European Journal of Pharmaceutics and Biopharmaceutics 121 (2017) 1–13

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Production of highly stable spray dried phage formulations for treatment of *Pseudomonas aeruginosa* lung infection

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ARTICLE INFO

Keywords:
Bacteriophage therapy
Biotherapeutics
Pulmonary infections
Powder aerosols
Antibiotic-resistant bacteria

ABSTRACT

The potential of bacteriophage therapy for the treatment of pulmonary infections caused by antibiotic-resistant bacteria has been well recognised. The purpose of this study was to investigate the effect of excipients on stabilisation and aerosolisation of spray dried powders of morphologically different phages – PEV podovirus and PEV myovirus. Seven anti-pseudomonal phages were screened against 90 clinical strains of bacterial hosts and three of the phages were selected for formulation study based on the host range. Design of experiments was utilised to assess the effect of different excipients on the stabilisation and aerosolisation of spray dried phages. Both podovirus and myovirus phages were stable in spray dried formulations containing trehalose or lactose and leucine as excipients with less than 1-$\log_{10}$ titre reduction during spray drying, with lactose providing superior phage protection over trehalose. Furthermore, the spray dried phage formulations dispersed in an Osmohaler at 85 L/min produced a high fine particle fraction of over 50%. The results showed that the phages in this study can form respirable dry powder phage formulations using the same excipient composition. Spray dried various types of lytic phages hold significant potential for the treatment of pulmonary infections.

1. Introduction

The emergence of multiple drug resistance (MDR) bacteria has become a critical problem in the treatment of respiratory infections. Cystic fibrosis patients suffer from chronic bacterial infections by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenza*, and *Burkholderia cepacia* complex [1]. Chronic lung infection, especially *P. aeruginosa* shortens the life span of cystic fibrosis patients [2]. Many of these bacteria are intrinsically resistant or have developed resistance to many currently used antibiotics [3,4]. Additionally, immunocompromised patients in hospital intensive care units are at risk of respiratory infection from MDR bacteria. Despite the efforts to develop new antibiotics to fight emerging antibiotic-resistant bacterial infection, drying pipeline of new antibiotics urges the need for an alternative therapy [5].

Bacteriophage (phage) therapy has been gaining renewed interests for its ability to eradicate MDR bacteria [6]. Phage therapy exploits the lytic life cycle of phages, which causes bacteriolysis followed by subsequent release of progenies. The released phage progenies then target nearby bacteria and the cycle is repeated. Potential advantages of phage therapy over conventional antibiotic treatment are due to the facts that phages are (i) naturally occurring antibacterials with low inherent toxicity, (ii) self-amplifying agents, and (iii) highly specific limiting unnecessary damage to non-targeted bacteria [7,8]. The activity of phages against MDR bacteria has been shown in *in vitro* studies [9,10] as well as their efficacy in animals [10–12] and humans [13–15]. A recent study used intranasal administration of phages to reduce the infective burden and inflammation in a *P. aeruginosa* lung infection model in mice [11]. In addition, bacterial load and inflammatory response reduction were observed with prophylactic treatment. Other *in vitro* studies have demonstrated the ability of phages to penetrate and disrupt bacterial biofilms [16], and the synergistic effect of using combinational therapy with antibiotics [17,18]. The potential use of phages for the treatment of infectious disease is being extensively discussed.

Over the past decade, great efforts have been put into delivering therapeutic dosage forms of phage for treatment of respiratory infections. Successful phage therapy requires phages to remain viable during

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http://dx.doi.org/10.1016/j.ejpb.2017.09.002
Received 10 July 2017; Received in revised form 5 September 2017; Accepted 6 September 2017
Available online 07 September 2017

the production and delivery in aerosolised form so that clinically significant dose can reach the lower airways. Nebulisation has been utilised by earlier studies in Europe for inhalation of liquid phage formulation [19]. More recent studies have confirmed that, depending on the types of nebuliser, phages can withstand the stress and remain viable during nebulisation process with high titre reaching the lower respiratory tract [20–22].

Powder formulations have the potential to provide easy storage, transport and administration with long shelf-life over liquid formulations. Several studies have shown processing liquid phage formulation into dry powders using lyophilisation [23], spray drying [24–26] and spray-freeze drying [25]. Vandenheuvel and colleagues [26] produced highly stable spray dried anti-Pseudomonal *Podoviridae* phage powders using trehalose as an excipient with less than 1 $\log_{10}$ unit reduction in phage titre. On the other hand, powders containing anti-*Staphylococcal* phage from *Myoviridae* family resulted in 2.5 $\log_{10}$ loss. This apparent difference in titre reduction seems to confirm that phage stabilisation is phage dependent. Matinkhoo et al. [24] reported that myovirus phages spray dried with trehalose, leucine and a surfactant resulted in less than 1 $\log_{10}$ unit titre reduction with superior aerosol performance. A more recent study by Leung et al. [25] compared the performance of spray drying with spray freeze drying in producing stable anti-Pseudomonal *Podoviridae* phage powders using trehalose, mannitol and leucine. Spray drying provided better phage protection over spray freeze drying; however, the three-excipient combination failed to give less than 1 $\log_{10}$ unit titre reduction which is generally considered as a desirable loss incurred during the inhalation process.

For the commercialisation of sprayed dried phage formulations for pulmonary infections, the physical stability of inhalable powders and biological viability of phages are the critical parameters. This study aimed to explore the effect of various excipients on stabilisation and aerosolisation of PEV podovirus and myovirus in spray dried powders, using comprehensive formulation screening, followed by further refinement of excipient composition and ratios within.

2. Materials and methods

2.1. Bacteriophages

Seven bacteriophages comprised of myoviruses and podoviruses, active against *P. aeruginosa*, were supplied by AmpliPhi Biosciences AU at a high titre of 10^{10} PFU/mL, stored in phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl), with pH adjusted to 7.2). These phages were originally isolated from the sewage treatment plant in Olympia (WA, USA) by the Kutter Lab (Evergreen Phage Lab) using *P. aeruginosa* dog-ear strain PAV237. This strain was used as a reference bacterial strain to assess phage titre.

2.2. Host range and efficiency of plating

In this study, spot test [27] was used to test each phage for host spectrum of activity against 90 clinical and MDR *P. aeruginosa* isolates collected in Australia. The top-agar plates with the targeted host lawn were prepared by mixing overnight culture of the target host strain (approximately 2×10^8 CFU) with 5 mL of 0.4% nutrient broth top agar and overlaying onto a 1.5% nutrient agar plate. Then, 10 µL of a phage stock solution (10^9 PFU/mL) were spotted on the top agar plate, left to dry for 20 min and incubated at 37 °C for 24 h. After incubation, the appearance of the lysis zone was examined for phage-susceptibility. Each phage was tested against each bacterial strain in duplicate. Phages that are active against the clinical isolates were assessed for efficiency of plating by calculating the ratio of the phage titres obtained with the test clinical isolates to those obtained with the host strain, PAV237. The phage titre was determined using plaque assay [25].

2.3. Design of experiment

The Taguchi experimental design method was adopted to identify the dominant excipients responsible for phage protection during spray drying. A total of 4 factors were considered for formulations containing trehalose or lactose (Supplementary Table 1) and 5 factors for those containing mannitol (Supplementary Table 2). All factors were evaluated at three levels – L_9 orthogonal array for trehalose and lactose, and L_{27} orthogonal array for mannitol. Taguchi's 'smaller-is-better' criterion (Eq. (1)) was utilised to optimise excipients ratios with least titre loss. For analysis of the results, Minitab® 16 software was used.

Minimise the performance characteristic ('smaller-is-better') equation:

$$(S/N)_i = -10 \cdot \log_{10}\left[\sum_{i=0}^{n} \frac{y_i^2}{\mathrm{n}}\right] \quad (1)$$

where S is signal, N is noise, y_i is the characteristic property and n is the number of experimental replicates.

2.4. Phage stability in liquid formulation

The stability of phages diluted in excipient solution was tested by adding 10 µL of phage lysate to 990 µL of trehalose, lactose, mannitol, leucine or plurnic F68 at two different concentrations, 50 mM and 0.5 M, except pluronic F68 which was tested at 5% and 15%. The concentrations were determined based on other studies on phage or protein stabilisation in dry powder formulations [23,24,26,28]. Phage viability was tested using plaque assay.

2.5. Spray drying

The liquid feed was composed of 30 mL of excipient solution in ultra-pure water and 0.3 mL of phage suspension (10^9 pfu/mL) with pH adjusted to 7.4. The phage viability in the feed solution was assessed prior to spray drying using plaque assay. The resulting phage-excipient suspension was spray dried using a Büchi 290 spray dryer coupled with a conventional two-fluid nozzle for atomisation. The suspension was fed at a constant feed rate 1.8 mL/min and an atomising airflow of 742 L/h with an aspiration rate of 35 m^3/h. The drying inlet air was heated to 60 °C and the outlet temperature ranged between 40 and 41 °C. Dried powder after passing through the cyclone was collected in a vial. A small amount of powder was resuspended in PBS to give a concentration of 50 mg/ml and the phage titre was assessed using plaque assay. Spray dried powders were stored over silica beads at 20 °C before use.

2.6. Formulation screening criteria

Fig. 1 shows the system we developed for screening the phage formulations (Fig. 1). Screening Criteria Level 1 involved powder analysis by optical microscopy and laser diffraction. Using optical microscopy, the morphology, approximate size and the solid state (amorphous or crystalline) of powders were assessed. Particle size distribution was measured using laser diffraction. Powders having particle size (D_{50}) greater than 5 µm were discarded from the list as they are less likely to be inhalable. Powders that passed Screening Criteria Level 1 proceeded to Level 2: plaque assay, followed by scanning electron microscopy (SEM). Formulations that met the target specification for titre loss (< 1.0 log) were imaged with SEM for more detailed morphology. Screening Criteria Level 3 involved aerosol performance testing. An Osmohaler™ and reduced Next Generation Impactor coupled with a validated weighing method were utilised as an initial screening tool, followed by full characterisation using a multi-stage liquid Impinger (MSLI). Target specification for fine particle fraction (FPF) is 40%.