate dehydration) during storage releasing water into the amorphous mixture [19]. The formation of mannitol hemihydrate is influenced by several factors, including the temperature profile utilized during the freezing step as well as the temperature used for annealing [20]. Preventing the formation of mannitol hemihydrate is critical as once formed, it is difficult to ultimately convert the hydrate to the anhydrous form during secondary drying, even if high temperatures and longer durations are used [20].

Formulation design, or the selection of excipients as well as their respective concentrations, can be utilized to mitigate, as least to an extent, the impact of the stresses encountered during freezing. While exposure to the surface of ice crystals during the freezing process is essentially inevitable, the use of nonionic surfactants, which compete with proteinaceous antigens for adsorption at the ice–water interface, has been shown to mitigate or at least partially mitigate the stress resulting from exposure to the ice–water interface [21]. In one such study, Chang et al. [22] demonstrated that several different surfactants, including Tween 80, Brij 35, and Triton X-10, were able to prevent denaturation and aggregation of a model protein, lactate dehydrogenase. Although the selection of the appropriate buffer salts depending on the formulation target pH is important, the behavior of the buffer salt during freezing should also be considered. Sodium phosphate buffer salts are often avoided when possible, especially when the concentration exceeds 10 mM due to crystallization of the dibasic form, which can lead to an acidic pH shift to as low as 3.8 [23]. However, many buffer systems exhibit a shift in pH upon freezing, although the magnitude of the shift varies depending upon factors such as buffer salt concentration and the presence of other excipients, such as sucrose [21,24,25]. The impact of solution pH is often evaluated as it is a critical parameter affecting the physical and chemical stability of pharmaceutically relevant molecules, both during formulation in the liquid state before lyophilization and during the lyophilization process. Lang et al. [26] evaluated the impact of pH, as well as other formulation parameters, on the stability of a VLP-based vaccine against nicotine addiction (NicQb). Across the pH range, which was evaluated, 4.6–8.2, it was observed that cleavage of the succinate linkage that coupled nicotine to VLP and VLP fragmentation increased significantly as the formulation pH was increased.

Conversely, it was observed that VLP aggregation increased as the pH was decreased, indicating an optimal pH range of 6.2–6.6 should be targeted to minimize degradation. Another factor for consideration during formulation design is the formation of distinct phases during the freezing process resulting in the formation of antigen (e.g., protein)-rich and sugar-rich phases. Phase separation is likely to occur when it is both thermodynamically favored (e.g., at low temperatures and high concentrations) and kinetically allowed (e.g., product temperature exceeds the T_g) [27]. Evaluating the propensity of a formulation to remain intimately mixed upon freezing can be an important consideration as a lack of intimate mixing or phase separation may impact the stability of APIs, such as protein-based antigens. The stability of a model IgG protein was evaluated in

formulations containing trehalose, inulin (1.8 and 4 kDa), and dextran (1.5, 5, and 70 kDa) after lyophilization and storage at 60°C [28]. A lower level of aggregation upon storage was observed in formulations containing smaller, more molecularly flexible sugars [28]. The smaller sugars were thought to be less inhibited by steric hindrance and configurational inflexibility resulting in a higher propensity to hydrogen bond with the model protein, IgG [28].

Further work is needed with pharmaceutically relevant proteins, excipients, and stresses to understand better the implications of phase separation on long-term storage stability. Formulation design can play a role in the occurrence of phase separation as some excipients, particularly polymers such as PVP and dextran, but also potentially more pharmaceutically relevant excipients may undergo phase separation. Thus, an understanding of the freezing process and the physical state of the components comprising the formulation along with the relevant antigens is important, particularly when trying to understand the impact of the entire freeze-drying process on product quality, i.e., both freezing and drying.

Primary drying

Upon completion of the freezing step, primary drying is initiated by increasing the shelf temperature and decreasing the chamber pressure. The collapse temperature of the product dictates the maximum shelf temperature, which can be utilized during the primary drying step, with the product temperature typically 5–40°C less than the shelf temperature [9]. Generally, the shelf temperature should be selected to dry the lyophile efficiently without exceeding the T_c . However, other factors such as chamber pressure, heat transfer coefficient of the vial, and the model of the lyophilizer itself also impact the temperature of the product. A conservative starting point for setting the shelf temperature during primary drying can be estimated using coupled steady-state heat and mass transfer theory [9]. Chamber pressure during primary drying should be optimized to facilitate homogeneous heat transfer and a high sublimation rate. A chamber pressure, which is too low, may be difficult to maintain consistently, risk back-streaming may result in inconsistent heat transfer while a pressure, which is too high, may result in a low sublimation rate due to a small difference in the vapor pressure of ice and the chamber pressure [9]. Determining the end of primary drying can be accomplished using several different approaches, including comparative pressure measurement (Pirani vs. capacitance manometer), pressure rise testing, and TDLAS (tunable diode laser absorption spectroscopy) (e.g., analysis of water vapor concentration profiles) among others [29]. The stresses imparted by primary drying have been evaluated in a limited number of studies. In one such study, the solution state stability, as well as the impact of freezing, primary, and secondary drying stresses on lactate dehydrogenase, was evaluated. Although collapse was evident in some of the formulations which were assessed,

cake collapse did not correlate with degradation or instability [11]. As the main stress, APIs are exposed to during drying associated with dehydration or loss of the hydration shell. Because primarily bulk ice is undergoing sublimation during primary drying, the potential impact of dehydration will be discussed about secondary drying.

Secondary drying

Secondary drying, which removes water from the amorphous freeze concentrate, is initiated by increasing the shelf temperature, usually for a limited duration, such as 4–6 h. As the rate of secondary drying is mainly limited by the diffusion of water molecules within the solid and evaporation or both at the solid/vapor interface, the chamber pressure is not a significant factor, and often the chamber pressure used during primary drying is maintained [30]. At the beginning of secondary drying, approximately 10% of the initial water or ice in the product remains [11]; however, by the end of secondary drying, the residual moisture content is typically less than 1%.

By conducting freeze–thaw studies, as well as studies that assess the product before and just after lyophilization, it is possible to elucidate if the freezing step, the drying step, or both are contributing to degradation occurring during the lyophilization process. During secondary drying, as associated or bound water molecules are removed via sublimation, a complex API molecule, such as a large protein, may be subjected to dehydration stresses or loss of the hydration shell. Specifically, for molecules, such as proteins, perturbation of the hydration shell has been associated with decreased stabilization of the native state resulting in an increased propensity for aggregation [23]. It has been argued that sugar molecules form hydrogen bonds with APIs, in place of water molecules (i.e., water substitution hypothesis), and that the formation of an immobilizing glassy matrix, which limits molecular mobility (i.e., vitrification hypothesis), can aid in preserving the native state of proteins or other complex macromolecules such as nucleic acids [23,30–32]. In one study, FTIR was utilized to assess dehydration-induced conformational changes of several model proteins such as basic fibroblast growth factor (bFGF), interferon- γ (IFN- γ), granulocyte colony-stimulating factor (G-CSF), and lactate dehydrogenase (LDH) [33]. For lyophilized (dehydrated) formulations, a difference in amide I region was observed relative to comparable aqueous formulations. The marked differences in the FTIR spectra were attributed to the general disordering of the protein backbone indicative of conformational changes or multiple conformations [33]. Additionally, the effect of including excipients, such as disaccharides, monosaccharides, and polyhydric alcohols, in the model protein formulations was evaluated. Although the results were often protein specific, typically the disaccharide stabilizers, such as sucrose, prevented conformational changes, while other excipients either had no impact or increased the observed conformational changes.

Vaccine-specific considerations

Conjugates and multivalent conjugate vaccines

Conjugate vaccines, in which capsular polysaccharides are covalently bound to a protein-based carrier, are of particular interest as they are highly effective in the prevention of disease [34]. Conjugate vaccines, which are currently available, commercially include the *Hemophilus influenzae* type b (Hib) vaccine as well as the meningococcal group C conjugate (MnCC) vaccine and the pneumococcal conjugate (PnC) vaccine [34,35]. Much interest in conjugate vaccines continues as this class of vaccines are capable of eliciting T cell–dependent responses that have been particularly effective in preventing disease in infants and children. For example, capsular Vi polysaccharide and type 5 or type 8 capsular polysaccharide have been evaluated as conjugate vaccine candidates to prevent infection by *Salmonella typhi* and *Staphylococcus aureus* [34]. Also, to develop vaccines aimed to avoid infection by pathogens, which have posed a challenge for decades, there is an opportunity to develop vaccines for low-income countries against pathogens such as Hib [35].

Production of conjugate vaccines is a complex multistep process [36]. At a high level, the creation of a conjugate vaccine involves the individual production of each polysaccharide followed by purification and chemical modification to generate reactive groups to facilitate linkage to the carrier protein. It has been reported that conjugation efficiency can be relatively low, around 20%, but with the optimization of the conjugation reactions, efficiency can be improved to approximately 50%. Upon production of the polysaccharide–carrier protein conjugate, further purification is typically required. Following purification, if necessary, as with a product containing multiple serotypes, such as Menveo and Prevnar, the individual polysaccharide–carrier protein conjugates can be combined during the formulation process [36]. Although conjugate vaccines can be targeted at preventing infection by a particular virus or bacteria (i.e., *H. influenzae* type b and the Hiberix vaccine), multivalent conjugate vaccines have also been successfully developed. Most of the information regarding the lyophilization of conjugated vaccines is related to the vaccine products which are available commercially, such as Hiberix.

Although the development and commercialization of multivalent conjugate vaccines can be challenging (e.g., complex purification and production process, characterizing and minimizing interference caused by the coadministration of multiple antigens) [36,37], decreasing the incidence of various diseases with a single vaccination is advantageous, both in terms of creating a significant opportunity to impact public health positively but also from a marketability perspective. Menveo is an example that highlights not only the positive impact a product can have on public health but also some of the challenges that can arise during the development of a lyophilized multivalent

conjugate vaccine. Menveo is available as a two-vial presentation with a lyophilized vial containing MenA [38]. The lyophilized MenA vial is reconstituted with an aqueous solution containing MenCWY. As demonstrated by Beresford et al. [39], there is a serogroup-specific stability pattern with MenA degrading more readily (e.g., aggregation, the formation of free saccharide, and depolymerization) relative to the CWY components in solution. MenA is particularly susceptible to degradation under acidic conditions due to the presence of acid labile phosphodiester groups, which is particularly significant as the structural integrity of the MenA polysaccharide is correlated with immunogenicity [39]. During the development of a vaccine, it is essential to consider the potential for shifts in pH, which can be observed during freezing due to the crystallization of buffer salts. Another potential challenge could be encountered during the lyophilization of conjugate vaccine products when hydrolysis of saccharides from the surface of the protein result in exposure of hydrophobic patches on the carrier proteins [39]. Considered in combination with the freeze concentration that occurs during the freezing step, exposure of hydrophobic patches could impact carrier protein aggregation. Currently, very little is available to inform the scientific community about the development and optimization of lyophilization processes specifically for multivalent or conjugated vaccines [40,41].

Live-attenuated

Live-attenuated vaccines have the advantage of generating both a humoral and cellular response, thereby offering long-term immunity after only one to two immunizations and without an adjuvant. They consist of viruses that have been “attenuated” by growing in cells that they usually would not grow in, thereby losing virulence for the intended target species while still maintaining the ability to confer protective immunity, as they mimic the natural route of infection [2,42]. The production of these vaccines is often easier than inactivated vaccines. However, they may present more formulation and stability challenges. Many of the marketed live-attenuated vaccines are stored in the dry state or require low-temperature storage to ensure shelf life. Thus, the lyophilization of these antigens is an excellent area to explore and understand [2].

Many components of a live viral vaccine antigen may be impacted by freezing. As previously discussed in this chapter, during the freezing process, the solute concentrates in the interstitial space upon nucleation and growth of ice crystals. As such, the antigens will be exposed to low temperatures and high concentrations of excipients, resulting in increased tonicity and osmolality, which may detrimentally impact the stability of the viral membrane. Additionally, proteins at the virus surface may also be affected by these factors, along with the formation of ice or crystallization of components in the matrix. The membrane or lipid bilayer of a live-attenuated virus is a fragile component of the antigen and is often susceptible to freezing-related damage. The external and internal environment of the virus will vary significantly through the process, and the impact is likely more significant during the freezing process. The nature of the excipient, e.g., amorphous versus crystalline, along with the freezing rate and temperature, may be

used to tailor the conditions that are observed by the antigen during this step of the freeze-drying process. Also, it is necessary to avoid ice crystallization and growth inside the virus, as this will result in disruption of the viral membrane and, ultimately, loss in potency.

A method for freezing of attenuated viral vaccines that may be employed involves loading the filled vials onto precooled shelves. This method will typically allow for more rapid freezing, and as the temperature cools, the water will leave the interior of the virus at a rate which prevents internal ice formation and thus disruption of the virus [2,42]. For instance, in the stabilization of Takeda's tetravalent dengue vaccine (TDV), the authors used a precooled shelf at -45°C , followed by a slow ramp to -37°C for primary drying [43]. For lyophilization of live-attenuated gdhA derivative *Pasteurella multocida* B:2, vials were loaded on a precooled shelf at -30°C , followed by primary drying at -30°C [44]. The freezing process may also be optimized for live-attenuated vaccines by utilizing a slow cooling rate as well, as this has demonstrated improved in-process stability for some antigens.

Carefully selected formulation components can help mitigate the impact of lyophilization process-related stresses and the loss of potency in live-attenuated vaccines [2,43,45,46]. Virus activity can be impacted by deviations in physiological pH [2]. Such pH shifts during lyophilization have been highlighted as a concern in the development of lyophilized products for decades [23]. This concern can be eliminated by proper choice of buffer and appropriate concentrations of the buffer components. By formulating phosphate at low concentrations and in the presence of amorphous excipients at sufficient concentrations to prevent crystallization of phosphate, the potential for a pH shift or one that deviates enough to impact viral stability is restricted.

Other formulation opportunities for stabilizing live virus antigens employ carefully selected stabilizers. Stabilizers could involve the selection of amorphous sugars, small molecule excipients (plasticizers), proteins such as HSA, gelatin, and more. Ideally, the selection of these stabilizers would mostly rely upon a thorough understanding of the degradation of these antigens, as is the case for many protein pharmaceutical systems [18]. However, given the complex nature of live viruses, the selection of stabilizers is mainly empirical. A design of experiments (DOE) approach performed on a tetravalent dengue viral vaccine utilized a variety of additives (sugar alcohols, surfactants, chelators, amino acids, polymers, and more) in combination with the more common sucrose and trehalose sugar stabilizers [43]. The authors sought to evaluate the interplay between these additives, trehalose, sucrose, and viral antigens. Through a series of experiments, they identified that formulations containing certain combinations of amino acids and urea-containing formulations conferred the highest stability for the vaccine candidate. Also, when mannitol was included, as a bulking agent, the highest potency was observed after 5 weeks of storage at 25°C . The predicted storage stability was confirmed with the model developed through real-time storage stability monitoring. Thus, the authors have demonstrated the potential importance of urea in conjunction with sucrose or trehalose as an additive for live viral vaccine stabilization [43]. Amino acids, in combination with

trehalose, were similarly found to be stabilizing for a live-attenuated camelpox vaccine, suggesting that a combination of amino acids and sugars is potentially a formulation combination of choice for such systems [46]. Amino acids, sorbitol, glycerol, and other small molecules fall into a category known as plasticizers or small molecules, which lower the free volume, and as the name suggests, reduce the glass transition in a solid [47,48]. Lovalenti et al. demonstrated the applicability of plasticizers in stabilizing a live-attenuated influenza vaccine through freeze drying, foam drying, and spray drying [42]. Not only did the plasticizers improve stability, but it was also demonstrated that spray drying and foam drying (both methods do not require freezing) were superior to lyophilization, suggesting further that not only formulation but also the freezing process is vital to live-attenuated vaccine stability.

Certainly, formulation and process development must yield a postlyophilization product with sufficient potency to elicit the necessary immune response. However, long-term stability is also vital to ensure that the product can be stored and shipped for adequate periods, allowing for distribution and immunization, potentially worldwide. The stability of live-attenuated vaccines is impacted by moisture content, similar to many other pharmaceutical products. Establishing a process that yields low moisture is essential to ensuring a longer shelf life for the product [30].

Subunit vaccines

Early vaccine development employed the use of live-attenuated vaccines, but due to potential safety concerns, new approaches using subunits of viruses or bacteria have been evaluated and developed. Subunits refer to an antigenic component of the virus or bacteria, such as a surface protein that elicits an immune response [49]. The benefit that is offered by subunit vaccines is that they are more easily characterized and can be produced with better consistency than traditional vaccines. However, this benefit is offset by lower immunogenicity. Thus, they may require the use of adjuvants to boost the immune response. Subunit vaccines are the minority in marketed vaccines but have made progress. Examples include human papillomavirus L1 protein and hepatitis B surface antigen, both of which form virus-like particles (VLP's) therefore inducing a stronger immune response than what would be expected from a free subunit protein [49]. More recently, herpes zoster recombinant subunit protein gE has advanced to market as a lyophilized product formulated with the liposome-based adjuvant system AS01, which contains 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and the saponin QS-21 as immunostimulatory components.

There are few examples of actual lyophilization development of subunit vaccines published in the literature, and likely this is primarily due to concerns around intellectual property. However, some academic and government laboratories have published data around the use of lyophilization to stabilize subunit products. At least one example of a ricin toxin subunit protein (without an adjuvant) is described in the literature [50]. In this study, RiVax was freeze-dried by freezing at -80°C and pulling a vacuum below

100 mbar at room temperature. The researchers evaluated several excipients, including sugars and amino acid with histidine buffer and 144 mL NaCl. Not surprisingly, many of the formulations were not capable of stabilizing the antigen, as observed by cloudiness upon reconstitution of the lyophilized formulations. The glass transitions were likely very low, and the moisture contents were probably too high based on the results described. However, given the lack of testing, it is difficult to conclude the cause of the cloudiness fully. The authors did find that trehalose formulations were more stable, and this is likely due to the higher glass transition of trehalose. Even if not completely dry, the T_g was likely sufficiently above room temperature storage [50]. Stability of the formulations was monitored via SDS-PAGE and SEC-HPLC over time to trend formation of fragments and aggregates. The methods for lyophilization and the analyses presented for this subunit protein make it challenging to extract generalizations about freeze-drying of these types of proteins. Thus, additional work needs to be done.

Influenza vaccines are inactivated whole virus vaccines, split-virus vaccines, or subunit vaccines. The subunit vaccine contains hemagglutinin (HA) and neuraminidase (NA), where HA is the major surface glycoprotein of the influenza virus and is a 225 kDa trimer of identical 75 kDa monomers. Amorij et al. investigated the effect of different buffers (phosphate and Hepes), sugars (trehalose, inulins, dextran), and lyophilization process parameters on the structural integrity of the HA protein [51]. They found that rapid freezing in a buffer that has a limited pH shift upon freezing resulted in maintaining the structure of the protein. When carbohydrates were incorporated, HA protein structure was kept irrespective of the type of carbohydrate and even in the presence of phosphate buffer, which alone resulted in conformational changes. They were not only able to demonstrate that optimization of the formulation and process resulted in favorable attributes but also that the optimized formulations were stable at room temperature for at least 26 weeks [51].

The application of lyophilization to subunit vaccines employs many of the same strategies as that used in the biotherapeutics space, and the examples illustrated here apply the strategies and learnings from that space. The sections of this chapter that refer to formulation and process equivalently apply to subunit vaccines, recognizing that all vaccine antigens are unique and that multivalency may present an additional challenge when thinking about antigen–antigen interaction and the accompanying analytical challenges for studying this.

Inactivated vaccines

The discovery of inactivated vaccines was stimulated by the need for safer vaccines due to the possibility of reversion of the virus in live-attenuated vaccines. Louis Pasteur, in his experiments, found out that the pathogens can be attenuated or entirely killed by exposing them to extreme environmental conditions [52]. These experiments encouraged research on live-attenuated vaccines; although, complete inactivation of the pathogen for vaccine development did not surface until mid-to-late 19th century

simultaneously, and independently, by Salmon, Smith et al., and Emile Roux from Louis Pasteur's team [53]. However, the first killed whole virus vaccine did not surface until the early 20th century by Enders et al. for their work on generating an inactivated poliovirus vaccine (IPV) by cultivating poliovirus in vitro in fibroblasts [54]. One of the most recent vaccines containing whole inactivated virus was hepatitis A which was published around 1995. Another form of inactivated vaccines was the inactivated toxins, also called toxoids that allowed for vaccines against diseases such as diphtheria, tetanus, etc. [53]. Inactivated vaccines have also revolutionized the prevention against influenza virus, especially the ever-changing seasonal strains. These vaccines are available as either whole-inactivated or split (disrupted using chemicals such as formaldehyde) [55]. More recently, whole-inactivated virus vaccines against Japanese encephalitis as well as tick-borne encephalitis have been developed [56].

Even though most of the live virus or bacterial vaccines are lyophilized drug products, inactivated vaccines continue to be formulated as liquid vaccines. Aside from dependence on cold chain, there is limited knowledge on the stability of liquid vaccines during transportation, or improper storage/lack of refrigerators in developing countries [57]. For vaccines such as the inactivated polio virus vaccine (IPV), stability in the liquid state is up to 4 years at 2–8°C, assuming no damage from transportation or at elevated temperatures. Lyophilization of such inactivated pathogen vaccines could provide better thermal stability. In such events, freeze drying of inactivated vaccines could offer stability, as well as possibly help extend the shelf life of the drug product. Among the inactivated vaccines, IPV and inactivated influenza virus vaccine are heavily studied. Kraan et al. were the first to lyophilize IPV (trivalent vaccine) with the preservation of all three serotypes of the D-antigen postlyophilization after unsuccessful attempts in the mid-20th century [58]. They compared vacuum drying with freeze drying to show that vacuum drying resulted in high (9%–12%) water content due to the relatively low specific surface area during the extended drying process under vacuum without freezing stresses. They also revealed that both sucrose and trehalose in freeze-dried formulations have weak antigen recovery resulting in reduced potency. After extensive excipient screening using design-of-experiment studies, a combination of sorbitol (10% w/v), magnesium chloride (8.5% w/v), and L-glutamic monosodium salt monohydrate (MSG) (8.5% w/v) resulted in a freeze-dried formulation that manifested better preservation of potency of IPV in terms of all three serotypes for up to 24 weeks at 25 and 37°C compared with the conventional liquid formulation. A more recent study shows an advanced method for screening Sabin-inactivated poliovirus vaccine (sIPV) using SE-HPLC. This assay accurately quantitated D-antigen more robustly and repetitively, unlike the ELISA that measured potency in terms of D-antigen units or DU [4]. Using SE-HPLC, they screened several surfactants to get a stable sIPV freeze-dried vaccine (containing 5% mannitol, 1% sorbitol, and 0.5% Pluronic F68 in 10 mM histidine buffer) with potency maintained at ambient temperatures. Additionally, they showed maintenance of neutralizing antibody titers, in transgenic mice expressing human CD155 receptor for virus entry, for lyophilized product incubated at 37°C for 4 weeks compared with 50%

reduction in titers for liquid product stored in similar conditions. Upon challenge, both lyo products—stored at 4 and 37°C—showed protection for up to 15 days compared with liquid formulation stored at 37°C that reduced the survival rate to 10%. In another study, Qi et al. focused on stabilizing the serotype 3 of the IPV vaccine during lyophilization using urea that helped preserve potency and prevent aggregation to facilitate long-term storage postlyophilization [59]. Thus, using the right excipients stability of IPV can be improved at ambient temperatures that can allow for adequate vaccine storage and outreach to developing countries.

Dry powdered influenza vaccine is also an essential area of research, for pre-pandemic preparedness, and to avoid cold chain dependence for stockpiling vaccines and better outreach to developing countries. For flu vaccines, both mucosal (pulmonary) and intramuscular routes have been popular. Inulin and trehalose were studied to stabilize the whole-inactivated H5N1 flu virus vaccine during freeze drying [60]. They showed that lyophilizing this pre-pandemic H5N1 vaccine allowed for at least 3 months of storage at 37°C without loss in characteristic th-1 type immune response in contrast to a liquid drug product that showed a significant loss in the immune response. In another study intended to create a pulmonary delivery for whole inactivated influenza (WIV) vaccine, Audouy et al. showed that although freeze drying designed stable drug product compared with a liquid formulation, only spray freeze drying (SFD) was able to create particles suitable for pulmonary administration by insufflation [61]. They showed that immunogenicity of the SFD formulation via the pulmonary route resulted in immune response like subunit protein formulation via the intramuscular route.

Whole inactivated vaccines are generally stable for several years at refrigerated conditions; however, they are also extremely temperature sensitive, making them highly dependent on cold chain maintenance. It might be prudent to create dried-powder vaccines that can withstand temperature fluctuations, thus improving the shelf life and preserving vaccine potency in extreme conditions. The dried powder could simplify stockpiling and facilitate pre-pandemic and pandemic preparedness, although the stability profile is longer in general at refrigerated conditions.

Antigen adjuvant lyophilization

Recombinant technology has allowed for the discovery of subunit protein antigens that have higher purity and safety compared with conventional vaccines; however, they lack the necessary pathogen-associated molecular patterns (PAMP) like structures rendering them less immunogenic [62]. Adjuvants are materials that help improve the immunogenicity of such protein antigens, thus playing an essential role in making modern vaccines safer [63]. Adjuvants such as aluminum salts, oil-in-water emulsions (MF59), and a liposomes adjuvant system (AS01) are currently available in marketed vaccines. In most adjuvanted vaccines, antigen and adjuvant are distributed in two or three vials—comprising of antigen, adjuvant, and buffer or both, due to difficulty in maintaining long-term stability of adjuvanted vaccines [64]. Currently, the vaccine antigen is either

lyophilized or available as a frozen liquid, thus requiring cold chain maintenance and distribution, making it difficult to distribute in developing countries [65,66]. Adjuvants are usually supplied at refrigerated temperatures, thus requiring a separate cold chain. A lot of effort and focus today is on stabilizing the adjuvanted vaccines by eliminating the need for cold chain distribution by direct or indirect means [67,68]. Reducing cold chain and the possibility of making the potent adjuvanted vaccine available globally can be achieved by developing a single vial adjuvanted vaccine. Since most of the currently used vaccine antigens are lyophilized, for reasons beyond stability as well [69,70], freeze drying of antigens with the adjuvant may seem like a possible solution.

Lyophilization of antigens, which are coformulated with adjuvants, can be difficult, as many adjuvants are sensitive to freezing stress [57,71]. Adjuvant physicochemical properties such as particle size, content, and conformation need to be preserved to maintain its potency in improving the immune response to an antigen. Additionally, when antigen and adjuvants are covalent, several interactions might impact the stability of the antigen and affect the magnitude of the immune response [72]. Initial investigations of covalent antigen and adjuvant for freeze drying were evaluated with aluminum-based adjuvants, owing to their vast history of safety in humans. It is well established, at least for suspensions of aluminum hydroxide and phosphate, that freezing stresses (i.e., freeze concentration) result in aggregation or coagulation [73,74]. Maintaining the size of the adjuvant may directly impact its ability to potentiate the immune response to an antigen [75]. Ideal single vial vaccine would maintain the properties of antigen and adjuvant during the freeze drying process and represent the liquid drug product after reconstitution with water or buffer. In the following discussion, we have reviewed the current literature for a single vial lyophilized vaccine.

Despite the aggregation issues with aluminum salts, there have been several studies demonstrating the freeze drying of vaccines with aluminum-based adjuvants. Since aluminum salts mainly existed as gels or suspension, freezing or freeze drying caused the collapse of the adjuvant, causing aluminum particles to be aggregate [76,77]. However, aluminum hydroxide–adjuvanted hepatitis B surface antigen (Alum-HBsAg) vaccine and aluminum phosphate–adjuvanted diphtheria toxoid (Alum-DT) vaccines were evaluated for feasibility of freeze drying, spray drying, air drying, or spray freeze drying [78]. In a comparison of the drying techniques, Maa et al. reported the coagulation of aluminum gels as well as loss in immunogenicity after freeze drying Alum-HBsAg formulation. One of the reasons aluminum particles aggregate after freeze drying could be during the freezing step where ice crystals help aluminum particles attract to each other and coagulate [76]. More recently, Hassett et al. attempted at stabilizing aluminum hydroxide–adjuvanted vaccine for anthrax using lyophilization [79]. Their antigen candidate for aluminum adsorption was a recombinant version of a protective antigen (PA) in *Bacillus anthracis*. Previous studies have established that DNI is a suitable model vaccine antigen (e.g., can elicit high PA antibody titers) and that deamidation is the primary degradation pathway for DNI. DNI was adsorbed on aluminum particles with or without glycopyranosyl lipid A (GLA), a TLR 4 agonist, as a liquid product, as well as

glassy lyophilized powder and compared *in vivo* in female BALB/c mice after formulation optimization and analysis. Before demonstrating the results for the lyophilized powdered vaccine, they showed that upon freeze–thaw, the liquid vaccine caused aggregation of aluminum particles, eventually affecting the immunogenicity. At 40°C, the liquid vaccine showed deamidation of the antigen and changes in DNI structure only after 1 week of storage, whereas the lyophilized powder preserved the antigen structure without any additional deamidation. Although after lyophilization, the particle size of the adjuvant increased from 2 µm to greater than 15 µm, the storage of the lyophilized product at 40°C up to 16 weeks did not affect the other physicochemical parameters of the vaccine.

Additionally, efficacy in mice was maintained for the lyo product after 16 weeks of storage at 40°C, in contrast to the liquid vaccine, where neutralizing antibody titers reduced after 8 weeks of storage at 40°C. This study confirmed the possibility of a single vial aluminum-adjuvanted freeze-dried vaccine, despite the detrimental effects of freeze–thaw on aluminum particles. The antigen used with an aluminum adjuvant can also dictate the possibility of lyophilizing aluminum-adjuvanted vaccines. Another study showed that thin-film freeze-drying with high aluminum salt content (~1% w/v) yielded stable, dried vaccine, with a slight increase in particle size and physicochemical properties independent on repeated freeze–thaw; unlike liquid vaccine [80]. Clausi et al. [73] evaluated the effect of excipients in inhibiting aggregation of aluminum particles during freeze drying. Unlike some other published work, Clausi et al. found slower freezing rates (~0.5°C/min) help in inhibiting aggregation of aluminum particles. In the past few years, several other studies have evaluated the effect of freeze drying on aluminum salt–based vaccines to manifest its feasibility and maintenance of efficacy post-lyophilization [74,81,82]. Further studies may be needed to evaluate the long-term effect of an increase in aluminum particle size on the stability of vaccine drug product as well as its efficacy in humans.

Emulsion adjuvants, second in chronology to aluminum, have been used in humans for about two decades (MF59 in Fluad and AS03 in Pandemrix) and demonstrate an acceptable safety profile. Like the aluminum-adjuvanted vaccines, emulsion-adjuvanted vaccines also require a two/three vial presentation, thus emphasizing dependence on cold chain distribution. Freeze drying of emulsions has been researched outside of the vaccine space [83]. For vaccines, the potency of the emulsion adjuvant may directly relate to the size of the adjuvant [75]. Additionally, stress during the freeze drying process can cause the oil droplets in emulsions to coalesce, leading to creaming and flocculation or both. For a single-vial freeze-dried emulsion-adjuvanted vaccine, antigen–adjuvant compatibility during the freeze drying process is vital. Conclusively, a stable freeze-dried emulsion-adjuvanted vaccine would solve the problem of cold chain dependence and facilitate distribution and storage for these vaccines, making them cost-effective. Two independent studies have evaluated the feasibility of freeze-drying covalent emulsion adjuvant and antigen by Orr et al. and Iyer et al. [64,84]. Orr et al. used a recombinant fusion protein ID93, comprising four subunits of mycobacterium tuberculosis and

adjuvant GLA-SE, which is a stable emulsion adjuvant delivering a TLR4 agonist GLA. They showed that covialing and lyophilizing ID93 with GLA-SE and subsequent storage at 50°C, as well as 4°C did not affect the significant physicochemical properties such as size (~10–20 nm increase in size), antigen integrity, adjuvant composition, and GLA concentration. Using C57BL/6 mice, they showed that lyophilized covialled formulation retains its efficacy in producing immune response after storage for 1 month at 4 and 50°C compared with liquid covialled vaccine at the same temperatures. Another study compared liquid and lyophilized single vial formulations containing a soluble form of RSV protein—RSVsF to show that apart from a minimal increase in particle size after reconstitution of the lyophilized formulation, all the other physicochemical properties remain unchanged. Using BALB/c mice, they also demonstrated that single vial freeze-dried vaccine containing emulsion adjuvant ME, with TLR4 agonists MPLA or PHAD (synthetic MPLA), and RSVsF antigen showed both humoral and cellular immune response like liquid vaccine. Both these studies show a slight increase in emulsion droplet size after lyophilization that does not seem to change upon further storage. The increase in droplet size may be due to the freeze drying process and may need further investigation. This minimal increase in particle size, however, does not seem to affect the immune responses. Barnes et al. have also successfully created a single vial, GLA-SE-adjuvanted ID93, tuberculosis vaccine in a cartridge format for ease of administration [85]. Recently, our colleagues at GSK lyophilized the adjuvanted malaria vaccine candidate—RTS,S/AS01. They showed preservation of immunogenicity and physicochemical properties of both the vaccine antigen and adjuvant at elevated temperatures [86]. These studies encourage the use of lyophilization to create thermostable adjuvanted vaccines that aid in storage and distribution and help eliminate cold chain distribution.

Lyophilization of covialled antigen and adjuvant is feasible with the right combination of excipients that can help maintain the physicochemical properties of both the antigen and adjuvant. More work in this area is needed to bolster the single vial vaccine platform, which may play a pivotal role in making vaccines cost-effective and improve outreach in developing countries.

Novel vaccine

Although the delivery nucleic acids (plasmid DNA and siRNA) have been investigated extensively in the past [87–90], in recent years, the interest in nucleic acid (DNA and RNA) has increased significantly as their potential to act as vaccine antigens has been realized [91]. For both DNA- and RNA-based antigens, often complexation with a delivery vehicle is required to protect the nucleic acid from enzymatic (i.e., DNase and RNase) degradation [91,92]. Although both DNA and RNA (e.g., nonamplifying mRNA and self-amplifying mRNA) have been studied, it appears that mRNA may have the greatest potential as the successful development and demonstration of positive clinical outcomes for DNA based antigens has been challenging [91,93]. For DNA-based

antigens, it appears that these challenges are likely related to inefficient intracellular (i.e., nuclear) delivery of DNA, resulting in the insufficient stimulation of the immune system [91]. Conversely, for mRNA-based antigens, intracellular, rather than intranuclear, delivery is required to facilitate translation.

Several reports relating the findings of well-designed, informative studies have been published demonstrating the potential for nucleic acid–based vaccines [94–96]; however, more research is needed to develop better and characterize a lyophilization process to produce nucleic acid antigen vaccine products. Petsch et al. [95] performed an extensive study of an mRNA-based vaccine targeted to prevent influenza A infection in multiple animal models and conducted a thorough assessment of the induction of both B and T cell responses, protection upon viral challenge, the potential for reduction of the vaccine schedule to a single immunization, and protection upon exposure to a heterologous virus. In one experiment, the ability of the antigen, PR8HA, to protect when administered 5 weeks before infection with a tenfold median lethal dose was evaluated. In this experiment, a frozen PR8HA antigen containing formulation was compared to a lyophilized formulation, which was stored at 37°C for 3 weeks before use. Although all the mice survived the viral challenge and did not display any deleterious clinical signs or weight loss, information regarding the lyophilization process and characterization of the lyophilized formulation were lacking.

Additionally, perhaps a longer storage duration would better reflect the improved thermostability of the lyophilized formulation. In another study, the stability of RNA lyophilized in water or the presence of 10% trehalose was explored [97]. The structural integrity of the RNA was maintained for a longer duration and at higher temperatures for the formulation containing trehalose. Although the lyophilized formulations were not well characterized, the lyophilization process used and the studies performed could serve as a critical starting point for future research in this area.

Considerations for lyophilization cycle scale-up

The technology transfer of a lyophilization process intended to produce a lyophilized vaccine product, which was developed at a relatively small scale (e.g., laboratory scale), necessitates the scale-up of the process, while maintaining product quality, in terms of both efficacy and safety. Ideally, a lyophilization cycle, which is being scaled up, is economical (e.g., not longer than necessary) and robust, yielding comparable product temperatures and cycle times [98]. Others have discussed at length the use of statistical and mechanistic mathematical modeling approaches that can be used to determine the lyophilization cycle design space [99,100]; thus, that will not be the focus of the subsequent discussion. Instead, this section will focus on how the differences in small (e.g., in the development laboratory) and large-scale (e.g., commercial) lyophilizers can result in differences in thermal properties and capabilities.

One point for consideration when scaling up a lyophilization cycle is related to the differences in radiative heat transfer and the corresponding edge vial effect in the laboratory and commercial-scale lyophilizers [98,101]. Radiative heat transfer from the environment to the interior of the sample chamber is related to the emissivity of the materials and surfaces that comprise a lyophilizer, such as a door and internal walls of the sample chamber. In one study, which compared the emissivity of the sample chamber walls and the doors of both laboratory- and commercial-scale lyophilizers, it was observed that the while the emissivity of the walls was fairly comparable (e.g., $\epsilon = 0.75$, laboratory scale and $\epsilon = 0.65$, commercial scale), larger differences were observed for the doors (e.g., $\epsilon = 0.90$, clear plexiglass door of a laboratory scale and $\epsilon = 0.35$, commercial scale). From this study, it is evident that the difference in the transfer of heat via radiation for laboratory- and commercial-scale lyophilizers should be considered. For example, in a laboratory-scale lyophilizer with a plexiglass door, it is expected that the radiative heat transfer is more significant for front vials resulting in higher heater transfer coefficients (Kv) for these vials. It was estimated that the Kv of front vials could be as much as three times greater relative to center vials, ultimately resulting in higher sublimation rates and shorter primary drying times at the laboratory scale [98]. These observations suggest that during the development and transfer of a lyophilization cycle, care should be taken to avoid underestimating the required primary drying time at the commercial scale, where smaller differences in heat transfer and heat transfer coefficients for edge (i.e., front) and center vials may be expected.

Another difference that has been observed is related to the degree of supercooling, the nucleation temperature of ice during freezing, and the resulting differences in primary drying times for batches produced in the laboratory-versus commercial-scale lyophilizers [102]. Relative to what is observed for batches produced in GMP or commercial sites, ice nucleation during freezing occurs at higher temperatures in development laboratories due to the presence of more particles in the air (i.e., more heterogeneous nucleation sites). Nucleation at higher temperatures leads to the formation of larger ice crystals and, consequently, larger pores in the amorphous matrix upon sublimation during primary drying. This relationship was evaluated in a study by Rambhatla et al. [102], which evaluated four different formulations containing either sucrose, dextran, hydroxyethyl starch, or mannitol. In this study, ice fog technology was used to decrease nucleation heterogeneity across the vials during freezing, and the BET method was used to assess the specific surface area of the lyophilized formulations [102]. Nucleation at lower temperatures (e.g., -11°C) resulted in a higher specific surface area relative to that measured for samples nucleated at higher temperatures (e.g., -1°C). The relationship between specific surface area and pore size was utilized to show that the relatively more significant surface area correlates with smaller pore sizes. Additionally, it was observed that increased specific surface area correlated with increased product resistance. Taken together, the results from this study suggest that for a lyophilized product produced at the commercial scale, a lower nucleation temperature, a higher specific surface area, and

a higher product resistance would be anticipated relative to the product produced in a development laboratory at a small scale.

Furthermore, the effect of product resistance on primary drying time was also evaluated [102]. Considering a representative lyophilized product manufactured in a GMP setting, with an estimated mean product resistance of $5.15 \text{ cm}^2 \cdot \text{Torr} \cdot \text{hr/g}$ and a lyophilized product produced in a development laboratory setting with a mean product resistance of $4.3 \text{ cm}^2 \cdot \text{Torr} \cdot \text{hr/g}$, an additional 3 h of primary time was estimated for the commercial-scale cycle [102]. Although 3 h does not represent a significantly longer primary drying time, a similar analysis could be utilized during scale-up to understand differences in primary drying times that could be observed.

Another difference that has the potential to impact the performance of a given lyophilization cycle in a commercial-scale lyophilizer relative to a small-scale lyophilizer is related to the use of a stainless steel guard rail during loading of the vials onto the shelf in the sample chamber [101,103,104]. In the development laboratory, vials are typically loaded onto the sample chamber shelf manually using a tray with a guard rail. Upon removal of the tray, the guard rail remains on the shelf within the sample chamber during the lyophilization cycle. However, on the commercial scale, vials may be automatically loaded without the use of trays or guard rails. The impact of the presence of the guard rail during the lyophilization cycle has been evaluated in several studies [101,103,104]. One such study investigated the ability of the guard rail, which is in contact or near the edge vials, to transfer heat to the edge vials. A higher sublimation rate was observed for edge vials in contact with the stainless-steel guard rail relative to vials in contact with a material that did not conduct heat efficiently (i.e., Styrofoam). However, the stainless steel rail decreased the rate of sublimation for vials in the front of the sample chamber shelf, which were exposed to the radiative heat originating from the plexiglass door ($\epsilon = 0.90$) [101]. Overall, it was concluded that the guard rail both transferred heat via conduction and blocked the radiative transfer of heat from chamber walls and the door with the net effect being a decrease in heat transfer [101]. A subsequent study [104] examined more closely the radiative heat transfer between the sample chamber wall, the stainless rail guard, and edge vials at large (i.e., commercial) and small (i.e., laboratory) scales. With the assumption of a few caveats, it was asserted that the radiative heat transfer from the wall to the guard rail and from the guard rail to the vial is predominately dictated by the guard rail emissivity. In the absence of a guard rail, it was concluded that the heat transferred to the vial is not significantly different because although more heat is transferred from the wall to the vial, heat is not being transferred by the guard rail to the vial. It was concluded that the edge vial effect can be calculated with sufficient accuracy and that edge vial effects should not be dramatically different with scale-up due to comparable wall and door temperatures with known emissivity.

Summary

Lyophilization is a means to create commercially viable, efficacious vaccines with an acceptable safety profile, but it is not without complexity. As the breadth of antigens encountered in vaccine development present such a wide variety of challenges, many of which are not well understood, the vaccine community has worked to try to identify improvements in how we freeze-dry vaccines. Through process-related optimizations to novel formulation strategies, scientists have uncovered some approaches that may offer solutions to historical challenges, but in many cases, it is not clear whether they will apply widely across many different antigens. Also, as the demand for lyophilization of vaccines continues and the populations needing vaccines are large in numbers, lyophilization capacity will likely become one of the industry's most significant constraints. Thus, scientists must continue to explore alternate ways of drying, novel liquid formulation strategies, and potentially new manufacturing strategies that do not limit batch size based on lyophilizer size. Lyophilization will likely always be needed, but learning from what we know and adapting for the future is essential to continue to deliver life-saving and life-altering vaccines to the world.

Conflict of Interest and Disclosure: Nicole Payton and Rushit Lodaya are employees of the GSK group of companies. Adora Padilla is a shareholder of the GSK group of companies. Adora Padilla is a former employee of the GSK group of companies while authoring this contribution.

Trademark Statement: Fluad is a trademark of Sequirus; Prevnar is a trademark of Wyeth LLC; Menveo, Hiberix, and Pandemrix are trademarks of the GSK group of companies.

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