AAPS PharmSciTech (2020) 21:133
DOI: 10.1208/s12249-020-01673-5

Review Article

Theme: Formulation and Delivery of Natural Products
Guest Editors: Harsh Chauhan, Abhijit Date and Sonali Dhindwal

The Stabilizing Excipients in Dry State Therapeutic Phage Formulations

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Received 22 December 2019; accepted 29 March 2020

Abstract. Phage therapy has gained prominence due to the increasing pathogenicity of "super bugs" and the rise of their multidrug resistance to conventional antibiotics. Dry state formulation of therapeutic phage is attractive to improve their "druggability" by increasing their shelf life, improving their ease of handling, and ultimately retaining their long-term potency. The use and selection of excipients are critical to stabilize phage in solid formulations and protect their viability from stresses encountered during the solidification process and long-term storage prior to use. Here, this review focuses on the current classes of excipients used to manufacture dry state phage formulations and their ability to stabilize and protect phage throughout the process, as discussed in the literature. We provide perspective of outstanding challenges involved in the formulation of dry state phage. We suggest strategies to improve excipient identification and selection, optimize the potential excipient combinations to improve phage viability during formulation, and evaluate new methodologies that can provide greater insight into phage-excipient interactions to improve design criteria to improve formulation of dry state phage therapeutics. Addressing these challenges opens up new opportunities to re-design and re-imagine phage formulations for improved efficacy as a pharmaceutical product.

KEY WORDS: phage therapy; dry state formulation; inhalation; excipients.

INTRODUCTION

Multidrug resistance (MDR) of antibiotics has become one of the major threats to human health globally (1,2); in the USA alone, there have been approximately 99,000 deaths annually from antibiotic-resistant, pathogen-associated hospital-acquired infections every year (3). There is a critical need to develop and/or re-introduce alternatives to antibiotics, with the World Health Organization reporting that the current rate of resistance in bacteria and tuberculosis exceeds the rate of discovery and development of anti-MDR drugs (4). Bacteriophage, or phage, has recently re-emerged as antimicrobial therapeutics from their initial discoveries to prevent bacterial infections by Twort and d'Herelle (5,6). While interest waned with the advent of small molecule antibiotics, the ever-increasing severity of antibiotic MDR and the rise of "super bugs" have led to the resurgence of phage therapy as a promising alternative or adjuvant therapy to antibiotics (7).

Phage are bacterial viruses that infect specific strains of a single bacteria species (8). During their life cycle (and therapeutic mode of action), phage infect their bacterial host, inject their genome, and using the host machinery, they assemble, produce, and propagate more phage until they leave and possibly lyse the host (*i.e.*, depending on the type of phage). Generally, the number of proliferated phage positively correlates with the number of its host bacteria, and without the bacterial host, phage is eventually eliminated by the reticuloendothelial system in mammals (9,10). Thus, phage have been endowed with desired properties by nature for therapy, including (1) strain-specific infection, (2) amplified: "auto-dosing" due to replication in target host, (3) non-disturbing to normal mammalian cells: low *in vivo* toxicity, (4) symbiotic relationship with and non-disruption to normal flora, (5) innate capability to penetrate and traverse tissues and barriers, (6) versatility in formulation and applications, (7) biofilm clearance, and (8) adaption to resistant bacteria (10–13).

One of the challenges to realize the promise of phage therapy is to translate the technology from the bench to a viable drug product that maintains long-term stability and potency (8). Initially, phage formulations are prepared as liquid suspensions, which limit their shelf life and safety and potency after storage. As a consequence, liquid dosage forms often require cold temperature storage at 2–8 °C and

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cold chain transportation. Since phage essentially consist of coat proteins (*i.e.*, capsid) encapsulating their genetic material, formulation strategies of phage have mostly been adopted from protein-based formulations. Typically, proteins are more stable in a dry state than in solution due to their sensitivity to the physical stresses in aqueous solutions, including temperature, pH, ionic strength, agitation, exposure to interfaces, the risk of microbial contamination, and water-related chemical degradation (14–16). In the dry powder state, the formulation typically has a longer shelf life, and there is more flexibility in its transportation and storage conditions. The dry powder form of phage enables its development as a pharmaceutical product and for clinical use. We and others have demonstrated that lyophilized and spray-dried phage retained their viability over different storage conditions upon resuspension and were more stable than liquid formulations stored over a same period (17–19). The Chan group demonstrated that spray-dried phage formulation containing 90% lactose maintained the viability of PEV61 phage over 12 months of storage at 20 °C at 60% relative humidity (RH) (17). Our group found that at least 100-fold more activity (*i.e.*, phage titer) was preserved as a lyophilized powder compared with its liquid preparation when stored in ambient condition and 4 °C for 2 months (18). To assess its ability for long-term storage, 25 lyophilized phages were stored at − 20 °C for 12 to 18 years, and only 8 of them (32%) exhibited ten-fold loss of titer, while 17 (68%) maintained the original titer (19). As a result, there have been extensive efforts to produce dry state phage formulations that retain potency and stability over different storage conditions.

The feasibility of drying phage offers more options for delivery to the target infection sites and enables the development of dosage forms with higher patient compliance, such as dry powder inhalation (DPI), oral tablets, topical paste, and dusting powders (20–22). For instance, formulation scientists have worked to develop inhaled phage therapeutics to treat lung infections, particularly chronic *S. aureus* and *P. aeruginosa* infections associated with cystic fibrosis (CF) (17,23,24). Dry powder inhalation (DPI) products offer better patient compliance and coherence compared with droplet nebulizers because they are smaller, portable, no need for electricity for operation or regular disinfection, and require less dosing time and minimum patient coordination between breathing and actuation of the device (25). Specifically, phage DPIs are stable phage powders with desirable aerosol performance. Chang *et al.* employed Taguchi experimental design with funneling approach and demonstrated that lactose provided the best phage stability among trehalose, lactose, and leucine, and the spray-dried formulations produced over 50% fine particle fraction desired for inhalation (26). Also, Golshahi *et al.* reported that endotoxin-removed bacteriophages KS4-M and FKZ lyophilized with 60:40 (*w*/*w*) lactose:lactoferrin matrix were successfully aerosolized and remained viable upon delivery to the lungs (27).

In these formulations, the excipients can greatly impact the stability of the phage in powder form and the characterization of these powders (17). As mentioned previously, knowledge and experience in protein formulations are good references in the selection of excipients for phage formulations (28,29). In dry formulations, these inactive ingredients should be able to help the protein or phage survive stresses encountered during processing and storage. During the process of dehydration, the gradual removal of water from the local environment of the protein can induce protein unfolding and subsequent aggregation (reversible or irreversible) and chemical degradation (15). If the protein is lyophilized, it can be subjected to stresses including lowered temperature, local supersaturation, changes in pH and ionic strength, and interfacial stresses from ice-water, ice-air, and water-air (30,31). In comparison, spray drying process causes instability at high temperatures and creates a mechanical stress by the vibration of the spray dry nozzle, which is not present in lyophilization (31). In addition to stabilizing the protein conformation, excipients are expected to satisfy the requirements for the physical properties of the powder needed for delivery and to meeti particular requirements for target dosage forms, such as maintaining the structure of the lyophilized cakes, achieving ideal dissolution rates, and/or obtaining particles in a desired size range. While the bioactivity may not be affected, the collapse of lyophilized cake results in an unacceptable appearance of the product and inconsistency in efficiency of drying, reconstitution time, and amount of residual water (32–34).

In this review, we provide an overview of excipients used in dry state phage formulations in the literature and their success to stabilize and retain the biological activity of these emerging therapeutics. We go on to address the unmet needs or outstanding questions that need to be further addressed to make advancements in formulating viable dry powder phage therapeutics.

STABILIZING EFFECT OF EXCIPIENTS USED IN DRIED PHAGE FORMULATIONS

Different excipients have been shown to be able to stabilize phage during different drying processes and are categorized into the following classes: sugar and sugar alcohol, polymer, surfactant, protein, salt, and amino acids (see Table 1). In this section, we reviewed the pharmaceutically accepted excipients (*i.e.*, appeared in approved drug products) used in solid phage formulations and their efficacy to stabilize phage.

Sugar and Sugar Alcohol

Sugar has been identified as one of the intracellular solutes (osmolytes) that stabilize microorganisms under harsh conditions such as dehydration and elevated temperatures (15). Sugars have been effective to stabilize a variety of therapeutic proteins and peptides and to protect them from aggregation, denaturation, and other degradation pathways in both dried and solution states (15,48). During dehydration, proteins are thought to form intermolecular hydrogen bonds to compensate for the loss of its interaction with water molecules, which may lead to their conformational change. In addition, the elimination of water is thought to perturb the local dielectric environment, and consequently, there is increased electrostatic attraction between proteins, leading to their aggregation. Water replacement and vitrification are the two leading hypotheses to explain how sugars stabilize these biological molecules during drying process and storage in solid state (49). According to the water replacement hypothesis, sugar can undertake the responsibility of water to network with proteins or phage *via* hydrogen bonding and

Table 1. Summary of Excipients Used in Current Published Papers. Only the Excipients that Have Been Used in FDA-Approved Products Are Included

Excipient class	Agent	Reference
Sugar and sugar alcohol	Trehalose	(17,18,23,24,26,35–42)
	Lactose	(24,26,27,43)
	Sucrose	(18,37,38,44)
	Mannitol	(18,23,26,35,38,42,45)
	sorbitol	(26)
Polymer	PEG 6000	(18,38,44)
	PEG 3000	(26)
	HPMC	(45)
	PLGA	(43,46)
	PVA, Pluronic-L92® PPO-PEO-PPO triblock copolymer	(46)
	Dextran 35	(24)
	Sodium alginate	(47)
	PVP	(38)
	Eudragit S100®	(41)
Protein	Casein sodium salt	(40)
	Lactoferrin	(27)
Amino acid	Leucine	(17,23,26,35,36,40)
	Glycine	(26,38)
Surfactant and others	Pluronic F68	(26,40)
	Tyloxapol	(40)
	Ammonium bicarbonate	(43)

prevent protein-protein interactions. Alternatively, in vitrification, sugars stabilize the proteins by restricting their local mobility and physically separating the protein molecules from each other (15,49). Sugar alcohols (*i.e.*, polyols), particularly mannitol, are frequently included in solid-state protein formulations. Compared with sugars, polyols act more as a bulking agent that maintain cake structure and form crystalline structures to facilitate water sublimation or evaporation (50,51).

Different sugar and sugar alcohols have been used to stabilize different phages during drying with varying and even conflicting degrees of efficacy. Merabishvili *et al.* freeze dried *S. aureus* phage ISP with six stabilizing excipients at different concentrations and found only 0.5 M trehalose and sucrose preserved activity of the phage with only 1 log titer loss (*i.e.*, activity) post-lyophilization and no residual loss after 37 months of storage at 4°C (38). Trehalose was also found to protect both LUZ19 and Romulus phage during spray drying; the transmission electron micrographs (TEM) showed that intact phage were visible only in trehalose while damaged phage head and dislocated phage tails were visible in samples formulated in lactose and dextran 35 (24). However, Chang *et al.* observed that actually both lactose and trehalose maintained the viability of spray-dried PEV phage; they comment that the lack of phage protection in the earlier study could be due in part of the hygroscopic nature of thermodynamically unstable amorphous lactose spray-dried powders (24) (26). While it is unclear whether lactose or trehalose achieves better stability, it is conclusive that sugar plays an indispensable role to stabilize dried phage. It is worth mentioning that the glass transition temperatures of lactose and trehalose are similar, at 108°C and 115°C, respectively (52), and it is thought they should possess similar ability to protect by vitrification mechanism.

The impact of lactose excipient on phage stability for dry inhalable powders has been widely tested, in part due to the fact that lactose has been the only sugar used in FDA-approved products for the inhalation route (53). As mentioned previously, Vandenheuvel *et al.* (24) demonstrated that higher concentrations of lactose and lactose-only formulations were not stable enough to maintain phage viability, and it has been suggested that this is due to the propensity of the amorphous powder to recrystallize (26). However, recent work suggests that increasing the percentage of lactose to greater than 80% in a lactose-leucine binary-excipient powder is needed to keep phage stable in the solid state for long-term storage (17). Lactose was amorphous in all formulations; interestingly, the glass transition temperature increased by 4 to 9 °C after 12-month storage at 20 °C/60% RH, which indicates that the formation of a rigid glass over time (17). In addition, lactose coupled with lactoferrin in a 60:40 ratio helped retain the viability of the phage after lyophilization (27). However, lactose is not more commonly used in biologics because it also acts as a reducing agent, which could alter the side chain functional groups of the amino acids and cause chemical degradation of proteins *via* the Maillard reaction or glycation (51). Other reports suggest the reduction may have damaged the intact phage, as evidenced by low titers and TEM of damaged phage structures (24).

Sucrose is popular in the formulation of protein therapeutics in solid forms, with approximately 60% of all commercialized solid protein parenteral products containing sucrose (51). However, sucrose is less commonly used in phage formulations, most likely due to the detrimental effect of sucrose reported in earlier research and the preferential sucrose-sucrose interactions that impedes the interaction between sucrose and protein, leading to failure in water replacement (54–56). For example, Dini and Urraza reported

that the lyoprotectant ability of lower concentration sucrose (0.1 M) was better than at higher concentrations (0.3 or 0.5 M), whereby phage destabilization occurred during freezing and drying processes and over 120 days of storage at 4 °C (37). Similarly, Puapermpoonsiri *et al*. demonstrated that high concentrations of sucrose caused a more rapid fall in phage stability over the first 7–14 days of storage. However, the low-concentration sucrose with phage failed to maintain the integrity of the cake and would unlikely be suitable for scaling and manufacturing (44). Contradicting the aforementioned studies, Merabishvili *et al*. saw that higher sucrose concentration (0.8 and 1 M) preserved phage activity better than at 0.3 M sucrose after lyophilization and storage (38). The lack of agreement correlating sucrose concentration with phage stability suggests that the ability of the excipient to protect the phage may depend on the phage type and its physicochemical properties (*e.g*., tertiary and quaternary structure, shape). Moreover, the structural variation between different phages could lead to change in predominant protection mechanism of excipients in different concentrations.

The representative sugar alcohol mannitol, has been extensively used in pharmaceutical formulations of both liquid and solid states. In solid products, mannitol routinely serves as a bulking agent for its crystallinity, high eutectic temperature, and matrix properties (57). These properties enable mannitol to maintain an intact structure of lyophilized cake or less merge in spray-dried particles, while at the same time allowing rapid drying under aggressive conditions (58,59). Furthermore, the already-crystalline form eliminates the recrystallization-induced risks during storage compared with amorphous excipients. Besides their physical advantages, mannitol was discovered to have therapeutic properties that synergistically functioned with phage to treat lung infections associated with cystic fibrosis (60). Finally, mannitol has been used as an additive in FDA-approved inhalable products. However, from previous studies, the performance of mannitol to preserve phage viability is highly questionable. Viable phage were detected in only 4 out of 27 mannitol and/or glycine formulations, with the remaining phage experiencing significant titer loss (26). A similar result was reported where there was negligible and 10,000-fold decrease in phage titer after lyophilization with 0.1 M and 0.5 M mannitol, respectively; here, it was suggested that mannitol should be used in combination with other stabilizers to effectively protect phage (38). However, mannitol can promote the recrystallization of an amorphous excipient partner in a multi-component system, which can ultimately lead to loss of phage activity. We previously observed a clear tendency of increasing titer loss with the increase of mannitol in dry phage formulations in a trehalose-mannitol binary-excipient system (18). Leung *et al*. also demonstrated increased loss of phage infectivity when mannitol concentrations was higher than 60% (35). Interestingly, the titer loss was consistent with the 20% and 40% mannitol formulations, which correlates with their earlier work where the titer loss was irrespective of the variation in excipients of the trehalose-mannitol-leucine tri-component system between 40:40:20 and 60:20:20 (23,35). This finding suggests that crystalline leucine and mannitol within a certain amount does not adversely affect phage stability and that the addition of mannitol could actually contribute to the structural integrity or shape of the solids.

Sugar alcohols have also been used in combination with sugars to formulate dry phage powders. *Pseudomonas aeruginosa* phage PEV2 was spray dried with a tri-excipient matrix of trehalose, mannitol, and leucine at different ratios, and there was improvement of phage viability after drying and during storage with increased amounts of trehalose (35). In contrast, no titer (*i.e*., amount of infectious phage) was detected in the mannitol and leucine only formulations. This finding suggests that trehalose is critical during processing and storage for phage to retain its viability, whereas the mannitol without disaccharides cannot protect phage. These results correspond well with our data that showed the phage stability decreased with the increase of mannitol in a mannitol-trehalose binary matrix and that the mannitol-only formulation showed the least stability in both post-lyophilization and storage (18). In another study, there was a ~ 1000-fold loss in titer of D29 phage after atmospheric spray freeze drying in a 1:1 ratio of trehalose to mannitol, whereas there was only a 4-fold reduction with an 7:3 ratio (42). The discrepancy in the stabilizing ability between mannitol and trehalose was thought to be caused by the crystallization of excipients. Using various analytical techniques including X-ray diffraction and dynamic vapor sorption, trehalose is amorphous in the formulations whereas mannitol remains in crystallized form (18,35). This finding suggests that there may be phase separation during processing where viable phage is surrounded and protected by trehalose in amorphous phase. Interestingly, the titer loss was found to be independent of the formulation compositions where the percentage of trehalose was slightly varied in tri-excipient compositions (*i.e*., trehalose:mannitol:leucine), and the formulations are partially crystalline (23,40). These observations could be due to that the changes in the composition ratio were not substantial to change the stability. In addition, Leung *et al*. reported that the moisture-induced recrystallization of trehalose resulted in the inactivation of phage during storage (35). This recrystallization under humidity was also later reported by Vandenheuvel *et al*. (39), who found that amorphous trehalose formed stable trehalose dihydrate crystals at 54% relative humidity (RH), regardless of the storage temperature. Collectively, these results indicate that phage stored at a temperature below the Tg of trehalose results in recrystallization and is insufficient to preserve phage activity. Consequently, it was proposed that phage products containing amorphous trehalose should be handled and stored under RH less than 20% (35,39).

Disaccharides in particular are critical in dry state phage formulations. Generally, trehalose and lactose are superior than sucrose and sugar alcohols in protecting phage from stresses during the solidification. It is suggested that the different abilities of trehalose and lactose to preserve phage viability is context-specific and is phage dependent. Even though there is some observed titer loss in lactose-containing phage formulations, no direct evidence has been shown to prove that lactose causes chemical reduction and degradation of the phage (*via* Maillard reaction) (24,27). Mannitol alone is detrimental to phage, and therefore is included as a component in a binary or tri-excipient system. However, the presence of mannitol could encourage the recrystallization of amorphous components, which would adversely affect phage activity. Consequently, the amount of mannitol added

would need be optimized, and a suggested maximum relative humidity of 20% is needed to reduce moisture-induced recrystallization during storage (36). While these proposed mechanisms were used to explain protein stability, it is a reasonable rationale to explain formulating and stabilizing phage.

Polymer

Polymers have also been used to stabilize phage in solid-state formulations. Similar to sugars, polymers raise the Tg of the formulation, thereby forming a viscous glass that immobilizes and protects the proteins. Here, preferential exclusion is the proposed mechanism to protect proteins as long as the water is still present in the system. However, while sugars have negligible effect on pH, some high viscosity polymers inhibit pH changes during freezing, presumably by impeding crystallization of buffer salts. However, the ability of polymers to hydrogen bond to the protein is less than sugars due to steric hindrance due to their larger molecular weight (15,61). This lack of hydrogen bonding would be more pronounced in phage formulations due to the larger molecular weight and higher order quaternary structure of the phage, which would result in greater steric hindrance. Besides stabilizing phage, polymers are also used as carriers or encapsulating agents to deliver phage.

From the protein formulation space, polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) have been rationally chosen as polymer excipients to stabilize phage formulations. However, these polymers are not as successful in preserving phage activity as in proteins. PVP inactivates phage, regardless of the concentration (38). There was a dramatic decrease in titer of ISP phage in both 1% and 5% PEG 6000 preparations immediately after lyophilization, with a gradual decrease during 37-month storage at 4 °C (38). Similar findings were noted by Puapermpoonsiri *et al*., where the addition of PEG 6000 abolished lytic activity of the phage irrespective of the concentration of PEG 6000 used and/or the lyophilization process. It was suggested that the destabilization could be attributed to the phase separation and protein precipitation caused by the interactions between protein residues and hydrophobic moieties on PEG (44). However, further studies are needed to confirm this hypothesis, as the steric hindrance between phage and PEG 6000 may be sufficient to prevent their hydrophobic interactions compared with protein and PEG. Multi-component excipient systems using PEG with other stabilizers have been tested but there was no marked improvement. We tested the stabilizing ability of PEG 6000 and 1:1 (*w*/*w*) PEG 6000: trehalose. Here, the goal was to take advantage of the sugar to inhibit local mobility of phage while using PEG to reduce the global mobility by steric hindrance. In combination, sugar and PEG adversely affected phage stability and viability, with a complete loss of phage titer was observed after 7 days of storage. Interestingly, the water residue in the two samples (formulated in PEG 6000 and trehalose:PEG 6000) was significantly different, whereby the addition of trehalose reduced water content by 1.9% (from 2.5 to 0.6%), indicating that there are interactions between trehalose, water, and PEG 6000. Presumably, trehalose replaced water to hydrogen bond with PEG 6000; here, PEG 6000 occupied trehalose to compensate the removal of water from nearby phage, leading to the titer loss (18). Chang *et al*. reported that the presence of PEG 3000 impacted the yield collected such that insufficient amounts of phage were collected for all PEG-containing spray-dried mixing formulations (26). Since PEG has been well established to precipitate phage, it is feasible that the loss of activity and amount of phage is simply due to the inability to completely resuspend the phage after PEG addition (62).

Additional polymers used in dosage forms for other purposes may also indirectly stabilize dry phage preparations. For example, hydroxypropyl methylcellulose (HPMC) was used in a lyophilized nasal insert phage formulation because of its high viscosity on rehydration *in situ*, which results in extended drug residence in the nasal cavity by attenuating mucociliary clearance (45). Even though stabilization is not the major aim of adding HPMC, it is thought to help phage survive from the stress during freezing, drying, and storage. However, no particular correlation was observed between the phage titers and the addition of mannitol or the concentration of HPMC in long-term stability studies. It was suggested that stability of the HPMC-embedded phage was due to possibly excluded volume effect from molecular crowding, polyol protection, and/or elevated Tg (45). It is unclear to ascertain the role of viscosity to preserve phage, but it could be critical given increased viscosity would confine and dramatically reduce mobility of both phage and mannitol. Therefore, in addition of the polymer to hydrogen bond with the phage capsid, it may be important to understand phage mobility with the polymer. Dextran is another bi-functional polymer used in pulmonary phage therapeutics. In addition to stabilizing phage during processing, it is thought to prevent *P. aeruginosa* from attaching to lung epithelial cells, thereby minimizing infection (63). However, others have shown that dextran 35 exhibits unsatisfactory protective ability, with no viable phage detected after spray drying using 4% dextran 35 solution (24).

In addition to preservation, polymers have been used as carriers to more efficiently deliver phage to its target site. While promising, it is necessary to ensure phage are stable in these formulations since the polymers used are not common excipients used to stabilize protein and phage therapeutics and may adversely impact activity. Vinner *et al*. encapsulated enteric bacteria Salmonella-specific phage Felix O1 with commercially available Eudragit S100® to generate a pH-responsive tablet for oral delivery (41). The formulation containing only Eudragit S100® nearly lost all phage activity whereas the addition of trehalose in the excipient matrix improved the preservation of phage titer by protecting the phage from the thermal and dehydration stresses during spray drying. The authors hypothesized that the protection effect is due to stabilizing phage protein conformation and vitrification of the phage powder. The higher proportion of trehalose in the polymer formulation yielded higher phage titers. However, this positive correlation between trehalose and phage titer was reversed when the powder was exposed to simulated gastric fluid at pH 2 since increasing the proportion of polymer enhanced acid protection. As a side note, the compression force from the tableting process had no adverse effect on subsequent phage viability (41). While the formulation needs further optimization to improve phage viability

throughout the spray drying process, this work underscores the feasibility of oral delivery of phage, which may be used to treat infections of the gastrointestinal tract and is a more patient-compliant alternative option to treat infections at other sites.

Phage have also been encapsulated in biodegradable polymers for controlled release and/or passive targeting. PLGA refers to a family of biocompatible and biodegradable polymers that are composed of poly lactic acid (PLA) and poly glycolic acid (PGA) (64). It has been extensively used and investigated to deliver small molecules, proteins, and nucleic acids. Building on PLGA's flexibility to deliver different scales of medicines, it has been used to deliver intact and active phage (64,65). Puapermpoonsiri *et al.* encapsulated two strains of phages into PLGA (PGA:PLA 50:50) *via* a modified w/o/w double emulsion-solvent extraction protocol and measured an initial partial loss of phage bioactivity; however, there was no titer after 7 days, which suggests that the microencapsulation did not stably maintain the activity of the phage (46). In another study, highly porous PLGA particles using ammonium bicarbonate as effervescent provided a large surface area for phage to deposit, and the resulting dry powder formulations of phage-loaded particles with lactose exhibited only minimal loss in titer after 16 days of storage at room temperature (43). The success of this approach implies that it is less harmful for phage to be absorbed onto the polymer surface than encapsulated, which can expose phage to different solvents. This finding suggests that the physical interaction and orientation of phage with polymers is critical to retain their potency.

Sodium alginate has been frequently used as a biopolymer matrix encapsulated and protects phage from the physiological environment (66,67). Sodium alginate is biocompatible and has been used in multiple FDA-approved oral products, including tablets, capsules, syrups, and suspensions (53,68) Recently, phage isolated from surrounding lake water samples was spray dried with 1.33% sodium alginate, which made phage resistant to gastric fluid and bile salts. Most types of phage survived the spraying and dehydration processes, with less than 2 log titer loss of activity (47). Regardless, further studies are warranted to understand long-term stability and phage-excipient combability in these polymer systems.

In summation, polymers have been used as stabilizers and/or carriers in phage formulations. The properties of polymers, such as pH tunability, endow them with the ability to be used in more dosage forms for different routes of administration compared to other excipients. From the analysis of the literature, while these multi-functional polymers can successfully encapsulate or adsorb phage, further work is needed to evaluate their feasibility for solid phage formulations and to optimize their use to satisfactorily stablize phage for long-term storage and activity.

Proteins

For their use in microbiology, phage are traditionally grown in liquid cultures of bacteria supplemented with appropriate nutrients. In additon, phage are freeze-dried with the nutrition broth and proteins including skim milk and peptone for long-term storage (69–71). From these insights, there have been recent efforts to use proteins as excipients to stably preserve phage. While the traditional reagents are not pharmaceutically acceptable, these findings provided some suggestions for excipient choices, *i.e.*, proteins can stabilize phage that survive thermal and desiccating stresses. In this section, two proteins used as additives in FDA-approved products are discussed in their use in phage formulations.

Casein is abundant as one of the major proteins in milk and has been incorporated as an inactive ingredient in approved oral tablets (53). To evaluate its application in dry phage formulations, one study showed that the addition of 2% of casein sodium salt to a spray-dried *Myoviridae* phage formulation with trehalose and leucine improved phage titers in fivefold. After 3 months of refrigerated storage, there was less than 0.15 log titer loss in formulations with the addition of casein (40). These observations demonstrated the feasibility of casein to stabilize phage.

Lactoferrin, a multi-functional iron-binding glycoprotein, is abundantly present in milk secretions and has also been approved in oral tablets by FDA (53,72). Lactoferrin shows a bactericidal effect against a broad spectrum of Gram-positive and Gram-negative bacteria by directly damaging bacterial membranes or chelating ferric iron, a nutrient, to inhibit bacterial growth (73–75). Particularly, lactoferrin is one of the abundant antimicrobials lining the airways and functions as a critical line of host innate immunity against respiratory pathogens (76). As a result, lactoferrin has been used in different phage formulations against cystic fibrosis–related bacteria. Here, phage formulated in 60:40 (*w*/*w*) lactose:lactoferrin generated titers similar to pure lactose formulation upon lyophilization, and there was no loss in titer at 4 °C or 22 °C storage after 3 months (27). The ability of lactoferrin to successfully retain phage activity after processing and during storage shows the opportunity that phage strains formulated with lactoferrin to achieve synergistic therapeutic efficacy.

Here, proteins have been added in phage formulations to help preserve the activity and possibly generate synergistic therapeutic effect. However, it is difficult to generalize the effects of protein on phage stability since only casein and lactoferrin have been investigated in a few studies. Further studies are warranted using these and other protein excipients to stably formulate phage.

Amino Acids

Interestingly, the selection of amino acid excipients between protein and phage formulations is different, and this is an instance where phage formulations do not follow the rationale for protein formulations. In protein formulations, histidine is the most popular amino acid excipient due to its multi-functionality as a buffering agent, antioxidant, cryo/lyoprotectant, and its ability to prevent protein aggregation (51). However, none of phage formulations uses histidine as an additive. Instead, leucine has been extensively used and investigated in phage preparations. This is most likely due to the fact that for dry powder inhalations, leucine is widely proven to outperform other amino acids in generating better dispersed respiratory particles (77–79). Here, in addition to the role of leucine to improve aerosol performance of phage, we also discuss how leucine impacts phage stability and viability.

Initially, Matinkhoo *et al*. demonstrated that the presence of 19% leucine in formulations with combination of trehalose, leucine, and casein sodium salt, with or without surfactants, did not adversely affect phage viability, with only 1 log titer loss across all formulations (40). But the main rationale for using leucine in dry powder formulations is to improve the dispersibility of the powders. Additional work demonstrated that 25% mass fraction of leucine is the threshold needed to enhance the particle dispersion in a binary component system; here the powder becomes completely crystalline, which dramatically reduces the powder density by forming predominantly hollow shells as opposed to solid spheres (80). As a result, in subsequent studies formulating spray-dried phage powders, at least 20% of leucine was added to achieve better aerosol performance. Nevertheless, the incremented proportion of leucine can cause stability issue. In Chang's work, minimal lytic activity loss was observed for phage formulations spray dried with 32% (*w*/*w*) leucine and the magnitude of titer reduction decreased with the increase of lactose or trehalose concentration. Interestingly, however, a higher sugar concentration than 80% did not lead to a further retaining phage bioactivity indicating the slight (0.3–0.4 log) titer loss was irrelated to leucine. Furthermore, there was significant reduction of titer in leucine-free formulations despite high concentrations of sugar, suggesting that a small amount of leucine is needed to improve phage stability in spray-dried powders (26). Chang *et al*. reported that the titer after spray drying was not affected by the excipient selection of lactose-trehalose combination; however, during long-term storage, the viability of phage decreased with increased amounts of leucine, especially when more than 20% leucine was present in the formulations (17). The same group also reported that titer loss in PEV2 and PEVE40 phage powders upon spray drying was independent on the combination of excipients (36).

The storage stability of leucine-containing formulations is related to the crystallinity, where leucine is a "double edged sword". On one hand, hydrophobic leucine supersaturates earlier at the surface of the droplet during the evaporation stage of spray drying and thus forms a partially ordered crystalline shell on the surface of the particles, thereby protecting other amorphous components against moisture from the storage environment (80–82); on the other hand, leucine could induce recrystallization of amorphous phage powder, leading to viability loss. Leung *et al*. confirmed the concentrated presence of leucine on the surface of spray-dried leucine-trehalose phage particles, and the coverage of leucine increased with the increase of leucine concentration (40% leucine achieved up to 89% coverage) (36). However, the dynamic vapor sorption data showed that the powders started recrystallizing at 50% RH regardless of leucine content (30% or 40%), indicating the leucine shell might not be continuous, which would result in water uptake of trehalose and subsequent recrystallization. In contrast, when lactose and leucine was used (instead of trehalose and leucine), the powders exhibited a higher relative recrystallization onset RH (60%), and this slight increase suggests that the combination of lactose and leucine is able to trap moisture better than the combination of trehalose and leucine (17). In some of the multi-component excipient systems, leucine could crystallize with mannitol. In multiple studies, the presence of 20% leucine could not form a crystalline shell to protect amorphous trehalose due to particle merging. Additionally, the amounts of recovered phage from *in vitro* aerosol dispersion studies were very low (20–53%), which suggests that the leucine shell does not offer sufficient protection (23,35).

While less commonly used in solid phage formulations, glycine is the only other amino acid that has been tested in formulating phage. Although it has been widely used in protein formulations as bulking and buffering agent (51), the use of glycine in phage formulations has been discouraging. The addition of 0.1 and 0.5 M of glycine completely inactivated ISP phage during lyophilization, as well as spray dried the PEV phages formulated with mannitol and glycine (26,38). Based on these initial findings and the fact that glycine does not have synergistic functions with phage to treat infections, glycine has not been further pursued.

In summation, leucine is the predominant amino acid in dried phage formulations for inhalation powders and is often used in combination with dissaccharides and/or mannitol. Generally, the proportion of leucine in the system negatively correlates with phage stability, even though small amounts of leucine are critical to phage stability in some formulations (26). From the literature, it is thought that 25% is the threshold for leucine to form complete crystalline shell to protect the amorphous components from moisture-induced degradation (80). The surface distribution of leucine in these phage-containing solid particles has been confirmed; however, the protective effect was unexpectedly not sufficient. It would be worthwhile to use other amino acids, especially when the formulation requires good buffering agent and antioxidants.

Other Excipients

Beside above-mentioned categories, there are other excipients that either exert special functions in formulation or being carried from the bulk phage solution. The stabilizing effect of surfactant Pluronic F68 has been evaluated (26,40). Chang *et al*. used Taguchi experimental design to demonstrate that Pluronic F68 has an insignificant effect on the stability of all the three tested phages in the dry powder formulations (26). Similarly, titer loss was not observed by Matinkhoo *et al*. in phage formulations upon the addition of Pluronic F68 in the trehalose/leucine excipient matrix (40). In the same report, another surfactant Tyloxapol contributed to higher titer loss when incorporated in the trehalose/leucine formulations (40). These initial studies warrant further investigation to understand the role of surfactants in the stability of phage formulations.

Only in few reports are salts added to phage solid formulations as excipients, which can be attributed to several reasons. First, unlike proteins which requires a pH deviated from its isoelectric point (pI) to achieve solubility and stability, phages commonly remain stable in a broad pH range (83,84). In addition, most of the solid formulations are intended for dosage forms that do not require tonicity adjustment. Finally, the existence of salt can exert unpredictable, adverse effects on the stability of biologics during the freezing process of lyophilization as a consequent of crystallization and the resulted dramatic pH shift and ionic strength change (85). Dini *et al*. found that addition of phosphate

buffer saline not only failed to improve the stability of phage during lyophilization in skim milk, trehalose, or sucrose formulations but also impaired the storage stability of phage in sucrose formulation. In contrast, SM buffer retained more viable phages after freeze drying process, especially combined with 0.1 M sucrose (37).

Typically, phage is suspended in buffer after purification, and consequently, there are residual salts in the final formulation prior to drying. However, in studies, phage are diluted 100–1000-fold upon the addition of excipients, and there is no report on the role of the diluted salts (23,26,41,42). Even though there is no evidence that these small amount of residual salt molecules affect the phage stability in final formulation, further research is needed to confirm that the impact is negligible, especially when to confirm the role of phage as a viable therapeutic drug product.

CHALLENGES AND OPPORTUNITIES IN DRY POWDER PHAGE FORMULATION

Solidification can stabilize peptides, proteins, and vaccines by reducing their molecular mobility and avoiding water-induced problems. Dry state formulations are generally less susceptible to temperature change, and as a result, these products are less dependent on maintenance of the cold chain. In addition, dry state improves the long-term storage stability as well as the ease in shipping and handling. Additionally, some particular dosage forms require drug to be in powder form, such as dry powder inhalation, tablets, and solid patches for wound therapy. The properties of produced powder impact the stability and performance. The interactions between excipients and the drying processes affect phage stability and activity. Therefore, selection of the proper excipient and understanding their effect on phage are both critical in formulation development.

Since the development of phage formulations is still in its infancy, the excipient selection remains limited, with a focus on a few chemicals such as trehalose, lactose, and leucine. Even though these excipients are attractive, it is a challenge to maintain phage viability, as evidenced by ubiquitous titer loss in every formulation immediately after drying and/or during storage. In addition, the ability of excipients to protect phage is strain specific, with variability among the different strains. These outstanding challenges nudge us to identify more candidates to expand our "excipient toolbox." To date, the main strategy in excipient selection is based on experiences from protein formulations. However, bacteriophage is structurally more complex than proteins and has larger dimensions than proteins. These bacterial viruses form organized, higher order protein assemblies with more repeated domains than single proteins (86,87). Mammalian viruses follow similar principles of assembly and organization (88,89). Therefore, we posit that there are opportunities to learn from the formulation of mammalian viruses to improve stability and potency of phage formulations.

Excipient Adoption from Vaccine Formulations

Compared with phage, mammalian viruses have been more developed for applications in vaccine and drug delivery. They are routinely dried to overcome the inherent thermostability issues to reduce the cold chain requirement. Kanojia *et al.* have extensively reviewed excipients in spray-dried vaccines and categorized the excipients into sugar, surfactant, divalent ions, protein, and polymers (90). Similar to phage formulations, sugars and polysaccharides are the most commonly used stabilizing excipients in spray-dried vaccines, and in particular, trehalose is most popular (90,91).

Besides the similarities, proteins and divalent ions used in mammalian virus vaccine formulations may help improve phage formulations. Typical protein stabilizers in vaccine products, such as albumin and gelatin, may be attractive for use in dry phage formulations. Gelatin could be efficient in spray-dried formulations attributing to its wide distribution in size. In theory, larger molecular components would slow down the migration of phage particle to the air-liquid interface during droplet drying while smaller components could take advantage of their steric flexibility to interact with phage, which is a proposed mechanism that skim milk preserved phage in the dry state (37,71). Even though the use of albumin and gelatin has been questioned due to their animal-derived source, the frequent appearance in commercialized vaccines suggests their safety and applicability (92,93). In addition, divalent ions like Ca^{2+}, Mg^{2+}, and Zn^{2+} have been shown to specifically interact with the capsid of mammalian viruses in both liquid and dried states (94–97).

Stabilizers and preservatives are routinely added in licensed vaccine products. Among the stabilizers, glutamate salts (usually sodium or potassium salts) are used in lyophilized vaccines and usually combined with disaccharides (mostly sucrose), phosphate buffer, and albumin, forming the commonly used matrix called SPGA (98). Adjustment of composition and modifications of the amount of each ingredient are made according to the type of vaccine (99–101); it is feasible to adopt this approach towards phage-specific formulation.

Formulation Screening

From the aforementioned studies, there is a comprehensive list of selective excipients used for formulation of phage, protein, and/or vaccine that would be considered General Recognized As Safe. In those previous studies, the correlation between formulation and phage bioactivity was quite phage strain dependent; as a result, it is necessary to customize formulations for each phage, which can be expensive and time-consuming.

To address this problem, high-throughput screening methods that have been widely used in protein and vaccine formulations can be adapted to screen excipients for phage-specific formulation (102–104). The availability of automation increases the throughput from tubes (*i.e.*, small number of samples) to microplates, minimizes manual preparation time, and eliminates operational error, thereby allowing a larger number of screening experiments. Using statistical analysis, we can efficiently design experiments that will provide relevant information needed to formulate phage. For example, Design of Experiment (DoE) is a statistical experimental design that handles a complex system with multiple variables and establishes an empirical relationship between the

variables and specific responses of the system using a minimum number of experiments (105). The acceptance and application of these two concepts can help formulation scientists to efficiently identify the optimal compositions for each individual strain of phage.

Yet some aspects should be considered when it comes to using DoE for determining dry state phage formulations. Since the screening is traditionally done in liquid, the compatibility of excipient-phage is reflective of the liquid state but does not represent interactions during the drying processes. It has been demonstrated that phage remained robust in different solution conditions; however, those same components did not protect phage to survive dehydration and other stresses during drying (18,26). Even though high-throughput platforms for screening freeze-dried formulations have been reported, the techniques need to be further investigated. Also, these platforms have not been shown to yet be suitable for spray drying (106). In addition, the impurity of residues should be taken into account since the pharmaceutical-level standardized production and purification steps for phage have not yet been established, and the impurities could be problematic especially when a high initial titer of phage is needed. Furthermore, the Critical Quality Attributes that are accurate, indicative, and can be efficiently determined should be scrutinized to evaluate both bioactivity of phage and characterizations of the whole formulation. For example, Chang *et al.* used Taguchi experimental design method to screen formulations and proposed a schematic formulation screening criteria, where formulations were evaluated by four selection levels to ultimately achieve stable and well-dispersed phage particles (26). This initial work is a good starting point that demonstrates the promise of DoE in phage formulation.

Expanding the Characterization Toolbox to Understand Phage-Excipient Interactions

Currently, evaluation of dry state formulations have concentrated on the characterization of powder properties (Fig. 1 and Table 2) using the following analytical techniques: dissolution; measurement of residual moisture by Karl Fischer titration and thermogravimetric analysis; testing crystallinity by X-ray diffraction, dynamic vapor sorption, dynamic scanning calorimeter, and Raman spectroscopy; visualizing phage morphology by TEM and powder morphology by scanning electron microscope and confocal laser scanning microscopy; measurement of particle size distributions by laser diffraction and impactors. These approaches are widely accepted to analyze the features of a powder. However, the mechanisms of how each excipient protects the phage are proposed without clear cut techniques to dissect the underlying interactions between phage and stabilizers.

Numerous techniques have been used to study the interaction between excipient and proteins. They are well described in the review by Kamerzell *et al.* and not repeated here (102). Unfortunately, there are limited options for analysis of solid forms. In summary, the higher order structural changes of proteins in solid formulations can be detected using FTIR and Raman spectroscopy, atomic force microscopy (AFM), confocal microscopy, and high-resolution ultrasonic spectroscopy (HR-US) (102). These methods have been primarily used for proteins and further studies are needed to validate their feasibility for dry state phage-excipient formulations.

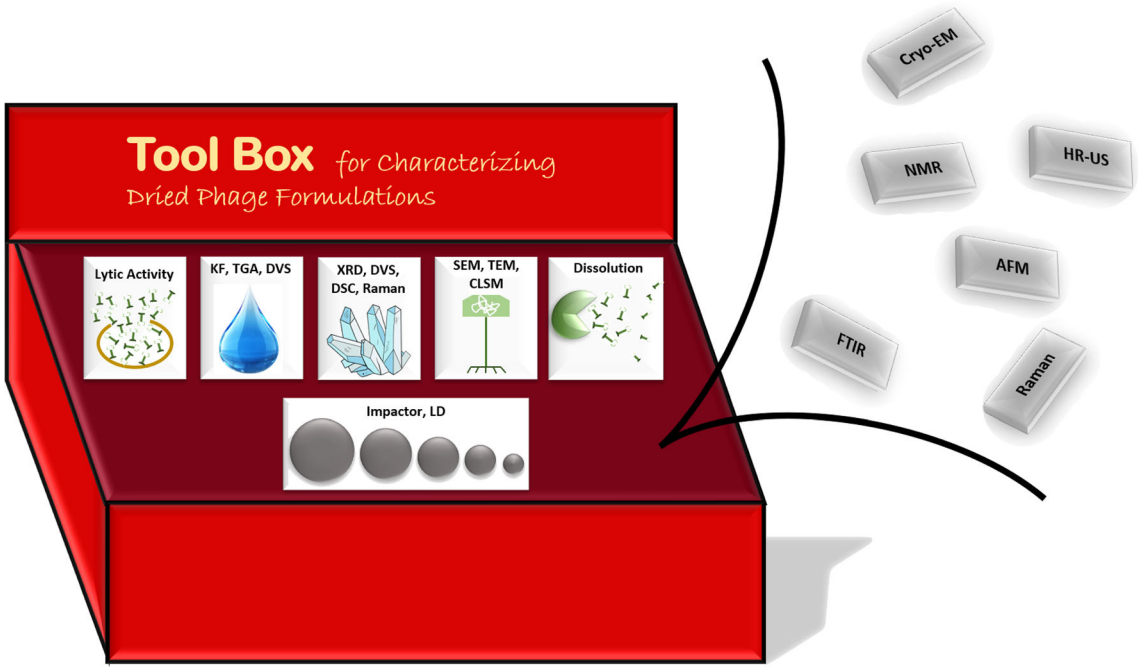

Fig. 1. Techniques used for characterizing dried phage formulations. Inside box: techniques already used in published papers. Acronyms: KF, Karl Fischer; TGA, thermogravimetric analysis; DVS, dynamic vapor sorption; XRD, X-ray diffraction; DSC, dynamic scanning calorimeter; SEM, scanning electron microscope; CLSM, confocal laser scanning microscopy; LD, laser diffraction. Outside box: techniques that can be used in future research: Cryo-EM, cryo-electron microscope; NMR, nuclear magnetic resonance; HR-US, high-resolution ultrasonic spectroscopy; AFM, atomic force microscope; FTIR, Fourier transform infrared spectroscopy

Table 2. Summary of Methods that Have Been Used to Characterize Phage Powders and Tools that Can Potentially Be Used to Investigate Phage-Excipient Interactions. N/A Represents Not Applicable; P/A Represents Potentially Applicable

Characterization method	Dry powder properties	Phage-excipient interaction	Example reference
Karl Fischer titration (KF)	Water content	N/A	(23,44)
Thermogravimetric analysis (TGA)	Water content/ thermal stability	N/A	(17,23)
Dynamic vapor sorption (DVS)	Water content/ crystallinity	N/A	(17,23,26,35)
X-ray diffraction (XRD)	Crystallinity	N/A	(17,23,26,39)
Dynamic scanning calorimeter (DSC)	Crystallinity	N/A	(23,35,39)
Transmission electron microscopy (TEM)	Morphology of phage	N/A	(24,38,44)
Scanning electron microscopy (SEM)	Morphology of powder	N/A	(17,23,26,39)
Laser diffraction	Geometric particle size distribution	N/A	(17,35)
Impactors/impingers	Aerodynamic particle size distribution	N/A	(17,23,27,35)
Confocal laser scanning microscopy (CLSM)	Phage encapsulation	P/A	(43,44,46)
Raman spectroscopy	Phage protein structure/crystallinity	P/A	(42,107,108)
Atomic force microscope (AFM)	Phage protein structure	P/A	(109,110)
Fourier transform infrared spectroscopy (FTIR)	Phage protein structure	P/A	(111,112)
Nuclear magnetic resonance (NMR)	Phage protein structure	P/A	(113–115)
High-resolution ultrasonic spectroscopy (HR-US)	Phage protein structure	P/A	(116–118)
Cryo-electron microscope	Phage protein structure	P/A	(119,120)
Atomic force microscopy-infrared spectroscopy (AFM-IR)	Phage protein structure	P/A	(121)

Several advanced molecular-level techniques can be used to study phage-excipient interactions on the cellular or molecular scale (Table 2). For example, recent work established the use of photothermal-induced resonance-enhanced atomic force microscopy-infrared spectroscopy to measure conformation change of phage proteins as a proxy for phage stability (121). Also, cryo-electron microscopy can be used to observe the steric interaction of phage with large molecules like protein and polymers (with a MW limit of 38 kDa) (119,122). In addition, by obtaining the morphology of phage at the atomic level by single particle analysis, the structural basis for deactivation could be determined (119,123). Alternatively, nuclear magnetic resonance (NMR), particularly solid-state NMR, could be used to test interactions in a less mobile state of a powder. NMR is a powerful instrument to detect protein motion and dynamic processes and has been used to understand protein-excipient interactions (102). It has also been repeatedly demonstrated that NMR is able to elucidate the 3D structures of phages at atomic resolution using experimental magic-angle spinning (MAS) solid-state NMR, where the structure of capsid protein and DNA inside the capsid were determined (124–126). Thus, it is highly possible that this technique can help us understand the interaction between the phage and its additives.

Another component of phage, the encapsulated genetic material, is neglected when testing the phage stability. However, the damage in DNA integrity can also lead to phage rupture and deactivation. Standard microbiological tools including quantitative PCR can be done to accurately enumerate phage in the presence of excipients (127,128).

Drying Techniques and Excipients

Currently, freeze drying and spray drying are the main solidifying techniques for phage formulations. In both methods, the added excipients are responsible to protect the phage from stresses generated during water removal, yet the excipients serve different functions during each process. In freeze drying, excipients are used to cryoprotect the phage, whereas in spray drying, excipients protect phage from thermal and mechanical stresses. Also, since spray drying is mostly used for preparing inhalable phage powders, excipients must confer desirable aerodynamic properties needed for the powders.

In spite of the difference in protective functions, sugars and sugar alcohols are the most popular additive in formulations manufactured by both methods, although there are differences in specific chosen excipients. For example, trehalose and mannitol were selected in large number of spray-dried formulations and several freeze-dried ones with or without combining other excipients (17,18,23,24,26,35–42,45). Lactose was also used in both types of formulations. However, lactose is more prevalently chosen in spray-dried formulations due to its ability to improve flowability of the powder (24,26,27,43). Sucrose was absent in spray-dried phage formulations, most likely as a result of its reported difficulty in disaggregating to primary particles for inhalation (129). The selection of excipients and drying method ultimately depends on the intended goal. For instance, when preparing inhalable phage powders, leucine is incorporated with other excipients for spray drying phage since it has been shown to improve the aerosol performances of the powders

(17,23,26,35,36,40). Polymers have also been used in both spray-dried and freeze-dried formulations to achieve modified release of therapeutic phage. Biodegradable PLGA has been freeze dried with phage to achieve a sustained-released phage inhalable powder (43,46). Puapermpoonsiri *et al.* demonstrated that rapid freezing of microsphere-encapsulating phage resulted in a smaller particle size compared with slower regular shelf freeze in lyophilizer. The titer loss was negligible after lyophilization, which indicated that the PLGA protects phage against stresses caused by rapid freezing (46). Additionally, it has been demonstrated that phage can be prepared as a tablet for oral delivery *via* spray drying with a pH-responsive anionic copolymer Eudragit S100® (41).

While there are no limitations to the use of excipients between the different drying methods, the methods can impact the stability of the phage formulations. For example, Leung *et al.* showed that spray freeze drying or spray drying the same formulation resulted in different phage titers (23). While the fast-evaporative drying process in spray drying resulted in some titer loss, there was a greater reduction in titer from spray freeze drying due to the use of the ultrasonic nozzle (23). In addition, since different types of phage require different formulations, a case-by-case strategy is required in deciding drying methods for solid phage formulation development.

CONCLUSIONS

Here, we have reviewed the pharmaceutically accepted excipients used in dry state phage formulations and discussed their current abilities to stabilize and maintain phage activity during processing and long-term storage. From overall analysis, disaccharides like lactose and trehalose showed superior preservation ability and most likely are the key determinant to achieve stable phage formulations. The use of other excipients, including the addition of mannitol and/or leucine, improve the physical properties of powders for dispersion but face challenges to retain therapeutic activity of the phage. Amino acids and proteins can stabilize and offer the promise of synergistic therapeutic effect with phage, and polymers can stabilize or encapsulate phage for desired delivery. However, further studies are needed to realize the promise of these less commonly used excipients.

Although dry phage formulations are rationally designed following the principles of protein formulations, there are challenges and opportunities that arise. With the current excipient space, it is possible to expand the number of candidate excipients by looking to formulation of similarly structured mammalian virus vaccines. Using other advanced analytical tools such as solid-state NMR and cryo-EM, it would be feasible to gain a greater understanding of stabilizing mechanisms between phage-excipient interactions. And using high-throughput methods with statistical analysis provide a smarter, streamlined approach to identify multiple excipients that present an optimized interaction with phage and each other to retain the phage activity and stability during drying and storage. Regardless, with the re-emergence of phage as antimicrobial therapeutics, there is great opportunity to re-think and improve their formulation for translation into a viable, attractive pharmaceutical product.

FUNDING INFORMATION

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH) under award number R01HL138251. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

REFERENCES

1. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. (1537–6591 (Electronic)).
2. Cars O, Diaz Hogberg L, Murray M, Nordberg O, Sivaraman S, Lundborg CS, So AD, et al. Meeting the challenge of antibiotic resistance. (1756–1833 (Electronic)).
3. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, et al. Antibiotic resistance: a rundown of a global crisis. Infect Drug Resist. 2018;11:1645–58. https://doi.org/10.2147/IDR.S173867.
4. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318–27. https://doi.org/10.1016/S1473-3099(17)30753-3.
5. D'Herelle F. On an invisible microbe antagonistic toward dysenteric bacilli: brief note by Mr. F. D'Herelle, presented by Mr. Roux. 1917. (0923–2508 (Print)).
6. Twort FW. An investigation on the nature of ultra-microscopic viruses. Lancet. 186(4814):1241–3. https://doi.org/10.1016/S0140-6736(01)20383-3.
7. Kingwell K. Bacteriophage therapies re-enter clinical trials. (1474–1784 (Electronic)).
8. Bodier-Montagutelli E, Morello E, L'Hostis G, Guillon A, Dalloneau E, Respaud R, et al. Inhaled phage therapy: a promising and challenging approach to treat bacterial respiratory infections. Expert Opin Drug Deliv. 2017;14(8):959–72. https://doi.org/10.1080/17425247.2017.1252329.
9. Patey O, McCallin S, Mazure H, Liddle M, Smithyman A, Dublanchet A. Clinical indications and compassionate use of phage therapy: personal experience and literature review with a focus on osteoarticular infections. Viruses. 2018;11(1). https://doi.org/10.3390/v11010018.
10. Huh H, Wong S, St. Jean J, Slavcev R. Bacteriophage interactions with mammalian tissue: therapeutic applications. Adv Drug Deliv Rev. 2019. https://doi.org/10.1016/j.addr.2019.01.003.
11. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. (2042–7158 (Electronic)).
12. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. (2159–7073 (Print)).
13. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, Balloy V, et al. Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. (1537–6613 (Electronic)).
14. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. (1474–1776 (Print)).
15. Ohtake S, Kita Y, Arakawa T. Interactions of formulation excipients with proteins in solution and in the dried state. Adv Drug Deliv. 2011;63(13):1053–73. https://doi.org/10.1016/j.addr.2011.06.011.
16. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. (1078–0467 (Print)).

17. Chang RYK, Wallin M, Kutter E, Morales S, Britton W, Li J, et al. Storage stability of inhalable phage powders containing lactose at ambient conditions. Int J Pharm. 2019;560:11–8. https://doi.org/10.1016/j.ijpharm.2019.01.050.
18. Zhang Y, Peng X, Zhang H, Watts AB, Ghosh D. Manufacturing and ambient stability of shelf freeze dried bacteriophage powder formulations. Int J Pharm. 2018;542(1–2):1–7.
19. Zierdt CH. Stabilities of lyophilized *Staphylococcus aureus* typing bacteriophages. (0099–2240 (Print)). doi: D - NLM: PMC204327 EDAT- 1988/10/01 MHDA- 1988/10/01 00:01 CRDT- 1988/10/01 00:00 PST - ppublish.
20. Garg T, Rath G, Goyal AK. Comprehensive review on additives of topical dosage forms for drug delivery. Drug Deliv. 2015;22(8):969–87.
21. Zhou Q, Leung SSY, Tang P, Parumasivam T, Loh ZH, Chan H-K. Inhaled formulations and pulmonary drug delivery systems for respiratory infections. Adv Drug Deliv Rev. 2015;85:83–99. https://doi.org/10.1016/j.addr.2014.10.022.
22. Monk R, Graves M, Williams P, Strange C. Inhaled alpha 1-antitrypsin: gauging patient interest in a new treatment. Copd. 2013;10(4):411–5. https://doi.org/10.3109/15412555.2012.758698.
23. Leung SS, Parumasivam T, Gao FG, Carrigy NB, Vehring R, Finlay WH, et al. Production of inhalation phage powders using spray freeze drying and spray drying techniques for treatment of respiratory infections. 2016;(1573-904X (Electronic)).
24. Vandenheuvel D, Singh A, Vandersteegen K, Klumpp J, Lavigne R, Van den Mooter G. Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. (1873–3441 (Electronic)).
25. Ibrahim M, Verma R, Garcia-Contreras L. Inhalation drug delivery devices: technology update. Med Devices (Auckl). 2015;8:131–9. https://doi.org/10.2147/MDER.S48888.
26. Chang RY, Wong J, Mathai A, Morales S, Kutter E, Britton W, et al. Production of highly stable spray dried phage formulations for treatment of *Pseudomonas aeruginosa* lung infection. Eur J Pharm Biopharm. 2017;121:1–13. https://doi.org/10.1016/j.ejpb.2017.09.002.
27. Golshahi L, Lynch KH, Dennis JJ, Finlay WH. In vitro lung delivery of bacteriophages KS4-M and ΦKZ using dry powder inhalers for treatment of Burkholderia cepacia complex and Pseudomonas aeruginosa infections in cystic fibrosis. J Appl Microbiol. 2011;110(1):106–17. https://doi.org/10.1111/j.1365-2672.2010.04863.x.
28. Malik DJ, Sokolov IJ, Vinner GK, Mancuso F, Cinquerrui S, Vladisavljevic GT, et al. Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. Adv Colloid Interface Sci. 2017;249:100–33. https://doi.org/10.1016/j.cis.2017.05.014.
29. Chang RYK, Wallin M, Lin Y, Leung SSY, Wang H, Morales S, et al. Phage therapy for respiratory infections. Adv Drug Deliv Rev. 2018;133:76–86. https://doi.org/10.1016/j.addr.2018.08.001.
30. Luthra S, Obert J-P, Kalonia DS, Pikal MJ. Investigation of drying stresses on proteins during lyophilization: differentiation between primary and secondary-drying stresses on lactate dehydrogenase using a humidity controlled mini freeze-dryer. J Pharm Sci. 2007;96(1):61–70. https://doi.org/10.1002/jps.20758.
31. Emami F, Vatanara A, Park EJ, Na DH. Drying technologies for the stability and bioavailability of biopharmaceuticals. Pharmaceutics. 2018;10(3):131. https://doi.org/10.3390/pharmaceutics10030131.
32. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: stability during storage at elevated temperatures. J Pharm Sci. 2012;101(7):2288–306. https://doi.org/10.1002/jps.23121.
33. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: stability after freeze-drying. J Pharm Sci. 2010;99(5):2256–78. https://doi.org/10.1002/jps.22000.
34. Rey L, May JC. Freeze-drying/lyophilization of pharmaceutical & biological products, revised and expanded: CRC Press; 2004.
35. Leung SSY, Parumasivam T, Gao FG, Carter EA, Carrigy NB, Vehring R, et al. Effects of storage conditions on the stability of spray dried, inhalable bacteriophage powders. Int J Pharm. 2017;521(1):141–9. https://doi.org/10.1016/j.ijpharm.2017.01.060.
36. Leung SS, Parumasivam T, Nguyen A, Gengenbach T, Carter EA, Carrigy NB, et al. Effect of storage temperature on the stability of spray dried bacteriophage powders. Eur J Pharm Biopharm. 2018;127:213–22.
37. Dini C, De Urraza PJ. Effect of buffer systems and disaccharides concentration on Podoviridae coliphage stability during freeze drying and storage. Cryobiology. 2013;66(3):339–42.
38. Merabishvili M, Vervaet C, Pirnay J-P, De Vos D, Verbeken G, Mast J, et al. Stability of Staphylococcus aureus phage ISP after freeze-drying (lyophilization). PLoS One. 2013;8(7):e68797. https://doi.org/10.1371/journal.pone.0068797.
39. Vandenheuvel D, Meeus J, Lavigne R, Van den Mooter G. Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. Int J Pharm. 2014;472(1–2):202–5. https://doi.org/10.1016/j.ijpharm.2014.06.026.
40. Matinkhoo S, Lynch Kh, Dennis JJ, Finlay WH, Vehring R. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. (1520–6017 (Electronic)).
41. Vinner GK, Rezaie-Yazdi Z, Leppanen M. Microencapsulation of salmonella-specific bacteriophage Felix O1 using spray-drying in a pH-responsive formulation and direct compression tableting of powders into a solid oral dosage form. 2019;12(1). https://doi.org/10.3390/ph12010043.
42. Ly A, Carrigy NB, Wang H, Harrison M, Sauvageau D, Martin AR, et al. Atmospheric spray freeze drying of sugar solution with phage D29. Front Microbiol. 2019;10(488). https://doi.org/10.3389/fmicb.2019.00488.
43. Agarwal R, Johnson CT, Imhoff BR, Donlan RM, McCarty NA, García AJ. Inhaled bacteriophage-loaded polymeric microparticles ameliorate acute lung infections. Nat Biomed Eng. 2018;2(11):841–9.
44. Puapermpoonsiri U, Ford SJ, van der Walle CF. Stabilization of bacteriophage during freeze drying. Int J Pharm. 2010;389(1–2):168–75. https://doi.org/10.1016/j.ijpharm.2010.01.034.
45. Alfadhel M, Puapermpoonsiri U, Ford SJ, McInnes FJ, van der Walle CF. Lyophilized inserts for nasal administration harboring bacteriophage selective for Staphylococcus aureus: in vitro evaluation. Int J Pharm. 2011;416(1):280–7.
46. Puapermpoonsiri U, Spencer J, van der Walle CF. A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. Eur J Pharm Biopharm. 2009;72(1):26–33.
47. Acar ES, Tayyarcan E, Evran Ş, Boyacı İ, Çakır İ, Khaaladi M, et al. Microencapsulation of phages to analyze their demeanor in physiological conditions. Folia Microbiol. 2019.
48. Back JF, Oakenfull D, Smith MB. Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry. 1979;18(23):5191–6.
49. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WL. How sugars protect proteins in the solid state and during drying (review): mechanisms of stabilization in relation to stress conditions. Eur J Pharm Biopharm. 2017;114:288–95. https://doi.org/10.1016/j.ejpb.2017.01.024.
50. Pramanick S, Chandel V, Singodia D. Excipient selection in parenteral formulation development. 2013.
51. Gervasi V, Dall Agnol R, Cullen S, McCoy T, Vucen S, Crean A. Parenteral protein formulations: an overview of approved products within the European Union. Eur J Pharm Biopharm. 2018;131:8–24. https://doi.org/10.1016/j.ejpb.2018.07.011.
52. Rowe RC, Sheskey PJ, Owen SC. Handbook of pharmaceutical excipients: pharmaceutical press London. 2006.
53. Inactive Ingredients Database 2019.
54. Tzannis ST, Prestrelski SJ. Moisture effects on protein—excipient interactions in spray-dried powders. Nature of destabilizing effects of sucrose. J Pharm Sci. 1999;88(3):360–70. https://doi.org/10.1021/js9800127.
55. Cox CS, Harris WJ, Lee J. Viability and electron microscope studies of phages T3 and T7 subjected to freeze-drying, freeze-

thawing and aerosolization. J Gen Microbiol. 1974;81(1):207–15. https://doi.org/10.1099/00221287-81-1-207.
56. Day JG, Stacey G. Cryopreservation and freeze-drying protocols: Springer Science & Business Media; 2007.
57. Akers MJ. Excipient–drug interactions in parenteral formulations. J Pharm Sci. 2002;91(11):2283–300. https://doi.org/10.1002/jps.10154.
58. Patel SM, Doen T, Pikal MJ. Determination of end point of primary drying in freeze-drying process control. AAPS PharmSciTech. 2010;11(1):73–84. https://doi.org/10.1208/s12249-009-9362-7.
59. Johnson RE, Kirchhoff CF, Gaud HT. Mannitol–sucrose mixtures—versatile formulations for protein lyophilization. J Pharm Sci. 2002;91(4):914–22. https://doi.org/10.1002/jps.10094.
60. Jaques A, Daviskas E, Turton JA, McKay K, Cooper P, Stirling RG, et al. Inhaled mannitol improves lung function in cystic fibrosis. Chest. 2008;133(6):1388–96. https://doi.org/10.1378/chest.07-2294.
61. Chi EY. Excipients used in biotechnology products. Pharmaceutical Excipients: Wiley; 2016. p. 145–98.
62. Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L, Treiber G. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology. 1970;40(3):734–44.
63. Feng W, Garrett H, Speert DP, King M. Improved clearability of cystic fibrosis sputum with dextran treatment in vitro. Am J Respir Crit Care Med. 1998;157(3 Pt 1):710–4. https://doi.org/10.1164/ajrccm.157.3.9703059.
64. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers. 2011;3(3):1377–97. https://doi.org/10.3390/polym3031377.
65. Kapoor DN, Bhatia A, Kaur R, Sharma R, Kaur G, Dhawan S. PLGA: a unique polymer for drug delivery. 2015.
66. Ma Y, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A, et al. Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. Appl Environ Microbiol. 2008;74(15):4799–805.
67. Colom J, Cano-Sarabia M, Otero J, Aríñez-Soriano J, Cortés P, Maspoch D, et al. Microencapsulation with alginate/CaCO 3: a strategy for improved phage therapy. Sci Rep. 2017;7:41441.
68. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012;37(1):106–26.
69. Cox C, Harris W, Lee J. Viability and electron microscope studies of phages T3 and T7 subjected to freeze-drying, freeze-thawing and aerosolization. Microbiology. 1974;81(1):207–15.
70. Ackermann HW, Tremblay D, Moineau S. Long-term bacteriophage preservation. 2004.
71. Clark WA. Comparison of several methods for preserving bacteriophages. Appl Environ Microbiol. 1962;10(5):466–71.
72. Masson P, Heremans J. Lactoferrin in milk from different species. Comp Biochem Physiol. 1971;1:119–29.
73. Orsi N. The antimicrobial activity of lactoferrin: current status and perspectives. Biometals. 2004;17(3):189–96.
74. Arnold R, Brewer M, Gauthier J. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect Immun. 1980;28(3):893–8.
75. Rr E, Giehl TJ, LaForce FM. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun. 1988;56(11):2774–81.
76. Travis SM, Conway B-AD, Zabner J, Smith JJ, Anderson NN, Singh PK, et al. Activity of abundant antimicrobials of the human airway. Am J Respir Cell Mol Biol. 1999;20(5):872–9.
77. Seville PC, Learoyd TP, Li HY, Williamson IJ, Birchall JC. Amino acid-modified spray-dried powders with enhanced aerosolisation properties for pulmonary drug delivery. Powder Technol. 2007;178(1):40–50. https://doi.org/10.1016/j.powtec.2007.03.046.
78. Chew NYK, Shekunov BY, Tong HHY, Chow AHL, Savage C, Wu J, et al. Effect of amino acids on the dispersion of disodium cromoglycate powders. J Pharm Sci. 2005;94(10):2289–300. https://doi.org/10.1002/jps.20426.
79. Yang X-F, Xu Y, Qu D-S, Li H-Y. The influence of amino acids on aztreonam spray-dried powders for inhalation. Asian J Pharm Sci. 2015;10(6):541–8. https://doi.org/10.1016/j.ajps.2015.08.002.
80. Feng A, Boraey M, Gwin M, Finlay P, Kuehl P, Vehring R. Mechanistic models facilitate efficient development of leucine containing microparticles for pulmonary drug delivery. Int J Pharm. 2011;409(1–2):156–63.
81. Cui Y, Zhang X, Wang W, Huang Z, Zhao Z, Wang G, et al. Moisture-resistant co-spray-dried netilmicin with L-Leucine as dry powder inhalation for the treatment of respiratory infections. Pharmaceutics. 2018;10(4):252.
82. Sou T, Kaminskas LM, Nguyen T-H, Carlberg R, McIntosh MP, Morton DAV. The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules. Eur J Pharm Biopharm. 2013, 83(2):234–43. https://doi.org/10.1016/j.ejpb.2012.10.015.
83. Guttman B, Raya R, Kutter E. Basic phage biology. Bacteriophages: Biology and applications. 2005;4.
84. Jepson CD, March JB. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine. 2004;22(19):2413–9.
85. Randolph TW. Phase separation of excipients during lyophilization: effects on protein stability. J Pharm Sci. 1997;86(11):1198–203.
86. Marvin DA. Filamentous phage structure, infection and assembly. Curr Opin Struct Biol. 1998;8(2):150–8. https://doi.org/10.1016/S0959-440X(98)80032-8.
87. Kutter E, Sulakvelidze A. Bacteriophages: biology and applications: CRC press. 2004.
88. Benson SD, Bamford JKH, Bamford DH, Burnett RM. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. Cell. 1999;98(6):825–33. https://doi.org/10.1016/S0092-8674(00)81516-0.
89. Bamford DH, Grimes JM, Stuart DI. What does structure tell us about virus evolution? Curr Opin Struct Biol. 2005;15(6):655–63. https://doi.org/10.1016/j.sbi.2005.10.012.
90. Kanojia G, Have R, Soema PC, Frijlink H, Amorij J-P, Kersten G. Developments in the formulation and delivery of spray dried vaccines. Hum Vaccin Immunother. 2017;13(10):2364–78.
91. Amorij JP, Huckriede A, Wilschut J, Frijlink HW, Hinrichs WLJ. Development of stable influenza vaccine powder formulations: challenges and possibilities. Pharm Res. 2008;25(6):1256–73. https://doi.org/10.1007/s11095-008-9559-6.
92. Common Ingredients in U.S. Licensed Vaccines. https://www.fda.gov/biologicsbloodvaccines/safetyavailability/vaccinesafety/ucm187810.htm: U.S. Food and Drug Administration; 2014 [updated December 31, 2014].
93. Vaccines Licensed for Use in the United States. 2019. https://www.fda.gov/biologicsbloodvaccines/vaccines/approvedproducts/ucm093833.htm. Accessed May 2019.
94. Ohtake S, Martin RA, Yee L, Chen D, Kristensen DD, Lechuga-Ballesteros D, et al. Heat-stable measles vaccine produced by spray drying. Vaccine. 2010;28(5):1275–84.
95. Newman J, Tirrell S, Ullman C, Piatti P, Brown F. Stabilising oral poliovaccine at high ambient temperatures. Vaccine. 1995;13(15):1431–5.
96. Chen C-H, Wu R, Roth LG, Guillot S, Crainic R. Elucidating mechanisms of thermostabilization of poliovirus by D2O and MgCl2. Arch Biochem Biophys. 1997;342(1):108–16.
97. Morefield GL. A rational, systematic approach for the development of vaccine formulations. AAPS J. 2011;13(2):191–200. https://doi.org/10.1208/s12248-011-9261-1.
98. Bovarnick MR, Miller JC, Snyder JC. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J Bacteriol. 1950;59(4):509–22.
99. McAleer HZMJ. Vaccine stabilizer containing L-glutamic acid and L-arginine. Merck and Co Inc. 1981.
100. Yannarell DA, Goldberg KM, Hjorth RN. Stabilizing cold-adapted influenza virus vaccine under various storage conditions. J Virol Methods. 2002;102(1):15–25. https://doi.org/10.1016/S0166-0934(01)00432-3.
101. Kang M, Jang H, Kim M, Kim M, Joh S, Kwon J, et al. Development of a stabilizer for lyophilization of an attenuated duck viral hepatitis vaccine. Poult Sci. 2010;89(6):1167–70.
102. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein–excipient interactions: mechanisms and biophysical characterization applied to protein formulation

development. Adv Drug Deliv Rev. 2011;63(13):1118–59. https://doi.org/10.1016/j.addr.2011.07.006.
103. Ausar SF, Espina M, Brock J, Thyagarayapuran N, Repetto R, Khandke L, et al. High-throughput screening of stabilizers for respiratory syncytial virus: identification of stabilizers and their effects on the conformational thermostability of viral particles. Hum Vaccin. 2007;3(3):94–103.
104. Schlehuber LD, McFadyen IJ, Shu Y, Carignan J, Duprex WP, Forsyth WR, et al. Towards ambient temperature-stable vaccines: the identification of thermally stabilizing liquid formulations for measles virus using an innovative high-throughput infectivity assay. Vaccine. 2011;29(31):5031–9.
105. Condra L. Reliability improvement with design of experiment: CRC Press. 2001.
106. Trnka H, Rantanen J, Grohganz H. Well-plate freeze-drying: a high throughput platform for screening of physical properties of freeze-dried formulations. Pharm Dev Technol. 2015;20(1):65–73. https://doi.org/10.3109/10837450.2013.871028.
107. Wen Z-Q. Raman spectroscopy of protein pharmaceuticals. J Pharm Sci. 2007;96(11):2861–78. https://doi.org/10.1002/jps.20895.
108. Thomas GJ Jr. Raman spectroscopy of protein and nucleic acid assemblies. Annu Rev Biophys Biomol Struct. 1999;28:1–27. https://doi.org/10.1146/annurev.biophys.28.1.1.
109. Dougan L, Feng G, Lu H, Fernandez JM. Solvent molecules bridge the mechanical unfolding transition state of a protein. Proc Natl Acad Sci. 2008;105(9):3185–90. https://doi.org/10.1073/pnas.0706075105.
110. Garcia-Manyes S, Dougan L, Fernández JM. Osmolyte-induced separation of the mechanical folding phases of ubiquitin. Proc Natl Acad Sci. 2009;106(26):10540–5. https://doi.org/10.1073/pnas.0902090106.
111. Goormaghtigh E, Ruysschaert J-M, Raussens V. Evaluation of the information content in infrared spectra for protein secondary structure determination. Biophys J. 2006;90(8):2946–57. https://doi.org/10.1529/biophysj.105.072017.
112. Barth A, Zscherp C. What vibrations tell us about proteins. Q Rev Biophys. 2002;35(4):369–430. https://doi.org/10.1017/s0033583502003815.
113. Mittermaier A, Kay LE. New tools provide new insights in NMR studies of protein dynamics. Science (New York). 2006;312(5771):224–8. https://doi.org/10.1126/science.1124964.
114. Ishima R, Torchia DA. Protein dynamics from NMR. Nat Struct Biol. 2000;7(9):740–3. https://doi.org/10.1038/78963.
115. Skinner AL, Laurence JS. Probing residue-specific interactions in the stabilization of proteins using high-resolution NMR: a study of disulfide bond compensation. J Pharm Sci. 2010;99(6):2643–54. https://doi.org/10.1002/jps.22055.
116. Ramsey JD, Gill ML, Kamerzell TJ, Price ES, Joshi SB, Bishop SM, et al. Using empirical phase diagrams to understand the role of intramolecular dynamics in immunoglobulin G stability. J Pharm Sci. 2009;98(7):2432–47. https://doi.org/10.1002/jps.21619.
117. Kamerzell TJ, Unruh JR, Johnson CK, Middaugh CR. Conformational flexibility, hydration and state parameter fluctuations of fibroblast growth factor-10: effects of ligand binding. Biochemistry. 2006;45(51):15288–300. https://doi.org/10.1021/bi061712q.
118. Kamerzell TJ, Ramsey JD, Middaugh CR. Immunoglobulin dynamics, conformational fluctuations, and nonlinear elasticity and their effects on stability. J Phys Chem B. 2008;112(10):3240–50. https://doi.org/10.1021/jp710061a.
119. Milne JLS, Borgnia MJ, Bartesaghi A, Tran EEH, Earl LA, Schauder DM, et al. Cryo-electron microscopy–a primer for the non-microscopist. FEBS J. 2013;280(1):28–45. https://doi.org/10.1111/febs.12078.
120. Cuervo A, Carrascosa JL. Observation of bacteriophage ultrastructure by cryo-electron microscopy. Methods Mol Biol (Clifton). 2018;1693:43–55. https://doi.org/10.1007/978-1-4939-7395-8_5.
121. Khanal D, Chang RYK, Morales S, Chan H-K, Chrzanowski W. High resolution nanoscale probing of bacteriophages in an inhalable dry powder formulation for pulmonary infections. Anal Chem. 2019;91(20):12760–7. https://doi.org/10.1021/acs.analchem.9b02282.
122. Henderson R. The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules. Q Rev Biophys. 1995;28(2):171–93.
123. Cuervo A, Carrascosa JL. Observation of bacteriophage ultrastructure by cryo-electron microscopy. In: Azeredo J, Sillankorva S, editors. Bacteriophage therapy: from lab to clinical practice. New York: Springer New York; 2018. p. 43–55.
124. Thiriot DS, Nevzorov AA, Zagyanskiy L, Wu CH, Opella SJ. Structure of the coat protein in Pf1 bacteriophage determined by solid-state NMR spectroscopy. J Mol Biol. 2004;341(3):869–79. https://doi.org/10.1016/j.jmb.2004.06.038.
125. Morag O, Sgourakis NG, Abramov G, Goldbourt A. Filamentous bacteriophage viruses: preparation, magic-angle spinning solid-state nmr experiments, and structure determination. Methods Mol Biol. 2018;1688:67–97. https://doi.org/10.1007/978-1-4939-7386-6_4.
126. Abramov G, Morag O, Goldbourt A. Magic-angle spinning NMR of intact bacteriophages: insights into the capsid, DNA and their interface. J Magn Reson (San Diego, Calif : 1997). 2015;253:80–90. https://doi.org/10.1016/j.jmr.2015.01.011.
127. Peng X, Nguyen A, Ghosh D. Quantification of M13 and T7 bacteriophages by TaqMan and SYBR green qPCR. J Virol Methods. 2018;252:100–7. https://doi.org/10.1016/j.jviromet.2017.11.012.
128. Peng X, Leal J, Mohanty R, Soto M, Ghosh D. Quantitative PCR of T7 bacteriophage from biopanning. J Vis Exp. 2018;(139):e58165. https://doi.org/10.3791/58165.
129. Naini V. Physical and chemical stability of spray dried sugars and protein-sugar molecuiar mixtures for inhalation. 1996.