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Review

Vaccine instability in the cold chain: Mechanisms, analysis and formulation strategies

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ABSTRACT

Instability of vaccines often emerges as a key challenge during clinical development (lab to clinic) as well as commercial distribution (factory to patient). To yield stable, efficacious vaccine dosage forms for human use, successful formulation strategies must address a combination of interrelated topics including stabilization of antigens, selection of appropriate adjuvants, and development of stability-indicating analytical methods. This review covers key concepts in understanding the causes and mechanisms of vaccine instability including (1) the complex and delicate nature of antigen structures (e.g., viruses, proteins, carbohydrates, protein-carbohydrate conjugates, etc.), (2) use of adjuvants to further enhance immune responses, (3) development of physicochemical and biological assays to assess vaccine integrity and potency, and (4) stabilization strategies to protect vaccine antigens and adjuvants (and their interactions) during storage. Despite these challenges, vaccines can usually be sufficiently stabilized for use as medicines through a combination of formulation approaches combined with maintenance of an efficient cold chain (manufacturing, distribution, storage and administration). Several illustrative case studies are described regarding mechanisms of vaccine instability along with formulation approaches for stabilization within the vaccine cold chain. These include live, attenuated (measles, polio) and inactivated (influenza, polio) viral vaccines as well as recombinant protein (hepatitis B) vaccines.

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1. Introduction

The dramatic success of vaccination in improving human and animal health is well established. For example, the US Centers for Disease Control (CDC) ranked vaccination as one of the top ten public health achievements in the United States in the 20th century "… resulted in the eradication of smallpox; elimination of poliomyelitis in the Americas; and control of measles, rubella, tetanus, diphtheria, *Haemophilus influenzae* type b, and other infectious diseases in the United States and other parts of the world." [1]. The past decade (2000–2010) witnessed the development and worldwide regulatory approval of many important new vaccines offering protection against bacterial (meningococcal and pneumococcal) and viral (rotavirus and human papillomavirus) infections. In addition, new vaccine formulations to protect against influenza (live, attenuated vaccine administered nasally) and varicella (for protection against zoster for adults) infections were also successfully developed and approved for use. Furthermore, new combination formulations of more well-established vaccines were commercialized to reduce the complexity of the vaccination schedule and to improve compliance including MMRV (measles, mumps, rubella and varicella), DTaP-HepB-IPV (diphtheria, tetanus toxoid, acellular pertussis, hepatitis B and inactivated poliovirus), and DTwP-HepB-Hib (diphtheria, tetanus toxoid, whole cell pertussis, hepatitis B and Haemophilus influenzae type B) vaccines. This availability of numerous new vaccines, combination vaccines, and improved formulations raises important challenges in terms of procuring and distributing them worldwide [2-4].

Along with these successes in introducing new vaccines to improve public health over the past decade, there have been concomitant major advances in our scientific understanding of the basic biological mechanisms of the human innate and adaptive immune systems as well as the molecular basis by which pathogens cause human disease. Despite these advances, the fulfillment of the

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Abbreviations

ADDC	antibody-dependent cell-mediated cytotoxicity
AFM	atomic force microscopy
ASO3	adjuvant system 03
CCID50	50% cell culture infective dose
D2O	deuterium oxide
DTP	diphtheria, tetanus, and pertussis
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FFA	fluorescence focus assay
FFU	fluorescent focus units
GRAS	generally regarded as safe
GSK	GlaxoSmithKline
HBsAg	Hepatitis B Surface Antigen
HBV	hepatitis B virus
Hib	haemophilus influenzae type B
HIV	human immunodeficiency virus
HPV	human papillomavirus virus
ICH	International Conference on Harmonization
IM	intramuscular
IN	intranasal
IPV	inactivated polio vaccine
LAIV	live attenuated influenza vaccine
mcg	microgram
MDCK	Madin–Darby Canine Kidney
MMR	measles, mumps, and rubella
MMRV	measles, mumps, rubella and varicella
MPL	monophosphoryl lipid A
NA	influenza neuraminidase
NVAC	National Vaccine Advisory Committee
OCC	outside cold chain
OPV	oral polio vaccine
PC	Percutaneous
RSV	respiratory syncytial virus
SC	subcutaneous
SRID	single radial immunodiffusion assay
TEM	transmission electron microscopy
TCID50	median tissue culture infective dose
TIV	trivalent inactivated vaccines
TT	Tetanus Toxoid
VERO	cells derived from kidney of African green monkey
VLP	virus like particle
VVM	vaccine vial monitor
VZV	varicella zoster virus
WHO	World Health Organization

promise of new vaccines is still lacking for many urgently needed, unmet medical conditions such as protecting against infectious diseases (e.g., malaria, HIV, RSV) as well as therapeutic treatments for cancer. In addition, the rapid emergence of new infectious disease threats to public health (e.g., H1N1 pandemic influenza) underscores the importance for rapidly moving from the identification of a new vaccine immunogen in the laboratory to a stable, commercially available vaccine formulation. Clearly, the need to develop, produce and distribute new vaccines to address unmet medical needs remains a high priority for improving public health.

Vaccine formulation development is an important part of the overall development cycle for producing, testing and approving new vaccine candidates. Vaccine formulation can be defined as "converting vaccine antigens to medicines" in which the commercial dosage form not only maintains the potency and stability during manufacturing and storage, but also is designed to be conveniently administered to patients. The modern vaccine formulation development path from the discovery of an immunogen to a usable vaccine includes: (1) physical and chemical characterization of the antigenic component, (2) development of stability-indicating assays including potency, (3) evaluation and optimization of the route of administration and adjuvants (in both animal models and in clinical trials), and (4) formulation design to maximize the candidate vaccine's (antigen and adjuvant) stability, shelf life, and immunogenic potential. A major focus of vaccine formulation development, in many cases, is the enhancement of potency through the use of vaccine adjuvants, since many candidate immunogens fail to transfer from the laboratory to the patient due to suboptimal efficacy in humans. One key approach to increase the success rate for new vaccine candidates is thus to ensure the appropriate formulation in the presence of conventional and/or novel adjuvants. Adjuvants not only promote the rate and extent of an immune response, but can potentially steer the immune response in the desired direction (e.g., humoral vs. cellular immune responses). In fact, a review of the five revolutions in the history of vaccine development [5] predicts the sixth revolution in vaccinology will be the introduction of novel vaccine formulations with novel delivery systems.

The purpose of this review is to raise awareness about the scientific and technical challenges encountered to successfully formulate and stabilize different types of vaccines, both in terms of stability of antigens, adjuvants and their complexes. These efforts are described in the context of maintenance of vaccine potency across the vaccine cold chain, during both clinical development and commercial distribution. Since vaccine stability and potency are defined by the analytical assays used to measure and monitor their physical, chemical and biological integrity, the design and development of accurate, precise and quantifiable analytical methods plays a key role in these stability assessments.

2. Overview of currently available vaccine formulations

A summary of the composition and stability parameters of some representative vaccines commercially available worldwide is provided in Tables 1 and 2 (e.g., FDA approved bacterial and viral vaccines [6], respectively). Based on published reviews [7–11] and publically available information online from various manufacturers and PATH [12], the vaccines listed in Tables 1 and 2 are summarized from the point of view of key pharmaceutical attributes including the vaccine type and manufacturer, formulation dosage form, route of administration, type of adjuvants, and storage considerations (e.g., shelf-life, heat and freeze sensitivities).

One general trend to note from Tables 1 and 2 is that live, attenuated vaccines do not contain adjuvants, but are more heat sensitive to potency loss during storage and distribution. These vaccines contain weakened, attenuated versions of infectious viruses and bacteria that must replicate *in vivo* (and therefore mimic natural infection). Some live, attenuated vaccines are administered orally (e.g., OPV and rotavirus) or nasally (influenza) which mimics the natural route of infection. Many live vaccines are administered by parenteral injection (e.g., IM or SC), yet still provide protection against infections often acquired naturally by other routes (e.g., oral-fecal). In addition, live, attenuated vaccines often require only one or two injections to generate a protective immune response. From a stability perspective, live, attenuated vaccines are often freeze-dried (lyophilized) in the presence of a complex mixture of

Table 1
Examples of vaccine types, formulations and stability parameters of FDA-approved bacterial vaccines. Information and data were adapted from Refs. [6,9,12].

Vaccine type	Product	Manufacturer	Formulation (admin route)	Adjuvant	Recommended storage	Shelf life	Freeze sensitive	Heat sensitivity
Live, attenuated bacteria								
BCG	TICE BCG	Organon	Lyo (ID, PC)	None	2–8 °C	≥2 years	No	≥9 days (54 °C)
	BCG Vaccine	Organon	Lyo (PC)	None	2–8 °C	N/A	No	48 h (23 °C)
Typhoid	Vivotif	Berna	Capsule (Oral)	None	2–8 °C	1 year	Yes	12 h (37 °C)
Conjugates polysaccharide-carrier								
Meningococcal	Menactra	Sanofi Pasteur	Liquid (IM)	None	2–8 °C	1 year	Yes	N/A
(Groups ACYW)	Menveo	Novartis	Lyo (IM)	None	2–8 °C	2 years	Yes	N/A
Pneumococcal (7-valent)	Prevnar	Pfizer	Liquid (IM)	Aluminum	2–8 °C	2 years	Yes	N/A
Pneumococcal (13-valent)	Prevnar-13	Pfizer	Liquid (IM)	Aluminum	2–8 °C	2 years	Yes	4 days (40 °C)
Pneumococcal (23-valent)	Pneumovax 23	Merck	Liquid (IM, SC)	None	2–8 °C	N/A	Yes	N/A
H. Influenzae	Hiberix	GSK	Lyo (IM)	None	2–8 °C	3 years	No	≥5 weeks (55 °C)
	ActHIB	Sanofi Pasteur	Lyo (IM)	None	2–8 °C	3 years	Yes	N/A
	PedvaxHIB	Merck	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	N/A
Subunit, purified bacterial antigens								
Tetanus Toxoid	[a]	Sanofi Pasteur	Liquid (IM, SC)	None	2–8 °C	N/A	Yes	N/A
Tetanus Toxoid Adsorbed	[a]	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
Anthrax	Biothrax	Emergent	Liquid (IM, SC)	Aluminum	2–8 °C	4 years	Yes	N/A
Typhoid	Typhim Vi	Sanofi Pasteur	Liquid (IM)	None	2–8 °C	3 years	Yes	N/A
Meningococcal (Groups ACWY)	Menomune	Sanofi Pasteur	Lyo (SC)	None	2–8 °C	3 years	Yes	6 weeks (60 °C)
Combination vaccines								
DTP w/Hepatitis B	Pediarix	GSK	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
DTP	Infanrix	GSK	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	N/A
	Tripedia	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
	Daptacel	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
DTP w/inactivated Polio	Kinrix	GSK	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
H. Influenzae, HepB	Comvax	Merck	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
Diphtheria and Tetanus.	[a]	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
	DecaVac	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
	TeniVac	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
	Diphtheria, Tetanus	Mass Biologics	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
Tetanus Toxoid, Diphtheria, & Acellular pertussis	Adacel	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	N/A
	Boostrix	GSK	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	N/A
Diphtheria and Tetanus, Acellular Pertussis, Inactivated Polio, Haemophilus b	Pentacel	Sanofi Pasteur	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
Meningococcal, Hib (Groups CY-Hib)	Menhibrix	GSK	Lyo (IM)	None	2–8 °C	N/A	No	N/A

N/A—not available.
[a] No trade name identified.

additives and excipients to provide sufficient stability during long-term storage. Freeze-dried vaccines necessitate reconstitution immediately prior to administration and thus require an appropriate diluent. Live attenuated vaccines tend to be heat sensitive during long-term storage in the solid state and during short-term storage upon reconstitution prior to administration. These vaccines thus tend to require careful maintenance of the vaccine cold chain. One challenging example is the varicella-containing vaccines, which often require frozen storage to ensure long stability, even in the lyophilized state, and can lose potency relatively rapidly when stored refrigerated.

A second general trend to highlight from Tables 1 and 2 is that non-replicating vaccines often contain adjuvants to enhance immune responses. These vaccines include inactivated viruses and bacteria, purified subunit protein and carbohydrate antigens, and recombinant subunit protein antigens. Since these vaccines cannot replicate *in vivo*, adjuvants are usually required to provide sufficient levels of protective immunity. In addition, inactivated and subunit vaccines usually require a series of three or more injections, often over a 6 month period. The need for multiple injections over such a long period presents multiple challenges not only to ensure patient compliance, but also in terms of maintaining vaccine supply and stability. From a long-term storage viewpoint, inactivated and subunit vaccines are generally more stable and are typically developed as liquid formulations. These vaccines, however, can be freeze sensitive to potency loss during storage and distribution.

3. Molecular properties of vaccine antigens

The goal of most vaccines is to simulate natural infections by pathogenic organisms. Thus, the use of both live, attenuated as well as killed bacteria and viruses provide natural agents for this purpose and have historically constituted the majority of the most successful vaccines. For example, attenuated viral vaccines are usually created by extended passage of the wild-type virus in cell culture to weaken the virus, including at different temperatures, in different cell lines, and/or in the presence of a mutagen. The

Table 2
Examples of vaccine types, formulations and stability parameters of FDA-approved viral vaccines. Information and data were adapted from Refs. [6,9,12].

Vaccine type	Product	Manufacturer	Formulation (admin route)	Adjuvant	Recommended storage	Shelf life	Freeze sensitive	Heat sensitivity
Live, attenuated virus								
Varicella[a]	Varivax	Merck	Lyo (SC)	None	Frozen	2 years	No	6 h (27 °C)
Zoster[a]	Zostavax	Merck	Lyo (SC)	None	Frozen	18 months	No	N/A
Rotavirus	Rotarix	GSK	Lyo (Oral)	None	2–8 °C	3 years	No	7 days (20 °C)
	RotaTeq	Merck	Liquid (Oral)	None	2–8 °C	2 years	N/A	N/A
Influenza[d]	Flumist	MedImmune	Liquid (Nasal)	None	2–8 °C	18 weeks	Yes	N/A
Adenovirus (Type 4 & 7)	[b]	Barr Labs	Tablet (Oral)	None	2–8 °C	N/A	Yes	N/A
Smallpox	ACAM2000	Sanofi Pasteur	Lyo (PC)	None	Frozen	18 months	No	N/A
Yellow Fever	YF-Vax	Sanofi Pasteur	Lyo (SC)	None	2–8 °C	3 years	Yes	N/A
Inactivated virus								
Hepatitis A	Vaqta	Merck	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	3 months (28 °C)
	Havrix	GSK	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	1-3 weeks (37 °C)
Influenza	Fluarix[d]	GSK	Liquid (IM)	None	2–8 °C	1 year	Yes	12 weeks (20 °C)
	Flulaval[d]	ID Biomed	Liquid (IM)	None	2–8 °C	1 year	Yes	N/A
	Agriflu	Novartis	Liquid (IM)	None	2–8 °C	1 year	Yes	N/A
	Fluvirin	Novartis	Liquid (IM)	None	2–8 °C	1 year	Yes	N/A
	Fluzone[d,f]	Sanofi Pasteur	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
	Flucelvax	Novartis	Liquid (IM)	None	2–8 °C °C	N/A	Yes	N/A
	Afluria	CSL Limited	Liquid (IM)	None	2–8 °C	1 year	Yes	N/A
	H5N1	Sanofi Pasteur	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
	H5N1	ID Biomed	Liquid (IM)	AS03[e]	2–8 °C	N/A	Yes	N/A
	[b]2009 (H1N1)	CSL Limited	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
	[b]2009 (H1N1)	MedImmune	Liquid (Nasal)	None	2–8 °C	N/A	Yes	N/A
	[b]2009 (H1N1)	ID Biomed	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
	[b]2009 (H1N1)	Novartis	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
	[b]2009 (H1N1)	Sanofi Pasteur	Liquid (IM)	None	2–8 °C	N/A	Yes	N/A
Rabies	RabAvert	Novartis	Lyo (IM)	None	2–8 °C	3 years	No	3 months (37 °C)
	Imovax	Sanofi Pasteur	Lyo (IM)	None	2–8 °C	N/A	Yes	N/A
Polio	IPOL	Sanofi Pasteur	Liquid (IM, SC)	None	2–8 °C	≥2 years	Yes	N/A
Japanese Encephalitis	Ixiaro	Intercell	Liquid (IM)	Aluminum	2–8 °C	1 year	Yes	N/A
	JE-Vax	Osaka	Lyo (SC)	None	2–8 °C	N/A	Yes	1 month (27 °C)
Recombinant vaccines								
Hepatitis B	Engerix B	GSK	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	72 h (25 °C)
	Recombivax HB	Merck	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	N/A
Human Papilloma Virus	Gardasil	Merck	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	3 months (45 °C)
	Cervarix	GSK	Liquid (IM)	AS04[c]	2–8 °C	4 years	Yes	7 days (37 °C)
Influenza	FluBlock	Protein Sciences	Liquid (IM)	None	2–8 °C	16 weeks	Yes	N/A
Combination vaccines								
MMR	M-M-R II	Merck	Lyo (SC)	None	Frozen	2 years	No	7 days (37 °C)
DTP w/Hepatitis B	Pediarix	GSK	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
DPT w/inactivated Polio	Kinrix	GSK	Liquid (IM)	Aluminum	2–8 °C	N/A	Yes	N/A
Hepatitis A & B	Twinrix	GSK	Liquid (IM)	Aluminum	2–8 °C	3 years	Yes	1 week (37 °C)
MMR w/Varicella	ProQuad	Merck	Lyo (SC)	None	Frozen	2 years	No	N/A

N/A—not available.
[a] Refrigerated formulation available.
[b] No trade name identified.
[c] Aluminum hydroxide and lipid A.
[d] Quadrivalent formulations available.
[e] Squalene, DL-α-tocopherol, and polysorbate 80.
[f] High dose intradermal formulation available.

resultant attenuated viruses retain the ability to replicate and are immunologically highly similar to the wild type agent, but display dramatically reduced pathogenicity. These live attenuated vaccines usually consist of a mixture of the infectious viral agent in the presence of an excess of inactive viral particles complicating their analysis. Both enveloped and non-enveloped viruses have been developed as vaccines, and the former (containing a lipid bilayer) are typically less stable and rapidly lose potency. In fact, live attenuated, enveloped viruses (e.g., measles, mumps, varicella) often require both complex formulations of numerous excipients and subsequent lyophilization to obtain adequate storage stability [7]. Some non-enveloped viruses such as polio and rotavirus, which possess higher intrinsic stability, have been formulated as liquid preparations for oral administration. Live attenuated vaccines may retain a low level of pathogenicity in a very small number of recipients. This disadvantage combined with their marginal stability has resulted in a search for alternatives.

Inactivated viruses and bacteria offer the advantage of little, if any, biological pathogenicity and often-improved stability. They are usually inactivated by chemical crosslinking with reagents such as formaldehyde. Although these "killed" antigens may be less immunogenic than their live counterparts, the addition of adjuvants such as aluminum salts can boost their effectiveness. Inactivated preparations of viruses and bacteria remain highly heterogeneous vaccine antigens, however, and their replacement by better defined antigens remains a desirable goal. Successful

examples of inactivated viral vaccines include influenza (flu), hepatitis A, and polio (IPV).

With the advent of recombinant DNA technology, individual components of these large complex bacteria or viruses have been employed. In the case of viral vaccines, surface proteins have been cloned and isolated, and in some cases, these viral proteins spontaneously form virus-like particles (VLPs). Although viral surface proteins that form VLPs have proven to be highly successful as vaccines (for example, hepatitis B and human papillomavirus), monomeric recombinant viral protein antigens are usually only weakly immunogenic and have yet to prove themselves useful vaccine candidates even when supplemented with potent adjuvants. The greater efficacy of VLPs appears to arise from their multi-epitopic nature. Most viruses and to some extent bacteria have coats of one or a few proteins present in a geometrically regular manner on their surface. The innate immune system possesses receptors that have evolved to recognize the patterns of such molecules [13,14]. Thus, VLPs better simulate the natural pathogen accounting for their increased effectiveness as immunogens compared to their monomeric counterparts. These complexes are often very stable, adding to their utility as immunogens. It is also possible to produce related molecular complexes in the form of membrane bound vesicles, sometimes referred to as "virosomes". They can be genetically engineered in a variety of different cell types. This approach has proven to be a clinically effective approach to prepare influenza vaccines (e.g., a Russian flu vaccine; [15]). Because virosomes contain a lipid bilayer, they are not only less stable than non-enveloped VLPs, they are also relatively heterogeneous compared to conventional VLPs [16,17].

Other vaccine antigens include polysaccharides, both free and conjugated to various proteinaceous carriers. Both polysaccharides and their conjugates have been used in highly successful vaccines [18,19]. Free polysaccharide based vaccines, such as those used in the prevention of adult pneumonia, are obtained in a relatively purified form from a wide variety of different bacterial subtypes (e.g., 23 different types) to produce vaccines with wide coverage. Such polysaccharide antigens are however insufficiently immunogenic in children and have therefore been conjugated to several different protein carriers (e.g., nontoxic mutants of diphtheria toxin, CRM_{197}, and a *meningococcal* outer membrane protein complex, OMPC, from *Neisseria meningitides*, etc.) to improve T-cell immunity [20]. Despite the complexity of such vaccines due to the large carbohydrate entity, the nature of the protein carrier, and the conjugative process, polysaccharide conjugate vaccines can be successfully manufactured at large scale and have become an effective vaccine strategy. For example, vaccines against *Haemophilus influenzae* type b [18] and *Streptococcus pneumonia* [19] are now widely available.

As shown schematically in Fig. 1, vaccine antigens range in size from the relatively small (such as recombinant proteins, ~5–10 nm) to the very large (e.g. attenuated bacteria, ~2000 nm). Most viruses and VLP antigens are in the size range of tens to hundreds of nanometers. This diversity of size and consequently internal structure present a significant challenge in terms of stabilization and formulation of vaccines. Although there is no direct relationship between size and stability, a few general comments can be offered. Large entities such as bacterial cells and enveloped viruses tend to display limited stability due to the number of their components and the complexity of their interactions. For example, enveloped viruses contain lipid bilayers, which usually display only moderate to low stability due to their osmotic sensitivity as well as the complex composition of their lipid, carbohydrate and protein components. The role of these individual macromolecular agents in providing overall viral stability is usually not known, adding to the difficulty in their analysis and understanding their role in designing stabilized formulations. Nonetheless, maintaining the structural integrity of lipid bilayer of a virus has been identified as the rate limiting structure in stability loss [7]. Small, non-enveloped viruses and VLPs are often much more stable due to the regular nature of the interaction between their protein subunits. VLPs usually consist of only one or a few viral surface proteins. The inherent stability of viral proteins, both individually and as part of the viral capsid, are highly variable, ranging from low to high, and requires extensive empirical characterization. While the RNA/DNA and carbohydrate components of viruses and bacteria are usually considered more stable, they are still subject to both physical and chemical degradation processes. Polysaccharide vaccine antigens are prone to hydrolytic degradation reactions over time [21,22]. Nucleic acids contain helical secondary structure while RNA may contain regions of tertiary structure, which can be particularly susceptible to unfolding. As one would expect, the nucleic acid bases are subject to a wide variety of chemical changes including oxidation. The subject of pharmaceutical stability of nucleic acids is delineated in detail elsewhere [23,24]. As discussed in more detail in the following section, it is always necessary, whatever the nature of an antigen, to conduct extensive stability studies, both under accelerated conditions (e.g., pH, temperature, ionic strength, mechanical, and freeze/thaw) as part of vaccine formulation development, as well as under actual storage conditions to establish shelf-life and expiry dating at the recommended storage temperature.

4. Vaccine adjuvants

Vaccine adjuvants are typically added to inactivated and subunit vaccines to boost protective immune responses. Although the precise immunological mechanism(s) of vaccine induced protective immunity remains an active area of research, vaccines have been

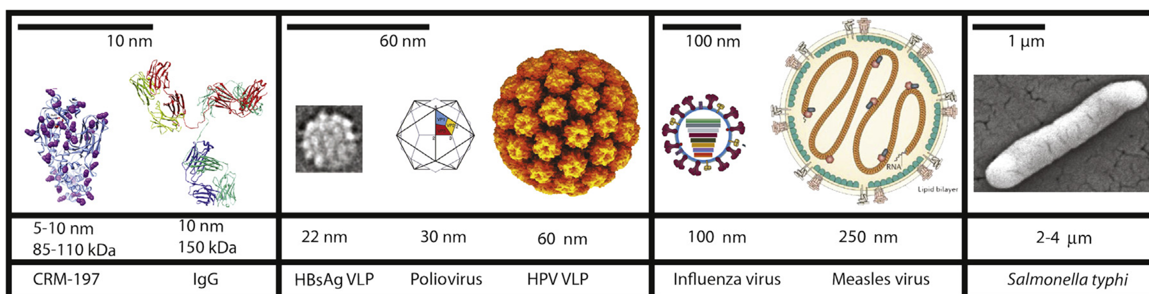

Fig. 1. Structure and size range of various viral and bacterial vaccine antigens. Adapted and reproduced from Refs. [173,194–198], with permission from Elsevier, American Society for Microbiology, Nature Publishing Group, and Cambridge University Press. Antibody structure was obtained from the RCSB Protein Data Bank (accession #1IGT), and the electron micrograph of the *Salmonella typhi* cell was kindly provided by the CDC (in public domain).

shown empirically to correlate with protection by the presence of neutralizing antibodies (as measured by antibody types, amounts, and affinity as well as by pathogen neutralization). In some cases, other correlates such as ADDC activity, complement activation and the presence of certain T-cells types linked with cellular immunity have been established [25]. Compared to live, attenuated vaccines, subunit vaccines are typically better tolerated and lack the risk of reversion and subsequent infection, yet they typically are not sufficiently immunogenic in terms of generating the breadth and depth immune responses needed for complete protection.

Only a small number of vaccine adjuvants have been approved for human use. Many novel candidate adjuvants, however, are in clinical development with the goal of eliciting specific immune responses, especially for urgent unmet medical needs such as therapeutic cancer vaccines as well as prophylactic preventative treatment for HIV, malaria and tuberculosis [26]. For example, activation of different subsets of helper T cells has been shown to be required for the most effective host immune response against different pathogens [25]. New adjuvants have also been explored for use with commercially available vaccines for dose sparing applications as well as for expanding use into poorly responding populations such as elderly and immunocompromised individuals.

Aluminum salts are by far the most commonly used adjuvants in human vaccines (see Tables 1 and 2). This is primarily due to their excellent safety record over ~70 years of use in a wide variety of childhood vaccines. Despite their long history, the biological mechanism of action of aluminum salt adjuvants has only recently become better elucidated. Aluminum adjuvants have been shown to facilitate and increase antigen uptake by immune cells as well as to act as an immune stimulant to attract immune cells to the site of injection [27]. Further, aluminum adjuvants appear to activate antibody responses in a manner independent of the toll-like receptors of the innate immune system [25]. From a formulation and stability perspective, aluminum salts can vary in terms of their chemical compositions, which in turn can affect other physical properties such as particle size, morphology, surface charge, and vaccine antigen binding capacity [28,29]. The physical properties of three different aluminum salt vaccine adjuvants are summarized in Table 3 [28]. These differences in physical properties greatly influence the interaction of the vaccine antigens with different aluminum adjuvants. For example, at pH 7, aluminum phosphate and aluminum hydroxide have opposite surface charges (negative and positive, respectively) and therefore bind different amounts of protein depending on the surface charge of the antigen (Table 3).

In the past decade, several new adjuvants have been approved for use by regulators in various countries. These include oil-in-water emulsions in flu vaccines (e.g., MF59 and AS03 produced by Novartis and GSK, respectively) as well as the addition of monophosphoryl lipid A (MPL) in combination with aluminum adjuvants to recombinant protein (virus-like-particle) vaccines such as HPV and HBV [26,30]. The MF59 adjuvant contains squalene-based emulsion particles suspended in citrate buffer and are stabilized with detergents (Span 85 and polysorbate 80). Although these particles do not appear to bind or associate with vaccine antigens, MF59 leads to enhanced antigen update and recruitment of various immune cells to the injection site [31]. MPL is a lipopolysaccharide derivative similar to those found on the surface of gram negative bacteria, and thus activates the innate immune system as a ligand for the TLR4 receptor [25]. There are many additional adjuvant candidates in preclinical and clinical development [32–34], many of which are toll receptor agonists targeting different pattern recognition receptors found on immune cells (e.g., dendritic cells in the immature state) of the innate immune system [34]. These in turn activate and regulate immune cells (e.g., mature dendritic cells, T and B cells) of the adaptive immune system. This leads to either an antibody and/or cell based immune response [25]. From a vaccine formulation perspective, adjuvants can fail not only due to insufficient levels of the correct immune response or excess reactogenicity, but also by causing destabilization of vaccine antigens during storage [26].

It should also be noted that the immune response generated from different adjuvants can be affected by the route of administration. Vaccine formulations can be administered by parenteral injection to different tissues (intradermal, subcutaneous, intramuscularly) or without injection by either oral delivery or nasal administration [35]. For example, commercial Hepatitis B (HBV) vaccines are administered by IM injection. This vaccine contains a recombinant, purified VLP viral surface protein in a lipid bilayer as well as an aluminum adjuvant. The development of needle free administration of HBV, however, remains an active area of research with the goal of the elimination of needles, improved compliance and enhanced immune responses. Further examples include the use of novel vectors, adjuvants or delivery devices [36]. There has also been ongoing interest in developing intradermal delivery, which has the potential for dose sparing. Clinical trials have been facilitated by new and improved devices for injection including specialized needle and syringes, microneedles and liquid injectors [37].

5. Vaccine formulation development

Subsequent to the selection of the vaccine antigen and adjuvant, a pharmaceutical formulation must be developed to ensure a predictable level of potency during long-term storage and distribution, as well as to provide convenient and appropriate administration to a target patient group. Not only does the nature of the interaction (or non-interaction) of the vaccine antigen with adjuvant need to be characterized, but the optimal state of this interaction must be preserved during storage and administration to ensure acceptable immune responses from the beginning to the end of the shelf-life of the vaccine.

Live, attenuated viral and bacterial vaccines are sensitive to environmental stress, especially elevated temperatures, and often require lyophilization to preserve potency during long-term storage and distribution (see Tables 1 and 2). This is not surprising since intact viruses and bacteria replicate *in vivo* and must maintain their ability to infect cells. This requires intact native structures of proteins, DNA/RNA and lipid bilayers. Freeze-dried (lyophilized) viruses and bacterial vaccines usually contain a mixture of cell culture

Table 3
Selected physical and chemical properties of aluminum-containing adjuvants. Table was adapted from Ref. [28] with permission from Springer.

Commercial designation	Chemical formula	Structure	Morphology	pI	Protein adsorptive capacity, mg/ml Al, pH 7.4	Solubility in citrate
Aluminum hydroxide	AlOOH	Crystalline boehmite	Thin Fibrils	11	1.8–2.6[a]	~0%
Aluminum phosphate	$Al(OH)_x(PO_4)_y$	Amorphous	Plate-like	5–7	0.7–1.5[b]	55%
Alum-precipitated	$Al(OH)x(PO_4)_y(SO_4)_z$	Amorphous	Plate-like	6–7	1.0–1.3[b]	Not determined

[a] BSA.
[b] Lysozyme.

components after isolation of the attenuated virus/bacteria as well as excipients that permit successful freeze-drying, stabilization of microorganisms, and acceptable pharmaceutical properties such as intact physical appearance (no cake collapse) and rapid reconstitution.

The lyophilization process involves controlled removal of water under decreased pressure and specific temperature conditions through various steps that include sample freezing, primary drying (sublimation of bulk water), and secondary drying (desorption of bound water) [38–40]. A variety of stresses are encountered during the freeze-drying steps (low temperature, phase separations, pH and ionic strength changes, and ice crystal formation among others) which necessitates the presence of lyo- and cryoprotectants as well as other stabilizing additives in the formulation to prevent or minimize the damaging effects of these stresses on the vaccine's stability and potency. A simultaneous optimization of the formulation composition and freeze-drying process is often required to obtain a stable vaccine product. It is also imperative that potency of the vaccine be assessed at each stage of freeze-drying process (e.g., freezing, primary drying, secondary drying). Successfully lyophilized vaccines should have a uniform cake appearance with no obvious signs of cake collapse and be able to be reconstituted easily, completely and quickly when mixed with a diluent. The factors that can impact the stability and potency of lyophilized vaccines include sample residual moisture content, sealing atmosphere composition and storage conditions such as temperature, humidity and light [39].

The lyophilized vaccine product is subsequently stored for months to years, under appropriate conditions, and then reconstituted immediately before administration to the patient. Reconstitution refers to the process of mixing of the freeze-dried vaccine powder with the diluent (liquid). Diluents for lyophilized vaccines vary in their composition and volume and are specifically designed to meet the pH, volume and chemical requirements of each vaccine [41]. While some diluents consist of sterile water, others may contain ingredients that not only help dissolve the freeze-dried powder but also stabilize (e.g., saline) and/or maintain the sterility of the reconstituted vaccine (e.g., preservatives). The volume of the diluent provided with a vaccine product is dependent on whether a single or multi-dose vial of vaccine will be reconstituted. The stability of reconstituted lyophilized vaccines is limited, both in terms of potency and ensuring sterility, and usually is used within hours or must be discarded. Therefore, once reconstituted, these vaccines should be stored under appropriate conditions (lower temperatures, protect from light, etc.) to better maintain the minimum required potency and sterility.

Although lyophilization technology has resulted in the development of many successful live, attenuated viral and bacterial vaccines, most of these vaccines still require storage at 2–8 °C or below which necessitates a cold chain for acceptable long term stability. A better understanding of the molecular basis or the origin of the instabilities of viruses and bacteria should result in the more rational design of stable vaccine formulations. In addition, alternative methods of processing such as spray drying may also lead to improved vaccine stability [40].

Non-replicating vaccines such as inactivated viruses/bacteria, purified subunit protein and carbohydrate antigens, VLPs, etc., are often more stable and can be formulated as liquid solutions stored in glass vials and/or prefilled syringes (it should be noted that WHO uses syringes only if they are auto-disabled). As discussed above, these vaccines also typically contain adjuvants to boost immune responses. Liquid vaccines are generally formulated to contain various compounds (excipients) that stabilize the antigen and prevent drastic conformational alterations over time that could lead to diminished potency. Optimization of buffer type, pH, ionic strength, and other stabilizing excipients is a key step in developing a successfully stabilized vaccine. Utilizing "generally regarded as safe" or GRAS excipients is the most straightforward place to start in identifying stabilizing excipients for vaccine formulations, since they have been classified as safe for food consumption [42]. It should be noted, however, that worldwide regulatory agencies have not necessarily approved GRAS compounds for use in injectable pharmaceuticals and vaccines. In this regard, the FDA provides a web-based database for excipients used in pharmaceuticals and vaccines (FDA inactive ingredient guide), including amounts and routes of administration [42,43].

Multi-dose vial formulations contain small amounts of preservatives that are bactericidal and bacteriostatic agents. Preservatives ensure the multi-dose vaccine can be used several times and over an extended period of time. One challenge with multi-dose presentations containing anti-microbial agents is the destabilizing effects many of these agents have on the potency and physical stability of some biological drugs and vaccines [44]. In this regard, single-dose prefilled syringes and vials, which are not formulated with preservatives, may be the only practical option.

Most liquid vaccine formulations of inactivated or subunit vaccines contain aluminum adjuvants to enhance immune responses. Although the generic term "alum" is frequently used in the vaccine literature, aluminum salt adjuvants are prepared by different processes and starting materials and therefore can actually vary dramatically in terms of their chemical composition, morphology and surface charge (Table 3). The adsorption mechanism of antigen to aluminum salts that is most frequently optimized in terms of vaccine formulation design is electrostatic interactions, although other physical mechanisms of interaction have been frequently reported (e.g., hydrophobic forces, ligand exchange, etc.) [45,46]. Briefly, adsorption is typically optimized by determining the isoelectric point (pI) of the antigen followed by selecting the oppositely charged aluminum salt, or selection of appropriate pH and buffering agents, resulting in an aluminum adjuvant with the opposite surface charge. Most commonly this involves addition of phosphate to aluminum hydroxide to lower its surface positive charge. Characterization and optimization of adsorption is conducted by obtaining an adsorption isotherm, which is produced by mixing increasing concentrations of aluminum salt with antigen or *vice versa*, centrifugation of the adsorbed antigen-adjuvant complex, followed by measurement of the amount of antigen that remains in solution, usually by UV-absorbance spectroscopy [47]. The need for careful optimization of the interaction of vaccine antigen and aluminum adjuvant is not always well recognized and it has been proposed that this become a key part of successfully designing vaccine formulations [48–50].

Intuitively, one might assume that the amount of antigen bound to an aluminum salt would be proportional to the robustness of the immune response, with perhaps a linear relationship. In practice, however, the amount of antigen adsorbed to the aluminum salt does not always correlate with a robust immune response since the soluble antigen itself, and the formulation components (e.g., phosphate) can play a role in immunological outcomes. Another important parameter, termed the adsorptive coefficient, is a measure of the strength of binding between the antigen and the aluminum adjuvant and can also play a role in immunogenicity [51]. It has been proposed that adsorption that is too strong could interfere with antigen processing and presentation by antigen presenting cells. Impaired antigen presentation would lead to poor B-cell activation, which would inevitably result in poor immunoglobulin secretion by plasma cells [51]. In some cases, weakened binding of antigen to aluminum produces an improved immune response [51,52].

Adsorption of vaccine antigens to aluminum adjuvants can also cause structural perturbations or changes in antigen conformational stability. For example, the thermal stability of several model antigens was decreased when adsorbed to aluminum adjuvant when compared to the soluble antigen alone [53]. These effects may be mitigated by addition of stabilizing excipients to the formulation [53]. Addition of excipients that increase the thermal stability (e.g., pH of buffer and phosphate concentration in the case of the Hepatitis B vaccine) can be used to generate more stable formulations [54,55]. Recent experimental work has also shown successful lyophilization of vaccines that contain aluminum adjuvants, with limited aggregation upon reconstitution [56,57]. Since aluminum adsorbed vaccines are sensitive to freeze-thaw damage, formulation strategies that include the addition of compounds that depress the freezing point (e.g., polyols) are an attractive solution to protect aluminum adjuvanted vaccines during exposure to freezing temperatures [10]. The formulation and stability of aluminum adjuvanted vaccines, such as HBV vaccines, are discussed in more detail in the case study section below.

6. Vaccine analysis and stability profiles

The key step to successfully monitor vaccine stability is identification of analytical test(s) with clinical relevance. This assay is referred to as the "potency test" and is defined as the "specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through administration of the product in the manner intended, to effect a given result" [58]. The potency assay not only permits a linkage between test results and the established safety and efficacy of the vaccine as determined in clinical trials, but also provides a mechanism to monitor subsequent commercial lots and evaluate their quality and stability from the perspective of pivotal clinical trials [58,59]. Due to their macromolecular complexity, it has not been possible to date to identify a physicochemical assay that can be directly related to vaccine's potency. Thus, biological assays (i.e., *in vivo* animal immunogenicity tests or *in vitro* cell-based or antibody binding-based assays) are the cornerstone of vaccine potency and stability testing. These biological potency assays typically have low precision (i.e., high variability) leading to numerous practical challenges in terms of establishing a vaccine's shelf-life and appropriate storage and handling conditions.

The overall goals of a vaccine stability program during clinical development are to (1) ensure that a vaccine remains clinically effective as defined by a lower potency limit, (2) assure a vaccine is clinically safe at the upper potency limit, and (3) confirm that a vaccine remains within the boundaries of the upper and lower potency limits throughout its shelf-life [58]. These goals are shown schematically in Fig. 2A where the potency value of a hypothetical vaccine at the time of manufacturing (i.e., the release potency) is determined from a combination of assay variability, stability loss rate (and its inherent variability), and the required minimum potency at the expiry date [60,61]. Due to the variability of biological potency assays, multiple vials of the vaccine are typically tested at any one-time point and a mean potency is reported. The associated errors of these measurements are typically determined during stability testing by determining the lower 95% confidence interval of the slope of the potency vs. time plots. For example, as shown in Fig. 2B, a linear regression line is fitted to the mean potency data (solid line) and the corresponding lower 95% confidence interval (dashed line) is used to establish the shelf-life (the timepoint at which minimum potency is reached).

One very important issue is the possibility that a mean potency value at a single time point may be below the minimum potency due to assay variability alone (see the 18 month time point in Fig. 2B). This situation has led to uncertainty for vaccine manufacturers and regulators on how to best define vaccine stability (i.e., the "compliance model" where vaccine expiry is determined by the first test result below minimum potency vs. the "estimation model" where statistical evaluation of all data points is used; in this case, 18 m vs. 24 m expiry dating). Although beyond the scope of this review, numerous scientific papers have appeared to better address these technical issues [58,62–64] as well as country specific and ICH regulatory

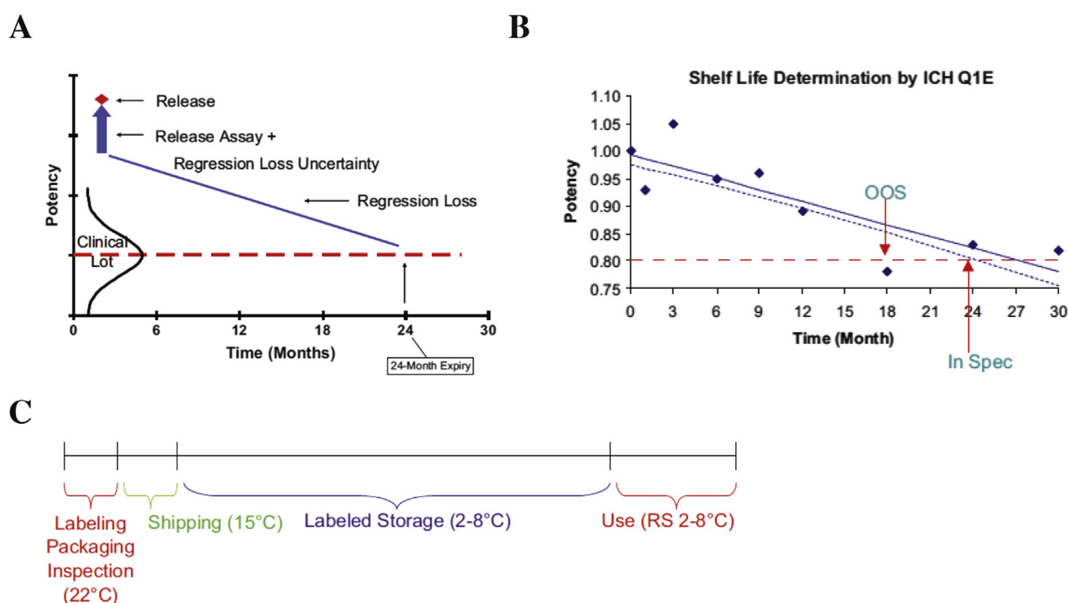

Fig. 2. Analysis of the stability profile of a hypothetical live virus vaccine. (A) Determination of release potency for a vaccine based on the stability profile and variability of the assay [199], (B) stability profile showing lower 95% confidence interval (dashed line) remains within specification for 24 months yet out-of-specification (OOS) result could imply a 18 month shelf-life [60], and (C) Handling and storage conditions during manufacturing, transport, storage and use [199]. Adapted and reproduced from Refs. [199,60] with permission from Elsevier.

guidelines including a 2006 WHO guideline on stability evaluation of vaccines [65]. As an example, for a live, attenuated viral vaccine whose potency is monitored by an imprecise viral plaque assay, one statistical evaluation estimated there is an ~30% chance over a 24 month stability study that the mean potency result for one timepoint during the study (e.g., at 0, 3, 6, 9, 12, 18, 24 months) will be below the minimum potency (due to assay variability) even though the vaccine is stable [60,61].

For a vaccine candidate in clinical trials, a vaccine stability program can differ from an approved vaccine in commercial distribution. For example, stability programs to support early-stage clinical trials are focused on establishing clinically effective ranges of vaccine potency while later-stage trials must establish a vaccine's safety and efficacy at the maximum release dose and minimum expiry dose to formally determine shelf-life and expiry dating. As shown in Fig. 2C, and as discussed in more detail below, these formal stability studies must not only account for losses of vaccine potency during manufacturing and storage, but also during shipping and handling of the vaccine immediately prior to administration. Post-approval stability programs include an annual stability lot to ensure consistency of manufacturing as well as comparability studies to support process changes [58,62]. The most crucial element of a vaccine stability program is the real-time stability determined at the actual storage temperature over the shelf-life of the vaccine (see Fig. 2B). Real time stability data are a requirement for regulatory approval and post-approval annual stability programs.

Accelerated stability studies are also performed for a variety of supportive reasons including the determination of causes of vaccine inactivation (e.g., effect of solution pH or excipients), to establish the stability-indicating nature of the potency assay, and to evaluate comparability between different vaccine lots and manufacturing processes [64]. In principle, accelerated stability data generated at different temperatures could be used to estimate stability profiles at lower temperatures using the Arrhenius relationship; however, due to the macromolecular complexity of vaccines, non-Arrhenius kinetics are often observed since the vaccine may inactivate by different mechanisms at different temperatures [64]. By definition, the Arrhenius relationship assumes that a vaccine inactivates by the same physicochemical mechanism across the temperature range being evaluated. For example, aggregation of viral particles is a multi-molecular event (i.e., it requires two or more viral particles to interact) while a conformational change, resulting in loss of protein structure or integrity of a lipid bilayer, is an inactivation event within individual viral particles. If one mechanism dominates at high temperature and another at lower temperatures, then Arrhenius kinetics would not be obeyed. Nonetheless, accelerated stability data have been successfully used to better understand vaccine stability under real time stability conditions with certain vaccines, especially over relatively narrow temperature ranges (e.g., 15° and 25 °C accelerated stability data may be able estimate vaccine stability profiles at 2–8 °C via an Arrhenius relationship while 45° and 55 °C accelerated stability data generally does not as accurately predict lower temperature behavior). A combination of real-time stability studies at the recommended storage temperature and accelerated stability studies at higher temperatures are outlined in regulatory guidelines for biotechnology products (e.g., ICH Q5C [66,67] to establish shelf-life and expiry dating [68,69]).

7. Monitoring vaccine stability in the cold chain

During commercial production and distribution, vaccine instability and potency loss can potentially occur across the entire vaccine supply chain from the manufacturer to patient administration. Because of the temperature sensitive nature of vaccines, the supply chain requires maintenance of either refrigerated or frozen temperatures, often referred to as the "vaccine cold chain". For example, potency loss can occur during long-term storage, shipping to various distribution centers, and the period immediately prior to administration. The final step, e.g., stability of a reconstituted lyophilized vaccine over the course of one day, can often be the most challenging in terms of maintaining appropriate storage and handling conditions.

Although the vaccine cold chain is typically thought of as a way to protect vaccines from inactivation due to exposure to elevated temperatures, it is important to note that low temperatures can also result in potency losses, e.g., inactivation of aluminum adjuvanted vaccines due to freezing. For example, a recent study in the United States found a high number of accidental exposures to freezing temperatures for vaccine vials labeled for storage at 2–8 °C due to inappropriate shipping or interim storage at health centers [70,71]. Unintended freezing of aluminum adjuvanted vaccines during transportation and distribution in the vaccine cold chain has become a concern in the developing world as well, since many WHO prequalified vaccines are freeze sensitive [72]. A detailed review of the scientific literature on the appearance of freezing temperatures across the entire vaccine cold chain found that accidental exposure to freezing temperatures at some point in distribution chain occurs for 75–100% of vaccine shipments, including 14–35% of refrigerators and/or transport shipments [10]. Even under well controlled storage conditions, the temperature can vary throughout a refrigerator placing vaccine supplies at risk of freezing during storage [73].

Ensuring the "vaccine cold-chain" is maintained from the factory to the end-user has led to an increasing awareness of critical challenges (and opportunities) in securing and monitoring the vaccine cold chain, both in the developed and developing world, to ensure end-users worldwide are receiving efficacious vaccines. Over the past few decades, there have been enormous efforts to implement the "vaccine cold chain" worldwide to ensure that vaccine potency is maintained during transportation and reception, storage at distribution centers, storage at health centers, and finally, at the point of use [71,74–76]. Vaccine Vial Monitors (VVMs) are an effective tool to reduce the risk that vaccines have been exposed to potentially damaging heat conditions [77,78]. Similarly, freeze indicators are used to monitor vaccines for exposure to freezing temperatures. Various VVMs are available depending on the type of vaccine and the target storage temperature requirements (Table 4). The VVM category used for a given vaccine is based on the stability data available for a specific vaccine. Therefore, the same vaccine type could have a different VVM category based on the manufacturer and the final presentation.

Table 4
Summary of Vaccine Vial Monitor (VVM) categories used during the distribution of various vaccines (Adapted from the website of World Health Organization, 2013). Since the VVM category used for a given vaccine is based on the stability data available for a specific vaccine, the same vaccine type could use different VVMs based on the manufacturer and the dosage form.

VVM category	Vaccine
2	OPV
7	DTP, (DTP + HepB + Hib), Hib, Influenza, MR, MMR, Japanese Encephalitis, IPV
14	DTP, TT, (DTP + Hep B), (DTP + Hib), (DTP + HepB + Hib), Japanese Encephalitis, Measles, MR, MMR, Rubella, Meningococcal, IPV, Rotavirus, Rubella, Yellow Fever, BCG, Cholera, DT, Hib, Hep A, Typhoid
30	DT, TT, Hep B, HPV, Meningococcal, Pneumococcal, Rabies, TT, Hib

Vaccine instability is of concern not only during commercial distribution, but also as part of clinical development. For example, during early clinical development when a new vaccine antigen has been identified, a series of studies are performed to ensure efficacy and safety in preclinical animal models. Frequently, very limited information is available on the stability of the vaccine antigen, especially in the presence of conventional and/or novel adjuvants, potentially leading to irreproducible results across different preparations of the experimental vaccine. During early clinical trials, strict cold chain requirements and surveillance of clinical supplies are important since due to limited stability data. For example, the FDA has identified instability of clinical candidates as one of the major reasons for suspension of clinical trials (i.e., clinical holds) with biological drugs [79]. This loss of potency of clinical material can not only occur due to temperature sensitive degradation events during storage, but also result from other environmental stresses. For example, inappropriate reconstitution and handling, excess agitation, and/or light exposure can also cause vaccine inactivation [7]. As discussed below, these examples of causes of vaccine inactivation can also play a key role in stability of commercial vaccines as well.

Many different approaches to better control temperature excursions in the vaccine cold chain are being pursued. These include new temperature monitoring technology to better identify thermal excursions, improved training procedures and infrastructure to reduce the frequency of their occurrence, better design and regulatory oversight of vaccine stability programs to address temperature excursions, and finally, adoption of testing procedures to directly evaluate vaccine vials accidently exposed to high/low temperatures. A recent review outlines currently available and emerging tools to improve the cold chain including improved temperature control strategies, better monitoring of temperature excursions, and regulatory oversight of the supply chain [80]. For example, additional stability studies to support product stability during internal handling (filling, labeling, and distribution) and external situations (unforeseen temperature excursions during distribution, handling and administration) are receiving more attention as part of regulatory filings [81–84].

Examples of additional stability data include temperature cycling studies, multiple freeze-thaws, and shipping simulations to help establish vaccine stability during temperature excursions that may occur after the product leaves control of the manufacturer [62,85,86]. Although it is difficult to design stability programs to

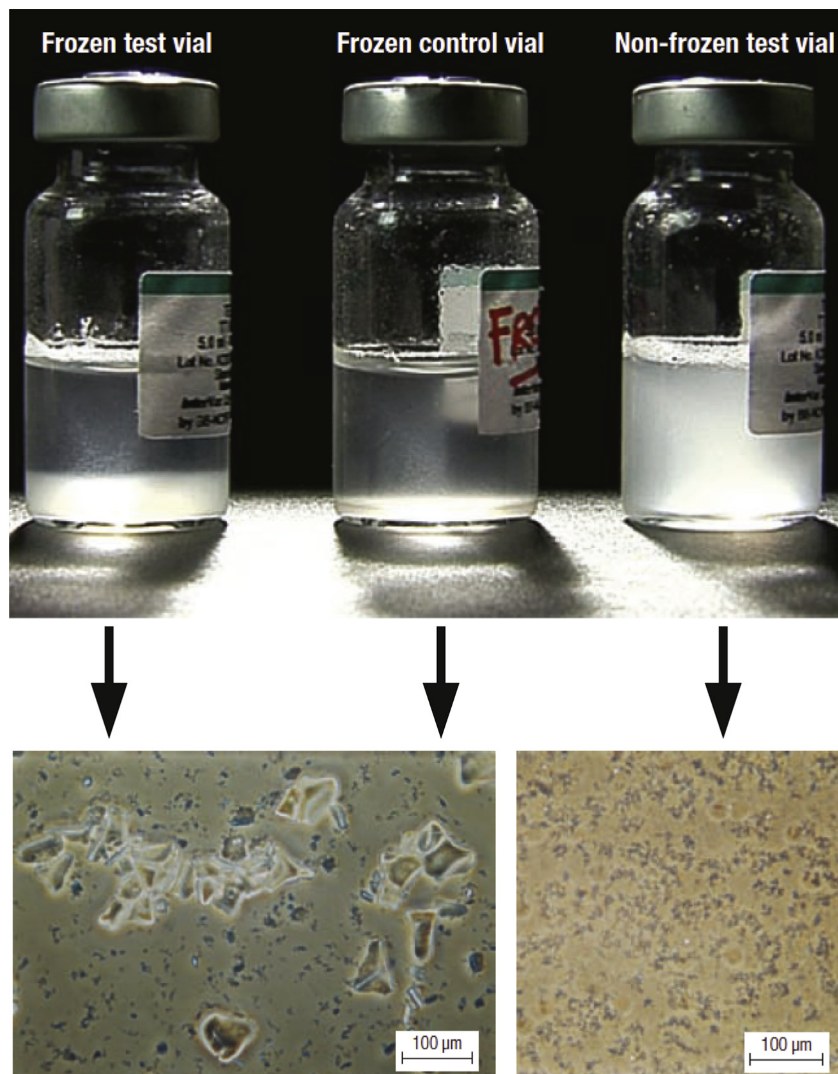

Fig. 3. Visual assessment and optical microscopy to detect freeze damage to an aluminum adjuvanted vaccine after a "shake test" followed by settling for 90 s [92]. Adapted and reproduced from Ref. [92] with permission from The World Health Organization.

cover all possible high temperature and freeze-thaw excursions, these types of studies can potentially lead to a stability database that can establish allowed temperature and time limits for excursions outside of recommended storage conditions [81]. For example, these types of stability data have been generated for live, attenuated [87] and subunit flu vaccines [88] as well as a human papillomavirus vaccine [89]. More recently, a meningococcal vaccine gained approval for storage and transport for up to four days without refrigeration or the use of ice packs [90]. A thermal stability test has been performed for specific lyophilized live, attenuated vaccines to provide an indicator of consistency of production of vaccine product in the context of lot release [9,91]. For example, in the freeze-dried state, measles vaccines were expected to retain a minimum potency of at least 3.0 log_{10} live virus particles per human dose after exposure to a temperature of 37 °C for at least one week. Additionally, the virus titer does not decrease by more than 1.0 log_{10} during incubation.

A "shake test" to determine if exposure to freezing conditions has changed the physical properties of the aluminum adjuvanted vaccine has been developed for field use, especially in the developing world [92]. When a vaccine supply is suspected of being exposed to freezing temperatures, selected individual vials from that lot (test samples) are compared to a control sample from the same vaccine (that have been intentionally frozen). The test and control samples are shaken and then placed on a flat surface and allowed to settle by gravity. Visual observation over several minutes reveals if the aluminum adjuvant has been physically altered. Some representative vials containing aluminum adsorbed vaccine, including vials that have not been frozen and were intentionally frozen, that were run in the shake test are shown in Fig. 3. The differences in the settling behavior of the aluminum adjuvant between the vials can clearly be detected by visual observation over a certain time period. A validation study has shown excellent agreement between results from the simple shake test performed by health care workers and detailed phase microscope examination of eight different types of WHO prequalified vaccines stored at both 5 °C and exposed to freezing conditions [92] [93,94].

8. Case studies: vaccine characterization, analysis of stability profiles, and examples of instability issues in the vaccine cold chain

8.1. Influenza vaccines

Influenza is the most commonly occurring respiratory disease caused by influenza viruses and is a leading cause of illness and death around the world [95,96]. Seasonal flu epidemics of influenza virus cause serious illness and millions of human infections worldwide especially among children, elderly as well as immune suppressed patients [97]. Influenza viruses are enveloped RNA viruses belonging to the Orthomyxoviridae family and contain eight single-stranded RNA segments encoding 11 proteins [96,98]. The viral particles are typically 80–120 nm in diameter and roughly spherical in shape (see Fig. 4A). The central core of the viral particles contains the segmented RNA in association with the viral proteins that package and protect the RNA. This core is surrounded by the host cell membrane derived lipid envelope that contains the two major envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The influenza viruses are divided into three types, A, B and C, with types A and B responsible for the annual human epidemics. Influenza A viruses are further classified into subtypes based on the antigenic nature of hemagglutinin and neuraminidase glycoproteins. The frequent and constant antigenic changes in the HA and to a lesser extent in the NA proteins as a result of point mutations and recombination events during viral replication (antigenic drift) necessitate the annual updating of the seasonal influenza vaccines. More dramatic changes (antigenic shift) in the HA subtype result in less frequent but more destructive influenza pandemics [98].

Multiple approved influenza vaccines (with the same antigenic composition) are currently available through different manufacturers (Table 2) [99]. Nearly all of the commercially available vaccines use HA and NA proteins as their primary antigens with the HA protein being responsible for eliciting the primary neutralizing immune response [98]. The seasonal flu vaccines are usually trivalent (or quadrivalent) and are composed of the influenza strains (or their HA proteins) most likely to circulate in the upcoming flu season. All current flu vaccines contain influenza A(H1N1), A(H3N2), and B viral antigens. There are two antigenically distinct strains of influenza B viruses referred to as the Victoria and Yamagata lineages [97]. Flu vaccines are available as live-attenuated influenza vaccines (LAIV) and trivalent inactivated vaccines (TIV), or more recently, quadrivalent vaccines. In addition, recombinant influenza vaccines based on recombinant HA based antigens have been in clinical development for the past decade [100-102], and a recombinant HA seasonal flu vaccine was recently approved for commercial use by the FDA (Flublock® produced by Protein Sciences). Although the virus has traditionally been grown in chicken eggs [97,103,104], mammalian cell lines (MDCK or Vero) have been approved for use for inactivated vaccines in many areas of the world [103,104] [105] and in the United States (Flucelvax® produced by Novartis).

We will first consider the formulation, analytical assays and stability profile of the live-attenuated influenza vaccines (LAIV) vaccine as shown in Fig. 4. The live-attenuated vaccine (FluMist®, MedImmune) is intranasally administered with a sprayer [97]. The live virus vaccine (See Fig. 4A) is supplied as a pre-filled refrigerated FluMist sprayer which contains a single 0.2 mL dose with 106.5-7.5 FFU (fluorescent focus units) of live attenuated influenza virus reassortants of the viral strains. Excipients including monosodium glutamate, hydrolyzed gelatin, arginine, sucrose, dibasic potassium phosphate, and monobasic potassium phosphate are included to optimize vaccine's stability at refrigerated temperatures. The potency of LAIV is evaluated by either a TCID50 assay (Fig. 4B) or an antibody-based fluorescence focus assay (FFA; see Fig. 4C) [87,104]. As shown in Fig. 4B, the TCID50 assay (also called an endpoint dilution assay) quantifies the amount of virus required to produce a cytopathic effect in 50% of inoculated tissue culture cells [106]. As shown in Fig. 4C, the FFA measures virus infectivity per dose using immunostaining of infected MDCK cells (fluorescent focus units). The assay utilizes hemagglutinin-specific primary antibodies followed by detection with a fluorescently labeled secondary antibody by manual counting of foci using a fluorescent microscope [87].

A representative stability profile of a live virus vaccine preparation of influenza at different temperatures using the TCID50 assay is shown in Fig. 4D. It is recommended that LAIV be stored at refrigerated temperatures (2–8 °C) before administration [87]. A recent study [87] evaluated the immediate and long-term impact on LAIV potency after real world temperature deviations including freeze-thaw cycles and exposure to heat. The stability study showed no significant loss of potency (by viral titers measured by the fluorescence focus assay) after three freeze-thaw cycles, warming of LAIV for 72 h at 15 °C, exposure to room temperature for 12 h or after heating to 37 °C for 6 h or less. There are, however, no published data to support the effect of long term freezing or exposure to room or elevated temperatures for longer periods of times on LAIV potency, and the manufacturer recommends that LAIV be stored only at refrigerated temperatures (2–8°C/35–46 °F) before administration.

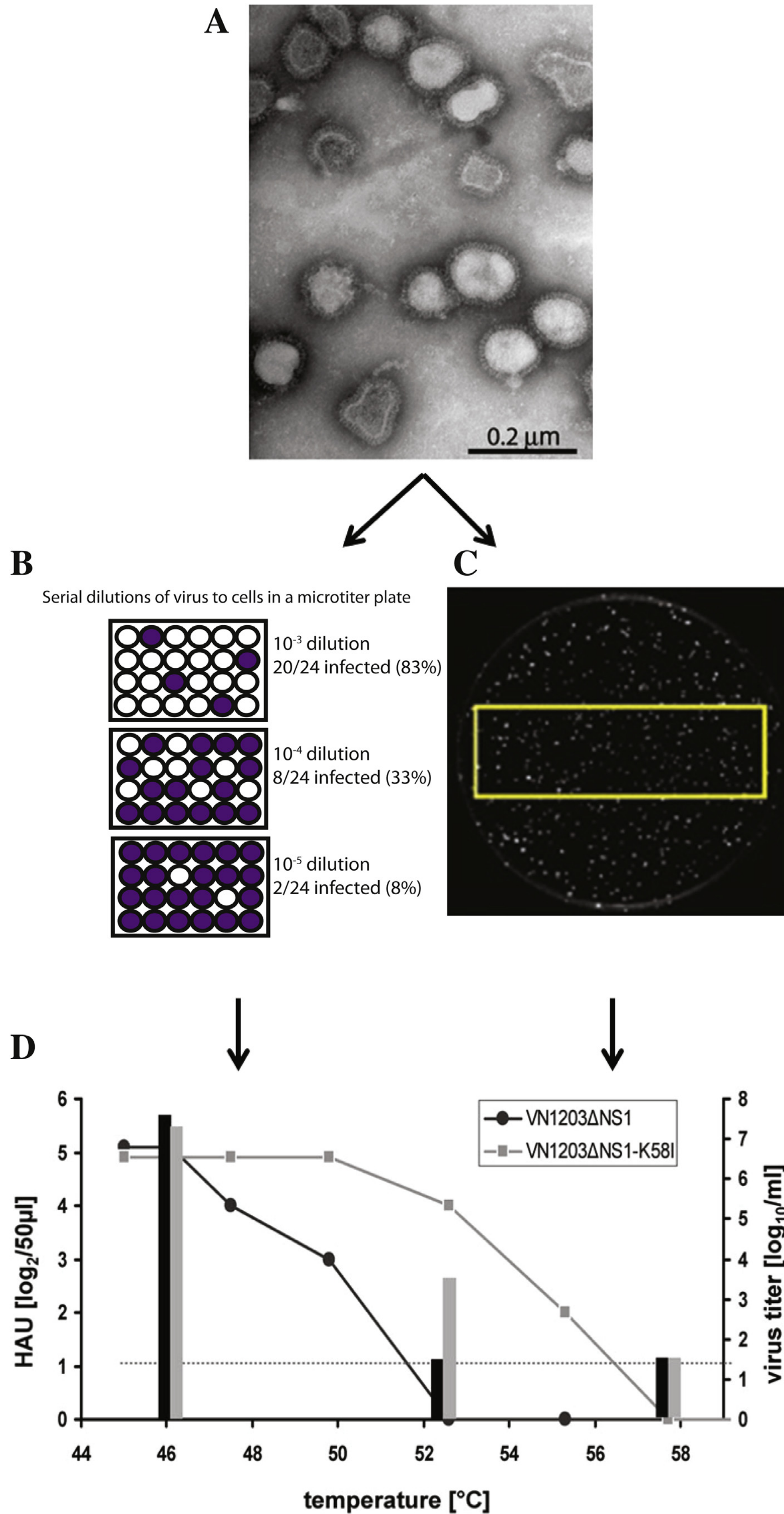

Fig. 4. Structure, potency assays and stability profiles of live influenza vaccine. TEM micrograph of (A) live-attenuated influenza vaccine (LAIV) [200]. Potency of LAIV vaccines as monitored by either (B) a TCID50 assay or [106] (C) a fluorescence focus assay (FFA) [201]. (D) The thermal stability of LAIV vaccine as monitored by TCID50 [202]. Adapted and reproduced from Refs. [201,202]. The image in Fig. 4C was provided courtesy of Molecular Devices, LLC.

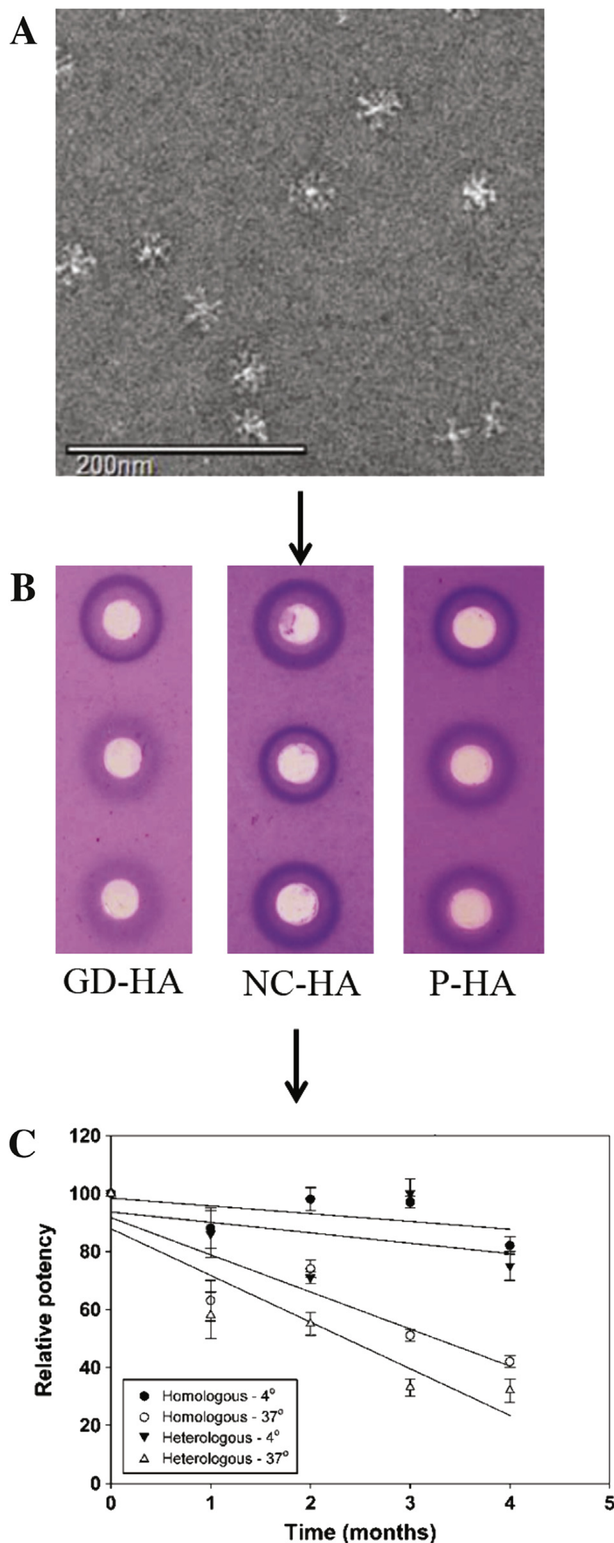

Fig. 5. Structure, potency assays and stability profiles of inactivated influenza vaccine. (A) TEM micrograph of trivalent inactivated vaccine (TIV) [203]. (B) The potency of TIV vaccines as monitored by SRID assay [109]. (C) The thermal stability of TIV vaccine stability as monitored by the SRID assay [204]. Adapted and reproduced from Refs. [109,203,204] with permission from Elsevier and Wiley.

We will now review the formulation, analytical assays and stability profile of the inactivated trivalent influenza vaccines (TIV), or more recently quadrivalent vaccines, as shown in Fig. 5. The inactivated vaccines (whole, split or subunit; see Fig. 5A) are administered intramuscularly (with the exception of Fluzone® Intradermal, Sanofi Pasteur). Although TIV contain varying amounts of different viral proteins (e.g., NA, M and NP) depending on the process methods, the hemagglutinin antigen (HA) is the main immunogen. A minimal amount of detergent, used to disrupt the viral envelope (in the case of split or subunit vaccines), may remain in the final vaccine preparation. Trace amounts of formalin or beta-propiolactone used to inactivate the virus may also be present in some vaccine preparations along with residual amounts of egg proteins and antibiotics. For example, Fluvirin® (Novartis Vaccines), a subunit (purified surface antigens) vaccine, is formulated in phosphate buffered saline. The 5-mL multi-dose vial formulation of Fluvirin® contains thimerosal as a preservative. Inactivated flu vaccines licensed in the United States do not contain an adjuvant, although adjuvants have a demonstrated capacity to enhance the protective antibody response of some inactivated influenza vaccines. These include aluminum based adjuvants and oil in water emulsions (MF59 from Novartis and ASO3 from GSK) among many other proprietary adjuvants [107]. Several seasonal flu vaccines with adjuvants have been approved and are used in Europe [105].

The potency of inactivated influenza vaccines, as well as the recently approved recombinant HA vaccines, is evaluated using a single radial immunodiffusion (SRID) assay that measures HA content per dose [100,108] as shown in Fig. 5B. The assay is based on the diffusion of detergent solubilized vaccine samples into an agarose matrix containing antibodies against the HA. The interaction between antigen and antibody produces a precipitation ring the size of which is directly proportional to the amount of antigen present. The vaccine (HA) potency is measured by assessing the diameter of the immunoprecipitin ring (Fig. 5B).

A representative stability profile of an inactivated influenza vaccine preparation at different temperatures using the SRID assay is shown in Fig. 5C. Inactivated influenza vaccines should be stored at refrigerated temperatures (2–8 °C for up to one year) and should not be frozen [97,103]. A stability study conducted on 27 full scale production batches of sub-unit influenza vaccines showed elevated storage temperatures increased the degradation rate of the hemagglutinin protein as measured by an SRID assay [88]. For example, degradation at 25 °C occurred 6–12 times faster than at 5 °C depending upon the influenza subunit vaccine strain. A statistical evaluation of the stability data obtained, however, showed that even for the most sensitive strain examined, exposure of the vaccine to room temperature for 2 weeks would not adversely affect the shelf life claim of the influenza subunit vaccine after one year in the refrigerator.

Additionally, mechanistic studies to evaluate the effect of environmental stress conditions on the structural stability of commercially available subunit influenza vaccines have shown that exposure to freeze-thaw events, low pH, and high temperatures all have adverse effects on the secondary and tertiary structure of the hemagglutinin protein [96,101,109,110]. In fact, exposure to freezing temperatures results in unfolding of HA caused by formation of ice and concentration of solutes [87,96,109]. Another recent mechanistic study demonstrated the partial loss of potency during storage of recombinant HA at elevated temperatures, as measured by the SRID assay, correlated with the formation of non-native disulfide bonds including cysteine residues located in the C-terminal region of the hemagglutinin protein [111].

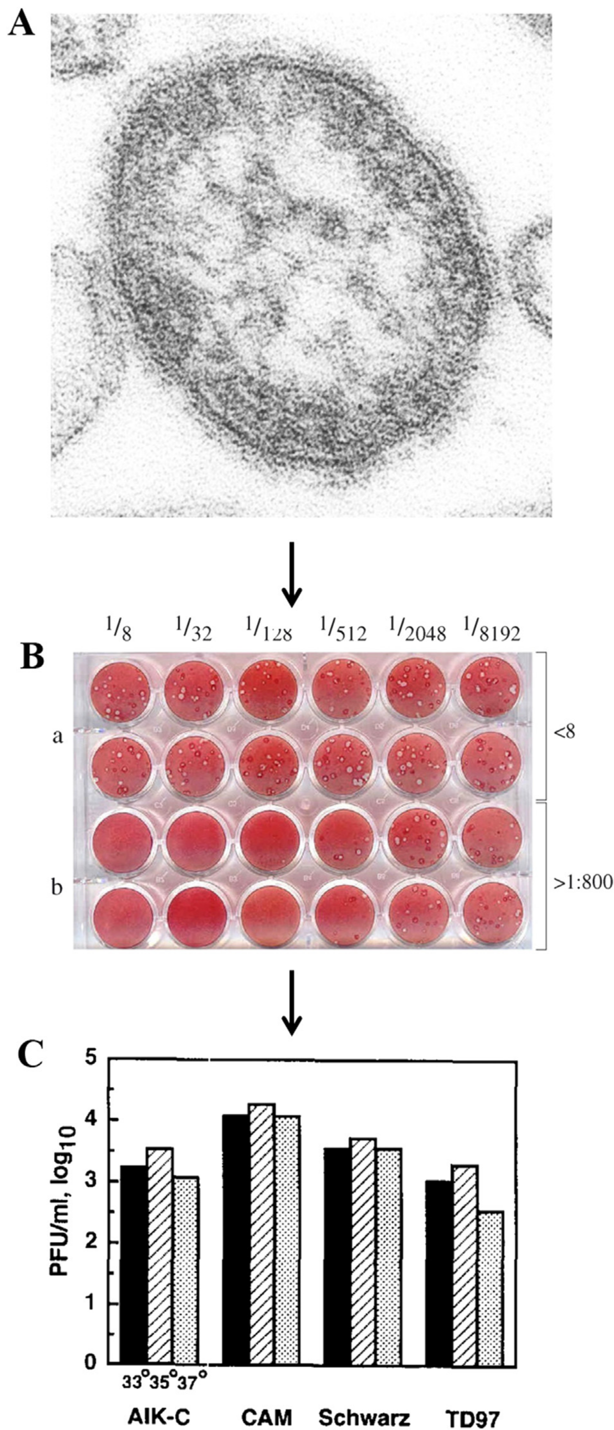

Fig. 6. Structure, potency assay and stability of live, attenuated measles vaccine. (A) TEM micrograph of measles virus (kindly provided by the CDC, in public domain) with diameter of ~250 nm, (B) representative measles virus plaques on VERO cells used for potency determination [124], and (C) Temperature sensitivity of Japanese measles vaccine strains as measured by a viral plaque assay [205]. Four vaccine strains of measles virus were assayed for plaques at 33, 35 and 37 °C. Adapted and reproduced from Refs. [124,205] with permission from Elsevier.

8.2. Measles vaccines

Measles, a highly infectious respiratory disease, continues to be a leading cause of global mortality among children [72]. The recommended age for measles vaccination varies between 6 and 15 months [112,113]. Although measles is considered to be eradicable due to the availability of efficacious live attenuated viral vaccines [114], these vaccines are unfortunately still underutilized in developing countries [115]. Measles virus is a member of the Morbillivirus genus of the Paramyxoviridae family [113]. The virus is spherical, enveloped, single stranded, containing negative sense RNA and is approximately 250 nm in diameter (Fig. 6A). The viruses contain six structural proteins, three complexed with viral RNA to form the nuclocapsid (P, L and N) while the other three (F, H and M) are associated with the envelope. The two viral transmembrane glycoproteins, the hemagglutinin (H) and the fusion protein (F) are essential for the fusion of the viral envelope with the host cell membrane, which leads to viral entry and subsequent infection.

Measles vaccines are one of the most unstable live, attenuated viral vaccines and thus require an uninterrupted cold chain for transportation and storage. Breaks in the cold chain can lead to less effective vaccination programs [116–118]. The commercial measles vaccines are lyophilized formulations of attenuated live viruses in the presence of various stabilizers (e.g., Attenuvax® produced by Merck; see Table 2) [113,115]. While most of the measles vaccines are produced in chick embryo fibroblasts, some vaccines currently used worldwide are produced in human diploid cells [112]. Attenuvax® is available in a lyophilized format where the virus is derived from the attenuated Edmonston strain and propagated in chick embryo cell culture [119]. Each 0.5 mL dose, which requires reconstitution, contains at least 1000 CCID50 (cell culture infectious doses) of measles virus along with stabilizer and media components (sorbitol, sodium phosphate, sucrose, sodium chloride, hydrolyzed gelatin, human albumin, fetal bovine serum, other buffer and media ingredients and neomycin). The live attenuated measles vaccines are administered subcutaneously or intramuscularly [113].

In the United States and many other developed countries, measles vaccine is administered as a combination vaccine (measles, mumps, rubella). In the United States, the only licensed MMR vaccine is produced by Merck & Company (MMR-II®), although other WHO recognized and internationally approved MMR vaccines are available in other countries. To maintain potency, MMR-II® must be stored between −50 °C and 2–8 °C [120]. In 2005, a quadrivalent vaccine produced was licensed in the United States that combined measles, mumps, rubella and varicella vaccines (MMRV, ProQuad® produced by Merck). The frozen formulation of ProQuad is starting to be more widely used in the United States and has been shown to be as immunogenic and well tolerated as its component vaccines, MMR-II® and Varivax® [121]. The use of frozen ProQuad® is, however, limited to geographical locations where a frozen cold-chain is available during transport and storage. A refrigerator-stable formulation of ProQuad® has also been recently developed to expand the utility of this vaccine and has been shown to have similar safety and immunogenicity profile to its frozen counterpart [121]. The stabilization of varicella, both monovalent and as a component of the quadrivalent vaccine, has proven to be especially challenging and has required extensive formulation development activities [7,122,123].

Two types of cell based tissue culture assays are used to monitor the potency of live, attenuated measles vaccines. The viral plaque assay measures "plaque forming units or pfu" formed in infected cells (See Fig. 6B). The second commonly used potency assay, TCID50 assay, was described in the previous case study. Both methods typically use Vero cells [117,124] and are widely used for measuring the potency of live virus vaccines during stability studies (Fig. 6C). In its lyophilized form, the current measles vaccines are generally stable for 2 years at 2–8 °C and retain satisfactory potency, up to 50% for at least 1 month at 22–25 °C storage temperatures. They are generally stable to freeze-thaw stress [125], but measles vaccines should be protected from light [120]. Measles

vaccines rapidly lose potency after reconstitution. For example, reconstituted vaccines lose 50% of their potency when stored for 1 h at 20–25 °C and almost all potency when held at 37 °C [113]. Moreover, these vaccines are also sensitive to light and colored glass vials have been shown to minimize potency loss [126]. The reconstituted product should be kept at 2–8 °C and used the same day due to both stability limitations as well as concerns for the potential of microbial contamination (in absence of a preservative). In contrast, the frozen MMRV vaccine must not only be transported and stored frozen, but once reconstituted, should be discarded if not used within 30 min [113].

Field evaluations of storage facilities and potency of lyophilized measles vaccines over the last two decades have demonstrated the importance of maintaining the cold chain during shipment and storage [125,127–130]. Several examples of breaches in the cold chain and subsequently its effect on measles vaccine potency are summarized and reviewed [125]. These case studies establish that an inefficient cold chain can cause potency loss of otherwise efficacious measles containing vaccines.

There is considerable interest in new formulations with improved thermal stability that can be administered by less invasive routes of administration during mass vaccination. For example, there are multiple ongoing efforts to develop and evaluate aerosol delivery of aqueous and dry powder forms of measles vaccines [117,118,131]. The dry powder inhalable vaccines have been shown to have enhanced thermostability and therefore may better maintain the potency of the vaccine during temperature excursions and also permit vaccine distribution under ambient conditions during segments of the cold chain [117,131]. The production of Measles vaccines delivered in a dry powder format would also eliminate the need for reconstitution. Aerosolized measles vaccines have been evaluated for use in school aged children and were shown to be more immunogenic than the subcutaneous route of administration [132], although they were less efficacious in young infants [133]. Novel formulations and excipients have recently been described with the long term goal of dramatically improving the stability of live virus vaccines and potentially allowing for the reduction in the number of steps, or even elimination, of the vaccine cold chain [134–136]. As one example, a recent study examined a novel excipient, silk fibroin protein (silk films), on the accelerated stability profile of a MMR vaccine during storage at elevated temperatures (25 °C, 37 °C, and 45 °C). The authors found the silk films significantly reduced the vaccine potency loss due to storage at elevated temperatures [137].

8.3. Poliovirus vaccines

Poliomyelitis is a devastating paralytic disease caused by infection with poliovirus. The virus can invade the central nervous system and causes destruction of motor neurons that can result in muscle weakness and acute flaccid paralysis that progresses to debilitating disease and possibly death [138]. Polioviruses belong to the family Picornaviridae, which are 27–30 nm in diameter, non-enveloped, and have a (+) sense single-stranded RNA genome 7.5 kb in length (see Fig. 7A). The genome is surrounded by four structural proteins: VP1, VP2, VP3, and VP4. There are 60 copies of VP1 and VP3, and 58-59 copies of VP2 and VP4 [7,139]. Three antigenic serotypes exist (Brunhilde, Lansing, and Leon), with the blueprint of the capsid proteins remaining constant and variability between serotypes residing in the neutralizing capsid epitopes themselves [140].

Development of widespread vaccination practices has eradicated the wild type virus in the Western Hemisphere [138]. In a small number of areas of the developing world, however, poliomyelitis remains a problem because it remains a challenge to vaccinate the entire population. Two different vaccines have been developed that both confer >90% protection to vaccinated individuals, inactivated poliovirus (IPV) and attenuated live poliovirus (OPV) [20]. Both types of vaccines (IPV and OPV) are derived from poliovirus grown in monkey kidney cells (VERO cells) and can either contain one serotype (monovalent) [141] or multiple serotypes (multivalent). The inactivated poliovirus vaccine (IPV) was developed by Jonas Salk in the 1950's and consists of poliovirus Types 1, 2, and 3 that were grown in cell culture and subsequently inactivated with formaldehyde [20]. Live attenuated poliovirus (OPV) was originally produced by successive passages through monkey tissue and was developed by many scientists including Albert Sabin [20,142].

IPV is administered via subcutaneous or intramuscular injection while OPV is given orally, to mimic infection with the wild type virus. Individuals vaccinated with OPV can shed the attenuated virus and non-vaccinated individuals can passively acquire the attenuated strain. This is a major advantage of OPV over IPV, along with intestinal immunity due to the oral route of administration. IPV is currently administered in the United States due to eradication of the disease in North America, although OPV remains the polio vaccine of choice in polio endemic areas [143].

One example of an inactivated polio vaccine (IPOL® produced by Sanofi Pasteur) is given in four doses to children aged 2, 4, 6–18 months as well as a booster at age 4–6. IPOL® is a trivalent vaccine in a liquid formulation that does not contain adjuvant. The vaccine also contains 0.5% 2-phenoxyethanol, 0.02% formaldehyde (as preservatives), and small amounts of antibiotics (<200 ng, from cell the culture medium) per dose. Polioviruses are resistant to many common inactivation reagents such as ethanol, ether, chloroform, and non-ionic detergents, but can be inactivated by UV light, heat (55 °C), chlorine, and formaldehyde [139,144]. Inactivated poliovirus is also formulated as a component of several combination vaccines (Table 2). The IPV vaccine is stored at 2–8 °C and should not be frozen [145].

OPV (monovalent, bivalent, and trivalent) is available from many different manufacturers worldwide. One example, OPVERO® manufactured by Sanfoi Pastuer, is a liquid formulation using human albumin, magnesium chloride, polysorbate 80, and HEPES as stabilizing formulation excipients. OPVERO® does not contain adjuvant and is stored at −20 °C, but can be stored at 2–8 °C for up to 6 months. Potency of OPV drops significantly when stored at elevated temperatures [144]. Heat inactivation of poliovirus is a major concern during the transport and storage of the vaccine prior to administration. Further increasing the thermal stability of poliovirus vaccines would have a positive effect by lessening the impacts of temperature excursions. Indeed, as described below, thermal stabilization of poliovirus vaccine has been the subject of considerable research over the past few decades.

During stability studies, the potency of live, attenuated poliovirus vaccines over time is monitored by viral plaque assay (a representative plaque assay shown in Fig. 7B). Biophysical studies have demonstrated that polioviruses can be inactivated by moderate heat treatment (42–45 °C) by first unfolding the capsid protein followed by degradation of the viral RNA [146]. Magnesium chloride ($MgCl_2$) has been shown to increase the thermal stability of OPV, and is included as a stabilizer in the vaccine [147]. In the late 1990s deuterium oxide (D_2O) was identified as a stabilizer of OPV [148]. Interestingly, D_2O has also been shown to stabilize other macromolecules [149] In a study by Chen et al., the OPV stabilization by D_2O and $MgCl_2$ was examined by spectroscopy and calorimetry. The authors showed that $MgCl_2$ and D_2O act in concert to rigidify the conformation of the virus, although the mechanisms of stabilization are different [150]. Biophysical studies with another picornavirus (Hepatitis A) established that $MgCl_2$

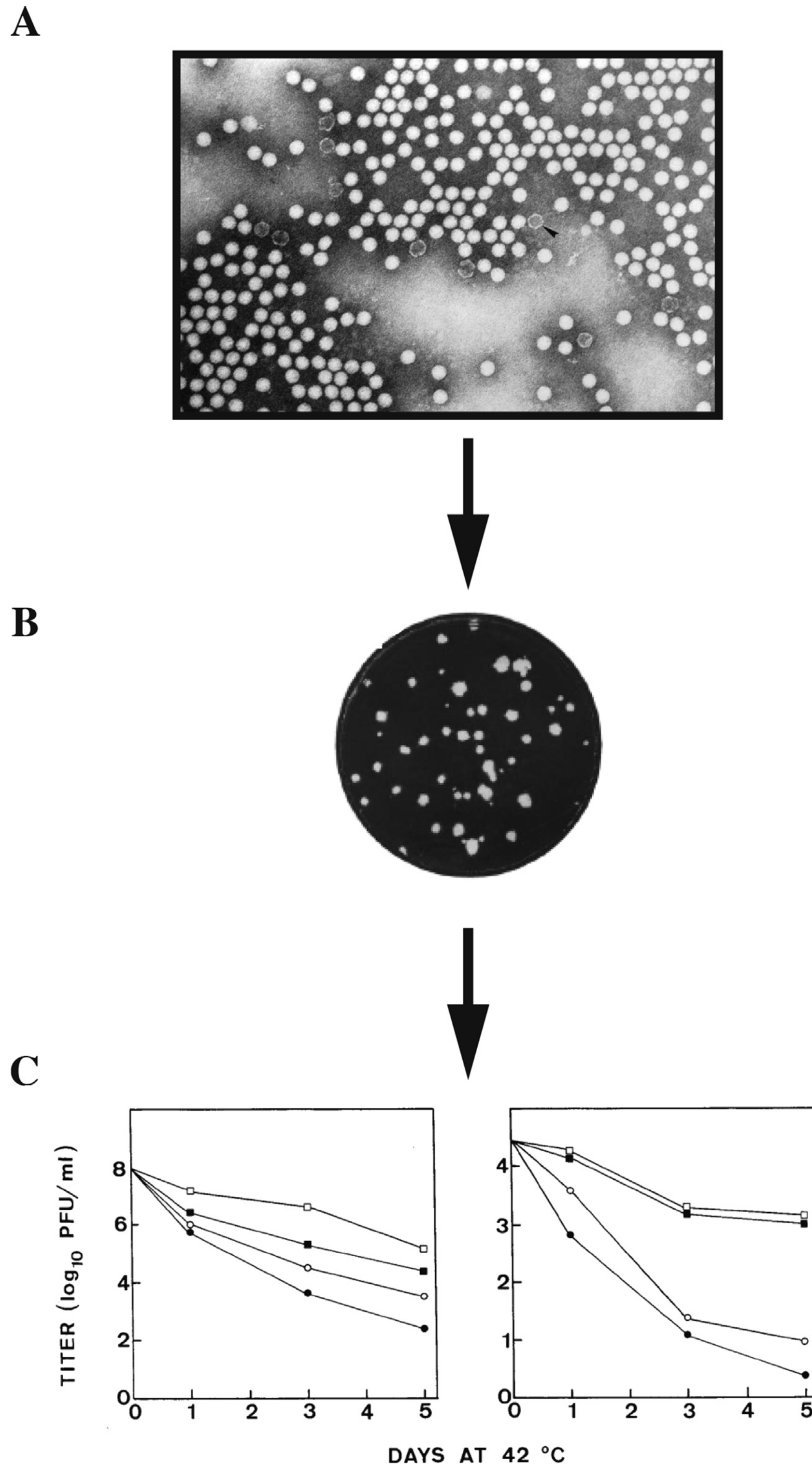

Fig. 7. Structure, potency assay and stability of live, attenuated polio vaccine. (A) TEM micrograph of poliovirus virions (provided kindly by the CDC, public domain) with diameter of ~30 nm, (B) Poliovirus plaques on a HeLa cell monolayer [206], and (C) Time dependent thermal inactivation of the Sabin strain in the presence of various stabilizers [153]. Adapted and reproduced from Refs. [206,153] with permission from Elsevier.

stabilization is due to conformational stabilization of the viral capsid [151]. The perceived harmful effects of D_2O in the formulations resulted in the exclusion of D_2O from consideration as a new OPV thermal stable formulation [152]. Another study by Verheyden et al., found an even greater increase in thermal stability when Pirodavir, a compound that binds the viral capsid, is included in the formulation [153]. These stability experiments were conducted by stressing each OPV containing formulation at 42 °C and measuring potency over time by a viral plaque assay (See Fig. 7C).

Several studies have evaluated the OPV vaccines in the cold chain in polio-endemic countries [128,154–156]. These studies

employed vaccine vial monitors (VVMs) to determine the severity and length of heat exposure of each OPV vial. A study by Samant et al. examined the integrity of the cold chain in a rural district in India. The authors focused on heat exposure of OPV at the last link of the vaccine cold chain, in this case, community health centers. They found 9% of the vials damaged by excessive heat that should not be used (by readout of VVMs). The authors found that the use of ice packs and the corresponding temperature of the vaccine carrier were the most important factors leading to temperature damage of OPV [154]. A study by Halm et al. investigated the effectiveness of using OPV vaccine stored in the cold chain and then taken outside-the-cold chain during an OPV vaccination campaign in a remote area of Mali. The authors found that limited out of cold chain storage of OPV is acceptable as long as the vaccine does not reach its discard point (assessed by reading a VVM) [155]. A separate study conducted in Chad drew similar conclusions [156].

8.4. Hepatitis B vaccines

Despite available vaccines, Hepatitis B virus (HBV) continues to cause significant disease worldwide with approximately 350 million people chronically infected [157]. HBV replicates in hepatocytes and causes major inflammation in the liver. Infection with HBV could lead to either acute or chronic infection, with chronic infection often resulting in cirrhosis and/or hepatocellular carcinoma [158]. HBV is a partially double-stranded DNA virus with a 3.2 kb genome. The virus is enclosed in a lipid envelope and is ~42 nm in diameter [159]. The HBV genome encodes three structural proteins and a DNA dependent DNA polymerase. The S-protein is the major lipid envelope protein (HBsAg) and contains 226 amino acids residues with a molecular weight of 26 kDa [158,160,161]. Free HBsAg is found in the plasma of infected individuals in the form of non-infectious virus-like particles (VLPs). These plasma derived VLPs are ~22 nm in diameter and were used to vaccinate individuals prior to the introduction of the recombinant HBsAg vaccines [162]. Upon purification from plasma, HBsAg VLPs were treated with heat and chemicals to ensure there were no residual viral contaminants [163–165].

The antigen component of recombinant Hepatitis B vaccines is the S-protein (HBsAg) which is typically expressed in yeast. A major structural difference between the plasma-derived and yeast-derived S-protein is the post-translational glycosylation pattern [166]. The HBsAg protein spontaneously self-assembles into virus-like particles (VLPs) during expression (Fig. 8A and B) [167]. HBsAg VLPs must also form disulfide-linked oligomers during purification that are essential for immunogenicity and conformational stability [168]. HBV vaccines are liquid formulations that contain 5–40 mcg of HBsAg per ml and are adsorbed onto or co-precipitated with aluminum adjuvants (Table 2). The two FDA approved vaccines (RECOMBIVAX® produced by Merck, and ENGENERIX-B® produced by GSK) have been formulated without preservatives in pre-filled syringes and single-dose vials [169]. Various other HBV vaccines are available worldwide and contain aluminum adjuvants. An additional formulation (Fendrix® produced by GSK) is formulated with MPL (a lipid A derivative) and aluminum phosphate as adjuvants [170,171].

To verify immunogenicity and monitor stability, two different potency assays are typically used (Fig. 8C). First, for the *in vivo* potency assay, BALB/C mice are inoculated with HBsAg and the antibody response is measured by ELISA. Alternatively, an *in vitro* ELISA based assay is also commercially available that measures the antigenicity of HBsAg surface antigen [172,173]. The composition of the vaccine formulation can have a significant impact on the stability of HBV vaccines throughout the cold chain as well tolerance to temperature excursions. Although HBV vaccines are reasonably stable at elevated temperatures [174], it is often desirable to formulate a vaccine to be as thermostable as possible [175]. Some representative stability profiles of HBsAg vaccines under different stress conditions, as monitored by the *in vivo* potency assays, are shown in Fig. 8D.

The primary concern with the transport, handling, and storage of HBV vaccines is avoidance of freezing. Freezing causes clumping of the aluminum salt that significantly reduces potency and alters the conformation of the HBsAg (see Fig. 3, optical microscope pictures). Scanning electron microscopy (SEM) and X-ray analysis on several different WHO pre-qualified vaccines containing aluminum adjuvant after subjecting them to freezing temperatures (-25 °C) were compared to vaccines that were stored at $+5$ °C. HBV vaccines that had been frozen showed significant aluminum salt agglomeration, rough structural features, and broken lattices that were not observed in the vaccines that were stored at $+5$ °C [93]. Similar observations using phase contrast microscopy have also been described using other aluminum containing vaccines [92] (Fig. 3). Another study by Chen et al. examined the freezing kinetics of the HBV vaccine and correlated freezing with an increase in particle size, changes in HBsAg protein tertiary structure, and changes in surface charge (zeta potential) resulting in reduced immunogenicity [176].

In practice, exposure of vaccines to freezing temperatures can occur in all areas of the world. Numerous studies conducted over the last decade highlight the importance of either maintaining the cold chain or putting into place different storage or transport practices that will not result in freezing aluminum salt containing vaccines [177,178]. A study in Indonesia, for example, conducted in 2001 and 2002, found inadvertent freezing of HBV vaccines occurred in 75% of shipments. Most freezing occurred during transport from province to district. Ice-free transport, air conditioned storage in districts (opposed to storage in a refrigerator), and room temperature storage at health care centers completely eliminated freezing of the vaccines [178]. Thus, in this case, an outside the cold chain (OCC) approach was feasible due to the stability of the HBV vaccine at ambient temperatures; however, the OCC approach is not always feasible, as a study by Ren et al., has demonstrated during transport to rural areas of Western China during the winter [127] where HBV vaccines were exposed to freezing temperatures. Although difficult to decisively establish, it is attractive to postulate that the increase of cases of Hepatitis B in 'vaccinated' populations is due to use of vaccine that has been frozen prior to administration [177,179]. A study conducted in Mongolia found a stark difference in anti-HBV antibody titers within vaccinated 2 year olds in urban areas (94.2%) and rural areas (70.4%) [179]. A subsequent study identified exposure of HBV vaccines to freezing temperatures during transport from province to rural health centers [180]. Finally, Davaalkham et al. [181] found significant differences in the effectiveness of the HBV vaccine when administered during the winter months. Taken together, these studies suggest that frozen vaccines were responsible for the differences in seroconversion rates. These studies also point out the importance of proper training of staff that will administer vaccines as well as the use of freeze indicators.

Many formulation studies have recently been undertaken to improve the stability of HBV vaccines to prevent loss of potency due to freezing [52,54]. The study by Braun et al., found addition of propylene glycol and other polyols protected HBV vaccines from freeze-thaw mediated damage through several cycles. Addition of propylene glycol depressed the thermodynamic freezing point of the formulation, thus preventing aluminum salt agglomeration [176]. Phosphate, histidine, and the pH of the buffer (pH 5.2), on the other hand, protected the vaccine from heat stress [54]. Addition of

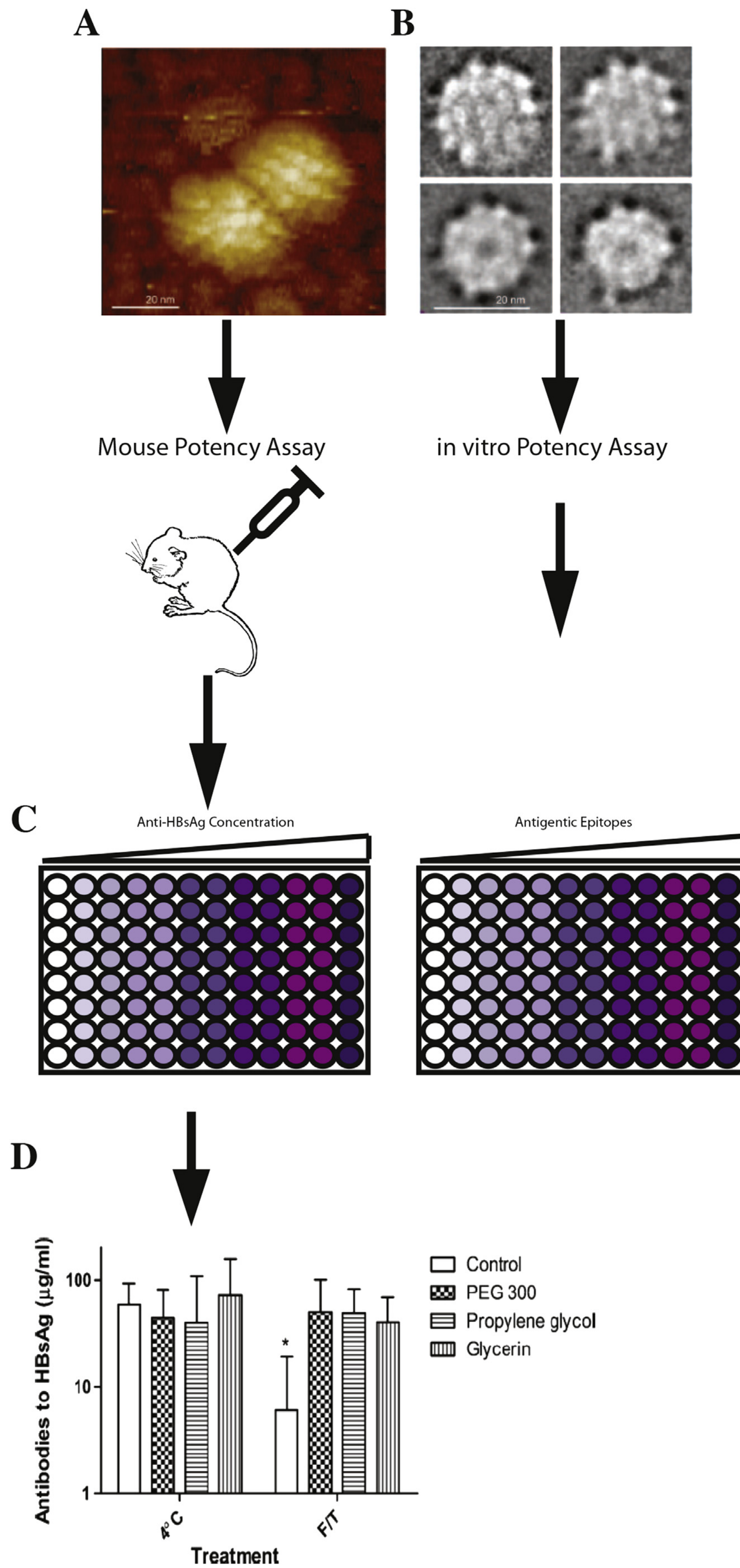

Fig. 8. Structure, potency assay and stability of an HBV vaccine. (A) AFM and (B) TEM micrograph of HBsAg VLPs produced from yeast [173]. (C) Potency is measured either by an *in vivo* mouse assay or an *in vitro* potency assay. Potency quantitation in both assays is measured by ELISA. (D) Effect of stabilizers added to an HBV vaccine formulation to maintain potency after freezing as measured by an *in vivo* mouse potency assay [189]. Adapted and reproduced from Refs. [173,189] with permission from Elsevier.

these excipients had no effect on the potency of the vaccine in a mouse immunogenicity assay (Fig. 8D).

9. Conclusions and future directions

Due to their inherent instability, vaccines require storage, transportation and administration under controlled temperature conditions. Thus, the vaccine cold chain must be carefully maintained across the entire supply chain during commercial distribution. Some vaccines are particularly sensitive to elevated temperatures (e.g., live, attenuated viral vaccines) while other vaccines lose potency if accidentally frozen (e.g., aluminum adjuvanted vaccines). In this review, the causes and mechanisms of vaccine instability were examined including the effects of exposure to elevated temperatures and freezing on the delicate nature of the three-dimensional structures of different types of macromolecular antigens as well as on the physical integrity of aluminum adjuvants. Moreover, analytical challenges to monitor vaccine potency over time were shown to play a key role in assessing vaccine stability, especially due to the inherent variability of commonly used bioassays often performed in cell-based formats or animal models. A major public health concern is that the loss of vaccine potency during storage and handling could lead to administration of subpotent vaccines. The inter-relationships between the nature of different vaccine antigens and adjuvants, their corresponding potency assays, and the determination of vaccine stability profiles in the cold chain were described with case studies of important commercially available vaccines including influenza, measles, polio, and Hepatitis B.

Organizations such as the World Health Organization (WHO) and PATH have developed strategies to better implement improvements in vaccine stability and the vaccine cold chain worldwide [182,183]. Based on experiences with 22 collaborations to improve stability of seven different vaccines antigens with 11 different vaccine manufacturers, PATH has summarized key "lessons learned" on vaccine stabilization projects including (1) integrate stabilization efforts into early (clinical) development, (2) circumstances where it makes sense to stabilize existing vaccines, (3) freeze stabilization is possible for aluminum adjuvanted vaccines, (4) heat stabilization requires a customized approach and results will be variable, (5) full benefits of heat stable vaccines will only be realized after changes are made in storage guidelines, (6) trade-offs may be needed between heat-stability and other format characteristics of a vaccine, and (7) vaccines with enhanced stability can benefit both vaccine producers and purchasers [184]. Some additional practical hurdles to implement new vaccine stabilization technologies include long and uncertain timelines for implementation due to costs and regulatory requirements [11].

In the United States, improving vaccine stability across the cold chain is a goal consistent with an emerging consensus over the past decade, by several advisory groups for the US Government (including the National Vaccine Advisory Committee, NVAC), of an urgent need to improve vaccine development, production and distribution capabilities. For example, a study by the NVAC examined potential weaknesses in the supply of childhood vaccines [185] and identified one the five major contributing factors to vaccine shortages as "… the high cost and complexity of the development, approval and manufacturing, and distribution of vaccines …". An earlier study by the NVAC examining vaccine development in the United States [186] points out, "It was clear that the critical step-up from bench scale to pilot lots and then to large-scale production, which depends on a small group of highly trained individuals, is often a particularly vulnerable point in the development process", and how "… scale-up or changes in formulation can result in a loss of potency or diminished efficacy". A review of the US vaccine system by a group of vaccine experts [187], which highlights the major components and participating groups in the US vaccine system, points out the key role of vaccine development and manufacturing. These general concerns about the ability of the vaccine community to rapidly develop, produce and distribute vaccines were also noted during the H1N1 influenza pandemic in 2009. For example, a US Department of HSS report [188] outlines the need to improve the ability of the United States to more rapidly develop, produce and distribute vaccines and medical countermeasures.

Despite the numerous vaccine stabilization challenges described in this review, there are also many opportunities for improvements. These range from short- to mid-term approaches as well as longer-term goals. For example, improvements such as better training and improved infrastructure are needed in the short term to improve compliance within the existing vaccine cold chain. In addition, there are opportunities to better employ current VVMs, along with developing new VVM technologies, to better monitor temperature excursions across each step of the vaccine cold chain. In the mid-term, there are opportunities to reformulate currently available vaccines to improve their stability in the vaccine cold chain. For example, the use of certain pharmaceutical excipients can potentially prevent freezing of aluminum adjuvanted vaccines such as Hepatitis B during transient exposure to low temperatures [182,189]. Longer term, by better understanding the physicochemical mechanisms of vaccine inactivation, mutations and modifications could be introduced into vaccine antigens (e.g., site-directed mutagenesis) to improve their inherent conformational stability. In addition, improvements in vaccine formulation technologies, including new adjuvants, are needed along with new physicochemical analysis approaches, to better preserve vaccine potency during both clinical development and commercial use [48,49]. For example, high throughput screening analysis of excipients for their ability to stabilize a variety of vaccine antigens by biophysical methods has been described [190–192]. In addition, high throughput robotics has been utilized to develop new liquid formulations of a measles vaccine using a cell-based assay [193]. Additional trends and novel approaches towards vaccine stabilization include use of novel excipients and evaluation of new drying technologies [11]. By developing a better basic understanding of the physicochemical mechanisms of vaccine inactivation [207], rational strategies to better stabilize new vaccines, both by molecular design and formulation strategies, will be possible in the long term.

Thus, a combination of longer-term research goals of improving the immunogenicity and inherent stability of vaccines along with shorter-term approaches to better utilize available vaccine cold chain technology are required. This combination approach will not only best ensure reduced wastage and improved vaccination rates with current vaccines, but will also play a key role in the future development of new vaccines to prevent and/or treat unmet medical needs due to infectious diseases worldwide.

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