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Manufacturing and ambient stability of shelf freeze dried bacteriophage powder formulations

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ABSTRACT

The severity of multidrug resistance to antibiotics has urged development of alternative treatment approaches, including bacteriophage therapy. Given the complexity of the bacteriophage structure, formulation and stability are primary concerns. Our present work optimized process and formulations of phage powder manufacturing and investigated the stability of lyophilized bacteriophage powders under ambient storage. The model phage M13 was formulated with trehalose, mannitol, sucrose and PEG_{6000} and lyophilized in different conditions. Bacteriophage viability was examined by titering and was considered as the assessment of phage stability. Less titer loss of trehalose and sucrose formulations were observed compared to mannitol and PEG groups both immediately after lyophilization and upon long term storage. When evaluating lyophilization conditions, an additional 1 log titer was preserved by reduction of product drying stress. Trehalose was stabilized in the amorphous state whereas mannitol stayed in crystalline state in lyophilized powders. Increased moisture content was demonstrated to have a positive impact on viability of phage after lyophilization and upon storage. Overall, 2% trehalose or sucrose (w/v) can sufficiently stabilize phage during lyophilization process and storage in ambient conditions. There is a positive correlation between residual water and stability of phage. These collective findings highlight the potential of long-term, ambient storage of bacteriophage towards their successful use in diverse healthcare settings.

1. Introduction

Recently, the increasing severity of multidrug resistant bacteria and the resulting decreasing efficacy of small molecule antibiotics has led to the re-emergence of bacteriophage (or phage) therapy (Boucher et al., 2009; Cars et al., 2008; Kingwell, 2015). Phage have evolved and been widely exploited as antimicrobial agents able to infect and weaken and/or kill specific, pathogenic host bacteria (Salmond and Fineran, 2015). Others (Loc-Carrillo and Abedon, 2011) (Debarbieux et al., 2010) have highlighted the attractiveness of phage therapy, including: 1) the specificity of bactericidal effect, 2) "auto-dosing" owing to host based replication, 3) low in vivo toxicity, 4) non-disruption to normal flora, 5) formulation and application versatility, 6) biofilm clearance and 7) adaption to resistant bacteria (Ryan et al., 2011).

Phage therapy has begun to make advances in clinical trials. A recently completed clinical trial found a phage cocktail was effective to treat *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus)* infections in burn wounds (Rose et al., 2014). They further collaborated in a larger European clinical trial investigating the safety and therapeutic efficacy of phage treatment of burn wounds infected with *E. coli* and *P. aeruginosa* (ClinicalTrials.gov, (2015) Identifier: NCT02116010). Additionally, there is a current Phase I clinical trial to evaluate the safety of AB-SA01 (AmpliPhi), a phage cocktail targeting *S. aureus* infections via topical administration to the intact skin of healthy adults (ClnicalTrials.gov, (2016) Identifier: NCT02757755). Moreover, preclinical results have shown the ability of another phage cocktail product, AB-PA01 (AmpliPhi), to successfully infect and kill *P. aeruginosa* clinical isolates from a global population of cystic fibrosis (CF) patients. Similar work has been done where a cocktail formulation containing 10 different phages were applied to investigate their effects on *P. aeruginosa* in sputum of CF patients (ClinicalTrials.gov, (2013) Identifier: NCT01818206). Several other therapeutic phage products

Abbreviations: HPMC, hydroxypropyl methylcellulose; PEG, polyethylene glycol; PBS, phosphate buffer saline; RT, room temperature; RH, relative humidity; OD_{600}, optical density at a wavelength of 600 nm; XRD, X-ray diffractometry; MW, molecular weight; Tg, glass transition temperature; SD, standard deviation; PLM, polarized light microscope; R1, recipe 1; R2, recipe 2

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are in development for treatment of venous leg ulcers (Rhoads et al., 2009) (ClinicalTrials.gov, (2008) Identifier: NCT00663091), ear infections (Wright et al., 2009), and dysentery (Sulakvelidze et al., 2001), amongst others.

To fully develop phage and advance their prospect as a feasible therapeutic option, their viability and potency must be demonstrated over time in various storage conditions. Since phage primarily consists of protein capsids encapsulating their genetic material, it is reasonable to formulate phage with similar formulation strategies applied to protein therapeutics. It is widely accepted that majority of proteins are more stable in a dry state than in solution (Frokjaer and Otzen, 2005; Mascarell and Ryan, 1997; Ohtake et al., 2011). Proteins are sensitive to physical stresses in aqueous solution, including temperature changes, agitation, pH changes, and exposure to interfaces or denaturants, which can lead to protein denaturation and aggregation. Moreover, exposure to water can induce chemical degradation such as hydrolysis and deamidation. During long term storage, protein aggregation in solution is an additional concern (Carpenter et al., 2002). In contrast, proteins in dry state can reduce physical and chemical stresses and improve stability.

Additionally, similar to proteins, phage can be manufactured with excipients into powder to enhance storage stability. Typically, different sugars and polymers, such as trehalose, sucrose, mannitol and PEG, are frequently used to stabilize protein formulations (Merabishvili et al., 2013) (Leung et al., 2017) (Alfadhel et al., 2011; Matinkhoo et al., 2011). For lyophilized samples, sugars help stabilize not only the lyophilizing process, including freezing, primary drying and secondary drying, but also shelf storage of solid products (Cao et al., 2003). In one study, a strain of Siphoviridae phage was formulated with hydroxypropyl methylcellulose (HPMC) with and without addition of mannitol, and lyophilized the formulation into powder. The phage titer (i.e. activity of phage) was checked periodically during storage at 4 °C and 100-fold titer loss was observed after 12 months. In another study, the titer of spray dried ΦKZ/D3 phage powder was measured after 3 months of refrigerated storage, and less than 0.15 log titer loss was found in all formulations (Alfadhel et al., 2011; Matinkhoo et al., 2011). Another study demonstrated that powder matrix containing no less than 40% trehalose preserved phage activity and aerosol performance well after storage at lower relative humidity (0 and 22%) at 4 °C for 12 months (Leung et al., 2017). Another study investigated the influence of different excipients on the stability of shelf freeze-dried phage. Their data demonstrated that 0.5 M trehalose was the best formulation and the phage titer lost 1.5 log in 4 °C storage condition after more than three years. To assess its feasibility for long-term storage, it is critical to validate the stability of phage post-lyophilization (Carne and Greaves, 1974; Clark, 1962; Merabishvili et al., 2013; Zierdt, 1988). A long term stability study was done on 25 lyophilized phages, which 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 (Zierdt, 1988).

These previous studies focused on long-term storage at refrigerated or frozen conditions, which limits the potential applications of phage as a drug product. Thus, to promote the development and application of phage therapy as a feasible therapy, it is necessary to study their ambient stability. The aim of this study is to develop formulations to stabilize bacteriophage and validate their storage stability at room temperature, or 25 °C, which will eliminate the cold-chain requirement of phage drug products. Excipients and lyophilization recipes are evaluated and screened in terms of stability by examining phage viability after storage at ambient conditions.

2. Material and methods

2.1. Materials

Model M13KE phage (denoted as “wild type”) and its host bacterial strain, *E. coli* ER2738, were purchased from New England BioLabs (MA, US). Purified phages were freshly obtained and stored in phosphate buffer saline (PBS) at a titer (i.e. activity assay of concentration) of 5×10^{11} or 3×10^{12} plaque forming units (pfu) per milliliter, following the amplification and purification methods as recommended by the manufacturer. Phage were added to formulations at 1000-fold dilution (Table 1). Excipients, including D-(+)-Trehalose dihydrate, sucrose and polyethylene glycol 6000 (PEG_{6000}) were all purchased from Sigma-Aldrich (MO, US). Mannitol was purchased from Pearlitol PF, US. Excipients were dissolved in Milli-Q water (Millipore, MA, US) and the solution was adjusted to pH 7.2–7.4. Table I presents formulations with given titer of phage and composition of excipients.

Table 1
Composition of formulations.

Formulation	Recipe	Initial Phage Titer (pfu/ml)	Excipient (w/v)			
			Mannitol	Trehalose	Sucrose	PEG_{6000}
F1	1	$\sim 5 \times 10^8$		2%		
F2	1	$\sim 5 \times 10^8$	0.4%	1.6%		
F3	1	$\sim 5 \times 10^8$	1%	1%		
F4	1	$\sim 5 \times 10^8$	1.6%	0.4%		
F5	1	$\sim 5 \times 10^8$	2%			
F6	2	$\sim 3 \times 10^9$			2%	
F7	2	$\sim 3 \times 10^9$		2%		
F8	2	$\sim 3 \times 10^9$				2%
F9	2	$\sim 3 \times 10^9$		1%		1%

2.2. Shelf lyophilization

Twenty milliliter glass serum vials with rubber stopper (VWR International, PA, US) containing 10 ml of phage solutions, were lyophilized in a VirTis AdVantage Freeze Dryer (VirTis Company Inc., NY, US). Samples were prepared for shelf lyophilization (Fig. 1). For lyophilization recipe 1 (R1), samples were loaded at 25 °C, and held frozen at a shelf temperature of −50 °C for 1 h after 75 min ramping. Primary drying was initiated at -40 °C over 1250 min, with a pressure of 50 mTorr. Secondary drying involved subsequent heating of the shelf to 25 °C using 1250 min, maintained under vacuum for a further 1250 min. As soon as the completion of lyophilization, vials were immediately sealed air tight (headspace filled with air) and stored in a plastic chamber at room temperature (RT). For recipe 2 (R2), the initial freezing steps involved equilibrating the samples at −5 °C, holding at that temperature for 20 min before further cooling to −30 °C at a ramping rate of 1 °C/min and maintaining temperature for 110 min. Primary drying was performed at −30 °C for 1000 min with a pressure of 230 mTorr. Secondary drying started with heating the shelf to 25 °C at a rate of 0.1 °C/min and maintained for 660 min under the same vacuum pressure. The headspaces of vials were filled with nitrogen. The plate in lyophilizer was lowered by using compressed air to seal the

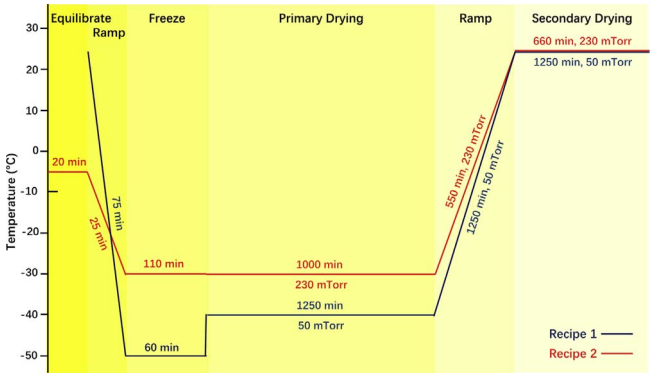

Fig. 1. Lyophilization steps. Note: length of lines does not represent time length; temperatures are shelf temperatures.