Freeze-drying of live virus vaccines: a review

L.J.J Hansen[a], R. Daoussi[b] C. Vervaet[c], J-P. Remon[c], T.R.M. De Beer[a]*

[a] Laboratory of Pharmaceutical Process Analytical Technology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium

[b]Zoetis, Hoge Wei 10,1930 Zaventem, Belgium

[c]Laboratory of Pharmaceutical Technology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

* Corresponding author: Tel.: +32 9 264 80 97

Fax: +32 2 222 82 36

Email: Thomas.debeer@Ugent.be

ABSTRACT

Freeze-drying is the preferred method for stabilizing live, attenuated virus vaccines. After decades of research on several aspects of the process like the stabilization and destabilization mechanisms of the live, attenuated viruses during freeze-drying, the optimal formulation components and process settings are still matter of research. The molecular complexity of live, attenuated viruses, the multiple destabilization pathways and the lack of analytical techniques allowing the measurement of physicochemical changes in the antigen's structure during and after freeze-drying mean that they form a particular lyophilization challenge. The purpose of this review is to overview the available information on the development of the freeze-drying process of live, attenuated virus vaccines, herewith focusing on the freezing and drying stresses the viruses can undergo during processing as well as on the mechanisms and strategies (formulation and process) that are used to stabilize them during freeze-drying.

INTRODUCTION

Since its discovery over 200 years ago by Edward Jenner, vaccination can be considered as one of the major steps in the fight against infectious diseases. Vaccination aims at controlling and ultimately eliminating infectious agents. The latter objective was obtained for smallpox, which was in 1980 certified as eradicated by the World Health Assembly [1].

Today, vaccines are estimated to avert approximately 2.5 million deaths from diphtheria, tetanus, pertussis (whooping cough), and measles every year and in all age groups. However, millions of people still die from vaccine preventable diseases [2]. This unfortunate finding can directly be linked to the difficulty to distribute vaccines in poorly-served remote rural areas, fragile states and strife-torn regions [3], but also to the often weak thermostability of vaccines [4]. Increasing the stability of vaccines has been at the forefront for decades. Vaccine development scientists aim to provide products preserving adequate quality (potency, titer, activity, and immunogenicity) during storage and after accidental exposure to exceptional conditions, until the vaccines are administered [5].

There are several factors (temperature, pH, suspension medium, exposure to light, freezing, thawing, anti-microbials and inactivating agents) influencing the stability of vaccines. However, the most important factor is the antigen itself as its antigenicity and infectivity are affected differently depending on the antigen's intrinsic stability [5]. According to the U.S. National Institute of Allergy and Infectious Diseases (NIAD), there are seven different types of vaccines, based on their antigen type: live, attenuated vaccines, inactivated vaccines, subunit vaccines, toxoid vaccines, conjugate vaccines, DNA vaccines and recombinant vector vaccines [6].

Live, attenuated vaccines generate both humoral (antibody) and cellular immune responses and are the most successful of all human vaccines because they are able to confer long-term immunity after one or two immunizations [7]. The antigens of such vaccines are bacteria (Tuberculosis (BCG), typhoid) or most often viruses (Measles, mumps, rubella, polio, yellow fever, varicella and rotavirus) [8]. Live,

attenuated virus vaccines consist of viruses that have lost their virulence but that are still able to confer a protective immunity against a virulent virus. Compared to inactivated vaccines, live, attenuated viruses are easier to produce, do not require the use of adjuvants in the formulation and only need minimal downstream processing [9].

The production process of virus vaccines is very complex because it uses living cells that make standardization difficult [9]. Live, attenuated vaccines also form a formulation challenge because of the macromolecular complexity of viruses and bacteria. Viruses can be enveloped or non-enveloped, and have a size ranging (diameter) from 30nm (poliovirus) to 300nm (vaccinia (Small Pox) virus) [7].

Protected in an adequate cryoprotection medium (e.g., buffer to avoid pH changes, non reducing sugars as cryoprotectants, non ionic surfactants to avoid adsorption and aggregation), most viruses are stable below -60°C. Other viruses are stable at higher temperatures but a temperature above 8°C is always harmful for any virus [5]. To maintain the vaccine temperature between safe margins, from the manufacturing location to the vaccine administration (patient), a cold chain is required.

The cold chain concept is applicable in several industrial fields (e.g., food and pharmaceutical industry) which require to maintain temperature-sensitive products at a protective temperature during processing, storage and distribution. The first significant efforts to ensure a continuous cold chain in the pharmaceutical field were deployed at the beginning of the 1980s with the development of insulated packages, temperature recorders and the improvement in training of the various parties involved in the distribution system [10]. Despite these numerous improvements, keeping the temperature between 2 and 8°C (the temperatures specified by the World Health Organization, the FDA and other governmental agencies in order to ensure an optimal quality of the vaccines) is not always feasible, even in industrialized countries: breakage of the cold chain due to handling errors occurs frequently [11-14] as well as equipment power failures [10,15]. However, maintaining a well-controlled cold chain is of major importance since a disturbance in it can lead to a decrease of the vaccine's therapeutic efficiency. In addition to the presented risks of disturbances, certain vaccines can't be delivered via the cold chain system as the requirements in term of minimal acceptable temperature are not met. This is the case for the oral vaccines against poliomyelitis [16] and the smallpox vaccine in dry form [17] which require a storage at -20°C. Therefore, reducing the dependency on the cold chain of the products would be very useful [18].

The limited stability of viruses in aqueous media above 8°C is well known [5]. This instability results from several types of water-mediated destabilization and degradation pathways.

Therefore, as is the case for proteins [19], the removal of the bulk water (to 1-2% total weight) can significantly increase the stability of viruses by inhibiting or sufficiently decelerating the degradation and destabilization pathways that can occur in aqueous media [7, 8, 9]. Except the oral polio vaccine (OPV), which is a stable vaccine in aqueous formulation [20], all live, attenuated viral vaccines are freeze-dried [4,21].

Freeze-drying offers many advantages over other drying processes: the low temperatures used during this process allow avoiding high destabilizing drying temperatures, the shelf life of the freeze-dried product is significantly enhanced, the dried product (cake) can be easily reconstituted and the aseptic processing operation meets the finished product sterility requirements without the stress of a terminal sterilization step.

Despite the numerous studies performed, the destabilization mechanisms as well as the protection mechanisms for live, attenuated virus vaccines during lyophilization are not well known. This is attributed to the already mentioned complex structure of viruses, the multiple degradation routes and the lack of analytical techniques allowing the measurement of physicochemical changes in the antigen's structure during and after freeze-drying. Because the destabilization pathways always result in a loss of bioactivity, potency assays are typically used during formulation development [22]. Unfortunately, such assays are characterized by weak accuracy and precision [23], and don't provide direct information to elucidate the mechanisms of destabilization and inactivation.

AIM

This review focuses on the freeze-drying of live, attenuated virus vaccines. The purpose is to review the available information on the development of the freeze-drying process of live, attenuated virus vaccines, herewith focusing on the stresses the viruses undergo during processing as well as on the mechanisms and strategies (process and formulation) to stabilize them during freeze-drying.

PART 1: LIVE, ATTENUATED VACCINE STRESS AND STABILIZATION MECHANISMS DURING FREEZE DRYING

To provide solutions for improving the stability of freeze-dried live, attenuated vaccines, understanding of the mechanisms of degradation and destabilization during freeze-drying is needed. A huge amount of information is available on the stress mechanisms and stabilization strategies of pharmaceutical pepides, proteins and DNA during freeze-drying [24-29]. This contrasts to the lack of detailed knowledge on viral-based vaccines. During the past decades, reported improvements of the freeze-drying of live, attenuated virus vaccines (formulation and process) have resulted mainly from empirical discoveries rather than from rational approaches [7]. This review aims at providing an overview of the available information.

The review will first describe the stresses that can occur during the freezing step and the strategies that are used to avoid these stresses. Next, the stresses occurring during the drying steps and the applied protection strategies are described.

Furthermore, this review will focus in more detail on the formulation and more precisely, on the most often used buffers and stabilizers. Finally, the process development strategy will shortly be discussed.

1. Virus structure:

Most live, attenuated viruses possess a lipid bilayer which is considered as the less stable virus component because of its high fragility. The enveloped viruses also have glycosylated or non glycosylated proteins that are essential to infect their host and must be protected [30]. Finally, the viral genome consists either of RNA or DNA and is considered as more stable, but is still subject to both physical and chemical stresses. Considering only this simplified structural description, loss of potency during freeze-drying can be due to protein destabilization (e.g., unfolding, degradation, aggregation), nucleic acid degradation, lipid layer alteration (e.g., phase transition, mechanical damage) and stresses related to changes in the internal (ice formation, osmolarity change) and external (pH and osmolarity change) virus environment. A schematic overview of the virus and the factors possibly affecting its potency is presented in fig.1.

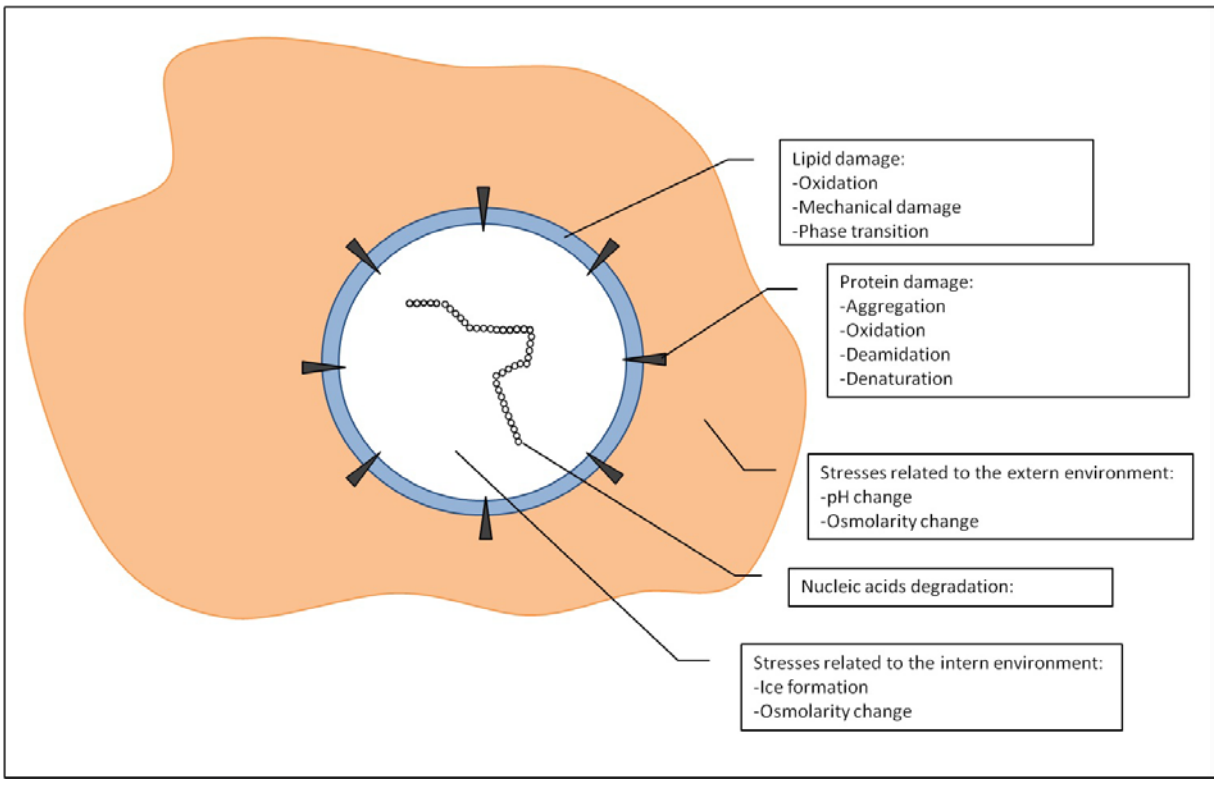

Figure 1: Schematic overview of the virus and the factors possibly affecting its potency.

2. Freezing and associated stresses

During the freezing step, the liquid formulation to be freeze-dried is cooled until ice nucleation starts, which is followed by ice crystal growth. Many biological structures and bio reactions are temperature dependent. Consequently, cooling creates conditions deviating from the normal physiological conditions [31] that might have detrimental effects on the viruses. Freezing can damage the individual virus components (viral coated proteins, viral lipid membrane), change the formulation buffer and osmolarity, all impacting the virus stability. Finally, based on cells cryopreservation knowledge, freezing could also cause intra-virus ice formation. The following paragraphs overviews these possible stresses.

Intra-virus ice formation:

Compared to viruses, the impact of freezing on cells has been more widely studied [31, 32]. Freezing might damage cells for several reasons (e.g., solution effects, extracellular ice formation, dehydration), but intracellular ice formation is the most important one [31-33]. Ice formation inside the cells increases the intracellular concentration of electrolytes which in turn affects ionic interactions that can be involved in the stabilization of the native state of intracellular enzymes [31,32]. Intracellular ice formation occurs when the cell cannot maintain its osmotic equilibrium with the environment, typically at high cooling rate [34]. At low cooling rate, providing water can leave the cells rapidly to maintain thermodynamic equilibrium across the cell membrane, the cytoplasm will not cool below its freezing point [34]. The effect of the cooling rate on cells is well illustrated in [32].
In addition, the interaction of water and ice with the hydrophilic surfaces of intracellular enzymes is different. This is potentially important because the surface tension of water is involved in maintaining the enzymes native state (=hydrophobic effect). If this water is replaced by ice, the stabilizing interaction via the hydrophobic effect can be lost [31]. Intracellular ice crystals can also directly cause mechanical damages to the cellular ultra-structure or the cells can also be affected by the volumetric expansion induced by ice formation [31].
Hence, it can be expected that ice formation within the live, attenuated virus might also result in significant loss of potency for similar reasons under fast cooling. To our best knowledge, there are no publications describing intra-virus ice formation or the consequences of intra-virus ice formation.

Changes in osmolarity:

Changes in ionic strength as well as in uncharged molecule concentration must be carefully evaluated, especially for vaccines containing antigens with semipermeable membranes like enveloped viruses because of the potential osmotic effects (contraction or swelling) [35].
Marek's disease vaccine can be dramatically affected by the osmolarity. Values of 745mOsm/kg and higher have been proven to markedly reduce the virus survival [36].

The vaccine integrity can be affected by osmotic changes. When grown in culture medium at specific ionic strengths, the virus must reequilibrate when introduced in solutions of different ionic strengths (e.g. during formulation). This results in osmotic swelling or contraction hence potentially damaging the virus.

When herpes simplex viruses are in a low osmotic pressure (50 mOsm) medium, a dramatic potency decrease (only 26% recovery) is observed, whereas a high osmotic pressure (840 mOsm) medium gives a recovery of 80-92% [37]. The reason for this is that in a hyper-osmotic medium, the internal water of the virus escapes and the protein concentration in the tegument (that anchors the lipid membrane and the capsid) increase. This lowers the nucleation temperature inside the virus membrane and prevents ice crystals formation and growth [37].

Both reported cases were not observed under freezing conditions. However, the freezing step implies the conversion of water into ice and hence might affect the ionic strength of the formulation. Such change could impact the virus integrity but it has, to our best knowledge, never been demonstrated.

Effect of the freezing step on pH:

During freezing, pH shifts by crystallization of buffer components can occur [38]. This possibility must be properly investigated as both chemical and physical degradations are strongly pH dependent [35].

Brandau and coworkers illustrated the importance of pH on virus activity for several live, attenuated vaccines [35]. The storage stability of the cold-adapted influenza virus liquid is dependent on the pH. At pH 7.1 the formulation retained its titer for 1 month upon storage at 4°C, whereas the same formulation stored at higher or lower pHs showed a significant titer decrease [39]. A pH change can affect viruses by activating or inhibiting certain enzymes (e.g., viral RNA polymerase) and/or by affecting the overall charge of the coated proteins leading to conformational changes of these proteins. The viral RNA polymerase of the live, attenuated oral polio vaccine (OPV) degrades RNA upon storage. However, keeping the formulation at a pH near 5-6 results in a lower enzyme activity [40]. The secondary and tertiary structure of the glycoprotein haemagglutinin (HA) of the influenza subunit virus has been shown to be sensitive to pH drops during freezing [41]. In the study, the magnitude of pH change of a phosphate-buffered solution (PBS) and a Hepes-buffered solution (HBS) was compared and the effects on the glycoproteins were evaluated. The pH change was higher when using PBS (because of the higher tendency of PBS to crystallize) and led to HA structural changes. The reversibility of HA changes upon acidification and subsequent neutralization is however an indication that structural change isn't caused by pH change (alone) [42,43] (see next paragraph).

Measles viruses are more stable at neutral pH. In an acidic environment, the physical stability of this virus is highly compromised. Light scattering and circular dichroism spectroscopy have shown evidence of aggregation at low temperature and precipitation in acidic environments [44].

Numerous studies have revealed that the optimal pH range to limit the loss of activity is generally between 6 and 8 [7,45,46] which is a convenient result regarding the needs for parenteral preparations which require a pH close to the physiological pH and confirms the conclusion by Peetermans that most viruses are affected by pH values outside the physiological limits [5]. In most cases, the glycoproteins are affected by a pH shift. Changes in their conformation can be reversible but can also lead to aggregation and precipitation of the viral particles. It is therefore highly recommended to examine with care the impact of pH changes and to select an appropriate buffer system.

Effects of the freezing step on the viral coated proteins:

The necessity to protect viral coated proteins during freeze-drying was already understood in 1968 in a study evaluating the stability of suspensions of influenza viruses dried to different residual moisture contents [47]. One year later, it was claimed that the surfaces of virus particles, containing coated proteins, were the first exposed to the phasic changes during freezing [48]. The kinds of changes occurring to the viral proteins during freezing, freeze-drying and long term storage is determinant for the activity of the re-hydrated virus particles [48].

Today, it is well known that proteins, in general, are subjected to different freezing stresses [49,50] during a freeze-drying process. There are three main stress factors: cold denaturation, ice formation and freeze concentration [50].

Change in osmolarity, pH and solute concentration have already been described in the previous paragraphs and are caused by the same stress factor, freeze concentration [24,25]. This stress factor (freeze concentration) can also lead to liquid-liquid phase separation [50,51] between incompatible excipients within the freeze concentrate and could negatively influence the preservation of virus by separating the virus from at least one of the protective excipients [50].

A second important stress factor is the formation of large ice-water interfaces due to ice crystallization that can promote surface-induced denaturation of surface-sensitive proteins [28]. High freezing rates result in small ice crystals and large ice-water interface [28]. Even if large ice-water interface has already been demonstrated to result in protein structure change and protein aggregation [24, 52], such a destabilization mechanism has, to the best of our knowledge, never been demonstrated for viruses. The mechanism of protein denaturation at the ice surface is still under discussion [50].

Finally, cold denaturation has been reported for a large number of proteins. This mechanism states that the protein's free energy of unfolding becomes negative at low temperature and may induce spontaneous unfolding of the protein [49]. However the contribution of this stress is frequently considered as negligible in lyophilization because the estimated cold denaturation temperatures are far below the lyophilization temperature especially in the presence of saccharides and polyols [50].

The freezing stresses inducing (possibly irreversible) protein conformation changes have been demonstrated in many case-studies performed on different model proteins [25, 49, 53,54]. In contrast, only a few studies provide information on the influence of freezing-stress upon viral proteins [41,55].
For herpes simplex viruses, a lipid-enveloped virus, immunogold labeling experiments have shown that the structural integrity of membrane glycoproteins can be lost during freezing [55].
But the most studied virus glycoprotein is the influenza haemagglutinin (HA). This protein can be found at the surface of several live, attenuated virus vaccines, but can also be used as antigen in the influenza subunit vaccine [41] as well as in influenza virosomes [56]. The secondary and tertiary structures of this viral protein are susceptible to freezing stresses [41]. Demonstrated by a proteolitic assay, the HA conformation might change because of pH change (acid-induced conformation change), but HA structural changes (exposed trypsin cleavage site) can also be observed despite moderate pH shift [41] and can be caused by a change of the ionic strength resulting from an increase in solute concentration in the non-frozen fraction, recognized as a protein destabilization factor [28].
Fluorescence and CD-spectroscopy of HA after freeze-thawing experiments also confirm that HA conformational changes after freezing are not only caused by a pH shift, but also by freezing-related stress [41]. Finally, haemagglutinin conformation change upon acidification was found to be reversible after subsequent neutralization [42, 43].

Effect of the freezing step on the lipid membrane:

The lipid membrane is very fragile and an important consideration since the loss of the viral lipid layer automatically results in viral inactivation [57].
Liposomes are considered as model for cell membranes and are extensively studied to understand the effects of lyophilization on the stability of the cell membrane. During the freezing step, the lipid layer must mainly be protected against ice crystal damage [58], fusion and thermotropic phase transitions [59].
More than fifty years ago, Greiff and co-workers observed a titer decrease of influenza A virus during freeze-thawing experiments [60]. According to the authors, the titer decrease was due to mechanical injuries caused by small ice crystals during freezing [60]. The first freeze-thawing cycles caused a relatively limited titer loss, but after the fifth freeze-thawing cycle a marked loss in infectivity titer was observed. These observations were linked to the ice crystal size. Large ice crystals and therefore large interstices (safety zones for the viruses) caused limited damage, whereas the formation of more but smaller ice crystals resulted in more mechanical injury and caused substantial titer decrease.
Conversely, in 2010, Chen and co-workers in their review about liposome lyophilization affirm that fast freezing results in smaller ice crystals which induce little damage to lipid bilayers [58]. In contrast, slow freezing generates bigger ice crystals which are detrimental to the membrane integrity. Other studies confirm the importance to avoid too large ice crystals [61, 62].

However, aiming for the smallest ice crystals can also lead to virus destabilization. Zhai and co-workers compared the effect of three different cooling rates on the retention of the viral infectivity after freeze-drying of a herpes simplex virus [37]. The fastest cooled samples (cooled in melting propane) surprisingly generated the highest virus loss. This loss was explained by recrystallization of the small ice crystals occurring when the rapidly frozen samples were warmed to a temperature at which lyophilization was practical. Ice recrystallization disrupted the virus, hence resulting in more loss of viral infectivity.

Although there are only a few studies available, it can be concluded that mechanical damages caused by ice crystals leading to viral inactivation can occur. Demonstration of the mechanism of action is lacking, but mechanical degradation is the most often proposed mechanism.

The mechanism of two other effects (fusion and thermotropic phase transitions) during the freezing step are less extensively explained in literature. Both result in an increase of membrane permeability, hence water-soluble molecules that were internally isolated are often leaked to the external medium. Furthermore, thermotropic phase transitions can lead to lateral phase separations of phospholipids and other constituents such as membrane proteins [59].

3. Formulation and process strategies to protect live, attenuated viruses during freezing.

This section overviews the strategies described in literature to protect live, attenuated viruses from the freezing stresses and their consequences discussed above (intra-virus ice formation, change in osmolarity, pH changes, glycoprotein structural changes, lipid membrane mechanical damages and fusion). To avoid these stresses, two protective actions are systematically taken: the use of cryoprotectants and an appropriate freezing rate. The effects of both parameters, especially the freezing rate, must be carefully studied as it might have a positive influence against a determined stress (e.g. a high freezing rate results in small ice crystals that are less detrimental to the membrane [58]) combined with a negative effect on another stress (ice recrystallization of the small ice crystals upon drying [37], larger ice-water interface leading to a larger extent of surface-induced denaturation of surface-sensitive proteins [28] or intra virus ice formation [34]).

Selecting a good cryoprotectant is also very important since this formulation component can protect live, attenuated viruses against: coated protein structural changes, fusion/aggregation, membrane damages caused by ice crystals, intracellular ice formation and pH shift. However, it should be emphasized that a good cryoprotectant does not necessarily act as a good lyoprotectant. Polyethylene glycol (PEG) is an excellent lactate dehydrogenase and phosphofructokinase protectant during freeze thawing experiments, however, PEG failed to protect both proteins during freeze drying unless associated with a supplemental stabilizer (i.e. glucose or trehalose) [63].

Sugars have been demonstrated to protect the HA glycoproteins against changes in secondary and tertiary structure caused by freezing [41]. Currently, in literature, two mechanisms are proposed to explain the stabilization of proteins: thermodynamic and kinetic [49].

The thermodynamic mechanism is related to the ability of the cryoprotectant to thermodynamically shift the equilibrium from the unstable, unfolded conformation toward a stable, native state [49]. The solute exclusion hypothesis is the most widely supported thermodynamic mechanism [49]. It suggests that during freeze concentration, the stabilizer is preferentially excluded from the surface of the protein, i.e., the vicinity of the protein contains a higher amount of water molecules that thermodynamically stabilize the native state of the protein. However, the validity of this solute exclusion hypothesis is questioned [29] in particular because of the low mobility in the glassy state. Thermodynamic stabilization requires a rapid equilibrium between native and unfolded states which isn't possible in the glassy state or even close to the glass transition temperature of the maximally freeze concentrate (Tg')[49].

The kinetic mechanism is rather related to the ability of the cryoprotectant to slow down the rate of inactivation. The vitrification hypothesis is the most widely invoked. During freezing, the solutes concentrate until the freeze-concentrate reaches a maximum and forms a glass at Tg', specific for each formulation.

The exact stabilization mechanism of protein is still under investigation and a subject of research.

In addition to the protection of the coated proteins, other virus structures must be preserved during the freezing step. The vitrification hypothesis during freezing, can also explain the protection of the viruses against other freezing stresses like aggregation, damages caused by ice crystals and pH shift.

It has been shown that the high viscosity and the limited molecular mobility in a glassy matrix prevents liposomes fusion/aggregation [58, 64, 65] and protects lipid membranes from damage by ice crystals [58].

In addition, cryprotectants could also prevent aggregation and fusion of live, attenuated viruses by isolating the virus particles in the unfrozen fraction, i.e., the particle isolation hypothesis. When frozen in low sucrose/DNA ratio, aggregation of lipid/DNA complexes was observed despite the glassy state of the sucrose under the experimental conditions. However, when frozen in high sucrose/DNA ratio, aggregation was prevented. The same protection was observed when glucose was used as stabilizer. Whereas a low glucose/DNA ratio did not prevented aggregation, a high glucose/DNA ratio provided particles comparable to unfrozen controls. Nevertheless, in contrast to the sucrose samples, no vitrification was observed when using glucose indicating that the immobilization of particles in a glassy matrix cannot explain the lipid/DNA complexes stabilization and that spatial separation of particles in the unfrozen fraction is sufficient to prevent aggregation [66]. Intra virus ice formation would also be a major concern during freezing of live, attenuated vaccines. Based on freezing studies of cells and liposomes, the use of non-penetrating cryoprotectants like hydroxyethyl starch (HES) or dextran can inhibit intracellular ice formation [31]. The stabilizers increase the osmotic potential of the extracellular

liquid, leading to an escape of water from the cells and thus preventing any intracellular ice formation [67].

To avoid pH shifts during freezing, crystallization of the buffer components in the formulation must be inhibited. This can be done by choosing optimal buffer salts, by keeping the buffer concentration at a minimum, by adding further solutes to maintain all buffer species in the amorphous state or by selecting an appropriate freezing rate [50]. Crystallization of buffer species can also be inhibited by agents such as sucrose and mannitol [38,68]. Finally, each buffer salt is characterized by a critical cooling rate, above which crystallization is inhibited and will not result in a pH shift [69].

In cellular cryobiology, the cooling rate is also important in order to manage the volumetric contraction due to the osmotic effect [70]. A slow cooling rate results in a high volumetric contraction caused by the escape of water from the cell [70]. A fast cooling rate allows little volumetric contraction because the intracellular water has no time to leave the cell rapidly [70]. Water inside the cell implies a less likely vitrification and intracellular ice formation [31]. Slow and high cooling rates depend on the cell and cytoplasm properties. By using a moderate cooling rate, it is possible to allow vitrification with a moderate volume contraction [31].

4. The drying step and the associated stresses:

The lyophilization drying step leads to dehydration of the sample. The consequences on live, attenuated viruses of ice sublimation (primary drying) and water desorption (secondary drying) have not been well studied and are even less described than the effects of freezing upon live, attenuated viruses. Therefore, reviewing the known drying stresses that proteins and liposomes face is the best option to reflect on the potential stresses that coat proteins and the lipid layer of the virus can undergo during the drying steps of freeze drying.

Effects of drying on the viral coated proteins:

Proteins in aqueous solution are fully hydrated and are surrounded by a monolayer of water which is termed the hydration shell [71]. Drying removes part of the hydration shell which can disrupt the native state of the protein and lead to denaturation [28]. During dehydration, exposure to a water-poor environment will cause a decrease of the charge density that may facilitate protein-protein hydrophobic interaction and cause aggregation [28] (during dehydration, the hydrated protein tends to transfer protons to the ionized carboxyl groups and thus abolishes as many charges as possible [71]). Water can also play a functional role in the active site of the protein. Removal of these water molecules can inactivate proteins [28].

Even if these stresses are not described in literature for viruses, they could destabilize the proteins in the virus coat, hence affecting the vaccine potency.

Effect of drying on the lipid membrane:

Similar to proteins, liposomes are also surrounded by a water layer (hydration shell) which is removed during drying and can cause fusion/aggregation of liposomes [58,72].
Another degradation caused by drying that might lead to viral inactivation is linked to the melting point (Tm) of the lipid layer. Above Tm lipids are in the liquid crystalline phase, while below its melting points, lipids are in the gel phase. During dehydration, water from the lipid headgroup region is removed which causes an increase in Van der Waal forces between lipids and results in a higher melting point [41]. When fully hydrated, egg phosphatidylcholine has a Tm of 0°C, but upon dehydration its Tm is 70°C [73, 74]. The changes in Tm between the hydrated and dehydrated state determine whether or not a phase transition will occur during lyophilization and rehydration [58]. The use of carbohydrates can prevent this phase transition which is explained in the next chapter.
If a phase transition from gel to liquid crystalline occurs, it can result in the release of the vesicle content in the environment. This phenomenon has been observed for liposomes, baker's yeast and cells.

5. Strategies to protect live, attenuated vaccines during drying:

The stabilizing capacity of sugars during drying is well known for viruses (see tables 1 and 2). However, as for freezing protection, hypotheses related to their protection mechanism of live, attenuated vaccines during drying are not available as such and must be derived from the freeze-drying protection mechanisms of sugars used for liposomes, cells or proteins.
There exist two sugar based drying stabilizing hypotheses: (i) the water-substitution hypothesis and (ii) the kinetic stabilization mechanism (vitrification theory). These two hypotheses can be invoked to explain protein stabilization as well as liposome stabilization.
The water-substitute hypothesis states that the sugar replaces the water molecules (that are removed in the hydration shell) in the hydrogen bonding interactions with the protein and offers a thermodynamic stabilization [75]. This hypothesis is also valid for liposomes and has the effect of reducing Tm of the membrane. By interacting with the phospholipid head group, carbohydrates maintain the spacing and reduce the van der Waals interactions among the acyl chains of the phospholipids. It reduces the interaction between water and phospholipids, replaces water and finally decreases the transition melting temperature [58,76].
This hypothesis could be relevant for viruses, as a sugar can be hydrogen bonded to the viral coated proteins (providing thermodynamic stabilization) and to the head group of the membrane phospholipids to protect the membrane from phase transition.

The vitrification hypothesis has the same mode of action as during the freezing step. For proteins the isolation into a glassy matrix lowers the molecular mobility needed for the degradation pathways like bimolecular interactions, unfolding and chemical degradation [26]. Similarly, drying can facilitate the aggregation of viral particles by removing the hydration shell surrounding them [77]. The dispersion of the viruses into a glassy matrix can also contribute to the protection by lowering the probability of aggregation.

The viruses appear to be protected by lyoprotectant in three different ways: (a) the dispersion of the virus in a glassy matrix reduces the probability of aggregation and the molecular mobility needed for the degradation pathways, the stabilization of (b) coated proteins and (c) lipid membrane by hydrogen bonding (i.e. water substitution) with the lyoprotectant.

PART 2: FORMULATION

Freeze dried live attenuated viruses often require complex formulations existing of numerous excipients [78]. Generally, such freeze-dried vaccines contains one or several antigens, a bulking agent to provide a satisfactory product appearance, a buffer to maintain the pH at a specific value, tonicity modifiers and stabilizers to provide protection to the organism against chemical and physical degradation during processing and storage [75].

Freeze-drying is performed to increase the stability of the vaccine but the freezing and the dehydration steps themselves can be stressful for the product, as described in the first part of this review.

To reduce the negative impact caused by the process, the use of an optimized formulation and process is essential. Optimizing the formulation means finding the best combination of excipients that will have a specific function related either to the process or to the protection of the active component during and after lyophilization.

Several compounds have been used in order to stabilize freeze-dried live, attenuated vaccines. Information gained from databases of licensed vaccines (table 1) as well as from published studies (table 2) reveal some trends and will further be exposed.

Among the different excipients, the buffer and the stabilizers are intended to play a critical role in the protection of the active ingredient and will be the main focus.

1. Formulation pH - Buffer:

In order to stabilize the pH of a vaccine formulation at a specific value (which guarantees an optimal stability of the antigens), buffers are added to the formulation. The buffer choice when developing a formulation to be freeze-dried is critical.

In protein formulation, the most commonly used buffers are phosphate salts [38]. For virus formulations, limited information is available. However, from table 2, it appears that potassium phosphate is the most frequently used buffer. The reason is the pH range of the buffer (6-8) which is particularly suitable to maintain satisfactory virus activity [7, 45, 46], and it is close to physiological pH.

Depending on the salt used, the crystallization tendency and the pH shift magnitude (see part 1, effect of the freezing step on pH) of phosphate buffer will vary. Sodium phosphate buffer demonstrated significant pH changes (increase ≈ 4 pH units) during freezing, as a result of crystallization of the buffer component, disodium hydrogen phosphate dodecahydrate, recognized to precipitate at low temperature [84, 87, 88]. The potassium phosphate buffer system was subjected to a smaller pH change due to the crystallization of the monobasic salt which is less soluble than the dibasic form [87].

However, the presence of a phosphate buffer in the formulation will not necessary result in a pH shift. This phenomenon appears to be concentration and freezing-rate dependent. Lower concentrations will result in smaller pH shift [41, 89]. This has been illustrated with a phosphate buffer [90] and with a Hepes buffered solution (pH 7.4) in an influenza formulation frozen at -20°C. A drop of 1.4 pH units has been determined for a concentration of 20mM [84], whereas at a concentration of 2mM the pH was reduced by less than 1 unit [41]. Variation in pH for a phosphate buffered saline depended also on the freezing rate with fast freezing (quenching) resulting in a more pronounced pH change compared to slow freezing [41,88, 91].

Finally, crystallization of buffer species can also be inhibited by agents such as sucrose and mannitol [38, 68]. For sodium phosphate buffer, sucrose and mannitol act by inhibiting the crystallization of dibasic sodium phosphate [92].

Several other buffers (tartrate, succinate, malate and citrate) present interesting properties for lyophilization purposes but are not used for freeze-drying of viruses because of their pH range, which is not appropriate for viruses.

2. Stabilizer:

Stabilizers are added to the formulation to provide protection to the active ingredient against process-induced stress [75]. Because neither the impact of each freeze-drying step on the live, attenuated vaccine, nor the stabilization mechanisms needed to protect the live viruses are fully understood or known, stabilizers are selected by trial-and-error screenings [8,49].

However, the large variability (size, shape, composition) in nature of antigens can make the optimal lyo- and cryoprotectant formulation difficult. Several classes of compounds (disaccharides, polyols, animal derived compounds, etc) have been used to stabilize freeze-dried live, attenuated vaccines. Information gained from published studies (table 2) as well as from databases of licensed vaccines (table 1) reveal some trends.

Disaccharides (i.e., sucrose and trehalose) are generally good stabilizers and are frequently used. In addition, sorbitol and animal-derived components (gelatin or albumin) seem to provide a satisfactory stability and are also frequently used.

Sucrose and trehalose:

Disaccharides like sucrose and trehalose have the capacity to remain amorphous during freeze-drying and are referred to in many publications as good stabilizers [75], with high Tg [90] and higher Tc (collapse temperature) than monosaccharides [93].

Even if never demonstrated, sucrose and trehalose probably protect the live viruses during freeze-drying by forming a glass (vitrification) and/or hydrogen bonds (water-substitution), hence avoiding several stresses presented in part 1 of this review. During the freezing step, dispersion of the antigen into a glass could prevent membrane fusion/aggregation and membrane damage caused by ice crystals. The stabilizers can also protect the viruses indirectly by inhibiting buffer crystallization. During the drying steps, the sugars can act as a water substitution agent by forming hydrogen bonds with the coated proteins or the lipid membrane, hence stabilizing the viruses. By lowering the melting temperature of the lipid membrane, the sugars can also prevent the membrane from phase transition during rehydration of the lyophilized product.

Only two formulations shown in tables 2 or 3 mention the use of trehalose as a stabilizer despite its more favorable properties. Sucrose is one of the best stabilizers but compared to trehalose, sucrose has been reported to undergo hydrolysis at low pH [75,94-96], forming reducing monosaccharides whereas no similar report is found for trehalose. Moreover, the viscosity of trehalose in solution has been determined to be nearly 2.5-fold higher than sucrose for all concentrations tested [97]. The viscosity increase during freezing restricts diffusion of reacting molecules [28], minimizing the rate of chemical degradation [29]. The glass transition temperature of trehalose is superior (118°C) [90] to sucrose (75°C) [90] which is particularly important for the long-term stability at ambient temperature. The glass transition temperature of maximally concentrated solutions (Tg') is higher for trehalose (-40°C) in comparison with sucrose (-46°C) [98] allowing a higher primary drying temperature and therefore a shorter primary drying time. For a detailed review on trehalose, readers are referred to [98].

Despite the favorable properties of trehalose, its limited occurrence in vaccine formulation is linked to the following aspects. Firstly, the superiority of trehalose as stabilizer is not universal. Better shelf-stability has been sometimes observed with formulations containing sucrose compared to similar formulations containing trehalose [26, 81, 93]. A combination of lactalbumin hydrolysate-sucrose was found to be a better thermal protectant of the live, attenuated *peste des petits ruminants* (PPR) vaccine than trehalose alone [78]. Conversely, in a stability study at various temperatures (i.e. 4°C, 25°C, 37°C and 40°C) trehalose, in combination with amino acids and divalent cations has been demonstrated to better protect a camelpox virus vaccine than a combination of sucrose and lactalbumine hydolysate [85].

Similarily, trehalose in combination with gelatin was demonstrated to be a better stabilizer than sucrose combined with sorbitol for the protection of a live-attenuated mumps vaccine [86]. Using Design of Experiment (DOE), sucrose, in combination with raffinose and compounds frequently observed to accumulate in developing seeds, demonstrated promising long-term stabilization of adenovirus (Ad5) in liquid and freeze-dried formulation stored at various temperature [99]. Although the use of trehalose in commercial products has been subjected for approval by several governing bodies, this compound has only recently been listed as a pharmaceutical reagent in the United States National Formulary (in 2010), European Pharmacopoeia (in 2010) and the Japanese Pharmacopoeia (in 2011) [98]. These approvals combined with the fact that the use of trehalose to stabilize live attenuated vaccines is recently off-patent, will undoubtedly increase the use of this stabilizer in vaccine formulations.

Sorbitol:

Sorbitol is a sugar alcohol which does not easily crystallize [81] despite being an isomer of mannitol (the most commonly used bulking agent) and has been demonstrated, when formulated together with gelatin, to be an effective stabilizer for measles virus [100], rinderpest virus [80], duck viral hepatitis virus [82] and others commercialized vaccines listed in table 1. Different sugars (lactose, sucrose and trehalose) and sorbitol were compared to stabilize duck viral hepatitis virus (DHV). The only formulations able to confer an acceptable stability were the sorbitol-containing formulations [89]. For measles virus, a sorbitol:gelatin ratio of 2:1 is sufficient to preserve the potency of the virus [100]. For DHV, higher sorbitol concentrations (sorbitol:gelatin ratio of 4:1, 8:1 and 16:1) reduce the stability [82]. This could be due to the low Tg of sorbitol (-1.6°C) [101] which implies that sorbitol cannot be used as a main formulation component. The low Tg of sorbitol limits its use, but is probably at the origin of its stabilizing success. Chang and co-workers studied the effect of sorbitol on the stability of lyophilized antibodies [102]. When added to a sucrose formulation, due to its low Tg, sorbitol acts as plasticizer, increasing the molecular mobility but improving the stability upon storage of an IgG1 (according to Chang, this surprising observation is due to the fact that “mobility” does not control protein stability at storage temperature well below the formulation Tg). In this same study, FTIR results showed that the sucrose formulation containing sorbitol produces a more “native-like structure” than the sucrose formulation without sorbitol [102].

Animal-derived components:

Animal-derived components such as gelatin and albumin have also been used as stabilizer in freeze-dried live, attenuated vaccines. Albumin, in combination with sucrose, was employed in 1950 by Bovarnick and coworkers to stabilize rickettsiae [103]. The gelling characteristics of gelatin are frequently used in vaccine formulation (tables 2 and 3) to prevent inactivation due to environmental

changes such as temperature [82]. However, animal-derived compounds like gelatin represent a potential danger due to the possible contamination with the scarpie-prion-protein (PrP^{SC}) and the new variant of the Creutzefeld-Jakob disease (vCJD) [104] and are therefore more and more requested to be replaced by stabilizers of non-animal origin. In an attempt to substitute gelatin as a stabilizer for a varicella virus liquid vaccine, other components including κ-Carrageenan and different plant proteins have been investigated [105]. Carrageenan, a polysugar synthesized by red algae, was the best choice regarding its capacity to retain the activity of the virus compared to a control formulation with gelatin as stabilizer [105].

A novel promising excipient, silk fibroin, a biologically-derived protein polymer purified from domesticated silkworm (*Bombys mori*) cocoons has recently shown interesting stabilizing properties on a MMR vaccine [106]. The encapsulation of measles, mumps and rubella viral particles in silk film enhanced their storage stability at 25, 37 and 45°C. The mechanism of stabilization seems to act by reducing residual moisture increase during storage at elevated temperatures and by increasing the denaturation temperature of the viral proteins [106].

PART 3: PROCESS DEVELOPMENT STRATEGY

The development of a freeze dying process is very important because of the impact the chosen parameters have on the final product (product appearance, virus potency/stability, residual moisture and freeze-drying cycle duration).

Several studies describing the freeze-drying of virus-based vaccines do not pay attention to the freeze-drying process optimization (process settings) [39,79-82,107-112]. These studies mainly focus on the formulation optimization by comparing different stabilizers and their effect upon virus titer under different storage conditions and on residual moisture of the finished product. The effects of the process conditions used are often unknown and the rationale behind their settings is almost never described. The choice for a specific set of parameters is often based on previous experience [83], or because it corresponds to a gentle cycle that allows the preservation of a large range of bacteria (for a bacterial vaccine) [113].

Table 3 presents some vaccine studies and their freeze drying process parameters used. Comparing the different process conditions (temperature, pressure and time) between the different studies is impossible because critical product parameters like Tg', Tc, filling volume or filling depth,… are formulation dependent. However, table 3 shows a high variability of freeze drying approach like the use of pre-cooled shelves and the freezing rate, which have a direct effect on the virus.

The use of pre-cooled shelves is conventional when freeze-drying vaccines [83]. However, based on Table 3, it is not systematically used (e.g. quench freezing) or when loading on pre-cooled shelves is not

desired. The placement of the vials on the shelves that have already been cooled to the desired freezing temperature (typically -40 or -45°C) results in higher nucleation temperature (-9.5°C), compared to the conventional shelf-ramped freezing (-13.4°C) [114]. With this pre-cooled shelf method, a large heterogeneity in supercooling between vials is observed [114], but this is also the case with the shelf-ramped freezing with differences up to 3°C resulting in important variability in product quality and process performance [115].

In order to obtain a more homogeneous freezing and avoid any ice formation on shelves prior loading, vials can be equilibrated at low shelf temperature (5-10°C) for 15-30min before linearly decreasing the shelf temperature [116].

A thorough study of both shelf-ramp and shelf pre-cooling freezing methods on virus potency could provide valuable process information and is, to our best knowledge, lacking.

As sufficiently demonstrated, the freezing rate is of major importance for virus protection.

Two studies [37,41] listed in table 3 compared different freezing rates and demonstrated the significant effect of this parameter on virus stabilization. Structural integrity of haemagglutinin of an influenza subunit vaccine is better preserved when the solution is rapidly frozen in liquid nitrogen [37]. Fast freezing allows the system to rapidly vitrify which may prevent the conformational change [37].

Herpes simplex virus-2 were better preserved when frozen in slush nitrogen [41]. In this case, the high freezing rate has a positive impact on the viruses by promoting the formation of small ice crystals.

From the two presented studies [37,41], it seems that quench freezing offers better preservation of viruses than slow freezing. Another emerging technique during freezing is the use of controlled nucleation, allowing the control of the nucleation temperature [50]. This method results in a more uniform ice crystal structure and maybe virus titer. This method is subjected to investigational studies in the pharmaceutical industry. To date, no study evaluating the impact of controlled ice nucleation on live attenuated viruses has been published.

Finally, Table 3 reveals that while the effect of freezing rate on viruses receives limited attention, the impact of primary and secondary drying parameters (temperature, time and pressure) on viruses receives almost no attention. These parameters can be easily calculated to obtain a freeze-drying cycle that promises an elegant product in the shortest time possible [117]. Rules and advise to select appropriate drying steps conditions for protein formulation are largely available in literature [28, 92, 115,116] and can be applied to viruses.

Primary drying is however very important as demonstrated by Schneid and co-workers on a bacterial vaccine formulation [117]. Whereas conservative primary drying conditions resulted after 28 days storage at 40°C in 50.10^6 living bacteria cells in the product, an increase of the primary drying temperature of 2 and 5°C resulted in 28.10^6 and 20.10^6 living bacteria cells, respectively [117]. This study highlights the importance of carefully selecting the primary drying temperature as a difference of 2°C can result in a 44% loss.

Residual moisture of the end product can impact vaccine stability and is dictated by the temperature, pressure and duration of secondary drying that are established via empirical studies [75]. Staying within a specified range of residual moisture levels can be vital for product integrity [4]. Whereas overdrying is to be avoided, too high residual moisture levels can cause structural collapse during storage [75] and can increase the product degradation rate as the sorbed water provides molecular mobility [4,75] that can induce chemical degradation (methionine oxidation of human growth hormone [118]) and aggregation (human serum albumin [119]).

CONCLUSION

Despite the high dependence on freeze-drying to stabilize live virus vaccines, their lyophilization process and especially their (de)stabilization mechanisms are still not well elucidated. The complexity of these formulations as well as the lack of techniques to evaluate the different virus degradation pathways are responsible for this gap [4, 7, 83]. Moreover, the large financial issues behind vaccine development and stabilization make that the available information is rarely published. However, other biopharmaceuticals like proteins, liposomes and cells have been well studied and provide valuable knowledge. Knowing the different stresses that can occur during freeze-drying is essential to provide stabilization solutions.

During the freezing step, the stresses that viruses can face are numerous and summarized in table 4. Although the stress and their causes are different, there are two strategies that can be used to protect the virus: an appropriate freezing rate and the use of a cryoprotectant that acts via the solid exclusion hypothesis and/or the vitrification hypothesis.

Information related to the destabilization mechanisms during the drying steps are less numerous. The lipid membrane and the coated viral proteins are the main attributes susceptible to degradation. Both can be protected by the use of a stabilizer via the vitrification hypothesis and the water replacement hypothesis. A typical property of lipids is their melting temperature (Tm) that must be taken into account in order to avoid phase transition. Disaccharides, via the water substitute hypothesis, can decrease the Tm and avoid such phase transition [73, 74].

Examination of the formulations used to stabilize viruses reveals a relatively constant use of sorbitol, sucrose and animal-derived components such as gelatin. However, other compounds (like trehalose and silk fibroin) are gaining interest in the freeze-drying field because of their superior stabilizing properties.

Finally, process parameters, despite being of high importance (freezing rate, primary and secondary drying temperatures) are not subjected to extensive studies. During optimization studies, the formulation

parameters often receive priority over the process settings while they however should be considered together.

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FIGURE CAPTION:

Figure 1: Schematic overview of the virus and the factors possibly affecting its potency.

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Table 1: Example of freeze-dried Vaccine formulations. Compiled from Physicians Desk Reference and the list of vaccines licensed for immunization and distribution in the U.S. (FDA)

Vaccine *Sponsor*	Description	Virus caracteristics	Excipients (not necessarily complete)
ACAM2000 *Sanofi Pasteur Biologics Co*	Live, Smallpox (vaccinia) Vaccine	DNA viruses. Envelopped	Mannitol, human serum albumin
Attenuvax *Merck*	Live attenuated measles virus	RNA viruses. Envelopped	Sorbitol, sucrose, gelatin, human albumin, fetal bovine serum
Biavax II *Merck*	Live attenuated rubella and mumps viruses	mumps: RNA viruses. Envelopped rubella: RNA viruses. Envelopped	Sorbitol, gelatin

MMR-II *Merck*	Live attenuated measles, mumps and rubella viruses	measles: RNA viruses. Envelopped mumps: RNA viruses. Envelopped rubella: RNA viruses.Envelopped	Sorbitol, gelatin
M-M-VAX *Merck*	Live attenuated measles and mumps viruses	measles: RNA viruses. Envelopped mumps: RNA viruses. Envelopped	Sucrose, glutamate, human albumin
M-R-VAX *Merck*	Live attenuated measles and rubella viruses	measles: RNA viruses. Envelopped rubella: RNA viruses. Envelopped	Sorbitol, gelatin
Meruvax II *Merck*	Live attenuated rubella virus	RNA viruses. Envelopped	Sorbitol, gelatin
Mumpsvax *Merck*	Live attenuated mumps virus	RNA viruses. Envelopped	Sorbitol, gelatin
Nasovac *Serum institue of India Ltd.*	Live influenza attenuated virus	RNA viruses. Envelopped	Sorbitol, gelatin, phosphate buffer
ProQuad *Merck*	Live attenuated measles, mumps, rubella and varicella viruses	measles: RNA viruses. Envelopped mumps: RNA viruses. Envelopped rubella: RNA viruses. Envelopped varicella: DNA viruses. Envelopped	Sorbitol, sucrose, gelatin, human albumin
Varivax *Merck*	Live attenuated varicella virus	DNA viruses. Envelopped	Sucrose, gelatin, glutamate
YF-Vax *Sanofi Pasteur Biologics Co*	Yellow Fever Vaccine	?	Sorbitol, gelatin
Zostavax *Merck*	Live attenuated varicella-zoster virus	DNA viruses. Envelopped	Sucrose, gelatin, glutamate

Table 2 : Compilation of the formulation composition of published studies on virus vaccines stabilization.

Vaccine	Formulations
Respiratory syncytial viruses (RSV) Human vaccine [79]	**SPGA (Sucrose phosphate glutamate albumin)** Sucrose Potassium phosphate buffer Na glutamate Bovine albumin
Rinderpest (RP)	**LGS**

Veterinary vaccine [80]	Lactobionic acid Hydrolysed gelatin Sorbitol Hepes buffer
	BUGS Potassium phosphate buffer Hydolysed gelatin Sorbitol
	LS Lactalbumine hydrolysate Sucrose
Live, attenuated *peste des petits ruminants* (PPR) vaccine Veterinary vaccine [81]	**LS** Lactalbumine hydrolysate Sucrose HBSS (Hank's balanced salt solution, used to maintain pH and osmotic balance)
	WBM (Weybridge medium) Lactalbumine hydrolysate Sucrose Na glutamate HBSS (Hank's balanced salt solution, used to maintain pH and osmotic balance)
	BUGS Potassium phosphate buffer Hydolysed gelatin Sorbitol
	TD Trehalose dihydrate
Duck viral hapatitis virus (DHV) vaccines Veterinary vaccine [82]	**SPGA (Sucrose phosphate glutamate albumin)** Sucrose Potassium phosphate buffer Na glutamate Bovine albumin
	LPGG (Lactose phosphate glutamate gelatin) Lactose Potassium phosphate buffer Na glutamate Gelatin
	TPGG (Trehalose phosphate glutamate gelatin) Trehalose Potassium phosphate buffer Na glutamate Gelatin

	SPGG (Sorbitol phosphate glutamate gelatin) Sorbitol Potassium phosphate buffer Na glutamate Gelatin
Influenza virus [39]	**SPG (Sucrose phosphate glutamate)** Sucrose Potassium phosphate buffer Glutamate Casitone
Respiratory syncytial viruses (RSV) Human parainfluenza 3 (hPIV3) Human vaccine [83]	Sucrose Phosphate buffer Pluronic F68
Herpes simplex virus (HSV) [37]	Tris-HCL buffer Sucrose Na glutamate
Camelpox virus (CMLV) [85]	**LS (Lactalbumine hydrolysate and sucrose)** Lactalbumine hydrolysate Sucrose HBSS (Hank's balanced salt solution, used to maintain pH and osmotic balance) **BUGS** Potassium phosphate buffer Hydolysed gelatin Sorbitol **TAA (Trehalose with amino acids and divalent cations)** Trehalose $_{L}$-Alanine and $_{L}$-Histidine Ca^{++} and Mg^{++}
Mumps virus [86]	**Trehalose** Potassium phosphate buffer Na glutamate Gelatin **Sucrose** Sorbitol Na glutamate Human serum albumin Gelatin

Table 3: Process parameters of some vaccine studies

Pre-cooled shelves	Freezing rate	Freezing (Temperature / Time)	Annealing (Temperature / Time)	Primary drying (Temperature / Pressure / Time)	Secondary drying (Temperature / Pressure / Time)	Organism	Remarks	Reference
-45°C	Unk	-45°C / 90min	-15°C / 33h	-30°C / 0.1mbar / 48h	2 steps: 1) 0°C / 0.1mbar / 30min 2) 10°C / 0.1mbar / 280min	Medi 534 Live attenuated virus vaccine	Annealing step not for all samples	83
No	Unk	-20°C / 24h (refrigerator) Liquid nitrogen / 5 - 10min	No	-35°C / 0.220mbar / 24h	20°C / 0.060mbar / 24h	Influenza subunit vaccine	Transferred to a vacuum dessicator at RT for 2 days	41
No	Unk	-75°C / unk. (cold air chamber)	No	-10°C / 0.15mbar / 18h	20°C / 0.15mbar / 15h	Bacteria		113
-50°C	10°C.sec^{-1}	-50°C / ~25sec	No	-50°C / 0.067mBar / 1000min	5°C / 0.067mBar / 120min	HSV-2 virus		37
No	20-50°C.sec^{-1}	Slush nitrogen / ~25sec	No	-180 to 20°C / 1.10^{-5}mBar / 14h				
No	200-400°C.sec^{-1}	Melting propane / ~25sec	No					
No	Unk	Liquid nitrogen / 5 - 10min	No	-35°C / 0.220mbar / 24h	20°C / 0.060mbar / 24h	Virosomes	Same research group as [41]	56
No	1°C/min	-50°C / 3h	No	2 Steps: 1) -50°C / 3Pa / 60h 2) 0°C / 3Pa / 4h	20°C / 3Pa / 8h	Adenovirus		119
No	1°C/min	-40°C / 3h	No	2 Steps: 1) -35°C / 0.045mbar / 25h 2) -20°C / 0.045mbar / 10h	20°C / 0.045mbar / 10h	Virus like particles		120
			No	-15°C / 0.045mbar / 20h	40°C / 0.007mbar / 10h			
			-15°C / 2h	-15°C / 0.045mbar / 20h	40°C / 0.007mbar / 10h			
No	Unk	-40°C / 3h	No	-40 to 37°C / 60mTorr / 40h		Duck viral hepatitis vaccine		82

Unk= unknown,

Table 4. Summary of the possible virus stresses during the freezing step, their cause and the strategy that can be used to avoid the stress.

Stress	**Cause**	**Strategy to protect**
Intra-virus ice formation	Fast freezing	Cryoprotectant and adequate freezing rate
Osmolarity change	Conversion of water into ice	Cryoprotectant and adequate freezing rate
pH shift	Buffer crystallization	Cryoprotectant Low buffer concentration Adequate freezing rate
Coated proteins destabilization	Freeze concentration	Cryoprotectant
Lipid destabilization -mechanical damage -fusion and phase transition	Ice formation	Cryoprotectant