

Development of Inhalable Phage-Encapsulated Liposomes for improved delivery against *Pseudomonas aeruginosa* Lung Infections

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Summary

The growing threat of multidrug-resistant respiratory infections has intensified the need for alternatives to antibiotics. Bacteriophages (phages) are natural viruses that can infect and kill bacteria and are considered potential alternatives to antibiotics. Inhaled phage delivery is a promising route that allows direct access to the infection site in lung airways for localized antibacterial action. However, phages are vulnerable to manufacturing and aerosolization stresses, which can compromise their viability. Moreover, phages can be internalized by the lung epithelial cells, reducing their availability to kill extracellular bacteria like *Pseudomonas aeruginosa*.

This study investigates phage encapsulation in liposomes as a strategy to protect phages during (i) formulation, (ii) nebulized delivery, and (iii) post-deposition cellular uptake in the lungs. Phage encapsulation was optimized by tuning the liposome surface charge and phage loading titer. Results indicate that hydrophobic interactions play a more critical role than electrostatic interactions in promoting encapsulation, with negative or neutral liposomes exhibiting limited loss in phage viability. Low phage loading titer favors encapsulation and minimize viability loss. Liposomal encapsulation protects phages from shear stress during nebulization and contributes to a higher fine particle fraction. Phage liposomes demonstrate controlled release, thereby limiting uptake by the epithelial cells and prolonging extracellular retention essential for the bacteria *P. aeruginosa*. Overall, liposome encapsulation represents an effective strategy to improve inhaled phage delivery.

Key Message

Encapsulation of phages in liposomes enhances inhaled phage delivery by protecting phages during manufacturing and nebulization, while also prolonging their extracellular retention in the lungs—crucial for treating infections caused by extracellular pathogens like *P. aeruginosa*.

Introduction

Antibiotic-resistant respiratory infections caused by bacteria *P. aeruginosa*, evade the action of antibiotics and remain difficult to treat [1]. Bacteriophages (phages), viruses that selectively infect and kill bacteria, are promising alternatives to traditional antibiotics. Inhaled phage delivery enables direct access to the lung infection sites, providing localized and targeted antibacterial action. However, phages are sensitive to manufacturing and aerosolization stresses, which can compromise phage viability [2]. Additionally, phage uptake by epithelial cells can further limit phage availability to treat infections caused by the extracellular bacteria, *P. aeruginosa* [3].

This study explores phage encapsulation in liposomes as a protective strategy to limit viability loss during formulation, inhaled delivery, and post-lung deposition. The study investigates: (i) factors influencing phage encapsulation – surface charge and phage titer, (ii) the effect on phage nebulization, and (iii) phage cellular uptake in an *in vitro* lung epithelial (H441) cell model.

Methods

Phage titer determination: *P. aeruginosa* bacteriophage 95 (ATCC 14211-B1) and its complementary host, *P. aeruginosa* 95 (ATCC 14211) were procured from American Type Culture Collection (Manassas, VA, USA). Brain Heart Infusion (BHI) broth used for bacterial culture and phage propagation was obtained from Becton Dickinson (Franklin Lakes, NJ, USA), and bacteriological agar was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Phage propagation and titer determination were performed using plaque assay. Briefly, an aliquot of the overnight culture (2.5% v/v) was mixed with melted soft agar (0.5% w/v agar in BHI media) and poured onto the surface of hard agar (1.5% agar w/v) in a Petri plate. Serially diluted phage samples (10 µL) were spotted onto the surface and incubated at 37°C for 16–18 h. Countable plaques (3–30) were used to calculate phage titer, expressed as plaque-forming units (PFU) multiplied by the dilution factor.

Preparation of phage liposomes and surface modification: Phage liposomes were prepared by the thin film hydration method [4]. Lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

For surface charge modification, neutral liposomes were prepared using phosphatidylcholine (PC, 112 mg) and cholesterol (Cho, 28 mg). Varying amounts of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were incorporated to yield positively charged liposomes. For anionic and PEGylated liposomes, DSPG-Na and DSPE-PEG were included, with a final lipid composition of PC: DSPG-Na: DSPE-PEG: Cho (mass ratio = 3:2:0.5:1.7).

Lipids were dissolved in methanol and subjected to rotary evaporation to obtain a thin lipid film. The lipid film was hydrated with 250 µL phage stock in 3.75 mL of sugar solution, containing 80:20 mannitol and sucrose (Fisher Scientific, MA, USA)) at 40°C for one hour. Phage loading titer for charge modification experiment was 2×10^7 PFU/mL. To understand the effect of loading titer on encapsulation, high and low loading titer were compared 2×10^7 and 5.49×10^8 PFU/mL, respectively. Liposomes were downsized using syringe filtration (0.44 µm and 0.22 µm), followed by extrusion through a 0.1 µm polycarbonate membrane at 40°C. Free phages were separated by size exclusion chromatography (SEC) using Sephadex G-25 column. The encapsulated phage titer was determined by lysing the liposomes using 0.01% v/v sodium deoxycholate, followed by centrifugation. Phage encapsulation was calculated, and particles were characterized (particle size, polydispersity index, and zeta potential) using Malvern Zetasizer Nano ZS90 (Malvern Panalytical, Worcestershire, UK).

Phage aerosolization and in vitro lung deposition studies: Phage viability loss on nebulization was tested using PARI LC Plus (Pari GmbH, Starnberg, Germany) air jet nebulizer. Phages (suspension/liposomes) were nebulized for 5 minutes, and recovered phages were quantified. The *in vitro* lung deposition of phages was tested using a Multi-Stage Liquid Impinger (MSLI; Copley, UK). The LC Plus nebulizer was connected to the MSLI via an induction port, with airflow set to 28 L/min using an HCP5 vacuum pump and DFM 2000 flow meter (Copley Scientific, UK). Fine particle fraction (FPF) was calculated as the percentage of phages deposited in Stage 4 and the filter relative to the total emitted phages [5].

Phage cellular uptake: An *in vitro* lung epithelial cell model, NCI-H441 (American Type Culture Collection, Manassas, VA, USA) was used to evaluate phage cellular uptake. Cells were seeded in the apical chamber at 500,000 cells/cm² and grown at the air-liquid interface [6]. When a favourable barrier and transepithelial electrical resistance (TEER) was achieved, the cells were treated with phage suspension/liposomes (2.82×10^6 PFU/mL) and incubated for 4 hours. Phage distribution across the cell monolayer was tested after incubation. For confocal microscopy, all fluorescent probes were purchased from ThermoFisher Scientific (Rockford, IL, USA). H441 monolayers were treated with SYBR-Gold-labeled phages or Rhodamine-labelled phage liposomes. After 4 h, cells were washed and stained with Hoechst 33342 and imaged using a Nikon-A1Rsi confocal microscope, 60× oil immersion lens (Nikon Instruments Inc., Melville, NY, USA).

Results and Discussion

Formulation – effect of liposome surface charge on phage viability and encapsulation

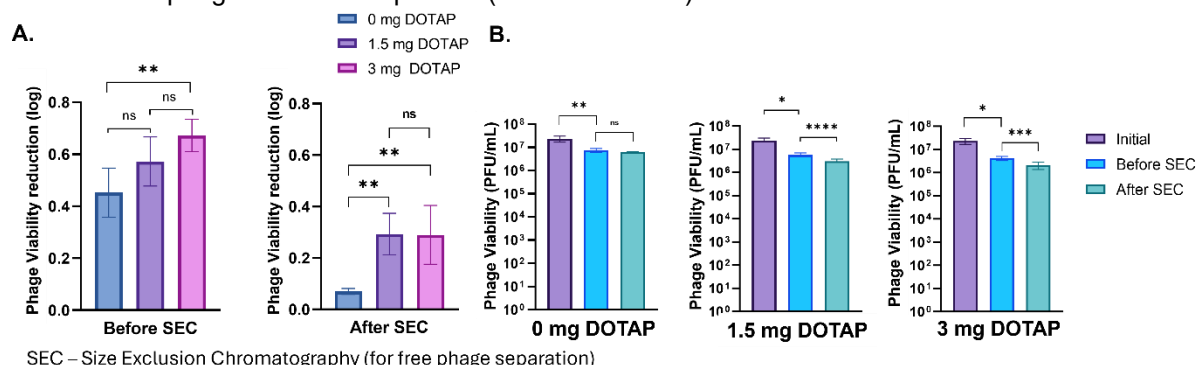
To address phage viability challenges, this study investigated factors influencing phage encapsulation in liposomes. Since phages are negatively charged, the surface charge of the liposomes was modulated to assess the role of electrostatic interactions on phage viability and encapsulation efficiency. The inherent surface charge of PC/Cho liposomes was slightly negative, while incorporation of DOTAP (1.5 and 3 mg) yielded cationic liposomes (**Table 1**). Phage encapsulation led to a marked reduction in zeta potential in DOTAP-containing formulations, indicating an electrostatic interaction between phages and cationic liposomes. This finding suggests that phages were predominantly localized on the liposome surface rather than the lipid core. Surface localization increases phage exposure to processing stresses (shear and thermal), thereby impacting phage viability.

Table 1: Particle characterization of charge modified blank and phage liposomes ($n = 3$, mean $\pm$ SD)

Formulation	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)	Encapsulation efficiency (%)
Blank PC/Cho	129.4 $\pm$ 1.01	0.066 $\pm$ 0.005	-13.49 $\pm$ 1.37	Not applicable
Phage PC/Cho	147.6 $\pm$ 1.70	0.090 $\pm$ 0.017	-17.69 $\pm$ 0.83	60.31 $\pm$ 2.79
Blank PC/Cho/1.5mgDOTAP	135.8 $\pm$ 1.21	0.050 $\pm$ 0.029	36.21 $\pm$ 1.88	Not applicable
Phage PC/Cho/1.5mgDOTAP	144.1 $\pm$ 0.40	0.099 $\pm$ 0.012	14.22 $\pm$ 0.88	39.67 $\pm$ 9.28
Blank PC/Cho/3mgDOTAP	141.9 $\pm$ 2.50	0.070 $\pm$ 0.016	50.83 $\pm$ 1.06	Not applicable
Phage PC/Cho/3mgDOTAP	143.9 $\pm$ 1.90	0.087 $\pm$ 0.007	20.65 $\pm$ 0.46	50.44 $\pm$ 5.14

Cationic liposomes with 1.5 and 3 mg DOTAP exhibited greater overall loss in viability, 0.91 ± 0.17 log and 1.07 ± 0.16 log, respectively. Consequently, the encapsulated titer (**Figure 1B**) was highest for

PC/Cho and reduced by 2-fold and 3-fold on addition of 1.5 and 3 mg DOTAP, respectively. Overall, neutral or slightly negative liposomes (PC/Cho) provide superior phage encapsulation ($60 \pm 2.79\%$) and stability compared to cationic formulations. This suggests that phage encapsulation is not only influenced by electrostatic interactions but also by hydrophobic interactions with lipid bilayer and localization of phages in the nanoparticle (core vs. surface).



SEC – Size Exclusion Chromatography (for free phage separation)

Figure 1: (A) Phage viability reduction (log), and (B) phage viability at different steps in processing liposomes consisting varying amounts of DOTAP in combination with PC/Cholesterol. Each data point represents mean $\pm$ SD, ($n = 3$), ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = no significant difference.

Formulation – effect of phage loading titer on phage viability and encapsulation

Negatively charged liposomes were selected for further studies and modified with PEGylated lipid (DSPE-PEG). PEGylation is known to reduce cellular uptake by providing a steric barrier on the liposome surface. To evaluate the effect of phage loading titer, liposomes were hydrated with low and high loading titers, yielding low (LT) and high titer (HT) liposomes, respectively.

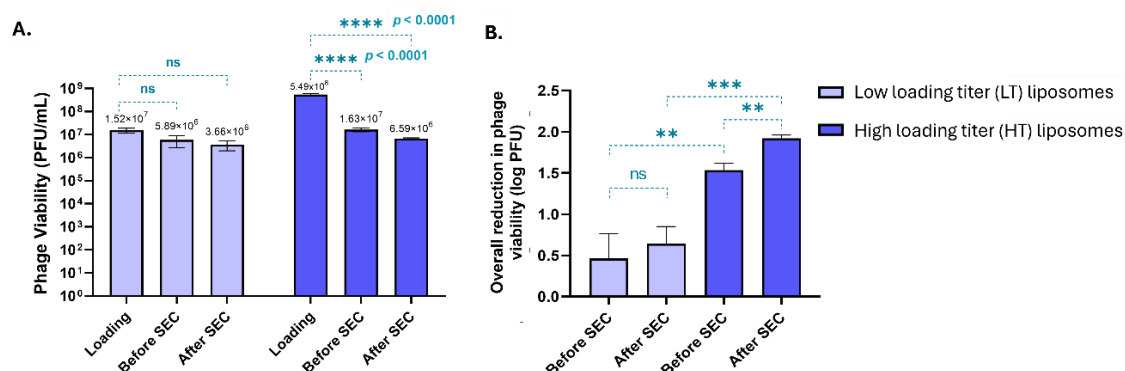


Figure 2: Comparison of (A) Phage viability and (B) log reduction in phage viability at different processing steps when using low and high phage loading titer. Each data point represents mean $\pm$ SD, ($n = 3$), ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = no significant difference.

Processing conditions such as temperature and shear stress impact phage viability (5). HT liposomes demonstrated a significantly higher loss in phage viability (1.92 ± 0.04 log) than the LT liposomes (0.64 ± 0.21 log) (Figure 2A). Two reasons contribute to phage loss: (a) loss due to processing conditions, and (b) separation of unencapsulated phages. Figure 2B, demonstrates that HT liposomes show significant loss ($p < 0.01$) of free phages on SEC compared to LT liposomes. Despite the observed loss, both HT and LT liposomes had same order of encapsulated titer 6.59×10^6 PFU/mL and LT 3.66×10^6 PFU/mL, respectively. Conclusively, LT liposomes exhibit higher phage encapsulation ($58.33 \pm 6.02\%$) than HT ($25.15 \pm 5.67\%$), hence LT liposomes were considered for further experimentation.

Impact of phage nebulization on viability and in vitro lung deposition

Table 2 summarizes phage viability loss during nebulization. Phage liposomes demonstrated a significantly lower viability loss (1.08 ± 0.05 log) compared to free phages (1.55 ± 0.04). Phage liposomes show a higher fine particle fraction, $37.32 \pm 2.94\%$ compared to the phage suspension $25.09 \pm 8.01\%$ (Figure 3).

Table 2: Phage viability loss during nebulization ($n = 3$, mean $\pm$ SD)

Sample	Log Phage titer (PFU/mL)			
	Loaded in nebulizer	Aerosolized	Recovered	Loss on nebulization
Phage suspension	6.18 ± 0.07	6.04 ± 0.01	4.49 ± 0.04	1.55 ± 0.04
Phage liposomes	5.92 ± 0.04	5.48 ± 0.05	4.40 ± 0.10	1.08 ± 0.05

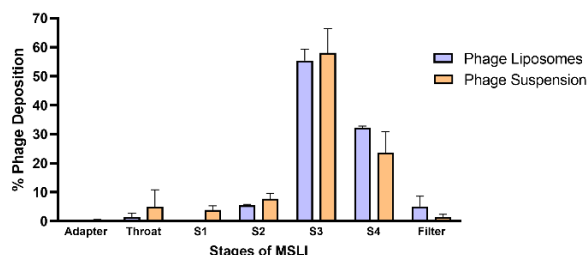


Figure 3: *In vitro* lung deposition of phage suspension and liposomes using Multi-Stage Liquid Impinger. Each data represents n=3, mean $\pm$ SD

Evaluation of phage cellular uptake in an *in vitro* lung epithelial cell model

Phage cellular uptake was studied in a lung epithelial cell monolayer, grown at air-liquid interface. After 4-hour incubation, more phage liposomes ($2.59 \pm 0.30 \times 10^6$ PFU/mL) were retained outside the cells as compared to free phages ($2.10 \pm 0.3 \times 10^6$ PFU/mL) (**Figure 4A**). Consequently, the cell lysates of phage liposomes ($1.42 \pm 0.16 \times 10^4$ PFU/mL) had significantly lower titer than free phage ($2.91 \pm 0.28 \times 10^4$ PFU/mL) (**Figure 4B**). This observation suggests that encapsulation of phages in liposomes can reduce their cellular uptake by 2-fold. This effect was also observed in confocal images (**Figure 4C**). Potential reason for this can be the slow and controlled release of phages from liposomes (observed but data not shown), which limits the cellular uptake in lung epithelial cells.

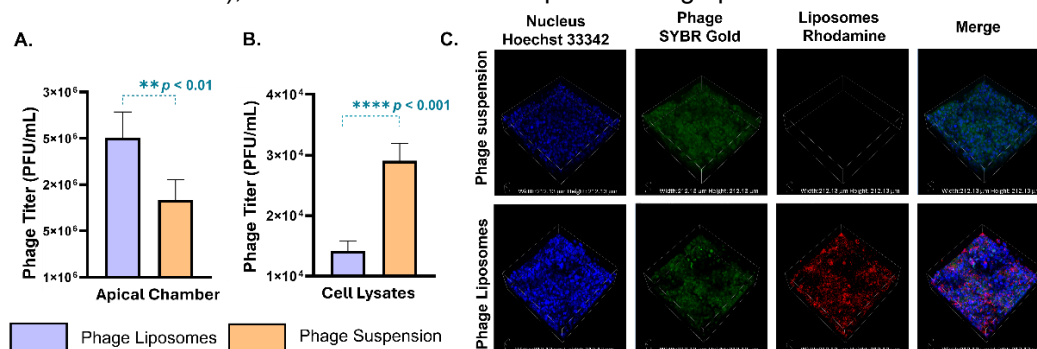


Figure 4: Phage distribution after 4-hour incubation with H441 lung epithelial cells (A) Apical chamber, (B) Cell lysates. (C) Confocal images showing lower phage cellular uptake in liposomes compared to phage suspension. Each data represents n=3, mean $\pm$ SD (** $p < 0.01$, and **** $p < 0.0001$).

Conclusion

Our results demonstrate the potential of liposomes for inhaled phage delivery. Lipid composition and loading titer strongly affect encapsulation and phage viability. Lower loading titer promoted higher encapsulation with minimal loss. Liposomes also protected phages during nebulization, improved aerosolization, deposition, and enabled sustained extracellular release ideal for targeting *P. aeruginosa*.

Acknowledgement

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