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Effects of storage conditions on the stability of spray dried, inhalable bacteriophage powders

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Abstract

This study aimed to develop inhalable powders containing phages active against antibiotic-resistant *Pseudomonas aeruginosa* for pulmonary delivery. A *Pseudomonas* phage, PEV2, was spray dried into powder matrices comprising of trehalose (0–80%), mannitol (0–80%) and L-leucine (20%). The resulting powders were stored at various relative humidity (RH) conditions (0, 22 and 60% RH) at 4 °C. The phage stability and *in vitro* aerosol performance of the phage powders were examined at the time of production and after 1, 3 and 12 months storage. After spray drying, a total of 1.3 log titer reduction in phage was observed in the formulations containing 40%, 60% and 80% trehalose, whereas 2.4 and 5.1 log reductions were noted in the formulations containing 20% and no trehalose, respectively. No further reduction in titer occurred for powders stored at 0 and 22% RH even after 12 months, except the formulation containing no trehalose. The 60% RH storage condition had a destructive effect such that no viable phages were detected after 3 and 12 months. When aerosolised, the total lung doses for formulations containing 40%, 60% and 80% trehalose were similar (in the order of 10⁵ pfu). The results demonstrated that spray drying is a suitable method to produce stable phage powders for pulmonary delivery. A powder matrix containing 40% trehalose provided good phage preservation and aerosol performances after storage at 0 and 22 % RH at 4 °C for 12 months.

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Graphical abstract

Phage Stability in F1 – F4 formulation after storage at 0%, 22% and 60% RH. Titer reductions at 0 months (Fresh), 1, 3 and 12 months are relative to the titer measured in the spray dryer liquid feedstock.

Keywords

Phage; PEV2; pulmonary infection; multi-drug resistance; MDR; spray drying

1 Introduction

The use of bacteriophages to treat bacterial infections has recently regained significant attention owing to the rapid emergence of multidrug-resistant (MDR) bacterial strains and the slow development of new antibacterial compounds. With these increased research efforts, a large number of novel phages have been isolated and characterised to target broad-spectrum antimicrobial resistance of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia* complex (BCC) (Ackermann, 2007; Larche et al., 2012; Essoh et al., 2013), which are major pathogens in chronically infected cystic fibrosis (CF) patients. In addition, the potential of phages to treat respiratory infections caused by MDR bacteria has recently been demonstrated in animal models (Chhibber et al., 2008; Carmody et al., 2010; Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Semler et al., 2014; Singla et al., 2015; Cao et al., 2015; Pabary et al., 2016). These promising results have made phage therapy one of the most promising alternatives to conventional antibiotics for respiratory infectious diseases.

Carmody et al. (2010) compared the efficacy of delivery routes in a mouse model of acute *B. cenocepacia* pulmonary infections. They reported that the bacterial density was significantly reduced in lungs of mice treated with intraperitoneal phages, while no significant difference was observed between untreated mice and mice treated with phages delivered using intranasal instillation. Chhibber et al. (2008) showed a significant reduction of *Klebsiella pneumoniae* in the lung to below the initial infectious dose, when the phages were administered intraperitoneally immediately or 3 h prior to the bacterial challenge. However, opposite results were reported by Semler et al. (2014) who showed significant bacterial reduction in the lung in BCC-infected mice treated with aerosolized phages using a jet nebulizer attached to a Nose-Only Inhalation Device, but not in the intraperitoneal treatment group. The different treatment outcomes in intraperitoneal phage delivery could be attributed

to different systemic clearance rates of phages used in these studies. On the other hand, the disparity of the pulmonary phage treatments may be accounted by the efficiency of delivery methods, with Semler et al.'s use of aerosol delivery achieving better pulmonary targeting efficiency. Traditionally, it has been considered that the more effective route for phage delivery should be the one that results in direct delivery to the site of infection, enhancing the chance of phages attaining and sustaining an efficacious concentration in the vicinity of target bacteria. Thus, for pulmonary infections, direct lung delivery would be a more rational and clinically relevant approach. The effectiveness of intranasal administration of phages to treat mice infected with *P. aeruginosa* has also been demonstrated (Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Cao et al., 2015). Debarbieux and coworkers (Debarbieux et al., 2010; Morello et al., 2011) showed that the survival rate of the infected mice was increased with higher phage-to-bacteria ratios, with 100% survival rate observed at a 10:1 ratio (with dose of the order of 10^8 pfu). Similar results were also reported by Cao et al (2015).

In addition to the delivery route and phage concentration, the timing of administration has an impact on the efficacy of phage therapy. Debarbieux and coworkers (Debarbieux et al., 2010; Morello et al., 2011) reported that the survival rate of *P. aeruginosa* infected mice was 100% when phages were given 2 h after the bacterial challenge, but it dropped to 75% and 25% in 4 h and 6 h post-treatment groups, respectively. Furthermore, for preventive treatment, 100% protection was achieved when a single dose of phages were given at 24 h (Debarbieux et al., 2010) and 4 day (Morello et al., 2011) before infection with *P. aeruginosa*. Similar results were reported in Pabary et al. (2016). Singla et al. (2015) compared the efficacy of intraperitoneal application of phages alone and when delivered in liposomes in a mouse model of *K. pneumoniae* lobar pneumonia. While the phages alone treatment was only able to prevent the infection up to 6 h prior to bacterial challenge, the liposome-entrapped phages provided complete protection up to 48 h prior treatment. Their results showed that entrapped phages could slow down the systemic clearance rate of phages in the absence of bacteria. It is noteworthy to mention that these animal models used were acute, rapidly progressive infection, and this does not resemble the chronic bacterial infections in CF patients. Therefore, the relevance of these results in translating to a chronic environment would still need to be assessed clinically.

While the quantity of phage research and well-defined preclinical trials has substantially increased, further research on pharmaceutical formulations and their long-term stability is important for both clinical and commercial application (Merabishvili et al., 2013). Currently, phage preparations for therapeutic use are limited to liquid formulations, which are generally recommended to be stored at 4 °C with one year shelf life (Merabishvili et al., 2013). As a result, most phage research for respiratory infections in the past has been confined to liquid aerosols using intranasal instillation (Carmody et al., 2010; Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Semler et al., 2014) and nebulisation (Sahota et al., 2015; Abedon, 2015). Since many bacteriophage strains may be stabilised in the powder form for years and so facilitate their global distribution (Golshahi et al., 2011), recent efforts have been devoted to develop stabilised dry dosage formulations for phage inhalation (Golshahi et al., 2011; Matinkhoo et al., 2011; Vandenheuvel et al., 2013, 2014; Leung et al., 2016).

Previously, we compared the phage viability and aerosol performance of two dry powder formation techniques, spray freeze drying and spray drying, for the production of inhalable phage powders (Leung et al., 2016). Multi-component excipient systems consisting of various amounts of trehalose, mannitol and leucine were used as the bulking and stabilising agents. We reported that the spray drying method caused less phage titer reduction during the powder formation process and achieved better aerosol performance. In this study, we focused on the effects of storage conditions on the stability of the spray dried powder matrix. After spray drying, the powders were immediately stored at various relative humidity (RH) conditions (0, 22 and 60% RH) at 4 °C. Since storage of biologics (proteins, phages) at ambient conditions is not recommended (Jo czyk et al., 2011) and there is no long term storage stability data for phage in powder form, 4 °C was chosen to maximize the chance of phage survival. The phage stability and *in vitro* aerosol performance of the powders were assessed at 0, 1, 3 and 12 months storage.

2 Material and method

2.1 Materials

The phage used in this study was an N4-type, lytic podovirus, PEV2. It was isolated from the sewage treatment plant in Olympia, WA, USA by students in the Evergreen State College Phage Laboratory, who made a detailed analysis of its infection-related properties and genome (Ceyssens et al., 2010). A PEV2 stock with a titer of 2.2×10^9 pfu/ml stored in salt-magnesium buffer (SMB, 5.2 g/l sodium chloride, 2 g/l magnesium sulfate, 6.35 g/l Tris-HCL, 1.18 g/l Tris base and 0.01% gelatin) was supplied via AmpliPhi Biosciences (AmpliPhi Biosciences AU, NSW Australia) and used without further purification.

Various amounts of D-(+)-Trehalose dihydrate, mannitol and L-leucine from Sigma–Aldrich (NSW, Australia) were co-spray dried with phage to form a powder matrix to protect the phage particles. Table 1 shows the composition of the five formulations prepared in the present study. The compositions of F2 and F3 were the same as that prepared in our previous study (Leung et al., 2016). Their long term storage stability was assessed in the present study.

2.2 Powder preparation

A volume of 50 ml excipient solution with a total solid concentration of 20 mg/mL was prepared in ultra-pure distilled water with the pH adjusted to 7–7.5 using 1 M hydrochloric acid and 1 M sodium hydroxide. Then 0.5 ml phage suspension was added to the mixture, resulting in 100 times titer dilution (starting titer = 2.2×10^7 pfu/ml). The phage viability in the sugar solution before spray drying was confirmed by using a standard plaque assay (Carlson, 2005). The mixtures were spray dried using a Büchi 290 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) using an open-loop setting at an drying gas flow rate of 35 m³/hr, atomizing air flow rate of 742 l/hr, and inlet temperature of 60 °C with a liquid feed rate of 1.8 mL/min. The outlet temperature was recorded to be 40 – 45 °C. Low outlet temperatures were used to minimize phage inactivation during the drying process (Chopin, 1980). The produced powders were collected inside a relative humidity controlled chamber

(RH < 20%), and stored at various humidity conditions (0, 22 and 60 % RH) at 4 °C before use.

2.3 Powder characterization

2.3.1 Particle morphology—Morphologies of the spray dried powders were examined using a field emission scanning electron microscope (SEM) (Hitachi S4500 FESEM, Japan) at 5 kV beam accelerating voltage. The samples were scattered on a carbon tape and sputter coated with ~15 nm of gold using a K550X sputter coater (Quorum Emitech, Kent, UK) before imaging.

2.3.2 Particle size distribution—Particle size distributions of the powder formulations were measured by laser diffraction using a Mastersizer 2000 (Malvern Instruments, UK). As leucine is expected to cover the particle surface during the spray drying process, the optical properties of leucine were used for analysis, with the real and imaginary refractive indices set at 1.52 and 0.1, respectively. The dispersant (air) refractive index was set to 1.0. The powders were dispersed through the measurement window with compressed air at 3.5 bar using a Scirocco 2000 dry powder module (Malvern Instruments, UK). All measurements were done in triplicate. The size distribution was expressed by the volume median diameter (VMD), and span that defined as the difference in the particle diameters at 10 and 90% cumulative volume divided by the VMD.

2.3.3 Particle crystallinity—An X-ray diffractometer (Model D5000; Siemens, Munich, Germany) was used to determine the crystallinity of the produced powders. The experiment was performed under ambient condition using Cu K α radiation at 30 mA and 40 kV, with an angular increment rate of 0.04° 2 θ / s from 5° to 40°.

2.3.4 Moisture sorption—The moisture sorption profiles of the powders were analysed using a DVS instrument (DVS-Intrinsic, Surface Measurement Systems, London, UK). Each sample (5 $\pm$ 1 mg) was subjected to a dual moisture ramping cycle of 0 to 90 % RH at a step increase of 10 %. Equilibrium moisture content at each RH was defined at less than dm/dt of 0.02 % per minute.

2.3.5 Thermal analysis—The thermal properties of the phage powders were analyzed using modulated differential scanning calorimetry (MDSC) (TA Instruments, Q1000 mDSC, New Castle, DE, USA) and thermogravimetric analysis (TGA) (Mettler Toledo, Greifensee, Switzerland). For mDSC measurement, 3 – 10 mg of powder was weighed into a TA aluminum hermetic pan (900793.901) and lid (900794.901) and then crimped. A pinhole was then added to the lid so that the moisture evaporation and dry glass transition temperature of the different formulation components could be examined. The samples were first equilibrated at 20 °C for five minutes before heating to 180 °C at a rate of 2 °C/min using a $\pm$ 0.5 °C/min temperature modulation with a continuous flow of nitrogen gas at 60 mL/min. In the TGA measurements, a sample mass of 5 $\pm$ 1 mg was weighed into an alumina crucible and heated from 30 to 400 °C at a rate of 10 °C/min with a dynamic nitrogen flow.

2.4 Powder dispersibility

In vitro aerosol performance of the powders stored at RH 22% was assessed at the beginning and after 3 and 12 months. An Osmohaler™ coupled to a USP stainless steel throat and a multi-stage liquid impinger (MSLI) was operated at 100 L/min for 2.4 s. A sample mass of 20 mg was loaded into a size 3 hydroxypropyl methylcellulose capsule (Capsugel, NSW, Australia). The capsule filling was done in a humidity controlled (RH: ~17%) box while the dispersion was conducted at RH of $50 \pm 5\%$ and $20 \pm 5\%$ °C. SMB was used as the rinsing solvent to determine the viable phage deposition profiles. Experiments were done in triplicate. The lower cutoff diameters of the MSLI stages 1–4 at 100 L/min are 10.1, 5.3, 2.4 and 1.32 μm , calculated with the adjustment equations given in Appendix XII C of the British Pharmacopoeia. The fine particle fraction (FPF) was defined as the mass fraction of particles with an aerodynamic diameter $\leq 5.0 \mu\text{m}$ with respect to the total recovered dose.

2.5 Phage powder stability

Freshly spray dried phage powders were transferred in a Perspex box at a RH of less than 17% into three 30 ml glass vials which were loosely capped. The samples were then stored at three RH conditions, 0, 22 and 60%, at 4 °C up to a year. The phage viability and powder were examined after 0, 1, 3 and 12 months storage. An amount of 15 mg of powder stored at each condition was dissolved in 300 μl SMB to give a concentration of 50 mg/ml. The Miles-Misra surface droplet technique (Carlson 2005) was employed to determine the amount of viable phages in the powder samples. Serial dilutions, 1:10, were performed by adding 20 μl samples to 180 μl SMB. A volume of 200 μl host bacteria containing $\sim 2 \times 10^9$ colony forming units (cfu) was mixed with 5 ml molten soft agar (0.4% Amyl agar, 48 °C). The mixture was then overlayed onto a solidified nutrient agar made of 1.5% Amyl agar and nutrient broth. A volume of 10 μL diluted phage samples were dropped onto the agar lawn in triplicate, air dried and incubated at 37°C overnight. Samples giving rise to 3–30 plaques were used for phage viability calculation.

2.6 Statistical Analysis

One-way analysis of variance (ANOVA) at a confidence level of 95% was employed to identify any statistically significant differences in mass deposition in the throats and FPFs. A *p* value of < 0.05 was considered statistically significant.

3 Results and Discussion

3.1 Powder characterization

3.1.1 Particle Morphology—Fig. 1 shows the SEM images of the produced powders. The particles are generally smooth and spherical. As spray dried trehalose is predominantly amorphous, significant particle merging was observed in F1, F2 and F3 due to their high trehalose content ($\sim 40\%$). Hence, the results suggest that the inclusion of 20% leucine is not sufficient to form a crystalline shell to protect the amorphous trehalose for the processing conditions chosen (Feng et al., 2011). The degree of particle merging was significantly reduced as the mannitol content increased to 60% (F4), and no particle merging was observed for F5. This was an expected outcome as spray dried mannitol is primarily

crystalline. The morphologies of the phage powders stored under desiccation were also examined and no significant changes were observed (data not shown).

3.1.2 Particle sizing—The volume median diameters of the spray dried particles are presented in Table 2. The results were in accordance with the SEM images, with most particles falling within the inhalable size fraction of less than 5 μm . No specific trends were observed with variation in formulation composition.

3.1.3 Powder crystallinity—Trehalose has been reported to be an excellent excipient in preserving the activity of protein or biological compounds (Wang 2000). However, the spray dried trehalose is predominantly amorphous and requires storage under moisture protection. Vandenneuvel et al. (2014) reported that high humidity (54% RH) could cause re-crystallization of the spray dried trehalose powder matrix and destroy the embedded phages. The X-ray diffraction patterns of the fresh spray dried samples and those stored under desiccation for 12 months are depicted in Fig. 2. No significant differences were noted. The results showed that the powders of F1 and F2 were predominantly amorphous. Stronger crystalline peaks were detected with increasing mannitol content. Suo et al. (2013) examined the XRD profiles of spray dried powders containing various amount of mannitol, trehalose and leucine, and the pure components showed that the $2\theta = 6^\circ$ peak was the primary peak for partially ordered leucine with the peak intensity increasing with the leucine content. They suggested that a fraction of 20% leucine was sufficient for self-assembly on the particle surface to form a coherent layer under the spray drying conditions they used, as the leucine peak size was considerable and a distinctive particle morphology was detected (Sou et al., 2014). Similar results were reported by Li et al. (2016) where addition of 10–20% leucine minimized moisture-induced deterioration in aerosol performance. As seen in Fig. 2, the $2\theta = 6^\circ$ peak appeared in all formulations of similar intensity, indicating the presence of leucine at the particle surface. However, the degree of particle merging appearing on the SEM images for F1 and F2 (Fig. 1) suggests that 20% leucine was not enough in our study to protect the powder from moisture sorption. The disparity between this study and previous reports could be attributed to the presence of the trace amount SMB and sodium hydroxide used to adjust the pH of the feed solution. Further studies are underway to investigate the effects of leucine content on the stability and dispersibility of phage powders. The XRD traces between the fresh samples and powders stored under desiccation for 12 months are similar (Fig. 2).

3.1.4 Thermal stability—As compared to conventional DSC, mDSC measurements apply two simultaneous heating rates to the sample: a linear heating rate to provide information equivalent to a standard DSC, and a sinusoidal (modulated) heating rate to determine the fraction of the total heat flow rate that responds to a changing heating rate. With the additional differentiation between reversing and non-reversing heat flows, mDSC measurement can resolve complex transitions into specific components to improve the interpretation of results. The total heat flow, reversing heat flow (due to a glass transition), and non-reversing heat flow (due to solvent evaporation or molecular relaxation) of each formulation are depicted in Fig. 3. Substantial endothermic peaks were observed at $\sim 20 - 80^\circ\text{C}$ for F1 and F2, and this was likely to be due to evaporation of residual moisture in the

powder matrix, which left through the pinhole into the dry nitrogen-purged environment. As shown in the XRD profiles (Fig. 2), formulations containing trehalose (F1–F4) were partially crystalline. Therefore, a dry trehalose glass transition was expected in their mDSC traces. On the other hand, mannitol glass transition would occur at temperatures for which moisture is still present and thus not present a dry glass transition temperature. The formulation containing 80% trehalose and 20% leucine (F1) showed a (~dry) glass transition at ~ 118 °C. This glass transition was also apparent in F2. No glass transition was detected in F5, indicating it was fully crystalline. A large endothermic peak was noted at 130 – 170 °C for F2–F5, with the peak height increasing with increasing mannitol content. This could be attributed to the melting of mannitol.

The TGA curves are shown in Fig. 4. Solvent evaporation was noted for F1 and F2 from 40 – 80 °C, which was consistent with the mDSC results. The components in the powder formulations started decomposition around 250 °C. As the trehalose content increased, the ash content also increased. This could be due to the high boiling point of trehalose (675 °C).

3.1.5 Moisture sorption—Fig. 5 shows the DVS weight change profiles of the powders as the RH increased from 0 to 90% at 25 °C. The absorption profiles of formulations containing trehalose (F1–F4) clearly demonstrated the sorption characteristics of a crystallization event, namely the overall mass decreased at a given RH as the material transited from amorphous to crystalline structure and expelled excess water. The onset RH for the re-crystallisation process depended on the formulation compositions with the onset values increasing with the mannitol content, except that the formulation containing 20% mannitol (F2) had a lower onset RH than the formulation containing trehalose and leucine only (F1). The results show that the inclusion of a small amount of mannitol promoted re-crystallisation of trehalose at a lower RH. The moisture sorption profile for F5 increased monotonically with increasing RH, demonstrating the powder was crystalline, consistent with the XRD data. In general, the moisture sorption capacity increased with increasing trehalose content, except F2 had a larger mass gain than F1 for RH between 30 – 40% and 90%. From the results, RH below 20% (ideally below 5%) would be recommended for handling and storage of the phage powders.

3.2 Phage stability

The studied PEV2 phage can efficiently infect *P. aeruginosa* strains isolated from CF patients under aerobic, anaerobic and biofilm conditions (Ceyssens et al., 2010). Upon spray drying, all formulations containing 40% trehalose (F1–F3) had a ~1.3 log titer reduction (Fig. 6) relative to the liquid feedstock. As the trehalose content reduced to 20% (F4), a 2.4 log loss was noted. The titer reduction further increased to 5.1 log for the formulation containing no trehalose (F5, data not shown), suggesting that mannitol and leucine alone were not sufficient to preserve the phages in powder form. These results were not surprising as it has been well reported that trehalose could stabilise proteins (Chang and Pikal, 2009; Jain and Roy, 2009) and phages (Merabishvili et al., 2013) in the drying process better than mannitol. Although the mechanisms of stabilisation in solid state are still unclear, the high T_g of trehalose and its capability as a water substitute are believed to play important roles (Jain and Roy, 2009; Wang et al, 2006).

To assess the effects of RH on the phage stability upon storage, the spray dried powders were transferred into three loosely capped glass vials and stored in at various humidity conditions (0, 22 and 60 % RH) at 4 °C. The titer reduction was determined relative to the titer measured in the liquid feedstock for spray drying. After 1 month storage (light grey columns in Fig. 6), no significant differences ($p > 0.05$) were noted among all storage conditions for individual formulation. The phage stability was different depending on the formulation compositions. Phages embedded in formulations containing 60% trehalose (F1 and F2) were stable with no further titer loss. A further loss of 0.7 log in titer occurred in F3. Formulations containing less trehalose content (20%) failed to preserve phages in the powder form, with the formulation containing 20% trehalose showing a further ~1.4 log loss, and no viable phage was detected for the formulation containing mannitol and leucine only (data not shown). The results again confirmed that the trehalose is a better agent in protecting phages in the solid state than mannitol for long term storage.

Powders stored at RH of 0% and 22% were able to protect phages from degradation after 3 months with no further phage reduction in all formulations. On the other hand, the phage stability was formulation dependent at 60% RH. No viable phages were detected in F1 and F4, while the titer in F2 and F3 remained unchanged. Gross morphological examination of the powder under light microscopy showed either small (F2–F4) or large crystals (F1) formation after storage at 60% RH. In contrast, all particles remained spherical at 0 % and 22 % RH. At the 12 month assessment point, no significant difference in viability occurred for phages stored at 0% and 22% RH. The excellent phage stability is correlated with the similarity in the powder morphologies and crystallinity (Fig. 2) between the fresh and stored samples. However, no viable phages were detected in all formulations stored at 60% RH. Our DVS data showed that the spray dried powders recrystallized at RH > 30%, which would result in phage inactivation as reported by Vandenheuvel et al. (2014). However, exposure to high relative humidity in spray dried pharmaceuticals is usually short term, when dosage formulations are taken out of moisture protective packaging prior to usage. In this context, 1 month stability at 60 % RH is adequate. Furthermore, these phage stability results confirmed that PEV2 phages embedded in the proposed formulations could achieve long term storage stability when the powders were stored at RH 22%.

3.3 *In vitro* aerosol performance

The deposition profiles, total recovery and viable respirable phages (FPF < 5µm) of F1–F4 after dispersing with an Osmohaler™ at 100 L/min for 2.4 s of the 0, 3 and 12 month old samples are depicted in Fig. 7. A low phage recovery (20 – 53%) was obtained for all the powders (F1–F4). Similar results were found in our previous study (Leung et al., 2016). It was hypothesized that phage particles tended to reside at the surface of the particle matrices during the spray drying process, and they were inactivated upon impaction with the interior wall of the Osmohaler™ DPI upon dispersion (Leung et al., 2016).

The overall trend of the phage deposition profiles was similar for all formulations. A significant fraction of viable phages was deposited on the capsule or device, which resulted an emitted (post-device) fraction below 70% in most cases. Negligible amounts of phage were found in the adaptor and the filter stage of the impactor. The majority of the emitted

phages were deposited on the S2, S3 and S4 stages of the impactor, giving reasonably good FPF (40 – 48%) for all fresh formulations.

The variation of the phage deposition profile of powders stored at 22% RH with time was found to be formulation dependent. The amount of viable phages deposited in the lower stages of the MSLI (S3, S4 and filter stage) were similar for F1, resulting in similar FPF values (~40%) at the three time points. While the deposition of viable phages on the capsule and device remained similar for F2 and F3, more phages were found on the upper stages (S1 and S2) and fewer phages were deposited on the lower stages (S3 for F2 and S4 for F3) of the MSLI. These resulted in a drop in the FPF after storage – the FPF of F2 reduced from 48% to 38% and 36% at three-month and twelve-month, respectively, and the FPF of F3 dropped from 44% to 32% and 38% at three-month and twelve-month, respectively. While the viability of phages retained in the capsule for F4 reduced from 31% to ~19% after storage, deposition of viable phages in the lower stages of the MSLI slightly increased, resulting in an augmentation of the phage FPF from 40% to ~48%, after storage.

The corresponding *in vitro* fine particle dose of phage for powders stored in 22 % RH is shown in Fig. 8. This was determined as the amount of viable phages embedded in particles smaller than aerodynamic diameter 5 μm i.e. the FPF after MSLI dispersion. Since there is not much difference observed in the phage viability in F1 – F3 after the powder production process (~1.3 log loss) and they have similar FPF values (~40%), the total lung dose of the three formulations before storage was comparable (F1 = $1.3 \pm 0.5 \times 10^5$ pfu, F2 = $1.3 \pm 0.2 \times 10^5$ pfu and F3 = $1.1 \pm 0.1 \times 10^5$ pfu). F1 had a slight increase in the total lung dose, where $1.6 \pm 0.3 \times 10^5$ pfu and $1.4 \pm 0.2 \times 10^5$ pfu were obtained at three and twelve months, respectively, due to the similar total recovery and FPF values. The total fine particle phage delivery of F2 doubled after three months ($2.7 \pm 0.2 \times 10^5$ pfu) due to the increased phage recovery (from 25% to 58%) despite the FPF dropping by 10%, but it decreased to $1.5 \pm 0.3 \times 10^5$ pfu at the twelve-month time point. The uncertainty of the plaque assay used to determine the phage titer is ± 0.5 log, which is quite likely to account for the increased viable phages obtained at the 6 month time point. As for F3, the drop in the titer FPF was counter balanced by the increase in phage recovery, resulting in no significant difference in the total lung dose at both 3- and 12-month time points ($\sim 1.1 \times 10^5$ pfu). Due to the low trehalose content, F4 had an inferior ability to preserve the embedded phages, resulting in a lower total fine particle dose (on the order of 10^4 pfu) compared with other three formulations (on the order of 10^5 pfu). Overall, powder matrices containing 40% trehalose provided good phage preservation and aerosol performances after storage at 22 % RH at 4 °C for 12 months.

A distinctive characteristic that differentiates phage therapy from conventional antimicrobial treatment is the ability of the phages to replicate *in situ* in the course of killing bacterial targets. This is likely to allow a wider dosing range to be used in phage treatment. Theoretically, only a low dose of phages may be required for them to self-amplify to reach the efficacious density (active treatment). However, a high enough initial phage number may be able to eradicate the bacteria in the first round of lysis without self-replication (passive treatment) (Payne and Jansen, 2003). Since inhaled phage therapy is in its infancy, there are no widely published data on the doses required for successful treatment. If the calculated

dose by Abedon (2011) is correct, i.e. a local concentration of about 10^8 phages per ml as the minimal effective phage density, then the phage titers of our powders (of the order of 10^5 pfu) are at the low end of effectiveness. Further studies to produce powders of higher titer by using phage stock of a higher titer, optimising the process parameters to reduce titer loss, and testing different excipient combinations are underway. Nonetheless, it will require preclinical pharmacokinetic (PK) and pharmaco-dynamic (PD) studies, and eventually, human clinical trials to establish the actual dose requirement for the PEV2 and other therapeutic phages.

4 Conclusions

The long term stability, in terms of phage viability and *in vitro* aerosol performance of spray dried inhalable powders stored at 0%, 22% and 60% at 4 °C were assessed. All powders were partially crystalline, with recrystallisation taking place at high humidity conditions (> 22%). No phage survived at the 60% RH condition after twelve months. Formulations containing high trehalose content (> 40%) suffered a ~1.3 log titer loss during the powder formation process and no further loss was noted upon storage up to twelve months. It was found that the addition of a large amount of mannitol (< 60%) had a destructive effect in the phage stability, although it improved the physical stability and dispersibility of the powders. All formulations were found to be dispersible after storage up to twelve months at 22% RH with FPF > 40%. The total fine particle delivery of phages for formulations containing 40% trehalose was of the order of 10^5 pfu. In summary, the proposed formulations preserved the PEV2 phages in the powder form and achieve reasonable aerosol performance during long-term storage period of twelve months at 4 °C. These powder formulations are easier to transport and administer compared with the liquid counterparts, making them more clinically viable method for delivery.

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Fig. 1. Morphologies of spray dried phage particles. F1: 80% trehalose and 20% leucine, F2: 60% trehalose, 20% mannitol and 20% leucine, F3: 40% trehalose, 40% mannitol and 20% leucine, F4: 20% trehalose, 60% mannitol and 20% leucine, and F5: 80% mannitol and 20% leucine. Scale bar is 5 µm.

Fig. 2.
XRD profiles of the spray dried formulations at 0 month (—) and after 12-month storage under desiccation (·····).

Fig. 3.
mDSC traces of the spray dried formulations.

Fig. 4.
TGA traces of the spray dried formulations.

Fig. 5.
Vapor sorption profiles for F1 – F5 under ramped RH from 0 to 90%.

Fig. 6.

Phage stability in F1 – F4 formulation after storage at 0%, 22% and 60% RH. Titer reductions at 0 months (Fresh), 1, 3 and 12 months are relative to the titer measured in the spray dryer liquid feedstock.'

Fig. 7.

The distribution profiles, total mass recovery and FPF of viable phage stored at 22% RH. Data presented as mean $\pm$ one standard deviation ($n = 3$). All formulations were dispersed at 100 L/min for 2.4 s using the Osmohaler™. The aerodynamic cutoff diameter of each stage is quoted in parentheses.

Fig. 8. Total fine particle dose (< 5.0 micrometers) of PEV2 phage from MSLI dispersion of powders stored up to 12 months in 0% RH. Data presented as mean $\pm$ one standard deviation ($n = 3$).

Table 1

Formulation compositions.

Formulation #	Contents % (w/w)		
	Trehalose	Mannitol	Leucine
F1	80	0	20
F2	60	20	20
F3	40	40	20
F4	20	60	20
F5	0	80	20

Table 2

Particle size distribution.

Formulation #	VMD (μm)	span
F1	1.82 ± 0.07	2.42 ± 0.03
F2	2.22 ± 0.14	2.32 ± 0.05
F3	1.83 ± 0.04	2.35 ± 0.04
F4	1.68 ± 0.06	2.41 ± 0.03
F5	1.56 ± 0.04	2.45 ± 0.01