Review Article

Scope of Phage Formulation Establishment Against Antibiotic Resistance

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Received: 11-10-2023; **Revised:** 20-12-2023; **Accepted:** 28-12-2023; **Published on:** 15-01-2024.

ABSTRACT

Antimicrobial resistance has become a growing concern, and it is essential to find new solutions to address this problem. Lytic bacteriophages, also known as phages, have emerged as a potential treatment for drug-resistant bacterial infections. Phage therapy is a new antibacterial approach gaining attention. Bacterial viruses possess antibacterial effector functions to combat infections. This review focuses on using phage therapy against intracellular bacteria. Recent research indicates that some phages can interact with mammalian cells, which prompts further investigation for harnessing therapeutic phages to target intracellular bacteria. The review provides an overview of the advances made to improve the capability of phages to attack intracellular bacteria using various formulation approaches. These approaches include encapsulating or conjugating phages into or with vector carriers such as liposomes, polymeric particles, inorganic nanoparticles, and cell-penetrating peptides. While promising progress has been made in this area, it is still in its infancy and warrants further attention. In conclusion, this review highlights the potential of phage therapy in targeting intracellular bacteria and the need for continued research to improve its efficacy. In this article, we aim to provide a comprehensive overview of the major challenges in phage therapy and the potential solutions that have been proposed to overcome them. We will delve into the scope of these solutions and place particular emphasis on the genetic modification of phages as a viable strategy to engineer bacteriophages to exhibit desirable biological properties. Our discussion will encompass the complexities involved in phage therapy and the limitations of traditional approaches, as well as the latest advancements in the field that have shown promise for addressing these challenges. By the end of this article, readers should have a thorough understanding of the current state of phage therapy research and the potential it holds as a novel approach for combating bacterial infections.

Keywords: Phage, Bacteriophage, phage formulation, ethics of phage, scope of phage formulation.

INTRODUCTION

Phages are viruses that infect bacteria and are separate from animal and plant viruses. They exhibit either a "lytic" or "lysogenic" life cycle. Among the two, lytic phages are the most ideal candidates for phage therapy as they multiply rapidly and cause lysis of the bacteria within their host range, thus increasing in number exponentially[1]. Frederick Twort and Félix d'Herelle discovered phages in the early 20th century. The genesis of phage therapy to treat infectious disease was around the year 1924 but the finding of antibiotics detested the usefulness of phage therapy. However, the dilemma of antibiotic resistance pulled phage therapy from its obsolete era [2]. The depletion of antibiotics has led to the revival of phage therapy. Bacteria have become resistant to most antibiotics, with some becoming resistant to all. Examples include vancomycin-resistant *Enterococcus faecium* (VRE) and vancomycin intermediate-resistant *Staphylococcus aureus* (VISA). Some hospital strains of methicillin-resistant *S. aureus* (MRSA) have become vancomycin-resistant. Experts predict that *S. aureus* will eventually become completely resistant to vancomycin, causing millions of deaths each year from infections that were once easy to control[1].

Phages are viruses found naturally that can infect both gram-positive and gram-negative bacteria. They are usually not affected by antibiotic resistance and, unlike most antibiotics, can target bacteria within biofilms[3]. Phage therapy is a more targeted and specific treatment compared to antibiotic therapy. It reduces the damage caused to the host's natural microbiota and is non-pathogenic to humans. Additionally, it is self-limiting as the phages only persist as long as the target bacteria are present, making it a safer and more efficient alternative. Due to the antibiotic crisis, we are now officially living in a post-antibiotic era which is a life-threatening scenario that needs more attention[2]. The World Health Organization's global priority for pathogens for research and development of new antimicrobials list includes ESKAPEE (Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter species*, and *Escherichia coli* pathogens. Based on various criteria, such as mortality, prevalence of resistance, treatability, and current pipeline, the situation is critical for most difficult-to-treat infections, including those acquired in the community, healthcare-associated, and nosocomial infections[3].

Phages are viruses that can kill antibiotic-resistant bacteria and have several advantages that make them safe and eco-friendly for clinical use. They have a high specificity that minimizes disruption of normal flora, are plentiful in the human gastrointestinal tract, and can self-replicate at the site of infection. Additionally, they are relatively non-toxic.

Table 1: The peculiarity of phages to advocate health-giving response. [1]

Issues	Antibiotics	Phages
•Experties to prevail over bacterial resistance	•The metamorphosis charachteristic of bacteria overpower the fixed and non transformable features of antibiotics leading to the disuse of it.	•The identity of phages itself gives them the upperhand as they are "living" and have the potentiality to mutate to a certain extent.
• MIC (Minimum Inhibitory Concentration)	•Low dosages that inhibit bacteria growth can promote gene expression of resistance.	•The case with phages in this issue is different as it is independent of MIC that is only one phage is sufficient to kill a given bacterium
•The escalation of the resistance	• The antibotics gravitate towrads the far-ranging side giving rise to resistance in several species and genera of bacteria.	•Phages loathe to conduct resistance with some exception.

The characteristics of a few selected intracellular bacteria and their susceptibility to phage therapy are briefly discussed[5]:

1.*Chlamydia* sp.

Chlamydia spp. are Gram-negative bacteria that live inside cells in various parts of the body. They have a unique two-phase developmental cycle consisting of an infectious form called elementary body (EB) and a non-infectious replicative form called reticulate body (RB). These bacteria rely on host nutrients for replication and evade phage attacks. Successful phage therapy against Chlamydia spp. would depend on the phage's ability to access the intracellular RB.

2. *Mycobacterium tuberculosis*.

Mycobacterium tuberculosis is a bacterium that can live inside and outside the body and can be transmitted through the air. It evades destruction by immune cells and can establish a niche inside different types of cells. Most people can keep the bacterium in check, but it can reactivate if the immune system is weakened. The BCG vaccine has limited effectiveness and does not prevent transmission of the disease. New strategies are needed to eradicate tuberculosis by targeting both the intracellular and extracellular forms of the bacterium.

3. *Salmonella enterica*

Salmonella enterica is a bacteria that can cause infections through contaminated food or water. It can invade cells and cause gut inflammation known as neutrophilic gastroenteritis. It can also spread to other organs and cause systemic infections. Overuse of antibiotics in farming animals and the bacteria's intracellular survival have contributed to antibiotic resistance.

These features make phages probably the safest and greenest technology available for clinical use. Many studies have provided convincing evidence of the safety and efficacy of phage therapy in animals and humans, and new phage-based companies and products have emerged recently[4].

❖ **Pathogenic Bacteria and phages reciprocation:** Traditionally, the pathogenic bacteria are categorized as either extracellularly or intracellularly. However, increasing evidence supporting the fact that extracellular pathogenic bacteria may exist both inside or outside of the cell made this categorization indistinct giving them the flexibility to establish survival zones that are beneficial for their growth. They can spread through direct cell-to-cell contact or by invading the surrounding extracellular space.

❖ **Interaction of phages with mammalian cells**:

Phage has traditionally been evaluated for its ability to kill bacteria in the absence of mammalian cells. However, recent studies have shown that phage can interact with human cells. Therefore, evaluating the antibacterial capability of phage in the presence of cells can help provide insights for in vivo studies[5]. One study confirmed the antibiofilm efficiency of a phage mixture (/NH-4 and /MR299-2) in removing P. aeruginosa biofilm growing on the surface of a cystic fibrosis bronchial epithelial (CFBE41o) monolayer. Although no comparison was made with human cell-free experiments, their in-vitro co-culture model provided a good prediction of the in vivo efficacy in treating lung infection in mice. P. aeruginosa biofilm formation could be initiated two hours after bacterial challenge[6].

Barr et al.[7] demonstrated in their work that Bacteriophages, also known as phages, are viruses that target and kill

bacteria. Since phages are not living organisms, they rely on diffusion to locate and attack bacterial cells. Recent studies have shown that a phage that loosely adheres to mucus surfaces exhibits sub diffusive motion, rather than normal diffusion. This means that the phage moves more slowly and randomly, increasing its chances of encountering bacterial cells. This sub diffusive search mechanism is unique to phages and is effective in reducing bacterial infection of mucosal surfaces. Adherent phages that are specifically engineered for this purpose could be used to manipulate microbiomes on mucosal surfaces, potentially offering protection from infection and other benefits. Opportunities for phage formulation and engineering could arise from the sub-diffusion phenomenon observed in the mucus layer. Encapsulating or conjugating phage into polymeric carriers that exhibit sub-diffusive motion in the mucus layer or engineering phage with Ig-like domains to enhance their interaction with the mucin, could significantly increase the rate of phage-host bacteria encounter and improve treatment efficacy.

- **The co-culture systems increase the efficacy of bacteriophages:**

[8]Understanding the fundamental dynamics of in vivo phage-bacteria interactions is crucial for the development of bacteriophage therapeutics. Such understanding can aid in the design of animal and human trials. Valuable insights can be obtained from human cell-line work. We have developed a human cell-based system that uses *Clostridium difficile*, a dangerous pathogen with limited treatment options, and the phage phiCDHS1, which has shown great efficacy in killing this bacterium in liquid culture. The human colon tumorigenic cell line HT-29 was used as it simulates the colon environment where *C. difficile* infection occurs. Our studies on the dynamics of phage-bacteria interactions revealed novel facets of phage biology. We found that phages can reduce *C. difficile* numbers more effectively in the presence of HT-29 cells than in vitro. Both planktonic and adhered Clostridial cell numbers were successfully reduced. We hypothesize that the strong phage adsorption to the HT-29 cells promotes phage-bacteria interactions. Our data also showed that the phage phiCDHS1 was not toxic to HT-29 cells, and phage-mediated bacterial lysis did not cause toxin release and cytotoxic effects. Using human cell lines to understand phage-bacterial dynamics offers valuable insights into phage biology in vivo. This approach can provide informative data for human trials, and it can help in the development of bacteriophage therapeutics.

- **Phage Selection**: Phages are viruses that target bacteria and are being considered as potential candidates for therapy. A good phage candidate can infect a wide range of bacterial strains. Myoviruses are known to exhibit a broader host range, as evidenced by a single phage lysing 17 out of 28 S aureus clinical isolates. However, narrow host range phages can also be effective, especially when targeting a specific strain of pathogenic bacteria to avoid adverse effects associated with host microbiome dysbiosis.

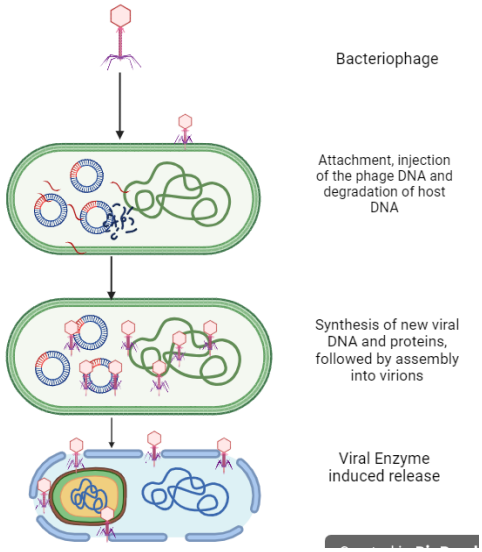

Figure 1: Lytic infection cycle of virulent phages. Step 1: adsorption: attachment of tail fibers onto a specific receptor on the bacterial cell wall. Step 2: injection: viral DNA penetrates the host through a hollow tube in the tail. Step 3: protein synthesis and host hijacking. Viral genes direct the synthesis of viral proteins using the machinery of the host. Steps 4 and 5: viral genome synthesis and assembly. Step 6: release: mature viral particle (virion) release occurs via viral peptidoglycan hydrolases (endolysins) that mediate host cell lysis, which liberates up to 200 infectious phages. [3]

In addition to host range, another important feature in phage selection is their ability to disrupt structured communities of bacterial cells that attach to surfaces and form biofilms. Biofilms are self-made exopolysaccharide matrices that provide an essential scaffold for biofilm development, promoting bacterial adhesion to surfaces and cohesion, while hindering diffusion. Many infectious diseases in humans are caused by or exacerbated by biofilms.

Although it is often assumed that biofilms confer resistance to phages, most phages can infect bacteria within biofilms. Certain phages have even coevolved with bacterial biofilms, making their infection of encased bacteria expected. Moreover, some phages carry depolymerases that degrade exopolysaccharides and disrupt the exopolysaccharide matrix of biofilms. In vitro studies showed that phages have reduced both single-species and dual-species biofilms. In an artificial CF sputum, phages could reduce P aeruginosa biofilm by 3-logs, indicating that phages can penetrate and lyse bacteria encapsulated in biofilms. Additionally, phages lyse distal bacteria, causing interior cells to awaken and become more metabolically active, which increases oxygenation and nutrient exposure, making them more sensitive to phage attacks. However, not all phages can penetrate the biofilm matrix, and some may even promote a mucoid phenotype of host bacteria that enhances biofilm formation. Although biofilm infections are common and cause clinically significant and potentially fatal infections,

much work remains in the quest for effective antibiofilm phages in clinical settings.

Lastly, extended storage of phages is another important consideration. Phages can be stored in short-term cold storage at 4°C when purified in a buffered solution, concentrated, and shielded from light. However, phage counts generally trickle down for months. Cryopreservation can minimize the loss of phages, but phage particles are inactivated with freezing and thawing cycles. Long-term storage is feasible with phage lyophilization (freeze-drying) using stabilizing additives. Although lyophilization is not compatible with most clinical applications, it may be suitable for inhaled phage therapies. But, specific storage conditions for each phage strain require evaluation[3].

❖ Initiating the conversation of Phage Formulation:

Even though phage can target intracellular bacteria, whether conveyed into the host cells by the bacteria or penetrated freely into the cells, the large number of phage studies has cut off to yield reasonable therapeutic outcomes making them unsuitable for targeting the eukaryotic intracellular infections. To overcome the paragon of phage, therapy several formulation approaches have recently been endeavored.

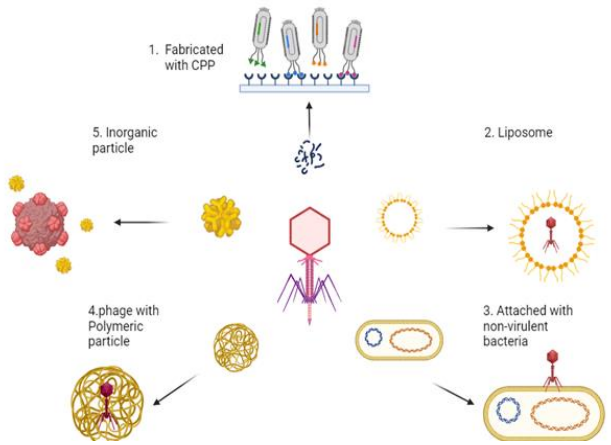

Figure 2: Different approaches for phage formulation.

1. **Fabricated phage**: Lytic phage therapy is a natural alternative approach to conventional antibiotics and has been proposed to combat infections caused by various pathogens in humans. Non-controlled clinical studies have shown that phages can be effective in this regard. There is an approach that uses genetically engineered phage to deliver the instructions for cell death to bacteria, with nonlytic phage. Unlike lytic phage therapy, where bacteria are killed following amplification and release of progeny phage into the environment, this approach uses nonlytic phage to deliver DNA encoding bactericidal proteins. Programmed cell death in bacteria is mediated through "addiction modules", which consist of two components - a stable toxin and an unstable antidote that antagonizes the toxic effect. Examples include the pemI-pemK genes of plasmid R100, the phd-doc genes of phage P1, and the ccdA-ccdB genes of plasmid F[9]. The technology of engineering phage with receptor-mediated internalization into eukaryotic cells capability is likely extendable for therapeutic phage against intracellular bacteria. Phages modified with surface-displayed cell-penetrating peptides (CPPs) have been found to promote the internalization of the intact phage into mammalian cells (Fig.1).

   - Common CCPs used to convert phage into a vector for gene delivery included the integrin-binding cyclic RGD (Arginine-Glycine-Aspartic acid) peptide. [10] A peptide displayed on the surface of bacteriophage fd makes it easier for the virus to enter and bind to cells. The cyclic peptide sequence GGCRGDMFGC was selected for the construction of the phage fdRGD due to its high affinity and integrin-binding properties. By displaying the ligand on the filamentous capsid in a hybrid mix of fusion and wild-type pVIII subunits, the phage can display multiple copies of the peptide - up to 300 or more - on each particle. This is a significant improvement compared to gIII adsorption protein phage display systems, which can only display four or five copies per particle. To confirm the display of integrin-binding RGD peptides on the phage surface, cell binding assays were performed. Cells added to wells coated with fdRGD were observed to flatten and spread out similarly to cells binding to wells containing fibronectin but did not attach to wells coated with fdREV. Cell binding was confirmed by staining the attached cells with crystal violet and analyzing the solubilized stain using a spectrophotometer. When cells were pre-incubated with the integrin-specific peptide GRGDSP, they were no longer able to attach to the phage, while the control peptide GRGESP did not affect binding. These results suggest that the hybrid phage fdRGD is displaying high-affinity, integrin-binding ligands that can efficiently attach cells to coated surfaces, and that cell attachment can be competitively inhibited by integrin-specific peptides.

2. **Attached with non-virulent bacteria**: Scientists have hypothesized that phages (viruses that infect bacteria) can be delivered into mammalian cells by the infected bacteria before they break open, which is called the Trojan horse hypothesis. *Mycobacterium avium* and *Mycobacterium tuberculosi*s are two bacteria that are difficult to treat with antibiotics because they can infect and grow within immune cells, and can also enter a dormant phase in the host[5.] Most antibiotics only work when the bacteria are actively replicating, which limits their effectiveness. Phage therapy, which uses phages to treat bacterial infections, has been attempted in the past with limited success. Phages are specific to certain strains of bacteria and need to be near the pathogen to be effective, which can make delivery difficult. However, a study by Sula et Al. showed that the phage DS-6A effectively killed M. tuberculosis in guinea pigs after being injected into the animals. It is possible that some *M. tuberculosis* cells were infected with the phage while outside the macrophages (the immune cells that engulf and kill bacteria), and these cells then delivered the

phage to other *M. tuberculosis* cells inside the macrophages.

- [11]Another study found that the phage TM4 was effective in killing both *M. avium* and *M. tuberculosis* when it was delivered directly into the macrophages where the bacteria were located. The phage was just as effective as macrolide antibiotics, which are frequently used to treat tuberculosis. Although the mycobacteria inhibit the fusion of phagosomes (the immune cells' "killing compartments") with lysosomes (the acidic compartments that break down cellular waste), they still retain the ability to fuse with endosomes. The study suggests that the TM4 phage may have reached the mycobacterial vacuole (the compartment where the bacteria are located inside the macrophages) through this mechanism or another unknown method. The researchers observed that the vacuoles containing both M. avium and another bacterium, M. smegmatis, had fused after four days, and were acidic like lysosomes. This suggests that the TM4 phage may have lysed the *M. avium* cells, causing the vacuole to become acidic. However, more research is needed to confirm this possibility.

3. **Liposomes**: Liposomes are tiny spherical structures made of one or more phospholipid bilayers surrounding an aqueous core. They are being extensively researched as a potential carrier system for drug delivery due to their excellent safety profile and the ability to protect a wide range of drug candidates from degradation. Liposomes are like eukaryotic cell membranes, which can help induce membrane fusion or other uptake mechanisms through surface functionalization. This provides unique opportunities to deliver phages into cells and sub-cellular compartments to target bacteria residing there[5].

- [12]Encapsulating bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 in liposomes was studied to determine their effectiveness in reducing Salmonella in poultry. The phages were encapsulated into liposomes that had a diameter between 309 to 326 nm and a positive charge ranging from +31.6 to +35.1 mV at pH 6.1. In simulated gastric fluid (pH 2.8), non-encapsulated phages decreased by 5.7 to 7.8 log units, whereas encapsulated phages were more stable with losses of only 3.7 to 5.4 log units. Liposome coating improved the retention of bacteriophages in the chicken intestinal tract. When broilers were administered cocktails of encapsulated and non-encapsulated phages, encapsulated phages were detected in 38.1% of the animals after 72 hours, whereas non-encapsulated phages were present in only 9.5%. The difference was significant. In addition, in an in vitro experiment, the cecal contents of broilers promoted the release of the phages from the liposomes. In broilers that were experimentally infected with Salmonella, the daily administration of both cocktails for 6 days post-infection resulted in similar levels of protection against Salmonella colonization. However, once treatment was stopped, the protection provided by non-encapsulated phages disappeared, whereas the encapsulated phages persisted for at least 1 week, showing the enhanced efficacy of encapsulated phages in protecting poultry against Salmonella over time. The methodology described here allows the liposome encapsulation of phages of different morphologies. The preparations can be stored for at least 3 months at 4°C and could be added to the drinking water and feed of animals. Among all carrier systems, liposomal encapsulation of active phages has been studied most widely to achieve better targeting and delivery efficiency, while also shielding phages from acid degradation and immune clearance. An unexpected bonus of this approach has been the improved bacterial killing efficiency of the liposomal phage over the unencapsulated counterparts[13]

- [12]The use of phages to treat infections caused by drug-resistant strains has been an area of interest for biomedical scientists. However, the ability of phages to act only on extracellular bacteria and the possibility of interference by anti-phage antibodies in vivo are considered important limitations of bacteriophage therapy. In this study, liposomes were used as a delivery vehicle for phages to overcome these hurdles. Anti-phage antibodies were raised in mice and pooled serum was evaluated for its ability to neutralize free and liposome-entrapped phages. The efficacy of phage and liposome-entrapped phages to enter mouse peritoneal macrophages and kill intracellular Klebsiella pneumoniae was compared. An attempt was also made to compare the efficacy of free phage and liposome-entrapped phage, alone or in conjunction with amikacin, in eradicating mature biofilm. The entrapment of phages in liposomes provided 100% protection to phages from neutralizing antibodies. On the contrary, unentrapped phages were neutralized within 3 hours of their interaction with antibodies. Compared to the inability of free phages to enter macrophages, the liposomes were able to deliver entrapped phages inside macrophages and cause 94.6% killing of intracellular *K. pneumoniae*. Liposome-entrapped phages showed synergistic activity along with amikacin to eradicate the mature biofilm of K. pneumoniae. Our study reinforces the growing interest in using phage therapy as a means of targeting multidrug-resistant bacterial infections, as liposome entrapment of phages makes them highly effective in vitro as well as in vivo by overcoming the majority of the hurdles related to the clinical use of phages.

4. **Phage with polymeric particles**: The main goal of encapsulating phages into polymeric microparticles was to protect them from being inactivated by the acidic and proteolytic environment of the stomach during oral delivery. Additionally, many of these polymeric systems could sustain the release of the phages, which increased their residence time at the site of infection, resulting in

improved therapeutic outcomes. To achieve this, both natural (alginate, chitosan, and pectin) and synthetic (polylactic-co-glycolic acid [PLGA] and EudragitÒ) biodegradable and/or biocompatible polymers were used to embed, encapsulate, adsorb, or conjugate phage. While the phage-loaded polymeric microparticles/nanoparticles' effectiveness in killing intracellular bacteria is rarely reported, they are useful against targeted bacteria with dual intracellular/extracellular life cycles, such as *S. enterica* and *S. aureus*. This review focuses specifically on two polymeric systems, alginate, and PLGA, as they have previously been used for intracellular drug delivery[5].

- Researchers attempted to combat Salmonella and *E. coli* outbreaks by incorporating bacteriophages and cinnamaldehyde onto sodium alginate emulsion-based films to give them antimicrobial properties. Results showed that increasing CNMA concentration yielded formulations with a higher amount of CNMA loaded. Sodium alginate films incorporated with EC4 and 135 phages displayed antimicrobial activity against *E. coli* and *S. Enteritidis*, respectively. Meanwhile, CNMA empowered the films with activity against both species. The combination of phages and CNMA resulted in a synergistic antimicrobial effect against *E. coli* and a facilitative effect against Salmonella. This technique holds promise for use in food packaging to prevent contamination[14].
- Jamaldin et al. Polymeric materials can be used to encapsulate bacteriophages and preserve their activity, extend their half-life, and control their release in the target environment. This study aimed to create customizable formulations of bacteriophages encapsulated in polymeric microparticles (MPs). We used a biocompatible and biodegradable polymer called poly(lactic-co-glycolic-acid) along with ammonium bicarbonate as a porogen to encapsulate bacteriophage that expresses OVA (257-264) antigenic peptide. Our findings demonstrated that the nano-engineered fdOVA bacteriophages that were encapsulated in MPs retained their structure and their immunological activity, inducing a strong immune response toward the peptide that was delivered. Furthermore, MP encapsulation prolonged the stability of bacteriophages over time, even at room temperature. Moreover, the study also revealed the ability of in silico-supported approaches to predict and regulate the release of bacteriophages. These results provide the basis for a versatile vaccine delivery system based on bacteriophages that could generate robust immune responses in a sustained manner, to be used as a platform against cancer and emerging diseases.

5. **Inorganic particles:** Inorganic nanoparticles have been used for treating bacterial infections either alone or in combination with antibiotics. These nanoparticles have multiple modes of action such as inducing oxidative stress, releasing metal ions, and non-oxidative mechanisms, which make it difficult for pathogens to develop resistance against them. Moreover, by functionalizing the surface of these nanoparticles with specific tissue-targeting moieties, the drugs can reach intracellular bacteria. Recently, phage has been conjugated to various types of inorganic nanoparticles to improve its antibacterial efficiency and extend its application to kill intracellular bacteria[5].

- The emergence of aggressive bacteria, combined with limited production of new antibacterial drugs, has led to inefficiencies in current antibiotic therapy, which poses risks to human health. Developing new antibacterial agents is a complex process due to the challenges of producing effective and safe drugs, high production costs, and long approval times, which can take up to 10-15 years. The key factors that determine vital processes such as intracellular uptake, biodistribution, and clearance are the physicochemical properties of nano-systems, which include particle size, surface charge, and solubility. Nanoparticles that are nano-meter-sized enable better drug-loading efficiency of both hydrophilic and lipophilic antibiotics, resulting in enhanced antibacterial effects. The zeta (ζ)-the potential of nano-systems and their surface charge drive interactions with proteins, tissues, or various components of the tissue, and thus affect cellular biodistribution and uptake. Host cells such as macrophages with anionic nature attract positively charged nano-systems compared to uncharged and negatively charged ones[15].

The hydrophobicity of nano-systems plays a great function in targeting drug delivery related to interactions with the phospholipid layer of the bacterial membrane. On the contrary, hydrophilic nano-systems interact less with opsonins, resulting in longer blood circulation compared with hydrophobic nano-systems. The enhanced actions of nano-systems as antibacterial drug delivery systems arise from various mechanisms, including their ability to optimize the physicochemical characteristics of entrapped antibacterial drugs, their favored accumulation near the cytoplasm, their electrostatic interactions with bacterial membrane, the high oxidizing power and production of reactive oxygen species, the prevention of unwanted interactions and protection of antibacterials against degradation, and the better clinical use of antibacterials through more patient-acceptable routes[15].

❖ Clinical Aspects of Phage therapy:

Phages have several unique features that make them effective antibacterial agents. They have a distinct mode of action that differs from antibiotics. They have a narrow range, which allows them to selectively eliminate harmful bacteria without harming normal microflora. Additionally, they can multiply at the site of infection, which enhances their effectiveness.

The first attempts to treat bacterial infections in humans with phages were made in the 1920s, just a few years after

their discovery. Since then, many uncontrolled "clinical" studies have been conducted to assess the efficacy and safety of phage therapy. However, these studies do not meet the current rigorous standards for clinical trials. Recently, the results of the first randomized controlled trials of phage therapy have been published.

Table 2: Summary of the benefits and limitations of different approaches for phage formulation.

Approach	Benefits	Limitations
Fabricated phage	Improve stability Enhanced antibacterial activity promote internalization of the intact phage into mammalian cells	Genetic modification doesn't enhance the cell penetration capability. Fabrication techniques are very complex, expensive, and difficult to scale up[16]
Liposome encapsulation	Protection of phages against in vivo conditions There have been extensive studies that demonstrate that compared to free phage, phage therapy has a therapeutic effect.	There are some issues of physical and chemical instability associated with liposomes. However, these problems can be minimized by adding antioxidants and/or freeze-drying. Another challenge with liposomes is their low loading capacity, which makes them less effective as an antibiotic delivery agent. To overcome this challenge, it is necessary to maximize the electrostatic attractions between liposomes and antibiotic molecules that have an opposite charge. [15]
Non-virulent bacteria	Efficient carrier for delivering anti-mycobacterial phages intracellularly.	reports on soft-tissue infections caused by this bacterium were occasionally reported. Therefore, its bacterial nature and associated mycobacterial antigens might pose a major safety concern in clinical settings[5].
Polymeric particles	Dual-responsive polymeric micelles. Better uptake of micelles by human astrocytes due to the presence of TAT.	Limited knowledge of dual-responsive micelles in anti-cancer drugs[15].
Inorganic particles	Inorganic nanoparticles induce oxidative stress, release metal ions, and operate through non-oxidative mechanisms. These properties hinder pathogen resistance.	The use of phage-conjugated AgNPs could potentially enhance the internalization of phages and act synergistically or additively to eliminate intracellular pathogens. However, safety concerns are associated with the therapeutic application of AgNPs[5].

Phage therapy has been performed on a relatively small scale in Poland for over 80 years. The first paper on the therapeutic use of bacteriophages was published in Poland in 1923. The Institute of Immunology and Experimental Therapy (IIET) in Wrocław, established by Professor Ludwik Hirszfeld in 1952, played a crucial role in the development of clinical phage therapy in Poland. They produced therapeutic phage preparations and coordinated PT conducted at different Silesian hospitals. In the 1980s and 1990s, about 2000 patients were treated with phage preparations produced by the IIET. The results of PT performed in Poland during this period were published in several articles, which are of primary importance to the English-language literature on clinical PT.[17]

- *Pharmacological aspects:*

Clinical studies have demonstrated the effectiveness of phage therapy. A study by Mie˛dzybrodzki et al. revealed that patients suffering from *Staphylococcus aureus* infections showed positive results, with bacterial eradication or improvement in patient health in over 36% of the cases. Similar studies have also shown the recovery of patients with orthopedic infections when phages were orally or topically administered. Phages can be considered selectively toxic antibacterial agents and are more targeted than antibiotics, which exhibit less specificity towards bacteria. This makes phage therapy advantageous as it also has minimal impact on the microbiome of the patient. The discovery and manipulation of the phage genetic elements are easier due to the technological tools available today.

The Pharmacokinetics of phages demonstrates the benefit of using phages compared to antibiotics as they are specific to the target bacteria. In other words, phages travel in the system only to adsorb onto the specific host where they exhibit their action, without affecting non-targeted bacterial strains and tissues. Moreover, the narrow host range of bacteriophages reduces the chances of a possible secondary infection because it will not affect non-target bacteria [18]

- *Immunological aspects:*

The immunological responses of the human body after exposure to phages by ingestion or presence in the bloodstream are important aspects of phage therapy. The immune system reacts to the presence of foreign particles, including microbial viruses. There is growing evidence that bacteriophages are part of the healthy human microbiota/microbiota, and many studies are evaluating their role in the microbiome.

It is unclear whether phages alter the immune response, including the clearance of microbial viruses. While bacteriophages are larger than some eukaryotic viruses, it is not clear whether they induce immune responses or how they interact with the immune system. However, immunization of humans or animals by injecting phages into their system has produced antibodies against phages, suggesting that natural antibodies may also be present in the human body for T-like bacteriophages[18].

[19]Research has shown that phages can interact with immune cells/mammalian cells and can stimulate innate and adaptive immune responses. Bacteriophages have been used as vaccine vehicles and have shown to be safe candidates for the immunization of patients with antibody-mediated immunodeficiency. However, it is critical to consider aspects of immunity when administering phage therapy, as the mode of administration can reduce the anti-phage response of patients.

Table 3: Summary of published results of randomized controlled trials of phage therapy.

Aim of the trial	Organizer/ sponsor	Time of trial	comments	Reference
A prospective, randomized, double-blind controlled study of WPP-201 for the safety and efficacy of treatment of venous leg ulcers	Southwest Regional Wound Care Center in Lubbock, Texas	September 2006–May 2008	Researchers conducted a Phase I study of a cocktail of eight lytic bacteriophages, WPP-201, developed by Intralytix Inc. The phage cocktail contained around 1x10^9 PFU/ml of each phage, and it was used topically on patients with full-thickness venous leg ulcers that had lasted for more than 30 days. Although WPP-201 did not have a significant effect on ulcer healing, no adverse events were reported by the patients after the phage administration.	20
A single-center, randomized, placebo-controlled trial on the safety and bioavailability measure of oral phage	Nestle´ Research Center, Nestec Ltd., Lausanne, Switzerland	June 2003	15 healthy volunteers were given purified T4 phage or a placebo in drinking water. No adverse events or significant changes in *E. coli* population were observed. Phages were detected in the stools of all volunteers given phage preparations, but not in their blood.	21
A double-blind, placebo-controlled initial phase I/II clinical trial targeting chronic ear infections caused by P. aeruginosa	Biocontrol Ltd., London, UK	July 2006–November 2007	Twelve patients with antibiotic-resistant *P. aeruginosa*-related otitis media were given either phages or a placebo. The bacteriophage-treated group showed a significant reduction in clinical symptoms and a 76% decrease in bacterial count after 42 days. No adverse events were reported.	22

❖ **Facet of Bacterial drug resistance plus Phage therapy from Ethical point of view**:

Drug resistance is a growing threat to global public health caused by drug distribution. It is an ethical issue and a matter of international justice. Medicine has shifted focus to chronic illnesses while the pharmaceutical industry focuses on profitable drugs for allergies, depression, and lifestyle issues. The lack of economic incentives has resulted in decreased research and development for antibiotics and vaccines. Infectious diseases like AIDS and tuberculosis have re-emerged and claimed millions of lives yearly. Medical research focuses on the wants of a minority of the world's population[17].

RESISTANCE: Drug resistance is a sign of our mishandling of disease-fighting drugs. Overuse in developed nations and underuse in developing nations has resulted in less effective drugs. The lack of new antimicrobial and vaccine development may come back to haunt us all, reversing medical progress. The emergence and spread of drug-resistant microbes may be passed on to others of the same or different species, driven by antibiotic usage.

The issue of drug resistance raises ethical concerns about the distribution of resources on two levels. Firstly, drug resistance affects the debate over access to existing drugs. Drug resistance reveals that markets do not promote utility in the context of antimicrobials, and this is a concern for the distribution of drugs. Secondly, drug resistance affects the debate over the allocation of research resources. Patents fail to provide incentives for the development of knowledge necessary for addressing the most significant medical problems in the world, which is another concern for drug distribution[23].

The development of new antimicrobial agents and drugs requires significant funds and decisions based on sound scientific data and public interest. A partnership between the private and public sectors may be the best approach, allowing for shared risks and benefits such as market exclusivity, tax incentives, and expedient approval

processes. The Orphan Drug Act, which provides extended tax credits and guaranteed market exclusivity for developers of drugs for rare conditions, is a useful model to follow.

Moreover, developing and using alternative therapies may provide an essential avenue for interrupting the cycle of resistance and obsolescence associated with new antimicrobial use. The authors conclude that immediate implementation of long-term solutions that do not rely on market forces to govern the production and consumption of antimicrobials is necessary[17]. Given the severity of drug resistance, relying on market mechanisms and the patent incentive system for solutions is not a viable option. Solutions will require governmental intervention and funding. However, the problem of drug resistance is global, and there is no global government, which is troubling. Therefore, the socialization of antimicrobials on a global scale is necessary. We cannot rely on profit-oriented commercial enterprises, patent incentives, and market mechanisms for the provision of treatment for infectious diseases. Different kinds of goods require different systems of economic distribution, and public goods warrant special treatment[23].

❖ Scope of Phage therapy:

1) **Phage for treating bacterial infections**: The emergence and increasing prevalence of multidrug-resistant bacterial pathogens have posed a significant challenge in the field of medicine, emphasizing the need for new and innovative antimicrobial strategies. While traditional antibiotics have been effective in the past, the rise of antibiotic-resistant bacterial strains has led researchers to investigate alternative methods of treatment. One such method is the use of lytic phages, which kill their host by amplifying and releasing progeny phages into the environment. However, in a recent study, researchers have explored the use of nonlytic phages as a potential alternative for combating bacterial infections by delivering DNA-encoding bactericidal proteins directly to the bacteria.

To test the concept of using phages as a lethal-agent delivery vehicle, the researchers used the M13 phagemid system and the addiction toxins Gef and ChpBK. The phage delivery of lethal-agent phagemids was found to significantly reduce the number of target bacteria by several orders of magnitude in vitro and a bacteremic mouse model of infection. This promising technology holds potential for use in antimicrobial therapies and DNA vaccine development, especially given the powerful genetic engineering tools available and the present knowledge in phage biology[9].

2) **Phage defends mucosal surfaces against bacterial infections:**

Bacteriophages, also known as phages, are helpful in defending mucosal surfaces against bacterial infections. However, their interactions with their bacterial hosts and the mucus-covered epithelium are not well understood. In our previous research, we found that T4 phages with Hoc proteins on their capsids can adhere to mucin glycoproteins and protect mucus-producing cells in vitro. This led us to propose the bacteriophage adherence to mucus (BAM) model of immunity.

To test this model, [7] developed a microfluidic device or chip, which mimics a mucosal surface that is constantly experiencing fluid flow and mucin secretion dynamics. We used mucus-producing human cells and Escherichia coli in the chip and observed similar accumulation and persistence of mucus-adherent T4 phages and non-adherent T4Δhoc phages in the mucus. Despite this, T4 phages reduced bacterial colonization of the epithelium by over 4,000 times compared to T4Δhoc phages. This suggests that phage adherence to mucus increases encounters with bacterial hosts via some other mechanism.

Phages are typically thought to depend entirely on normal diffusion, driven by random Brownian motion, to contact their hosts. However, we found that T4 phage particles displayed sub-diffusive motion in mucus, while T4Δhoc particles displayed normal diffusion. We conducted experiments and modeling and discovered that sub-diffusive motion increases phage-host encounters when bacterial concentration is low. By concentrating phages in an optimal mucus zone, sub-diffusion increases their host encounters and antimicrobial action.

Our revised BAM model proposes that the fundamental mechanism of mucosal immunity is sub-diffusion, resulting from phage adherence to mucus. These findings suggest interesting possibilities for engineering phages to manipulate and personalize the mucosal microbiome[7].

3) **Phage can prevent the lung infections in cystic fibrosis patients:**

Due to the increase in antibiotic resistance, there is a need for non-antibiotic therapies to treat infections. This is particularly true for patients with cystic fibrosis who are infected with Pseudomonas. Two bacteriophages (bacterial viruses) have been identified that can kill Pseudomonas in human lung cells and animal models of lung infection. Bacteriophages are an ideal choice because they can replicate on the target cell, producing more bacteriophages. Using two bacteriophages reduces the risk of resistant colonies developing at the site of infection. [6]This study demonstrates the efficacy of bacteriophages in validated infection models. Pseudomonas aeruginosa is a common cause of lung infections in patients with cystic fibrosis. Biofilm formation and antibiotic resistance of Pseudomonas are major challenges that can complicate antibiotic therapy. This study evaluated the effectiveness of using bacteriophages to kill Pseudomonas in both biofilms and in murine lungs. Two phages were isolated and characterized from a local wastewater treatment plant. Both phages were effective against clinical isolates of P. aeruginosa. The two phages together killed all nine clinical isolate strains tested, including both mucoid and nonmucoid strains. An equal mixture of the two phages

was effective in killing P. aeruginosa strains when growing as a biofilm on a cystic fibrosis bronchial epithelial CFBE41o- cell line. Phage titers increased almost 100-fold over 24 hours, confirming replication of the phage. Furthermore, the phage mixture was also effective in killing the pathogen in murine lungs containing 1x107 to 2x107 P. aeruginosa. Pseudomonas was effectively cleared (reduced by at least 3 to 4 log units) from murine lungs in 6 hours. This study supports the growing interest in using phage therapy for the treatment of multidrug-resistant Pseudomonas lung infections in cystic fibrosis patients.

4) Phage can fight food contamination:

Despite the implementation of good processing practices in food companies and the appropriate washing of food products by consumers, outbreaks of Salmonella and Escherichia coli continue to occur. [14]This study explores the use of different combinations of bacteriophages (phages) and cinnamaldehyde (CNMA) on sodium alginate emulsion-based films to make them antimicrobial against S. Enteritidis and E. coli. The films were prepared by casting and characterized in terms of thickness, moisture content, water vapor permeability (WVP), swelling index (SW), chemical interactions by FTIR, surface morphology by SEM, and antimicrobial performance. Results showed that phage incorporation was not compromised by CNMA and that increasing CNMA concentration yielded formulations less heterogeneous and had a higher amount of CNMA loaded.

Films characterization revealed that in general, phage incorporation did not introduce significant changes in film parameters while the presence of CNMA increased the roughness, thickness, and swelling ability of films. Sodium alginate films incorporated with EC4 and 135 phages displayed antimicrobial activity against E. coli and S. Enteritidis, respectively, while CNMA empowered the films with activity against both species.

The combination of both phages with a higher concentration of CNMA resulted in a synergic antimicrobial effect against E. coli and a facilitative effect against Salmonella. Overall, the incorporation of EC4 and 135 phages, together with CNMA on alginate emulsion-based films, holds great potential for use in food packaging to prevent food contamination.

5) Phage with nanotechnology:

According to recent reports from the World Health Organization, antibiotic resistance is becoming increasingly prevalent among bacteria, posing a significant threat to human health. The current antibacterial therapies are often ineffective due to poor solubility, stability, and side effects. This has prompted researchers to explore new and innovative strategies to combat these resilient microbes.

As a result, there is a high demand for novel antibiotic delivery systems. Nanotechnology has emerged as a promising solution due to its unique physicochemical properties, drug-targeting efficiency, enhanced uptake, and biodistribution. In this review, we will focus on the recent applications of both organic and inorganic nanosystems in the domain of antibacterial delivery.

Organic nanosystems, such as liposomes, lipid-based nanoparticles, polymeric micelles, and polymeric nanoparticles, have been widely studied for their antibacterial delivery potential. These systems offer a variety of characteristics that make them suitable for antibacterial delivery, including their biocompatibility, biodegradability, and low toxicity.

Inorganic nanosystems, such as silver, silica, magnetic, zinc oxide (ZnO), cobalt, selenium, and cadmium, have also shown promise in antibacterial delivery. These nanosystems offer unique physical and chemical properties that enable them to act as effective antibacterial agents.

In addition to discussing the characteristics of each nanosystem, we will highlight recent promising innovations used to overcome antibacterial resistance. These include the use of lipid polymer nanoparticles, nonlamellar liquid crystalline nanoparticles, anti-microbial oligonucleotides, smart responsive materials, cationic peptides, and natural compounds. By providing a comprehensive overview of the latest research in this field, we hope to contribute to the development of more effective antibacterial therapies[15].

❖ **Future Aspects:**

Phages, unlike antibiotics, have a wider range of mechanisms of action and can often be safer. However, incorporating phage therapy into existing regulatory practices and economic models for the distribution and use of antibacterial agents is a challenge. Phage therapy is more successful in regions with friendly regulatory climates and where phage-product suppliers receive minimal funding, such as in Poland and the former Soviet Union. As Western scientists and companies publish more well-controlled phage therapy studies, phage therapy is expected to gain increasing demand as an antibacterial agent[24]. However, the regulatory climate and primary economic models for drug development in the USA can be less favorable for phage therapy. In the next 5-10 years, phage therapy is expected to become prevalent in Western medicine to treat chronic bacterial infections that are resistant to current antibacterial drugs. Phage therapy is already being used in Poland within the EU. Over a longer period, phage therapy may have a larger role as an alternative, narrow-spectrum antibacterial treatment, especially given the risks associated with disruption of the human microbiome from broad-spectrum antibacterial agents. In this context, phage cocktails that can be modified as needed may be useful.

CONCLUSION

Phages are becoming a compelling alternative to chemical antibiotics. Proper phage selection, effective formulation, and greater clinician understanding can manage all concerns associated with phage therapy. In an era where

antibiotic-resistant bacterial infections are on the rise, phages provide numerous advantages with relatively few disadvantages[25]. Phage therapy is limited by narrow host range, cytotoxicity, and immunogenicity. Patenting and regulatory issues also hinder investment from pharmaceutical companies. Genetic manipulation through recombineering techniques can address these limitations by shaping biological properties of phages to increase efficacy and safety, while also solving patenting issues and increasing interest from larger companies. Alternative options should also be considered[26]. Due to the absence of well-controlled clinical trial data and complex regulatory frameworks, most recent human data generation has been on a single-patient compassionate use basis. Most data include the use of antibiotics, making it challenging to draw solid conclusions about the effectiveness of phages alone. [3] However, compassionate use of human data does support the exploration of the combination of phages and antibiotics, which is a promising avenue for near-term clinical development. Recent advancements in phage genomics, purification, and formulation have significantly contributed to improving the efficacy and reliability of phage therapy. However, there are still knowledge gaps regarding the appropriate routes of administration, phage selection, frequency of administration, dosage, phage resistance, and pharmacokinetic and pharmacodynamic properties of phages. Additionally, phages require a thorough investigation into the immunologic response they may elicit. The advancement of other technologies that support phage therapy, such as rapid and accurate phage assessment methods and criteria, standardization of phage manufacturing, phage banking, phage product stability during storage and transport, and new quantitative methods that allow for precise monitoring of pharmacologic parameters of phages, will need to follow suit and evolve. It is possible that some may decide the transition to phage therapy cannot be made due to the lack of necessary capabilities or financial resources or because of the belief that chemical antibiotics are superior and can defeat the resistance problem. Redoubling efforts to enhance ineffective antibiotics have, in many cases, succeeded in improving their performance.

Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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