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Review article

Paving the way for phage therapy using novel drug delivery approaches

Thomas Briot [a,*], Camille Kolenda [b,c,d], Tristan Ferry [c,d,e], Mathieu Medina [b,c,d], Frederic Laurent [b,c,d], Gilles Leboucher [a], Fabrice Pirot [f,g], On behalf of the PHAGEinLYON study group

[a] Pharmacy department, Hospices Civils de Lyon, Groupement Hospitalier Nord, Lyon, France
[b] Laboratory of bacteriology, French National Reference Centre for Staphylococci, Hospices Civils de Lyon, Lyon, France
[c] Reference Center for Complex Bone and Joint Infection (CRIOAc), Hospices Civils de Lyon, Lyon, France
[d] International Centre for Research in Infectiology, INSERM U1111, Université Claude Bernard Lyon 1, Lyon, France
[e] Infectious and Tropical Diseases unit, Croix-Rousse Hospital, Hospices Civils de Lyon, Lyon, France
[f] Plateforme FRIPHARM, Service pharmaceutique, Groupement Hospitalier Edouard Herriot, Hospices Civils de Lyon, Lyon, France
[g] Laboratoire de Recherche et Développement de Pharmacie Galénique Industrielle, Plateforme FRIPHARM, Faculté de Pharmacie, Laboratoire de Biologie Tissulaire et Ingénierie Thérapeutique - UMR 5305, Université Claude Bernard Lyon 1, Lyon, France

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ABSTRACT

Bacterial resistance against antibiotics is an emergent medical issue. The development of novel therapeutic approaches is urgently needed and, in this context, bacteriophages represent a promising strategy to fight multi resistant bacteria. However, for some applications, bacteriophages cannot be used without an appropriate drug delivery system which increases their stability or provides an adequate targeting to the site of infection. This review summarizes the main application routes for bacteriophages and presents the new delivery approaches designed to increase phage's activity. Clinical successes of these formulations are also highlighted. Globally, this work paves the way for the design and optimization of nano and micro delivery systems for phage therapy.

1. Introduction

Phages (short for bacteriophages) were described in 1896 by Ernest Hankin and in 1915 by William Twort. They are considered as non-living organisms, are largely ubiquitous in the environment, and constitute the most abundant organisms on Earth [1]. In 1917, Felix d'Herrelle classified phages as a class of viruses able to infect and kill bacteria. They are generally specific of a bacterial species and are unable to infect eukaryotic cells, even though their effect on mammalian immunity is not fully elucidated [2].

Most phages consist of a protein capsid surrounding a nucleic acid (RNA or DNA) and a tail. For example, the capsid size ranges from 45 to 185 nm among the members of the *Caudovirales* order [3]. Two different life cycles have been described: the lytic and lysogenic cycles. For both of them, infection begins with the attachment to bacteria and the injection of genetic material into the host. In the lytic (virulent) cycle, the phage genome is injected into the cytoplasm of the host bacterium and the cell machinery is used to manufacture phage proteins. Once phages replicate, the bacterium is destroyed by phage endolysins, and new virions are released in the external medium [4]. In the lysogenic cycle (temperate), the viral genetic material is incorporated into the bacterial chromosome with whom it is replicated, and then transferred to daughter bacteria without lysis of the host (Fig. 1).

At the beginning of the 20th century, phages were used to treat various infectious diseases, mainly bacterial diarrhoea by oral intake, or skin and soft-tissue suppurations by local application. However, due to the discovery of antibiotics, and the advances in research and their chemical synthesis, phage therapy was forsaken except in some countries of the USSR bloc, notably Georgia [5].

Currently, the emergence and progression of bacterial resistance against antibiotics have become an emergent issue, responsible for more than 700,000 deaths per year, and estimated to increase to 10 millions per year after 2050 [6]. Research on antibiotics is neglected, and the antibiotics pipeline is quite stagnant, as very few new drugs become available each year. In this context, phages may represent a promising therapeutic approach, especially in combination with antibiotics, as they have a synergistic activity [7]. In addition, phages are generally recognized as safe in clinical applications, without significant specific

* Corresponding author.
E-mail address: thomas.briot@chu-lyon.fr (T. Briot).

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adverse reactions described [8].

Some phages also present polysaccharide depolymerases, a class of enzymes able to disrupt bacterial biofilms, which constitutes a barrier to antibiotic activity and favours the resistance to antibiotics. This specific mechanism of action can be used in synergy with antibiotics to overcome bacterial resistances [9–11]. An increasing number of studies and reports on clinical cases on the subject were published, highlighting the therapeutic potential of this approach [12,13].

Despite their abundance in the environment and their easy amplification, phage therapy development is limited by the absence of clear guideline defining the manufacturing process necessary to obtain pharmaceutical-grade phage suspensions, including the selection of bacterial strain for production, purification, and quality controls [14,15]. Indeed, the regulatory status of phages has not been harmonized worldwide, and no phage product is currently approved for human use by the European Medicines Agency (EMA) or the Food and Drug Administration (FDA). EMA considers phages as medicinal products, but some differences exist within Europe. For example, in Belgium, phages are indicated as Active Product Ingredients (API), and as such a monography is present in the Belgium Pharmacopeia [16].

Currently, phages are mostly used as compassionate therapy and the therapeutic evaluation is mainly based on clinical reports comporting numerous biases [14]. Randomized controlled trials are relatively rare, although urgently required. In April 2022, on the clinicaltrials.gov website, 43 clinical trials including the term “bacteriophages” were registered in the interventional study category. Among these 43 clinical trials, only 27 really concerned bacteriophage therapy and were not under the unknown or withdrawn status (Table 1).

Moreover, phage utilization is not evident, and a careful consideration of this protein material has to be observed, as it could be an unstable material. For example, the clinical trial Phagoburn developed to test phages in the context of cutaneous infectious was unable to conclude and not completed due to the instability of phages in a cocktail formulation [17]. Indeed, patients were treated with a lower than expected dose that was actually too low and considered as ineffective to fight against bacterial infection on wound. This example highlights the urgent need to combine the research on phages with development on pharmaceutical formulations able to guarantee their stability, ensuring high phage titres. Moreover, some anatomic regions are not accessible by phages administered in liquid suspension, thus more appropriate and sophisticated delivery systems are required.

In this context, nanomedicines, defined as the application of nanotechnology to medicine, can be tailored to encapsulate them.

Taking advantage of the large knowledge generated using drug delivery nanosystems in the field of oncology and infectious diseases, one could now adapt and design nanotechnology systems for phage therapy, thereby overcoming the limitations related to phages and facilitate their use. For example, during the COVID-19 crisis, nanotechnology has played a pivotal role in the vaccination strategy. Indeed, lipid nanoparticles stabilize mRNA and facilitate the delivery of genetic material to the target site, which has led to the development of two mRNA-based vaccines (BNT162b2 and mRNA-1273) for which the efficacy reached 95% in phase III clinical trials [18].

The aim of this review was to present the current landscape in terms of pharmaceutical formulations available for phage therapy in infectious disease contexts, with a particular focus on the administration routes. Their advantages and disadvantages were detailed, and innovative nanomedicines delivery systems were also presented as a promising approach for phage therapy.

2. Pharmaceutical formulations to protect phages

2.1. To prolong the shelf-life

2.1.1. Overview

One of the main issues of phage-based therapies is their instability, which is related to their own biological properties, as well as to external environmental factors. In buffered media, single-phage suspensions are generally stable for years. Several internal (capsid size, tail length, contractile capability, etc.…) and external (high temperatures, acidity or alkalinity, salinity, etc.…) parameters can affect their stability, thereby causing phage titre reduction or loss of infectivity [19–21]. Environmental factors have thus to be controlled to preserve the high titre of phage products required for therapeutic purposes [22]. Phages are usually administered within a cocktail, corresponding to an association of several phages to get a better activity [23]. Indeed, under the

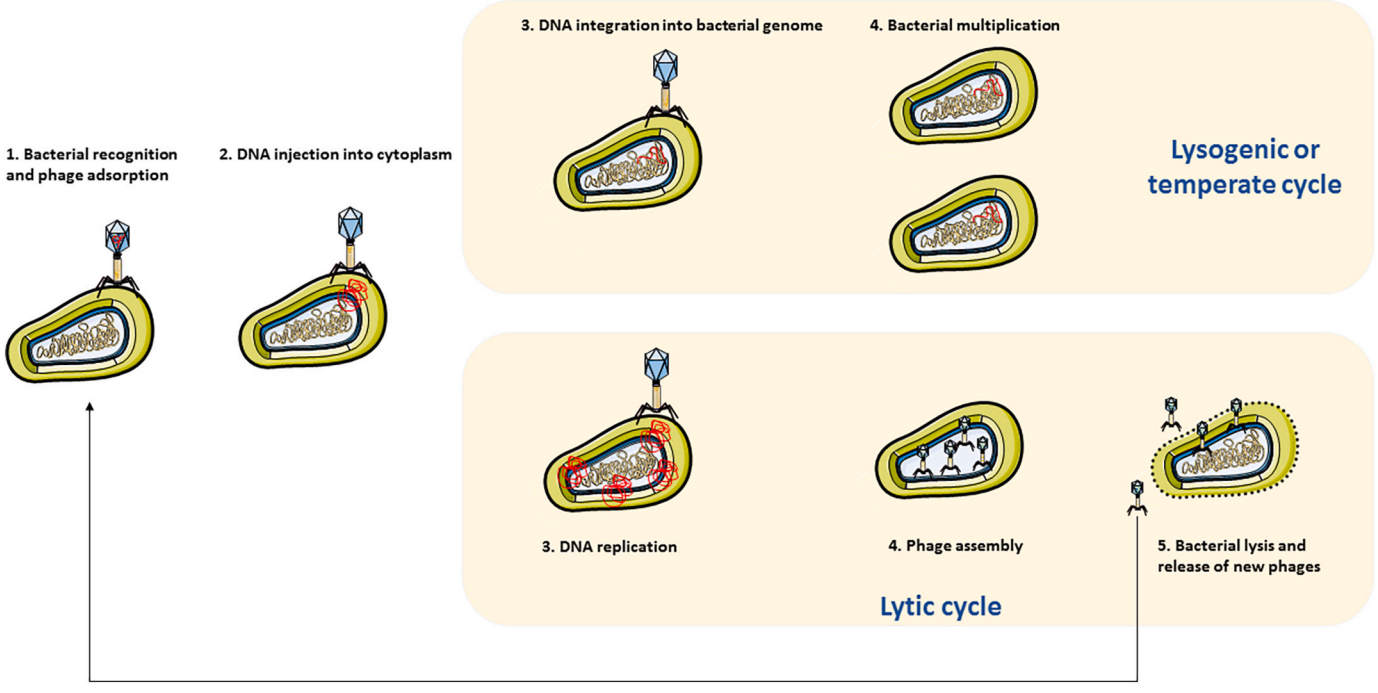

Fig. 1. Schematic representation of the lytic cycle and lysogenic (temperate) life cycle of phages.

Table 1
Clinical trials concerning phage therapy and interventional studies extracted from clinicaltrials.gov in April 2022.

Administration route	Pharmaceutical formulation	Phages	Pathology	Phase	Status	NCT number
Oral	Capsules	PreforPro (commercial bacteriophage product)	Mild gastrointestinal symptoms	Not applicable (Prebiotic)	Completed	NCT04511221
Oral	Suspension administered with sodium bicarbonate solution	A cocktail of lytic Shigella-specific bacteriophages	Shigellosis	1 2	Not yet recruiting	NCT05182749
Oral	Capsule	PreforPro (commercial bacteriophage product)	Gastrointestinal disorder	Not applicable (Prebiotic)	Completed	NCT03269617
Oral	Suspension	T4 phage cocktail (Anti-*E. Coli*)	Diarrhoea	Not applicable	Terminated	NCT00937274
Oral	Suspension	Cocktail	Crohn's disease	1 2	Recruiting	NCT03808103
Oral	Suspension	Cocktail of anti-*P. aeruginosa*	Oropharyngeal decontamination in patients under invasive mechanical ventilation	Not applicable	Recruiting	NCT04325685
Oral	Suspension	Cocktail of anti-*K. pneumoniae*	Healthy volunteers	1	Completed	NCT04737876
Oral	Suspension	Anti-*E. Coli*	Bacteremia	1	Not yet recruiting	NCT05277350
Oral (nasogastric tube)	Suspension (Faecal filtrate transfer)	Viruses, including bacteriophages	Necrotizing enterocolitis	1	Not yet recruiting	NCT05272579
Intravenous	Suspension	Cocktail of anti-*S. aureus*	Bacteremia	1 2	Not yet recruiting	NCT05184764
Intravenous Intravesical	Suspension	Anti-*E. coli* or *K. pneumoniae*	Urinary tract infections (neurogenic bladder and complicated urinary tract infections with risk of recurrence) due to *E. coli* and *K. pneumoniae*	1 2	Recruiting	NCT04287478
Intraarticular (after DAIR procedure)	Suspension	Anti-*S. aureus, Staphylococcus epidermidis, Staphylococcus lugdunensis, Streptococcus* spp., *Enterococcus faecium, Enterococcus faecalis, E. coli, P. aeruginosa, and/or K. pneumoniae*	Prosthetic joint infections (hip or knee)	1 2	Not yet recruiting	NCT05269121
Intraarticular (after DAIR procedure)	Suspension	Anti-*S. aureus, S. epidermidis, S. lugdunensis, Streptococcus* spp., *E. faecium, E. faecalis, E. coli, P. aeruginosa, and/or K. pneumoniae.*	Prosthetic joint infections (hip or knee)	2 3	Not yet recruiting	NCT05269134
Pulmonary (nebulized)	Suspension	Liquid piobacteriophage complex (anti-staphylococcus, enterococcus, streptococcus, *E. coli, Proteus vulgaris, Proteus mirabilis, P. aeruginosa, K. pneumoniae, and Klebsiella oxytoca*)	Acute tonsillitis	3	Active, not recruiting	NCT04682964
Pulmonary (nebulized)	Suspension	Cocktail of bacteriophages (BX004-A)	Cystic fibrosis with chronic *P. aeruginosa* pulmonary infection	1 2	Not yet recruiting	NCT05010577
Pulmonary	Suspension	Cocktail (Anti-*P. aeruginosa*)	Cystic fibrosis with chronic *P. aeruginosa* pulmonary infection	1 2	Recruiting	NCT04596319
Pulmonary (nebulized)	Suspension	YPT-01 (Anti-*P. aeruginosa*)	Cystic fibrosis with chronic *P. aeruginosa* airway infections	1 2	Recruiting	NCT04684641
Topical	Sterile compress dressings impregnated with a phage solution	Anti-*Staphylococcus*	Diabetic foot	1 2	Not yet recruiting	NCT02664740
Topical	Suspension	Cocktail: Anti-*P. aeruginosa,* anti-*S. aureus* anti-*Acinetobacter*	Diabetic foot ulcer	1 2	Recruiting	NCT04803708
Topical	Gauze pads saturated with the phage cocktail	Cocktail of anti-*S. aureus*	Healthy volunteers	1	Completed	NCT02757755
Topical	Gel	Cocktail of anti-*S. aureus*	Atopic dermatitis	1 2	Not yet recruiting	NCT05240300
Topical (spray)	Suspension	Anti-*Stapylococcus aureus*, *P. aeruginosa,* or *K. pneumoniae*	Wound infection (burned patients)	1	Not yet recruiting	NCT04323475
Topical (spray)	Microcapsule (obtained with an amino acid-based biodegradable polymer)	Cocktail of 14 bacteriophages against *S. aureus*, *P. aeruginosa,* or *K. pneumoniae*	Pressure ulcer	1 2	Not yet recruiting	NCT04815798
Topical (via an ultrasonic debridement device)	Suspension (phosphate buffer)	Cocktail of 8 against *S. aureus*, *P. aeruginosa,* or *E. coli*	Venous leg ulcers	1	Completed	NCT00663091
Topical ± Intravenous	Suspension	Anti-*S. aureus*	Diabetic foot osteomyelitis	2	Recruiting	NCT05177107
Intraurethral	Suspension	Cocktail of anti-*E. coli*	Urinary tract infections	1	Completed	NCT04191148
Intravesical bacteriophage	Suspension	Anti-*P. aeruginosa* (Pyophage cocktail, commercially available). Not produced under good manufacturing practice conditions	Complicated or recurrent urinary tract infections	2 3	Completed	NCT03140085

Clinical trials with an "Unknown status" or "Withdrawn" status were rejected.
Abbreviations: DAIR, debridement, antibiotics and implant retention; NCT, national clinical trial.

pressure of phages, bacteria develop multiple strategies to prevent phage infections, and a resistance to phages may emerged [24]. The administration of a cocktail of phages can be effective in preventing bacterial resistances and improving clinical outcomes.

However, cocktail-phage suspensions may be less stable than single-phage suspensions, as observed in the Phagoburn clinical trial. This instability is an important limitation for pharmaceutical preparations and further clinical applications, especially in the case of standard preparations, leading to difficulties in developing standardized industrial formulations [25].

2.1.2. Pharmaceutical formulations developed

To counteract the instability due to environmental factors, several strategies have been developed. Some of them rely on the i) modification of the viscosity of the medium to maintain the phage morphology, using gelatin or the ii) modification of the osmotic pressure by adding ions (sodium chloride) or buffer (phosphate buffered saline) or iii) use of dry formulations of phages, obtained by freeze-drying or spray-drying [15,21,26,27]. However, spray-drying induces shear-stress to the raw material requiring careful processing, and freeze-drying may affect protein conformation [28].

When freeze-drying or spray-drying processes are used for the production of phage formulations, as for other protein structures, the preservation of conformations and the ability of the product to be completely dissolved after reconstitution have to be taken into careful consideration. Sugars (mainly disaccharides) can help to maintain the protein structure during high-temperature transition and desiccated phases [29,30]. In this perspective, the effect of various cryoprotectants on phage stability during freeze-drying have been previously studied [31]. More precisely, the impact of different excipients (such as glucose, sucrose, mannitol, sorbitol, gelatin, polyethylene glycol (PEG),) on phage stability (anti-*E. coli*, anti-*Klebsiella*, anti-*Enterobacter*) and the capability of phages to replicate after freeze-drying and reconstitution were studied. Using an association of gelatin and sucrose, *E. coli* and *Enterobacter* phage stability was maintained up to 20 months in a dry form, and after reconstitution, the phage activity was recovered (the phage titre decrease was less than 1-unit log).

When stored in dry formulations, phages are susceptible to the relative humidity (optimal relative humidity range: 4–6%) and temperature variations affect their titre and infectivity [27,32,33]. To overcome the issues related to spray-drying or lyophilization technics, Gonzalez-Menendez et al. have developed alginate capsules and microparticles to encapsulate four different *Staphylococcus* phages [21]. The capsules (5 mm) were able to protect from degradation one of the four phages tested, for which the titre was retained for six months at 4 °C. For the three other phages, a decrease in titre was observed after three months. When encapsulated in alginate micro-particles, the titres were more stable as a reduction of less than 1 to 1.5-unit log after three months at 4 °C was observed. Petsong et al. developed microparticles to encapsulate a cocktail of three phages targeting *Salmonella* by mixing whey proteins and trehalose. The mixture was then freeze-dried and stored at 4 °C. After three months, the titre decrease was less than 1-unit log [34].

In summary, some pharmaceutical strategies to optimize the storage of individual phages have been investigated. However, optimizing storage conditions for cocktails of phages remains a challenge, although cocktails are preferred for clinical applications.

2.2. To protect phages from harsh environments

2.2.1. Oral administration

2.2.1.1. Overview. Gastrointestinal infections are the most frequent infectious diseases, and are responsible for a high mortality worldwide, with an estimated number of 1.9 million deaths each year [35]. The oral administration of phages has thus been evaluated to treat locally gastrointestinal infections. The administration was safe and did not modify the faecal microbiota in healthy controls and in patients (diarrhoea due to *E. coli* infection), thereby opening the way for their application for gut infections [36–38]. In addition, faecal phage detection was correlated with the phage dose orally administered, suggesting a passive gut transit [37]. However, in another randomized clinical trial including paediatric patients, phage therapy failed to control acute diarrhoeas and no phage replication was observed [38]. This clinical failure could be explained by the high sensitivity of phages to acidic conditions and an important loss of infectivity within few minutes in the stomach [39]. Finally, a decrease in the phage concentration along the gastrointestinal tract or a too weak intestinal host titre limiting the phage replication were probably the causes of clinical failures of oral phage treatments.

After oral or rectal administration, phages were not detected in liver, suggesting that their bioavailability after an enteral administration is limited, restraining oral phage administration for systemic infections [40]. In fact, the intestinal mucus constitutes a physical barrier, mainly due to the presence of glycoproteins such as mucin acting as a size-exclusion filter due to its mesh size of 200–500 nm, and thus prevents the organism from intoxication and infections due to foreign substances, including exogenous phages [41]. Moreover, intestinal peristalsis and clearance can prevent drug activity, contributing to their rapid elimination and affecting the residence time of delivery devices [42]. In rat animal model, the regular mean transit time is 80 min, 3 h, and 11 h in the jejunum, small intestine, and total gastrointestinal, respectively [43]. In this scenario, the use of phages to treat intracellular (enterocyte) infections could thus be very limited.

On the other hand, some phages are naturally present and resident in the gut (namely phagome) and adhere to the mucus, protecting epithelial cells from invasive bacteria [44,45]. This gastrointestinal phagome is investigated as it is involved in the regulation of commensal bacteria. It is assumed that it is also involved in inflammatory intestinal diseases in which the bacteriome is altered, suggesting a disturbance of the gut phagome [46,47]. Phage therapy, via an oral administration, could be considered to treat gastrointestinal inflammatory diseases. However, on the Clinicaltrials.gov database, a single clinical trial is registered (in the recruiting phase) for oral phage therapy (NCT03808103) for the treatment of Crohn's disease (Table 1), highlighting the interest of studying the phagome in the context of inflammatory intestinal diseases and also confirming the difficulties in using phages via enteral administration.

Despite the challenges related to this mode of administration, the oral route is the preferred and most convenient one, as it offers several advantages such as the possibility of self-administration, the high patient compliance, and the avoidance of sterile conditions for product manufacturing thereby reducing production costs.

In order to develop oral pharmaceutical formulations of phages, two main objectives have to be fulfilled: (1) protect phages from acidic degradation or enzyme inactivation during the gastrointestinal transit and (2) prolong their residence time in the intestine to be effective against the targeted bacteria.

2.2.1.2. harmaceutical formulations developed. To protect phages and to

maintain elevated phage concentrations in the gut, pharmaceutical formulations have been developed (Fig. 2).

For example, tablets made of gastro resistant microparticles encapsulating anti-*Salmonella* phages have been produced [48]. First, microparticles composed of a pH-responsive anionic copolymer based on methacrylic acid and methyl methacrylate (Eudragit® S-100) were produced by spray-drying. Trehalose was added into the formulation to protect phages during high-temperature transitions and desiccation. Then, dried microparticles were compressed to obtain tablets. As a result, an increased stability of phages was observed when in contact with a simulated gastric fluid (pH 2).

Other polymers such as alginate, a polysaccharide stable in acidic media and forming a gel in association with calcium, has also been used. Abdelsattar et al. encapsulated an anti-*E. coli* phage in calcium-alginate beads (size range between 2.3 and 2.8 mm) coated with chitosan [49]. Smaller alginate microparticles (size range between 50 and 200 μm) encapsulating an anti-*E. coli* phage have also been reported [39]. These particles were coated with methacrylic acid and methyl methacrylate co-copolymer to obtain a pH-responsive formulation able to release phages at pH 2. In another study, titre decrease in simulated gastric fluids was studied for phages encapsulated in particles of alginate, alginate-chitosan, alginate-carrageenan, or alginate combined with whey protein [50]. They concluded that all encapsulation strategies were able to protect phages from the acidic medium (pH 2.5) for at least 2 h, except for alginate-chitosan particles in which phages were undetectable after only 1 h. Finally, pectin has also been used to develop microparticles able to encapsulate phages and to protect them in in vitro acidic medium [51].

To enhance the residence time of drugs or phages in the intestine, one approach consists in optimizing pharmaceutical formulations able to interact with the intestinal mucus. Mucoadhesive polymers, such as alginate, chitosan, pectin, and carboxymethylcellulose have been largely used to coat micro and nanoparticles for oral applications [52–55]. For example, alginate microparticles associated to antacid $CaCO_3$ and encapsulating a cocktail of three phages active against *Salmonella* were able to prolong the gut residence time of phages in a chicken model compared to free phages, and were associated with a lower caecal *Salmonella* inoculum [56]. In another study, cationic liposomes (about 300 nm) have been designed to encapsulate anti-*Salmonella* bacteriophages [57]. These nanoparticles were able to protect phages against acidic conditions as the loss of titre was decreased. Moreover, in vivo experiments in chickens demonstrated that the encapsulation in positively-charged liposomes increased the intestinal residence time of phages compared to free phages. These mucoadhesive strategies open novel perspectives to enhance the residence time of phages in the intestine while improving bacterial clearance.

2.2.2. *Administration directly into the blood stream*

2.2.2.1. Overview. Due to the low bioavailability of phages after oral administration, intravenous (IV) administration is preferred to treat systemic or severe infections. The IV administration of phages is generally recognized as safe and has been employed against multidrug-resistant infections with poor prognosis [58]. To be safe, the administration of phages in the blood stream must follow the general considerations related to the IV administration of drugs: in brief, the suspension has to be sterile (bacteria-free), endotoxin-free, and free of residual products of preparation (solvent, bacterial residues, bacterial metabolites).

In a recent study, Cano et al. reported the case of a 62-year-old man suffering from a recurrent knee prosthesis infection for which an amputation of the limb was recommended [59]. Phages were IV administered daily, for a total of 40 injections, and associated with an oral antibiotic (100 mg oral minocycline twice per day). A clinical improvement was noted, and the patient remained asymptomatic up to 34 weeks after completing the treatment. Another example: a 68-year-old man suffering from necrotizing pancreatitis complicated by an *Acinetobacter baumannii* infection was treated with a percutaneous and IV administration of a phage cocktail [23]. This cocktail was administered for several weeks, which led to clinical improvement all along the treatment. Phage-resistant bacteria emerged during the course of treatment; however, a resolution of the infection was finally obtained.

If phage IV administration is generally well tolerated, some side effects have also been described. For example, a suspension of phages was used to treat a 72- year-old man suffering from a chronic methicillin resistant *Staphylococcus aureus* prosthetic joint infection [60]. After three days of IV administration, the treatment was discontinued because

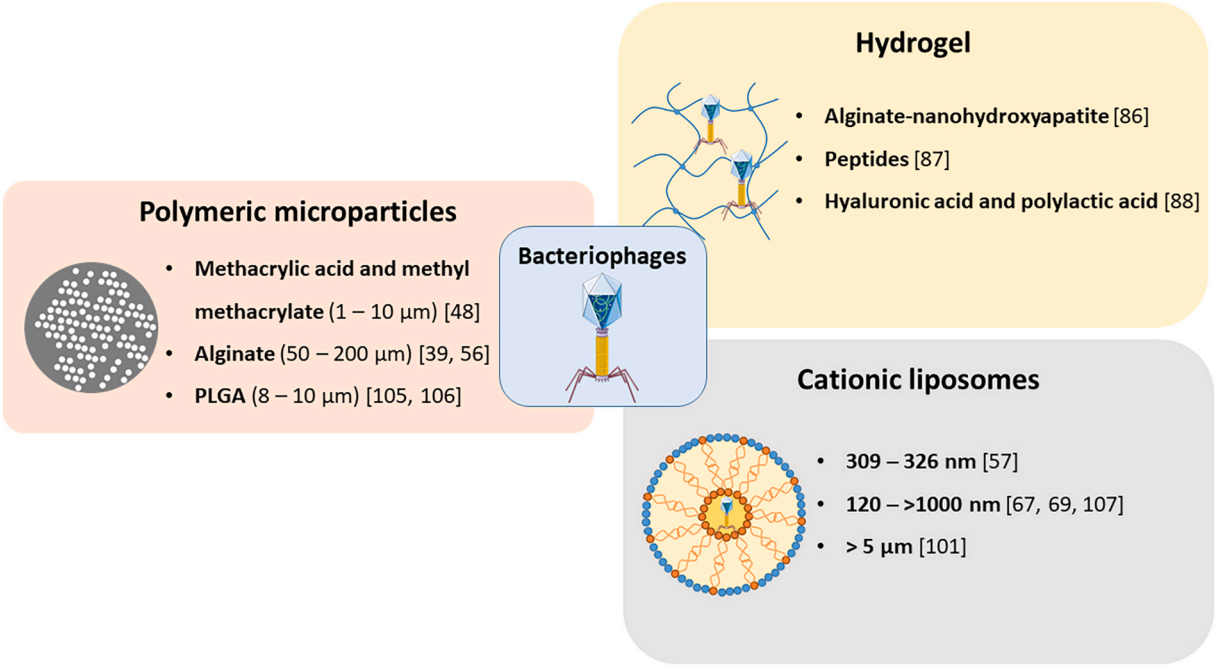

Fig. 2. Schematic representations of novel drug delivery approaches for phage therapy.

of hepatic impairment (elevation of transaminase levels). This effect was transient and reversible, suggesting it was a direct consequence of the phage IV administration.

After injection, phages have to quickly reach their bacterial host to start self-amplification, otherwise their blood titre rapidly decreases [23,61]. Following an IV administration, phages accumulate in the liver, spleen, and lymph nodes where the mononuclear phagocyte system (MPS) eliminates them, but also in the lungs and muscles. Their renal clearance is however limited [40,61]. Phage-neutralizing antibodies constitute another source of inactivation. They are naturally present and abundant in the blood stream [59,62,63]. When the regimen consisted in repeated doses, the level of specific phage-neutralizing antibodies increased [64,65]. However, their ability to inactivate phages is not full elucidated [22,65], and favourable clinical outcomes can occur even in the presence of phage-neutralizing antibodies. Indeed, a 15-year-old man suffering from a disseminated drug-resistant *Mycobacterium abscessus* who received an IV cocktail of three phages twice a day for 32 weeks displayed no evidence of phage neutralization, despite the detection of phage-specific antibodies [66].

In addition, considering pharmacokinetic parameters, phage concentration seems to be a key point in achieving successful clinical outcomes, because of a non-linear elimination of phages [61]. Pharmaceutical formulations, by modifying the pharmacokinetic parameters of encapsulated drugs, can thus improve the rate of successful clinical outcomes.

2.2.2.2. Pharmaceutical formulations developed. Two major advantages are ascribed to the association of nanoparticles with phages: i) overcoming side effects, and ii) increasing phage activity while reducing inactivation mechanisms. Among the different nanocarriers developed, liposomes constituted of a phospholipid bilayer have been mostly employed [57,67–70]. When loaded in cationic-liposomes, phages are protected from neutralizing antibodies, which preserves their bacteria-killing capacity [69]. As phages are specific to bacteria, they are not able to infect eukaryote cells even if an uptake of fluorescent phages by human macrophages has been observed after IV administration [40].

Drug delivery nanosystems are used to control the release of bioactive compounds to the site of interest (targeting) or in the blood circulation to obtain a prolonged effect, enhancing their half-life. For example, in the study of Singla et al., when used in suspension, phages were rapidly uptaken by the MPS and became undetectable, while when encapsulated or associated to nanoparticles, they remained detectable for a longer time in the blood [70]. Chadha et al. administered via intraperitoneal injection a cocktail of five phages against *Klebsiella pneumoniae* associated with cationic liposomes and reported a prolonged circulation time into the blood and various organs [67]. Indeed, when trapped into liposomes, phages were detected into the spleen, liver, and blood for almost 48 h, whereas free phages were rapidly cleared after 24 h. The efficacy of the system was then assayed on a mice model of burn wound infection caused by *K. pneumoniae*. Because of a prolonged circulation time, a higher reduction in the blood bacterial count was observed in mice treated with cationic liposomes compared to mice treated with free phages and, finally, a faster resolution of the infection was associated with the used of phages trapped into cationic liposomes, demonstrating the relevance of modifying the pharmacokinetic parameters of phages.

To decrease the MPS capture and increase the mean blood residence time of nanoparticles, polyethylene glycol , an hydrophilic and inert polymer was used to decorate the external layer of nanoparticle surfaces, thus conferring stealth properties [71]. PEG coating on nanoparticle shield limits their aggregation, and their opsonization by reducing the hydrophobic interaction with opsonins and therefore phagocytosis, leading to a prolonged circulation time [72].

3. Pharmaceutical formulations to target infection sites

Phages are able to self-replicate only in presence of a host; they are therefore cleared when their host is eliminated. This specificity constitutes one major advantage of their use in comparison to antibiotics. On the other hand, to actively fight an infection, phages have to quickly target their bacterial host, and the administration of phages has thus to be performed as close as possible to the infection site [73]. Moreover, penetrating eukaryote cells can be promising for the treatment of intracellular infections, and when loaded in nanoparticles, phages can be uptaken by animal cells [69]. In fact, once phages are encapsulated in nanoparticles, their internalization pathway depends on the particle (cargo) properties and no longer on the phage properties [74]. In this context, as previously reported for cytotoxic or anti-infective drugs, nanomedicines represent a promising strategy to target drugs to a site of interest and controlling the release of active substances [75–77].

3.1. Administration in contact to bone and joints

3.1.1. Overview

Bone and joint infections constitute a heterogeneous group of infections comprising septic arthritis, osteomyelitis, and device-associated infections. Prosthetic joint infections, representing more than 42% of all bone and joint infections [78], are the most dramatic complications impacting patients with prosthesis and are encountered in 1–2% of all prosthesis implanted [79]. In most cases, the bacteria responsible for these infections are coagulase-negative staphylococci and *S. aureus* [80]. The best therapeutic option is generally the surgical removal of the infected implants and tissues. However, for old patients or patients with comorbidities, the prosthetic replacement is sometimes not an option, and antibiotic therapy remains the only solution: the Debridement, Antibiotics and Implant Retention (DAIR) procedure could thus be performed. The main cause of therapeutic failure in the antibiotic treatment of joint infections is related to biofilm formation. Within the biofilm, bacteria are engulfed and are growing in a well-organized polymeric matrix: they are thus protected from the host immune system and the antibiotic access is hampered. In contrast, as previously explained, phages are able to degrade and enter into biofilms thanks to enzymes (polysaccharide depolymerases) [81]. Thus, the action of phage therapy and antibiotic therapy can be synergistic. Nowadays, joint infections represent one of the main therapeutic indications for the use of phage therapy in combination with antibiotic therapy, especially for patients for whom antibiotic therapy alone is no longer an option and amputation considered.

3.1.2. Pharmaceutical formulations developed

Due to short residence time of phages in the blood after IV administration, as detailed above, a local delivery of phages is combined with the IV, when possible. Moreover, if administered in close proximity to the host, phages self-replicate immediately. An association of systemic antibiotherapy (IV or oral administration) and a local phage administration in a suspension form can be performed directly in an infected joint cavity during the DAIR procedure with favourable clinical outcomes [60,82]. However, the administration of phage suspensions can lead to the rapid dispersion of phages through the body and rapid clearance, and pharmaceutical formulations can be helpful to favour the contacts between phages and the bacteria adhering to the bone or prosthesis and avoid this rapid clearance [83].

Hydrogels, corresponding to chemical or physical three-dimensional networks of hydrophilic polymers and gums are widely used in biomedical applications [84]. In the medical technology field, injectable and non-injectable hydrogels are mainly used to locally control the release of bioactive compounds, but also in the context of reconstructive surgeries, tissue regeneration, device coatings, adoptive cell therapy, wound healing, cell delivery, hemostatics [85].

Ribeiro Barros et al. have developed an alginate-nanohydroxyapatite

hydrogel able to encapsulate phages. A prolonged release of phages was obtained over time, 97% were released after 24 h. Both the in vitro and ex vivo antimicrobial activities of phages were enhanced when these were loaded into the hydrogel, and the improved osteogenic capability of alginate-nanohydroxyapatite hydrogels was maintained regardless the presence of phages [86]. Additionally, Wroe et al. have developed various hydrogels using adhesive peptides (GRDGSPC or GGYGGGPC $(GPP)_5$ GFOGER$(GPP)_5$GPC) in order to encapsulate phages against *P. aeruginosa* [87]. The in vitro activity of phages against biofilm formation was enhanced compared to free phages. When used in vivo in a mice model of bone infection (radius infection), phages encapsulated into hydrogels had a better ability to reduce *P. aeruginosa* infection compared to free phages [87]. In addition to these successful pre-clinical results, clinical studies have been conducted using phages entrapped in hydrogels. For instance, in order to treat a 49-year-old man suffering from a multidrug resistant *S. aureus* knee infection, Ferry et al. have associated phages to a hydrogel commercially available composed of hyaluronic acid and polylactic acid. Phages were thus embedded into the hydrogel matrix and rapidly released from it, which allowed their deposition on the infected prosthesis [88].

Phages may also be associated with the prosthetic material before its implantation in order to prevent post-operative infections. By loading a phage specific for *E. coli* (λvir-phage) on tricalcium phosphate, a commonly used ceramic prosthetic material, and coating it with calcium alginate hydrogel, Ismail et al. have enhanced the retention of phages on the ceramic surface, thereby inducing higher levels of lytic activity. Moreover, phages were released in a controlled and extended manner, compared to those loaded on tricalcium phosphate without coating [89]. Bouchart et al. have also developed calcium phosphate-based ceramic pellets loaded with a cocktail of phages infecting *S. aureus* and *E. coli* strains in order to reduce bacterial colonization and biofilm synthesis on prosthetic implants [90].

Although phages seem to constitute a promising strategy for the treatment of bone and joint multi-resistant infections, very few pharmaceutical formulations, except for hydrogels, are currently developed.

3.2. Pulmonary delivery

3.1.2. Overview

Acute respiratory tract infections comprise upper (from nostrils to vocal cords) and lower respiratory tract infections (LRTIs; from the trachea to bronchioles and alveoli) [91]. LRTIs include bronchitis, bronchiolitis, pneumonia, pulmonary tuberculosis, and tracheitis and are responsible for frequent hospitalizations and deaths worldwide: pneumonia for example represents the fourth cause of death worldwide [92].

Among LRTIs, tuberculosis is a major bacterial pulmonary infection that has spread worldwide and that requires long-duration antibiotic treatment, hence leading to the development of resistances. The World Health Organization estimates that half a million of tuberculosis cases were caused by multidrug-resistant or rifampicin-resistant strains in 2019 [93]. Moreover, *Mycobacterium tuberculosis* are intracellular pathogens, anti-tuberculosis agents have thus to penetrate cells to eradicate bacteria.

As another example of pulmonary disease, cystic fibrosis (CF) is a recessive genetic disease whose incidence has been estimated to 1/300 to 1/4000 live births, which corresponds in the US to 1000 births each year [94]. CF affects mostly the lungs, pancreas, and intestine, and CF patients are largely susceptible to LRTIs. Indeed, CF is characterized by a large production of a thick and viscous pulmonary mucus contributing to increase the bacterial adherence and biofilm formation. To prevent or treat these recurrent LRTIs, patients generally receive long-duration antibiotic treatments. Over time, bacteria become resistant, and therapeutic strategies become more and more complex [95].

In these contexts, phage therapy has been tested as its actions can synergize with those of antibiotics in order to increase the activity of the latter or to disrupt biofilms.

3.2.2. Pharmaceutical formulations developed

Prazak et al. have developed a nebulization method for the administration of phages to mechanically-ventilated patients, because of their high risk to contract a multidrug-resistant *S. aureus* pneumonia [96]. The authors suggested that the application through nebulization concentrates phages in the lungs and thus increase their activity. Nebulized phages (in a suspension form) were delivered via an inhalation device (vibrating mesh aerosol drug delivery system used in human therapy) in an in vivo rat model of multi-resistant *S. aureus* pneumonia. Phages remained active after nebulization, were uniformly distributed through lungs, and did not spread all over the organism. The survival of infected rats was improved thanks to the administration aerosolized phages compared to placebo; however, some *S. aureus* strains were not fully eradicated from the lungs. A similar study has been conducted by Guillon et al. in order to evaluate the feasibility of a phage nebulization system to treat *P. aeruginosa* ventilator-associated pneumonia in mechanically-ventilated pigs [97]. A cocktail of five phages was prepared as a suspension form in a sodium chloride solution and was aerosolized during 15 min through a static-mesh nebulizer. The distribution of phages was homogenous in both lungs. Finally, phage nebulization seemed to contain *P. aeruginosa* pneumonia in this model.

As previously mentioned, some bacteria are intracellular. To be effective, antibiotics, and more generally anti-infective therapies, must be able to penetrate cells, which constitute a major challenge. Phages alone do not have the ability to penetrate eukaryotic cells: in order to improve their internalization, novel delivery strategies based on nanomedicines have been developed and showed interesting results [69]. Nanotechnologies have been previously used to administer anti-inflammatory drugs and antibiotics directly in the lung compartment with favourable clinical outcomes [98,99]. Moreover, nanoparticles, either polymeric or lipid-based, can be internalized into cells via several endocytic pathways, depending mainly on their size and surface structure [74,100]. Using this nanotechnology approach, Singla et al. have developed phage-loaded liposomes in order to enhance the cell endocytosis of phages and target intracellular *K. pneumoniae* [69]. When loaded into cationic liposomes, phages (KPO1K2) were able to kill 94.6% of intracellular *K. pneumoniae* whereas free phages were able to kill about 20% of intracellular bacteria. In another example, Nieth et al. have developed giant liposomes (mean size inferior to 5 μm) for pulmonary delivery (inhalation) and the targeting of mycobacteria [101]. Liposomes were composed of three lipids: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and a fluorescent lipid Red®-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE). The authors demonstrated, in vitro, that phage-loaded liposomes penetrate more efficiently than free phages into cells (monocyte, THP-1 cells).

As previously mentioned, CF constitutes a major drug resistance concern, due to resident bacteria embedded in a thick and viscous pulmonary mucus. Several formulations to deliver phages into this mucus have been developed and a recently published review inventories phages in dry powders, administered via an inhalation route to treat *P. aeruginosa* in CF patients [102]. This review highlighted the fact that very few studies were published, that most of them were pre-clinical studies, and that more research was required before the safe and effective administration of phages in dry forms to humans.

Cationic liposomes or positively-charged polymers interact with mucin via electrostatic bounds and may be used to deliver drugs in a controlled manner. For example, chitosan, a naturally cationic polymer, is largely used to adhere to mucosal layers [103]. However, very few publications developing phage encapsulation with such a strategy are reported. Gondil et al. have encapsulated a phage lysin into chitosan nanoparticles to treat *Streptococcus pneumoniae* infections, and the authors demonstrated the muco-adhesive properties of particles in an ex vivo gut loop surface [104].

Poly(lactic-*co*-glycolic acid) (PLGA) nano and microparticles are largely used for pharmaceutical applications because of the biocompatibility and biodegradability of PLGA, which is FDA-approved for IV administration. The used of 10-μm PLGA microspheres able to encapsulate phages against *S. aureus* or *P. aeruginosa* has been reported [105]. Once formulated, microspheres were freeze-dried to improve their stability. However, the phage lytic activity was reduced, probably due to the double emulsion method based on the use of organic solvent such as dichloromethane. Indeed, organic solvents are required to solubilize the polymers, however they can destroy phages, and the residues present in pharmaceutical formulations can be toxic in clinical application. Agarwal et al. have developed a cocktail of phages loaded in 8-μm PLGA microspheres to treat *P. aeruginosa* lung infections [106]. The formulation was also prepared using a double emulsion technique (using dichlorotethane as organic solvent) followed by freeze-drying to keep the microspheres stable over time. Interestingly, in vitro, phage-loaded microspheres were able to kill *P. aeruginosa* in biofilms. Phage-loaded microspheres were then aerosolized and administered to an in vivo mouse model; compared to the administration of free phages, high titres of phages were recovered in the lungs, and phages were able to self-amplify in the presence of bacteria. Finally, phage-loaded microspheres were able to reduce the count of bacteria whereas free phages had no effect on the bacterial count. To overcome the reduction of phage titre during particle formulation, Cinquerrui et al. have developed phage-loaded liposomes 100 to 300 nm in mean size using a microfluidic process. However, the organic solvent used during the process (isopropanol) was once again responsible for a decrease in phage titre. An important encapsulated phage yield was obtained (10^9 PFU/mL) by increasing the initial free phage titre used during the microfluidic mixing [107].

Several pharmaceutical strategies are thus under evaluation to treat pulmonary infections, and most of them involve dry formulations. Smaller particles may be required to target deeper lung structures (i.e. pulmonary alveoli), and increasing the encapsulation yield in nanovectors is aimed by researchers.

3.3. Cutaneous applications

3.3.1. Overview

Dermatological infections are superficial infections and comprise notably burn, wound, and ulcer infections. They are often complicated by the occurrence of antibiotic resistances and phages may represent an option to overcome this issue. In the context of dermatological infections, a wide variety of pathogens is encountered, and *S. aureus*, *P. aeruginosa*, *Streptococcus species*, *Enterococcus species, K. pneumoniae,* and *E. coli* are the most frequent of them [108,109]. In some pathologies, such as diabetes mellitus, acute bacterial skin infections are more frequent, are associated with a high morbidity and mortality, and their management is complicated by the diabetes-related complications (neuropathies, vasculopathies) and the drug-drug interactions [110]. Moreover, chronic wound infections are often associated with biofilm formation (prevalence superior to 78%) and poor clinical outcomes [111]. Finally, wound infections represent a major risk of sepsis due to the proliferation of microorganisms and their progressive invasion of deeper tissues. Therefore, anti-bacterial therapy has to be urgently initiated, especially in burned patients, but their efficacy may be compromised because of the spread of multi-drug resistance.

In the particular context of burned patients, a phase-1/2 clinical trial (Phagoburn, NCT02116010) involving a phage therapy has been published [17]. An alginate template was soaked with a cocktail of 12 natural anti-*P. aeruginosa* phages and was daily applied for 7 days onto the wounds. This experimental treatment was compared to the standard of care, corresponding to a 1% sulfadiazine silver emulsion cream applied daily for 7 days. This study demonstrated the safety of the application of phages to burned patients. However, the proportion of successful treatment outcome was lower among the phage-treated patients compared to those who received the standard treatment. This issue could be related to the important decrease in the phage titre that was observed during the study, demonstrating the major role that pharmaceutical technologies have to play in order to improve the phage clinical success.

3.3.2. Pharmaceutical formulations developed

Numerous studies have evaluated the use of phages for the treatment of skin infections, demonstrating the great interest for this topical route of administration, mainly in an attempt to overcome the issue of multidrug resistance. These studies are presented in already interesting published reviews [112,113]. Currently, very few clinical trials have been published, and reviews mainly report case series.

Nanotechnologies are used to treat wound infections as they can reduce antibiotic dependence due to their intrinsic antimicrobial properties. They are, for example, metallic nanoparticles of silver, copper, or zinc [114]. Nanoparticles can also be responsible for a decreased inflammation, they are able to promote angiogenesis and cell proliferation, which improves wound healing [114]. In the context of phage therapy, Chhibber et al. developed a cocktail of free phages or phages loaded into cationic liposomes ~200 nm in size to treat mice suffering from *S. aureus* methicillin-resistant wound infections [68]. A significant increase in the phage titre into the wound was observed in the group of mice treated with phage-loaded liposomes compared to the group of mice treated with free phages, and was related to a faster wound healing in the liposome-treated group. Nanospheres encapsulating phages against *S. aureus* were originally designed by copolymerization (precipitation polymerization) of a thermally-responsive polymer, poly(N-isopropylacrylamide), with allylamine [115]. The size of nanospheres decreased when the temperature was over 34 °C, from 400 nm to ~170 nm. An in vitro assay has showed that phages were released when nanospheres collapsed (over 34 °C) by measuring the clearance of *S. aureus* according to the temperature. This demonstrates the interest of the use of temperature-sensitive formulations for the treatment of wound infections.

In studies reporting the use of phages to treat cutaneous infections in humans, phages were systematically impregnated in dressings, gauzes, bandages, or filter paper discs, and then applied onto wounds [112,113]. Indeed, liquid formulations are not suitable for topical application since they can easily run off from the infection site. Semi-solid preparations such as hydrogels therefore appear to be more appropriate in this context. Sodium alginate is a natural polymer widely used in commercial wound dressings due to its capacity to create a moist wound environment favourable to a better wound healing while reducing microbial proliferation [116]. A hydrogel composed of polyvinyl alcohol cross-linked to sodium alginate was formulated to absorb both a suspension of phages against *S. aureus* and a solution of antibiotic (minocycline) [117]. The stability of phages in the hydrogel was confirmed for 28 days. Moreover, in an in vivo murine model, phage-loaded hydrogel formulations were effective to control a drug-resistant bacterial skin infection and enhanced wound healing. Hydrogel can also be obtained by 3D printing [118]. By mixing an alginate solution containing phages against *E. coli* and a $CaCl_2$ solution, a hydrogel was obtained and enabled a slow release of phages for at least 24 h, thereby maintaining a high phage titre on the site of infection. However, the authors noted a significant decrease of the phage lytic activity during the encapsulation process, depending of the amount of phages embedded in the hydrogel, and the residual activity was comprised between 84.9% and 89.5% of the initial lytic activity. In fact, once embedded in hydrogels, phages must diffuse in the matrix before reaching their host and exert their lytic activity, which could explain this decreased activity.

A previous review focusing specifically on hydrogel formulations for phage encapsulation has been published by Kim et al. who made an exhaustive list of the hydrogels used to treat cutaneous infections [83].

The treatment of some dermal infections requires the access to deeper tissues, hence facilitating skin penetration is of interest. Sodium

alginate-based hydrogel obtained by ionotropic gelation associated with a skin permeation enhancer (choline oleate) to encapsulate a cocktail of phages against *A. baumannii* was tested [119]. Ex vivo experiments using porcine ear skin on a Franz diffusion cell performed to assess phage penetration showed that the addition of choline oleate induced an exponential increase in the permeation of phages through ear skin, whereas without choline oleate no permeation was observed.

4. Discussion

Phage therapy is regaining interest for the treatment of multidrug-resistant bacterial infections and to reduce antibiotic dependence. Currently, phages are mostly administered under suspension forms or, in the case of skin infections, associated with dressings. However, suspensions are not suitable to target some sites of interest, as phages are rapidly cleared from the organism. To overcome these limitations, several pharmaceutical technologies are under investigations.

Pharmaceutical formulations must be able to encapsulate and to protect phages from the environment while preserving their biological activity, especially their lytic activity. Moreover, the excipients selected must be biocompatible and able to release phages at the desire site of infection.

Another problem concerns the status of phages and their availability. Currently, phages are mainly used as compassionate therapy, and each cocktail is adapted after performed a phagogram [16], i.e. corresponding to a personalized therapeutic approach. This is a strength as it is highly adapted for each clinical context, but also a weakness as phages are not immediately available, since they first have to be selected via phagogram and then produced. Once obtained, they have to be rapidly associated with a pharmaceutical formulation adapted to the site of interest and the administration route. This delay is critical for the success of the therapy.

Phages are generally negatively charged structures. More precisely, capsids, corresponding to heads, are negatively charged while tails are generally positively charged [120,121]. It has to be kept in mind that a loss of viability of phages may be related to changes in their structure, due for example to electrostatic interactions. The solvents used in drug technology processes could also decrease the phage activity and may be toxic in clinical application. Excipients recognized as safe and FDA-approved have to be chosen.

On the other hand, numerous hydrogels or particles designed to load phages rely on divalent ions (Ca^{2+} or Mg^{2+}) in their formulations as cross-linking agents. They are moreover able to enhance the phage stability while promoting their activity against their host [122].

Moreover, phage endolysins (peptidoglycans hydrolases extracted from phages and able to disrupt cell membranes and walls) as well as biofilms are also largely studied, and are currently administered as protein-based solution [123,124]. As for phage delivery, pharmaceutical technologies aiming at increasing the protein stability dealing with the endolysin activity are under evaluation. For example, they correspond to dendrimer formulations or alginate nanoparticles [125,126].

Clinical trials must now go on and confirm the interest of using phages in the current context of bacterial resistance of the antibiotic era. It is also an imperative necessity to determine how to use phages, and more numerous clinical evidence must be established regarding treatment regimens, in terms of treatment duration and schedule, route of administration, concentration and quantity of phages to administer, as well as methods to boost their efficacy.

5. Conclusion

While phages appear to constitute a promising strategy to overcome antibiotic resistances, few pharmaceutical technologies are currently developed. Phage therapy is also suffering from the lack of methodologically rigorous clinical trials that would confirm the results of observational studies, and this could slow down the pharmaceutical research on drug formulations. Further studies aiming at optimizing treatment regimens (duration and schedule) and therapeutic dose (or phage concentration) are needed to gain insight into the effectiveness of phage therapy as anti-infectious disease treatment.

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