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Stabilization of bacteriophage during freeze drying

U. Puapermpoonsiri[a], S.J. Ford[b], C.F. van der Walle[a,*]

[a] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 27 Taylor St, Glasgow, Strathclyde G4 0NR, UK
[b] Cancer Research UK Formulation Unit, University of Strathclyde, 27 Taylor St, Glasgow, UK

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ABSTRACT

With preliminary clinical trials completed for the treatment of antibiotic resistant infections using bacteriophages, there is a need to develop pharmaceutically acceptable formulations. Lyophilization is an established technique for the storage of bacteriophage, but there is little consensus regarding drying cycles, additives and moisture content specific to phage. Here, the addition of sucrose or poly(ethylene glycol) 6000 yielded stable freeze-dried cakes only from high concentrations (0.5 M and 5%, respectively), with addition of bacteriophage otherwise causing collapse. Gelatin, which is added to storage media (a solution of salts), played no role in maintaining bacteriophage stability following lyophilization. A secondary drying cycle was most important for maintaining bacteriophage activity. The addition of high concentrations of PEG 6000 or sucrose generally caused a more rapid fall in bacteriophage stability, over the first 7–14 d, but thereafter residual activities for all phage formulations converged. There was no distinct change in the glass transition temperatures (T_g) measured for the formulations containing the same additive. Imaging of cakes containing fluorescently labeled bacteriophage did not show gross aggregation or phase separation of bacteriophage during lyophilization. However, the moisture content of the cake did correlate with lytic activity, irrespective of the formulation, with a 4–6% moisture content proving optimal. We propose that residual moisture is followed during lyophilization of bacteriophage from minimal concentrations of bulking agent.

1. Introduction

Bacteriophage therapies are known to be clinically useful and safe in both man and animals (Weber-Dabrowska et al., 2003; Bruttin and Brussow, 2005), with the first, successful Phase I/IIa clinical trial recently being reported—targeting *Pseudomonas aeruginosa* infections of the human ear (Wright et al., 2009). An understanding of the various potential routes of formulation of bacteriophage will become key to further progression of bacteriophage therapy, moving away from simple solution formulations for oral or topical administration, or inhalational therapy (Golshahi et al., 2008). Previously, we encapsulated bacteriophages within biodegradable poly(lactic-co-glycolic acid) microspheres, retaining the lytic activity of bacteriophage despite the emulsification process (Puapermpoonsiri et al., 2009). These microspheres were developed as dry powders, suitable for the pulmonary delivery of macromolecules (Rouse et al., 2007). However, the lytic activity of the encapsulated bacteriophages was short lived, up to 7 d at either 4 or 22 °C, and bacteriophage formulations with long-term stability, i.e. retaining lytic activity up to several months, are required. Such formulations may include freeze-dried powders of bacteriophage applicable to dry-powder inhalers or reconstitution for nebulization.

Exceptionally long-term storage of bacteriophage held with reference centers has been reported (Ackermann et al., 2004). Comparison of nine bacteriophage families stored in solution at 4 and −80 °C and in the freeze-dried state over 21 years, was made. Despite morphological loss and loss of activity, tailed phages were relatively stable at −80 °C for up to 10 years in storage, with an average decline in titer of 1 $\log_{10}$ per year. A greater decline was observed for phages which has been frozen and subsequently lyophilized, though this was over a 21-year period. Lyophilization of bacteriophages is therefore a useful route to formulation. However, the needs of a reference center (long-term stability) are very different to those for formulation of bacteriophage as an acceptable medicinal product. Notably, the additives used for stabilization of the freeze-dried bacteriophage have skimmed milk (Clark and Geary, 1973), peptone (Carne and Greaves, 1974), sodium glutamate with gelatin (Engel et al., 1974) and 50% glycerol (Ackermann et al., 2004); all of which are pharmaceutically unacceptable on account of being either immunogenic or of high viscosity upon reconstitution. Clearly, if a pharmaceutically acceptable, freeze-dried bacteriophage formulation is required then alternative stabilizers need to be investigated, particularly pay-

* Corresponding author. Tel.: +44 0141 548 5755; fax: +44 0141 552 2562.
E-mail address: chris.walle@strath.ac.uk (C.F. van der Walle).

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ing attention to the need for gelatin which is commonly added to bacteriophage storage media (Fortier and Moineau, 2009).

Lyophilization is an important step in many pharmaceutical formulations, including therapeutic proteins and vaccines, and much work has been published in the area (Carpenter et al., 1997). In addition to the key consideration of protein conformational and chemical stability, are practical considerations of the appearance of the lyophilized 'cake', its physical stability and rapid, complete dissolution upon reconstitution. Lyophilization of proteins and vaccines is generally considered to confer stabilization through the solid state, reducing molecular mobility, hydrolysis and microbial contamination; though there remain numerous routes of physical and chemical instability (Wang, 2000). Lyophilization of bacteriophages with the appropriate bulking agents and stabilizers is therefore of direct importance, as demonstrated by similar studies for viral gene delivery vectors and vaccines (Croyle et al., 2001; Amorij et al., 2008). Specific additives generally include trehalose, sucrose or polyethylene glycol (PEG), chosen as representatives of commonly used protein stabilizers. Accordingly, we describe the lyophilization of bacteriophages in the presence of PEG and sucrose as stabilizers generating pharmaceutically acceptable freeze-dried bacteriophage formulations. Two lyophilization protocols were investigated to compare the bioactivity and physical property of the formulations following only primary drying and following both primary and secondary drying. The formulations were characterized for shelf-life in terms of residual lytic activity of bacteriophage compared to fresh bacteriophage preparations, and also for their physical and thermal properties.

2. Methods and materials

2.1. Materials

Potassium chloride, polyethylene glycol MW 6000 (PEG 6000), sucrose, gelatin and fluorescein isothiocyanate (FITC), were purchased from Sigma–Aldrich Chemical Company (Dorset, UK). PEG 6000 was chosen for its previous proven utility in protein formulation (Wang, 2000). Water was purified to >17 MΩ cm. Sodium and potassium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate, analytical grade, were supplied by Fisher Scientific, UK. Tryptone, yeast extract, granulated agar, peptone, sodium chloride, and Tris–HCl were purchased from Melford Laboratories Ltd., UK. All other chemicals and solvents were purchased from either Sigma–Aldrich or Fisher Scientific at analytical grade or equivalent.

2.2. Bacterial and bacteriophage strains

Bacteriophage selective for *Staphylococcus aureus* (strains 9563 and 8588, NCIMB, respectively) was kindly provided from laboratory of Prof. Mattey, University of Strathclyde, UK. Mucoid *P. aeruginosa*, clinical isolate, strain 217 M, was kindly donated by Dr. Tyrone Pitt, Laboratory of Health Care Associated Infection, Health Protection Agency, Colindale, London, UK. The bacteriophage selective for this *P. aeruginosa* strain was isolated from Clyde river water by Fiona McColm, University of Strathclyde, UK.

2.3. Bacteriophage preparation and harvest

S. aureus and *P. aeruginosa* were grown in Luria Bertani (LB) broth (1% tryptone, 1% yeast extract, 0.5% NaCl) at 37 °C overnight and 0.3 ml of this culture was mixed with 0.45 ml of bacteriophage stock solution (10^9–10^{10} plaque forming unit (pfu) per ml). This mixture was incubated at 37 °C for 10–20 min and 200 μl added to 4 ml of partially cooled LB agar (LB broth containing 1.5% agar) which was poured onto a cooled LB agar plate and incubated at 37 °C overnight. The resultant bacterial lawn was inspected for the presence of plaques and 5 ml of 'storage medium' (1 M Tris–HCl, 0.1 M NaCl, 8 mM $MgSO_4$, 0.1 g/l gelatin, pH 7.5) was used to flood the plates, which were placed at 4 °C for 3–4 h with gentle swirling every 0.5 h. Storage medium containing bacteriophage was then decanted and extruded through a 0.22 μm sterile filter. The lytic activity of the bacteriophage in this solution was determined by plaque assay.

2.4. Plaque assay

A serial dilution of the bacteriophage was made and a 100 μl aliquot of each dilution added to an equal volume of overnight bacterial culture. Each mixture was added to 4 ml of partially cooled LB agar and poured onto an agar plate and kept at 37 °C for 12 h. A bacterial culture without bacteriophage and a bacterial culture with a known concentration of bacteriophage were prepared in the same manner as negative and positive controls. Following overnight incubation, the numbers of plaques was counted for each dilution and used to calculate the number of pfu.

2.5. Transmission electron microscopy (TEM)

Negative staining of bacteriophage was employed for TEM, with 10 μl of sample dropped onto the surface of a Formvar/carbon coated 300 mesh grid. The sample was allowed to settle for 30 s and excess sample was then drained away from the grid carefully. The sample was then stained with 10 μl methylamine vanadate negative stain (NanoVan®, Nanoprobes), with excess stain removed by wicking and the sample left to dry before imaging.

2.6. Fluorescence labeling

A 0.5 g excess of fluorescein isothiocyanate (FITC) was added to 1 ml of purified bacteriophage equilibrated in 46 mM $NaHCO_3$, pH 9, in 10 ml total volume, and agitated continuously for 2 h. Following agitation, the resulting suspension of bacteriophage was dialyzed in phosphate buffered saline (PBS) pH 7.4 in a dialysis bag having a MW cut-off of 12,400 Da (D9777, Sigma–Aldrich, UK). The dialysis buffer was exchanged every 4 h for 24 h to remove free FITC.

2.7. Lyophilization and stability

One milliliter of bacteriophage solution ($\sim 3.3 \times 10^8$ pfu/mL) composed of 700 μl of additives and 300 μl of bacteriophage stock solution was lyophilized in a 10 mL freeze-drying vial using an AdVantage benchtop freeze dryer (VirTis, US). The initial freezing steps involved cooling of the shelf holding the samples to 5 °C, held for 30 min, before further cooling to −5 °C at 1 °C/min for 30 min, and then to −30 °C at a rate of 1 °C/min, and maintained for 1 h. Primary drying was initiated at −30 °C over 1000 min, with a chamber pressure of 100 mTorr. Secondary drying involved subsequent heating of the shelf to 25 °C at 0.1 °C/min, maintained under vacuum for a further 6 h, with final equilibration of pressure and temperature. Following the completion of lyophilization, vials were immediately sealed air tight and stored in containers with silica gel at 4 °C. To test the lytic activity of bacteriophage, lyophilized bacteriophage selective for *S. aureus* and *P. aeruginosa* were stored in this manner for 2, 7, 14 and 30 d. Following storage at each point of time, the dried cake was reconstituted in 1 mL sterile water and lytic activity for bacteriophage was determined by plaque assay as above. For the purpose of comparison, negative control samples included storage medium, with and without gelatin and sucrose or PEG 6000. Experiments were repeated for three independently prepared batches.