Synthesis of Nanolipid Carriers Encapsulated Phage Enzymes Against Bacterial Biofilms

Sahana Senthilkumar1 , S.Sagar1,a)

1Senthil Home Health Solutions, Vijayawada, AndhraPradesh, India

Corresponding Author: a)[shashvatsagar5@gmail.com](mailto:shashvatsagar5@gmail.com)

**Abstract:** The persistent challenge of bacterial biofilms, known for their high resistance to conventional antibiotics, necessitates innovative solutions. Nanolipid carrier (NLC) encapsulated phage enzymes have emerged as a promising approach to combat biofilm-associated infections. This study investigates the synthesis and application of NLC-encapsulated phage enzymes, highlighting their potential to enhance the stability, delivery, and antibacterial efficacy of bacteriophages.NLC encapsulation protects phage enzymes from environmental degradation and provides a controlled release mechanism, significantly improving their ability to target and disrupt biofilm structures. Experimental results demonstrate that NLC-encapsulated phage enzymes exhibit superior penetration and disruption of biofilms compared to their non-encapsulated counterparts, resulting in enhanced antibacterial efficacy. These findings suggest that NLCs can significantly improve the therapeutic potential of phage enzymes against biofilm-forming bacteria.However, the implementation of this technology faces several challenges. Ensuring the long-term stability of NLC formulations and optimizing production processes for large-scale manufacturing are critical issues. Additionally, achieving precise targeting of NLCs to infection sites and understanding the interactions between NLCs and the human immune system are areas that require further investigation. Regulatory and safety concerns also need to be addressed to facilitate the clinical application of NLC-encapsulated phage enzymes.

**Keywords**: nanolipids, phage enzymes, Anti microbial function,biofilms

# INTRODUCTION

Controlling unwanted spoilage and/or harmful microbes has always been difficult in a variety of businesses, including the food, pharmaceutical, and medical fields.Many naturally occurring antibacterial compounds, however, are extremely sensitive to certain manufacturing and/or storage conditions. Volatile antimicrobials evaporate when these materials are exposed to high pressure and temperature, which causes them to break down quickly [(Kovacs et al., 2024)](https://paperpile.com/c/d48CvH/JzqrP)[(Aparna et al., 2021; Poornima et al., 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/d48CvH/2mDCS+on6f2+YZOtp). Reduced or even lost antibacterial activity may arise from unfavorable interactions with formulation ingredients (lipids, proteins, etc.) or from alteration by natural enzymes . For instance, the low bioavailability of antimicrobial peptides, which has been linked to their poor stability against proteolysis and hydrolysis, low permeability across barriers, and brief half-life in the circulatory system, limits their application in medicine [(Pinilla et al., 2021)](https://paperpile.com/c/d48CvH/5zDbI). Be obtained through encapsulation, which also protects natural antibacterial chemicals from some of the most extreme production and storage conditions. Solid lipid nanoparticles (SLNs), nanostructured lipid nanocarriers (NLCs), liposomes, and other lipid-based nanostructures can all be used to deliver and release natural antimicrobial drugs under regulated circumstances. These nanostructures have been used to deliver antimicrobial enzymes, essential oils, antimicrobial phytochemicals, bacteriocins, and other antimicrobial peptides. Most studies use liposomes, even though SLNs and NLCs have not been thoroughly demonstrated to be antimicrobial nanocarriers [(Chakraborty et al., 2024)](https://paperpile.com/c/d48CvH/zQVkr)[(Adel et al., 2023)](https://paperpile.com/c/d48CvH/VxQbg) [(Merchant et al., 2022; Pandiyan et al., 2022)](https://paperpile.com/c/d48CvH/LFNhS+9C2ag).

The capacity to create biofilms is a feature shared by all microorganisms. Because they can proliferate on practically any surface, bacteria are able to construct these intricately designed colonies. Within biofilms, bacterial extracellular matrix envelops the growing cells in multicellular aggregates.[(Ganapathy 2022)](https://paperpile.com/c/d48CvH/LFNhS+9C2ag+1At7o) They can arise in industrial, medicinal, or natural environments and have a variety of effects on people [(Kovacs et al., 2024)](https://paperpile.com/c/d48CvH/JzqrP). For instance, the buildup of biofilms on implants or catheters can lead to chronic infections that are challenging to cure. This book focuses on new ideas in the field of bacterial biofilm research, such as the burden of infections linked to biofilms and the many ways by which Gram positive and Gram negative bacteria produce biofilms. It also emphasizes the different anti-biofilm tactics that can be applied to reduce infections linked to biofilms [(Pinilla et al., 2021; Varney et al., 2024)](https://paperpile.com/c/d48CvH/5zDbI+gzrLM). Environmental problems are rising as a result of microbiological contamination producing water reservoirs and biofilm growth in shale gas fracturing flowback. The initial 500 bbl (oil barrels, or 21,000 gal or 79,500 L) fracturing water tanks have been increasingly replaced with lined or unlined clay pits, which are exposed to the surrounding environment and can contain surface water, dust, rain, and bioaerosols. This is due to the rise in oil output. These source ponds have a significant level of bacterial contamination [(Cate et al., 2024)](https://paperpile.com/c/d48CvH/KObbh).

It is acknowledged that encapsulation is a useful strategy for protecting and efficiently delivering natural antimicrobials [(Lopes & Brandelli, 2018)](https://paperpile.com/c/d48CvH/uF5FY). The structural integrity of biofilms, characterized by a dense matrix of polysaccharides, proteins, and extracellular DNA, complicates the treatment of infections. This matrix not only acts as a physical barrier but also creates microenvironments with varying conditions that can lead to phenotypic variations within the biofilm, further contributing to antibiotic resistance. As a result, biofilm-associated infections often require prolonged and aggressive treatment regimens, which can lead to increased healthcare costs and adverse effects [(Rai et al., 2024)](https://paperpile.com/c/d48CvH/N9Gx8). Phage enzymes are added after the lipids have been dissolved in organic solvents in the encapsulation process. Lipid vesicles or nanoparticles containing the enzymes are created using methods such as solvent evaporation, thin-film hydration, and high-pressure homogenization. [(Jain & Verma, 2022; Marya et al., 2022)](https://paperpile.com/c/d48CvH/HENeX+Pnjys)

To guarantee their size, stability, and encapsulation efficiency, the carriers are frequently put through purification and characterisation procedures after encapsulation [(Das & Kaur, 2021)](https://paperpile.com/c/d48CvH/AXHrQ).

Enzymes generated from phages called endolysins cleave peptidoglycan, which is an essential part of bacterial cell walls. Bacterial lysis is caused by endolysins, which compromise the integrity of cell walls [(M. Hasan et al., 2024)](https://paperpile.com/c/d48CvH/v6hr1). Conversely, depolymerases specifically target polysaccharides within the biofilm matrix, thereby diminishing structural support and facilitating the dispersal of the biofilm. Although these enzymes have a well-established selectivity and efficacy against biofilms, issues with their stability, administration, and penetration through the biofilm matrix hinder their practical utilization [(Belete et al., 2024; M. Hasan et al., 2024)](https://paperpile.com/c/d48CvH/v6hr1+rxTU9).

The aim of this study is to analyse the synthesis of nanolipid carrier encapsulated phage enzymes against bacterial biofilms.

# Materials and methods

## Extraction of Phage Enzyme

The extraction of the phage enzyme was conducted using a method involving cold acetone precipitation. Initially, a mass culture of the phage was grown in a nutrient-rich media that contained its host organism. This culture was incubated at 37°C for 2-3 days to allow sufficient enzyme production. After incubation, the host cells were separated from the culture supernatant using ultracentrifugation, which involved spinning the sample at high speeds to pellet the cells. Following cell removal, acetone was added to the supernatant to precipitate the enzyme, and the mixture was centrifuged at 7000 x g for 15 minutes to collect the enzyme pellet. The pellet was then re-suspended in phosphate-buffered saline (PBS) to stabilize the enzyme. The quality and purity of the extracted enzyme were confirmed by running the sample on SDS-PAGE, where the enzyme bands were compared to a protein marker to identify and validate the enzyme based on molecular weight and band pattern.

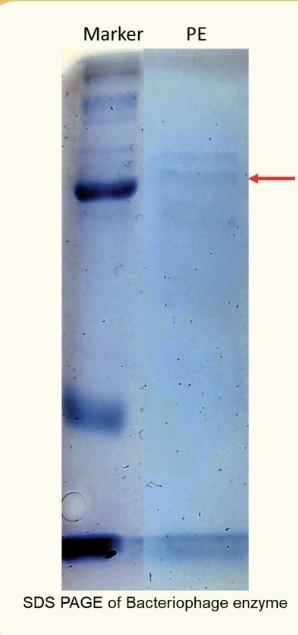
## Preparation of Nano-Lipid Carriers (NLC)

The NLC were prepared utilizing a microemulsion technique. Two separate solutions were prepared in Tris buffer: one containing a combination of liquid and solid lipids, and the other containing surfactants, Tween 80 and SDS. The lipid solution and surfactant solution were heated to 70°C and mixed using a magnetic stirrer for 2 hours to ensure complete emulsification and homogenization. After mixing, the solutions were allowed to cool to room temperature. The enzyme sample was introduced to the surfactant solution, and both solutions were combined simultaneously to form the NLCs. The mixture was then placed at -20°C overnight to solidify the carriers. The following day, the sample was lyophilized (freeze-dried) to remove any residual solvents and to obtain a dry powder of NLCs for subsequent analysis and applications.

## Antibiofilm Activity

The antibiofilm activity of the NLCs was assessed against *Staphylococcus aureus* bacteria. The NLC sample was prepared at a concentration of 10 mg/mL. To determine the efficacy of the NLCs in inhibiting biofilm formation, a serial dilution of the NLC sample was performed. For each dilution, 100 µL of the NLC sample was diluted two-fold, and 10 µL of an overnight culture of *S. aureus* was added to each dilution. The mixtures were incubated at 37°C for 24 hours to allow biofilm formation. After incubation, unattached bacterial cells were removed by washing the wells, and the biofilm was fixed. The biofilm was stained with crystal violet, a dye that binds to bacterial cells and extracellular matrix, for 20 minutes. The wells were then washed to remove excess dye and destained with 90% ethanol to quantify the amount of biofilm. Absorbance at 590 nm was measured using a spectrophotometer, providing an indication of the biofilm density and the effectiveness of the NLCs in inhibiting biofilm formation.

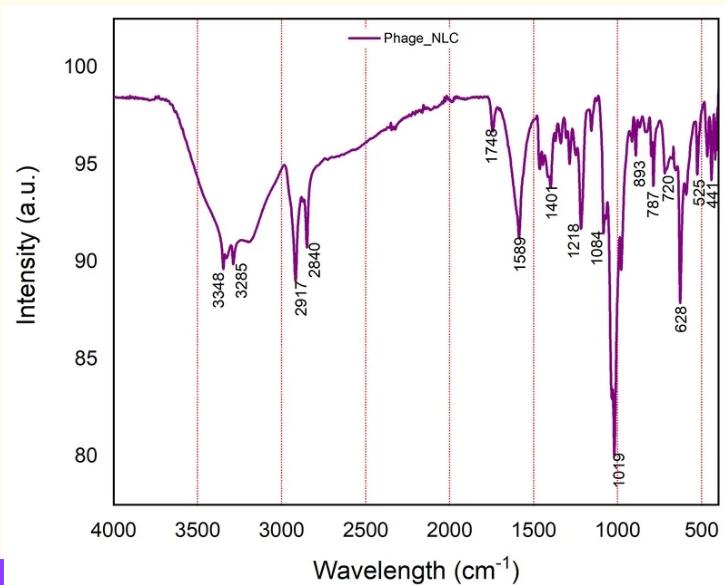
# Results & Discussion



**Figure 1:** SDS-PAGE analysis of depolymerase enzymes showing a distinct band on the gel.



**Figure 2:** Antibacterial activity assay showing inhibition zones against S. aureus using different concentrations of NLC-encapsulated phage enzymes.



**Figure 3:** FTIR spectroscopy analysis showing the incorporation of NLC-encapsulated phage enzymes.

## SDS-PAGE Examination

The existence and purity of the depolymerase enzyme were confirmed by the SDS-PAGE gel, which showed a distinct band for it.

## Antimicrobial Test

There were notable zones of inhibition against *S. aureus* in the antibacterial assay. The enzyme was present in zones of inhibition of 11 mm for 50 µL, 13 mm for 75 µL, and 30 mm for 100 µL. It shows that the negative control (NC) displayed a 10 mm zone of inhibition, while the positive control (PC) had a 12 mm zone.

## Spectroscopic Examination

FTIR examination of the phage enzyme added to NLC revealed a number of peaks that were suggestive of the molecular interactions present in the mixture. The stability and delivery of the enzyme depend on these interactions.

The outcomes show that the phage enzyme's antibacterial activity is considerably increased by the NLC formulation. When comparing the NLC-incorporated enzyme to the pure enzyme, the enlarged zones of inhibition indicate better stability and delivery. The presence of important functional groups is confirmed by the FTIR spectrum, which supports the improved performance of the NLC formulation.

Phage enzyme encapsulation using nanolipid carriers (NLCs) presents a viable strategy against bacterial biofilms. Because biofilms are resistant to antibiotics and other antimicrobial agents, they pose a serious threat(Rafi et al., 2024). However, phage enzymes—specifically, depolymerases—have demonstrated promise in breaking down biofilms. Based on current research discoveries, we address here the manufacture, advantages, and uses of NLC-encapsulated phage enzymes [(Wang et al., 2024)](https://paperpile.com/c/d48CvH/AAcPK).

Because SLNS and NLCs are more recently discovered than liposomes in the field of food science, there has not been much development in the encapsulation of natural antimicrobial phytochemicals for food applications [(Shah et al., 2014; Wang et al., 2024)](https://paperpile.com/c/d48CvH/AAcPK+D0Pxb). According to certain studies on the use of NLCs for plant extract encapsulation, the antimicrobial activity of turmeric extract against S. aureus. has improved, green tea stability has increased, and some natural antimicrobial compounds' toxicity on human cultured cells has decreased following NLC encapsulation [(Lipid-Based Nanostructures for Food Encapsulation Purposes: Volume 2 in the Nanoencapsulation in the Food Industry Series, 2019)](https://paperpile.com/c/d48CvH/rO3yE). Additionally, another recent study encapsulates the same antimicrobial extract using both liposomes and NLC, providing an intriguing opportunity to examine the advantages of each method (Tuluwengjiang et al., 2024). In these studies, both nanostructures demonstrated comparable sizes (about 100 nm) and particle size distributions (about 0.4), high EE (95 and 98%), and superior antimicrobial activity in comparison to the free turmeric extract; turmeric extracts loaded in liposomes (TNL) and NLC (T-NLC) were reported in it . [(Jafari & Silva, 2022)](https://paperpile.com/c/d48CvH/9i6ge). But as compared to the TNL (0.58 mg/mL and 0.07, respectively), the T-NLC had greater MIC values against E. Coli (18.5 mg/mL) and S. aureus (0.079 mg/mL). The behavior and velocity of each lipid-based structure's bioactive release, which in some circumstances tends to be lower in NLC, may be connected to this discrepancy [(Cortesi et al., 2017)](https://paperpile.com/c/d48CvH/rVkXm).

Regarding mechanism and effectiveness Bacteriophages are viruses that infect bacteria and have demonstrated potential in breaking down biofilms, the protective coatings that bacteria produce to ward off antibiotics [(Sherry et al., 2013)](https://paperpile.com/c/d48CvH/wLoP4). Phage penetration and disruption of these biofilms can increase the sensitivity of treated bacteria, as studies have shown. For example, by enzymatically breaking down the components of the biofilm, phages such as MA-1 have greatly decreased Pseudomonas aeruginosa biofilms.​ [(Cortesi et al., 2017)](https://paperpile.com/c/d48CvH/rVkXm).

Phages can work better when combined with other therapies. A study on Pseudomonas aeruginosa biofilms showed that by halting the emergence of phage-resistant bacterial mutants, employing a combination of various phages enhanced biofilm removal. [(Tiwari & Jain, 2023)](https://paperpile.com/c/d48CvH/Pb1GC)[(Graf et al., 2023)](https://paperpile.com/c/d48CvH/NMJRM)

The stability and distribution of phage enzymes can be enhanced by encasing them in nanolipid carriers [(Bhattacharyya, 2023)](https://paperpile.com/c/d48CvH/aTi0b). The phages' capacity to target biofilms is improved, their protection from external variables is increased, and a regulated release mechanism is provided by this encapsulation [(N. Hasan et al., 2023)](https://paperpile.com/c/d48CvH/DSwBd). It has been demonstrated that using this technique greatly increases the antibacterial effectiveness of phage enzymes.​[(Sabarathinam & Madhulaxmi, 2021)](https://paperpile.com/c/d48CvH/i6txA)[(Sushanthi et al., 2021)](https://paperpile.com/c/d48CvH/8n1B0)[(Harsha et al., 2022)](https://paperpile.com/c/d48CvH/Pm5SD)

Phage therapy has gained more attention as a substitute for conventional antibiotics because of its ability to treat illnesses that are resistant to drugs. Scholars are investigating diverse formulations and delivery strategies, such as NLC encapsulation, in order to maximize the therapeutic efficaciousness of phages against infections linked with biofilms [(Khan et al., 2023)](https://paperpile.com/c/d48CvH/fRXyj).

Phage enzyme stability, release kinetics, and targeting capabilities can all be enhanced by further honing NLC formulations [(Dong et al., 2024)](https://paperpile.com/c/d48CvH/vE3vt)[(Neha et al., 2021)](https://paperpile.com/c/d48CvH/jcpXs)[(Maliael et al., 2021)](https://paperpile.com/c/d48CvH/Z9lDe)[(Lakshmi, 2021)](https://paperpile.com/c/d48CvH/Bh3Lc). The best lipid compositions and surface alterations to improve enzyme encapsulation and activity can be the subject of future research.

To increase treatment efficacy and reduce adverse effects, targeted delivery devices that can administer NLCs precisely to tissues or surfaces contaminated with biofilms should be developed. For targeted delivery, methods such as magnetic targeting and ligand attachment can be investigated.​​[(Dharman 2021)](https://paperpile.com/c/d48CvH/pglhh)

The synergistic effects of combining NLC-encapsulated phage enzymes with antibiotics or other antimicrobial treatments can enhance biofilm breakdown and decrease the risk of resistance development. Research may concentrate on finding the best dosage schedules and combinations [(Bayat et al., 2024)](https://paperpile.com/c/d48CvH/7ftsi). It is possible to enhance biofilm eradication and stop the emergence of resistant bacterial strains by using cocktails of several phages encapsulated in NLCs. The optimal phage combinations and their interactions within NLCs can be investigated in future studies.[(Chokkattu et al., 2022; Ramamurthy et al., 2022)](https://paperpile.com/c/d48CvH/IDZOq+78Crm)

Even though NLCs increase the stability of encapsulated enzymes, they might eventually degrade, especially under some storage situations. [(Bayat et al., 2024; Ioannou & Baliou, 2024)](https://paperpile.com/c/d48CvH/7ftsi+Rn08d). One major problem is ensuring the encapsulated phages remain stable and active over the long term [(Sears, 1996)](https://paperpile.com/c/d48CvH/Nl72). Temperature, humidity, and light are examples of environmental variables that may have an impact on the stability and effectiveness of NLC formulations. It can be challenging to scale up the complex, multi-step methods used in the production of NLC-encapsulated phage enzymes for large-scale manufacturing [(Johannesman et al., 2024; Sears, 1996)](https://paperpile.com/c/d48CvH/Nl72+S3O7). It is difficult to guarantee consistency and quality on a commercial level.​ NLC-encapsulated formulations can be expensive to produce, which could prevent them from being widely used, particularly in environments with limited resources.

# CONCLUSION

Phage enzymes encapsulated in nanolipid carriers (NLCs) offer a novel and intriguing solution to the enduring problem of bacterial biofilms, especially in light of antibiotic resistance. By efficiently targeting and upsetting biofilm formations, the encapsulation of phage enzymes within NLCs increases their stability, permits regulated release, and boosts their antibacterial activity. Applications for this technique could include environmental bioremediation, industrial biofilm control, and therapeutic therapy of persistent illnesses.

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