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Research paper

# A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres

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ABSTRACT

With the emergence of widespread antibiotic resistance, there has been renewed interest in the use of bacteriophages. While their potency, safety and specificity have underpinned their clinical potential, to date, little work has been focussed on their formulation with respect to controlled release and/or passive targeting. Here, we show that bacteriophages selective for *Staphylococcus aureus* or *Pseudomonas aeruginosa* can be encapsulated into biodegradable polyester microspheres via a modified w/o/w double emulsion-solvent extraction protocol with only a partial loss of lytic activity. Loss of lytic activity could be attributed to the exposure of the bacteriophages to the water-dichloromethane interface, with the lyophilization process itself having little effect. The microspheres were engineered to have an appropriate size and density to facilitate inhalation via a dry-powder inhaler and fluorescently labeled bacteriophages were distributed entirely within the internal porous matrix. The release profile showed a burst release phase (55–63% release within 30 min), followed by a sustained release till around 6 h, as appropriate for pulmonary delivery. Despite the poor shelf-life of the formulation, the work is proof-of-concept for the formulation and controlled delivery of bacteriophages, as suitable for the treatment of bacterial lung infections.



## 1. Introduction

The clinical benefit of bacteriophage therapy in man has been verified by more recent reports of clearance of antibiotic-resistant pathogens following oral dosing [1], and has begun to attract the attention of the wider research community [2]. Bacteriophages have also been reported to treat antibiotic-resistant ear infections in pet dogs, [3] and importantly, a Phase I/IIa clinical trial targeting *Pseudomonas aeruginosa* infections of the human ear was completed in November 2007 (Biocontrol Ltd., UK). The interest in bacteriophage therapy arises from the increasing problem of antibiotic resistance, particularly of the vancomycin-resistant Pseudomonal and Staphylococcal strains [4,5]. A good clinical example of this problem, and where bacteriophage may be of clinical potential, is the treatment of chronic lung infections seen in cystic fibrosis (CF) patients. Antibiotic therapy for CF patients is continually under review in response to emerging antibiotic resistance as a consequence of the high selection pressures on bacteria [6]. Chronic *P. aeruginosa* colonization also leads to the appearance of mucoid strains which grow as biofilms producing an alginate exopolysaccharide [7].

Clinical use of bacteriophage in animals and man commonly appears to involve simple liquid formulations for oral or topical administration [1,8]. Oral dosing has been shown to be safe in humans; resulting in the maintenance of the natural balance of the gut flora and the absence of phage-specific antibodies in serum samples [9]. However, a theoretical pharmacokinetic study has suggested that the timing of bacteriophage dosing for the treatment of a bacterial infection could be critical: with earlier inoculations being conversely less effective [10]. Given this, there is a strong argument for the development of robust controlled release delivery vehicles for bacteriophages. Furthermore, passive targeting with controlled release of bacteriophages would be beneficial in the treatment of localized bacterial infections; for example, bacteriophages have been encapsulated in biodegradable poly(ester amide) along with an antibiotic as a wound–healing preparation (PhagoBioDerm™, Intralytix Inc.), which is used clinically to treat topical infections of patients with antibiotic-resistant bacteria [11,12]. Other than this example, however, there would appear a good opportunity to develop further bacteriophage formulations in anticipation of their emerging clinical use.

In this study, we have focussed on biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microspheres which are approved delivery vehicles for human use and, through various synthetic routes, can be adapted for specific controlled release profiles alongside active or passive drug targeting [13]. Although the majority of commercialized PLGA microsphere formulations are for peptide hormones,

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there is a well-established research track record of their potential for the controlled release of proteins [14,15] and DNA vaccines [16,17]. While the initial 'burst' release of encapsulated protein from microspheres undermines the full potential for controlled release [18], this is conversely of benefit to pulmonary delivery where rapid drug deposition must occur due to muco-ciliary clearance and phagocytosis of PLGA particles [19]. The problem of conformational changes to DNA and proteins during emulsification [20,21] clearly demands that encapsulation of bacteriophages via the double emulsion–solvent evaporation technique must proceed with caution. We have previously shown that the choice of emulsifier influences protein encapsulation and microsphere morphology, [22] and have developed novel dimpled microcapsules suitable for pulmonary delivery of macromolecules [23]. One advantage of freeze-dried powders for lung inhalation is their simple application for dry-powder inhalers. Other potential routes for the pulmonary delivery of bacteriophages include nebulization [24]. In the present study, we have modified the double emulsion–solvent extraction protocol for the fabrication of dimpled microcapsules, in order to investigate the lytic activity, stability and release of bacteriophages selective for *Staphylococcus aureus* or *P. aeruginosa* following encapsulation and lyophilization.

## 2. Methods and materials

### 2.1. Materials

PLGA (50:50 Poly(DL-lactide:glycolide), inherent viscosity 1.05 dl/g, RG5010) was purchased from Purac Biochem, Netherlands. Poly(vinylalcohol) (PVA) (MW 25,000 88% hydrolyzed), gelatin and fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich Chemical Company (Dorset, UK). Water was purified to >17 MΩ–cm. Dichloromethane (DCM), analytical grade, was obtained from Fisher Scientific, UK. Pluronic-L92® PPO-PEO-PPO triblock copolymer (MW PPO:3000–3600) was received as a kind gift from BASF, USA. Tryptone, yeast extract, granulated agar, peptone, sodium chloride, and Tris–HCl were purchased from Melford laboratories Ltd. UK. All other chemicals were purchased from either Sigma–Aldrich or Fisher Scientific at analytical grade or equivalent.

### 2.2. Bacterial and bacteriophage strains

Bacteriophage selective for *S. aureus* (strains 9563 and 8588, NCIMB, respectively) was kindly provided from the 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 HealthCare 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

*Staphylococcus 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 units (pfu) per ml). This mixture was incubated at 37 °C for 10–20 min and 200 μl was 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 was 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 number of plaques was counted for each dilution and was used to calculate the number of pfu. For the calculation of lytic activity of the bacteriophage following encapsulation into PLGA microspheres, 100 mg of dried microspheres (immediately after lyophilization) was reconstituted with 1 ml of storage medium for 1 h with end-to-end rotation, the suspension was diluted 10-fold with storage medium and was tested by plaque assay as described above.

### 2.5. Bacteriophage concentration and purification

In brief, 0.5 g cesium chloride was added to each milliliter of harvested bacteriophage solution (6 ml total in this study) and was placed on a rocking platform until the CsCl was completely dissolved. A modified method of Sambrook et al. was followed [25]. Briefly, a step gradient was initially made by carefully pouring 2 ml CsCl solutions of decreasing density (1.7, 1.5, and 1.4 g/ml in that order) on top of one another in a thick-walled 38 ml polycarbonate centrifuge tube (Beckman–Coulter, UK). The 6 ml bacteriophage solution in $CsCl_{(aq)}$ (above) was then carefully poured on top of the step gradient and the interface of each CsCl density step was marked on the outer surface of the tube. Following centrifugation (64,000*g*) in a Beckman SW 28 rotor for 2 h at 4 °C, a bluish band appeared at the interface between CsCl densities of 1.4 and 1.5 g/mL. This layer was carefully collected by pipette and was used immediately for labeling or formulation.

### 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. Emulsification, lyophilization and fabrication of microspheres

A water-in-oil-in-water (w/o/w) double emulsion–solvent evaporation method was employed [23] to fabricate the bacteriophage-containing microspheres; with modifications including the addition of the bacteriophages during the secondary emulsion step to minimize their exposure to the organic solvent. Briefly, for each batch, the primary emulsion was prepared by adding 200 μl 1% w/v aqueous Pluronic L92 into 2 ml 5% w/v PLGA (total mass 100 mg) in DCM and by homogenizing for 15 s at 22,000 rpm using a Ultra Turrax IKA T18. This primary emulsion was added to 22 ml secondary aqueous phase composed of 1% PVA in storage medium containing $10^9$ pfu/ml bacteriophage and was homogenized for 3 min at 14,000 rpm. This w/o/w emulsion was immediately injected into 'hardening tank' composed of 200 ml 1% w/v aqueous

PVA and was stirred using an overhead stirrer (IKA RW11, 500 rpm). Stirring was continued for 4 h (per total volume of 2 ml DCM), and the microcapsules were then washed with 500 ml of storage medium. Fabrication was performed at room temperature. Following washing, the microsphere slurry was frozen in liquid nitrogen and lyophilized over 72 h using a MicroModulyo, ThermoSavant, UK (vacuum of 30 mTorr, condenser −40 °C, sample compartment 10 °C). Lyophilized microspheres were kept at 4 °C in a sealed container with silica gel. For the purpose of comparison, blank polymeric microcapsules were also fabricated. Experiments were repeated for four independently prepared batches.

For experiments investigating the effect of emulsification alone on the lytic activity of bacteriophage (selective for *S. aureus*), addition of bacteriophage ($10^9$ pfu/ml) to the aqueous phase of either the primary or secondary emulsions was performed as described above, but for the omission of PLGA from the oil phase and omission of the hardening tank step. Experiments were repeated for four independently prepared batches.

To test the effect of lyophilization alone on the lytic activity of bacteriophage (selective for *S. aureus*), 0.5 ml of bacteriophage solutions of $5 \times 10^9$, $5 \times 10^7$ and $5 \times 10^3$ pfu/ml was frozen and lyophilized as described above over periods of 24 and 96 h, and each dried powder was then reconstituted in storage medium for measurement of lytic activity by plaque assay.

### 2.8. Morphological characterization of microspheres

Non-lyophilized microspheres were routinely sized prior to freeze-drying by laser diffractometry using Mie scattering theory (Mastersizer 2000, Malvern Instruments Ltd., UK). The microsphere suspension was dispersed into the aqueous chamber till a laser obscurity between 10% and 20% was reached and the size was reported as the mode diameter. The median diameter was measured for three independent batches, by calculating the mean mode diameter and standard deviation.

The morphology of microspheres was examined by Scanning Electron Microscopy (SEM). Microspheres were mounted onto metal stubs using double-sided adhesive tape, vacuum coated with a thin layer of gold (100–150 Å), and the microcapsules were imaged using a Jeol JSM-6400 operating at 6 kV, 20 °C and $10^{-5}$ Torr.

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

Fluorescein-labeled bacteriophages were encapsulated as described above and the lyophilized microspheres were viewed with 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) 20× and 40× dry objectives were used and the pinhole was set automatically for optimal performance. The images were converted with Volocity software. The fluorescence was transient and photo-bleaching was rapid and no quantitative analysis of encapsulation was made.

### 2.10. In vitro release study

The release of encapsulated fluorescein-labeled bacteriophage was measured *in vitro*. Approximately 10 mg of lyophilized microspheres was accurately weighed and suspended in 500 μl PBS in Eppendorf tubes with a triplicate sample per batch of microspheres ($n = 3$ in each batch). The tubes were rotated end-to-end at room temperature for 2–3 weeks. At the predetermined time points (30, 60, 90, 120, 150, 180, 210, 240 min, then 5, 6, 7, 8, 10, 12, 14, 19, 24 h), Eppendorf tubes were centrifuged (12,000*g*, 5 min), and the supernatant was removed and clarified by filtration (0.45 μm Whatman VectaSpin Micro™). Bacteriophage yields were measured by fluorescence using an excitation wavelength of 495 nm and an emission wavelength of 525 nm (Varian Cary Eclipse fluorometer, Oxford, UK).

### 2.11. Stability study

Microspheres encapsulating bacteriophage selective for *S. aureus* were prepared as described above, and following the completion of the lyophilization cycle these were immediately stored in sealed containers with silica gel at 4 °C or room temperature (22 °C). After 1, 3 and 7 days, the dried microspheres were redispersed in 1 ml storage medium and lytic activity was determined by plaque assay as described above. Experiments were repeated for four independently prepared batches.

### 2.12. Cascade impaction

Briefly, three independently prepared batches were tested for each formulation using 30 hard gelatin capsules (type 3, Capsulgel, Belgium), each loaded with 30 ± 2 mg of dried PLGA microcapsules. A Multi-stage Liquid Impinger (MSLI) (Copley Scientific, UK) was assembled and used as described [13], with 20 ml of water dispersed onto each stage and the glass filter. The microsphere suspension from each stage was collected after completion of the 30 capsule tests and lyophilized in order to measure the weight fraction of dried microspheres collected at each stage and the glass filter. Calculation of geometric mean weight diameter ($d_g$) and the geometric standard deviation ($\sigma_g$) was done from the log-probability plot for cumulative % frequency undersize versus particle size, as described by Martin [26]. The density of lyophilized microspheres was calculated from the volume occupied by a known mass of microspheres loaded into a measuring cylinder and tapped 10,000× (Tap Density Volumeter, Copley Scientific, UK).

## 3. Results and discussion

### 3.1. Measurement of the activity of the formulated bacteriophages

It is important to note that the formulation of bacteriophage via emulsification, lyophilization or encapsulation into microspheres will result in either biologically active or inactive bacteriophage. The plaque assay that is described allows enumeration of the lytic activity of a sample of bacteriophages, with each plaque arising from a single infectious bacteriophage when co-incubated with appropriately viable bacterial cells. However, the plaque assay will only yield quantitative data for lytic activity over a narrow range wherein the number of plaques for a particular dilution of a bacteriophage sample can feasibly be counted. Making further dilutions of a bacteriophage sample to bring the number of plaques into a countable range following the initial overnight co-incubation carries uncertainty due to the drop in bacteriophage activity over time. Thus, the approaches to measure and interpret the efficiency of bacteriophage encapsulation and the rate of release are inherently more complicated when compared to the well-established spectroscopic assays that can be employed for protein- or DNA-loaded microspheres (e.g. [20,21]). Measurement of the total encapsulated bacteriophage via dissolution of the microspheres followed by a protein- or DNA-based assay will not distinguish between active and inactive populations. In this study, two approaches were adopted to characterize the bioactivity and release profile of the bacteriophage-loaded microspheres: (1) semi-quantitative measurement of the lytic activity of the bacteriophages released within a 1 h period from the microspheres, and (2) quantitative measurement of fluorescently labeled bacteriophages

released as a percentage of the total encapsulated bacteriophages, making no distinction between active and inactive populations. As regards the semi-quantitative measurement of the lytic activity of the bacteriophages in this study, the symbols used in Tables 2–5 are first defined in Table 1.

### 3.2. Effect of lyophilization and the emulsion type on bacteriophage activity

Our primary concern in encapsulating the bacteriophages using the double emulsion protocol was to minimize exposure of the protein coat of the bacteriophages to the oil–water interface. This is because the dichloromethane–water interface is well known to be strongly denaturing to proteins [27]. Several approaches have been used to address this problem such as the type of surfactant or emulsifier used [22]; solid protein dispersions versus liquid emulsions [28]; and buffering of the mildly acidic interior of the polyester matrix [29]. However, given that the objective in this study was to fabricate microspheres suitable for inhalation [23], the approach adopted was to investigate the effect of the addition of the bacteriophages during either the primary or secondary emulsion steps, a two-step secondary emulsion was used in the latter.

As shown in Table 2, the method wherein bacteriophages selective for *S. aureus* were added to the external aqueous phase resulted in the maintenance of a greater lytic activity when compared to the method wherein bacteriophages were added to the internal aqueous phase. Since the primary emulsion is a water-in-oil system wherein the bacteriophages are concentrated within the small aqueous fraction, it is reasonable to assume a higher degree of exposure to the solvent interface. In contrast, the secondary emulsion is an oil-in-water system wherein the bacteriophages are more dispersed throughout the larger aqueous phase and less likely to come into contact with the solvent interface. However, the encapsulation efficiency will also decrease with increasing dispersion of the bacteriophages in the secondary aqueous phase. Therefore, a two-step secondary emulsion was adopted wherein a balance between bacteriophage dispersion and entrapment was maintained. The results are consistent with the work reporting that the efficacy of bacterial infection by bacteriophage was attenuated in proportion to the concentration of the water-miscible organic solvent within a given system [30]. Thus, our results show that the lytic activity of the bacteriophage can be maintained during encapsulation into PLGA microspheres by simple modification of the double emulsion-solvent evaporation protocol to minimize the exposure at the solvent interface.

Since the fabrication of PLGA microspheres involves freeze-drying, before investigating the effect of the encapsulation process on the lytic activity of the bacteriophages, we first determined the effect of lyophilization alone. The integrity of the protein coat of the bacteriophage will presumably be challenged by the lyophilization process, as is the case for many other proteins (e.g. [31,32]). For bacteriophage selective for *S. aureus*, a range of concentrations in 0.5 ml storage medium were lyophilized as described in the methods over periods of 24 and 96 h. Reconstitution of the dried powder with water and measurement of lytic activity by plaque assay showed good retention of lytic activity of the bacteriophages for both lyophilization periods (Table 3). As commonly observed, the number of plaques decreased for decreasing concentrations of bacteriophage. Therefore, these results demonstrate that the lyophilization step following microsphere fabrication does not significantly affect the lytic activity of the bacteriophages used in this study.

**Table 2**
Effect of emulsification type on lytic activity of bacteriophage.

| Emulsion formulation | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
|---|---|---|---|---|
| Bacteriophage added to internal aqueous phase | P(7) | P(56) | P(45) | −P |
| Bacteriophage added to external aqueous phase | P(360) | +P | ++P | +P |
| Non-emulsified bacteriophage solution (control) | ++P | ++P | ++P | ++P |

**Table 3**
Effect of lyophilization on lytic activity of bacteriophage.

| Time of lyophilization (hours) | Bacteriophage concentration (pfu/ml) | | |
|---|---|---|---|
| | $5 \times 10^9$ | $5 \times 10^7$ | $5 \times 10^3$ |
| 24 | ++P | +P | P(6) |
| 96 | ++P | +P | −P |

### 3.3. Lytic activity and stability of encapsulated bacteriophage

Before encapsulating the bacteriophages, we first considered what a suitable 'dose' of (formulated) bacteriophages would approximate initially albeit with reference against one animal model of bacterial infection. Capparelli et al. reported that $10^9$ pfu of bacteriophages ($M^{Sa}$) could rescue mice infected with *S. aureus* strain A170, for both lethal bacterial loading at $10^8$ cfu/mouse and non-lethal loading at $5 \times 10^6$ cfu/mouse [33]. In brief, all the mice treated with the highest bacteriophage dose of $10^9$ pfu/mouse were protected from death (5/5 mice), whereas 90–100% of the mice injected with $10^8$ pfu/mouse died within 4 days. Therefore, the number of bacteriophages that were considered a target for encapsulation into a given, single dose of microspheres (here, 30 mg microspheres tested by cascade impaction) was in the order of $10^9$ pfu; though we note that quantitative measurement of the encapsulation efficiency of active bacteriophage by plaque assay cannot be made, as explained in Section 3.1. For *in vivo* testing, the solid dose would obviously need to be adjusted as appropriate to the species. Similarly, the reproductive capacity and pharmacokinetics of the bacteriophage, and the use of more that one bacteriophage strain would need to be considered [10].

Following initial experiments investigating the effect of emulsification and lyophilization on the lytic activity of bacteriophages, the fabrication protocol described above was used to encapsulate bacteriophages selective for *S. aureus* and *P. aeruginosa*. As shown in Table 4, bacteriophages retained lytic activity following encapsulation and release but those selective for *P. aeruginosa* appeared to show a greater loss of lytic activity than those selective for *S. aureus*. However, while the bacteriophages may indeed have differing susceptibility to the encapsulation process, direct comparison is complicated by the differing phenotype of the bacteria themselves. In contrast to *S. aureus* strain 8588, *P. aeruginosa* strain

**Table 1**
Definition of the symbols used to describe the 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) |

**Table 4**
Lytic activity of bacteriophage following encapsulation and release.

| Formulation | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
|---|---|---|---|---|
| *P. aeruginosa* bacteriophage, microspheres | P(200) | +P | P(300) | P(300) |
| *P. aeruginosa* bacteriophage, solution (control) | ++P | ++P | ++P | ++P |
| *S. aureus* bacteriophage, microspheres | P(400) | +P | +P | ++P |
| *S. aureus* bacteriophage, solution (control) | ++P | ++P | ++P | ++P |
| Blank microspheres (negative control) | – | – | – | – |

217 M is a mucoid strain/phenotype. The exopolysaccharide produced (an alginate) by mucoid *P. aeruginosa* strains functions as a physical or electrostatic barrier, conferring resistance to antibiotics and phagocytes [7]. Digestion of the exopolysaccharide and a reduction in alginate viscosity have been shown to facilitate phage penetration of biofilms [34]. With regard to the results here, the difference in lytic activity observed therefore maybe in part due to exopolysaccharide secretion by the *P. aeruginosa* strain.

The stability of the bacteriophage in the lyophilized microspheres was followed over a period of 7 days, after which no further lytic activity was observed at either 4 or 22 °C (Table 5). The results are somewhat unexpected given the apparent stability following lyophilization of the phage alone (Table 3), and the previous work showing that the half-life of bacteriophage $\lambda$ at 20 °C was 20 days following lyophilization of a simple aqueous suspension [35]. However, it is interesting to note that the loss of stability did not appear to be strongly dependent upon the storage temperature, suggesting that the loss of integrity of the bacteriophages maybe due to a physical interaction at the surface of, or encapsulation within, the PLGA porous matrix.

### 3.4. Morphological characterization of the microspheres

Microspheres encapsulating phage selective for *S. aureus* or *P. aeruginosa* had mode diameters around 10 μm (Table 6). There was no statistical difference between the mean mode diameters ($P > 0.05$ using Friedman's analysis with Dunn's post test); therefore, microsphere size was independent of the bacteriophage encapsulated. For each batch of microspheres, the size-distribution profiles showed one major peak with a span between 1.14 and 1.22. SEM micrographs were obtained for at least three different batches of microspheres to ensure that the morphologies produced were consistent. The external morphology of microspheres encapsulating the bacteriophage was markedly different from that of the blank microspheres; the latter being consistent with the previous study showing that Pluronic L92 generates dimpled, non-porous surfaces [23] (Fig. 1A). Microspheres fabricated in storage medium and encapsulating bacteriophage showed smooth to slightly irregular surfaces with some external pores (Fig. 1B–D). Also, while a fraction of the blank microspheres showed complete internal collapse, indicating a hollow interior as observed previously, similar collapse of the microspheres harboring bacteriophages was not observed, suggesting an internal matrix. Thus, the microsphere morphology was altered by the modified fabrication protocol used to accommodate encapsulation of bacteriophage. The shift away from an entirely hollow interior indicates an increase in the stability of the double emulsion system immediately prior to microsphere hardening [36]. The use of storage medium as the aqueous phase, rather than water, would have imparted an osmotic pressure between the primary and secondary aqueous phases. The addition of salts to the secondary phase is known to increase the density of the internal matrix and the movement of water between the two phases [37], and would also explain the morphological features of the bacteriophage-loaded microspheres.

The low apparent density of the bacteriophage-loaded microspheres was in agreement with our previous data for dimpled microspheres [23], yielding a calculated aerodynamic diameter ($d_a = d.\sqrt{\rho}$ where $d$ is the physical diameter and $\rho$ is the apparent density) of between 3.30 and 3.83 μm for the blank and phage-loaded microspheres (Table 6). The geometric mean weight diameter ($d_g$) was determined by cascade impaction to be 6.60 μm for phage-loaded microspheres, nearly 2-fold greater than the corresponding value of $d_a$ and the value of $d_g$ for the blank microspheres. The higher value of $d_g$ for the phage-loaded microspheres was most likely due to the smooth surfaces of the microspheres, which would increase the surface area for particle–particle contact by van der Waal's interactions (cf. dimpled surfaces on the blank microspheres). With respect to delivering particles to the lung periphery via inhalation, aerosols are generally manufactured to meet an aerodynamic diameter of around 3–5 μm. Thus, the phage-loaded particles described here would ideally have a smaller span (and corresponding $\sigma_g$) and smaller value for $d_g$, but nevertheless a fraction would likely approach the lung periphery on inhalation. Further testing of the phage-loaded microspheres in animal models of lung infection would be required to more fully characterize their therapeutic activity.

### 3.5. Distribution of the bacteriophage in the microspheres and their release profile

In the SEM micrographs (Fig. 1), the bacteriophage-loaded microspheres did not appear to be entirely hollow as seen for the

**Table 5**
Lytic activity of the bacteriophage following storage of microsphere formulations.

| Storage time (days) | Storage at 4 °C | | | | Storage at room temp. (22 °C) | | | |
|---|---|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
| 1 | ++P | ++P | ++P | ++P | ++P | ++P | ++P | ++P |
| 3 | +P | ++P | +P | +P | +P | +P | +P | +P |
| 7 | −P | −P | −P | −P | −P | −P | −P | −P |

**Table 6**
Physical characteristics of the microspheres.

| Microsphere formulation | Median diameter ($d$) (μm) | Span | Apparent density ($\rho$) (g/cm$^3$) | Calculated aerodynamic diameter ($d_a$) (μm) | Geometric mean weight diameter ($d_g$) (μm) | Geometric standard deviation ($\sigma_g$) |
|---|---|---|---|---|---|---|
| *P. aeruginosa* | 9.4 ± 0.6 | 1.22 ± 0.02 | 0.12 ± 0.004 | 3.30 | nd | nd |
| *S. aureus* | 10.6 ± 0.3 | 1.19 ± 0.08 | 0.11 ± 0.003 | 3.57 | 6.6 | 1.5 |
| Blank | 10.9 ± 0.6 | 1.14 ± 0.12 | 0.12 ± 0.004 | 3.83 | 3.4 | 1.5 |

Data are expressed as the mean ± standard deviation for three independent batches; nd, not determined.

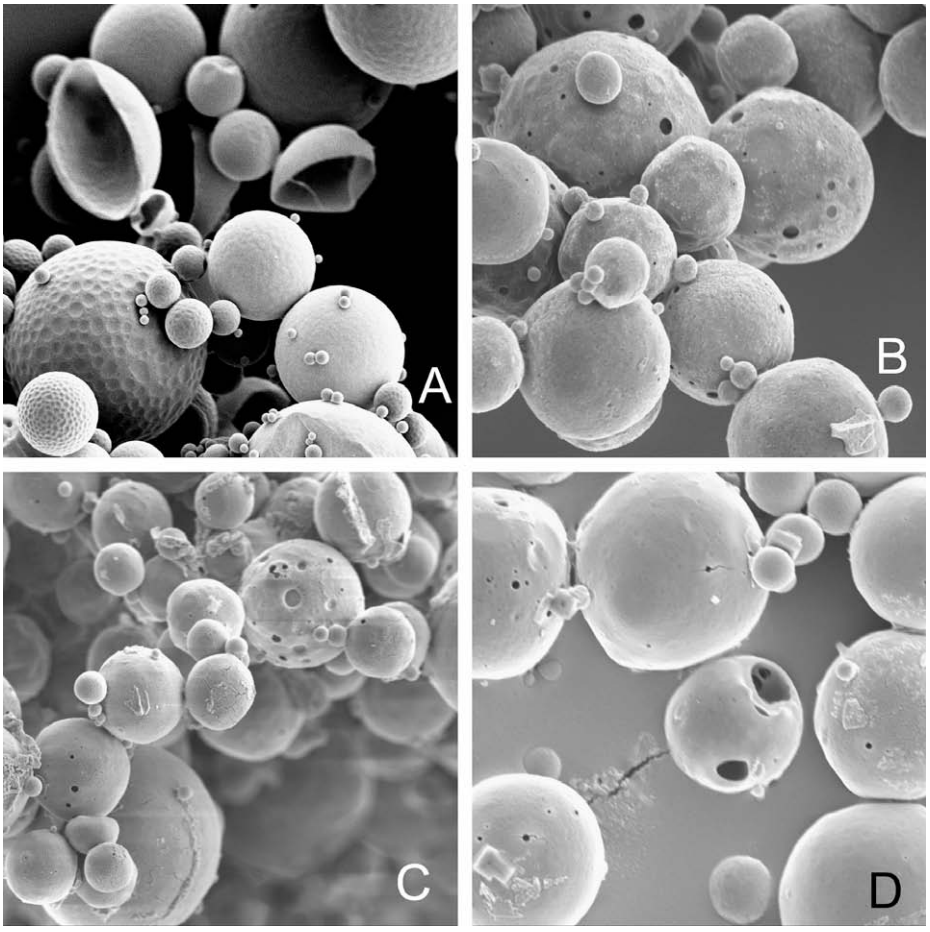


**Fig. 1.** Scanning electron micrographs of PLGA microspheres. (A) Prepared with 1% PVA as an aqueous phase (magnification 2000×); (B) prepared with storage medium as the aqueous phase (magnification 3000×); and (C and D), prepared with bacteriophage selective for *S. aureus* in storage medium as the aqueous phase (magnification 2000× and 3500×, respectively).

blank microspheres. In relation to the release of the bacteriophages, it was necessary to determine their initial distribution – either surface bound or homogenously distributed. Confocal photomicrographs showing an equatorial slice of the microspheres demonstrated that the bacteriophages were entirely encapsulated within the internal matrix, rather than simply being surface adsorbed (Fig. 2). The photomicrographs are also consistent with the interpretation of the SEM micrographs, showing that the interior of the bacteriophage-loaded microspheres was not hollow; the punctuate distribution seen at higher magnification photomicrographs (40×) may reflect the porosity of the internal matrix.

The release profile for encapsulated bacteriophages selective for *S. aureus* showed an initial burst release (Fig. 3): the percent cumulative release after 30 min was, as a fraction, 0.55 of the final percent cumulative release over 24 h. A similar burst release was observed for bacteriophages selective for *P. aeruginosa* (the equivalent fraction being 0.63). Following the burst release, for both bacteriophage formulations a slower sustained release phase was observed, approaching plateau after ∼6 h. The encapsulation efficiencies, inferred from the release profiles near plateau, were around 18% and 27% for bacteriophages selective for *S. aureus* and *P. aeruginosa*, respectively. While this would suggest that the majority of the bacteriophages were lost during encapsulation, the apparent losses observed here are consistent with the encapsulation efficiency of some proteins [22], though values for encapsulation efficiency are known to vary considerably depending on the emulsification protocol and the nature of the drug entrapped [28]. Since this is the first study for encapsulation of bacteriophage into PLGA microspheres, there are no known comparators. However, since bacteriophages that remain active naturally undergo self-replication following infection of a suitable bacterial host, it could be considered that a wider range of encapsulation efficiencies may be permissible as regards the clinical efficacy of a bacteriophage formulation.

The two-phase bacteriophage release profiles reflect those commonly observed for proteins with similar non-homogenous distributions within the internal matrix as observed by confocal photomicrographs [38]. The non-homogenous deposition may have facilitated the burst release of bacteriophages if water was freely able reach the localized areas of entrapped bacteriophage. This would suggest a release dominated by diffusion of the bacteriophage within a given mesoporous matrix. This interpretation is consistent with the previous work by Sandor et al. investigating the effect of protein MW (size) and protein loading on the subsequent release profile [39]; bacteriophages would be expected to behave in a manner similar to the larger proteins studied by Sandor et al., and therefore be dependent on pore interconnectivity and diffusion, rather than on degradation of the polyester matrix. However, assuming a simple diffusion-mediated release is likely to be too simplistic since the release of drug distributed heterogeneously within a PLGA microsphere matrix has been modeled to a percolation process dependent on swelling of the matrix and evolution of the pore structure [40].

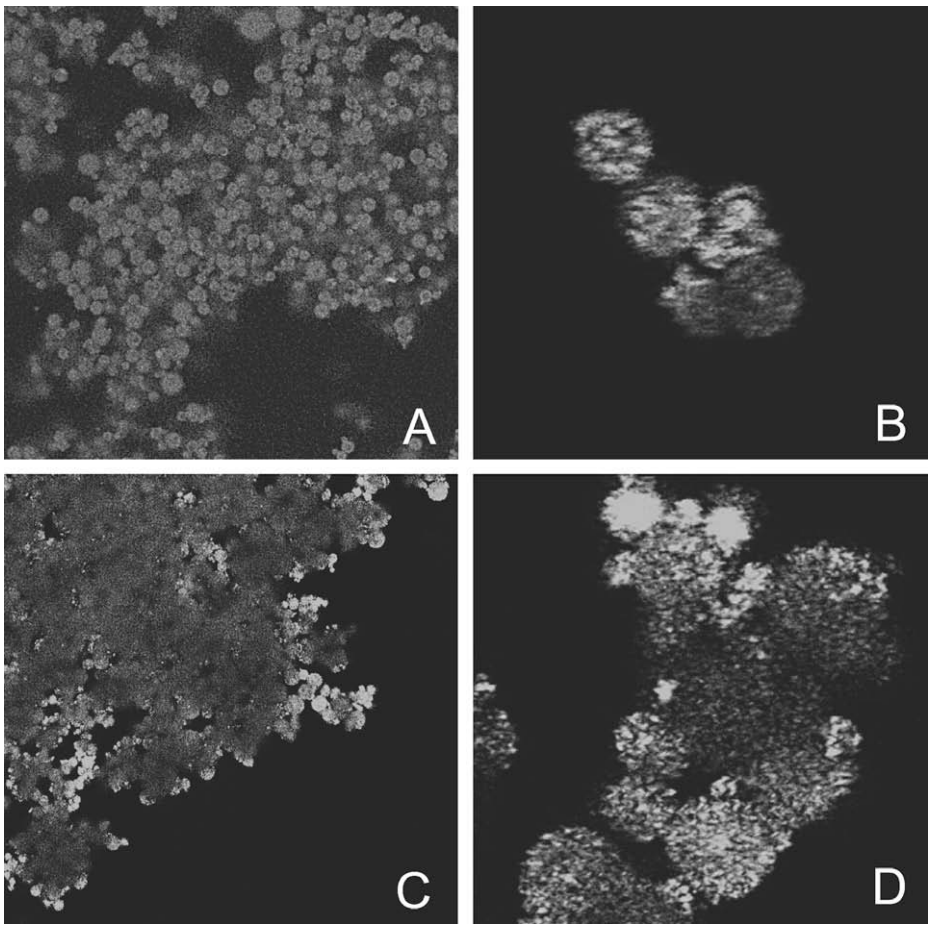


**Fig. 2.** Confocal photomicrographs of microspheres encapsulating fluorescein-labeled bacteriophages selective for *S. aureus* (A and B) and *P. aeruginosa* (C and D) at 20× (A and C) and 40× (B and D).

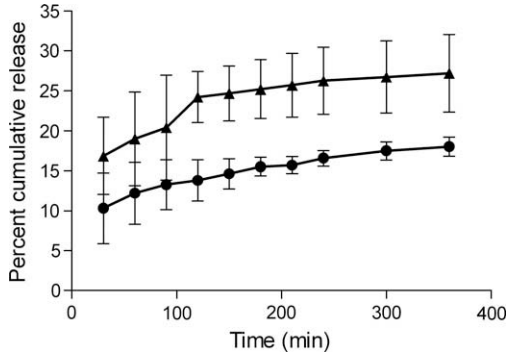


**Fig. 3.** *In vitro* release profiles of bacteriophage selective for *S. aureus* (circles) and *P. aeruginosa* (triangles), shown as the cumulative percentage of the total added during the encapsulation process.

## 4. Conclusions

We demonstrate that bacteriophages are quite resilient to encapsulation into biodegradable matrices via emulsification and freeze-drying. Optimization of the protocol, for example, substituting supercritical $CO_2$ for organic solvent [41], or the use of typical lyo- and cryo-protectants for proteins/DNA, may increase the shelf-life of the bacteriophage formulation. This work opens the way towards the passive targeting and controlled release of bacteriophages that may facilitate wider clinical applications for bacteriophage therapy.

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