European Journal of Pharmaceutics and Biopharmaceutics 127 (2018) 213–222

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Effect of storage temperature on the stability of spray dried bacteriophage powders

Sharon S.Y. Leung[a], Thaigarajan Parumasivam[a,b], An Nguyen[a], Thomas Gengenbach[c], Elizabeth A. Carter[d], Nicholas B. Carrigy[e], Hui Wang[e], Reinhard Vehring[e], Warren H. Finlay[e], Sandra Morales[f], Warwick J. Britton[g], Elizabeth Kutter[h], Hak-Kim Chan[a,*]

[a] Faculty of Pharmacy, University of Sydney, Sydney, NSW, Australia
[b] School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia
[c] CSIRO Manufacturing, Bayview Avenue, Clayton, Melbourne, Victoria, Australia
[d] Vibrational Spectroscopy Core Facility & The School of Chemistry, University of Sydney, Sydney, NSW, Australia
[e] Department of Mechanical Engineering, University of Alberta, Edmonton, Alberta, Canada
[f] AmpliPhi Biosciences AU, 7/27 Dale Street, Brookvale, Sydney, NSW, Australia
[g] Tuberculosis Research Program, Centenary Institute, and Sydney Medical School, University of Sydney, Sydney, NSW, Australia
[h] The Evergreen State College, Olympia, WA, USA

ARTICLE INFO

Keywords:
Phage
PEV2
PEV40
Pulmonary infections
Phage dry powder
Antibiotic resistance

ABSTRACT

This study aimed to assess the robustness of using a spray drying approach and formulation design in producing inhalable phage powders. Two types of *Pseudomonas* phages, PEV2 (*Podovirus*) and PEV40 (*Myovirus*) in two formulations containing different amounts of trehalose (70% and 60%) and leucine (30% and 40%) were studied. Most of the surface of the produced powders was found to be covered in crystalline leucine. The powders were stored at 4 °C and 20 °C under vacuum. The phage stability and *in vitro* aerosol performance of the phage powders were examined on the day of production and after 1, 3 and 12 months of storage. A minor titer loss during production was observed for both phages (0.2–0.8 $\log_{10}$ pfu/ml). The storage stability of the produced phage powders was found to be phage and formulation dependent. No further reduction in titer occurred for PEV2 powders stored at 4 °C across the study. The formulation containing 30% leucine maintained the viability of PEV2 at 20 °C, while the formulation containing 40% leucine gradually lost titer over time with a storage reduction of ~0.9 $\log_{10}$ pfu/ml measured after 12 months. In comparison, the PEV40 phage powders generally had a ~ 0.5 $\log_{10}$ pfu/ml loss upon storage regardless of temperature. When aerosolized, the total *in vitro* lung doses of PEV2 were of the order of 10^7 pfu, except the formulation containing 40% leucine stored at 20 °C which had a lower lung dose. The PEV40 powders also had lung doses of 10^6–10^7 pfu. The results demonstrate that spray dried *Myoviridae* and *Podoviridae* phage in a simple formulation of leucine and trehalose can be successfully stored for one year at 4 °C and 20 °C with vacuum packaging.

1. Introduction

Phage therapy has recently been demonstrated as a promising alternative to conventional antibiotics to treat and prevent pulmonary infections caused by multidrug resistance (MDR) bacterial strains [1–7]. Early research on pulmonary delivery of phage was confined to liquid sprays using intranasal instillation and nebulization as minimum formulation development was required [8]. Nebulization has been a popular choice because of its high efficiency in delivering phage to the lung at a low inhalation flow rate and minimum requirement in inhalation coordination [3,9]. Recently, increasing efforts have been devoted into developing respirable phage powder formulations for improved storage stability, easy transport and administration. Various dry powder formulation techniques, including freeze drying followed by deagglomeration in a mixer mill [10], spray drying [11–14] and spray freeze drying [13], have been reported to be capable of producing inhalable phage dry powders. Among them, the spray drying technique is favorable because it is a single-step method to produce fine phage powders with the potential of causing only moderate titer loss ($\leq$1 $\log_{10}$ pfu/ml) upon processing. Yet, there has been no report on the effect of temperature on long term storage of the spray dried phage powders. This study is the first to assess the storage stability, in terms of both the

* Corresponding author.
E-mail address: kim.chan@sydney.edu.au (H.-K. Chan).

https://doi.org/10.1016/j.ejpb.2018.02.033
Received 30 October 2017; Received in revised form 14 February 2018; Accepted 23 February 2018
Available online 24 February 2018
0939-6411/

phage viability and in vitro aerosol performance, of the produced phage powders at room temperature and under refrigeration.

As phage are essentially composed of a stable protein capsule enclosing genomic materials, current solid-state phage formulation strategies have largely adapted the knowledge obtained in the development of protein-based pharmaceuticals and viral vaccines [15,16]. While a 1–10 $\log_{10}$ pfu titer reduction was noted when phage was dried with buffer only [17,18], addition of sugars, such as sucrose [17,19], lactose [10,17] and trehalose, provided excellent protection for phages during the drying process and storage. It is believed that the high transition temperatures (Tg) of these sugars (60 °C for sucrose, 108 °C for lactose and 115 °C for trehalose) and/or their capability as a water substitute play important roles in stabilizing protein/phage in the solid-state [20]. Upon drying, the hydrogen bonding between water molecules and the phage protein coat could be replaced by bonding with the excipients to prevent protein unfolding and immobilize phages within the amorphous glassy matrix [20]. However, crystallization of trehalose kills phage [21]. Therefore, it is crucial for amorphous sugar components to remain well below the glass transition temperature during storage.

In addition to the sugar, L-leucine has been included as a second excipient to improve the powder dispersibility for pulmonary delivery [11,14]. L-leucine also has surfactant-like properties, in that it is surface active and crystallizes early due to low solubility, thus forming the outer shell of the particles, which could encapsulate the phage and other excipients. The *in vitro* aerosol performance of a trehalose-leucine formulation produced by Matinkhoo et al. [11] was not assessed. However, they showed formulations with a third excipient, either a surfactant or casein sodium salt could achieve an *in vitro* lung mass up to 82.7% of the loaded mass, with a lung dose of the order of 10^6 pfu/mg. These spray dried phage powders were stable with less than 0.15 titer loss under refrigeration for a period of three months. In our recent study [14], we showed that sugar (trehalose and/or mannitol) powder matrix containing 20% leucine could achieve reasonably good aerosol performance (~40% fine particle fraction based on the recovered phage) with satisfactory stability such that no further titer loss was noted after 12 months storage at RH ≤ 22% and 4 °C (≤1 $\log_{10}$ pfu/ml loss upon storage). Vandenheuvel et al. [21] also showed that phages embedded in trehalose powders were most stable when stored at 4 °C and 0% RH. A 54% RH storage condition caused powder crystallization and significant phage inactivation, with the impact being more pronounced for large phage virions (*Myovirus romulus*) than smaller one (*Podovirus* LUZ19). Thermal instability was also reported for the phage powders stored at 25 °C [21]. Since dry powder inhaler products are usually administrated at room temperature and cold-chain storage is limited in most developing countries, more information on the stability of phage powders at higher temperatures is important for future development of phage powder formulations.

Although leucine is a well-studied aerosolization enhancer, its capability of protecting spray dried powders against moisture was recently reported [22,23]. Li et al. [22] showed an addition of 10–20 wt% of leucine could minimize the moisture induced deterioration of the aerosol performance of spray dried disodium cromoglycate powders, which absorbed a significant amount of water without recrystallization under higher relative humidity (≥60%). In their follow-up work [23] on spray dried salbutamol sulfate, which recrystallized at high RH condition (≥60%), a higher mass fraction (≥40%) of leucine was required to eliminate the effect of moisture on the aerosolization performance. Based on a mechanistic model and experimental results, Feng et al. [24] suggested that a minimum leucine threshold must be exceeded to obtain crystalline leucine to form low-density and well-dispersing particles. For trehalose-leucine powders produced with a Büchi B-90 Nano Spray Dryer, a mass fraction ≥ 25% of leucine at a total feed concentration of 28.9 mg/mL was required to ensure 100% leucine crystallinity. Our previous studies [13,14] suggested that the amount of leucine (20%) may not be sufficient to form a crystalline shell to protect the amorphous trehalose. In the present study, we extended our work to investigate the effects of leucine content and storage temperature on the long term stability of spray dried phage powders. In addition, two types of *Pseudomonas* phages, PEV2 (*Podovirus*) and PEV40 (*Myovirus*), were studied to elucidate the robustness of the spray drying approach and formulation design in producing inhalable phage powders. The phage stability and *in vitro* aerosol performance of the powders were assessed after 0, 1, 3 and 12 months storage at 4 and 20 °C with vacuum packaging. This is the first successful study demonstrating the long-term room temperature stability of spray dried, inhalable phage powders with less than 1 $\log_{10}$ pfu/ml loss and no deterioration in the *in vitro* aerosol performance after one year.

2. Methods and materials

2.1. Materials

Two *Pseudomonas* lytic phages of different morphologies, a N4-type P*odovirus* (PEV2, 1.4×10^{11} pfu/ml stock titer) and a PB1-like *Myovirus* (PEV40, 2.2×10^{10} pfu/ml stock titer), were used. The phages were isolated from the sewage treatment plant in Olympia, WA, USA by students in the Evergreen State College Phage Laboratory. Phage stocks 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) were supplied via AmpliPhi Biosciences (AmpliPhi Biosciences AU, NSW Australia) and used without further purification. D-(+)-trehalose dihydrate and L-leucine (Sigma–Aldrich, NSW, Australia) were co-spray dried with phage to form a powder matrix to protect the phage particles. Adapting the mathematical model developed by Feng et al. [24] for a Büchi 290 spray dryer, it was estimated that a leucine content of ≥30% was required to form a fully crystalline leucine shell under the spray drying conditions used in our previous studies [13,14] to produce the phage powders. Table 1 shows the compositions of the four formulations prepared in the present study.

2.2. Powder preparation

The powder preparation was the same as that documented in our previous study [14]. Briefly, an aliquot of 500 µl of the phage stock was added to 50 ml excipient solution of trehalose and leucine at a total solid content of 20 mg/ml prior to spray drying. The mixtures were spray dried using a Büchi 290 spray dryer (Buchi Labortechnik AG, Flawil, Switzerland) using an open-loop setting at a drying gas flow rate of 35 m^3/h, atomizing air flow rate of 0.742 m^3/h, and inlet temperature of 60 °C with a liquid feed rate of 1.8 ml/min. The outlet temperature was between 40 and 45 °C. The produced powders were aliquoted into scintillation vials and packed inside a vacuum sealed bag using a Westinghouse vacuum food sealer inside a relative humidity controlled chamber (RH < 20%). The vacuum packed vials were then stored at 4 and 20 °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) (Zeiss Ultra Plus

Table 1
Formulation compositions.

Formulation #	Phage	Starting titer (pfu/ml)	Contents (w/w %)	
			Trehalose	Leucine
F1	PEV2	1.4×10^9	70	30
F2	PEV2	1.4×10^9	60	40
F3	PEV40	2.2×10^8	70	30
F4	PEV40	2.2×10^8	60	40