Vaccines against the cold chain

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Vaccines require continuous refrigeration to retain their efficacy as most vaccine components are derived from mammalian or microbial origin which are thermally unstable. Continuous refrigeration, known as the vaccine cold chain, comes at a cost that directly correlates to the standard of infrastructure that is available. A break in the cold chain caused by poor infrastructure can result in direct loss of functionality in these lifesaving medicines. Therefore, several approaches have been suggested to mitigate these losses. Not only will these benefit general healthcare via improved shelf-life but also enhanced resistance to thermal fluctuations and, in some cases, improved drug target localisation. In this minireview, we highlight trends in vaccine thermal stabilisation and look to the future for cold chain logistics.

Vaccines and thermal stability

Vaccines, as with most biologically derived therapeutics, are essentially made from proteins, lipids, metal ligands and other small moieties that play their part in overall functionality. Many of them are liquid formulated or reconstituted before being administered to patients. Pharmaceutical formulations are designed to target drug uptake and prevent in-solution events such as flocculation, aggregation, and denaturation. Salts, osmolytes, buffers and viscosity modulators improve the overall stability of the formulation giving a nominal shelf life of, in the best case, several years in the refrigerator (Brandau et al., 2003).

Freeze-drying or lyophilisation provides an excellent alternative for those vaccines that are able to be processed in this way. This methodology has dramatically improved the shelf-life and thermal stability of numerous vaccines by simply removing the aqueous phase, thereby eliminating the possibility of detrimental in-solution events (Brandau et al., 2003). However, there are caveats when it comes to freeze-drying, as not all vaccines can be processed through this method due to issues such as incompatible excipients, adjuvants, and freeze-sensitivity. As a result, many other alternatives have been developed over the last 15-20 years.

Thermal stabilisation inherently means improving the resistance of a particular compound to thermal stress and degradation. This stress can come from the environment, such as tropical or cold

climates, or from equipment i.e., fridges and cooled boxes etc., and is referred to as heating or freezing (Kumru et al., 2014). Both these variations can have a detrimental effect on the functionality of a vaccine. Heating encompasses an increase in vibrational energy, faster moving molecules, and once this passes a certain threshold specific to the entity, it will result in degradation. Freezing affects vaccines indirectly via its medium. Liquid formulated vaccines are always based in an aqueous solvent which forms ice crystals upon freezing. These crystals can impair hydrophobic interactions which are essential for protein-based vaccines (Matthias et al., 2007). Upon thawing, these weakened bonds cannot reform into their original state thereby causing the vaccine to lose its potency. The alum adjuvant ($AlOH_4^-$) commonly used in many vaccines also contributes to freeze-thawing problems via crystal salt formation that impairs protein stability (Martin and Lloyd, 2005). For these reasons, vaccines must be protected from both freezing and heating.

The vaccine cold chain

The current system of ensuring the retention of pharmaceutical functionality is to use continuous refrigerated logistics, also known as the cold chain infrastructure (Figure 1). Vaccine cold chain is a ~ 50-year-old technology, originally designed to keep vaccines cold during the start of Expended Program for Immunisation (EPI) by the World Health Organisation (WHO) (Keja et al., 1988).

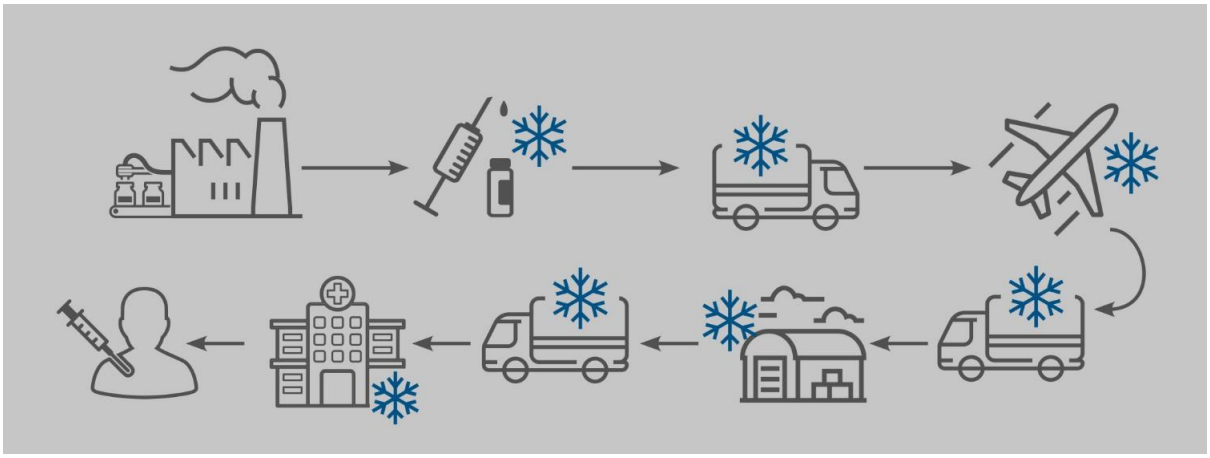

Figure 1 Vaccine cold chain, starts when vaccine is manufactured and continues until it can be administered to a patient. From (Sartbaeva, 2018).

When the EPI was initiated in 1974, fewer than 5% of children in low-income countries were receiving DTP and poliomyelitis vaccines in their 1st year of life. These coverage levels have now surpassed 50% in many low-and middle-income countries, and millions of cases and deaths have been prevented. Despite the successes of EPI, currently about 2 million infants are still dying from vaccine-preventable diseases worldwide every year, indicating that vaccine cold chain on its own is not enough in our fight against infectious diseases (Liu et al., 2012).

The benefits of thermally stable vaccines are highlighted by the successful eradication of smallpox in humans in the 1970s and rinderpest in cattle in the 1980s. Both diseases have vaccines formulations that are stable without refrigeration. A third disease that is close to eradication is polio. Only three countries around the globe have not yet been declared polio free, but the WHO aims to eradicate polio by 2025. The oral polio vaccine is stable at room temperature for up to 3-4 weeks. Thankfully, scientists have been developing innovative solutions to keep vaccines from spoiling outside refrigerators. Many of these are now established methodologies or are highly promising for their intended use. We highlight some of the prominent methodologies in the following section.

Towards thermal stabilisation of vaccines

Vaccine thermal stabilisation has been a prominent trend (Figure 2) in the last two decades following the conclusion that cold chain logistics and storage are inherently flawed, and alternatives are required especially for developing regions around the world (Brandau et al., 2003, Dumpa et al., 2019). With the world currently focusing on mitigating SARS-CoV-2 infections and trying to increase vaccination rates, one of the hurdles is vaccine thermal instability. The required storage temperature for the Pfizer-BioNTech and Moderna COVID-19 vaccines is −65 °C due to the active compound being messenger-RNA (mRNA) which is produced between DNA transcription and protein translation as an unstable intermediary in gene expression (Baden et al., 2020, Polack et al., 2020). Only specialised refrigerators and cooled boxes are able to attain such a level of cooling as the vaccine degrades rapidly at room temperature. Development of room temperature stable vaccine formulations, either dried or liquid, that can overcome this barrier could speed up the return to society as it was before the pandemic hit in 2019. In addition, it could help humanity with stockpiling vaccines thereby

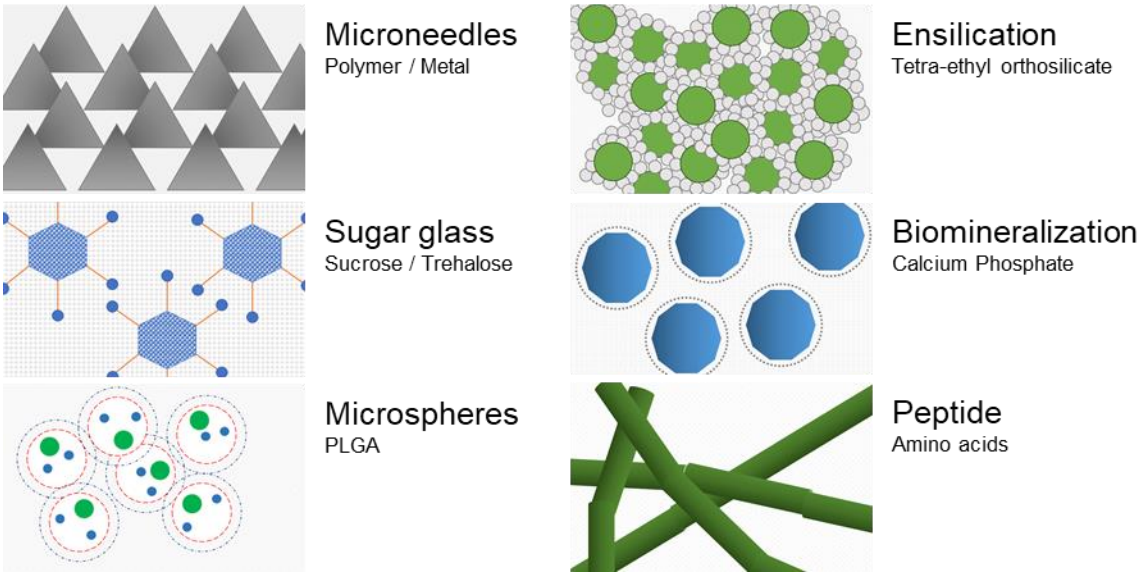

Figure 2. Graphical representation of various methods suitable for thermal stabilisation of vaccines and being developed today.

providing a first line of defence and preparedness to future outbreaks of emerging diseases and enable remote locations without good infrastructure to receive these life-saving therapeutics.

One of the prominent methods that improves thermal stability of vaccines are microneedles. These have been proposed as an alternative due to their improved thermal stability compared to liquid formulated vaccines. Microneedle patches containing vaccine antigens require less space, are solid formulated, use a lower antigen dosage, and can achieve immunity via intra dermal vaccination as shown in several studies described by Prausnitz (Prausnitz et al., 2009). These have been widely researched specifically towards vaccination against influenza (Choi et al., 2012). The most recent development presented a dried vaccine formulation against dengue fever with a shelf-life of 3 weeks at ambient temperatures (Turvey et al., 2019).

Other research on thermal stabilisation using carbohydrate glass, a mixture of trehalose and sucrose, demonstrated the improvement in shelf-life of stabilised adenoviruses up to 6 months at 45 °C (Alcock et al., 2010). This promising technique is now being used as a vaccine platform that can be tailored to provide immunity against other targeted mammalian viruses. The most recent development showed a formulation using thermally stabilised simian adenovirus vector targeted at rabies which is currently progressing towards clinical trials (Wang et al., 2018).

Microspheres made of poly-lactic-co-glycolic acid (PLGA) have been utilised mainly for target drug localisation and delivery. However, a recent study discussed the thermal stabilisation of inactivated polio virus (IPV) within these microspheres. The researchers found increased stability at 37°C with 2.4-fold increased retention of antigen after 1 month of two pulsatile releases (Tzeng et al., 2016). This meant that their formulation could provide the initial and booster shot in one injection, which could lead to drastic savings in vaccinations and logistics.

Method	Starting material	Formulation	Target	Type	Thermal Stability	T_m
Microneedles	Poly-lactic acid	Solid	Dengue virus[1]	Live attenuated	1 month at 25 °C	-
Sugar matrix	Sucrose & trehalose	Solid, reconstitute upon use	Rabies adenovirus[2]	Live attenuated replication deficient	30 days at 45 °C	-
Microspheres	Poly-lactic-co-glycolic acid	Solid, in solution or can be reconstituted upon use	Poliovirus[3]	Inactivated	1 month at 37 °C	-
Ensilication	Tetraethyl orthosilicate	Solid, release required before use	Ag85B-Sbi[4] TTCF[5]	Recombinant protein	1 month at RT	> 95 °C Ag85b > 80 °C TTCF
Biomineralization	Calcium phosphate	Liquid	Enterovirus[6]	Live attenuated	1 month at 26 °C	-
Peptide	Amino acid fibrils	Solid and liquid	$ESAT6_{51–70}$[7]	Epitope	6 months at 45 °C	-

Solvent free biofluid	Glycolic acid ethoxylate lauryl ether	Liquid, non-aqueous	Avidin[8]	Recombinant protein	56 days at 40 °C	139 °C
Microneedles + Sugar matrix	Nanopatch	Solid	Adenovirus[9]	ChAd63	10 weeks at 37 °C	-

Table 1. Overview of various technologies for vaccine thermal stabilisation. [1](Turvey et al., 2019), [2](Wang et al., 2018), [3](Tzeng et al., 2016), [4](Wahid et al., 2019), [5](Doekhie et al., 2020a), [6](Wang et al., 2013), [7](Sun et al., 2016), [8](Bui-Le et al., 2021), [9](Pearson et al., 2013).

Another material shown to be suitable for vaccine thermal stabilisation is silica, silicon dioxide, SiO_2, the main constituent of sand. This inorganic material is surprisingly biocompatible with several organisms using silica to stabilise their environment. With its wide use in society, chemical inertness, and ceramic properties, it is an interesting material for thermal stabilisation. We developed a method of encapsulating proteins in silica known as ensilication (Chen et al., 2017). This method uses soluble (prehydrolysed) silica to create a soft shell around the biological target, by utilising silica's ability to grow in a network thus surrounding the biological target. Because silica grows as a network, the shell it creates tailor-fits the biological target, supporting and protecting the target from unwanted unfolding or aggregation. Silica shells, each with a target inside, aggregate together and create particles, that can precipitate out of the solution. After drying, the particles appear as a white powder that can resist up to 100 °C heating and several freeze-thaw cycles (Doekhie et al., 2020b). Our studies on vaccine antigens are posing an interesting future for ensilicated vaccines that do not require cold chain refrigeration (Wahid et al., 2019, Doekhie et al., 2020a). Our latest *in vivo* data on the tetanus toxoid C fragment (TTCF) show that ensilicated TTCF samples transported without refrigeration and released before injection can produce a similar immune response to native samples. Embedding antigens within porous silica has also been proposed for thermal stabilisation of vaccines by Montoya (Montoya et al., 2020). Additionally, a study where the Japanese encephalitis vaccine (JEV) was incorporated into PEI-silica nanoparticles showed good immune response *in vivo* with enhanced thermal stability showing 1-log decrease in titre after 7 days compared to 6-log reduction for the unprotected vaccine at 25 °C (Wang et al., 2016).

Peptide induced biomineralization using calcium phosphate has also shown its competence in thermal stabilisation of a live virus vaccine via self-assembly (Wang et al., 2013). This was done via surface modification to express specific peptides that promote biomineralization which resulted in a 4-log fold increase in stability after 18 days of storage at 26 °C.

Nanofiber peptide vaccines also demonstrate increased stability up to 6 months at 45 °C (Sun et al., 2016). These peptides form self-assembled fibrils containing specific epitopes, antigenic regions of immunological significance, which can be tailored to target a specific pathogen. Similar stability of these antigenic peptides in powder or suspension formulation has been shown.

Another method proposed as an alternative are solvent free biofluids. Here, researchers showed that by chemical protein surface modification, they could increase the unfolding temperature (T_m) of

avidin, a ligand binding protein, by 73 °C (to 139 °C) passing the boiling point of normal aqueous solvents (Bui-Le et al., 2021). This is due to the water free nature of the solvent-free biofluid. Whether this will work *in vivo* is yet to be determined.

Combinations of the mentioned methodologies have also been presented in the scientific literature. Microneedles coated with sugar glass stabilised adenovirus ChAd63 retained single-dose immunogenicity after 10 weeks storage at 37 °C (Pearson et al., 2013).

The future of vaccine storage

Methodologies such as microneedles, sugar glass matrices, ensilication, encapsulation, biocompatible polymers and others have the potential to eliminate vaccine cold chain, or at least reduce the dependency on this fragile system. We believe that evolution dictates change, not revolution. To improve vaccine availability, scientists working on this challenge of thermal stabilisation must understand that (novel) methods should be easy to implement into the current system of vaccine manufacturing, especially focusing on formulation development. Additionally, untrained medical professionals need to be able to utilise the novel vaccine formulations if they require unorthodox ways of reconstitution and administration.

In summary, thermal stabilisation of vaccines is well on its way with several methodologies showing promising results in *in vivo* studies. We believe that the increase in shelf-life at ambient temperatures and novel formulations will result in reduced wastages thereby improving the vaccine accessibility for developing countries struggling to maintain vaccine stockpile levels or requiring emergency aid. Health workers will be able to reach all those in need of vaccines even in remote regions of the globe which is essential in protecting our global health.

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