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# Stabilization of bacteriophage during freeze drying

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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-

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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.

**Table 1**
Definition of the symbols describing lytic activity of bacteriophage by plaque assay.

| Symbol | – | –*P* | *P* (number) | +*P* | ++*P* |
|---|---|---|---|---|---|
| Definition | Negative control (no bacteria plated out) | Bacterial lawn, no plaques observed | Number of individual plaques counted on plate | Individual plaques too many to count (>400 per plate) | Confluent lysis (fragmented bacterial lawn) |

### 2.8. Confocal laser scanning microscopy (CLSM)

For each formulation, a drop of 40 μl of fluorescein-labeled bacteriophage on a glass coverslip was lyophilized as above and imaged on a Leica DM6000B microscope equipped for epifluorescence and TCSSP5 confocal laser scanning systems. The fluorescein conjugates were excited with an Argon laser line set to 488 nm and with an emission bandwidth of 521–616 nm which was tailored to provide the best image. Leica (HCX Plan Fluotar) 10× dry objectives were used and the pin-hole was set automatically for optimal performance. The images were converted with Volocity® software.

### 2.9. Particle size measurements

The hydrodynamic diameters of the bacteriophage following reconstitution of the freeze-dried cakes in distilled water were determined by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, UK). Values are given as the average of two measurements. It should be noted that the analysis of DLS assumes a perfect sphere, which is not the case for the tailed bacteriophages; nevertheless, the data presented were of a sufficient quality to allow determination of aggregation or dispersion.

### 2.10. Moisture content

From the stored samples following primary and secondary lyophilization cycles, the dried cakes were redispersed in 3 ml anhydrous methanol and the water content of each cake determined by Karl Fischer titration (Mettler Toledo DL 37, Leicester, UK). The apparatus was calibrated with Karl Fisher water standard (2-methoxy ethanol) and blanked with the addition of anhydrous methanol. The water content was determined for each formulation from three independently prepared samples.

### 2.11. Differential scanning calorimetry (DSC)

The glass transition ($T_g$) for the lyophilized samples was determined with a Mettler Toledo DSC822e calorimeter. Temperature-modulated DSC (TM-DSC) was used to determine the mid-point of the $T_g$, observed without superposition of the enthalpy relaxation and so circumventing the need to undergo a quench-cool step to remove thermal history for the material (Rouse et al., 2007). Specifically, the $T_g$ is represented by the reversing signal which describes a kinetic event, whereas the relaxation endotherm is represented by the non-reversing heat flow. Samples were prepared by carefully weighing ~3–5 mg of powder into a 40 μl aluminium pan, which was then hermetically sealed with a pin-hole in the lid. An empty pin-holed 40 μl aluminium pan was used as a reference. To determine the $T_g$ the pans were heated at a rate of 2 °C/min from 40 to 100 °C for sucrose samples, or from −55 to 0 °C for the PEG 6000 samples. The accuracy of the DSC sensor is 0.1 °C and measurements were performed in duplicate with the results analyzed using Mettler STAR software.

## 3. Results and discussion

### 3.1. Lytic activity of freeze-dried bacteriophage

In this study, to characterize the lytic activity and release profile of the freeze-dried bacteriophage formulations we adopted a semi-quantitative measurement, employing the symbols defined in Table 1. A semi-quantitative approach is required since lyophilization will result in either biologically active or inactive bacteriophage and while the plaque assay allows enumeration of the lytic activity of a sample of bacteriophages (in pfu) this can only be over a narrow range for practical purposes of counting the number of plaques for a particular dilution (Puapermpoonsiri et al., 2009). In the initial stability study (Table 2), bacteriophages were lyophilized from storage medium (SM) with or without gelatin, comparing the lytic activity against non-lyophilized bacteriophage, both liquid and freeze-dried bacteriophage being stored at 4 °C. Extending the primary drying cycle with a secondary drying cycle was seen to be beneficial for the stability of both bacteriophages; particularly phage active against *S. aureus*, which was seen to rapidly loose lytic activity if not further dried. The freeze-dried formulations here are clearly much more stable than for bacteriophage encapsulated in a polyester matrix, which lost lytic activity after 7 d (Puapermpoonsiri et al., 2009). For both bacteriophages, lytic activity was seen to begin to fall after 30 d, irrespective of whether gelatin was added into the storage medium or not.

A loss of stability over this period is consistent with other work showing that the half-life of bacteriophage λ at 20 °C was 20 d, although assessment of the stability of bacteriophage λ at 4 °C was not performed (Jepson and March, 2004). As noted by Ackermann, differing families of bacteriophage show distinct stabilities, with the 'tailed phages' seen to be most stable during long-term storage (Ackermann et al., 2004). Electron microscopy morphologically classified the NCIMB 9563 bacteriophage into the Siphoviridae family, with an isometrically hexagonal head, ~80 nm in diameter, and a long, noncontractile tail of ~200 nm in length, with a

**Table 2**
The lytic activity of bacteriophages lyophilized from storage media (SM)[a].

| Freeze-dry cycle | Phage selective for: | SM with gelatin | | | | SM without gelatin | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | d 2 | d 7 | d 14 | d 30 | d 2 | d 7 | d 14 | d 30 |
| Primary | *S. aureus* | +*P* | +*P* | *P* < 100 | *P* < 100 | ++*P* | ++*P* | +/++*P* | +*P* |
| | *P. aeruginosa* | ++*P* | ++*P* | +*P* | +*P* | ++*P* | ++*P* | +/++*P* | +*P* |
| Primary & Secondary | *S. aureus* | ++*P* | ++*P* | +/++*P* | +*P* | ++*P* | ++*P* | +/++*P* | +*P* |
| | *P. aeruginosa* | ++*P* | ++*P* | ++*P* | +*P* | ++*P* | ++*P* | ++*P* | +*P* |
| Control[b] | *S. aureus* | ++*P* | ++*P* | ++*P* | +*P* | ++*P* | ++*P* | ++*P* | +*P* |
| Control[b] | *P. aeruginosa* | ++*P* | ++*P* | ++*P* | +*P* | ++*P* | ++*P* | ++*P* | +*P* |

[a] Where variation in lytic activity for the three independent experiments was observed, this is shown as +/++*P* (high titers) or *P* < 100 (low titers).
[b] Bacteriophage in SM at 4 °C.

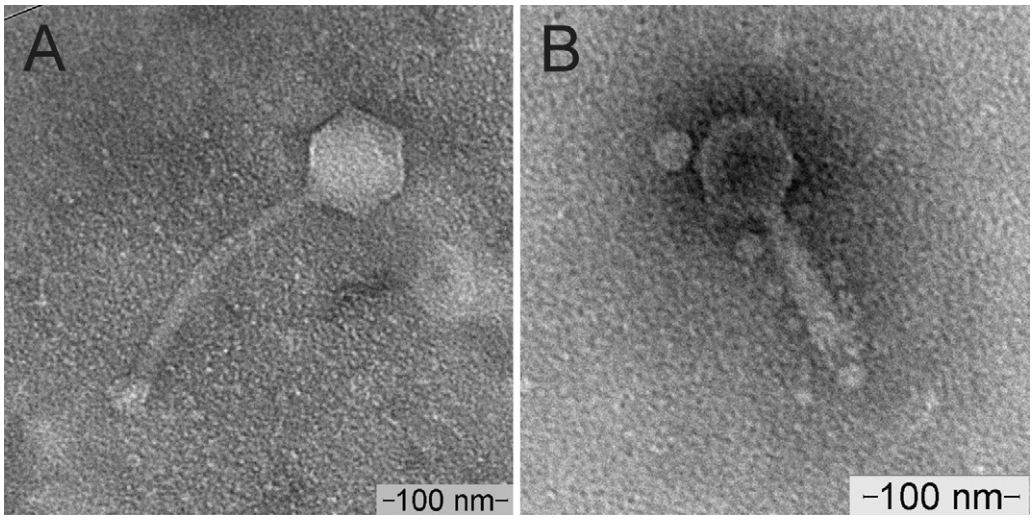


**Fig. 1.** Transmission electron micrograph of the two bacteriophages used in this study and selective against *S. aureus* (A) and a mucoid *P. aeruginosa* (B).

knoblike structure at its distal end (Fig. 1A). The phage isolated against *P. aeruginosa* strain 217 M was classified into the family Myoviridae, with a smaller icosahedra ~55 nm in diameter and a shorter, contractile tail of ~110 nm in length, consisting of a sheath and central tube (Fig. 1B) (Ackermann, 2005). It was interesting to note that the inclusion of gelatin did not appear to be beneficial in these lyophilization studies; rather than acting as a stabilizer to lyophilization, the inclusion of gelatin was slightly detrimental to the stability of the bacteriophage, particularly phage active against *S. aureus*. From previous lyophilization studies (Engel et al., 1974), the role of gelatin as a stabilizer is not entirely clear, and it is most likely that the inclusion of 5% sodium glutamate stabilized the freeze-dried bacteriophages. Therefore, there was no sound reason to include gelatin in the media immediately prior to lyophilization of the bacteriophages under study here, allowing the effect of sucrose and PEG 6000 to be studied in isolation.

The results for the lytic activities of bacteriophage lyophilized in the presence of PEG 6000 showed poor stabilizing activity. Indeed, the addition of PEG 6000 at both concentrations tested appeared to be detrimental, irrespective of the lyophilization cycle adopted; although we note that secondary drying yielded a relatively greater lytic activity (Table 3). Nevertheless, the final measurement of lytic activity at day 30 showed an activity similar to that observed at the same stage for freeze-dried bacteriophage in storage media alone. Thus, while PEG 6000 caused a greater loss of activity over the first 14 d, thereafter the relative stabilities of the various bacteriophage formulations appeared to converge. Data for the stabilizing effect of sucrose also showed, in general, a detrimental effect on bacteriophage lytic activity, though it was possible to retain good lytic activity through optimization of sucrose concentration and lyophilization cycle (Table 4). A high sucrose concentration of 0.5 M caused a decrease in the lytic activity of the reconstituted bacteriophage, relative to phage lyophilized from 0.1 M sucrose or storage media alone. Lower concentrations of sucrose maintained good lytic activity of the bacteriophage over the 30-day period, similar to activities seen in Table 2. Sucrose would therefore appear to be favored over PEG 6000 for the stabilization of bacteriophage during lyophilization.

The effect of different excipients may be explained by comparison of their structure and mechanism of stabilization. Lyo- and cryo-protection afforded by sucrose is thought to occur through its preferential exclusion from the protein/polypeptide surface (Kendrick et al., 1997), thermodynamically favoring the folded state (Lee and Timasheff, 1981), or via hydrogen bonding with polar

**Table 3**
The lytic activity of bacteriophages lyophilized using PEG 6000 as a stabilizer.

| Freeze-dry cycle | Phage selective for: | SM, no gelatin, 1% PEG | | | | SM, no gelatin, 5% PEG | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | d 2 | d 7 | d 14 | d 30 | d 2 | d 7 | d 14 | d 30 |
| Primary | *S. aureus* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* |
| | *P. aeruginosa* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* |
| Primary & Secondary | *S. aureus* | ++*P* | ++*P* | +*P* | +*P* | +*P* | +*P* | +*P* | *P* < 100[a] |
| | *P. aeruginosa* | ++*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* | +*P* |

[a] Variation in lytic activity between the three independent experiments is shown as in Table 2.

**Table 4**
The lytic activity of bacteriophages lyophilized using sucrose as a stabilizer.

| Freeze-dry cycle | Phage selective for: | SM, no gelatin, 0.1 M sucrose | | | | SM, no gelatin, 0.5 M sucrose | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | d 2 | d 7 | d 14 | d 30 | d 2 | d 7 | d 14 | d 30 |
| Primary | *S. aureus* | ++*P* | ++*P* | +*P* | nd | +/++*P*[a] | +*P* | +*P* | nd |
| | *P. aeruginosa* | ++*P* | ++*P* | +*P* | nd | +*P* | +*P* | +*P* | nd |
| Primary & Secondary | *S. aureus* | ++*P* | ++*P* | ++*P* | +*P* | +*P* | +*P* | +*P* | +*P* |
| | *P. aeruginosa* | ++*P* | ++*P* | +*P* | +*P* | ++*P* | +*P* | +*P* | +*P* |

[a] Variation in lytic activity between the three independent experiments is shown as in Table 2; nd = not determined.

groups on the protein surface (Allison et al., 1999). The level of stabilization afforded by sugars is usually dependent on their bulk concentration. The stabilization mechanism of PEG may also occur through preferential exclusion wherein the PEG molecules remain randomly coiled and excluded from the protein surface, which in turn in preferentially hydrated (Bhat and Timasheff, 1992). However, PEG also appears to undergo hydrophobic interactions with non-polar protein residues, in contrast to sucrose (Lee and Lee, 1981). This leads to destabilization of the system, phase separation and protein precipitation. The same underlying PEG-phage non-polar interactions may therefore have a similar destabilized effect, though this requires further investigation.

### 3.2. Physical characterization of freeze-dried bacteriophage/cakes

The appearance of the cake for bacteriophage lyophilized from storage media alone was poor, being entirely collapsed, consistent with the lack of bulking agent. The intention of adding a non-

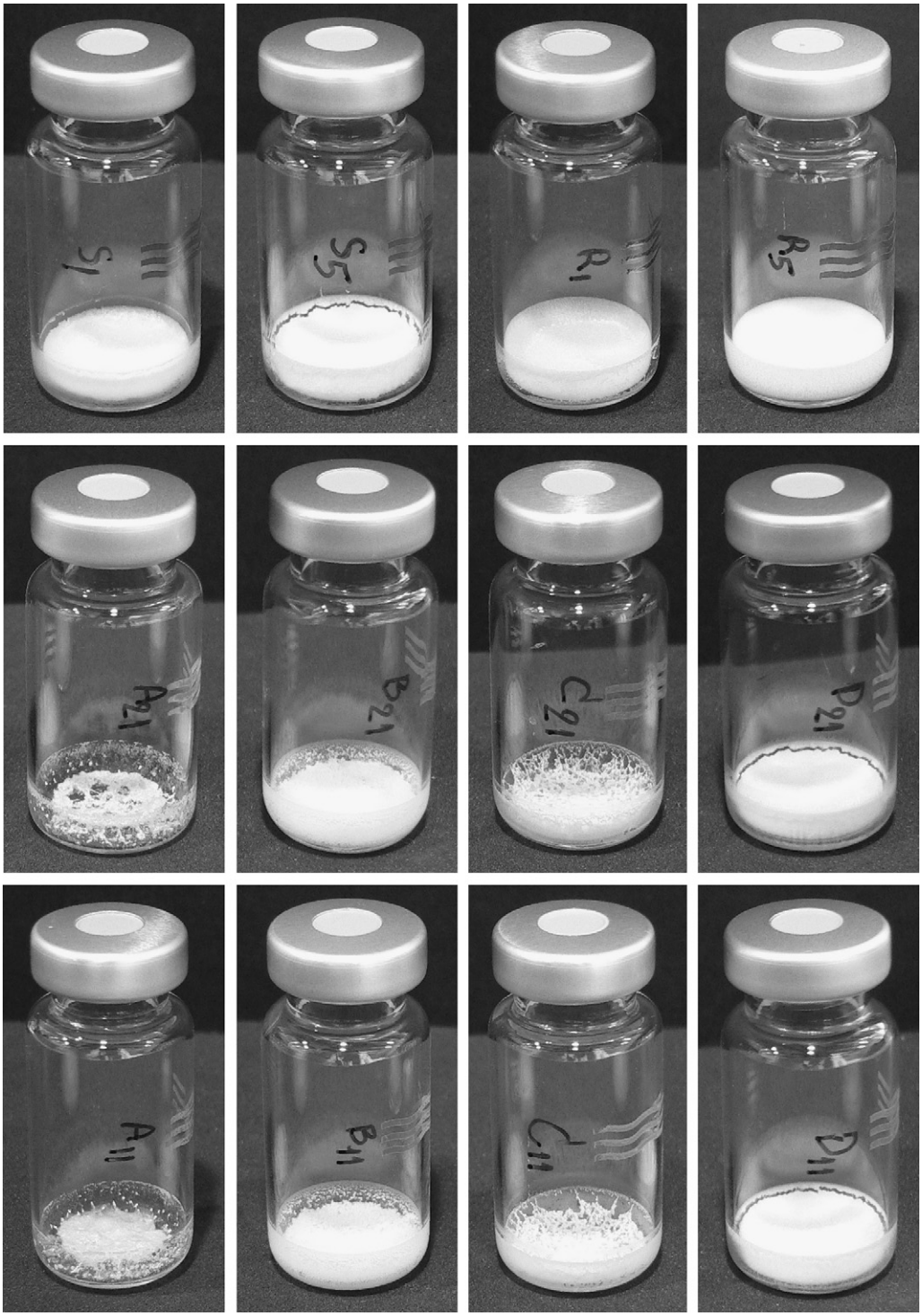


**Fig. 2.** Images of the freeze-dried cakes following primary and secondary drying cycles. Top row from left to right: 0.1 M sucrose, 0.5 M sucrose, 1% PEG, and 5% PEG. Middle row from left to right: additives as above but containing bacteriophage selective for *P. aeruginosa*. Bottom row from left to right: additives as above but containing bacteriophage selective for *S. aureus*.

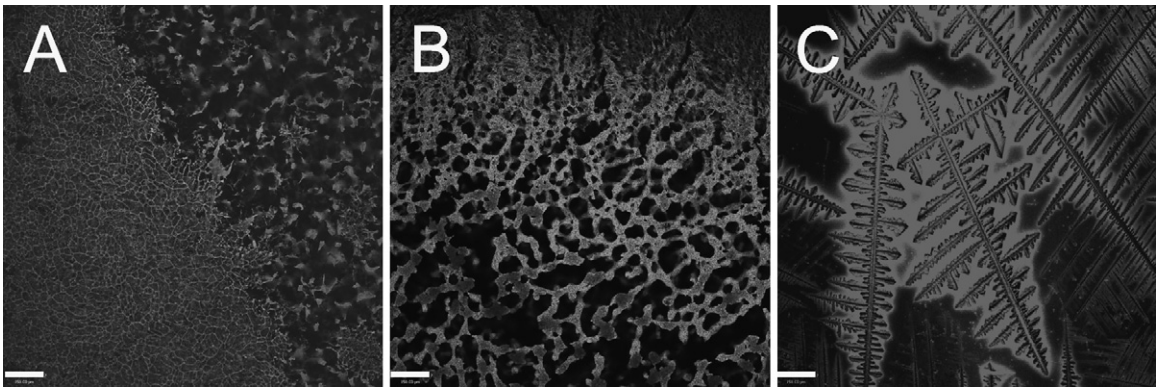


**Fig. 3.** CLSM images of freeze-dried thin films of 1% PEG (A), 5% PEG (B) and storage media (C) containing fluorescein-labeled bacteriophage selective for *P. aeruginosa*. Bar = 150 μm.

protein stabilizer was to produce a pharmaceutically acceptable, elegant freeze-dried cake for reconstitution. The cakes observed for bacteriophage lyophilized from 0.5 M sucrose and 5% PEG 6000 remained stable after pressure and temperature equilibration (Fig. 2). It was noted that the bacteriophage themselves caused collapse of the cake for lower concentrations of sucrose, and the same effect was seen for 1% PEG 6000. Therefore, high concentrations of PEG and sucrose were required to stabilize freeze-dried cakes containing bacteriophage. Similar behavior was seen for lyophilized protein formulations (Pereira et al., 2007).

The $T_g$ of the sucrose samples determined by TM-DSC was $72 \pm 1$ °C, typical of previous data for similar samples acquired by DSC (Saleki-Gerhardt and Zografi, 1994). Therefore, although the bacteriophage caused collapse of the lyophilized cake this was not through a plasticizing effect wherein the $T_g$ of the sucrose sample was markedly lowered. Similarly, the inclusion of bacteriophage did not alter the $T_g$ of the samples containing PEG 6000, which remained at $-25 \pm 1$ °C, close to that determined for lyophilized PEG 8000 (−16 °C) using DSC and thermally stimulated current spectrometry (Amin et al., 2004). In these amorphous samples, residual water would in any case be expected to lower the $T_g$, consistent with previous work (Rouse et al., 2007).

To investigate the possibility that loss of lytic activity of the bacteriophage was caused through aggregation or phase separation during lyophilization, we labeled the phage with fluorescein and imaged their distribution throughout freeze-dried thin films, for the same formulations. Thin films were required for imaging on the particular microscope stage used. Images are shown for labeled bacteriophage (selective for the *P. aeruginosa*) lyophilized from PEG 6000 and storage media alone, clearly showing their amorphous and crystalline nature, respectively (Fig. 3). Very similar images were acquired for labeled bacteriophage selective for *S. aureus*. Images for freeze-dried sucrose containing labeled bacteriophages showed thin, homogenous films, unrepresentative of the porous nature of the corresponding cakes in Fig. 2. Nevertheless, for all freeze-dried films, the bacteriophage was dispersed throughout the matrix suggesting that there was no phase separation or aggregation. Evidence of aggregation was tested further by particle sizing of the reconstituted bacteriophage using DLS. Although data quality was poor for samples containing PEG 6000 or sucrose (null data not shown), reliable data could be acquired for samples lyophilized from storage medium, with or without gelatin. In these cases, no evidence of aggregation of the bacteriophage could be found (Table 5), since the mean particle diameter measured corresponded to the approximate sizes of the bacteriophages seen by TEM (note that DLS assumes a sphere during data interpretation). No particle sizing data could be acquired for the blank solutions of storage media, suggesting that the presence of low concentrations of gelatin did not interfere with particle sizing of the bacteriophage.

Note that an initial supercooling step was included wherein the shelf (not sample) temperature was lowered to −5 °C prior to further cooling to −30 °C at a moderate rate of 1 °C/min, slightly above the $T_g'$ of sucrose (−32 °C). The intention of this step was to improve the homogeneity of ice crystallization. Previous work has shown that supercooling and ice nucleation temperatures, but not cooling rates between 0.05 and 1 °C/min, are linked to the rate of primary drying (Searles et al., 2001). The effect of this supercooling/ice nucleation step on phage adsorption to the ice/water interface and lytic activity needs to be determined. Ideally, the temperature during primary drying should remain below the $T_g'$ of sucrose but this also increases cycle time/cost. Collapse phenomenon can occur during primary drying above the experimentally determined collapse temperature, due to loss of porous structure of an amorphous sample, but the stability of a protein may not always be effected by the collapse of the cake (Schersch et al., 2009).

**Table 5**
Particle sizing data for bacteriophage following reconstitution of the freeze-dried cake.

| | Phage selective for: | Mean diameter (nm)[a] | Peak width (nm)[b] |
|---|---|---|---|
| Before lyophilization | | | |
| SM without gelatin | *S. aureus* | 134 | 48 |
| SM without gelatin | *P. aeruginosa* | 140 | 78 |
| SM with gelatin | *S. aureus* | 133 | 45 |
| SM with gelatin | *P. aeruginosa* | 131 | 62 |
| After lyophilization | | | |
| SM without gelatin | *S. aureus* | 202 | 149 |
| SM without gelatin | *P. aeruginosa* | 124 | 54 |
| SM with gelatin | *S. aureus* | 148 | 72 |
| SM with gelatin | *P. aeruginosa* | 126 | 38 |

[a] Measured using the intensity distribution plot, the % intensity for each peak was ≥96%.
[b] Equivalent to the standard deviation of the peak.

### 3.3. Residual moisture content of the freeze-dried formulations

An important parameter for lyophilized product development is the residual moisture content in the final product. There is no single, optimal residual moisture content for all biomolecules. Some proteins show increased chemical stability as moisture content decreases with little change in physical stability (Breen et al., 2001). In contrast, for other proteins, lyophilization resulting in a moisture content of the cake which is below 10% results in protein denaturation and loss of activity (Jiang and Nail, 1998). Clearly, when studying lyophilized biomolecules, including bacteriophage, a case-by-case assessment is required which aims to balance chemical stability (e.g. amino acid isomerization) with physical stability (e.g. unfolding). We attempted to control the final moisture content of the cake by simply extending the primary drying cycle, during which bulk water sublimes, with a secondary cycle performed at elevated temperatures to remove unfrozen water adsorbed to the product surface (Pikal et al., 1990). Given that the lytic activity for bacteriophage lyophilized from storage media only appeared to be dependent on the lyophilization protocol (Table 2), we investigated the residual moisture content of the corresponding freeze-dried cakes. Note that for bacteriophage lyophilized in the presence of PEG 6000 or sucrose, the moisture content was measured only following the secondary drying cycle, since lytic activity appeared to be more dependent on the additive concentration, in general (cf. Tables 3 and 4).

In Fig. 4A it can be seen that a secondary drying cycle consistently decreased the moisture content of the cake produced from storage media from *ca.* 7–9% to 5–6%. (The outlying sample at ~11% may be due to the relatively low cake mass generating larger deviations than may be expected, though this does not change the overall trend.) A clear correlation between moisture content and lytic activity can therefore be drawn. Thus, water content can be controlled by the process and robust control should be achievable on scale-up: keeping the residual moisture to a level $\leq$6% may represent a good starting point for formulation of freeze-dried bacteriophage, since other formulation variables such as particle aggregation and $T_g$ show no clear correlation with lytic activity. The usefulness of moisture content as an indicator of stability of freeze-dried bacteriophage is also supported when comparing formulations containing sucrose or PEG (Fig. 4B). Here however, we see that the very low moisture contents (*ca.* 2–3%) of the cakes containing high additive concentrations (5% PEG 6000 and 0.5 M sucrose) correlate with the lower lytic activities of the reconstituted bacteriophages (Tables 3 and 4). Therefore, reducing the moisture content below 4% is detrimental to bacteriophage stability, most likely due to the removal of non-frozen water adsorbed to the bacteriophage tail/head. Consistent with this argument, is that the moisture content of bacteriophage lyophilized from 1% PEG and 0.1 M sucrose was *ca.* 4–6% and correlated with the higher lytic activities observed for each additive. Comparing data for the moisture content of the cakes with the corresponding lytic activities of the bacteriophages highlights the upper and lower limits for a 'target' moisture content for optimal retention of bacteriophage activity, which is broadly in agreement with previous data for lyophilized proteins (Jiang and Nail, 1998; Breen et al., 2001).

It is interesting that higher concentrations of PEG or sucrose had lower residual moisture content of the final freeze-dried cakes following lyophilization. This must indicate that the drying process for the higher concentration additives was more efficient. Similarly, the residual water content of the cakes containing PEG alone (the controls) was much less than for PEG with bacteriophage. In both cases this can be explained by reference to the final appearance of the corresponding cakes (cf. Fig. 2). Clearly, in cases of collapse of the cake, that is, for addition of bacteriophage to 1% PEG and for 0.1 M sucrose, there will inevitably be a loss of porosity within the cake. Since efficient loss of non-frozen water during the secondary drying cycle is dependent on the maintenance of the porous structure of the cake, loss of porosity will result in higher residual moisture contents under the same lyophilization procedure. Therefore, optimization of freeze-dried bacteriophage products would ideally include monitoring of the residual moisture content *in situ*, to a final moisture content of 4–6%, in this case.

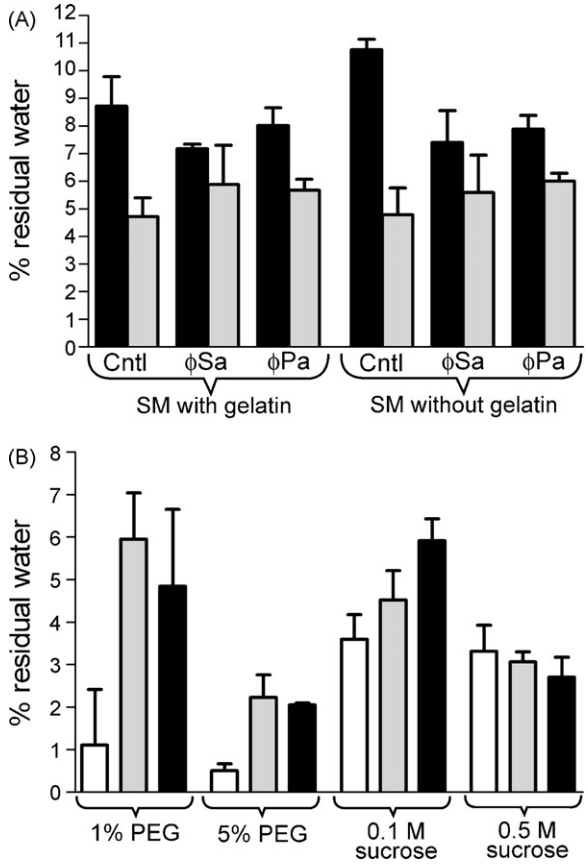


**Fig. 4.** (A) The moisture content of cakes lyophilized from storage media (SM) following only a primary drying cycle (black bars) or following primary and secondary drying cycles (grey bars) with and without gelatin and containing either bacteriophage selective for *P. aeruginosa* (ΦPa) or *S. aureus* (ΦSa), or control (Cntl). (B) The moisture content of cakes lyophilized from high/low sucrose or PEG 6000 concentrations, following primary and secondary drying cycles, and containing bacteriophage selective for *P. aeruginosa* (black bars) or *S. aureus* (grey bars), or neither (control, white bars).

## 4. Conclusion

Consistent with long-term storage studies, bacteriophages were stable to lyophilization in the presence of low concentrations of simple salts, without the need for sucrose stabilizers commonly added to solid state protein formulations. Obtaining elegant lyophilized cakes of bacteriophage which did not collapse required high concentrations of sucrose or PEG 6000. High concentrations of additive caused a more rapid inactivation of the bacteriophages compared to low concentrations of additive. However, complete attenuation of bacteriophage activity did not occur and after 30 d of storage reasonable lytic activities remained for all formulations tested. Changes in the activity of the lyophilized bacteriophages correlated with the moisture content of the cake; the interpretation being that a 4–6% moisture content is optimal for the bacteriophages studied here. Further studies on freeze-dried bacteriophage should focus on achieving non-collapsed cakes for as low an additive concentration as possible, possibly monitoring residual

moisture content and the collapse temperature during lyophilization *in situ*. These lyophilization studies will therefore underpin the development of novel formulations which include a drying step, for example, immobilization of phage into dry polymer/powders for inhalation. Similarly, lyophilization may be necessary prior to non-refrigerated transport, followed by reconstitution for oral administration, injection or nebulization, for example.

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