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Lyophilized inserts for nasal administration harboring bacteriophage selective for *Staphylococcus aureus*: In vitro evaluation

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ABSTRACT

Nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) poses an infection risk and eradication during hospitalization is recommended. Bacteriophage therapy may be effective in this scenario but suitable nasal formulations have yet to be developed. Here we show that lyophilization of bacteriophages in 1 ml of a viscous solution of 1–2% (w/v) hydroxypropyl methylcellulose (HPMC) with/without the addition of 1% (w/v) mannitol, contained in Eppendorf tubes, yields nasal inserts composed of a highly porous leaflet-like matrix. Fluorescently labeled bacteriophage were observed to be homogenously distributed throughout the wafers of the dried matrix. The bacteriophage titer fell 10-fold following lyophilization to 10^8 pfu per insert, then falling a further 100- to 1000-fold over 6 to 12 months storage at 4 °C. This compares well with a total dose of 6×10^5 pfu in 0.2 ml liquid applied into the ear during a recent clinical trial in humans. The residual water content of the lyophilized inserts was reduced upon the addition of mannitol to HPMC, but this did not have any correlation to the lytic activity. Mannitol underwent a transition from its amorphous to crystalline state during exposure of the inserts to increasing relative humidities (as would be experienced in the nose), although this transition was suppressed by higher HPMC concentrations and the presence of buffer containing gelatin and bacteriophages. Our results therefore suggest that lyophilized inserts harboring bacteriophage selective for *S. aureus* may be a novel means for the eradication of MRSA resident in the nose.

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1. Introduction

Bacteriophages have long been known to be clinically effective and safe to use in animals and humans (Weber-Dabrowska et al., 2000). Their administration commonly involves only simple solutions for oral or topical administration (Soothill et al., 2004). For example, concurrent bacterial infection including septicemia in patients was reportedly treated after oral administration of bacteriophages three times a day (Weber-Dabrowska et al., 2001, 2003). Controlled release formulations of bacteriophages have included the development of microencapsulated bacteriophage Felix O1 for oral delivery using chitosan-alginate-$CaCl_2$ systems (Ma et al., 2008), and polyester microspheres as dry powders for pulmonary delivery (Puapermpoonsiri et al., 2009). Pulmonary delivery of a *Burkholderia capacia* complex (BCC) bacteriophage by nebulization has demonstrated that bacteriophages can successfully reduce bacterial lung infection (Golshahi et al., 2008). However, it is only recently that a controlled clinical trial has been reported, investigating the efficacy and safety of bacteriophages in ear drop preparations for the treatment of antibiotic resistant *Pseudomonas aeruginosa* in chronic otitis (Wright et al., 2009).

The current emergence and spread of profoundly antibiotic resistant pathogens is well documented. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major source of antibiotic resistant infections in hospitals and the community, though nasal decolonization offers an effective treatment strategy (Critchley, 2006). The nasal cavity is a common route for local and systemic drug delivery, including poorly bioavailable polar drugs such as peptides, since the 'first pass' hepatic metabolism is avoided (Illum, 2003). The nose is highly vascularized and the tight junctions of the nasal epithelium may be transiently loosened during dehydration to delivery vehicles (Arora et al., 2002). However, mucociliary transport is rapid, which decreases the residence time to short periods of less than 30 min (Mygind and Dahl, 1998). It is for this reason that oligosaccharide bioadhesive polymers are attractive (Henriksen et al., 1996; Soane et al., 1999), since their hydration at the mucosa

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promotes extensive hydrogen bonding and physical entanglement with mucin chains in the mucus layer (Mortazavi, 1995).

In this study, hydroxypropyl methylcellulose (HPMC) was selected because of its high viscosity on rehydration *in situ* which may explain why, in animal models, HPMC formulations have been observed to result in longer drug residence times in the nasal cavity (McInnes et al., 2005). HPMC nasal inserts lyophilized from HPMC solutions between 1 and 3% are tolerated in humans; with the 1 and 2% HPMC formulations showing good hydration and spreading properties and, for the 2% HPMC formulation, extending nasal residence times to over 4 h (McInnes et al., 2007b). It is hypothesized that, upon application of the HPMC nasal insert, water sorption from the nasal mucosa results in a higher HPMC gel concentration than that prepared prior to lyophilization; the highly viscous gel then attenuates mucociliary clearance.

Although lyophilization is one of several methods for long term bacteriophage storage (Ackermann et al., 2004; Fortier and Moineau, 2009), the process may cause protein denaturation (Ma et al., 2008). This is of concern given the complex nature of the head and tail structure of bacteriophages of the Siphoviridae family used in this study (Puapermpoonsiri et al., 2010). For this reason, mannitol was investigated as a stabilizer since it is a non hygroscopic material used extensively as an excipient (Rowe et al., 2009) and as a lyoprotectant during lyophilization of complex biopharmaceuticals including plasma (Bakaltcheva et al., 2007) and immunoglobulin-G (Schule et al., 2007). However, characterization of the amorphous/crystalline forms of mannitol following lyophilization is required since this can affect solid state stability (Torrado and Torrado, 2002). The present study evaluates the viability and stability of bacteriophages in lyophilized HPMC/mannitol formulations, characterizing the residual water, water sorption and solid state of the lyophilizates.

2. Materials and methods

2.1. Materials

Hydroxypropyl methylcellulose (HPMC) powder K4MP grade was obtained from Dow Chemicals, Michigan, IL, USA. D-mannitol powder was purchased from VWR International, Lutterworth, UK. Granulated agar, tryptone, yeast extract and sodium chloride were all purchased from Melford Laboratories, Ipswich, UK. Trizma base, magnesium sulphate heptahydrate, gelatin, fluorescein isothiocyanate (FITC) and methanol were purchased from Sigma–Aldrich, Dorset, UK. Dichloromethane (DCM) and methanol were purchased from Fisher Scientific, Leicestershire, UK. All chemicals were at analytical grade or equivalent.

2.2. Bacterial and bacteriophage strains

The bacterial strain used as a model for MRSA was *S. aureus* FDA209 P variant, acquired from the NCIMB, Aberdeen, UK (strain 8588; ATCC ref: 11522). The bacteriophage selective for this *S. aureus* strain is of the family Siphoviridae and was acquired from the NCIMB, Aberdeen, UK (cat. no. 9563; ATCC ref: 6538-B).

2.3. Culture conditions

S. aureus bacterial cultures were grown in Luria Bertani (LB) medium (1% tryptone, 1% yeast extract, 0.5% NaCl). Frozen stocks were initially streaked and grown on LB medium containing 1.5% agar, to select for individual colonies. Cells were cultured by inoculating 5 ml of LB medium with a single colony of *S. aureus* bacteria and incubating overnight at 37 °C with vigorous shaking. These overnight cultures were then used for bacteriophage preparation.

2.4. Bacteriophage preparation

A mixture of 300 μl bacterial culture and 450 μl bacteriophage stock solution (10^9–10^{10} plaque forming units per ml (pfu/ml) was incubated for 20 min at 37 °C, and 200 μl was then added to 4 ml of partially cooled LB agar (LB broth containing 1.5% agar). This partially cooled LB agar containing bacteriophage was poured onto a LB agar plate which was then incubated at 37 °C overnight. Bacteriophages were collected by adding 5 ml of storage medium (SM) buffer (1 M Tris–HCl; 2 g/l $MgSO_4 \cdot 7H_2O$; 0.1 M NaCl; 0.1 g/L gelatin, pH 7.5) to flood the plates which were kept at 4 °C for 3–4 h and swirled gently every 0.5 h. Bacteriophages were then collected by decanting the storage medium from the plates which then filtered by using 0.22 μm pore size filter (Millipore Ltd., Watford, UK), and stored at 4 °C.

2.5. Bacteriophage titration (plaque assay)

The lytic activity of the bacteriophage (the phage titer) was determined by plaque assay. To determine the phage titer, a bacteriophage stock solution was titrated by 10-fold serial dilutions in SM buffer and 100 μl of each dilution was mixed with 100 μl of overnight bacterial culture. The mixture was added to 4 ml of partially cooled LB agar and poured onto LB agar plates. Plates were then incubated overnight at 37 °C. The number of plaques was counted the following day and used to calculate the concentration of bacteriophage (pfu/ml). A negative control (bacterial culture without bacteriophage) and positive control (bacterial culture with a known concentration of bacteriophage) were also prepared for comparison. To determine the residual lytic activity of the lyophilized bacteriophage in the nasal inserts, each lyophilizate was reconstituted in 1 ml sterile water and the plaque assay was performed from the serial dilutions as above. Phage titers were calculated from the dilutions giving a countable number of plaques (<300) per plate, this was generally around a dilution of 10^6. Bacteriophage formulations before lyophilization were also tested for lytic activity, in order to evaluate the effect of lyophilization and storage on phage integrity.

2.6. Bacteriophage purification

Purified bacteriophages were prepared using a modified method (Sambrook and Russell, 2001). Briefly, 0.5 g caesium chloride (CsCl) was added to each ml of bacteriophage solution and allowed to dissolve. A step gradient was prepared by pouring 2 ml of CsCl solutions of decreasing density (1.7, 1.5, and 1.4 g/ml) on top of one another in a thick-walled 38 ml polycarbonate centrifugation tube (Beckman–Coulter, High Wycombe, UK). The aqueous solution of bacteriophage in CsCl was then carefully poured on top of these layers, and the interface between the layers was marked on the outside of the tube. After centrifugation at 22,000 rpm in a Beckman SW 28 rotor (64,000 × *g*) at 4 °C for 2 h a visible bluish band was formed at the interface between the CsCl solutions of 1.4 and 1.5 g/ml densities. This band was carefully collected using a syringe with small bore needle (21G) and stored at 4 °C to use for labeling or formulation. The lytic activity (titer) of the purified bacteriophage solution was tested by plaque assay as above.

2.7. Fluorescein labeling

Fluorescein isothiocyanate powder (FITC), 0.5 g, was added to 1 ml of purified bacteriophage solution and diluted in 10 ml of 46 mM $NaHCO_3$ (pH 9) and shaken gently for 2 h. The resulting suspension was centrifuged briefly and dialyzed extensively in phosphate buffered saline (PBS), pH 7.4, by 3× exchange over

24 h, using a cellulose dialysis bag with a MW cut-off of 12,400 Da (D9777, Sigma–Aldrich, UK).

2.8. Preparation of formulations

Control samples (labeled C) were prepared by dissolving the required amount of HPMC powder in one third of the final volume of sterile distilled water (at 80–90 °C), by slow addition of HPMC with stirring until a consistent dispersion was obtained. The remaining amount of sterile water was then added (using water at room temperature) and stirring was continued until a uniform gel of 1% and 2% (w/v) HPMC was obtained. The required mass of mannitol powder was also added, where appropriate, to produce a concentration of 1%. The gel was stored at 4 °C overnight to partially degas the gel and allow complete hydration of the polymer chains. Blank samples (labeled B) were prepared as above but using sterile storage media (SM) instead of sterile distilled water. Formulated samples (labeled F), for inserts harboring bacteriophages, were prepared as for the blank samples, but adding the required amount of stock phage solution to the aqueous HPMC dispersion with gentle stirring.

2.9. Lyophilization of formulations

Nasal inserts were prepared by lyophilizing 1 ml of formulation solution (Table 1) in a 1.5 ml Eppendorf tube using a VirTis AdVantage freeze-dryer (VirTis, USA). The freeze-drying protocol was carried out following a method previously devised for bacteriophage (Puapermpoonsiri et al., 2010): freezing was initiated by cooling to 5 °C for 30 min, followed by cooling to −5 °C at a rate of 1 °C/min, held for 30 min, and then to −30 °C at 1 °C/min, held for 1 h; primary drying was started at −30 °C with a chamber pressure of 100 mTorr for 1000 min, with secondary drying following heating to 25 °C at 1 °C/min, maintained for 6 h under vacuum. After lyophilization, the samples were collected and stored in a container with silica gel at 4 °C. The lytic activity of the bacteriophage from the lyophilizate was tested at day 1, 4, 7, 14, 30, 60, 180, 240 and 360.

To investigate the effect of the freezing and drying protocol on lyophilizate morphology, a simple rapid freeze/dry protocol was also used, involving freezing of gels to −80 °C over 30 min and subsequent drying at 10 °C for 24 h under vacuum (MicroModulyo, Thermo Scientific Ltd., UK).

2.10. Scanning electron microscopy (SEM)

Lyophilized samples were prepared by cutting a section of the sample with a sharp scalpel blade, fixed onto metal stubs using double-sided adhesive copper tape and coated with gold under vacuum. Samples were imaged using a Jeol JSM-6400 scanning electron microscope at 10 kV intensity, at the electron microscopy facility at the University of Glasgow, UK.

2.11. Confocal laser scanning microscopy (CLSM)

Lyophilizates for CLSM were prepared as above but using fluorescein labeled bacteriophage during formulation. A small section of the lyophilizate for each formulation was placed on a glass slide and covered with a cover slip. Samples were analyzed and imaged on a Leica DM 6000B microscope at the Centre for Biophotonics, University of Strathclyde, using an Argon laser line at 488 nm with emission bandwidth of 521–616 nm. Scans were performed using 10× and 20× objectives and images were converted with Volocity® software (Improvision, PerkinElmer, Cambridge, UK).

2.12. Residual water content

Gel samples of 1 g were formulated as above but lyophilized in 10 ml, tared glass vials. The mass of the dried cakes was obtained by difference and the vials were then stored in an air-tight box at 4 °C. For residual water determination, the dried cakes were reconstituted with 4 ml solvent mixture (dichloromethane and methanol 50:50). The mass of the solvent for each sample was obtained and a blank sample containing a known solvent mass was also prepared. The water content of each cake was determined by the Karl Fischer (KF) titration method (Mettler Toledo DL 37, Leicester, UK). After calibration with a Karl Fischer water content standard (Hydranal, Sigma–Aldrich, Gillingham, UK), the apparatus was used to determine the water content of the blank solvent mixture. The water content for each formulation was determined twice from four independent samples and the result of the titration was expressed as a percentage.

2.13. Dynamic vapor sorption (DVS)

The isothermal sorption behavior was studied using a DVS 1000, Surface Measurement Systems, Cheshire, UK, consisting of a highly sensitive Cahn microbalance to measure any changes in the mass of the sample as a result of sorption or desorption of water vapor. The method of McInnes et al. (2007a) was followed. Briefly, samples were subjected to a controlled cycle of relative humidity (RH) ranging from 0 to 95% in stepwise increments of 10%. RH was then decreased through the same steps. Changes in the mass of the sample due to sorption or desorption of moisture were expressed as a percentage of its dry mass.

2.14. Differential scanning calorimetry (DSC)

Samples of 4–6 mg were accurately weighed into 40 μl aluminium pans, hermetically sealed with pin-hole lids, and heated under a nitrogen purge in a Mettler Toledo DSC822e (Mettler–Toledo, Leicester, UK). Thermal transitions for the blank and test lyophilized samples were analyzed using a modulated DSC method for determination of the glass transition (T_g) of HPMC, and a standard linear DSC method to determine the crystallization and melting events of mannitol. The T_g of HPMC was represented in the reversing signal and the mid-point of the T_g determined at a heating rate of 2 °C/min from 25 to 240 °C. A quench-cool method was used for determination of the enthalpy of crystallization (ΔH_c) for mannitol from 25 to 200 °C at a heating rate of 10 °C/min. The data analysis was performed using Mettler STARe software.

3. Results and discussion

3.1. Bacteriophages remain relatively stable over one year storage in the lyophilized inserts

There is no current guidance on the bacteriophage titer that may be clinically effective against MRSA resident in the (human) nose, though some indications can be obtained from previous related studies of bacteriophage therapy. For example, the bacteriophage titers for respirable powders developed by Golshahi et al. (2010), were *ca.* 10^8–10^9 pfu per 100 mg of powder, with a single dose capsule load for inhalation of *ca.* 10^7–10^8 pfu. Furthermore, in a clinical trial testing the efficacy and safety of bacteriophages targeting *Pseudomonas aeruginosa* in otitis in humans, 10^5 pfu of each of six bacteriophages in 0.2 ml liquid was applied into the ear (Wright et al., 2009). On consideration of these two studies, we aimed for a bacteriophage titer of 10^9 pfu per nasal insert, pre-lyophilization, with the expectation that the titer would fall following lyophilization. Loss of titer has been previously reported (Puapermpoonsiri

Table 1
Notation and composition of the test and control formulations described in this study.

Notation/formulation	C1	C2	C3	C4	B1	B2	B3	B4	F1	F2	F3	F4
HPMC	1%	1%	2%	2%	1%	1%	2%	2%	1%	1%	2%	2%
Mannitol	–	1%	–	1%	–	1%	–	1%	–	1%	–	1%
Phage stock 10^{10} pfu/ml	–	–	–	–	–	–	–	–	10%	10%	10%	10%
	Diluent = sterile water				Diluent = storage media				Diluent = storage media			

et al., 2010) and is most likely associated with conformational changes to the bacteriophage protein coat, since proteins are well known to experience stress during freezing and drying. The plaque assay was used to determine the lytic activity of the bacteriophages in the formulations by enumeration of observable plaques, wherein each observed plaque represents a single phage.

The results presented in Table 2 show the lytic activity of the bacteriophages before and after lyophilization of the formulations. Enumerating the plaques for a given dilution enabled calculation of the bacteriophage titer that would have been present in each undiluted sample tested. A titer of 10^9 pfu/ml was calculated for the HPMC/mannitol gel formulations prior to lyophilization, indicating that there was no loss of lytic activity during mixing of the bacteriophages into the gels (since the titers added to the gel were consistently 10^{10} pfu/ml and a 10-fold dilution of the bacteriophage stock was made). In contrast, lyophilization of formulations resulted in a titer of 10^8 pfu/ml, which implied that 90% of the original lytic activity had been lost. Although this may seem high, this loss was less than or equivalent to previous losses following encapsulation (Puapermpoonsiri et al., 2009), or lyophilization with high concentrations of sucrose (Puapermpoonsiri et al., 2010). A titer of 10^8 pfu/ml is still considerable, comparable with bacteriophage doses of *ca.* 10^7–10^8 pfu for inhalation (Golshahi et al., 2010) and 6×10^5 pfu for dosing of the ear (Wright et al., 2009); this warranted long term stability testing of the lyophilized inserts.

From Table 2, the lytic activity was seen to decrease around 10- to 1000-fold over 1–12 months storage at 4 °C: lytic activity decreased from 10^8 pfu/ml on day 1 to around 10^7, 10^6 and 10^5 pfu/ml after 1, 2 and 12 months, respectively. The stability of the bacteriophage in the lyophilized nasal inserts was therefore far greater than for encapsulated phage in polyester matrices, wherein no lytic activity was observed after 1 week (Puapermpoonsiri et al., 2009). This was encouraging, particularly since the bacteriophage titer in the inserts after 12 months may still represent a therapeutic dose (Wright et al., 2009). No particular correlation could be observed between the formulations and the bacteriophage titers; *i.e.* neither the addition of mannitol nor the higher HPMC concentration conferred any additional benefit as regards long-term stabilization of the bacteriophages. The addition of HPMC at either 1 or 2% brings about 'molecular crowding' through excluded volume effects experienced by the bacteriophage, a phenomenon well understood through studies using macromolecules such as polyethylene glycol (PEG), dextran and Ficoll, or sol–gels (Eggers and Valentine, 2001). The concentrations required to bring about molecular crowding using PEG or dextran are equivalent to the HPMC concentrations used here (Dominak et al., 2010). While the excluded volume experienced by the bacteriophage increases for further addition of HPMC and/or mannitol, this does not necessary imply further change to the lytic activity since the effect of a concomitant increase in viscosity in these systems remains unknown. A 1% HPMC concentration would therefore appear to be sufficient as regards cryo- and lyoprotection of the bacteriophages. The stabilizing effect of HPMC is greater than the stabilization of bacteriophage by PEG 6000 (Puapermpoonsiri et al., 2010), and would suggest that high molecular weight, polyol polymers are useful formulation excipients for bacteriophages.

However, a simple explanation involving stabilization of protein (bacteriophage coat) structure via polyols, is not complete. This is because sucrose conferred no additional stability to lyophilized bacteriophages tested over 30 days (Puapermpoonsiri et al., 2010). Similarly, the lytic activity of the bacteriophages lyophilized from 1% solutions of mannitol, *i.e.* without the addition of HPMC, showed a large loss of around 6 log cycles by day 1 (Supplementary Data, Table S1). A further consideration involves the possible adsorption of the bacteriophages to crystalline domains of mannitol within the lyophilizate. This method of surface stabilization would reflect that proposed for protein coated microcrystals which have been shown to stabilize both enzymes and DNA through surface adsorption to the crystalline carriers during dehydration (Kreiner et al., 2005; Moore et al., 2010). However, given that mannitol conferred no additional stabilizing effect, this mechanism is unlikely to play a significant role in these studies. The addition of mannitol is therefore only justified in order to improve the strength of nasal inserts, as previously described (McInnes et al., 2007a). Stabilization conferred through immobilization of the bacteriophages onto the HPMC matrix is also unlikely since immobilization onto polyester matrices retained lytic activity for only 7 days (Puapermpoonsiri et al., 2009). Molecular crowding is well known to increase the conformational stability of

Table 2
Calculated phage titer for each phage formulation during storage at 4 °C.

	F1 (titer ± SD)[a], pfu/ml	F2 (titer ± SD)[a], pfu/ml	F3 (titer ± SD)[a], pfu/ml	F4 (titer ± SD)[a], pfu/ml
Before lyophilization	$(8.90 \pm 7.5) \times 10^9$	$(5.53 \pm 8.5) \times 10^9$	$(2.90 \pm 8.4) \times 10^9$	$(4.00 \pm 4.5) \times 10^9$
Day(s) after lyophilization				
1	$(4.40 \pm 5.2) \times 10^8$	$(8.53 \pm 1.4) \times 10^8$	nd	$(8.70 \pm 1.2) \times 10^8$
4	$(4.27 \pm 2.2) \times 10^8$	$(1.47 \pm 4.7) \times 10^8$	$(3.93 \pm 2.6) \times 10^8$	$(7.37 \pm 4.0) \times 10^8$
7	$(3.53 \pm 1.7) \times 10^8$	$(3.20 \pm 1.2) \times 10^8$	$(3.90 \pm 1.1) \times 10^8$	$(4.20 \pm 7.9) \times 10^8$
14	$(5.00 \pm 2.0) \times 10^7$	$(1.03 \pm 1.0) \times 10^8$	$(5.77 \pm 1.5) \times 10^8$	$(1.21 \pm 8.8) \times 10^9$
21	$(8.00 \pm 1.4) \times 10^7$	$(1.33 \pm 7.5) \times 10^8$	$(1.53 \pm 1.0) \times 10^8$	$(6.60 \pm 7.0) \times 10^8$
30	$(2.60 \pm 2.7) \times 10^7$	$(6.00 \pm 4.3) \times 10^7$	$(4.33 \pm 2.5) \times 10^7$	$(4.00 \pm 1.4) \times 10^8$
60	$(7.33 \pm 1.1) \times 10^6$	$(9.00 \pm 5.5) \times 10^6$	$(8.67 \pm 5.1) \times 10^6$	$(3.47 \pm 8.5) \times 10^7$
180	$(8.07 \pm 9.5) \times 10^6$	$(4.13 \pm 2.3) \times 10^6$	$(3.25 \pm 3.5) \times 10^6$	$(8.87 \pm 1.0) \times 10^6$
240	$(5.33 \pm 8.5) \times 10^6$	$(5.67 \pm 8.9) \times 10^6$	$(6.00 \pm 1.9) \times 10^6$	$(1.32 \pm 3.1) \times 10^7$
360	$(2.23 \pm 1.2) \times 10^6$	$(4.67 \pm 1.5) \times 10^5$	$(8.00 \pm 4.5) \times 10^5$	$(2.30 \pm 7.9) \times 10^6$

[a] Each value represents an average from three independent experiments; nd, not determined.

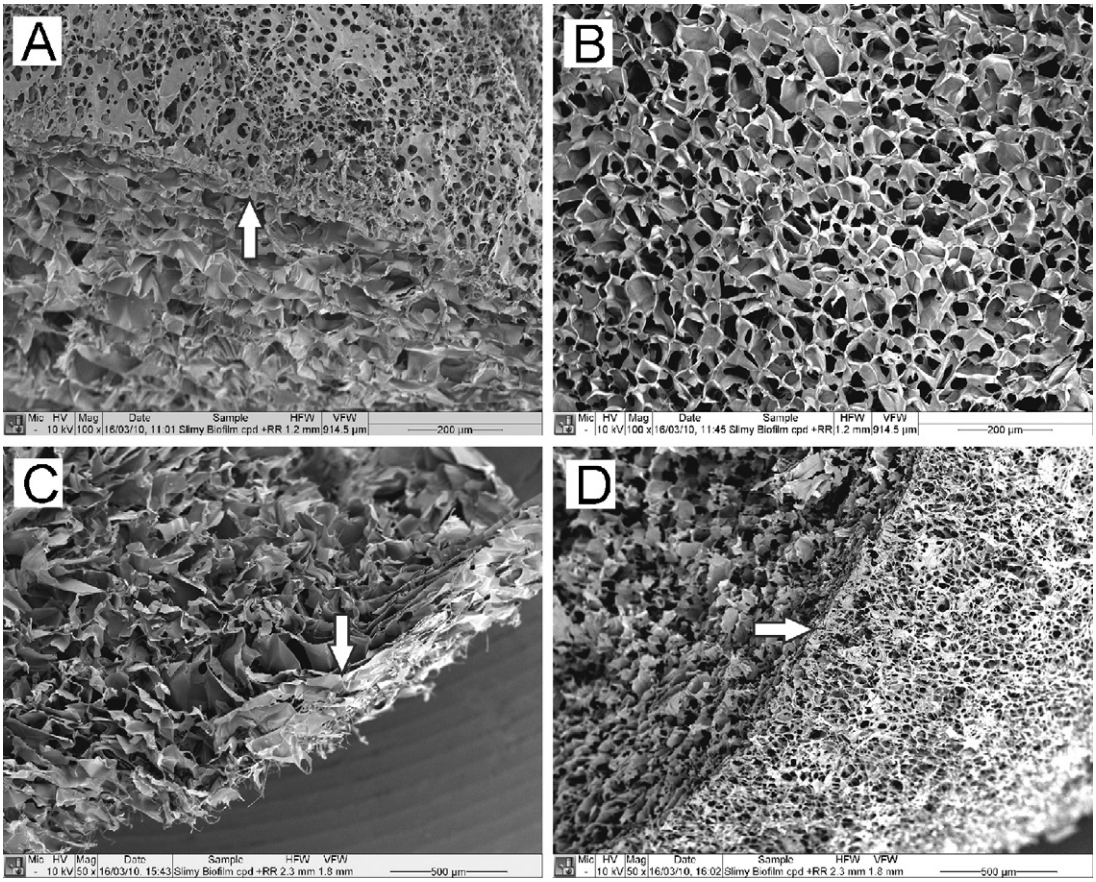

Fig. 1. Representative scanning electron micrographs for lyophilized nasal inserts, comparing the controls against formulations containing bacteriophage. (A) 1% HPMC; (B) 1% HPMC/1% mannitol; (C) 1% HPMC containing bacteriophage; (D) 1% HPMC/1% mannitol containing bacteriophage. The white arrows point from the internal matrix to the boundary with the surface of the insert. The scale bar is shown in bottom right of each pane.

proteins (Eggers and Valentine, 2001) and this effect may have contributed to the observed stabilization of the bacteriophage by HPMC.

Another explanation for the observed lytic activities is to compare the T_g of the amorphous materials: the T_g of the polyester used was ~45 °C (Rouse et al., 2007), while the T_g for sucrose is ~72 °C (Puapermpoonsiri et al., 2010). Differential scanning calorimetry (DSC) is widely used for the determination of the T_g of amorphous materials and modulated temperature DSC (MTDSC) has been shown to be applicable to the study of HPMC blends (Nyamweya and Hoag, 2000). We also chose MTDSC since the T_g of HPMC can be obscured by an associated enthalpic relaxation peak, which for some amorphous materials can be considerable (Rouse et al., 2007). The midpoint of the T_g of HPMC for formulations without mannitol was consistent, varying around 186 ± 7 °C (Table 3). The T_g values are also in agreement with previous data, reporting the T_g of HPMC K4M powder to be 184 °C (Doelker, 1993). The range in the T_g values reported is reasonable given that the change in the heat capacity of HPMC during the transition event was small, making the extrapolation of the baseline less certain. The difference in the values may also reflect the difference in the water content of the samples, since water is well known plasticizer of amorphous materials, with a general tendency to decrease the T_g (Hagens et al., 2004; Bouissou et al., 2005). It was not possible to observe the small T_g for HPMC when mannitol was added to the formulations. The T_g of the excipient may therefore relate to the resultant stability of the formulated bacteriophage in the solid state; *i.e.*, the more stable the excipient's amorphous state, the more stable the lyophilized bacteriophages remain.

3.2. Bacteriophages are distributed homogenously throughout the porous lyophilizates

Lyophilization of the formulated gels produced a lyophilizate with a sponge-like structure wherein individual 'leaflets' with smooth surface textures were layered. This gave rise to a highly porous interior which could be readily distinguished from the less porous external surface for all formulations (Fig. 1). The addition of mannitol into the gels also had no effect on the structure of the lyophilizates, cf. Fig. 1A/B and C/D. The porous structures were largely indistinguishable for the lyophilized 2% HPMC formulations (data not shown). In all cases, the highly porous structure is suggested to provide an ideal route for rehydration of the lyophilizate within the nasal cavity (McInnes et al., 2007a). The formation of the pores can be attributed to the space that was occupied by ice crystals during the freezing step. Fast and slow cooling protocols have been used for the lyophilization of carboxymethyl cellulose in order to control the pore structure and strength of the resultant lyophilizate (Yuan et al., 2009). The cooling rate adopted here (1 °C/min) was very similar to the slow cooling rate of Yuan et al. (0.83 °C/min), who reported 60–100 μm pore sizes for carboxymethyl cellulose. The same size range can be observed for the lyophilizates in this study: Fig. 1B showing pore sizes ranging from *ca.* 50 to 100 μm. Imaging at the boundary of the inner matrix and surfaces, shows that the individual leaflets forming the pore walls can be much closer together (Fig. 1A and C). The addition of bacteriophage did not appear to change pore size when comparing Fig. 1A/C and B/D, and further analysis using a technique such as Brunner Emmet Teller (BET) analysis would be required to make

Table 3
Glass transition temperature (T_g) of HPMC polymer for different formulations.

Formulation	HPMC powder[a]	C1	C3	B1	B3	F1	F3
T_g (°C)	187	190	188	192	188	179	186

[a] As received.

further distinction. To investigate the effect of the lyophilization protocol on lyophilizate morphology, a rapid freezing/rapid drying protocol was used. The structure of the lyophilizate remained composed of individual leaflets but the porous structure had been changed to a structure better described as lamellar (Supplementary Data, Fig. S1). This presumably represents the formation of large sheet-like ice crystals during the rapid freezing step, since similar structures have been described as a consequence of changes in ice nucleation (Searles et al., 2001). Thus, the morphology of the nasal insert would appear to be reproducible so long as careful control over freezing and drying steps can be maintained throughout the lyophilization process.

To examine possible aggregation and/or localization of the bacteriophages during the freeze-dry steps, the distribution of fluorescein-labeled bacteriophages was visualized by CLSM. Confocal micrographs show the bacteriophages to be distributed in a homogenous manner throughout the lyophilizate (Fig. 2), which reflects the overlapping of the leaflets and pore structure observed by SEM (cf. Fig. 1). Although it is not possible to resolve nanometer-sized aggregates of small numbers of bacteriophages in these images, there is no evidence of gross aggregation or localization of the bacteriophages, *i.e.* the freezing step would appear to have avoided slow formation of ice crystals and supersaturated regions of liquid.

3.3. Residual water content and water sorption of the lyophilizates

Residual moisture content is an important consideration for both the stability of the bacteriophage and amorphous polymeric materials. Fig. 3 shows that the addition of mannitol to the HPMC gels appeared to promote the drying of the lyophilizate, particularly

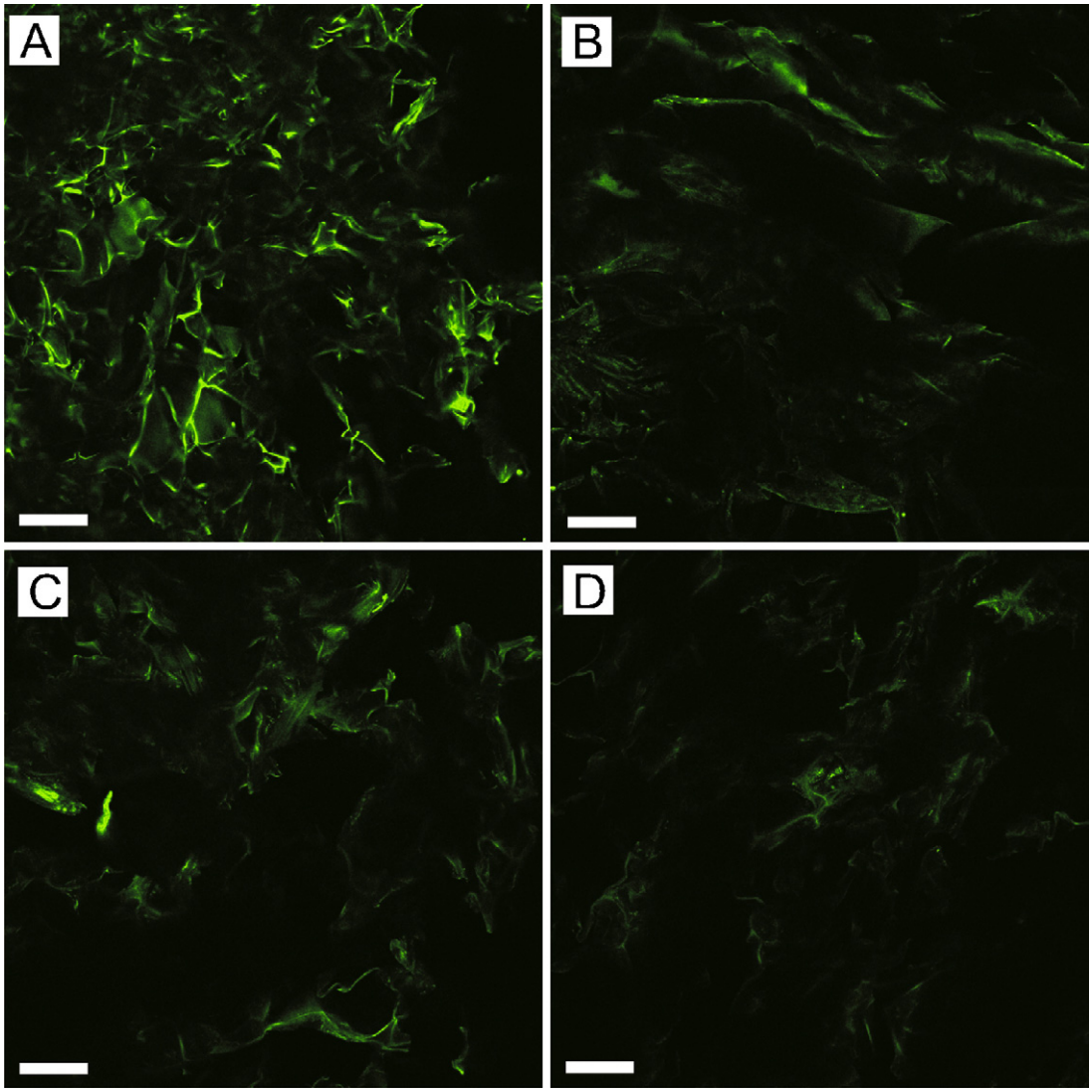

Fig. 2. CLSM images of fluorescein-labeled bacteriophage distributed through the lyophilized formulations. (A) 1% HPMC; (B) 1% HPMC/1% mannitol; (C) 2% HPMC; (D) 2% HPMC/1% mannitol. Bar = 250 μm.

for the 1% HPMC formulations, maybe due to its non-hygroscopic nature; although the difference between formulations F3 and F4 was not statistically significant ($P > 0.01$). Previous studies with lyophilized bacteriophages suggested that a more porous cake resulted in better mass transfer and lower residual moisture, with a value of 4–6% correlating to maximum retention of bacteriophage titer (Puapermpoonsiri et al., 2010). Except for the 1% HPMC/1% mannitol lyophilizate, the residual moisture content in these formulations was comparatively high. The resultant stability of the formulations over 12 months storage would therefore not have been predicted, and corroborates the suggestion above that the high T_g of the HPMC is more important in maintaining the stability of the bacteriophages. These HPMC/mannitol formulations are therefore not comparable to lyophilized formulations designed for reconstitution to yield non-viscous fluids for injection or nebulization, for example.

DVS was used to mimic the water sorption that may be experienced by the lyophilized inserts in the nose. The water sorption profiles showed an increase in mass as a result of exposure to increasing relative humidities (RH) to 95%, and corresponding drying as RH returned to zero (Fig. 4). For lyophilizates containing mannitol, a small but noticeable mass loss was observed during the water sorption phase between 5 and 10% RH. This was due to the transformation of amorphous mannitol to the crystalline form, with concomitant loss of water from the lyophilizate. Mannitol itself adsorbed very little water vapor during the same cycle: showing only a 0.3 and 1% mass increase for the powder as received and lyophilized, respectively. In contrast, HPMC powder (as received) displayed a 30% increase in mass for the same cycle. The addition of mannitol reduced water sorption only for the control samples and, to a lesser extent, the blank and bacteriophage formulations containing 1% HPMC (Fig. 4). However, the water sorption profile was not determined only by HPMC since a marked difference was observed between inserts formulated with water (28–52% mass increase), storage media (67–102% mass increase) and bacteriophage (101–143% mass increase). Since the storage medium contained buffer salts and gelatin, the gelatin component is most likely responsible for the difference between the control and blank samples, since proteins are well known to retain 'bound' water and gelatin powder is deliquescent. This was confirmed by DVS analysis for gelatin powder alone, which demonstrated a 41% mass increase over the same cycle. The difference between the blank and bacteriophage samples is therefore due to the bacteriophage themselves, presumably for the same reason of bound water associated with the protein capsule coat. Our results agree well with those for comparable lyophilized formulations in the study by McInnes et al. (2005), who showed that HPMC absorbs more than 50% of its dry mass while mannitol demonstrated very low water vapor sorption.

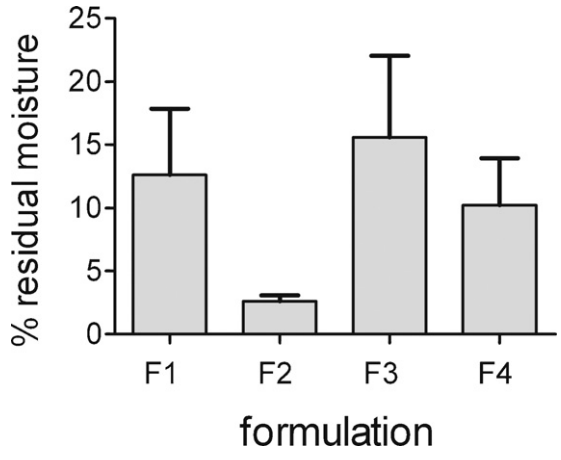

Fig. 3. Residual moisture content of the lyophilized bacteriophage formulations.

Table 4
Heat of crystallization (ΔH_c) values for mannitol in different formulations.

Formulation	ΔH_c (mJ)[a]
Mannitol powder[b]	1120
Mannitol lyophilizate	798
HPMC/mannitol physical mixture 1:1	488
HPMC/mannitol physical mixture 2:1	251
C2	360
C4	99
B2	231
B4	44
F2	58
F4	9

[a] Calculated from the integral of the exothermic ΔH_c peak for thermograms normalized by the mass of sample analyzed.
[b] As received.

Since it has been reported that polymeric excipients inhibit crystal transformation by slowing crystal growth or by removing excess water by absorption (Tian et al., 2007), we investigated if this was also true for the lyophilizates. Following a quench-cool cycle in order to transfer the mannitol into its amorphous state, DSC thermograms showed an exothermic re-crystallization peak followed by an endothermic melting peak at 166 °C, corresponding to previous data (Torrado and Torrado, 2002). The heat of crystallization (ΔH_c) was determined for each formulation after normalization of the thermograms (Table 4). A clear decrease in the ΔH_c for mannitol was observed upon the addition of HPMC in simple powder blends, dependent on the relative amount of HPMC added. ΔH_c values for the corresponding lyophilized mixtures (C2 and C4) also indicated that re-crystallization of mannitol was directly inhibited by HPMC.

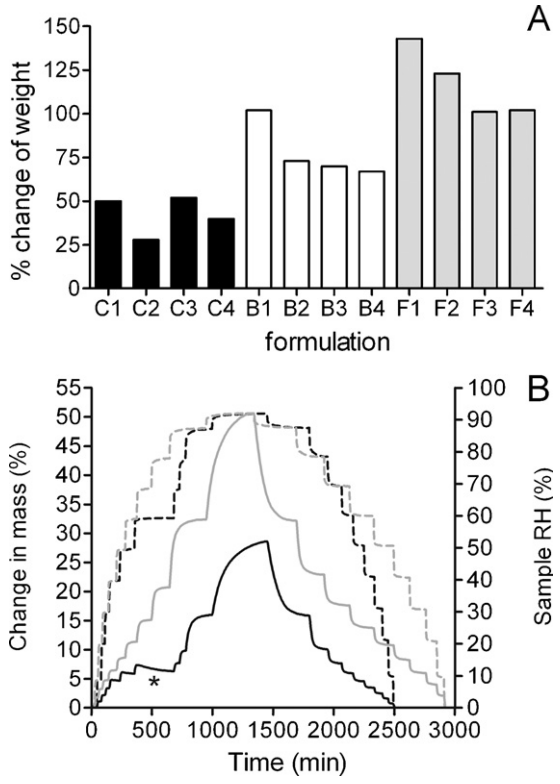

Fig. 4. (A) Percentage change in mass obtained during dynamic vapor sorption for the lyophilized formulations (at 95% RH). (B) Example profiles of the percentage change in sample mass (solid lines) versus RH (dashed lines) for formulations C1 (grey lines) and C2 (black lines); the * indicates the loss of mass as a consequence of the change from amorphous to crystalline mannitol.

The addition of storage media containing gelatin further decreased the ΔH_c (B2 and B4), with the presence of bacteriophage in the 2% HPMC formulation (F4) almost completely attenuating mannitol re-crystallization. The inhibition of mannitol re-crystallization by proteinaceous material has been previously reported (Hawe and Friess, 2006), consistent with the respective changes to ΔH_c seen between the control and blank/bacteriophage formulations. Given these data, it is reasonable to assume that the amorphous form of mannitol is stabilized to varying extents in the lyophilizates, dependent on the original percentage of HPMC in the gel and the presence of gelatin and bacteriophages.

4. Conclusions

Lyophilized nasal inserts harboring doses of bacteriophage corresponding to doses used in previous clinical trials of bacteriophage therapy can be formulated and remain stable in the fridge for up to 1 year. The addition of mannitol to the lyophilizate does not confer added stability to the bacteriophages and may be omitted if this was the sole consideration. However, given that lyophilization can be a costly step, formulations which dry to a defined percentage more rapidly may be preferred on these grounds, in which case the 1% HPMC/1% mannitol formulation would be preferred. Increasing the relative amount of HPMC in the blend with mannitol would appear to stabilize the amorphous form of mannitol in the lyophilizate, though transition of mannitol from its amorphous to crystalline form does not affect bacteriophage stability, the structure of the internal matrix, or the distribution of the bacteriophages with the matrix.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.07.006.

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