Advances in Colloid and Interface Science 249 (2017) 100–133

Contents lists available at ScienceDirect

Advances in Colloid and Interface Science

journal homepage: www.elsevier.com/locate/cis

Historical perspective

Formulation, stabilisation and encapsulation of bacteriophage for phage therapy

Danish J. Malik[a,*], Ilya J. Sokolov[a], Gurinder K. Vinner[a], Francesco Mancuso[a], Salvatore Cinquerrui[a], Goran T. Vladisavljevic[a], Martha R.J. Clokie[b], Natalie J. Garton[b], Andrew G.F. Stapley[a], Anna Kirpichnikova[c]

[a] Chemical Engineering Department, Loughborough University, LE11 3TU, UK
[b] Department of Infection, Immunity and Inflammation, University of Leicester, University Road, Leicester LE1 7RH, UK
[c] Department of Mathematics and Computer Science, Liverpool Hope University, Hope Park, Liverpool L16 9JD, UK

ARTICLE INFO

Keywords:
Antibiotic resistance
Bacteriophage
Encapsulation
Phage therapy
Pharmacodynamics

ABSTRACT

Against a backdrop of global antibiotic resistance and increasing awareness of the importance of the human microbiota, there has been resurgent interest in the potential use of bacteriophages for therapeutic purposes, known as phage therapy. A number of phage therapy phase I and II clinical trials have concluded, and shown phages don't present significant adverse safety concerns. These clinical trials used simple phage suspensions without any formulation and phage stability was of secondary concern. Phages have a limited stability in solution, and undergo a significant drop in phage titre during processing and storage which is unacceptable if phages are to become regulated pharmaceuticals, where stable dosage and well defined pharmacokinetics and pharmacodynamics are *de rigueur*. Animal studies have shown that the efficacy of phage therapy outcomes depend on the phage concentration (i.e. the dose) delivered at the site of infection, and their ability to target and kill bacteria, arresting bacterial growth and clearing the infection. In addition, in vitro and animal studies have shown the importance of using phage cocktails rather than single phage preparations to achieve better therapy outcomes. The in vivo reduction of phage concentration due to interactions with host antibodies or other clearance mechanisms may necessitate repeated dosing of phages, or sustained release approaches. Modelling of phage-bacterium population dynamics reinforces these points. Surprisingly little attention has been devoted to the effect of formulation on phage therapy outcomes, given the need for phage cocktails, where each phage within a cocktail may require significantly different formulation to retain a high enough infective dose.

This review firstly looks at the clinical needs and challenges (informed through a review of key animal studies evaluating phage therapy) associated with treatment of acute and chronic infections and the drivers for phage encapsulation. An important driver for formulation and encapsulation is shelf life and storage of phage to ensure reproducible dosages. Other drivers include formulation of phage for encapsulation in micro- and nanoparticles for effective delivery, encapsulation in stimuli responsive systems for triggered controlled or sustained release at the targeted site of infection. Encapsulation of phage (e.g. in liposomes) may also be used to increase the circulation time of phage for treating systemic infections, for prophylactic treatment or to treat intracellular infections. We then proceed to document approaches used in the published literature on the formulation and stabilisation of phage for storage and encapsulation of bacteriophage in micro- and nanostructured materials using freeze drying (lyophilization), spray drying, in emulsions e.g. ointments, polymeric microparticles, nanoparticles and liposomes. As phage therapy moves forward towards Phase III clinical trials, the review concludes by looking at promising new approaches for micro- and nanoencapsulation of phages and how these may address gaps in the field.

1. Introduction

The discovery of antibiotics and the subsequent control of bacterial infections may be regarded as a significant achievement of modern medicine. Surgery, transplantation and chemotherapy rely heavily on the control of bacterial infections. Broad spectrum antibiotics are highly

* Corresponding author.
E-mail address: d.j.malik@lboro.ac.uk (D.J. Malik).

http://dx.doi.org/10.1016/j.cis.2017.05.014
Received 17 March 2017; Received in revised form 11 May 2017; Accepted 11 May 2017
Available online 14 May 2017

attractive since they may be used against a wide range of bacteria without the need to identify the infection causing bacterial agent. This advantage has also resulted in significant misuse and overuse of antibiotics contributing to the emergence of antibiotic resistance [1]. Bacterial resistance to antibiotics has become a significant problem in the treatment of a wide range of infections where the bacteria commonly causing the infections have become highly resistant to many classes of antibiotics including third generation cephalosporins, carbapenems and fluoroquinolones [2]. Recent evidence of plasmid mediated colistin resistance in Enterobacteriaceae is particularly troubling [3]. Between 1940 and 1962 > 20 new classes of antibiotics came to market; since then the antibiotic pipeline has produced only two new classes [4]. Coates et al. [4] estimate that to stem the rising tide of antibiotic resistance 20 new classes of antibiotics are urgently needed. It is highly unlikely that this goal will be achievable even if governments provided stronger economic incentives to industry to develop new broad spectrum antibiotics. Czaplewski et al. [5] call for a sustained, concerted and coordinated international effort and discuss the need to invest in the development of alternatives to antibiotics (non-compound approaches) including probiotics, vaccines as well bacteriophage and phage derived lysins.

2. Bacteriophage as novel antimicrobials

Bacteriophages (viruses that infect bacteria) are highly abundant in the environment and may be the source of low cost antimicrobials. The focus of recent phage therapy approaches is on the use of lytic tailed phages all of which belong to the *Caudovirales* and include the *Myoviridae*, *Siphoviridae* and *Podoviridae* families [6]. Members of the *Caudovirales* have an icosahedral capsid head that contains double-stranded DNA (15–500 kbp), and a tail with surface receptor proteins that interact with surface features on the host bacterium [7]. Successful phage therapy requires interaction between the phage and the bacterium resulting in adsorption of the phage to the host bacterium followed by injection of the phage DNA. The pharmacodynamics of this process has been modelled using mathematical models based on colloidal interactions [8–10]. Upon infection, the phage replication cycle ensues culminating (after a short latency period) in cell lysis and the liberation of multiple phage virions (with a burst size that is typically between 10 and 100 [11]). Gill and Hyman [12] and Weber-Dabrowska et al. [13] provide an overview of key considerations related to phage choice, isolation and purification for phage therapy. Phage therapy practice relies on the isolation of naturally occurring phage abundantly found in the environment [14]. Typically, phage are isolated from the environment and screened against commonly occurring pathogenic bacterial strains (to identify host ranges) and then evaluated using in vitro and in vivo animal models. The limitation of host ranges is overcome through the use of phage cocktails to ensure sufficient coverage of commonly occurring strains and the use of phage mixtures targeting different receptors reduces the probability of encountering phage resistant bacterial mutants. Phages are generally manufactured using standard fermentation process technology. In brief, host bacteria are grown in liquid culture in a bioreactor. During the log growth phase, phages are added to the bioreactor to infect the bacteria. Incubation of phage with bacteria results in phage adsorption to the bacteria, infection and following a short lag period, release of bacteriophage virions. The resulting lysate contains the product (the amplified phage) along with bacterial debris and residual fermentation media. Removal of cellular debris is typically done using centrifugation and/or filtration. Ion exchange, gel filtration etc. can be used to further purify the bacteriophage (e.g. for the removal of host cell proteins, host cell DNA or endotoxin for Gram negative bacteria). Typically phage may then be re-suspended in simple saline or buffer and stored under refrigerated conditions or processed further e.g. spray dried to improve storage shelf life or encapsulated in micro- or nanoparticle formulations.

Phage are unique antimicrobials in that in the presence of host bacteria, they are able to increase their numbers by infecting the bacteria and producing virion progeny whilst minimally affecting the overall microbiota and body tissues. Phage carrying polysaccharide depolymerases (polysaccharide degrading enzymes) in their structure may be able to disrupt biofilms [15–17]. For example *Enterococcus* and *Staphylococcus* phage capable of destroying biofilms have been reported [18,19]. In addition to their potential as human biotherapeutics, phage are being developed for agricultural use to rid the environment and domestic animals of pathogens that could contaminate the food supply chain [20], in aquaculture for the treatment of fish pathogens [21] and for the control of infections in intensively farmed poultry [22,23]. Recent advances in molecular biology and sequencing technology have improved our basic understanding of how bacteriophage interact with bacteria and have opened new possibilities for utilising phage, including genetically engineered phage, for potential therapeutic and diagnostic applications [7,24].

3. Phage therapy for acute and chronic infections

Most of the recent phage therapy studies (using small vertebrate animals) have investigated treatments focusing on acute infections (Table 1). In acute infections the specific infection causing bacterium may be identified using suitable rapid diagnostic methods (e.g. lateral flow assays, PCR, MALDI-TOF Mass Spectrometry). In such instances, narrow spectrum antimicrobials such as bacteriophages may provide a suitable therapeutic alternative where organisms are resistant to frontline antibiotics or to reduce the use of broad spectrum antibiotics as part of a global effort towards antibiotic stewardship. An example of this is in cases of urinary tract infections where a significant proportion of the cases are caused by a particular pathotype *Escherichia coli* with specific virulence factors [25]. Animal studies have shown that phage may be effective in certain instances e.g. in treating acute respiratory infections caused by *Pseudomonas aeruginosa* [26–28] and in the treatment of systemic infections caused by *S. aureus* [29]. A significant focus of phage therapy studies in animals has been around respiratory infections, gastrointestinal infections and infections of the skin and wounds (Table 1). Phage therapy studies with animals has shown that in certain instances, it may help in reducing the densities of the infecting bacterial populations to levels that may allow the host immune response to mount a successful defence and clear the infection [26,27,30].

A number of in vivo phage studies (with animals and humans) have suggested that phage therapy may be beneficial in the treatment of difficult to treat antibiotic resistant pulmonary infections (e.g. cystic fibrosis [26] and pneumonia [31,32]), topical and wound infections [33,34] and gastrointestinal infections [35].

3.1. Challenges of antibiotic resistance for respiratory infections

Cystic fibrosis (CF) is a genetic disease of the lung resulting in reduced hydration and thickening of secretions covering the respiratory epithelium. Highly viscous mucus is not cleared by the epithelial cells and eventually leads to chronic inflammation and bacterial infections.

Two common bacterial strains isolated from sputum are *Staphylococcus aureus* and *Haemophilus influenzae*, which are also one of the first to infect the lungs during childhood [36]. *S. aureus* can generally be controlled with antibiotics but infection usually predisposes chronic colonization. The main pathogen infecting the pulmonary epithelium is *Pseudomonas aeruginosa*, which grows in biofilms within the CF lungs [37]. Several pulmonary pathogens are capable of forming biofilms in which the bacteria are encased in exopolysaccharide (EPS) and are less metabolically active [38]. The extracellular matrix in the biofilm impairs the action of antibiotics acting as a diffusional barrier. The low metabolic activity of the bacteria in the biofilm also limits the efficacy of many classes of antibiotics that target various metabolic pathways. Some bacteriophage may be able to degrade biofilms and infect bacteria residing within them [39,40]. *Burkholderia cenocepacia* are opportunistic pathogens in CF causing acute pulmonary disease and sepsis or chronic infection characterised by accelerated decline in lung function. Multidrug resistant phenotypes are exhibited by most strains. There is a need for alternative therapies to treat *B. cenocepacia*; several virulent phages for *B. cenocepacia* have been characterised. A number of in vivo phage therapy studies have looked at *P. aeruginosa* [26,27,28,41] and *B. cenocepacia* [42] infections in animal models.

Ventilator-associated-pneumonia (VAP) occurs often in patients in an intensive care unit setting. A clear respiratory airway is a necessity for patient survival; after being intubated and receiving mechanical ventilation, patients suffer from pneumonia caused by infection with *P. aeruginosa*, *S. aureus*, *Streptococcus pneumoniae*, *H. influenzae* and *Acinetobacter baumannii* which take advantage of the weakened immune system of the patient. One of the major problems is antibiotic resistance. *Klebsiella* sp., *Enterobacter* sp., *Serrati* sp., and *Burkholderia* sp., have also been reported as infecting bacteria. *Klebsiella pneumoniae* is an opportunistic bacterial pathogen responsible for much community-acquired pneumonia and a significant proportion of hospital-acquired pneumonia [31]. It is a leading cause of pneumonia morbidity and mortality with resistance to carbapenems and other antibiotics [43]. A number of in vivo phage therapy studies have looked at *B. cepacia* [42] and *K. pneumonia* [31,32] infections in animal models.

Tuberculosis (TB) is a global health problem; one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb), the agent of TB, and at risk of developing disease and transmitting infection. Annually there are over 10 million new cases of TB and an estimated 1.4 million deaths [44]. Multidrug resistant tuberculosis cases are a significant healthcare problem with extensively drug resistant *M. tuberculosis* practically untreatable in low-income countries [45]. Multi-drug resistant (MDR) Mtb strains are resistant to isoniazid and rifampicin, two of the frontline antibiotics used to treat TB. The World Health Organisation (WHO) estimate there were nearly 500,000 new cases of MDR TB in 2015 [44]. Most alarming are the development of extensively drug resistant (XDR) strains, which are additionally resistant to second line antibiotics. Although antibiotic treatment rapidly reduces the infectiousness of patients, those with drug-resistant infections remain infectious during therapy until the drug resistance is identified and the treatment regimen is changed. Treatment for TB is protracted, requiring antibiotics to be taken for at least 6 months, rising to two years for MDR infections. The antibiotics can have undesirable side effects, and interruptions to therapy (intermittent supply or patient non-compliance) provide the opportunity for the development of drug resistance and treatment failure. There is therefore, an urgent need for new therapies to treat TB and also to shorten the length of treatment. The Mtb bacillus is transmitted in aerosol droplets and therapies which reduce patient infectiousness or prevent the establishment of new infections will also have great impact on TB control. Infection is established in a new host when inhaled Mtb bacilli in the lower respiratory tract are engulfed by alveolar macrophages [46]. The bacilli resist usual macrophage killing mechanisms and replicate, until such time that the immune response triggers recruitment of systemic mononuclear cells which surround the focus of infection and form a granuloma. The centre of the granuloma becomes hypoxic, pH drops and conditions no longer support bacterial growth. Instead the Mtb bacilli remain in a dormant state with low metabolic activity, until such time that the immune system weakens, the granuloma breaks down and with this the bacilli reactivate and replicate once more. During this active TB disease the patient excretes large number of bacilli when they cough and can transmit the infection to new susceptible hosts. A number of recent studies have started looking at phage therapy for the treatment of TB infections [47,48] and strategies to tackle the challenge of phage delivery to these intracellular bacteria.

3.2. Challenges of antibiotic resistance for wounds

Chronic wounds are a severe worldwide problem. A recent prevalence study estimated 2.2 million wounds managed by the UK National Health Service (NHS) in 2012/2013 costing the UK NHS £5.3 billion [49]. Studies suggest that wound management accounts for over half of community health nurse resources in European settings [50]. The majority of chronic wounds are colonized with bacteria that form biofilms, thereby compromising wound healing by making them more refractory to treatment and slow tissue repair by stimulating chronic inflammation at the wound site [51]. Antimicrobials including antibiotics and antiseptics are used clinically to decrease the bacterial load and promote wound healing [52]. However, systemic antibiotic therapy may be of little benefit due to poor diffusion of antimicrobials through the biofilm and the poor efficacy of antibiotics against bacteria with reduced metabolic activity. This reduced activity increases antibiotic tolerance since many classes of antibiotics are only effective against actively dividing bacteria by targeting peptidoglycan produced in the cell wall (β-lactams), protein (aminoglycoside) synthesis, or DNA replication (quinolones) [53]. Polymicrobial biofilms in chronic wounds are recognised as an important factor in the failure to achieve wound healing in a timely fashion. As an example, diabetic foot infections (DFI) typically tend to be polymicrobial with aerobic Gram-positive cocci, especially staphylococci, the most common causative microorganism [54]. *S. aureus* and *P. aeruginosa* are considered important pathogens in antibiotic resistant wound infections. A number of animal studies have looked at phage therapy for treating *S. aureus* and *P. aeruginosa* wound infections [33,34].

3.3. Challenges of antibiotic resistance for gastrointestinal infections

Enteric pathogens form a large part of the healthcare burden around the globe. The most common infection causing bacteria are *Clostridium difficile* causing *C. difficile* associated diarrhoea [55], *Shigella dysenteriae* causing shigellosis [56], *Escherichia coli* causing gastroenteritis [57], *Vibrio cholerae* causing cholera [58], *Salmonella enterica* causing salmonellosis [59], *Listeria monocytogenes* causing listeriosis [60] and *Helicobacter pylori* causing chronic gastritis [61]. The primary route of infection in low income countries is through the faecal-oral route or from contaminated food and water [62]. Treatment usually involves antibiotics however, with emerging antibiotic resistance, infections with antibiotic resistant organisms is on the rise, hence the need for alternative therapy options. In addition to humans, these enteric

pathogens are also capable of infecting farm animals resulting in high antibiotic use for animal husbandry, emerging resistance and risk of contamination of the food chain. Numerous studies have evaluated the potential of phage therapy for the treatment of gastrointestinal infections caused by *E. coli*, *S. enterica*, *V. cholera* and *C. difficile* (Table 1).

3.4. Phage-bacterium population dynamics - the need for bacteriophage encapsulation

There are a number of specific features regarding phage-bacterium dynamics that are worth highlighting in the context of phage therapy. Bacterial killing by phage is dependent on phage particles adsorbing to the target bacteria [9]. Phage-bacterium binding kinetics may be modelled as a simple first order process with respect to the concentrations of bacteria and phage populations respectively [8,63]. Payne and Jansen [63] developed a mathematical model focusing on the dynamics of phage infection of bacteria during the bacterial exponential growth phase. Levin and Bull [8] extended the model to include the effect of bacterial mutation leading to phage resistant populations and the effect of the immune response in combating bacterial infections. Cairns et al. [9] fitted in vitro phage-bacteria population dynamics (time series data for *Campylobacter jejuni*) to extract interaction parameters; the model included the rate at which resistant cells arise by mutation from a susceptible population. Their model and that of Levin and Bull [8] helps explain the in vitro observations (see Tanji et al. [64]) showing an increase in concentration of resistant bacteria in the presence of high concentrations of phage. In in vitro studies, the rate of growth of resistant bacteria tends to be similar to that of the original susceptible population. The fitted rate of mutation was found to be of the order of $10^{-5}\,h^{-1}$ (which is relatively fast). An alternative explanation may be the presence of a small number of resistant cells present at the start of the culture [9]. Measures to suppress growth of resistant bacteria would include use of multiple phage strains incorporating phage that bind to different receptors. The purpose of the modelling is to illustrate a number of important features that provide insights that may help inform in vivo phage therapy. Modelling can be used to determine the effects of parameters such as the dose of phage delivered, the rate at which it is delivered (e.g. controlled release) or bacteria growth rate, on phage-bacteria population dynamics and on phage therapy outcomes. It may be used to help interpret results of in vivo studies and inform the design of future studies.

We have undertaken numerical simulations of phage-bacterium population dynamics for an acute infection model to highlight key features including the dynamics of phage interaction with bacteria during the exponential growth phase (model equations and parameters used for simulations are laid out in Appendix A). Our model is similar to that of Levin and Bull [8] for phage-bacteria interactions incorporating phage resistant bacteria arising from bacteria mutation (we have used the mutation rate used by Cairns et al. [9]; we haven't included a model of the immune response). Phage growth is determined by a number of parameters, the phage-bacteria adsorption rate constant, burst size (the number of phage released for each infected bacteria), latent period (following infection how long it takes for the phage replication cycle to be completed in a bacterial cell), the bacteria growth rate, phage and bacteria elimination rates due to host response and the initial concentrations of bacteria and phage [65]. A unique feature of our model is that we have also incorporated the effect of controlled release of phage which to our knowledge has not previously been done.

Our simulations demonstrate the conditions under which phage therapy may be able to limit the proliferation of bacterial populations. High phage densities are needed in order to arrest the growth of phage susceptible bacteria (Fig. 1 a, b). We have indicated an arbitrary upper bacterial concentration threshold of 10^9 CFU/ml as an aid to guide the reader's eye. Adsorption of phage to bacteria results in an initial drop in phage concentration (see experimental results by Cairns et al. [9] with *Campylobacter jejuni* showing this). The timing of rapid in situ phage amplification is highly dependent on the concentration of the bacteria [9]. Successful amplification of phage is dependent on high bacterial concentration hence efficient killing is not achieved until both phage and bacteria concentration increase significantly (Fig. 1 b). See also experimental results in support of this in the work by Tanji et al. [64]. Resistant bacteria quickly become the dominant population (Fig. 1 a, b) unless a phage mixture (cocktail) is used containing high doses of the different phage strains capable of killing the resistant mutant population (Fig. 1 c, d). It is known that phage clearance mechanisms rapidly reduce the phage concentration in the absence of host bacteria [58].

Using high initial bacterial concentration, a high phage dose is quickly able to arrest the rise of bacterial growth (Fig. 2 a). Tanji et al. [64] show this for *E. coli* using an in vitro chemostat experimental system. However, if low starting concentrations of bacteria are present, phage concentrations decay significantly and phage are unable to amplify and achieve a sufficiently high concentration to eradicate the infecting bacteria until the bacteria have had time to grow bacterial numbers substantially (Fig. 2 b). In such circumstances it takes time for the bacterial population to grow before phage are able to adequately multiply in situ and then arrest bacterial growth (experimental results by Cairns et al. [9] show this). During this delay phage concentrations may decay significantly thereby resulting in poor phage therapy outcomes. If phage are administered prophylactically too early prior to infection or at the early onset of infection (when the bacteria concentration is low), clearance of phage by the host immune system or by other mechanisms (e.g. shortening of intestinal transit times during diarrhoea) may result in lowering of the in situ phage concentration resulting in poor phage therapy outcomes (Fig. 2 b). Under such conditions encapsulation may be a good strategy (Fig. 2 c). Encapsulation of phage and their slow controlled release may help in ensuring that the in situ phage concentration remains at a therapeutically effective level (over a realistic time period) allowing phage to amplify once the bacteria concentration increases to levels sufficient for in situ phage amplification (Fig. 2 c).

Our simulations show that the in vivo phage concentration at the site of infection is an important parameter governing the efficacy of phage therapy. Loss of phage activity during formulation and storage, or phage inactivation due to the in vivo environment (e.g. acidic pH of the stomach or enzymatic activity) may result in poor phage therapy outcomes. These issues have received little attention in phage therapy literature. Research is therefore needed in the area of phage formulation to ensure phage titre remains stable during storage (e.g. over a 24 month period). Phage cocktails necessitate careful consideration of formulation conditions as each type of phage making-up the cocktail may require individually tailored formulations to ensure phage viability and stability during storage.

3.5. Phage therapy animal studies – acute infections

3.5.1. Key points arising from reviewing phage therapy literature focusing on acute infections in animals

Most in vivo phage therapy studies (in animals) have delivered the phage and bacteria dosed simultaneously together (Table 1). Typically, a high ratio of phage to bacteria has been used (phage concentrations

10–1000 times higher than the infective dose of bacteria). Phage treatment of acute infections caused by both Gram positive and Gram negative bacteria have been studied (Table 1).

Phage have been administered using different delivery routes including intramuscular [34,66–69], subcutaneous [29,31–34,42], intraperitoneal [31,34,67,70–72], intravenous [29,68], intranasal [26–28,42], orogastrically [57,58,64,70,73–81], intracranial [69] or added to animal feed [75,82] or water [83]. The route of phage administration has been shown to affect efficacy outcomes [34,42,66]. In a number of studies intraperitoneal delivery was shown to result in higher concentrations of systemic phage, delivered earlier, remaining in circulation for longer and allowing phage access to numerous host organs including lungs, spleen and kidneys [34,32]. Better therapy outcomes were seen for lung infections when phage were dosed using the intraperitoneal or systemic route instead of using pulmonary delivery [31].

A number of in vivo animal studies have shown dose dependent recoveries with high doses of phage resulting in better clinical outcomes [33,66–68]. Delaying the start of phage treatment post-infection typically resulted in poor outcomes [33,67,68]. This was attributed to high growth rates resulting in high concentrations of replicating bacteria overwhelming the immune system with bacterial toxins [68].

Inoculation of control animals (not infected with bacteria) with high phage doses was shown not to result in adverse health symptoms in animals [67]. A number of in vivo studies reported rapid drop in phage titre in the absence of host bacteria suggesting clearance by the host immune response or other removal mechanisms [32,58]. Encapsulation of phage in liposomes (for treating gastrointestinal infections) was shown to increase the retention time of phage in the host with better therapeutic efficacy [31].

In situ amplification of phage (at high phage doses) has been observed in some instances [71]. Frequent administration of high phage doses was found to result in better outcomes [64,76,78,80]. This is an important point considering in vivo phage-bacteria dynamics. Better phage therapy outcomes were noted when both phage and bacteria concentrations tend to be high. Phage titre reduction in vivo may result in poor phage therapy results when bacterial concentrations may be too low for rapid phage amplification. Reduction in phage titre may also explain results of a number of studies that evaluated prophylactic phage therapy with mixed results [70,73,75,79,80,83]. Use of phage cocktails rather than single phage was found to yield better results [58,64] and in some instances reduced the chances of finding phage resistant mutants. A number of studies did find phage resistant mutants [66,69]. The complex architecture of the intestinal tract was found to present a challenge for phage therapy. Temporary reduction in bacterial faecal shedding attributed to phage treatment was typically followed by an increase in faecal bacterial concentration without clearance of gastrointestinal infections [57,58,75,77,83].

Results of a number of animal studies suggest that stopping rapid amplification in bacterial numbers is an important factor in therapy outcomes [26–28]. Arresting infection by early administration of high phage dose when the bacteria are also highly susceptible to phage infection was found to correlate with better phage therapy outcomes. Administration of high concentrations of phage may result in multiple phage particles adsorbing per bacterial cell which may lead to bacterial cell lysis due to phage binding without phage virion production. Delbruck [84] coined the term "lysis from without". Large numbers of phage adsorbing to the bacterial cell wall may lead to cell wall damage due to the enzymatic action of tailed phage enzymes acting on the cell envelope/wall [85].

Infection is generally induced in animal models with a single bacterial species, however, even single bacterial triggered infections necessitate the use of phage cocktails rather than single phage treatments. This is because phage mixtures targeting different receptors reduce the probability of encountering phage resistant bacterial mutants (see modelling section above). Denou et al. [86] suggest that cocktails containing between 10 and 16 phage may be needed to cover between half and two thirds of *E. coli* strains (representing the five main pathogenic types isolated from patients suffering from diarrhoea) in order to cure *E. coli* related diarrhoea. Cocktails of phage may also provide synergistic effects such as access to bacteria in mucus and biofilms with a phage possessing biofilm/mucus degrading enzymes enhancing access within mucus/biofilms for other phage better suited to killing the bacteria [87]. This point has significant implications for phage formulation since different phage within a cocktail may need different formulation ingredients to aid shelf life during storage in order to retain infectivity. A number of animal studies (discussed below) have shown that regular repeated doses of high phage titres resulted in better outcomes rather than a single dose. Encapsulation of phage in controlled release systems may allow constant release (zero order release) of phage e.g. using readily available technologies such as osmotic pumps or constant reservoir systems used in the pharmaceutical industry. Vandenheuvel et al. [88] discuss the importance of phage stability and the unacceptability of phage titre drop in view of pharmaceutical regulations. The results of animal studies (above) provide strong evidence of the need to deliver high titres of phage at the site of infection. Phage dose-response studies in animals suggest that a drop in titre of 1-log during storage could result in phage therapy failure. Reduction in phage titres due to phage inactivation attributed to stomach acidity was partly blamed for failure of a recent clinical trial aiming to show reduction in acute bacterial diarrhoea symptoms in children using phage therapy [35].

The pharmacokinetics of phage delivery to various animal organs depended on the route of administration. In a number of studies, intraperitoneal delivery of phage resulted in higher concentrations of phage in systemic circulation and those found in different organs including lungs. Encapsulation of phage in liposomes was found to increase the length of systemic circulation of phage. There is some evidence in literature that phage encapsulated in liposomes may enhance delivery to intracellular infections (discussed later).

Bacteriophages have a narrow host range. They are specific not only in terms of killing a particular species, but often they only target a sub set of strains within the species. In an outbreak situation where the causative agent and strain information is available, selection of a relevant phage for therapy may not be a problem [26]. However, as phage host range is narrow, rapid diagnosis of the causative agent and the strain would benefit accurate phage selection. Most phage therapy studies in model animal systems (discussed above) have used simple phage solutions prepared immediately prior to use. Phage banks may need to regularly screen phage against common infection causing strains and manufacture these in batches to make them readily available. The phage would need to be processed in such a manner so as to ensure long shelf life (typically 12 months) e.g. formulated in excipients and spray dried or freeze dried to ensure accurate dosage at the time of therapy.

Characterisation of the kinetics of phage binding to target bacterium and in vitro evaluation of phage-bacterium population dynamics as part of the selection tests (e.g. host range, presence of integrase genes) ascertaining the suitability of phage for therapeutic use is often overlooked and needs greater attention.

A detailed list of literature studies covering phage therapy in various animal models is provided in Table 1 and more detailed commentaries on some selected papers are now given as follows.

Table 1
Summary of phage therapy animal studies to treat acute local, systemic, gastrointestinal and pulmonary bacterial infections.

Ref.	Host organism: *E. coli*	*E. faecium*	*V. vulnificus*	*S. aureus*	*P. aeruginosa*	*K. pneumonia*	*C. jejuni*	*V. cholera*	*B. cenocepacia*	*S. enterica*	*C. difficile*	Animal: Mouse	Chickens	Rabbit	Cattle	Rat	Hamster	Pig	Infection: Systemic	Septicaemia	Meningitis	Local	Wound	Gastrointestinal	Pulmonary	Delivery: Intramuscular	Intravenous	Intracranial	Intraperitoneal	Subcutaneous	Intranasal	Orogastrically	Phage added to feed water	Aero	Injected into thoracic air sac	Tracheotomy	Instillation	Bacteria Log(CFU)	Phage Log(PFU) E – encapsulated R – repeated dosage	Single phage (*) / Cocktail (⁂)	Phage therapy Outcome: Amplification	Clearance of infection	Reduction in concentration of bacterium or mortality	Delayed phage treatment post infection	Dose related studies	Phage resistant mutants	Prophylactic treatment
[66]	Ec																		Sy							bp												7	2-8	*	●	●	●	○	●	◐	●
[69]	Ec																			Se							bp												6	2-6	⁂		●	●		●		◐
[69]	Ec																				M								bp									3	6-8	*	●	●	●		●		◐
[67]		Ef																	Sy										bp									9	8	*		●		◐	●		
[68]			Vv																Sy			L					p			b								2-6	4-8	*		●	●	○	●		
[71]				Sa															Sy										bp									9	9-11	*	●	●		●	●		
[33]				Sa																			W							bp								8	6-9	*	●		●	○	●		
[34]					Pa																		W			p			p	b bp b								2	8	⁂		●	●			●	
[29]				Sa															Sy			L					bp			bp								8	7-9(R)	*	○	●			●		
[31]						Kp													Sy										pΦ		b							4	9, 7(E)	*		●	●	●			●
[69]	Ec																							G		p						b						10	10	*	●	○	○			◐	
[83]	Ec																							G								b	p					4-8	5-6	⁂	●	○	●				○
[64]	Ec																							G								bp*						9	8-10(R)	⁂		●	●	●	●		
[73]								Cj																G								bp						5	9-10	*/⁂	●	○	●	○			○
[70]					Pa																			G					bp			bp						6-10	10	*		○	◐	○			○
[74]										Se														G								bp						4	8	⁂		○	◐				
[75]	Ec																							G								bΦ						11	10-11	⁂		○	○				○
[57]	Ec																							G								bΦ*						5	~5(R)	⁂		○	●				
[76]											Cd													G								bp*						3	~8(R)	*/⁂		○	●			○	
[78]										Se														G								bΦ						7	10(R)	⁂		○	●			●	
[77]										Se														G								bΦ						7	10(R)	⁂		○	●			●	
[58]								Vc																G								bp*						5, 8	5-7, 8	*/⁂		●	●		◐	◐	◐
[58]								Vc																G								bp*						8	9	*/⁂		●	●			◐	◐
[59]										Se														G								bΦ						9	~10(R)	⁂		○	●				○
[80]										Se														G								bp						6	~10(R)	⁂		●					○
[80]										Se														G								bp						5	~10(R)	⁂		○	●				
[92]	Ec																								P	p						p		p					6-8	⁂				●				
[90]	Ec																								P									p	b			4	7-9	⁂		○	●	●			◐
[42]									Bc																P				p		p					b	b	7-9	7-9	*	◐	○	◐	◐			
[26]					Pa																				P												bp	7	7-9	*	◐	●	●	●	●		●
[27]					Pa																				P												bp	7	8	⁂		○	●				
[28]					Pa																				P												bp	6	6-8	*	◐	○	●		●		●
[31]						Kp																			P					pΦ							b	4	2-4	*	◐	●	●	●			●
[32]						Kp																			**P**					p							b	7-8	9-11	*		●	●	○			○

3.5.2. Parenteral phage delivery (animal studies)

A number of animal studies have looked at parenteral delivery of lytic bacteriophage following delivery of an infectious dose of bacteria (Table 1). Early work by Smith et al. [66] demonstrated the potential of intramuscular and intravenous administration of phage (10^8 PFU/mouse) against *E. coli* infection (intramuscular injection of LD_{50} dose of 10^7 CFU/mouse) in a mouse model. The effect of delaying treatment (8 h delay in treatment) showed favourable results. Prophylactic treatment up to 2 days before induced infection resulted in significant protection in animals. Intramuscular injection of bacteria resulted in high concentration of bacteria at the site of infection and in the blood, spleen and liver ~24 h post-inoculation. Within 5 min of phage administration, high phage numbers were found in the muscle, blood, spleen and liver. They persisted for shorter durations in the blood and liver compared with the inoculated muscle and spleen. Phage resistant mutants were found at the site of infection but were considered much less virulent.

Barrow et al. [69] inoculated (intramuscularly (septicemia model) and intracranially (meningitis model)) newly hatched and 3 week old chickens with *E. coli* and phage and showed dose dependent recovery. Administration of higher concentration of phage resulted in significantly better outcomes. The same study also evaluated oral *E. coli* administration to Aberdeen Angus Calves with concomitant intramuscular phage delivery. The phage were unable to arrest the intestinal growth of *E. coli* and the presence of phage resistant mutant strains were found in the gut one day following *E. coli* inoculation and two days later in the blood (indicating translocation of phage from the gut into blood e.g. through intra-abdominal abscesses) of the infected calves. This study showed the importance of phage dose and route of delivery on therapy outcomes (mortality).

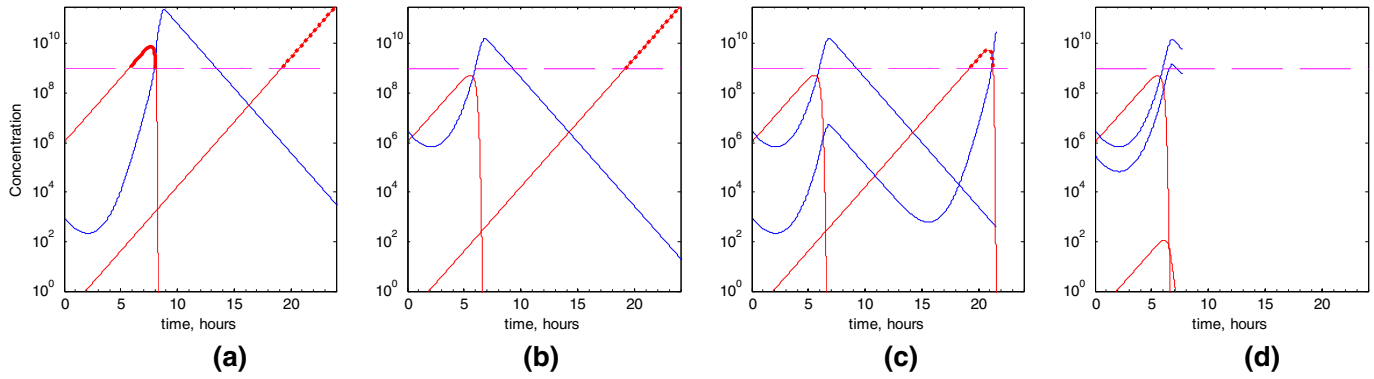


Fig. 1. Population dynamics of bacteria and phage.
Blue lines – phage P1 (solid line) and P2 (dashed line). Red lines – bacteria B1 (solid line) and B2 (dashed line); B2 is resistant to P1.
(a) – low dose of single phage ($P1_0 \approx 10^3$ PFU/ml) is insufficient to prevent bacteria concentration exceeding upper threshold;
(b) - high dose of phage ($P1_0 \approx 10^6$ PFU/ml) arrests B1 growth, but phage resistant mutant bacteria B2 grows unchecked;
(c) and (d) - mixture of two different phage; (c) - low dose of P2 is insufficient to prevent resistant bacteria B2 concentration exceeding upper threshold; (d) - high dose of both phage prevents both bacteria concentrations from reaching upper threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Using a *Enterococcus faecium* bacteremia mouse model Biswas et al. [67] investigated the effect of phage dose in *E. faecium* infected mice (concomitant delivery of phage and bacteria delivered via intraperitoneal injection) as well as the effect of delaying the start of phage treatment (5 h, 8 h, 12 h, 16 h and 24 h after inoculation with bacteria). The severity of illness symptoms exhibited by mice was correlated with the bacterial concentration in the blood and this was shown to be dependent on the concentration of phage used for treatment. Mice inoculated with high titres of phage suspension (without infection with bacteria) showed no adverse symptoms. 24 h delay in phage treatment resulted in 60% (3 out of 5) of the mice dying after 96 h. The dosage and the timing of the delivery of phage were found to be important in arresting bacterial growth and in mice recovery.

Cerveney et al. [68] evaluated the efficacy of phage (used locally and systemically) against the Gram negative bacterium *Vibrio vulnificus* (causes septicemia after ingestion of contaminated oysters and necrotising fasciitis in contaminated wounds) in an iron-dextran-treated mouse model. Bacteria were injected subcutaneously whereas phages were injected intravenously. A high phage dose (10^8 PFU) was given (the animal was subcutaneously given a dose of 10^6 CFU of a highly virulent clinical isolate of the bacterium to create a skin lesion) and shown to reduce the bacterial burden of the lesion and in the bacterial CFU in the liver (14 h post-infection). Experiments with delayed treatment (≥ 6 h) post-infection showed lack of efficacy and resulted in treatment failure. Dose response studies with animals infected with 10^3 CFU of bacteria and immediately given either 10^4, 10^6 or 10^8 PFU of phage showed only the higher concentration of phage dose at 10^8 PFU afforded significant protection to the treated mice. The need for high phage dosages was attributed to the short doubling time of the bacterium (45 min) which resulted in a high growth rate of bacteria post-infection. The study also evaluated the half-life of phage (2.2 h at 10^8 PFU) injected intravenously and found that phage concentrations in blood rapidly decreased due to clearance by the reticuloendothelial system. Phages are known to induce antibodies aiding their removal from circulation as well as neutralising their interaction with bacteria [30,42,89].

Matsuzaki et al. [71] showed dose dependent pharmacodynamics using concomitant intraperitoneal injection of *S. aureus* followed by phage (delivered within 60 min of bacterial inoculation) with high concentrations of phage observed in blood (at high phage doses 10^{11} PFU to treat 10^9 CFU of *S. aureus*) indicating in situ amplification of phage. In a rabbit model of wound infection induced by *S. aureus*, Wills et al. [33] used a high dose of phage (10^9 PFU, delivered subcutaneously) whilst simultaneously infecting with *S. aureus* (~10^8 CFU) to show protection against abscess formation. The study also carried out a dose response study and showed that only at the highest phage dose

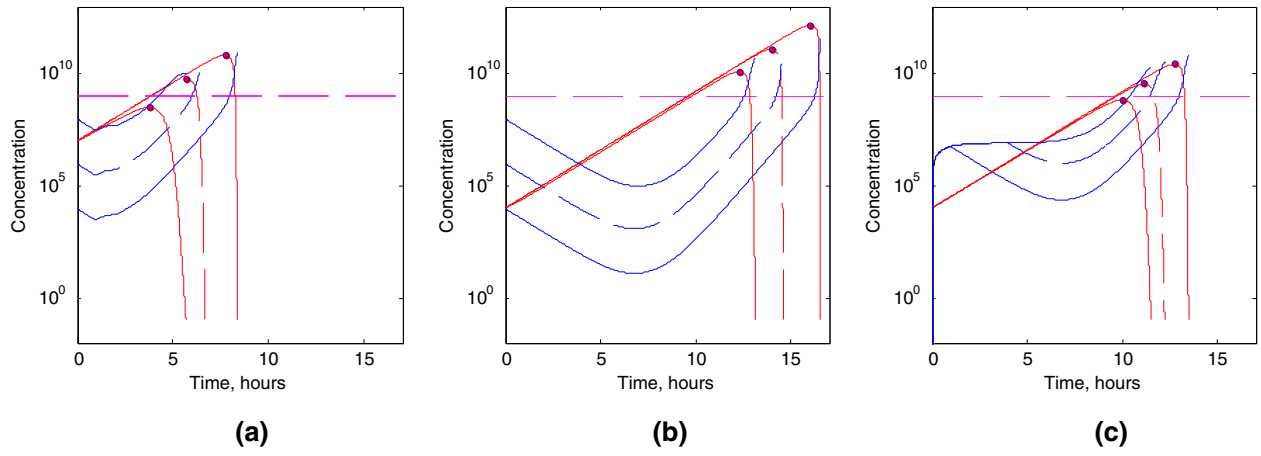


Fig. 2. Population dynamics of bacteria and phage.
Blue lines – phage concentration; red lines – bacteria concentration.
(a), (b) – effect of phage dose (10^4–10^8 PFU/ml) and initial bacteria concentration (10^4, 10^7 CFU/ml);
(c) - effect of continuous sustained delivery of phage for 1 h, 4 h and 10 h (solid, broken and dashed lines accordingly). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was there an absence of abscess formation. The median area of abscess formation and the concentration of bacteria found in the abscess (CFU/ml) showed an inverse correlation with the dose of phage given. The effect of delaying treatment (by giving a high phage dose (10^9 PFU) 6 h, 12 h and 24 h after bacterial infection) resulted in abscess formation in all animals.

McVay et al. [34] in a mouse burn wound model compared intramuscular, subcutaneous and intraperitoneal routes of phage delivery using a cocktail of *Pseudomonas aeruginosa* phage. Phage administered by the intraperitoneal route ($\sim 10^8$ PFU) afforded better protection against *P. aeruginosa* infection ($\sim 10^2$ CFU injected subcutaneously at the burn site) with higher concentrations of phage found in the blood, spleen and liver of animals. Phage resistant mutants were not found even in animals that had died. Intraperitoneal delivery of phage was found to result in delivery of higher phage dose, delivered earlier into the blood and other tissues, and delivered for a more sustained period of time.

Capparelli et al. [29] intravenously infected mice with *S. aureus* (dose, 10^8 CFU/mouse) followed by immediate intravenous injection of phage (10^7, 10^8 or 10^9 PFU/mouse). All the mice in the control group (infected but not treated with phage) and those treated with the lowest phage dose (10^7 PFU/mouse) died within 4 days (10/10 mice). The mice treated with the intermediate dose (10^8 PFU/mouse) were only partially protected (2/5 mice survived) whilst mice treated with the highest dose (10^9 PFU/mouse) all survived (5/5 survived). 4 days after phage injection, high concentrations of bacteria (in kidney, heart, spleen and lung) were recorded for the untreated controls whereas no bacteria were found in the same organs for mice treated with 10^9 PFU/mouse. Lack of phage amplification in blood (attributed to a replication threshold) was in all likelihood down to the low concentrations of bacteria in blood making it difficult to accurately monitor phage amplification under such conditions. At low levels of systemic infection (*S. aureus* dose 10^6 CFU/mouse) a 10 day delay in phage therapy (10^9 PFU/mouse) cleared *S. aureus* infection in the animals. Capparelli et al. [29] also evaluated the efficacy of phage therapy for local *S. aureus* infections (subcutaneous injection of bacteria and phage). Concomitant injection of phage prevented formation of abscess in the mice. Delayed phage treatment (phage administration 4 days after onset of infection) did not prevent abscess formation. There was a reduction in the bacterial load in the abscess and the weight of the abscess when multiple injections (4 injections/day) were used. Repeated doses were found to be more effective than a single dose in reducing the bacterial load in the abscess and the weight of the abscess. *S. aureus* can adopt an intracellular lifestyle. Capparelli et al. [29] also evaluated the efficacy of phage activity in a *S. aureus* intracellular infection mouse peritoneal macrophage cell line model. Phages were not able to penetrate the macrophages and kill the intracellular *S. aureus*. Using a Trojan horse approach, *S. aureus* infected with phage were able to access the intracellular compartment of the macrophages and kill *S. aureus* cells residing within. This study suggests the possibility of encapsulating phage in systems that may facilitate access to intracellular compartments where infecting bacteria may be targeted (more on this later in the section on encapsulation of phage in liposomes to target intracellular pathogenic bacteria).

3.5.3. Oral phage delivery (animal studies)

Animal studies using oral delivery of bacteriophage have focused on acute gastrointestinal infections (Table 1). The main concern here is phage inactivation due to the acidic and proteolytic environment of the stomach. The use of antacids e.g. calcium carbonate or bicarbonate prior to infection challenge or with phage delivery in order to neutralise the stomach acidity has been common practice in a number of studies [23,58,64,76,78]. A few studies have employed encapsulation of phage in polymer or liposome encapsulation systems [57,75,77,78]. Colom et al. [77] showed that encapsulation of phage in liposomes resulted in significantly longer periods of phage retention (several days longer) in the caecum of chickens and lower number of bacteria colonizing the gut.

Chibani-Chennoufi et al. [83] showed survival of T4-like *E. coli* phage through the gastrointestinal tract of mice when added to drinking water. The minimal oral dose for consistent faecal recovery was 10^4 PFU/ml of drinking water. *E. coli* infected mice showed 10^5 fold amplification of phage with concomitant drop in faecal *E. coli* counts from 10^8 to 10^4 CFU/ml. However, phage sensitive *E. coli* concentration recovered post-treatment even when residual high faecal phage titres were present, suggesting phage sensitive *E. coli* were able to replicate in the gut and that the concentration of phage present in the gut was not high enough to eliminate the *E. coli*. The authors suggested sites in the gut where *E. coli* may be protected from the phage (cells growing as microcolonies in the mucin layer). Other studies have also reported an initial reduction in faecal shedding followed by recovery of bacterial numbers [64,73,74].

Tanji et al. [64] used a chemostat (in vitro studies) to investigate the dose and frequency of phage administration on phage-*E. coli* population dynamics. The chemostat experiments suggested repeated high doses of phage were needed to keep the *E. coli* concentration in check. The results informed phage therapy in mice where *E. coli* was orally administered (dose 10^9 CFU/mouse) using a plastic sonde directly into the stomach followed 2 days later with phage cocktail delivery to the stomach in a similar manner (single dose of 10^8 PFU/mouse or 10^{10} PFU/mouse or repeated daily doses of 10^{10} PFU/mouse). Daily administration of phage resulted in a stable concentration of phage in the faeces and significant reduction in the *E. coli* concentration in the faeces. A cocktail containing three phage was employed however, the concentration of phage in the faeces suggested one phage dominated and was present at a significantly higher concentration in comparison with the other two (indicating differences in the pharmacodynamics of the three phage). Nine days after the start of phage therapy with daily administration of phage, no *E. coli* were detected in the lower small intestine and the colon of treated mice. A number of studies have used repeated dosing of phage and shown reduction in intestinal colonization with bacteria [57,76–80]. This study stands out as one of very few where elimination of *E. coli* was observed for treatment of gastrointestinal infections.

Wagenaar et al. [73] evaluated the prophylactic or therapeutic efficacy of phage treatment for control of *Campylobacter jejuni* (*C. jejuni,* which colonizes young broiler chickens, is an important human pathogen and is the most common cause of bacterial gastroenteritis worldwide). A modest 1-log reduction in bacterial CFU counts ($\sim 10^9$ CFU/g faeces) was observed in both the prevention and the therapeutic groups compared with the control group (not given phage). Phage selection was on the basis of activity across a wide range of *C. jejuni* strains.

Watanabe et al. [70] investigated the efficacy of phage therapy using a murine model of gut-derived sepsis caused by *P. aeruginosa* (resembling the clinical pathophysiology of septicemia in humans). Administration of cyclophosphamide (to suppress the immune system) and ampicillin (to disrupt the gut microflora) was used before oral inoculation with *P. aeruginosa* to achieve gut-derived sepsis. Intestinal overgrowth in the gut resulted in *P. aeruginosa* crossing the gastrointestinal mucosal barrier by bacterial translocation and spreading systemically in the mice. Timing of phage treatment was found to influence the efficacy of phage therapy. Mice treated with a high dose of phage 1 day post-infection with *P. aeruginosa* showed improved survival rates over controls (mice not treated with phage). Average numbers of viable bacteria in blood, liver, spleen and mesenteric lymph nodes were

significantly lower in phage treated mice compared with controls. Faecal counts were also lower in phage treated mice as were cytokine levels in serum and liver. Compared to oral administration, intravenous and intraperitoneal administrations of phage were found to result in better outcomes. The infectious dose was 10^6 CFU/mouse with phage dose of 10^{10} PFU/mouse. Only simultaneous intraperitoneal administration of phage and bacteria resulted in improved mice survival.

Andreatti Filho et al. [74] showed short term (24 h after infection) reduction in *Salmonella enterica* detection (dose ~ 10^4 CFU/chick) from caecal tonsils following phage administration (phage cocktail containing 45 different phage administered using oral gavage, 10^8 PFU/chick). However, no significant differences were observed between the phage treated and the control groups after 48 h. The cocktail used wasn't sufficiently well characterised in terms of individual phage concentrations and the kinetics of individual phage binding and their lytic activity; a recurring theme in the reading of literature in this area.

Stanford et al. [75] encapsulated *E. coli* phage in a commercial pH responsive methacrylate polymer using a spray drying process (a 1 log reduction in activity due to spray drying was noted). Oral inoculation of 10^{11} CFU/steer of a five strain mixture of *E. coli* O157:H7 was followed either by encapsulated phage given as a single oral bolus (10^{10} PFU/steer) or mixed-in with feed (10^{11} PFU/steer). Higher concentration of faecal shedding of phage (2-log higher) was noted for encapsulated phage given as part of the feed rather than bolus suggesting differences in in situ phage concentrations. Phage were shown to replicate in situ over a period in excess of ten days following treatment and continued to be shed 42 days post-treatment (*albeit* at lower concentrations in comparison with the first ten days) however, no significant differences in reduction in *E. coli* shedding was observed between the phage treated steer and the controls. Insufficient understanding of the in vivo pharmacodynamics of the phage against the *E. coli* strains may have resulted in poor outcomes of phage therapy.

To address the issue of human gastroenteritis caused by *E. coli* transmitted through farm animals, meat and poultry Abdulamir et al. [57] studied the effect of a cocktail of 140 lytic *E. coli* specific phage encapsulated using a hydroxypropyl methylcellulose (HPMC) based enteric coating. The capsules (ZeinPharma Germany, GmbH, Germany) encapsulating the 140 *E. coli* specific phage were administered orally in rats. A concentration of 10^7 PFU/ml phage suspended in an aqueous solution of sodium bicarbonate and an injectable master mix in lambda buffer were compared with HPMC capsules carrying the phage cocktail in lambda buffer. HPMC encapsulated phage were found to give better results with a reduction in *E. coli* shedding of up to 4-log.

Nale et al. [76] used a hamster model for the oral delivery of either single or a mixture of phage against *Clostridium difficile*. A hamster model of acute *C. difficile* infection (CDI) was used. The model reflected some of the clinical features of CDI including toxin-mediated diarrhoea and tissue pathology. Animals were given sodium bicarbonate orogastrically to reduce stomach acidity. Animals were infected with ~10^3 CFU/hamster of spores and subsequently treated with ~10^8 PFU/hamster of either a single phage or a cocktail of phage. The first dose was given at the time of infection with further doses every 8 h until the scheduled end-point of 36 h (toxin production in untreated animals is limited up to this time point in the cecum and colon thereby allowing assessment of the effect of phage treatment on colonization in the absence of toxin related symptoms). At this time point, animals were culled and enumeration of total bacterial load (spores and vegetative cells) in the cecum and colon was undertaken. Phage treatment with certain combination of phage mixtures was shown to reduce bacterial load in the cecum and colon however, results were variable. This variability was attributed to the ratio of phage to bacteria present at various locations in the cecum and colon during the initial phase of bacterial outgrowth. In a separate experiment using the earlier protocol but continuing regular 8 h phage treatments delayed onset of symptoms by 33 h (due to toxin production) in phage treated animals compared to untreated animals was observed. Complete protection was not achieved. The amount of phage delivered to the cecum or colon was not quantified however, high titres of phage 10^7 PFU/ml were recovered from the cecum and colon.

Recently Colom et al. [77] used a cocktail of three *Salmonella* phage individually encapsulated in cationic liposomes to treat *Salmonella* infection in commercial broilers under farm-like conditions. Bacteriophage retention in the chicken caecum was investigated by giving an oral dose of phage (10^{10} PFU/chicken) of either free phage (1:1:1 mixture of the three phages ϕ20, ϕ78 and ϕ87) or liposome encapsulated phage cocktail to 63 1-day old chickens. Caecum samples collected from euthanized animals showed significantly higher numbers of chickens treated with encapsulated phage had phage in their caecum 48 h and 72 h later. Bacteriophage therapy against *Salmonella* was evaluated over 17 days in chickens orally administered either the free phage cocktail or encapsulated phage cocktail (bacterial dose at day 0: 10^7 CFU/chicken, phage dose 10^{10} PFU/chicken). Oral doses of phage were administered daily for 9 days (from day −1 to day 7 after *Salmonella* infection). Over the course of daily phage therapy (days 1, 3, 6) both groups receiving phage treatment showed lower (~3 log reduction) *Salmonella* concentration in the cecum compared with the control group. Once daily phage treatment had stopped (day 8 onwards), *Salmonella* concentration in the free phage treated group returned to levels similar to the control group. However, the liposome encapsulated phage treated group continued to show lower *Salmonella* concentration in the cecum compared with the control group up to day 15. *Salmonella* infection was not eradicated by either treatment which was attributed to high initial *Salmonella* concentration in the cecum (10^6 CFU/g of cecum), which was considered higher than naturally occurring levels.

In a separate study, Colom et al. [78] used a cocktail of three *Salmonella* phage individually encapsulated in alginate microparticles containing $CaCO_3$ to treat *Salmonella* infection in commercial broilers under farm-like conditions. Bacteriophage retention in the chicken caecum was investigated by giving an oral dose of phage (10^{10} PFU/chicken) of either free phage (1:1:1 mixture of the three phages ϕ20, ϕ78 and ϕ87) or alginate/$CaCO_3$ encapsulated phage cocktail to 63 1-day old chickens. Caecum samples collected from euthanized animals showed significantly higher numbers of chickens treated with encapsulated phage had phage in their caecum 48 h and 72 h later. Bacteriophage therapy against *Salmonella* was evaluated over 17 days in chickens orally administered either the free phage cocktail or encapsulated phage cocktail (bacterial dose of day 0: 10^7 CFU/chicken, phage dose 10^{10} PFU/chicken). Oral doses of phage were administered daily for 9 days (from day −1 to day 7 after *Salmonella* infection). Over the 15 days, a significant reduction (between 2 log and 3 log) in *Salmonella* concentration was noted for the group treated with encapsulated phage. At days 8, 10 and 15 the encapsulated phage treated group had lower *Salmonella* concentration compared with the free phage treated group showing better therapy outcomes using the encapsulated phage. The mucoadhesiveness of the microparticles and the slow sustained release kinetics were thought to be important factors in explaining these results. Similar concentration levels of phage were noted in the caecum for both encapsulated and free phage (~10^3–10^4 PFU/g). Phage persisted in the caecum even after phage treatment was stopped with higher phage concentrations noted for the free phage treated animals. *Salmonella* infection was not eradicated by either treatment. The authors reported that they did not find any phage resistant bacteria in the phage treated groups. Hence the persistence of *Salmonella* infection was not attributed to phage resistant mutants.

A different study using alginate microencapsulated *Salmonella* phage to treat pigs [59] showed that co-administration of Salmonella

with a phage cocktail resulted in reduction in pig intestinal *Salmonella* concentration (~2 log reduction). Yen et al. [58] recently reported studies with mice and rabbits on the prophylactic effect of phage therapy to treat cholera. Oral administration of a cocktail of three *Vibrio cholera* lytic phage up to 24 h prior to infection with *V. cholera* reduced colonization and cholera-like diarrhoea. Complete elimination of bacteria was observed at short prophylaxis times (3 h), high phage dose (10^6–10^7 PFU/mouse) and low bacterial infection dose (10^5 CFU/mouse). Phage administration in negative controls (i.e. not inoculated with bacteria) showed a significant drop in phage titre (3 log reduction in mice) 12 h following phage treatment, indicating phage elimination by the host.

3.5.4. Pulmonary phage delivery (animal studies)

Pulmonary delivery of phage has been achieved using a variety of methods including nasal instillation [26–28,42], aerosolization of liquids using a nebulizer [90,91] and intraperitoneal injection [31,32]. Carmody et al. [42] found intraperitoneal delivery of phage resulted in reduction in an acute lung *Burkholderia cenocepacia* infection in mice compared with nasal instillation whereas Oliveira et al. [92] found intramuscular injection better than oral administration or using a spray.

The dose of phage, concentration of bacteria and the timing of phage therapy were found to have a significant impact on phage therapy outcomes. In most cases delivering high doses of phage immediately or soon after inoculation of lungs with bacteria resulted in better outcomes [26,28,32]. Singla et al. [39] compared treatment of induced acute *K. pneumonia* infection in mice with free phage versus liposome encapsulated phage and showed that high dose of encapsulated phage gave better outcomes given up to 48 h prior to *K. pneumonia* infection. A number of studies reported in situ amplification of *P. aeruginosa and K. pneumonia* phage [26,28,39]. Phages were rapidly cleared from the lungs of mice [28] hence high phage dose was needed for prophylactic treatment 24 h prior to bacteria inoculation [26]. Effective phage therapy reduced the host inflammatory response [26,28,39].

Phages for treating respiratory infections caused by *P. aeruginosa*, *B. cenocepacia*, *E. coli* and *K. pneumonia* are available. Efficacy of phage activity against clinical strains obtained from CF sputum and in relatively early stage biofilms has been shown [27,39]. Phages with lytic activity have been shown to possess hydrolases that degrade bacterial exopolysaccharides. Animal studies have shown that a number of phage delivery routes are effective for phage delivery to treat acute lung infections. However, further work needs to be done to show the efficacy of phage therapy in chronic infections against more mature biofilms containing well established polymicrobial communities in both in vitro and in vivo systems.

Oliveira et al. [92] evaluated the effect of the route of administration (oral, direct spray on beaks and intramuscular injection) and the concentration of phage in the dose (10^6, 10^7 and 10^8 PFU/chicken) in the dissemination of 3 lytic *E. coli* phage in the organs of chickens (lungs, liver, duodenum and spleen). The focus of the study was on controlling avian respiratory infections. Administration of phage using intramuscular injection was found to be the best means of ensuring phage dissemination in all tissues (including lungs). In the work by Oliveira et al. [92], aerosol spray administration (details regarding the spray generation were not given) allowed phage to be directly delivered to the lungs. Sufficient details regarding the formulation of phage for spray dose were not given, e.g. whether the spray droplet size distribution was suitable to aid droplet distribution in the lower respiratory tract. The phage type, administration route and dose delivered were all found to affect the distribution of phage in the animal tissues. Phages were rapidly cleared from the organs 10 h after administration.

Huff et al. [90] determined the efficacy of aerosol administration (using an atomising nozzle and exposing the birds to the aerosol in a closed chamber resulting in uptake by inhalation) of a cocktail containing two bacteriophage (isolated against avian pathogenic *E. coli*) to prevent an *E. coli* respiratory tract infection in broiler chickens. A severe dose of 10^4 CFU/chicken (age 1 week) followed by a cocktail of phage (a mixture of two phage at 10^7:10^8 PFU/chicken) resulted in reduction in observed mortality (at age 3 weeks) compared with untreated controls. The dose of phage was found to be important with lower concentrations of aerosolized phage affording worse protection. The timing of phage administration was also evaluated with phage treatment immediately following *E. coli* infection showing positive efficacy results. However, results were mixed when phage were administered prior to or 3 days following *E. coli* challenge. Bacteria were cultured from swabs of liver and air sacs but data on phage titres from the tissue samples were not given.

Carmody et al. [42] investigated the therapeutic potential of bacteriophage in an in vivo mouse model of acute *B. cenocepacia* pulmonary infection. Carmody et al. [42] compared intranasal inhalation of bacteriophage with intraperitoneal injection of phage. 9 to 12 week old mice were infected with 10^7 or 10^8 CFU *B. cenocepacia* (via tracheotomy). 24 h after infection, mice were treated with either intranasal inhalation (spray details not given) or intraperitoneal injection of phage suspended in buffer (100 times higher dose of phage with respect to the bacterial dose was given). 48 h after phage treatment, mice were euthanized and lung tissue samples were analysed for *B. cenocepacia* and phage. Mice treated with phage delivered via intraperitoneal injection had significantly lower lung bacterial counts compared with untreated control mice 48 h after treatment. The phage concentration in the lungs of mice was not statistically significant from mock infected mice with similar dose of intraperitoneal treated phage. The intranasal phage treatment did not result in a statistically significant reduction in lung bacterial counts, however, phage had amplified in the lungs and were present at concentrations above those in mock infected mice with similar dose of inhaled phage. 24 h after infection (but before phage treatment), bacteria were primarily found in lung parenchyma in peribronchiolar and perivascular areas, occasionally co-localised with alveolar macrophages. Relatively few bacteria were found in the airway lumen. Bacteria were found in microcolonies in lung parenchyma 72 h after infection (without phage treatment). The concentration of phage in lungs of mice treated intranasally was 2 log higher in comparison with intraperitoneal treated mice. Intranasally administered phages were found co-localised with alveolar macrophages 48 h after treatment. This suggested that phage delivered intranasally were sequestered by lung macrophages and therefore may be unavailable to kill bacteria found in the lung parenchyma. Inhaled phage did replicate in bacteria as the titres were found to be higher compared with the control group. Phage may have been able to replicate in bacteria taken-up by the macrophages. The phage in the macrophages remained viable as determined by the high phage titres. In intraperitoneally treated mice, phages were found in vascular and perivascular areas and alveolar septa where phages were found co-localised with degraded bacteria. Results from the study indicated better access of phage delivered intraperitoneally via systemic circulation to bacteria than those delivered via pulmonary delivery. The site of greatest bacterial killing may have been within the lung parenchyma rather than the airway lumen. Phage delivered via the airway may not have been able to effectively penetrate the respiratory epithelium where bacteria may have translocated from the lumen to the lung interstitium. The lower concentration of phage in the lung following intraperitoneal delivery was attributed to a rapid decrease in bacterial density and subsequent clearance of phage thereafter in the absence of sufficient bacterial hosts.

Semler et al. [91] found aerosol delivery of *B. cenocepacia* phage in a

mouse lung infection model to be an effective means of phage delivery resulting in reduction in *B. cenocepacia* concentration over a three day period post-infection. They also evaluated intraperitoneal delivery of *B. cenocepacia* phage which resulted in higher concentrations of phage delivered to the lung supporting the findings of Carmody et al. [42] that intraperitoneal delivery of phage allows phage to access the lung. There was however a discrepancy between the results of Semler et al. [91] and Carmody et al. [42] with regard reduction in mouse lung bacteria concentrations for phage delivered using the intraperitoneal route. This may be explained by low concentrations of bacteria present in the lung for experiments carried out by Semler et al. [91] which may have resulted in poor in situ phage amplification.

Debarbieux et al. [26] evaluated pulmonary phage therapy using a fluorescently labelled *P. aeruginosa* strain (to monitor in real-time the spatial and temporal development of infection) in an acute mouse lung infection model. The infectious dose was 10^7 CFU/mouse. In preventive experiments, phages were given intranasally 24 h before infection. Page lysates were simply purified using caesium chloride ultracentrifugation and then suspended in phosphate buffered saline. Untreated mice died within 48 h of infection. Mice treated with a phage to bacterium ratio of 1:10 died within 5 days after infection. Mice treated with a higher phage to bacterium ratio 1:1 (80–100%) and 10:1 (100%) survived until the end of the experiment (12 days post-infection). Subsequent experiments were carried out using the higher phage dose i.e. phage to bacterium ratio of 10:1. Phages were administered 2 h post-infection. Within 6 h post-infection differences were observed in the amount of luminescent bacteria in the lungs of the infected mice suggesting rapid killing by the phage. After 24 h untreated mice were either dead or showed high levels of luminescence whereas phage treated mice showed no or weak spots of light with tissue cultures showing bacterial load of 10^8 CFU/ml in untreated mice versus 10^2 CFU/ml in phage treated mice at 24 h post-infection. Phage concentrations in bronchiolar lavage fluid were lower in uninfected controls (10^6 PFU/ml) versus infected and phage treated mice (10^7 PFU/ml) indicating phage amplification within the infected lungs. Measurement of inflammatory cytokines IL-6 and TNF-alpha indicated attenuation of the host inflammatory response due to phage treatment. Delaying phage treatment by 2 h, 4 h and 6 h post-infection resulted in all mice surviving after 24 h (treated with phage 2 h post-infection) but only 75% of mice surviving following phage treatment 4 h and 6 h post-infection. At 72 h post-infection survival was as follows 100% (2 h delay), 75% (4 h delay) and 25% (6 h delay). Phages were seen to remain effective in mice 24 h following treatment. Infection of mice 24 h post-phage treatment (intranasal administration of 10^8 phages/mouse: 10^7 CFU/mouse) resulted in 100% of mice surviving 16 days post-infection whereas all untreated mice had died. Non-invasive bioluminescence data suggested that rapid bacterial replication kinetics was an important factor in arresting infection by early administration of phage when the bacteria were also highly susceptible to phage infection. High dose of phages administered early during the onset of infection rapidly reduced bacterial numbers and the associated inflammatory response in the host.

Alemayehu et al. [27] compared phage therapy efficacy in a murine model using both mucoid and non-mucoid *P. aeruginosa* strains isolated from CF patients. Lungs of 6- to 8-week old mice were infected intranasally with *lux*-tagged *Pseudomonas* (dose 10^6 CFU/mouse). 2 h following infection a binary mixture of phage (podovirus and myovirus) was delivered intranasally at a dose of 10^7 PFU/mouse. Phages were effective in reducing considerably the amount of bacteria associated bioluminescence in the first 6 h period post-infection.

Morello et al. [28] evaluated the efficacy of phage treatment in a mouse lung-infection model (a dose of 10^6 CFU/mouse resulted in animal death 2 days following infection) against a multidrug resistant *P. aeruginosa* mucoid strain isolated from a cystic fibrosis patient. Within 20 h post-infection, bacteria had multiplied in the lungs by 2-log compared with the initial dose (found in broncho-alveolar lavages). A dose dependent curative effect was observed when phage (dose 10^6 PFU/mouse or 10^7 PFU/mouse was given intranasally 2 h post-infection). 20 h post-infection bacteria were detected in macrophages, alveolae and extracellular spaces of lungs from untreated animals. Phage treatment resulted in lower concentrations of bacteria, high concentrations of phage (suggesting in situ amplification) and low concentration of markers of host immune response (cytokines and lactate dehydrogenase) with histological examination of lung tissue samples indicating less severe damage to lung tissues for the phage treated mice. Uninfected mice given phage intranasally cleared these at the rate of 0.5 log/day. Phage given 4 days prior to mice being given an infective dose of bacteria needed high preventive phage doses (10^8 PFU/mouse) to achieve 100% survival.

The use of bacteriophage as a therapeutic strategy to fight *K. pneumoniae* infection has previously been tested; a murine model of infection could be cleared [32]. However, in this study (as in other animal studies reviewed above), clearance of infection only occurred when bacteriophage were administered immediately after *K. pneumoniae* infection which limits the potential of phage therapy for clinical use.

Singla et al. [31] used liposome encapsulation of the lytic *K. pneumoniae* bacteriophage (belonging to the *Podoviridae* family). They investigated treatment efficacy of liposome-encapsulated phage compared with free phage, to treat established *K. pneumoniae* infection in a murine pneumonia model. Delay in phage treatment administered via intraperitoneal injection (6 h, 24 h and 48 h) post-intranasal administration of *K. pneumonia* was investigated. Free phage administration 6 h post-administration of *K. pneumonia* was shown to result in complete elimination of *K. pneumonia* from lung samples (this was not the case for the 24 h (~2 log reduction in CFU noted) and 48 h (CFU levels similar to untreated positive controls) treated animals) delay is phage treatments. Phages were observed in the vascular and perivascular areas and along alveolar septa; similar results were reported by other researchers [42]. Phages were observed in lungs for longer (up to ~3–4 days) for animals infected with *K. pneumonia* compared with negative controls. Phage amplification was noted in infected animals treated with free phage. Animals treated with liposome encapsulated phage 6 h (infection cleared in 24 h post-treatment), 24 h (infection cleared in 24 h post-treatment), 48 h (reduction in CFU) and 72 h (reduction in CFU) after *K. pneumonia* infection showed variable levels of efficacy of treatment. Earlier treatment was better than delaying treatment 48 h or 72 h. In the *K. pneumonia* mouse infection model, bacterial concentrations in control animals (untreated) were seen to peak at day 3 and fell significantly by day 5. Phage amplification in lung samples was noted for phage encapsulated liposome treated animals. Liposome entrapped phage persisted in lungs for 5 days post-treatment. Prophylactic phage treatment (3 h, 6 h and 24 h prior to intranasal *K. pneumonia* infection) was studied [31]. No significant therapeutic effect was observed for mice treated with free phage 24 h prior to infection. The 3 h and 6 h treatment resulted in absence of infection. For phage encapsulated in liposomes no infection developed when mice were treated 6 h, 24 h and 48 h prior to administration of infection suggesting encapsulation had a beneficial prophylactic therapeutic effect due to retention of the phage at therapeutic concentrations in the lung.

Singla et al. [31] showed evidence of reduction in levels of pro-inflammatory cytokines IL-6 and TNF-α with free phage treatment (0 h and 6 h prior to intranasal bacterial inoculation) and with liposome treated animals (24 h and 48 h prior to intranasal bacterial inoculation). Debarbieux et al. [26] showed reduction in host inflammatory response due to reduction in the number of bacteria upon phage

treatment.

Although the majority of *K. pneumoniae* bacilli during infection are thought to be extracellular, it is recognised that *K. pneumoniae* can survive within macrophages and epithelial cells [93,94]. Liposome encapsulation may enhance access of phage to intracellular *K. pneumonia*. Using an ex vivo model of cultured murine peritoneal macrophages infected with *K. pneumonia*, Singla et al. [95] showed that cationic liposome encapsulated phage were able to kill greater numbers (~95%) of intracellular *K. pneumonia* compared with free phage (~20%). A potential limitation of phage therapy for treatment of infection is the development of phage-neutralising antibodies which may result in reduction of the concentration of phage at the site of infection or in systemic circulation [30]. Singla et al. [95] demonstrated using ex vivo experiments that liposome encapsulation was effective at completely protecting *K. pneumonia* phage from the neutralising effect of murine antibodies, whereas free phages were inactivated by serum antibodies raised against the phage in mice. Liposome encapsulated phage were able to inactivate *K. pneumonia* following 3 h exposure to mouse serum containing antibodies.

3.6. Phage therapy for chronic infections

Results of animal studies (discussed above) relate to acute, rapidly progressive infections. In chronic infections, bacterial populations may have a greater diversity of bacterial strains as polymicrobial colonies encased within biofilms with significant bacterial populations maintained over long periods of time, years even decades. Although a measureable specific immune response to the presence of the antigens expressed by the infecting bacteria may be present, however, on its own, the host may be unable to clear the infection.

Alemayehu et al. [27] investigated the ability of phage to penetrate and kill *Pseudomonas* cells growing as biofilms on the surface of a layer of human lung cystic fibrosis bronchial epithelial cells. Phage applied to 24 h old biofilms (bacteria concentration ~ 10^7 CFU/well; phage dose ~ 10^8 PFU/well) were able to increase phage numbers by replicating in situ whilst killing the resident *Pseudomonas* (2-log reduction in observed bioluminescence and 3- to 4-log reduction in cell concentration after 24 h incubation of phage with cells). It took longer to clear *Pseudomonas* grown in biofilms in comparison with planktonic cells in the lung model. Singla et al. [95] evaluated phage activity against young (4 day old) and more mature (7 day old) *K. pneumonia* biofilms. Both free phage and liposome encapsulated phage showed similar levels of antimicrobial activity (~2 log reduction in CFUs) in the 4 day old biofilm model compared with the antibiotic amikacin. For 7 day old biofilms the effect was less marked (~1 log reduction).

Cocktails of phage covering multiple species and having the correct host range for different strains may be needed to facilitate clearance of chronic infections. Examples of such chronic infections include infections in cystic fibrosis (*P. aeruginosa*) and tuberculosis (*M. tuberculosis*). Having suitably diagnosed the polymicrobial constitution of the infection causing bacteria, tailored phage formulations would need to be prepared and administered. Phage banks with well characterised phage lines with long shelf life would be needed in order to prepare such formulated phage cocktails and supplied for clinical use. The challenge here is delivery of precise concentrations of formulated phage cocktails at the site of infection e.g. for cystic fibrosis, which may require the phage to penetrate biofilms to gain access to the bacteria. Furthermore the phage would need to be phagocytosed into macrophages to target *M. tuberculosis* cells present in granulomas which poses an additional challenge.

3.7. Phage therapy case studies with humans and recent clinical trials

There are relatively few recent published randomised control trial studies on the therapeutic use of phage involving humans. Typically these tend to be case studies [96] however, recently two small randomised controlled trials have been undertaken [35,97]. The formulations used for these trials tend to be simple phage lysates that have been purified using crude laboratory methods including centrifugation, microfiltration, ultracentrifugation and in some instances endotoxin removal through affinity chromatography [98]. Phage cocktails used for the trials were simple suspensions in buffer without microencapsulation. The titre of phage s used was typically low and reliant on in situ amplification which may have resulted in therapy failure in at least one of the studies [35].

Wright et al. [97] conducted a small (24 patients) randomised (two groups of 12), double blind Phase I/II clinical trial to evaluate the safety and efficacy of a therapeutic phage preparation against chronic otitis due to antibiotic resistant *Pseudomonas aeruginosa* infection. Each 0.2 ml dose contained 10^5 PFU/ml each of six bacteriophages suspended in 10% glycerol in phosphate-buffered saline and was applied to the infected ear using a standard 1 ml syringe equipped with a 27 gauge spinal needle. Patients re-visited the clinic on 7, 21 and 42 days following treatment. Patient inclusion criteria included checking that the *P. aeruginosa* strain causing infection was susceptible to at least one phage in the therapeutic cocktail. Bacteriophage amplification (~2 log) was noted for bacteriophages in human subjects under trial conditions [97]. There were no reported side effects and no evidence of local or systemic toxicity due to phage therapy. Phage therapy was shown to result in a modest reduction (from the baseline) in median *P. aeruginosa* counts. Results were not overwhelmingly conclusive. In cases where infection was treated phage counts decreased to below the limit of detection. At day 42, 3 out of 12 patients in both the phage therapy group and the placebo group had levels of *P. aeruginosa* below the limit of detection. High levels of *P. aeruginosa* were present in patients ~10^9 CFU/g.

A recent randomised phage therapy trial to reduce *E. coli* associated diarrhoea in children (6–24 month old males) failed to improve diarrhoea outcomes. This was attributed to low intestinal *E. coli* concentration resulting in lack of significant phage amplification in vivo [35]. Half of the patients contained phage susceptible *E. coli* in stool samples however, the stool CFU titres were low (median titre 10^5 CFU/g stool). Phage-bacteria pharmacodynamics dictates that conditions where there is a low in situ bacterial concentration are not conducive to significant phage amplification. Phage cocktails used in the study were given orally without antacid and were not protected from the stomach acidity by encapsulation hence phage titres reaching the site of infection may have been low. Rapid elimination of the phage from the host may also have further lowered the efficacy of the treatment.

Fish et al. [96] recently published results of six case series where bacteriophage treatment of intransigent diabetic toe ulcers was successful. The study used a commercially available (Eliava Biopreparation, Tbilisi, Georgia) single lytic phage Sb-1 (broad host range against *S. aureus*) in sterile solution (10 ml per vial containing ~10^7–10^8 PFU/ml). Treatment involved standard wound care, including weekly soft tissue debridement if necessary [96]. Phage preparation was applied topically by dripping the phage solution into the wound cavity; gauze soaked with phage solution (0.1–0.5 ml depending on ulcer volume) was packed into the cavity and the wound was wrapped with dry gauze. The actual dose given was variable depending on the size of the toe ulcer. The patient was instructed to leave the gauze in place for 48 h. The protocol was repeated weekly in a hospital-based wound clinic setting until the ulcer became too small to pack. All six cases reported healed with an average time to heal of ~6 weeks.

Merabishvili et al. [98] prepared a well-defined cocktail (designated BFC-1) of lytic phage (containing a mixture of three broad host range lytic phages; two *Myoviridae* (against *P. aeruginosa* and *S. aureus*) and one *Podoviridae* (against *P. aeruginosa*)) designed for treatment of *P. aeruginosa* and *S. aureus* infections in burn wound patients as part of a pilot human clinical study. The cocktail contained 10^9 PFU/ml of each bacteriophage applied (application by spraying using a syringe with a spray adapter fitted at the end) in doses of 1 ml/50 cm^2 wound bed with the assumption that this would result in a ratio of phage to bacterium of 100:1. The cocktail formulation consisted of endotoxin free phage (pyrogenicity evaluated using a rabbit animal model) suspended in physiological buffer and stored at 2–8 °C in 3 ml 'single use only' vials. Stored at 4 °C, the phage suspension titre remained stable over 12 months. A safety trial on infected burn wounds of eight patients had shown no adverse events following application. No efficacy data was reported in the paper. However, a recent paper [99] presented results from the application of BFC-1 cocktail on colonized burn wounds in a small clinical trial (nine acute burn wound patients, 10 applications). Punch biopsies were taken to determine bacterial species identification and antibiotic susceptibility tests. 1 ml of the BFC-1 solution was applied per 50 cm^2 of burn wound area. The other half received standard treatment protocols. Patients also received systemic antibiotic treatment. Following BFC-1 treatment, the wound was covered with dressings, gauze and bandages according to standard treatment protocols. 2–5 h later, the burn wound was uncovered and two biopsies taken next to (within 2 cm) of the previous ones. Bacterial load determination of the biopsies was undertaken. Despite initial indications of wound colonization or infection, bacterial cultures taken before and after treatment showed low levels of bacteria (in 8/10 applications). In all cases bacterial load remained unchanged after BFC-1 applications as well as after standard treatment. The low bacterial load in the wound was attributed to topical and systemic antibiotic treatment of patients prior to enrolment of the patients in the study (which took 7 days from initial assessment of colonization/infection with target pathogen). Rose et al. [99] found that the sprayed BFC-1 solution had a tendency to run off the burn wound. Better formulation e.g. as a gel or part of a dressing (e.g. with controlled release properties) was suggested for future trials.

3.8. Summary

For phage therapy to work, bacteriophage concentrations at the site of infection must be sufficiently high for significant in situ phage amplification to occur and as a result reduce the rate of replication of the infecting population of bacteria. The phages need to be delivered to the site of infection and they need to have access to replicating bacteria susceptible to phage infection. Even when susceptible bacterial concentrations are high at the site of infection, delivery of low concentrations of phage may not be sufficient to increase phage concentration rapidly enough to achieve rapid killing of bacteria. Self-amplification will not occur when concentrations of bacteria are low [8]. In vivo reduction in phage concentration due to the host immune response or other mechanisms also needs consideration. Under these conditions, steady or frequent delivery of high concentrations of phage may be needed for successful phage therapy. Phage resistant populations are highly likely and require use of a number of phage strains that bind to different receptors thereby killing the mutant bacteria. Use of different phage strains for therapeutic use requires careful formulation of phages to retain phage stability such that viable phages at a sufficiently high concentration are present at the time of use. Rational design of micro- and nanocapsules loaded with precise dose of encapsulated phage in stimuli responsive controlled release systems could be engineered to do this.

4. Bacteriophage encapsulation

Developing formulations that incorporate bacteriophage for therapeutic applications requires an appreciation of the chemical and physical stresses phage may encounter both during processing as well as during storage once formulated. Phage inactivation and long term reduction in phage titre upon storage is highly undesirable. The physical and chemical properties of the formulation need careful consideration when selecting a technique for encapsulation. Accurate loading of phage per particle requires particle monodispersity which is rarely achieved in practice however, some techniques are considerably better than others (discussed below). The particle morphology should be without deformities and the particles shouldn't aggregate or uncontrollably fuse together with material in the surrounding environment. A polymer or lipid may be used to coat an existing structure containing the phage, for example Murthy and Engelhardt [82] sprayed phage on dried skimmed milk and then encapsulated them in a lipid coating. Alternatively, phage may be incorporated in the formulation within the droplet which upon drying or crosslinking results in phage entrapment in the particle core. Depending on the technique used, polymer gelling may be part of the technique or it may be a process occurring downstream. There are many techniques and processes that may be used for stabilising, immobilising and encapsulating phage. The most common methods are spray-drying, spray freeze drying, freeze drying, extrusion dripping methods, emulsion and polymerisation techniques. The literature focusing on phage encapsulation, formulation and storage stability is reviewed below and summary information is presented in Tables 2–4.

4.1. Formulation of bacteriophage for preservation and storage

Phages are protein structures and they are therefore susceptible to factors known to denature proteins; these include exposure to organic solvents [100,101], high temperatures [102], pH [102,103], ionic strength [103], and interfacial effects. Additionally, mechanical stresses during formulation or encapsulation including shear stresses during mixing and agitation, atomisation during spraying [104] and, desiccation stresses during drying [105], need careful consideration. Drying and lyophilization of bacteriophage with suitable carriers and bulking agents to improve storage life have been the focus of numerous studies (Table 2). A number of encapsulation techniques (reviewed further on) have been used, followed by drying [75] or incorporating solvent evaporation (e.g. during electrospinning, [106]) to improve phage storage. One of the most common and successful modes of long-term preservation of bacteriophage is storage at 4 °C in Trypticase Soy Agar and Brain Heart Infusion broth whereas storage at − 80 °C requires 50% glycerol as a cryoprotectant [107] or freeze drying with excipients (e.g. sucrose or trehalose) as lyophilization and cryoprotectants [108,109]. Ackermann [109] observed that phage titres for one of the largest collections of tailed phages typically tended to drop by 1 log over the course of a year but then remained fairly stable (although there were many individual variations).

Clark [107] working for the American Type Culture Collection (ATCC) evaluated storage stability of a wide variety of bacteriophages (16 in total) for long term preservation and distribution. Phage specimens were treated and stored for two years at room temperature and at 4 °C as (i) broth lysates; (ii) lysates diluted with 50% glycerol; (iii) saturating filter paper with lysate and then drying; (iv) freeze dried by mixing lysates with an equal volume of double strength skim milk (routine practice at that time at the ATCC). Phage titres measured immediately post-processing indicated that freeze drying resulted in a significant loss of titre (between 1 log and 2 log reduction). However,

freeze drying did produce stable phage titres over the course of 2 years when stored refrigerated. After two years, the titre of phage in the broth lysates were found to be generally higher than those of glycerol or freeze dried preparations. Thermally dried preparations generally did not prove satisfactory. Preparations stored at 4 °C showed higher titres than those kept at room temperature. All titres declined with time regardless of the conditions of preservation.

4.1.1. Effect of thermal and environmental stresses on phage stability

Phage may show different susceptibility to ingredients used in formulations. Knezevic et al. [103] examined the effects of environmental factors on adsorption and inactivation of four *P. aeruginosa* phages (one *Podoviridae* (designated δ) and three *Siphoviridae* phages (designated J-1, σ-1 and 001A). Factors investigated included temperature (range 7–44 °C, exposure for 30 min in SM buffer), pH (pH 1.5–pH 9, at 37 °C for 30 min), phage neutralisation by exposure to carbohydrates and amino acids (potential phage receptor constituents; incubation at 37 °C for 30 min in SM buffer containing either glucose, rhamnose, mannose, galactose, glucosamine, glutamine or alanine), exposure to exopolysaccharides and lipopolysaccharides, silver nitrate (e.g. for combination therapy with phage), povidone-iodine (for combination therapy) and exposure to serum of rats. All phage were completely inactivated after 30 min exposure at pH 1.5. Maximum viability was observed at pH 7. Significant differences in susceptibility for the phage were observed at pH 3, 5 and 9. Phages σ-1 and δ were found to be less susceptible to low pH than J-1 and 001A. Survival at pH 9 was higher than at pH 5. Phage δ was considerably neutralised by glucose, rhamnose, glucosamine, mannose and alanine. None of the phage survived at working silver nitrate concentrations of 0.3%. Phages δ and 001A were highly sensitive to povidone-iodine; exposure for 30 min to even the lowest concentration 0.5% completely inactivated them. For phages σ-1 and J-1 the PhI50 (i.e. concentration to achieve a 50% reduction in titre) concentration values were ~4%.

Briers et al. [102] evaluated the stability of the structural peptidoglycan hydrolase gp181 of the *P. aeruginosa Myoviridae* bacteriophage ϕKZ to temperature (range 25–90 °C), ionic strength (20–320 mM) and pH (5–8). The protein is supposed to degrade the peptidoglycan layer locally during the infection process. Gradual loss of enzymatic activity due to thermal inactivation was observed from 100% activity at 25 °C down to 0% at 70 °C when exposed for 60 min and down to < 20% activity when exposed for 10 min to 90 °C. Optimal enzymatic activity at 25 °C was observed at pH 6.2 and ionic strength 140 mM with variation in pH and ionic strength in either direction resulting in loss of activity.

4.2. Freeze drying of bacteriophage for storage and encapsulation

Freeze drying (lyophilization) is routinely used in the pharmaceutical industry [110] to dry proteins, vaccines, peptides or colloidal carriers such as liposomes, nanoparticles and nanoemulsions [111]. Freeze drying is typically done from solution and involves a freezing step followed by a drying step. During the freezing step, the phage containing solution is cooled and ice crystals of pure water form resulting in concentration of the remaining liquid. This typically results in an increase in viscosity as well as osmolarity of the concentrated solution which inhibits further crystallization and eventually results in a frozen amorphous/crystalline phase. Preventing aggregation of bacteriophage and inactivation due to high osmotic pressure changes needs careful consideration here. At the end of the freezing stage, drying begins as a result of switching on a very low temperature condenser which draws water from the sample over time. A vacuum is also pulled to remove air which slows mass transfer to the condenser. A primary drying stage results in direct sublimation of the ice crystals, and remaining adsorbed water is removed in a secondary drying phase. In the freeze-dried state, the rate constants of most chemical and physical degradation reactions are dramatically reduced thereby permitting long term storage under refrigerated or ambient temperatures (25 °C). Traditional freeze drying is a slow process since the lyophilization is carried out at temperatures below the glass transition temperature of the maximally freeze-concentrated bacteriophage-excipient solution (Tg′) and below the collapse temperature (Tc) of the sample thereby yielding a porous material with microstructure that is conducive for drying. Freeze dried material typically results in a cake that needs further processing e.g. milling to achieve fine particles suitable for loading in dry powder inhalers (DPI) [112].

A brief summary of the main issues surrounding the freeze drying of phage is now given. Papers on phage and virus freeze drying have mainly focused on evaluation of formulations in order to stabilise phage for storage [105,109,113–115]. A number of papers have shown that addition of amino acids (e.g. sodium glutamate [113]), peptides (e.g. peptone [108]) and proteins (casein and lactoferrin [116]) to the formulation improves phage viability upon freeze drying and following rehydration (Table 2). Literature suggests that disaccharides e.g. lactose [116], sucrose [105,114,115,117] and trehalose [77,104,105,114,118] improve phage survival during freezing as well as subsequent lyophilization. Osmotic damage is an important factor that reduces phage surviving the freeze drying process [108]. This includes hydration stresses which may be ameliorated through addition of sugars to the hydration medium [119], something often overlooked in recent studies that use simple buffer or saline for rehydrating freeze dried samples [105,114,120]. A number of papers have shown that the concentration of sucrose in the formulation affects phage viability during freeze drying and subsequent rehydration [105,114], although different phages would appear to prefer either high or low concentrations. Rapid rates of freezing at relatively low temperatures (< − 20 °C) have been found to result in better phage survival compared with slow freezing. This has been attributed to having less time for osmotic damage to occur [108].

A detailed listing of literature studies is provided in Table 2 and more detailed commentaries on some selected papers are now given as follows.

Engel et al. [113] investigated the storage stability of mycobacteriophages using freeze drying. The effect of various cryoprotectants and lyoprotectants at varying phage titre (10^4, 10^5 and 10^9 PFU/ml, for phage BK1) was investigated including sodium glutamate (with and without gelatine), gelatine, peptone (with and without sorbitol), dextran (with glucose), calf serum and skim milk. 53 different mycobacteriophages were finally tested using sodium glutamate (5 wt% with 0.5 wt% gelatine). Storage of freeze dried phage at room temperature in the dark for as long as 2.5 years resulted in only a modest change in phage viability.

Puapermpoonsiri et al. [117] carried out a qualitative study evaluating the effect of sucrose, PEG and gelatine during freeze drying on short term viability of *S. aureus* and *P. aeruginosa* bacteriophage over a 30 day period. Primary drying (freezing and sublimation of frozen water at − 30 °C for 1000 min) was followed by a secondary drying cycle (at an elevated temperature of 25 °C for 6 h) to desorb adsorbed water. The residual moisture content (4–6 wt%) of the freeze dried powder was found to correlate with better phage viability; but due to the qualitative nature of this work, it is difficult to accurately assess the magnitude of this effect, which needs further exploration. Gelatine was found to play no role in maintaining phage viability following lyophilization. Control of the residual moisture content in the lyophilized material was found to be important in order to balance chemical (reactivity of amino acids) and physical stability (e.g. unfolding of tail proteins) of bacteriophage resulting in loss of lytic activity. High

concentrations of sucrose and PEG were needed to prevent collapse of freeze dried cakes due to secondary drying at 25 °C. High concentrations of additives (e.g. sucrose) lead to a decrease in phage titre with better results at lower sucrose concentrations. After 30 days of storage at 4 °C viable phage were still present. Dini and Urraza [105] investigated the effect of buffer systems (PBS and SM), buffers with/without skimmed milk and addition of disaccharides (sucrose or trehalose) on a Podovirus coliphage stability during freeze drying (at − 80 °C for 24 h) and storage. The coliphage freeze dried in SM buffer gave the highest titre compared with skimmed milk and PBS respectively. Addition of trehalose or sucrose to PBS did not improve phage titre outcomes. This may have been due to hydration stresses due to osmotic shock rather than the freeze drying process. Freeze dried samples were rehydrated using a simple buffer rather than a buffer with added sucrose to prevent osmotic shock. Addition of sucrose to PBS did stabilise the phage titre upon storage for 120 days (at 4 °C) with phage freeze dried in PBS alone showing a progressive decrease in phage titre over 120 days of storage (~2.5 log loss compared with the initial lyophilized product). Addition of sucrose at low concentrations (0.1 M) in SM buffer was found to result in a high phage titre post-freeze drying (only a ~0.5 log drop in titre attributed to the drying step rather than freezing step) with the titre remaining stable for up to 120 days upon storage. Increasing the sucrose concentration in SM buffer to 0.3 M or 0.5 M resulted in a ~0.5 log lower titre compared with the 0.1 M sucrose sample. Sucrose containing powders had a higher residual moisture content, compared with samples dried using PBS or skimmed milk. Only a small portion of the overall loss of phage titre (~0.3 log) was attributed to the freezing step (for the 0.5 M sucrose-SM buffer sample) with lyophilization accounting for the majority of the ~1.5 log phage titre loss. Whether this could be attributed to the rehydration step needs further exploration.

One of the only papers to look at rehydration was Cox et al. [119] who looked at loss of phage viability arising from detachment of tails from capsid heads due to rapid rehydration following freeze drying. Slow rehydration of freeze dried phage by exposure to a controlled humidity environment (100% RH at ambient temperature) for 60 min followed by rehydration using broth or sucrose containing broth resulted in significantly higher phage titre. The effect of hydration is one area that definitely needs further exploration.

Merabishvili et al. [114] investigated the stability of a *S. aureus* myovirus phage ISP after freeze drying. Different formulations containing two different concentrations of phage titres (10^8 and 10^9 PFU/ml) were prepared with different excipients (each excipient at either 0.1 M or 0.5 M concentration: sucrose, trehalose, mannitol, glycine, and polyvinylpyrrolidone (PVP) and PEG 6000 at 1% and 5%). Primary drying was carried out at − 30 °C whereas for secondary drying, the temperature was gradually increased to 25 °C over a period of ~10 h followed by isothermal drying. Some of the excipients (PVP, mannitol and PEG 6000) proved to be unsuccessful. PVP was found to inactivate the phage even before freezing. Complete inactivation of phage ISP after lyophilization was observed using glycine as excipient (glycine crystallizes during freezing). Complete loss of phage ISP activity using the sugar alcohol mannitol at 0.1 M was observed with a 4 log titre reduction using 0.5 M concentration. The phage titre thereafter remained stable over a 27 month period with a 2 log reduction after 37 months of storage. Phage ISP titre in PEG 6000 showed a 1.8 log (1% PEG) and 5 log (5% PEG) reduction following lyophilization. After 37 months of storage, further loss of titre was 3 log (1% PEG) and 1.7 log (5% PEG). High concentrations (0.5 M) of trehalose and sucrose were found to be the most effective stabilisers for phage ISP. A 1 log loss in phage titre was observed immediately after lyophilization with a further 1 log loss following 37 months of storage. Different concentrations of trehalose and sucrose (0.3 M, 0.5 M, 0.8 M and 1.0 M) were studied further. Phage ISP freeze dried from 0.8 M and 1.0 M sugar solutions showed the smallest titre reduction immediately after drying (~0.5 log). Over the next 27 months of storage phage ISP titre remained stable (variability within ~1 log, measured at time period 3, 7, 12 and 27 months). Phage ISP stability was also recorded for phage stored in LB broth (1 log reduction) and 0.9% NaCl (~2 log reduction) over the same period (after 37 months) stored at 4 °C. Malenovská [115] also reported good results with sucrose in combination with gelatine during lyophilization of structurally different animal viruses. It is noteworthy that a commercially available freeze dried phage cocktail preparation contains sucrose and gelatine [121].

Golshahi et al. [116] determined the feasibility of preparing stable freeze dried formulations of *B. cepacia* and *P. aeruginosa* phage for respiratory delivery. A 2 log reduction in phage titre was achieved following freeze drying in a formulation of lactose/lactoferrin (60:40 w/w). Freeze dried phage titre remained stable over a 3 month storage period at 4 °C and 22 °C. Addition of lactoferrin resulted in powder that did not require milling to make it respirable (size < 5 μm) with ~30 wt% of powder available in the free particle fraction (FPF) upon aerosolization.

4.2.1. Freeze drying of encapsulated phage

A number of papers have looked at freeze drying phage incorporated in polymer matrices [77,100,118,121,122]. Puapermpoonsiri et al. [100] lyophilized microencapsulated *S. aureus* and *P. aeruginosa* phage (dispersed in a salt buffer with gelatine) in PLGA (poly(lactic-*co*-glycolic acid)). The microspheres were frozen in liquid nitrogen followed by lyophilization. All phage viability was lost after 7 days of storage regardless of storage temperature. Loss of phage viability was attributed to the use of an organic solvent in the emulsification process (discussed later). Alfadhel et al. [123] used freeze drying as part of the encapsulation process. *S. aureus* phage containing nasal inserts were prepared by freeze drying (freezing at − 30 °C and drying at 10 °C) phage formulations containing hydroxypropyl methylcellulose (HPMC) as the encapsulating polymer. Freeze drying of phages formulated in the polymer solution resulted in ~1 log drop in titre immediately after lyophilization and between 3 and 4 log reduction over a year of storage at 4 °C. Addition of mannitol or increasing the HPMC concentration was not found to improve phage stability during storage.

Dai et al. [118] undertook encapsulation of *E. coli* phage in water soluble polyvinylpyrrolidone (PVP) nanofibers prepared using electrospinning. Phage containing PVP solutions (with and without trehalose in SM buffer) were frozen in liquid nitrogen and then freeze dried (drying conditions not given). Immediately after drying the PVP and PVP-trehalose solutions showed no significant drop in phage titre. However, after 8 weeks of storage at room temperature (humidity < 40%) the PVP sample showed a 3 log drop in titre whereas the PVP-trehalose sample showed no significant titre drop. Korehei and Kadla [106] encapsulated *E. coli* phage using emulsion electrospinning and co-axial electrospinning. In emulsion electrospinning, the phages were pre-encapsulated in alginate nanoparticles which were suspended in polyethylene oxide (PEO)/chloroform solution for electrospinning. In co-axial electrospinning the aqueous phage suspension remained in the core of the fiber whereas the shell was made from polyethylene oxide (PEO). The phage containing fibers were freeze dried (details not given). Fibers prepared using co-axial electrospinning were stored at 20 °C, 4 °C and − 20 °C for up to 4 weeks. High phage titre was observed immediately after freeze drying of fibers prepared using emulsion (10^6 PFU/ml) and co-axial (10^8 PFU/ml) electrospinning. Over 4 weeks of storage the co-axial fibers showed no loss of phage titre when stored at − 20 °C and 4 °C whereas a significant drop in tire (> 6 log reduction) was observed for the samples stored at 20 °C.

Recently Colom et al. [77] showed promising freeze drying results for trehalose containing liposomes with encapsulated *S. enterica* phage. Lyophilization resulted in a modest drop (< 1 log) in phage titre for the liposome encapsulated phages. Trehalose containing liposomes showed no loss of phage titre when stored at 4 °C in 10 mM $MgSO_4$ buffer (pH 6.1) for a period of 3 months.

Table 2
Summary of studies looking at effects of formulation, drying and storage on phage viability.

Ref.	Host Bacteria									Formulation				Excipients											Method						Result / PFU Log reduction ● good ◐ moderate ○ poor / ③ value				
																									( ) -encapsulated							Storage conditions brown/blue – low/high relative humidity			
	B. cepacia	*C. ulcerans*	*E. coli*	*Mycobacteriophage s*	*P. aeruginosa*	*S. aureus*	*S. eneterica*	*V. choleraе*	Others	Broth / lysate;	Buffer	Saline	Water	Gelatine	Glycerol	Proteins. (casein, whey etc.)	Amino acids or peptides	Trehalose	Mannitol	Sucrose	Polyvinylpyrrolidone	Lactose	Leucine	Others	Freeze drying,	Air drying	Drying on filter paper	Spray drying	Spray freeze drying	Stored as liquid	Before Storage	Storage duration, months (**LT** – long term)	ambient temperature ~25 C	refrigerated ~4 C	sub-zero temperatures
[107]			Ec	M	Pa	Sa			12	B																			L		24	◐	●	
										B					Gl														L			◐	●	
										B						P									FD						◐		◐	●	
										B						P											FP				○		○	○	
[104]					Pa								W					T	M				Le						SF		●	NA			
													W					T	M				Le					SD			●				
[113]				M						B				Ge			A								FD						●	30	●		
										B						P									FD						●				
										B														O	FD						○				
[108]		Cu								B							A								FD						●	3	●		
										B							A			S					FD						●		●		
[119]			Ec							B										S					FD						●	NA			
[109]			Ec		Pa	Sa		Vc	>400	B																			L	NA	LT		●	
										B					Gl														L					●
[105]			Ec								Bu														FD						①	4			
											Bu									S					FD						①			●	
[114]						Sa				B										S					FD						①	37		●	
										B								T							FD						①			●	
										B											PV										○				
										B							A								FD						○				
												Sa																		L			②		
										B																			L			①		
										B									M					FD						④			⑥	
										B														O	FD						②			⑥	
[123]						Sa					Bu			Ge					M					(FD)						①	12		③-④	
											Bu			Ge					M				O	(FD)						①			③-④	
[118]			Ec								Bu							T			PV				FD						●	2	⓪		
													W					T			PV				FD						●		②		
											Bu										PV				FD						●		④		
													W								PV				FD						●		⑧		
[106]			Ec								Bu														(FD)						●	1	○	●	●
[120]					Pa								W					T					Le				SD			①	12		①/⑦	
																			M				Le				SD			②			③/⑦	
[100]					Pa	Sa					Bu														(FD)						◐	0.25	○	○	
[117]					Pa	Sa					Bu									S					FD						◐	1		◐	
											Bu														FD						●			◐	
											Bu			G										FD						●			◐	
											Bu													O	FD						○			◐	
[75]			Ec								Bu																	(SD)			●	12	●		
[128]					Pa	Sa					Bu												La					(SD)			④-⑧	N/A			
											Bu								T									(SD)			⓪-②				
											Bu													O				(SD)			⑦-⑧				
[132]					Pa	Sa					Bu								T									SD			N/A	12	○	⓪-③ ①-④	
[77]							Se						W					T							(FD)						●	3		●	
[131]	Bc				Pa						Bu					P		T					Le	O				SD			●	3		●	
[116]	Bc				Pa								W			P						La			FD						②	3	●	●	
[157]						Sa					Bu					P								O			(AD)				-	0.5	●	●	
						Sa					Bu					P								O						(L)		1.5		●	

Intensity of shading differentiates vertical sections of the table.

4.2.2. Spray freeze drying of bacteriophage

Spray freeze drying may offer the possibility of a single step process to produce bacteriophage loaded porous powders with controlled particle-size distribution without subjecting the phage to high thermal stresses encountered during conventional spray drying (discussed below).

Leung et al. [104] recently spray freeze dried (using an ultrasonic nozzle to atomise the solution) a *P. aeruginosa* podovirus (PEV2) by spraying the phage solution (formulations containing different concentrations of trehalose, leucine and mannitol in combination) directly into a dewar of liquid nitrogen; the frozen droplets were subsequently lyophilized (primary drying at − 30 °C for 24 h and secondary drying at 25 °C for 48 h) in a conventional freeze dryer. The ultrasonic nozzle employed high frequency vibration (48 kHz) to produce fine droplets; a significant 2 log reduction in phage titre was noted just due to the ultrasonic spraying process itself (without freeze drying). The sprayed droplets froze immediately upon immersion in liquid nitrogen. Subsequent lyophilization resulted in highly porous dry powder (mean geometric particle diameter ~ 30 μm corresponding to the droplet size generated by the ultrasonic nozzle). Particles with high trehalose content (60%) were found to be highly hygroscopic and collapsed during storage whilst particles with lower trehalose content (40%) retained their porous structure (examined using SEM). The higher trehalose containing particles adsorbed more water at lower relative humidity values. Leung et al. [104] suggested handling and storage of powders below 20% RH to avoid moisture related recrystallization of excipients damaging encapsulated phage. The loss in phage titre post-atomisation due to spray freeze drying was negligible; this suggested that phage activity was preserved by the excipients during the freeze drying step as well as the rehydration step. The high phage titre in the spray freeze dried powders was attributed to rapid freezing of the droplets in liquid nitrogen resulting in good distribution of the phage within the frozen particles. Spray freeze dried powder with the higher trehalose content also had the higher phage titre. The aerosol performance (using a commercial DPI device, an Osmohaler™) of the freeze dried powders showed a reasonable fine particle fraction (size < 5 μm having 44% phage dose ~ 10^4–10^5 PFU dose delivered) with high total recovery of aerosolized viable phage dose (88%–97%).

4.3. Spray drying of bacteriophage for storage and encapsulation

Spray drying processes atomise a liquid containing dissolved solids, converting it into a fine mist which is contacted with a hot dry gas (typically hot dry air) inside a drying chamber. Due to the high surface area to volume ratio of the very small atomised droplets, the solvent rapidly evaporates with each droplet forming a particle comprising the non-volatile components (bacteriophage and excipients) along with a small amount of residual moisture. The particles are separated from the airstream exiting the dryer, and cyclones or bag filters are typically used for this. Spray drying is a scalable industrial process technology and is widely used to produce fine powders for pharmaceutical applications including pulmonary delivery via dry powder inhalers (DPI) [124]. Dry powders are favoured for respiratory drug delivery because they show relatively long storage stability without requiring refrigeration [125,126] and DPI are simple to use and do not require regular cleaning and disinfection [127].

Phages have been shown not to be able to withstand high shear stress for too long. The nebulization process has been shown to result in loss of phage titre [104,128]. Phages are also sensitive to thermal stresses and have been shown to partially lose activity at temperatures higher than 60 °C [108,129]. Low spray drying temperatures (~40 °C outlet air temperature) have been shown to result in higher phage titre [120] with high loss of phage titre observed at higher temperatures [104,128,130,131]. Literature on spray drying of phage suspensions typically includes excipients in the formulation to protect the phage from desiccation and thermal stress (Table 2). Upon spray drying, sugar excipients form amorphous structures with high glass transition temperatures (Tg), e.g. in their anhydrous amorphous form Tg of trehalose is 115 °C and Tg for lactose is 108 °C. Below the glass transition temperature, vitrification due to the excipient stabilises the encapsulated bioactive agent and permits storage over long periods at ambient or lower temperatures. Adsorption of moisture results in lowering of the Tg and compounds that are hygroscopic e.g. lactose require handling under controlled atmospheric conditions (low relative humidity) [131]. Refrigerated storage of spray dried powders at relative humidity exceeding 20% has been shown to result in loss of phage titre [120,132] whereas samples stored at low humidity were stable. Trehalose is the most frequently reported excipient used in the spray drying of phage and it has been shown to result in spray dried powders with high phage titre and good storage stability [104,120,131,132]. Trehalose has low toxicity and has been shown to protect biological material including proteins, probiotics and vaccines, against desiccation and thermal stress. Crowe et al. [133] suggested that the efficacy of trehalose as an excipient in drying is partly due to its high glass transition temperature at all water contents but also because trehalose binds residual water left over from the drying process to form a dihydrate which might otherwise participate in lowering the glass transition temperature to below ambient [132]. Other excipients that have been used to improve the dispersibility of spray dried phage containing powders include dextrans [128], lactose (common excipient used for DPI powders; [112,128]), glucose, sucrose, mannitol [104] and, leucine [104,131] (Table 2). Reducing sugars such as lactose may be unsuitable excipients for bacteriophage [128]. Alternatives such as mannitol have been suggested in the literature [134]. Phage spray drying studies using mannitol have reported improved particle dispersion characteristics however, phage viability results have been mixed [104,120]. Addition of proteins e.g. casein in combination with trehalose have shown good results [131]. Refrigerated storage of spray dried powders was reported to yield higher titres of phage during long term storage compared with storage under ambient conditions [120]. Different phage strains formulated and spray dried under identical conditions showed significant differences in the resulting phage titre suggesting the need for individually formulating each phage to be used in a phage cocktail [128,132]. A number of studies have reported spray dried phage powders to have a suitable mass median aerosolized diameter for pulmonary delivery of phage to treat respiratory infections [104,128,131,132,135].

Stanford et al. [75] took a cocktail of 5 *E. coli* phage (as lysates) which were added to an aqueous pH responsive methacrylate polymer (Eudragit S100) containing an unspecified stabiliser, then spray dried (co-current spray dryer fitted with a rotary atomiser) into a powder (with 4% moisture content). No drying conditions were reported in the paper. The resulting encapsulated phages (described as Ephage) were stored at 20 °C. Spray drying of the aqueous phage-polymer solution resulted in a 1 log reduction in phage titre post-drying. Encapsulated phages were shown to survive 20 min exposure to an aqueous solution at pH 3. Storage at 20 °C for one year resulted in a decline in phage titre of between 1 log and 3 log depending on the phage. Walbeck [130] in a recent patent (assigned to Omnilytics, USA) reported a 7 log loss in phage viability post-spray drying using *E. coli* O157:H7 specific phage (the phage suspensions were unprocessed crude lysates including phage, lysed bacteria and fermentation media, no excipients were added, a rotary atomiser was used to generate the spray). The loss in phage viability may be attributed to the high air inlet temperature (range 200–220 °C) used which resulted in the dry phage powders being

exposed to high air outlet temperatures (90–130 °C).

Vandenheuvel et al. [128] spray dried *S. aureus* and *P. aeruginosa* phages using lactose, trehalose and dextran as excipients. Dextran was used not only for its effect as an excipient in the spray drying process; in the treatment of respiratory infections it may prevent *P. aeruginosa* from attaching to lung epithelial cells and may increase CF sputum clearance. The effect of atomisation air flow rate and drying air inlet temperatures were investigated (using inlet air temperatures of either 85 °C or 100 °C). At a relatively low inlet air temperature of 85 °C, the *P. aeruginosa* podovirus was found to be more resistant to spray drying stresses in comparison with a *S. aureus* myovirus. Lactose and dextran were found to be unsuitable excipients, whereas phage viability results with trehalose (4% wt) were more promising with < 0.5 log reduction for the podovirus and a 2 log reduction for the myovirus. The effect of air inlet temperature was investigated further using trehalose only. The viability of the myovirus was found to be strongly affected by the higher air temperature. At an air inlet temperature of 100 °C a ~5 log reduction in phage titre was observed in comparison with a ~3 log reduction at 85 °C using the same air inlet flow rate. The mean particle size (D50) was between 2 and 6 μm. Increasing atomisation air flow (which reduced particle size and would have increased atomisation stresses had a mildly adverse effect on phage tire). This paper showed that two different types of bacteriophage (a myovirus and podovirus) processed under the same conditions show significant differences in stability to spray drying stresses and result in significantly different loss of titres.

The same group then assessed the storage stability (over 12 months) of the spray dried *S. aureus* (myovirus) and *P. aeruginosa* (podovirus) phage using trehalose as excipients [132]. They principally investigated the effect of relative humidity (RH 0% and RH 54%) and temperature (0 °C and 25 °C). Within 3 months of storage, the *S. aureus* myovirus titre fell by 2 log (4 °C, 0% RH) and 4 log (4 °C, 54% RH) respectively but then stabilised thereafter. At 25 °C almost complete loss of phage viability was observed (7 log) for both 0% RH and 54% RH samples after 4 months of storage with a 2 log loss observed after the first month. The podovirus was more stable with < 1 log reduction over the 12 months for the 4 °C sample irrespective of the RH, and between 1 log and 2 log reduction at 25 °C. Powder X-ray diffraction analysis (phage free powders dried under identical conditions to the phage containing powders) showed that spray dried trehalose powder was non-crystalline. Differential scanning calorimetry data showed that the spray dried trehalose had a Tg between 116 °C and 117 °C i.e. the trehalose was in an amorphous state suitable for phage vitrification. After 8 months of storage at 54% RH, the presence of an endothermic peak between 86 and 91 °C on DSC scans indicated the presence of stable trehalose dihydrate crystals. Spray dried trehalose powder samples stored at 0% RH did not show changes in DSC endotherms or XRD spectra. Trehalose is known to protect bioactive agents from thermal and dehydration stresses through structural and conformational stabilisation of proteins by direct hydrogen bonding and the process of vitrification. Over time, at 25 °C, phage titre dropped even where no crystallization of the amorphous trehalose was observed.

Leung et al. [104] spray dried a *P. aeruginosa* phage (*Podoviridae*) designated PEV2 focusing on making phage containing powder for pulmonary delivery. The lytic podovirus (supplied by AmpliPhi Biosciences, Australia) was used at a titre of 10^7 PFU/ml suspended in dilute (2 wt% solids) aqueous mixtures of trehalose, mannitol and leucine (respective ratios either 60:20:20 or 40:40:20). The formulations were designed for use in dry powder inhalers (DPI), as leucine helps improve the dispersibility of the powders by reducing cohesive interactions between particles. Mixing phage with excipients in solution resulted in a ~0.5 log titre reduction, which was attributed to changes in ionic strength when the phage buffer was diluted in excipient solution. Leung et al. [104] used a two fluid nozzle for liquid atomisation which itself resulted in a ~1 log loss in phage viability. The air inlet temperature was 60 °C which resulted in low outlet temperatures of 40–45 °C. The low outlet temperature was chosen to minimize thermal stress for the phage. However, titres were lower (due to the drying process) than recorded for spray freeze drying process reported using the same phage and excipients in the same paper. Spray dried powders were collected inside a relative humidity controlled chamber (RH < 20%) and stored at 4 °C. Spray drying resulted in a further ~1.5 log drop in phage titre. Leung et al. [120] further investigated the effect of humidity (0%, 22% and 60% RH) on long term storage (over 12 months) of spray dried *Pseudomonas aeruginosa* phage (at 4 °C) dry powders containing different amounts of trehalose (40%, 60% and 80%). Storage in a low humidity environment (0% and 22%) was found to result in no loss of phage titre. High humidity (60% RH) resulted in complete loss of viable phage after 3 months.

Matinkhoo et al. [131] spray dried *Myoviridae* bacteriophage for *P. aeruginosa* (phiKZ and D3) and for *B. cepacia* (KS4-M and KS14) using trehalose, leucine and casein (a mixture of four similar phosphoproteins with MW between 19 kDa and 24 kDa) as excipients. Similar to Leung et al. [120], leucine was added to improve the dispersibility of the powder. Surfactants were added to some formulations to aid in the dispersion of the bacteriophage in the feed solution [136]. A vibrating mesh atomiser was used resulting in atomised droplet diameters around 7 μm. Mild spray drying conditions were used to provide a low outlet temperature of between 40 °C–45 °C (air inlet temperature of 75 °C) and an outlet relative humidity of < 7% RH. The spray dryer was housed in a controlled humidity chamber with glove ports to allow collection of and storage of powder without exposure to moisture. In vitro evaluation of the deposition of the phage loaded spray dried powders was carried out using an experimental idealized throat and lung system (Alberta Idealized Throat attached to an Anderson cascade impactor) using a commercially available DPI. Four formulations were tested: (i) Leucine (77 wt%):trehalose (19 wt%) (LT); (ii) leucine (76 wt%):trehalose (19 wt%):tyloxapol (2 wt%) (LTX); (iii) leucine (76 wt%):trehalose (19 wt%):pluronic (2 wt%) (LTP); (iv) leucine (76 wt%):trehalose (19 wt%):casein (2 wt%) (LTC). All powders had a well dispersed appearance. Small batches of material were produced for each formulation; the manufacturing yield of the surfactant containing formulations was significantly higher (~80 wt%) compared with LT and LTC (~60 wt%). Between 20 and 30 wt% of aerosolized dose (LTX, LTP and LTC) did not reach the lung and was entrained in the mouth-throat fraction. In all cases the total lung fraction exceeded 50 wt%. All *Myoviridae* spray dried phages showed a titre loss of < 1 log. The formulation containing trehalose, leucine and the milk protein casein was found to be the best formulation. All the spray dried powder formulations prepared by Matinkhoo et al. (2011) delivered > 50 wt% of dose in the correct (< 5 μm) size range using the DPI. The LTC formulation was found to most consistently deliver single phage doses and cocktails of phage.

A number of in vitro studies have evaluated the use of nebulizers for aerosolization of liquid phage formulations for pulmonary delivery. Sahota et al. [137] carried out an in vitro study delivering an aerosol containing two different *Pseudomonas aeruginosa* bacteriophages PELP20 and PELI40 (isolated from the commercially available Pyophage and Intestiphage preparations from the Eliava Institute, Tbilisi, Georgia). A commercial nebulizer was evaluated to deliver the aerosolized phage dose into a cascade impactor (phage in the droplet size range < 5 μm were deemed suitable for respiratory delivery); 12% of the nominal dose was aerosolized with < 0.8% of dose found to be in the correct size range for delivery to the lung. Golshahi et al. [138] undertook a study where they employed two commercial nebulizers (a

jet nebulizer (Pari LC star) and a vibrating mesh nebulizer (Pari eFlow)) and estimated that around 50% of nebulized *Burkholderia cepacia* phage dose was in the droplet range (measured using Phase Doppler Anemometry) that would result in deposition (estimated using mathematical modelling) in either the tracheobronchial or alveolar region of the lung.

4.4. Drying bacteriophage on filter paper

A couple of early studies looked at stabilising phage by drying phage suspensions by filtering onto filter paper and then drying the filter paper [107,139]. Prouty [139] investigated the long term storage of bacteriophage of lactic acid *streptococci* by saturating filter paper with whey filtrate (pH 4.5–4.7) containing high concentration of phage. Phage titre was not reported however, phages were found to remain viable for up to 78 months stored at ambient temperature. Further work is needed to look at stabilising phage in porous substrates.

4.5. Key points regarding drying of bacteriophage formulations

Phage drying and encapsulation can be performed using spray drying, freeze drying and spray freeze drying techniques. Freeze drying appears to be more successful that spray drying at maintaining high titre values, although the ability of spray drying (or spray freeze drying) provides very small particles thereby allowing them to be used in DPI for pulmonary delivery, without the need for further milling. The best conditions for the spray drying of phage have tended to be with low outlet air temperatures (40–60 °C) which are much lower than typically used in the spray drying of biological materials (70–100 °C). Some of the viability loss in spray drying may be due to the atomisation process. Survival rates during subsequent storage additionally depend on temperature and humidity during storage and are important considerations for the long term storage of phage. A review of published work on drying of phage clearly indicates that phage survival varies from phage to phage and is highly dependent on drying conditions and formulation parameters, which need to be optimised for each phage (although formulations involving disaccharides tend to be most successful). This presents a considerable challenge for phage therapy since cocktails of phage are typically needed. These need to be formulated for storage and the active titre of individual phage may be highly variable if all phage are formulated together.

4.6. Methods used for bacteriophage encapsulation in micro- and nanoparticles

Bacteriophages may be encapsulated in protective micro- and nanoparticles to overcome adverse storage and physiological conditions *en route* to delivering the phage load at the site of infection (e.g. lungs, intestinal delivery). Controlled release and sustained release strategies for phage delivery applications may be achieved using a diverse array of strategies. These include systems based on diffusion controlled release (e.g. solvent diffusion based osmotic pumps), matrix dissolution and erosion-controlled systems, ion exchange swelling based systems. Phage compatible formulation and encapsulation processes need to be carefully designed to prevent damage to viral capsid and DNA/RNA components and stabilisation of the structure of viral capsid and tail proteins to prevent loss of phage viability during manufacturing operations. It is important that the carrier encapsulating the phage is able to withstand adverse environmental conditions for the duration of exposure and is capable of delivering the bacteriophage to the site of infection upon arrival [140,141].

A number of phage encapsulation studies using synthetic and natural polymers have utilised the emulsification route followed by solvent removal (Table 3). The dispersed phase usually comprises of the core material and solvent carrying the active agent (bacteriophage) and the polymer and a second phase which allows for the breakup of the inner phase into droplets. The emulsion may be water-in-oil (W/O) more typical for phage encapsulation [100], but could also be oil-in-water (O/W) [142] and in some cases a third phase may also be present. Once the droplets have formed, the solvent carrying the core material may be removed, which leaves behind solid particles containing the active agent in the core. Different mechanical emulsification techniques may be used to break-up the inner phase into droplets, such as high-pressure homogenisation, rotor-stator homogenisation, and ultrasonication. When two immiscible liquids are put together, shearing force breaks up the inner phase into small droplets. The droplet size uniformity however, is low as the shear applied does not remain uniform throughout the container resulting in droplet heterogeneity with some droplets being larger and some smaller. More advanced methods to produce uniform droplets based on microfluidic and membrane based techniques have thus far not been used for phage encapsulation (these methods are discussed at the end of the paper).

Alternatively, phage containing polymer droplets have been produced via the extrusion technique [143]. The droplet may form in the air or into another liquid. The dispersed phase containing the polymer and phage is extruded through a needle with a specific nozzle diameter determining the size of the droplet [144]. Atomisation nozzles have also been used to generate smaller droplets [78]. If the polymer requires a gelling or crosslinking agent then the droplet may be collected in a bath of the gelling agent [143,145,146]. Examples of this method are presented by the work of Ma et al. [143,145] and Dini et al. [122] where the extrusion technique was employed to extrude alginate containing phage droplets into a bath of calcium chloride which caused ionotropic gelation. The process of gelation can occur in a number of ways depending on the properties of the polymer/hydrogel. Other gelling triggers apart from ionotropic gelation include, heating, cooling and covalent crosslinking. Other techniques that have been used for phage encapsulation in synthetic and natural polymers include polymer precipitation [147], photopolymerisation [148] and thermal phase inversion [142,149].

Most papers on phage encapsulation in liposomes have used the thin-film hydration method [31,39,77,95,150]. Phage have been encapsulated in nanofibers using electrospinning techniques [101,106,118,151–153], in films e.g. for wound healing and food biocontrol of pathogens [154,155] and in thin film structures [148].

4.7. Polymers used for bacteriophage encapsulation

Studies on phage encapsulation have used a variety of hydrophilic and hydrophobic polymers including agarose [148], alginate [78,122,143–145,149,155,157,158], chitosan [143,156,158], pectin [122], whey protein [149,154,157,159], gelled milk protein [149], hyaluronic acid methacrylate [148], hydroxypropyl methyl cellulose (HPMC) [57,123], poly(*N*-isopropylacrylamide) [147], Poly(DL-lactide:glycolide) [100], polyesteramide [121,160], polyvinyl pyrrolidone [101,118], polyethylene oxide/polyvinyl alcohol [106,151–153], cellulose diacetate [153], polymethyl methacrylate [75,161]. Phages have been encapsulated in different morphologies including nanospheres, microspheres, nanofibers, microfibers, membranes and thin film structures (Table 3). Triggers for phage release include polymer solvation, polymer dissolution and erosion [153], polymer hydrolysis [100,162], phase inversion induced by temperature [147], and pH triggered dissolution of polymer and enzyme driven polymer degradation [148].

Table 3
Summary of bacteriophage microencapsulation literature in polymer carriers.

Ref	Host Organism											Carrier system	Encapsulation method	Additives	Target application	Release studies	Dimensions	Structure	Key Highlights
	E. coli	*L. lactis*	*L. monocytogenes*	Proteus	*P. aeruginosa*	*S. enterica*	*Salmonella* Typhimurium	Staphylococcus	*S. aureus*	Streptococcus		hydroxypropyl methylcellulose (**HPMC**); hyaluronic acid methacrylate (**HYMA**); Poly(DL-lactide:glycolide) (**PLGA**); Polyvinyl alcohol (**PVA**); Polyvinyl pyrrolidone (**PVP**); cellulose diacetate (**CDA**); polyethylene oxide (**PEO**); whey protein (**WP**)	water-in-oil emulsion (**W/O**); water-in-oil-in-water double emulsion (**W/O/W**); extrusion (**E**); gelation (**G**); electrospinning (**ES**); wet spinning (**WS**); solvent evaporation (**SW**); precipitation polymerisation (**PP**); liquid phage filled capsules (**LPFC**)	polysaccharides (**PS**); sucrose (**Suc**); other sugars (**S**); chloroform as solvent (**Chl**); others (**O**); aqueous solvent (**Aq**)	food contamination (**FC**); nasal carriage of infectious organism (**NC**); gastrointestinal / respiratory / wound infections (**G / R / W**);	simulated gastric fluid (**SGF**); intestinal fluid (**SIF**); phosphate buffer saline (**PBS**);		• sphere ⦚ fibre □ film	acid exposure (**AE**); cascade impaction study (**CI**); storage studies (**SS**); nasal inserts (**NI**); clinical case studies (**CCS**); thermally responsive polymer (**TR**); edible coating (**EC**); exposure to degrading enzymes (**EEnz**); exposure to bile salts (**BS**); animal studies (**AS**); exposure to bile salts (**BS**); +/- indicates phage protection / damage
[100]					Pa				Sa			PLGA; PVA	W/O/W – SE	-	R	Buffer	~ 10 μm	•	CI; SS
[123]									Sa			HPMC	Freeze drying	S + O	NC	Sterile	N/A		NI; SS;
[155]	Ec		L									Alginate	E + G	-	FC	N/A	N/A		SS;
[160]	Ec			P				S				Polyamide	Casting	Suc	W	PBS	N/A		SS;
[121]				P	Pa			S				Polyamide	Casting	Suc + O	W	N/A	~ 100 μm	□	CCS
[147]						Se						Polyamide	PP	-	W	Water	~500 nm	•	TR
[154]	Ec											WP	Imbibition	O	FC	SGF/SIF	~100 μm	⦚	EC; SS;
[158]	Ec											Chitosan + alginate	E + G	-	G	SGF/SIF	N/A		AE(+); EEnz (+); BS (+)
[149]		Ll										Skim milk + rennet	W/O + G	-	G	SGF/SIF	~ 100 μm	•	AE(+); EEnz (+);
[149]		Ll										Alginate	E + G	-	G	SGF/SIF	~ mm	•	AE(-); EEnz(-);
[149]		Ll										Alginate + WP	E + G	-	G	SGF/SIF	~ mm	•	AE(+); EEnz(+);
[159]									Sa			Alginate + WP	E + G	Suc; S; PS; O	G	-	< 1 mm	•	AE (+); SS;
[145]									Sa			Alginate	E + G	$CaCO_3$	G	SGF/SIF	< 1mm	•	AE(+); EEnz(+); BS (+);
[122]	Ec											Alginate; Pectin	E + G	-	G	SGF/SIF	~ mm	•	AE (+); EEnz (+)
[143]						Se						Chitosan + alginate	E + G	-	G	SGF/SIF	< 1mm	•	AE(+); EEnz (+); BS (+); SS;
[78]						Se						Alginate	E + G	$CaCO_3$	G	SGF/SIF	~100 μm	•	AE (+); EEnz (+); BS (+); SS; AS
[59]						Se						Alginate	E + G	Other	G	-	-		AS;
[75]	Ec											Eudragit S100	Spray drying	-	G	SGF	-		AS; SS;
[57]	Ec											HPMC	LPFC	-	G	-	-		AS
[157]						Se						Alginate + WP	E + G	PS	G	SGF/SIF	< 1 μm	•	AE (+); EEnz (+); BS (+); SS;
[101]	Ec											PVP	ES / WS	Aq	-	Buffer	Ø~100nm/ ~10μm	⦚	
[152]	Ec											PVA	ES	Aq	-	Broth	Ø ~500 nm	⦚	SS;
[151]	Ec											PVA	ES	Aq	-	Broth	Ø ~500 nm	⦚	SS;
[118]	Ec											PVP	ES	Trehalose; O	-	Buffer	Ø ~100 nm	⦚	SS;
[106]	Ec											Alginate + PEO	W/O + E + G + ES	Chl	FC	Buffer	Ø ~ μm	⦚	SS
[106]	Ec											PEO	ES (coaxial)	Chl	FC	Buffer	Ø ~ 1 μm	⦚	Core-shell; SS
[153]	Ec											PEO; CDA	ES (coaxial)	Chl	FC	Buffer	Ø ~ 1 μm	⦚	Core-shell
[148]									Sa			HYMA	photo-crosslinking	-	-	Buffer	-	□	Enzyme triggered release
[142]									Sa			Soya bean oil	O/W	surfactant	W	-	-	•	Nano-emulsions

4.8. Encapsulation of bacteriophage in polymeric microparticles

Polymer phage encapsulation literature has focused largely on gastrointestinal infections (Table 3). The drivers for encapsulation are the need to protect phage from the harsh stomach environment rendering free phage inactive or at any rate resulting in reduction in phage titre. High doses of bacteriophage need to be delivered in a controlled manner at the site of infection in order to effectively reduce the concentration of infectious bacteria present there. This poses a considerable formulation and delivery challenge. Animal studies focusing on gastrointestinal infections (discussed above) have shown poor efficacy outcomes due to the challenges associated with in vivo phage therapy. Oral administration of bacteriophage for human or animal use requires careful consideration of a number of factors including: the acidic pH of the stomach, digestive enzymes (pepsin, proteases, lipases, amylase and trypsinogen), bile salts, pancreatic juices, residence time in different intestinal compartments (duodenum, jejunum, ileum) and phage permeability into the mucosal lining where the infection may reside. Without proper formulation, phage may easily be inactivated due to exposure to adverse environmental conditions. Carriers designed to protect the active agents e.g. from the harsh gastrointestinal environment may also be used to trigger their release. An important example of this is pH dependent release of encapsulated phage [75], exploiting the variation in pH throughout the GI tract; carriers may be designed to respond to specific pH which differs from the stomach (pH 1–3) to the small intestine (pH 5.5–6.5) and the colon (pH 6.5–7.2) [141]. Gastric emptying rates are another important factor and need to be considered in the design of phage encapsulation systems for oral use. Previous studies indicate that in humans gastric emptying of small microcapsules (< 2 mm) is rapid (~0.5–1.5 h) and is not greatly affected by the digestive state of the individual [163]. Gastric emptying times and environmental conditions in other animals may be quite different to those in humans e.g. gastric emptying times in pigs is longer (~1.4–2.2 h) [164]. A suitable biocompatible carrier encapsulating the phage may be required to protect and circumvent natural processes of the biological system e.g. the acidity of the stomach environment [75]. Triggered release of the phage at the site of infection needs to be engineered into the delivery system. For example, the gastrointestinal tract may be modelled as a distinct set of compartments (duodenum, jejunum, ileum etc.) with each compartment having different environmental conditions (with different pH and transit times) [165]. Release of phage in the infected colon may be the desired outcome. High capsule erosion rates triggered by a high pH early in the gastrointestinal tract may result in phage release too early with poor phage therapy outcomes. The harsh environment of the digestive system renders many sensitive therapeutic agents e.g. proteins and phage inactive when administered without due consideration to proper formulation [122,166–168]. Owing to the nature of the infection, fluctuations in physiological conditions may also need to be considered and may indeed be put to use to trigger release at the site of infection [141]. Consideration of particular infection specific symptoms that might influence the delivery of the phage needs to be taken into account. For example, the onset of

diarrhoea in humans causes the osmotic gradient between the epithelia and colon to decrease resulting in increased fluid leakage and shorter transit times [169]. Changes in pH have been observed in the intestine during infection which can directly affect the microbial population as well as transit times in the GI-tract [170].

Pathogens that cause systemic diseases may spread by the faecal/oral route and may cross the intestinal epithelial barrier by translocation. The small intestine and the ileum are scattered with lymphoid tissue that forms the Peyer's patch (PP) nodules. The PP nodules are overlaid by the follicle-associated epithelium (FEM) displaying Microfold cells (M cells). A large number of pathogens infecting the human body take advantage of this route to gain access to the host tissues. *Salmonella typhimurium* [171], *E. coli* [172] and many other pathogens have been shown to adhere preferentially to M cells. Targeting the M cells in the PPs may offer the opportunity to deliver phage accurately at the site of infection. Using anti-M-cell monoclonal antibodies as specific ligands, adsorption of phage containing micro- or nanoparticles (e.g. liposomes) into PPs may be used to target the M-cell membrane [173].

The micro/nanoparticles must possess specific physicochemical properties such as size, zeta potential and hydrophilicity/hydrophobicity balance contained within a narrow range of values [174]. Enhancing phage residence time inside the intestine may exploit the mucoadhesive property of certain molecules e.g. alginates [78,175,176]. This approach has not met with universal acceptance since the superficial mucus layer in the intestine is cleared at a rate between 50 and 270 min, potentially removing anything bound to it. Nanocarriers coated with polyPEG, hyaluronic acid or decorated with vitamin B12 have been shown to have a greater ability to diffuse through the mucus layer [177] thereby avoiding mucoadhesion.

There is scope for the development of innovative strategies to deliver phage encapsulated in micro- and nanoparticles for different target clinical applications. Chemically modified biopolymers provide many opportunities for phage encapsulation including aliphatic polyesters, polyamides, polycarbonate and poly(amino acids). Synthetic polymers, like Eudragit® are a family of pH responsive polymethacrylate polymers; Eudragit® L100 dissolves at pH 6 and Eudragit® S100 at pH 7. These polymers are commercially available (Evonik, Darmstadt, Rohm Pharma Germany) and are widely used in the preparation of microspheres or for their coating. Eudragit® has been successfully used for the production of microspheres encapsulating drugs for colon delivery [178–182]. Combining Eudragit® with other biodegradable polymers (mentioned above) may allow tailored systems for phage intestinal delivery. “Smart” stimuli responsive polymers with modifiable properties have been studied for various applications with tunable pharmacokinetics [147,148,183].

Particle size, surface charge and presence of ligands on the carrier surface play an important role in delivery and have so far not been extensively exploited for targeted phage delivery. A vast choice of natural and synthetic polymers is available offering considerable opportunities to tailor the encapsulation and subsequent release characteristics of phage for different biomedical applications. Many carrier systems have been explored for controlled release of therapeutic agents; these include polysaccharide polymers, synthetic polymers, liposomes, and micelles, thereby permitting use of a multitude of different strategies for encapsulation and release based on different stimuli.

Polymer based controlled release systems have been engineered for the delivery of therapeutic agents for enteric applications including treatment of ulcerative colitis, Crohn's disease [184], colorectal cancer and food poisoning as well as many others [185]. The concentration of bacterial species in the upper GI tract is approximately 10^3–10^4 CFU/ml, largely composed of Gram positive facultative anaerobic bacteria. In the colon however, this increases significantly to 10^{11}–10^{12} CFU/ml [186] and is largely composed of anaerobes such as *Bacteroides*, *Bifidobacteria*, *Eubacteria*, *Clostridia*, *Enterococci* and *Enterobacteria*. To survive in this environment bacteria utilise undigested substrates present in the small intestine. Carbohydrates like di- and tri-saccharides and polysaccharides are fermented by these organisms [187]. Encapsulation using natural polysaccharides is attractive due to their biodegradability by human enzymes. Microbes produce enzymes such as β-glucuronidase, α-arabinosidase, β-xylosidase, β-galactosidase, nitroreductase or urea dehydroxylase [188]. These enzymes metabolise polysaccharides which the body is unable to process. Naturally occurring polysaccharides are popular because they are low-cost and readily available offering a wide variety of structures and properties [189,190]. Other polysaccharides used for drug delivery applications include; cellulose derived biopolymers e.g. hydroxypropyl methylcellulose, pectin derived biopolymers e.g. polygalacturonic acid, guar gum, carrageenans, carob bean gum (galactomannans) and dextrin. Amylose, a derivative of starch has been used to develop commercially available drug delivery systems, COLAL® and COLAL-PRED® for colonic diseases. These polysaccharides utilise the role of enzymatic action by microflora to release the encapsulated agent [191,192].

Alginate and chitosan have been used in a number of studies to encapsulate bacteriophage (Table 3). Sodium alginate is an anionic linear polysaccharide composed of alternating blocks of β-(1 → 4)-linked D-mannuronic acid (M) and α-(1 → 4)-linked L-guluronic (G) residues. The encapsulation of phage in alginates is achieved using mild processing conditions; alginate gelation is achieved by crosslinking of carboxylate anions of guluronic acid and calcium ions. Chitosan is a cationic hydrophilic polysaccharide obtained by partial deacetylation of chitin; it has been studied for its muco-penetrative and tight-junction penetration properties [193,194]. Penetration of the mucosal anionic surface by chitosan is determined by the amino groups [195]. Lameiro et al. [196] showed encapsulation of an adenoviral vector; for mucosal delivery of vaccines; encapsulation efficiency over 84% was achieved with a chitosan coating. Premature release in aqueous media as a consequence of the swelling characteristic of chitosan was observed. Takeuchi et al. [197] showed that chitosan coated sub-micron liposomes interacted with the gut mucosa and had longer residence times in the GI tract. Pectin has been used for the encapsulation of phage [122]. Pectin is a polysaccharide of partial methyl ester of α-D-galacturonic acid (poly-Gal) interrupted with (1 → 2)-α-L-rhamnose units. Pectin forms rigid gels through crosslinking of galacturonic acid with multivalent cations. Pectin incorporated in liposome nanocomplexes has been shown to result in increased intestinal mucoadhesion and prolonged retention in the intestinal mucosa [198].

4.8.1. Protection of bacteriophage from stomach acid, pepsin and bile salts

Alginate and chitosan have been widely used to encapsulate a variety of bacteriophage for oral delivery applications (Table 3). The porosity of alginate gel micro-beads affects phage susceptibility to acid damage (when encapsulated phages have been exposed to SGF) due to diffusion of acid into the microparticles [122,143,145]; hydrogel pores tend be in the 5–200 nm range depending on the degree of crosslinking [175]. Dini et al. [122] showed that acid exposure of *E. coli* phage (pH 1.6, 30 min exposure) encapsulated in pure alginate microparticles resulted in complete loss of viable phage. *L. lactis* phages encapsulated in alginate were rapidly inactivated upon exposure to SGF at pH 2 [149]. A number of studies have shown that incorporation of calcium carbonate (antacid) within alginate micro-beads helps protect encapsulated phage from acid damage [78,145]. The antacid containing encapsulated phage were significantly more stable exposed to SGF acidic conditions in comparison with free phage.

Blending alginates with other polymers such as neutral gums (e.g. Guar gum [122]), pectin [122], chitosan [122,143,145,158], whey protein [149,157,159] and Eudragit [178] has been used to improve the acid stability of alginate microparticles.

A number of studies have indicated that phage encapsulated in alginate-chitosan microparticles improves phage stability upon exposure to acid conditions however, the protection was not complete [122,143,145]. Ma et al. [143,145] showed that *Salmonella* and *S aureus* phages are sensitive to the acidic pH of stomach and therefore required protection for enteric treatment; this was afforded by encapsulating phage in composite microparticles containing chitosan and alginate. Only a ~2.5 log decrease in *Salmonella* phage Felix O1 was shown after 1 h exposure to simulated gastric fluid (containing pepsin) at pH 2.4 compared with release of phage at pH 6.8 [143]. Kim et al. [158] reported promising results for *E. coli* (*Myoviridae*) phage encapsulated in chitosan-alginate beads exposed to SGF (containing pepsin, 1 h exposure at pH 2 and pH 2.5). For free phage, complete loss of viable phage was noted within 5 min of acid exposure at pH 2 and after 60 min exposure at pH 2.5. Additives such as maltose (disaccharide) have been added to formulations to improve storage stability of alginate encapsulated phage to prevent phage loss during air drying of microparticles. Ma et al. [143] exposed *Salmonella* Felix O1 phage encapsulated in chitosan-alginate micro-beads to simulated bile (exposure for 1 h and 3 h). Free phage showed a 1 log (1 h exposure) and 2 log (3 h exposure) reduction in phage titre. Microencapsulation in chitosan-alginate beads resulted in complete protection of phage to bile salt inactivation. Dini et al. [122] showed that acid exposure of *E. coli* phage (pH 1.6, 30 min exposure) encapsulated in pectin afforded improved resistance to acid exposure and protection from pepsin inactivation.

The concentration of encapsulating agent was found to be an important factor in affording phage protection from SGF. Increasing whey protein concentration in alginate-whey protein microparticles resulted in better acid protection by reducing permeation rates of acid and digestive enzymes [159]. Whey protein amino groups may sequester protons diffusing through the polymer matrix. Tang et al. [157] encapsulated *Salmonella* Felix O1 phage in alginate-whey protein microparticles (different alginate: whey protein compositions) with high phage loading 10^{10} PFU/g wet particles. Solutions with high polymer concentrations (~ > 5% w/v) were found to have high viscosity and posed difficulties in extrusion and lead to a wider distribution of particle sizes [157]. Alginate-whey protein microspheres were found to be better at protecting *Salmonella* Felix O1 phage in SGF compared with alginate chitosan microspheres [143] prepared by the same group. Exposure of encapsulated Felix O1 to bile acid (1 h and 3 h duration) did not result in significant change in phage titre [157].

4.8.2. Release kinetics of encapsulated bacteriophage

Most studies with alginate containing phage show slow release of encapsulated phage over a 2 h–6 h period upon exposure to SIF [143,158]. Most systems swell upon exposure to SIF and phage release is due to diffusion and encapsulation matrix degradation. Ma et al. [143] showed that *S. aureus* phage K encapsulated in chitosan-alginate micro-beads showed a sustained release rate over a 6 h period (exposure to SIF pH 6.8). Microspheres were seen to swell and disintegrate. Addition of calcium carbonate in alginate-chitosan micro-beads slowed down the release rate. This was attributed to excess Ca^{2+} ions slowing the alginate hydrogel dissolution [145].

Kim et al. [158] exposed *E. coli* (*Myoviridae*) phage encapsulated in chitosan-alginate beads to SIF (0.1% bile and 0.4% pancreatin, pH 7.5, 37 °C, 6 h exposure). Sustained release of *E. coil* phage encapsulated in chitosan-alginate beads over a 6 h period was noted [158]. Sustained release of phage (from beads) was shown to result in a ~3 log reduction in the in vitro concentration of *E. coli* 157:H7 after 5 h (compared with ~4 log reduction by free phage). The starting concentration of bacteria was 10^6 CFU/ml. After 10 h of incubation in SIF, both free phage and encapsulated phage were unable to arrest bacterial growth (comparison with untreated controls) possibly due to presence of phage resistant mutant bacteria. Colom et al. [78] showed the in vitro release kinetics in SIF (40 min exposure, pH 8, 1 mg/ml pancreatin, 10 mM bile salts, 42 °C) for three *Salmonella* phages (ϕ20 (podovirus), ϕ78 (podovirus) and ϕ87 (myovirus)) individually encapsulated in alginate microparticles containing $CaCO_3$. Almost complete phage release was reported within 40 min of exposure to SIF.

Microparticles with different compositions of alginate-whey protein encapsulating *Salmonella* Felix O1 phage were exposed to SIF (pH 6.8) for 6 h [157]. The microparticles were found to swell and eventually disintegrate. Encapsulated *Salmonella* Felix O1 phage showed sustained release profiles which varied depending on the amount of polymer content of the particles. For the same alginate content, increasing the whey protein concentration was found to slow the phage release rate. Whey protein hydrogels tend to swell readily at pH values above the protein isoelectric point (IEP ~pH 5.1) [199]. Pancreatin may also degrade whey protein provided it is able to diffuse into the hydrogel structure. This may explain increasing erosion rates after an initial period of exposure to SIF. Both whey protein and alginate possess negatively charged functional groups which would result in electrostatic repulsion and swelling of the gel upon exposure to alkaline SIF. Alginate-whey protein encapsulated *S. aureus* phage K released phage over a 2 h period upon exposure to SIF (pH 6.8) [159]. The microspheres were seen to swell and eventually disintegrate with sustained release of phage. Changes in formulation resulted in modest differences in the release profiles. Dried microspheres resulted in slower release of phage. Increasing concentrations of either alginate or whey protein retarded the release of phage K. *L. lactis* P008 phage encapsulated in alginate-whey protein showed a sustained release rate upon exposure to SIF over a 2 h period [149]. Pre-exposure to SGF followed by exposure to SIF resulted in a ~1 log reduction in total phage released. Gelled milk protein microspheres were also found to be suitably protective of P008 phage and showed sustained release of phage upon exposure to SIF over a 2 h period.

4.8.3. Effect of size of beads

Particle size was found to be an important factor influencing phage protection from SGF for acid permeable beads [159]. Alginate-whey protein microparticles having the same composition but different mean sizes showed larger particles protected phage better compared with smaller microparticles [159]. Larger particles keeping phage further away from the acid environment were better at protecting phage from acid damage [157]. However, larger particles are expected to experience more mastication [159] in the mouth and longer gastric retention [163]. Samtlebe et al. [149] encapsulated *L. lactis* P008 phage in enzymatically gelled milk protein microcapsules with a small mean particle size (~130 μm) which was shown to protect phage exposed to SGF at pH 2 for up to 2 h. Colom et al. [78] showed that three *Salmonella* phage individually encapsulated in alginate microparticles (size ~ 150 μm) containing $CaCO_3$ were able to protect the phage from exposure to SGF although reduction in phage titre was noted for phage ~3.1 log reduction ϕ78 and ~2.4 log reduction ϕ87. No loss of titre was noted for phage ϕ20.

Future studies may want to focus on developing methods to control and tailor the porosity of the microcapsule shell to protect encapsulated phage from SGF acidity whilst controlling the phage release rate.

4.8.4. Storage stability

Wet alginate-whey protein microspheres containing encapsulated *S. aureus* phage K showed no loss in phage viability over a six week storage period (stored at 4 °C) [159]. Dried microspheres (with added maltose) were shown to retain phage viability over a two week storage period (no reduction in phage titre when stored at 4 °C and 20% reduction in phage titre when stored at 23 °C). Colom et al. [78] stored *Salmonella* phage encapsulated in alginate/$CaCO_3$ microparticles at 4 °C

for six months and showed only a slight (~25%) reduction in phage titre. Tang et al. [157] air dried (at 22 °C) *Salmonella* Felix O1 phage encapsulated in alginate-whey protein microparticles and found an 8-log reduction in phage titre. Addition of maltodextrin to the alginate-whey protein microspheres stabilised the phage resulting in a < 0.5 log reduction in titre post-air drying (drying time 30 h, at 22 °C). No significant loss in *Salmonella* Felix O1 phage titre was observed for air dried particles over a 2 week storage period (stored at 4 °C and 23 °C). Phage encapsulated in wet alginate-whey protein microspheres stored at 4 °C for 6 weeks showed no significant loss in phage titre.

4.8.5. Bacteriophage encapsulation in synthetic polymers

Surprisingly few studies are available on phage encapsulation in synthetic polymers. Puapermpoonsiri et al. [100] freeze dried lytic *Staphylococcus aureus* and *Pseudomonas aeruginosa* phage encapsulated in PLGA (poly(lactic-*co*-glycolic acid)) microspheres prepared using an W/O/W double emulsion technique (using dimethylformamide, DMF as organic solvent). The microsphere size was designed to deliver the encapsulated phage via inhalation using a dry powder inhaler (1-5 μm). The phage encapsulated microparticles had low bulk density and aerodynamic diameter ~ 4 μm suitable for respiratory delivery. Phage activation was retained post-particle formation and sustained phage release kinetics was noted upon exposure of particles to PBS buffer over a 6 h period. Phage release showed an initial burst release (within 30 min for microparticles suspended in buffer) followed by a longer release over ~6 h suggesting either inhomogeneous distribution of phage within the microparticles or reflecting the dissolution kinetics of the polymer microparticles [100]. The formation of phage loaded PLGA microspheres necessitated a solvent evaporation step (accomplished using freeze drying). No viable phage were recovered from the particles after 7 days of storage at either 4 °C or 20 °C. Phage inactivation due to exposure to organic solvent (dichloromethane) posed a problem. Matsubara et al. [200] evaluated the kinetics of the loss of viability of a filamentous phage exposed to four water miscible organic solvents ethanol, acetonitrile, dimethylsulfoxide and DMF; these solvents disrupt protein conformation and enzymatic activity [201].

Stanford et al. [75] used Eudragit ES100 (methyl methacrylate-*co*-methacrylic acid polymer) for *Escherichia coli* specific phage encapsulation for cattle treatment. They showed high sensitivity of the phage to the acid in the gastric environment. However, encapsulated phage showed improved viability and in vivo reduction in faecal shedding of *E. coli* following induced infection (discussed earlier in the review). The potential of ES100 for encapsulation of phage is also reported in a patent [202]. A number of other published studies have looked at phage encapsulation in polyester amide films for wound healing applications [121,160]. Thin biodegradable polymer films impregnated with a mixture of lytic bacteriophage (containing a mixture of lytic phage (trade name, Pyophage) against *P. aeruginosa*, *E. coli*, *S. aureus*, *Streptococcus*, and *Proteus mirabilis*, sold under the trade name PhagoBioDerm) was employed Markoishvili et al. [121] to treat skin infections.

Hathaway et al. [147] encapsulated *S. aureus* phage K in poly(*N*-isopropylacrylamide), a thermally responsive polymer. Phage were encapsulated in PNIPAM nanospheres (~500 nm) which undergo a fully reversible temperature dependent phase transition at a lower critical solution temperature (LCST) which results in a change in the particle size due to polymer shrinkage and expulsion of water from the nanospheres along with the phage contained therein. The LCST for PNIPAM can vary between 32 °C to 36 °C depending on polymer properties [147]. The PNIPAM encapsulated phage nanoparticles were grafted on to non-woven polypropylene films (mimicking a wound dressing film) [147]. Evidence of temperature dependent phage release data was somewhat limited in this paper.

Bean et al. [148] encapsulated *S. aureus* phage K in an agarose slab covered in a thin film of a photo-cross-linkable hyaluronic acid methacrylate (HAMA) polymer. The HAMA polymer was shown to degrade in the presence of hyaluronidase enzyme secreted by *S. aureus* (a rare example of using a virulence factor secreted by the pathogen as a trigger for phage release).

Milo et al. [203] describe the use of a pH responsive coating for urinary catheters which may immobilise phage and release these upon onset of infection by *Proteus mirabilis*. Urinary tract infections may result in an increase in pH due to bacterial urease which could be used as a trigger to dissolve an upper layer of pH responsive polymer (e.g. methyl methacrylate-*co*-methacrylic acid; Eudragit S100) as part of the construction of the catheter hydrogel coating.

4.9. Bacteriophage encapsulation in liposomes

Relatively few published studies have looked at encapsulation of bacteriophage in liposomes (see Table 4). In nearly all cases the thin-film hydration method has been used for liposome preparation. Briefly, this entails dissolving lipids in an organic solvent (typically chloroform) followed by solvent evaporation under vacuum leaving a dry lipid film. Addition of an aqueous phage suspension to the dry lipid film results in the stacks of liquid crystalline lipid bilayers becoming fluid and swelling. Agitation of the solution results in detachment and closure of the lipid layers thereby forming relatively large and heterogeneous multilamellar liposomes. Extrusion of the liposome mixture through porous membranes has been be used to form smaller liposomes [77,150]. Nieth et al. [47] employed a novel method proposed by Weinberger et al. [204] to prepare phage encapsulated in giant unilamellar vesicles (GUV) using gel-assisted formation of GUV by rehydrating lipid layers on polyvinyl alcohol gels. This technique afforded a number of advantages over the traditional thin film hydration method. Firstly, faster hydration kinetics due to aqueous solvent penetration through lipid film stack was achieved due to the difference in chemical potentials between the dry PVA supporting the lipid stacks and the liquid phase above [47]. Secondly, better encapsulation phage efficiency was achieved with the phage incorporated within the cast PVA gel films instead of the rehydration buffer. Confocal microscopy showed visual evidence that this method allowed good encapsulation of a fluorescently labelled *E. coli* λ phage. When the *E. coli* λ phage was provided externally with the swelling buffer, no encapsulation was observed suggesting that the lipid stacks themselves were impermeable to the phage present in the external solution. The GUV prepared using this technique resulted in large vesicles (5 μm–50 μm). Extrusion of GUV through a 5 μm pore size membrane resulted in reduction in vesicle size to < 5 μm however, *E. coli* λ phage concentration was below the sensitivity of the fluorescence microscope. No quantitative phage titres were carried out by breaking the liposomes and releasing their phage cargo. Nieth et al. [47] also employed the inverse emulsion liposome preparation method of Pautot et al. [205] to encapsulate the fluorescent *E. coli* λ phage. GUV prepared using the inverse emulsion method had an average diameter between (10 μm–15 μm). Nieth et al. [47] also showed significantly greater intracellular uptake of liposome encapsulated fluorescent *E. coli* λ phage (compared with free phage) in a macrophage-like human monocytic cell line.

Colom et al. [77] encapsulated *S. enterica* phage in cationic liposomes (liposome size range ~ 200–800 nm). They suggested that cationic liposome loaded bacteriophage would achieve two objectives: (i) to protect the bacteriophage from gastric acids and (ii) act as a promoter for mucoadhesiveness owing to their positively charged surfaces thereby prolonging intestinal residence time of phage [77]. The *Salmonella* phages were encapsulated in unilamellar liposomes using the thin film hydration method. In vitro studies showed that encapsulated phage were released upon exposure to SIF within 60 min. Exposure to SGF resulted in loss of phage viability with modest protection of *S. enterica* phages afforded by encapsulation in cationic liposomes compared with free phages. The intestinal residence time of liposome encapsulated phages in the cecum of newly hatched chickens was

considerably longer compared with free phage (refer to earlier section on animal studies). This was attributed to the small size and mucoadhesive properties of the cationic liposomes. Nieth et al. [47] also encountered problems in terms of bacteriophage encapsulation in liposomes. Encapsulation efficiency, control over the size, phage titre, were found to be key issues that need addressing in future studies. Balcao et al. [206] encapsulated lytic bacteriophage (against *Salmonella* and *E. coli*) by emulsifying an aqueous phage solution (water phase) in a lipid melt (oil phase) using homogenisation technique resulting in nanoparticles in the size range (85–200 nm). The size of the nanoparticles showed stability over a 3 month storage period at room temperature. Quantitative results on the antibacterial activity of encapsulated phage were not provided although lytic activity of encapsulated phage was observed following phage release.

Singla et al. [39] evaluated the encapsulation of *K. pneumonia* phage (belonging to the *Podoviridae* family) in liposomes prepared using different phospholipid: cholesterol ratios with additional charge inducing agents to prevent liposome aggregation. The liposomes were prepared using the film-hydration method and ranged in size between 500 and 1000 nm. Phage encapsulation efficiency was shown to be higher for the electrostatically stabilised positively charged liposomes (containing stearylamine) with higher lipid content. Phage encapsulated liposomes stored at 4 °C for 9 weeks were stable both in terms of size as well as phage titre. Storage at ambient temperature or 37 °C resulted in changes in size (increase) as well as loss of phage titre. In vivo toxicity testing and biodistribution of phage was carried out in mice. Briefly, $\sim 10^8$ PFU dose was delivered to mice via the intraperitoneal route. Rectal temperatures were taken hourly (for the first 5 h) and daily (for 4 days post-phage treatment). No adverse change in temperature or symptoms of lethargy or sickness was noted for the liposome/phage treated animals. Phage distribution in lungs, liver, spleen and kidney were quantified. Blood titres of phage reached ~10 PFU/ml shortly after injection (within 1st hour) for both free phage and liposome encapsulated phage. Liposome encapsulated phage remained in blood and other organs for longer periods compared with free phage treated mice. Free phage were cleared from the blood and organs 48 h post-treatment whereas liposome encapsulated phage were detectable in blood and all organs up until day 3 (albeit at low concentrations ~ 1 PFU/ml) post-treatment and remained detectable in the spleen up until day 14.

Liposomes are spherical structures delimited by a double layer of bulky amphiphilic lipids which encloses an aqueous phase. Charge, size, fluidity of the bilayer, lamellarity and surface functionalization are the major parameters that regulate liposomes fate in vivo and in vitro. Size and lamellarity vary as a result of the manufacturing process. Fluidity and superficial charge depend on the chemical nature of the lipids and their relative ratio. Liposomes are also highly biocompatible and they have been widely used in the last fifty years to encapsulate hydrophilic or hydrophobic therapeutic agents in their aqueous core or within the bilayer thereby enhancing drug bioavailability and stability over time. Encapsulated liposomes are a promising drug delivery system and pharmaceutical carrier of choice for numerous applications [207].

From a clinical viewpoint the potential ability of liposome encapsulated phage to enter live cells containing intracellular pathogens is of crucial importance e.g. *M. tuberculosis* or other intracellular bacteria e.g. *S. aureus* or *E. coil*. The surface of liposomes may be functionalized to achieve various goals: (i) attachment of specific ligands for targeted delivery of liposome cargo; (ii) attachment of hydrophilic polymers to increase systemic circulation time and slow release of bacteriophage cargo; (iii) attachment of various labels to monitor the fate of the bacteriophage loaded liposomes; (iv) incorporation of positively charged lipid derivatives or positively charged polymers e.g. to aid mucosal adhesion thereby improving residence time in the gastrointestinal tract; (v) protection of bacteriophage from physical and chemical stresses; (vi) liposome encapsulated bacteriophage may result in modulated immune response to the presence of the bacteriophage in the body.

Table 4
Summary of bacteriophage microencapsulation literature in liposome carriers.

Ref	Host organism	Carrier system	Encapsulation method	Additives	Target infection	Release studies	Size	Of note PE = phage encapsulation outcome
[206]	*S. enteritidis*	Softisan 100™; soybean PC	Water-in-oil-in-water double emulsion	Tween 80; glycerol; polyoxamers (Lutrol F68)	Respiratory	–	50–250 nm	PE (modest)
[150]	*E. coli*; *M. Smegmatis*	DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine); DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine)	Thin film hydration + PVA gel assisted hydration	–	Intracellular	–	5–50 μm	Giant unilamellar vesicles; PE (good)
[77]	*S. enterica*	Mixture of DLPC:Chol-PEG600:cholesterol:cholesteryl molar ratio (10:1:2:7)	Thin film hydration + extrusion	Chloroform as solvent; trehalose added as cryoprotectant	Gastrointestinal	Simulated gastric fluid/buffer	~200–800 nm	Freeze drying; storage studies; PE (good)
[31]	*K. pneumonia*	Phosphatidylcholine:cholesterol	Thin film hydration	Tween 80; stearylamine	Respiratory	–	~100 nm	Animal studies
[39]	*K. pneumonia*	Phosphatidylcholine:cholesterol	Thin film hydration	Tween 80; stearylamine; dicetylphosphate	Respiratory	–	~500–1000 nm	Storage studies; PE (good)
[95]	*K. pneumonia*	Phosphatidylcholine:cholesterol	Thin film hydration	Tween 80; stearylamine	Respiratory	–	~100 nm	Ex vivo biofilm model; intracellular macrophage model; animal studies

4.9.1. Liposome size, charge and surface modification e.g. PEGylation

Previous studies have found that by reducing the size of particles below 1 μm (i.e. nm scale) enhancement in liposome nanoparticle access to infection sites deep in intestinal mucosa may be achieved thereby improving phage therapy efficacy by targeting bacterial refugees residing in these difficult to access niches [197,198]. The residence time of nanoparticles has been shown to be longer compared to larger microparticles; larger particles are more prone to peristaltic action and rapid transit through the colon. Nanoparticles have been shown to interact with epithelial cells and show increased retention enabling selective delivery of therapeutic agents into the colitis tissue [208]. Conventional formulations have so far not exploited this ability causing deposition in regional areas. Smaller particle sizes are able to take advantage of endocytosis for internalization into the epithelial cells of the colon. They attach to specialised epithelial M cells (discussed above) which are responsible for antigen transport from the lumen to the immune system. These M cells are also responsible for uptake of nanoparticles by transcytosis [209]. Microparticles have been shown to adhere to the inflamed mucosal wall whereas nanoparticles are absorbed across the epithelial barrier [210].

Interactions between charged particles and the mucus may be an appropriate strategy for intestinal delivery. Cationic delivery systems adhere to the negatively charged intestinal mucosa thereby promoting cellular uptake and subsequent drug release [211]. The mucins which comprise the mucosa are also known to be negatively charged. Anionic systems adhere to inflamed tissue via electrostatic interactions. The presence of positively charged proteins in the inflamed regions typically increases. Some authors suggest that unlike cationic particles which get immobilised on the mucus, anionic particles are able to diffuse through the mucus network due to decreased electrostatic interactions [170]. Other studies suggest that particles are entrapped within the mucin mesh no matter what charge they carry since the mucus displays negatively and positively charged proteins. Hence, if a non-adherent approach is needed, the nanoparticle surface could be decorated with specific moieties that confer so called 'stealth' properties [212]. Incorporating poly(ethylene glycol) (PEG) on the surface of nanoparticles has been shown to result in a hydrophilic surface chemistry that allows unobstructed diffusion of nanoparticles through the epithelium. An explanation for this is that surface modification prevents strong interaction of nanoparticles with the mucus which typically prevents diffusion to the colitis tissue [213].

Active targeting by surface functionalization of phage containing nanoparticles e.g. liposomes with ligands for selective phage accumulation at the site of infection may help increase the local concentration of phage delivered thereby increasing their therapeutic efficacy. Monoclonal antibodies and peptides specific for target sites have been used to increase specificity and enhance muco-penetrative properties [214]. It is thought that by incorporating ligands targeting receptors present at the site of infection may improve binding and thereby intensify endocytosis. Possible receptors for targets in the inflamed colon may include, ICAM-1 which is upregulated during inflammation, mannose receptors and macrophage galactose-type lectin (MGL) (expressed by activated macrophages). The combined effect of the small particle size of nanoparticles, PEGylation and integration of mannose ligands has recently been shown to increase penetration ability for macrophage targeting [208].

Functionalization of liposomes for specific targeting may have an attractive future for phage delivery applications. Some pathogenic microorganisms such as *M. tuberculosis* and *S. enterica* (serovar Typhimurium) are able to survive specific antimicrobial machinery causing persistent infections. Long-term residence is achieved by internalization in mononuclear phagocyte system (MPS). It is not fully understood how these bacteria inhibit their clearance by the immune system but common mechanisms include prevention of the normal maturation of the phagosomal environment or blockage of the fusion of the phagosome with endosome and lysosome. What happens afterwards is even less clear; bacteria can remain stored for years in an asymptomatic state that can suddenly be triggered into a potentially life-threatening active state, especially for people with a weak or compromised immune system. Liposomes innately accumulate in MPS due to their interaction with serum proteins that accelerates phagocytosis. It makes bacteriophage-encapsulated liposomes an appealing approach to treat antimicrobial resistant bacterial infections that spread from within the MPS. The rate of clearance depends on the physical/chemical characteristics of the vesicle` surface. It has been shown that uptake by MPS for liposomes is enhanced when phosphatidylserine incorporating PEG is used. On the other hand, long-circulating liposomes with high PEG value can accumulate at the site of infection due to increased permeability. Agrawal et al. [215] have demonstrated that tuftsin, a tetra-peptide, used as a homing-device improves liposomes uptake by MPS cells but also stimulates these cells to take action against infections. Leishmaniasis, *Aspergillus fumigatus* and *Mycobacterium tuberculosis* infections have been treated with drug-loaded tuftsin-bearing liposomes and tuftsin-free liposomes and in all cases the treatment lead to enhanced effectiveness.

Phage therapy has recently been considered as an alternative to, or a supplement for, antibiotic treatment for mycobacterial respiratory infections. However, phage therapy for TB and other respiratory infections caused by non-tuberculosis mycobacteria (NTM) is complicated by the intracellular nature of the infection. It is generally recognised that phage do not diffuse across eukaryotic cell membranes and therefore would be unable to infect the intracellular mycobacteria [48]. Using a non-pathogenic mycobacterium, *Mycobacterium smegmatis*, infected with a mycobacteriophage as a 'Trojan horse' to deliver phage to the macrophage compartments in which *M. avium* resides, showed promise when tested in vitro with infected macrophages [216]. However, when used in a murine model of *M. avium* infection, this approach was less effective. It was deemed that the numbers of infected *M. smegmatis* cells required, would deliver too large a dose of antigenic material to be acceptable [217]. Alternative methods of delivery of phage to intracellular mycobacteria have been sought. Looking to the relatively routine use of liposome encapsulation for intracellular delivery of small molecule drugs [218], Nieth et al. [150] recently suggested encapsulation of phage within liposomes may result in uptake of phage and delivery to the required intramacrophage compartment to initiate lytic infection of intracellular pathogens. To this end, they demonstrated successful encapsulation of mycobacteriophage TM4 in giant unilamellar vesicles. Furthermore, by encapsulation of a lambda phage expressing yellow fluorescent protein and fluorescent labelling of macrophage endosomal proteins, they were able to demonstrate intracellular delivery and co-localisation of the lambda phage and the endosomes; in Mtb infected macrophages, the mycobacterial phagosome fuses with early endosomes. This demonstration that liposome-encapsulated phage could gain access to the target mycobacteria satisfies a requirement for effective therapeutic use of bacteriophage to treat intracellular mycobacterial infections. A previous study using liposome encapsulation of frontline antituberculosis antibiotics, rifampicin and isoniazid, showed enhanced treatment in a murine infection model of Mtb compared with treatment with antibiotics alone [219]. The efficacy of a liposome-encapsulated mycobacteriophage to treat Mtb infection in macrophages or animal models remains to be demonstrated.

Encapsulation of bacteriophage in liposomes may be a potential approach to tackle antibiotic resistant bacterial infections in wounds. Previous studies have shown that topical applications of buflomedil hydrochloride loaded liposomes in mice significantly accelerate wound epithelialization [220] in normal and ischemic tissue. Furthermore, by modifying the liposome surface properties (e.g. charge), liposomes can be trapped in gauzes [221] reducing the number of applications and consequent costs. The surface of liposome vesicles can be modified with hyaluronic acid (HA) in order to confer bioadhesive properties [222]. Indeed, HA-anchored liposomes were found to bind to the extracellular

matrix (ECM) without compromising any of the liposome properties. A marked enhancement of liposomal adhesion (in vitro) to a monolayer of human epidermoid carcinoma cells was observed. Topical delivery of bacteriophage-loaded liposomes would constitute a phage reservoir localised in the area of the wound releasing phage at a high concentration over an extended period of time.

4.10. Bacteriophage encapsulated in electrospun fibers

Fabrication of phage encapsulated polymer fibers (diameters ranging from tens of nanometers to microns) may readily be achieved using electrospinning, by drawing a charged polymer-in-solvent solution onto a grounded electrode whilst evaporating the solvent. A number of studies have shown the feasibility of encapsulating phage in nanofibers using the electrospinning technique (Table 2). A number of different polymers have been evaluated for encapsulating phage in fibers including cellulose diacetate [153], polyethylene oxide [106,153], polyvinyl alcohol [151,152] and polyvinyl pyrrolidone [101,118]. Good phage encapsulation results have been reported using water soluble or chloroform soluble polymers [106,118]. Rapid dehydration during electrospinning was shown to damage phages. Use of buffer and incorporation of sugars e.g. trehalose was shown to improve protection of phage [118]. Protection of phages in core-shell fibers using co-axial electrospinning technique was shown to be particularly promising [106,153]. Using immiscible solvents for the core (phage contained in an aqueous core) and shell (shell polymer dissolved in an organic water immiscible solvent) was found to stabilise the polymer jet resulting in the formation of a stable core-shell fiber structure [106,153]. In this process, aqueous solvent evaporation from the core is very slow thereby overcoming drastic changes in osmotic pressure in the phage containing core. Encapsulation of phages in nanoparticles formulated in an emulsion followed by electrospinning of the emulsion was another approach that was shown to result in improved phage protection during electrospinning [106]. Increasing polymer molecular weight or blending different polymers (e.g. cellulose acetate/PEO) was shown to result in changes in the phage release kinetics from electrospun fibers [153]. The main reported mechanism of phage release from encapsulated fibers is typically swelling of fibers upon exposure to solvent followed by polymer erosion accompanied by phage diffusion and release. Encapsulating phage in hydrophilic water soluble polymers results in rapid phage release due to polymer dissolution whereas use of hydrophobic polymers or blends may allow tailoring of phage release to ensure slower release over a prolonged period.

The polymer and solvent combination used for fiber production were found to be important factors in the formation of nanofibers and retention of phage viability therein. Lee and Belcher [101] spun fibers (diameters 100–200 nm) containing *E. coli* M13 phage which retained infectivity against bacterial host when re-suspended in buffer. The phage containing fibers were spun from an aqueous polyvinyl pyrrolidone buffered solution containing phage; the fibers were captured as a non-woven fibrous mat. Concentration of viable phage in the fibers was not reported. Salalha et al. [152] and Kuhn [151] encapsulated T4, T7, and λ *E. coli* phage in electrospun nanofibers (diameter 250–400 nm) prepared from aqueous poly(vinyl alcohol) (PVA) solutions. Nanofiber encapsulated phage were shown to retain viability and remained stable for three months stored at − 20 °C and − 55 °C and nearly so at 4 °C. Salalha et al. [152] suggested that this method was a facile way of preserving phage. Immediately after electrospinning, a 2 log loss in phage viability was reported (T4 ~ 1%, T7 ~ 2%, and λ ~ 1%). The losses in phage viability during electrospinning may be attributed to drying stresses due to rapid evaporation of solvent and drastic changes in osmotic pressure experienced by phage. Dai et al. [118] investigated electrospinning (diameter 100-200 nm) of *E. coli* T7 phage in SM buffer using PVP as polymer (15% w/v) and trehalose (5% w/v) as excipient as a means to improve phage stability for long term storage. Inclusion of SM buffer had a significant positive effect on phage viability post-electrospinning. The phage titre of PVP/SM buffer samples (with and without trehalose) fell by no more than ~1 log following electrospinning. Electrospinning phage-PVP solutions in deionised water resulted in a 4 log reduction in phage titre. Addition of trehalose to deionised water resulted in some improvement with a 2 log reduction post-electrospinning, showing that trehalose had a protective effect. Dai et al. [118] also compared phage stabilisation using electrospinning with freeze drying. Phage encapsulated in electrospun fibers (made from SM buffer with trehalose) were found to have similar titre compared with freeze dried powder samples. Storage of phage T7 in electrospun fibers for eight weeks (stored at 20 °C) resulted in a 3 log reduction in phage titres for samples prepared from SM buffer with/without trehalose.

Korehei and Kadla [106] evaluated emulsion electrospinning (pre-encapsulating phage in ~800 nm alginate nanoparticles followed by lyophilization and then re-suspension in a polymer containing solvent followed by electrospinning) and coaxial electrospinning (incorporating the phage in a protective core in a core-shell fiber structure) processes to encapsulate *E. coli* T4 phage in polyethylene oxide fibers. The process of encapsulating phage in alginate nanoparticles resulted in a 1 log drop in phage titre. Freeze drying the phage encapsulated in nanospheres did not result in a drop in phage titre. Electrospraying the alginate suspension on its own (without polyethylene oxide) resulted in a large drop in phage titre from 10^8 PFU/ml to 10^3 PFU/ml. Polyethylene oxide (PEO) is soluble in both aqueous and organic solvents making it a suitable polymer for electrospinning. Polyethylene oxide fibers encapsulating alginate nanoparticles were found to result in fibers with a bead-on-string morphology (fiber diameter ~ 1 μm). Dissolution of fibers in SM buffer showed a phage titre of 10^6 PFU/ml (a 2 log reduction). Fibers with diameter ~ 2 μm were prepared using a core/shell approach with the fiber shell made using PEO in chloroform and the core containing T4 phage in SM buffer [106]. Good lytic activity was retained post-electrospinning with a high phage titre obtained (10^8 PFU/ml) with fibers dissolved in SM buffer. Storage stability of T4 phage in the core-shell fibers after freeze drying (freeze drying conditions were not reported in the paper and no details were given on whether any excipients were added) was investigated (stored at − 20 °C, 4 °C and 20 °C) over a period of 30 days. No significant loss in phage titre was observed for samples stored at − 20 °C and 4 °C. Storage at 20 °C resulted in a significant loss (> 6 log reduction) in phage titre. Upon exposure of PEO fibers to SM buffer, fibers were seen to swell followed by disintegration (within 10 min) resulting in release of phage for the alginate-emulsion based fibers and the core-shell fibers. Initial rate of phage release was faster from the core-shell fibers (the resulting final titre was also higher) compared with the alginate nanoparticle encapsulated fibers. Korehei and Kadla [153] varied the molecular weight (MW) of PEO (100 kDa–600 kDa) and blended hydrophilic PEO with the hydrophobic polymer cellulose diacetate (CDA). *E. coli* T4 phage encapsulated in fibers produced using different PEO/CDA showed slow release of phage depending on the PEO MW and the ratio of PEO/CDA. Higher PEO MW (results in increased polymer entanglements) and greater proportion of hydrophobic CDA (slower rate of polymer swelling and erosion) resulted in slower release of phage.

4.11. Immobilisation of bacteriophage on surfaces including thin films

Microbial biofilms on medical devices e.g. urinary and central venous catheters, stents, implants, invasive health monitoring devices, are a significant healthcare problem and are responsible for a vast majority of medical device associated infections. Bioactive packaging materials are also attractive in other areas including food packaging e.g. ready-to-eat meat, ready cut fruits and poultry [155]. Antibiofilm formation strategies include oriented immobilisation by covalent attachment of bacteriophage to polymer surfaces [223] or immobilisation of bacteriophage in stimuli responsive materials where release is triggered in response to an external stimulus [148]. Although phage immobilisation on surfaces may readily be achieved through passive adsorption [224],

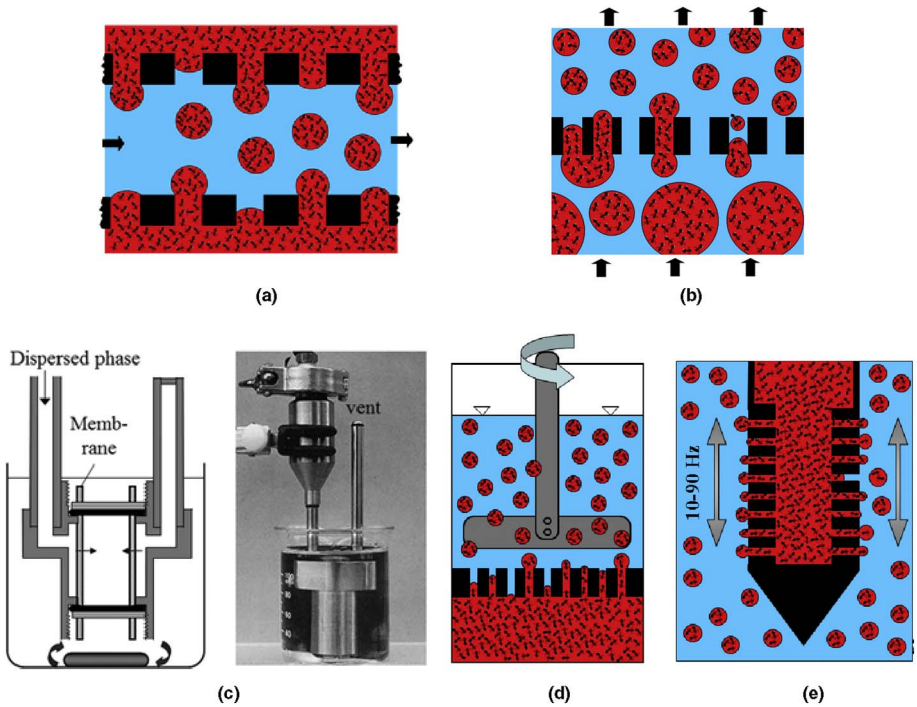


Fig. 3. Membrane emulsification (ME) methods that can be used for phage encapsulation: (a) Cross flow ME; (b) premix ME; (c) Shirasu Porous Glass (SPG) microkit; (d) Micropore Dispersion Cell; (e) axially oscillating membrane tube.

the process is inefficient with loss of phage activity due to poor orientation of phage tails needed to recognise and infect target bacteria. Chemical biotinylation of the phage capsid head has been used for oriented immobilisation (to retain phage lytic activity by leaving the tail fibers free in order to capture bacteria) of a *Salmonella enteritidis* phage on streptavidin-labelled magnetic beads [225]. Tolba et al. [226] used phage display technology to introduce affinity tags on the capsid head to immobilise *E. coli* phage on streptavidin coated magnetic and cellulose beads. Pearson et al. [223] covalently attached lytic *E. coli* and *S. aureus* phage through reaction of primary amine groups on the capsid head with R-COOH surface groups (generated using microwave plasma in the presence of maleic anhydride) on polyethylene and polytetrafluoroethylene surfaces. Anany et al. [227] and Lone et al. [155] utilised the electrostatic interaction between the anionic capsid head of *E. coli* O157:H7 and *Listeria monocytogenes* phage cocktails and polyvinylamine treated cellulose membranes. Modification of cellulose membranes resulted in greater numbers of infective phage oriented correctly. A recent patent focuses on controlled covalent attachment of bioactive bacteriophage to a hydrogel coating material for regulating biofilm development with examples focusing on biofilm reduction on urinary catheters [228]. An earlier patent gave examples of phage immobilisation on nylon sutures for wound healing applications [229]. Cooper et al. [230] used carbodiimide oriented covalent attachment of a tailed *P. aeruginosa* bacteriophage to magnetised multiwalled carbon nanotubes. Preliminary results showed antimicrobial efficacy of bacteriophage-nanocomposite conjugates against *P. aeruginosa*. Handa et al. [231] employed chemical vapour deposition to make a smooth aminosilane monolayer on a glass substrate. Subsequently, monolayers of covalently bound *Salmonella* specific phage were attached to the aminosilane monolayer using well-established sulfo-NHS (*N*-hydroxysulfosuccinimide) and EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) chemistry [231]. Techniques for attachment of phage to gold substrates has been investigated to increase their diagnostic utility as part of quartz crystal microbalance (QCM), surface plasmon resonance (SPR) transduction based diagnostic platforms [232] or direct detection as part of virus electrodes [233]. Recently, Richter et al. [234] applied an electrical potential to align *E. coli* phage T4 and drive them to the electrode surface resulting in head down, tail-up orientation resulting in increased sensitivity of phage for bacteria and a low detection limit for *E. coli* of 10^3 CFU/ml. Tawil et al. [235] reported on the synthesis of colloidal phage-gold nanoparticle complexes for the detection of a single *S. aureus* bacterium in a mixture of *S. aureus* and *E. coli* using Dark Field Microscopy. The potential of phage for biosensing applications including low-cost medical diagnostics may require their coupling with novel materials (thin film constructs, magnetic, metallic, polymer nanoparticles, quantum dots) [24].

5. Future trends in bacteriophage formulation and encapsulation

5.1. Phage encapsulation using novel stimuli responsive materials

There is significant scope for future research on encapsulation of phage in novel stimuli responsive polymers utilising different triggers for phage release including light, temperature, pH, enzymes. A wide range of external stimuli may be used to trigger self-assembly of phage containing micro- and nano-particle structures (e.g. in micelles, nanogels, capsules, vesicles, core-shell particles, hybrid particle-in-particle structures, emulsions and foams) and may induce their reversible or irreversible disintegration, aggregation, swelling. Micro- and nanoparticles with surface grafted ligands may be used for targeted delivery of phage cargo released at the site of infection. Stimuli responsive homo-polymer and mixed-polymer brushes grafted on to microparticles may be used to trigger phage release in response to changes in environmental or interfacial conditions. Phage may be encapsulated in different architectures e.g. thin film structures as multi-layered films, hybrid films combining polymers and nano- or micro-particles.

5.2. Phage encapsulation using membrane emulsification

Advanced techniques may be applied for phage encapsulation to precisely control the size and architecture of phage containing micro- and nano-particles. To-date methods used for phage encapsulation have not utilised state-of-the art processing techniques that could be applied for the encapsulation of phage to achieve controlled dosage forms in uniform micro- and nanoparticles. Membrane emulsification (ME) is one such process that allows formation of uniform drops by injecting a

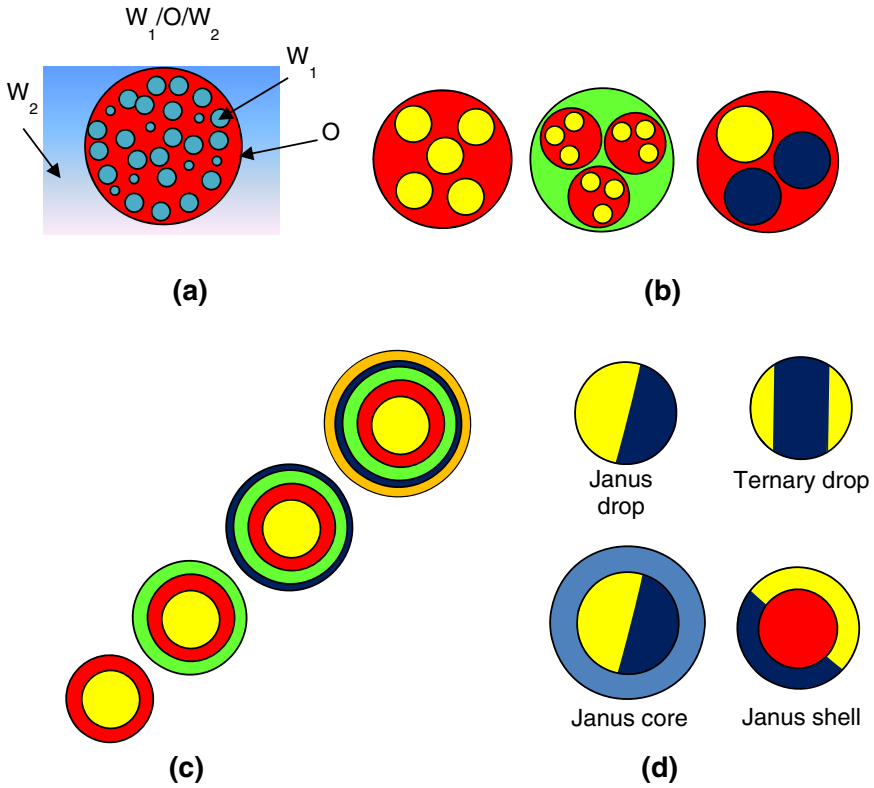


Fig. 4. Morphologies of complex drops: (a) Conventional multiple emulsion drop; (b) multiple emulsion drops generated in microfluidic channels with controlled number of inner drops [246] and distinct inner drops [247]; (c) drops generated in microfluidic channels with multiple concentric shells [249]; (d) Janus and ternary drops generated in microfluidic channels [250,251].

dispersed phase liquid through a microporous membrane into the continuous phase (Fig. 3 a). Alternatively, a pre-emulsified mixture of the dispersed and continuous phase may be repeatedly injected through the membrane (Fig. 3 b). To encapsulate bacteriophage, the formed drops, typically composed of a mixture of the wall forming materials, solvent(s) and phage, could be solidified under mild agitation using various solidification reactions or processes, such as free-radical polymerisation, polycondensation, ionotropic/thermal gelation, cooling crystallization, and molecular or particle self-assembly triggered by solvent evaporation [236]. The advantages of ME over standard emulsification procedures using high-pressure valve homogenisers or rotor-stator devices are in higher drop size uniformity and lower energy inputs and applied shear, which can be useful to preserve phage integrity. It is well known that bacteriophages are sensitive to mechanical shearing [104,128]. In direct ME, the shear rate on the membrane surface is $\sim 10^3$–$10^4\ s^{-1}$ however, uniform drops may be obtained without any shear by spontaneous droplet formation driven by Laplace pressure gradients [237]. The shear rate in high-shear mixers and colloid mills is typically $\sim 10^5\ s^{-1}$ and can exceed $10^7\ s^{-1}$ in microfluidizers. The most common membranes used in ME are Shirasu Porous Glass (SPG) (Fig. 3 c) and microsieve metallic membranes [238]. Shear on the membrane surface required for drop detachment can be generated in various ways including; (i) using a paddle stirrer placed above the membrane surface (Fig. 3 d) [239]; (ii) rotating membrane [240] and (iii) oscillating membrane [241]. In the oscillating ME system, the tubular membrane can oscillate tangentially clockwise or counterclockwise [242] or radially upward and downward (Fig. 3 e) [241], with frequencies ranging from 10 to 90 Hz.

5.3. Phage encapsulation using microfluidic emulsification

Bacteriophage containing microcapsules could be fabricated using microfluidic devices with unprecedented uniformity and control over the size, shape and morphology [243,244]. The process involves creation of drops in a variety of geometries consisting of micrometre length scale channels designed to bring two or more immiscible fluid streams into contact with one another. This is followed by conversion of the generated drops into solid particles (using techniques discussed earlier). The variation of drop sizes in microfluidic devices is negligible (< 3%) and encapsulation efficiency of bacteriophage can reach 100%. The drop productivity can exceed 10,000 drops/s [245], however, the flow rate of the dispersed phase is modest (0.01–10 ml h^{-1}/drop generation unit - DGU). The most common planar DGUs are co-flow drop makers in which immiscible fluids meet in parallel streams, cross-flow drop makers or microfluidic junctions in which the immiscible fluid streams meet at an angle to one another, and flow-focusing drop makers in which there is a geometric element, such as a constriction, that causes the streams to accelerate, narrowing the inner fluid thread and causing it to break into drops [243].

Microfluidic emulsification allows for precise fabrication of structured multiple emulsions with controlled drop morphology. Conventional multiple emulsions are composed of numerous drops of the inner phase dispersed within larger drops of the middle phase, which itself is dispersed in an outer phase (Fig. 4 a). The middle phase must be immiscible with both the inner and outer phase. A $W_1/O/W_2$ emulsion consists of drops of the inner water phase W_1 dispersed within the oil drops, which are then dispersed in the outer water phase W_2. Bacteriophage typically would be suspended in W_1 phase and the oil phase would contain a suspended or dissolved encapsulating shell material such as a polymer. Microfluidic methods can be used to produce multiple emulsion drops consisting of a controlled number of inner drops encapsulated within each large drop (Fig. 4 b) [246]. The number and content of individual inner drops can be controlled thereby allowing the loading of bacteriophage to be precisely manipulated [246,247]. The distinct inner drops can be used to achieve simultaneous encapsulation of two or more types of bacteriophage in separate microenvironments and to control the loading for each of them. Thus phage cocktails may be individually formulated to overcome the limitations of one formulation having to suit all.

Microfluidic methods can be used to prepare multiple emulsion drops with concentric onion-like shells around the core drop (Fig. 4 c). Droplets with different numbers of shells may be synthesised depending

of the number of immiscible phases within each drop e.g. double ($W_1/O/W_2$ or $O_1/W/O_2$), triple ($W_1/O_2/W_3/O_4$ or $O_1/W_2/O_3/W_4$), quadruple ($W_1/O_2/W_3/O_4/W_5$ or $O_1/W_2/O_3/W_4/O_5$), and quintuple ($W_1/O_2/W_3/O_4/W_5/O_6$ or $O_1/W_2/O_3/W_4/O_5/W_6$) [248]. High-order multiple emulsions may be useful in the production of complex capsules for co-encapsulation and simultaneous or sequential release of several multi-component actives (Fig. 4 c) [249]. Multiple shells would allow tailored formulations within a single capsule e.g. an outer acid stable shell may protect phage from the stomach acidity, an inner burst release shell for bolus delivery of phage with an innermost sustained release shell resulting in slow sustained phage release over a significant time period negating the need for repeated phage dosing.

Other approaches might include phage encapsulation in Janus particles composed of two hemispheres with various physical and chemical properties (Fig. 4 d). Janus drops typically have two distinct regions of roughly equal surface area, however, the term has expanded to include all multi-segment droplet structures with regions of different composition co-existing in asymmetric geometries [250]. Janus particles may be used to encapsulate bacteriophage in different polymers having different in vivo degradation rates to achieve burst release followed by controlled sustained release.

6. Conclusions

Implementation of phage therapy necessitates the use of phage cocktails to overcome the limited host range of an individual phage and the risk of phage resistant bacterial mutants. High doses of phage need to be reliably delivered at the site of infection. Repeated frequent dosing of phage may be impractical hence, encapsulation of phage in sustained release systems is needed. Bacteriophage susceptibility to chemical and physical stresses differs among phage families and also within a family. Careful consideration needs to be given to ensure the formulation chemistry and processing conditions are suited to phage making-up the cocktail in order to ensure reproducible retention of high phage titres and stability during long term storage. Further research is urgently needed in the field of phage formulation and encapsulation to adequately support any future phage therapy field.

Acknowledgement

We would like to acknowledge EPSRC support (Grant no. EP/M027341/1) Tackling Antimicrobial Resistance: An Interdisciplinary Approach.

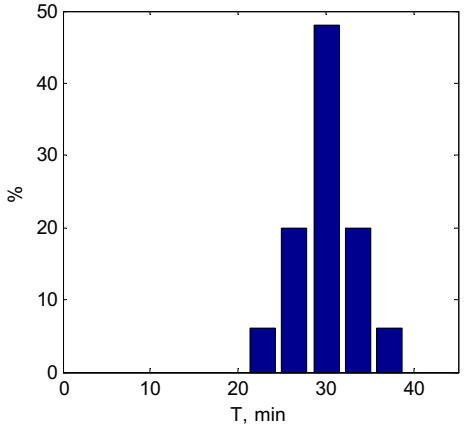
Appendix A. Mathematical model of bacteria/phage interaction

$$\begin{aligned}
\frac{dS}{dt} &= g\cdot S - a\cdot S\cdot(P_1 + P_2)\\
\frac{dR}{dt} &= g\cdot R + m\cdot g\cdot S - a\cdot R\cdot P_2\\
\frac{dP_1}{dt} &= b\cdot a\cdot S_T\cdot P_{1T} - n\cdot a\cdot S\cdot P_1 - d\cdot P_1\\
\frac{dP_2}{dt} &= b\cdot a\cdot (S+R)_T\cdot P_{2T} - n\cdot a\cdot (S+R)\cdot P_2 - d\cdot P_2
\end{aligned} \tag{A1}$$

Here

S, R - concentrations of susceptible and resistant bacteria accordingly [CFU/ml],
P_1 - concentration of the phage affecting susceptible bacteria only [PFU/ml],
P_2 - concentration of the phage affecting both susceptible and resistant bacteria [PFU/ml],
T - latent period [min]; index 'T' indicates the value of the variable T minutes in the past,
g - bacteria growth rate coefficient [min^{-1}],
a - (bacteria/phage) adsorption coefficient [$\text{ml min}^{-1}\ \text{PFU}^{-1}$],
n - average number of phages adsorbed by an individual bacterium,
b - burst size,
m - probability of bacteria mutation resulting in resistance to phage P_1,
d - phage death/removal coefficient [min^{-1}].

For both figures in the paper the following values were used: $g = 0.02\ \text{min}^{-1}$, $n = 1$, $b = 30$, $m = 10^{-5}$, $d = 0.02\ \text{min}^{-1}$.
For the results shown in Fig. 1: $a = 10^{-10}$ ml/min, $T = 40$ min.
For the results shown in Fig. 2: $a = 0.5 * 10^{-10}$ ml/min, $T = 60$ min.
Also, for any given latency period T a discrete distribution in the range $0.76T$–$1.24T$ was used instead of the fixed T. It was T for 48% of infected bacteria, $0.88T$ for 20%, $1.12T$ for 20%, $0.76T$ for 6% and $1.24T$ for 6% - as the figure below illustrates for $T = 30$ min.



References

[1] O'Neill J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Rev Antimicrob Resist 2016:1–84. http://dx.doi.org/10.1016/j.jpha.2015.11.005.

[2] Moellering RC. N Engl J Med 2010;363:2377–9. http://dx.doi.org/10.1056/NEJMp1002530.

[3] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016;16:161–8. http://dx.doi.org/10.1016/S1473-3099(15)00424-7.

[4] Coates AR, Halls G, Hu Y. Novel classes of antibiotics or more of the same? Br J Pharmacol 2011;163:184–94. http://dx.doi.org/10.1111/j.1476-5381.2011.01250.x.

[5] Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, et al. Alternatives to antibiotics — a pipeline portfolio review. Lancet Infect Dis 2016;16:239–51. http://dx.doi.org/10.1016/S1473-3099(15)00466-1.

[6] Wittebole X, De Roock S, Opal SM. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. Virulence 2014;5:226–35. http://dx.doi.org/10.4161/viru.25991.

[7] Young R, Gill JJ. Phage therapy redux — what is to be done? Science 2015;350:1163–4. http://dx.doi.org/10.1126/science.aad8222.

[8] Levin BR, Bull JJ. Population and evolutionary dynamics of phage therapy. Nat Rev Microbiol 2004;2:166–73. http://dx.doi.org/10.1038/nrmicro822.

[9] Cairns BJ, Timms AR, Jansen VAA, Connerton IF, Payne RJH. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog 2009;5:1–10. http://dx.doi.org/10.1371/journal.ppat.1000253.

[10] Bull JJ, Gill JJ. The habits of highly effective phages: population dynamics as a framework for identifying therapeutic phages. Front Microbiol 2014;5:1–12. http://dx.doi.org/10.3389/fmicb.2014.00618.

[11] Hadas H, Einav M, Fishov I, Zaritsky A. bateriophage T4 development depends on the physiology of its host *Escherichia coli*. Microbiology 1997;143:179–85.

[12] Gill JJ, Hyman P. Phage choice, isolation and preparation for phage therapy. Curr Pharm Biotechnol 2010;11:2014.

[13] Weber-Dabrowska B, Jonczyk-Matysiak E, Zaczek M, Lobocka M, Lusiak-Szelachowska M, Gorski A. Bacteriophage procurement for therapeutic purposes. Front Microbiol 2016;7:1–14. http://dx.doi.org/10.3389/fmicb.2016.01177.

[14] Mattila S, Ruotsalainen P, Jalasvuori M. On-demand isolation of bacteriophages against drug-resistant bacteria for personalized phage therapy. Front Microbiol 2015;6:1–7. http://dx.doi.org/10.3389/fmicb.2015.01271.

[15] Sutherland IW, Hughes KA, Skillman LC, Tait K. The interaction of phage and biofilms. FEMS Microbiol Lett 2004;232:1–6. http://dx.doi.org/10.1016/S0378-1097(04)00041-2.

[16] Cornelissen A, Ceyssens PJ, T'Syen J, van Praet H, Noben JP, Shaburova OV, et al. The t7-related pseudomonas putida phage φ15 displays virion-associated biofilm degradation properties. PLoS One 2011;6. http://dx.doi.org/10.1371/journal.pone.0018597.

[17] Pires DP, Oliveira H, Melo LDR, Sillankorva S, Azeredo J. Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. Appl Microbiol Biotechnol 2016;100:2141–51. http://dx.doi.org/10.1007/s00253-015-7247-0.

[18] Gutiérrez D, Vandenheuvel D, Martínez B, Rodríguez A, Lavigne R, García P. Two phages, phiIPLA-RODI and phiIPLA-C1C, lyse mono- and dual-species staphylococcal biofilms. Appl Environ Microbiol 2015;81:3336–48. http://dx.doi.org/10.1128/AEM.03560-14.

[19] Khalifa L, Brosh Y, Gelman D, Coppenhagen-Glazer S, Beyth S, Poradosu-Cohen R, et al. Targeting *Enterococcus faecalis* biofilms with phage therapy. Appl Environ Microbiol 2015;81:2696–705. http://dx.doi.org/10.1128/AEM.00096-15.

[20] Smith HW, Huggins MB, Shaw KM. The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. J Gen Microbiol 1987;133:1111–26. http://dx.doi.org/10.1099/00221287-133-5-1111.

[21] Nakai T, Sugimoto R, Park K-H, Matsuoka S, Mori K, Nishioka T, et al. Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. Dis Aquat Organ 1999;37:33–41.

[22] Smith HW, Huggins MB. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. J Gen Microbiol 1983;129:2659–75. http://dx.doi.org/10.1099/00221287-129-8-2659.

[23] Atterbury RJ, Van Bergen MAP, Ortiz F, Lovell MA, Harris JA, De Boer A, et al. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl Environ Microbiol 2007;73:4543–9. http://dx.doi.org/10.1128/AEM.00049-07.

[24] Peltomaa R, López-Perolio I, Benito-Peña E, Barderas R, Moreno-Bondi MC. Application of bacteriophages in sensor development. Anal Bioanal Chem 2016;408:1805–28. http://dx.doi.org/10.1007/s00216-015-9087-2.

[25] Ulett GC, Totsika M, Schaale K, Carey AJ, Sweet MJ, Schembri MA. Uropathogenic *Escherichia coli* virulence and innate immune responses during urinary tract infection. Curr Opin Microbiol 2013;16:100–7. http://dx.doi.org/10.1016/j.mib.2013.01.005.

[26] Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. J Infect Dis 2010;201:1096–104. http://dx.doi.org/10.1086/651135.

[27] Alemayehu D, Casey PG, Mcauliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophage phiMR299-2 and phiNH-4 can eliminate *Psuedomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway. MBio 2012;3:1–9. http://dx.doi.org/10.1128/mBio.00029-12.Editor.

[28] Morello E, Saussereau E, Maura D, Huerre M, Touqui L, Debarbieux L. Pulmonary bacteriophage therapy on pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention. PLoS One 2011;6. http://dx.doi.org/10.1371/journal.pone.0016963.

[29] Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against *Staphylococcus aureus* in mice. Antimicrob Agents Chemother 2007;51:2765–73. http://dx.doi.org/10.1128/AAC.01513-06.

[30] Smith HW, Huggins MB. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. Microbiology 1982;128:307–18. http://dx.doi.org/10.1099/00221287-128-2-307.

[31] Singla S, Harjai K, Katare OP, Chhibber S. Bacteriophage-loaded nanostructured lipid carrier: improved pharmacokinetics mediates effective resolution of *Klebsiella pneumoniae*-induced lobar pneumonia. J Infect Dis 2015;212:325–34. http://dx.doi.org/10.1093/infdis/jiv029.

[32] Chhibber S, Kaur S, Kumari S. Therapeutic potential of bacteriophage in treating *Klebsiella pneumoniae* B5055-mediated lobar pneumonia in mice. J Med Microbiol 2008;57:1508–13. http://dx.doi.org/10.1099/jmm.0.2008/002873-0.

[33] Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against *Staphylococcus aureus* abscesses in a rabbit model experimental bacteriophage protection against *Staphylococcus aureus* abscesses in a rabbit model. Antimicrob Agents Chemother 2005;49:1220–1. http://dx.doi.org/10.1128/AAC.49.3.1220.

[34] McVay CS, Velásquez M, Fralick JA. Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. Antimicrob Agents Chemother 2007;51:1934–8. http://dx.doi.org/10.1128/AAC.01028-06.

[35] Sarker SA, Sultana S, Reuteler G, Moine D, Descombes P, Charton F, et al. Oral phage therapy of acute bacterial diarrhea with two coliphage preparations: a randomized trial in children from Bangladesh. EBioMedicine 2016;4:124–37. http://dx.doi.org/10.1016/j.ebiom.2015.12.023.

[36] Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15:167–93. http://dx.doi.org/10.1128/CMR.15.2.167.

[37] Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004;2:95–108.

[38] Peters BM, Jabra-Rizk MA, O'May GA, William Costerton J, Shirtliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. Clin Microbiol Rev 2012;25:193–213. http://dx.doi.org/10.1128/CMR.00013-11.

[39] Singla S, Harjai K, Raza K, Wadhwa S, Katare OP, Chhibber S. Phospholipid vesicles encapsulated bacteriophage: a novel approach to enhance phage biodistribution. J Virol Methods 2016;236:68–76. http://dx.doi.org/10.1016/j.jviromet.2016.07.002.

[40] Chan BK, Abedon ST. Bacteriophage and their enzymes in biofilm control. Curr Pharm Des 2015;21:85–99.

[41] Furusawa T, Iwano H, Hiyashimizu Y, Matsubara K, Higuchi H, Nagahata H, et al. Phage Therapy Is Effective in a Mouse Model of Bacterial Equine Keratitis. Appl Environ Microbiol 2016;82:5332–9. http://dx.doi.org/10.1128/aem.01166-16.

[42] Carmody LA, Gill JJ, Summer EJ, Sajjan US, Gonzalez CF, Young RF, et al. Efficacy of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection. J Infect Dis 2010;201:264–71. http://dx.doi.org/10.1086/649227.

[43] Martin RM, Cao J, Brisse S, Passet V, Wu W, Zhao L, et al. Molecular epidemiology of colonizing and infecting isolates of *Klebsiella pneumoniae*. mSphere 2016;1:1–12. http://dx.doi.org/10.1128/mSphere.00261-16.

[44] WHO. Global tuberculosis report. 2016; 2016.

[45] Jassal M, Bishai WR. Extensively drug-resistant tuberculosis. Lancet Infect Dis 2009;9:19–30. http://dx.doi.org/10.1016/S1473-3099(08)70260-3.

[46] Russell DG, Barry CE, Flynn JL. Tuberculosis: what we don't know can, and does, hurt us. Science (80-) 2010;328:852–6.

[47] Nieth A, Verseux C, Barnert S, Süss R, Römer W. A first step toward liposome-mediated intracellular bacteriophage therapy. Expert Opin Drug Deliv 2015:1–14. http://dx.doi.org/10.1517/17425247.2015.1043125.

[48] Nieth A, Verseux C, Römer W. A question of attire: dressing up bacteriophage therapy for the battle against antibiotic-resistant intracellular bacteria. Springer Sci Rev 2015;3:1–11. http://dx.doi.org/10.1007/s40362-014-0027-x.

[49] Guest JF, Ayoub N, Mcilwraith T, Uchegbu I, Gerrish A, Weidlich D, et al. Health economic burden that different wound types impose on the UK's National Health Service. BMJ Open 2015;5:e009283. http://dx.doi.org/10.1111/iwj.12603.

[50] Lindholm C, Searle R. Wound management for the 21st century: combining effectiveness and efficiency. Int Wound J 2016;13:5–15. http://dx.doi.org/10.1111/iwj.12623.

[51] Clinton A, Carter T. Chronic wound biofilms: pathogenesis and potential therapies. Lab Med 2015;46:277–84.

[52] Wiegand C, Abel M, Ruth P, Hipler UC. HaCaT keratinocytes in co-culture with *Staphylococcus aureus* can be protected from bacterial damage by polihexanide. Wound Repair Regen 2009;17:730–8. http://dx.doi.org/10.1111/j.1524-475X.2009.00536.x.

[53] Peterson LR. Review. Squeezing the antibiotic balloon: the impact of antimicrobial classes on emerging resistance. Clin Microbiol Infect Suppl 2005;11:4–16. http://dx.doi.org/10.1111/j.1469-0691.2005.01238.x.

[54] Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJG, Armstrong DG, et al. 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. Clin Infect Dis 2012;54:132–73. http://dx.doi.org/10.1093/cid/cis346.

[55] Nale JY, Shan J, Hickenbotham PT, Fawley WN, Wilcox MH, Clokie MRJ. Diverse temperate bacteriophage carriage in *Clostridium difficile* 027 strains. PLoS One 2012;7:1–9. http://dx.doi.org/10.1371/journal.pone.0037263.

[56] Curtis V, Cairncross S. Effect of washing hands with soap on diarrhoea risk in the community: a systematic review. Lancet Infect Dis 2003;3:275–81.

[57] Abdulamir AS, Sabah J, Bakar Fatimah A. Novel approach of using a cocktail of designed bacteriophages against gut pathogenic *E. coli* for bacterial load biocontrol. Ann Clin Microbiol Antimicrob 2014;13:1–11.

[58] Yen M, Cairns LS, Camilli A, Zuckerman JN, Rombo L, Fisch A, et al. A cocktail of three virulent bacteriophages prevents *Vibrio cholerae* infection in animal models. Nat Commun 2017;8:14187. http://dx.doi.org/10.1038/ncomms14187.

[59] Wall SK, Zhang J, Rostagno MH, Ebner PD. Phage therapy to reduce preprocessing *Salmonella* infections in market-weight swine. Appl Environ Microbiol 2010;76:48–53. http://dx.doi.org/10.1128/AEM.00785-09.

[60] Oliveira M, Viñas I, Colàs P, Anguera M, Usall J, Abadias M. Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. Food Microbiol 2014;38:137–42. http://dx.doi.org/10.1016/j.fm.2013.08.018.

[61] Hua Zou WW. Phage therapy: promising for *H. pylori* infection. Clin Microbiol Open Access 2013;2:10–1. http://dx.doi.org/10.4172/2327-5073.1000112.

[62] Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 2013;382:209–22. http://dx.doi.org/10.1016/S0140-6736(13)60844-2.

[63] Payne RJH, Jansen V. Understanding bacteriophage therapy as a density-dependent kinetic process. J Theor Biol 2001;208:37–48. http://dx.doi.org/10.1006/jtbi.2000.2198.

[64] Tanji Y, Shimada T, Fukudomi H, Miyanaga K, Nakai Y, Unno H. Therapeutic use of phage cocktail for controlling *Escherichia coli* O157:H7 in gastrointestinal tract of mice. J Biosci Bioeng 2005;100:280–7. http://dx.doi.org/10.1263/jbb.100.280.

[65] Leung CY, Weitz JS. Synergistic elimination of bacteria by phage and the immune system. bioRxiv 2016:57927. http://dx.doi.org/10.1101/057927.

[66] Smith HW, Huggins MB. Successful Treatment of Experimental Escherichia coli Infections in Mice Using Phage: its General Superiority over Antibiotics. J Gen Microbiol 1982;128:307–18. http://dx.doi.org/10.1099/00221287-128-2-307.

[67] Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, et al. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect Immun 2002;70:204–10. http://dx.doi.org/10.1128/IAI.70.1.204.

[68] Cerveny KE, DePaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. Infect Immun 2002;70:6251–62. http://dx.doi.org/10.1128/IAI.70.11.6251.

[69] Barrow P, Lovell M. Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. Clin Diagn Lab Immunol 1998;5:294–8.

[70] Watanabe R, Matsumoto T, Sano G, Ishii Y, Tateda K, Sumiyama Y, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by *Pseudomonas aeruginosa* in mice. Antimicrob Agents Chemother 2007;51:446–52. http://dx.doi.org/10.1128/AAC.00635-06.

[71] Matsuzaki S, Yasuda M, Nishikawa H, Kuroda M, Ujihara T, Shuin T, et al. Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage phi MR11. J Infect Dis 2003;187:613–24. http://dx.doi.org/10.1086/374001.

[72] Tsonos J, Vandenheuvel D, Briers Y, De Greve H, Hernalsteens JP, Lavigne R. Hurdles in bacteriophage therapy: Deconstructing the parameters. Vet Microbiol 2014;171:460–9. http://dx.doi.org/10.1016/j.vetmic.2013.11.001.

[73] Wagenaar JA, Bergen MAPV, Mueller MA, Wassenaar TM, Carlton RM. Phage therapy reduces *Campylobacter jejuni* colonization in broilers. Vet Microbiol 2005;109:275–83. http://dx.doi.org/10.1016/j.vetmic.2005.06.002.

[74] Andreatti Filho RL, Higgins JP, Higgins SE, Gaona G, Wolfenden AD, Tellez G, et al. Ability of bacteriophages isolated from different sources to reduce *Salmonella enterica* serovar *enteritidis* in vitro and in vivo. Poult Sci 2007;86:1904–9.

[75] Stanford K, Niu YD, Johnson R. Oral delivery systems for encapsulated bacteriophages targeted at *Escherichia coli* O157:H7 in feedlot cattle. J Food Prot 2010;73:1304–12.

[76] Nale JY, Spencer J, Hargreaves KR, Buckley AM, Trzepin P, Douce GR, et al. Bacteriophage combinations significantly reduce *Clostridium difficile* growth in vitro and proliferation in vivo. Antimicrob Agents Chemother 2016;60:968–81. http://dx.doi.org/10.1128/AAC.01774-15.Address.

[77] Colom J, Cano-Sarabia M, Otero J, Cortés P, Maspoch D, Llagostera M. Liposome-encapsulated bacteriophages for enhanced oral phage therapy against *Salmonella* spp. Appl Environ Microbiol 2015;81:4841–9. http://dx.doi.org/10.1128/AEM.00812-15.

[78] Colom J, Cano-Sarabia M, Otero J, Aríñez-Soriano J, Cortés P, Maspoch D, et al. Microencapsulation with alginate/CaCO3: a strategy for improved phage therapy. Sci Rep 2017;7:41441. http://dx.doi.org/10.1038/srep41441.

[79] Zhang JY, Kraft BL, Pan YY, Wall SK, Saez AC, Ebner PD. Development of an Anti-Salmonella Phage Cocktail with Increased Host Range. Foodborne Pathog Dis 2010;7:1415–9. http://dx.doi.org/10.1089/fpd.2010.0621.

[80] Bardina C, Spricigo DA, Cortés P, Llagostera M. Significance of the bacteriophage treatment schedule in reducing salmonella colonization of poultry. Appl Environ Microbiol 2012;78:6600–7. http://dx.doi.org/10.1128/AEM.01257-12.

[81] Teixeira AP, Oliveira R, Alves PM, Carrondo MJT. Advances in on-line monitoring and control of mammalian cell cultures: supporting the PAT initiative. Biotechnol Adv 2009;27:726–32. http://dx.doi.org/10.1016/j.biotechadv.2009.05.003.

[82] Murthy K, Engelhardt R. US 2009/0130196 A1: bacteriophage composition. 2009;2009.

[83] Chibani-Chennoufi S, Sidoti J, Bruttin A, Kutter E, Sarker S, Bru H. In vitro and in vivo bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy. Antimicrob Agents Chemother 2004;48:2558–69. http://dx.doi.org/10.1128/AAC.48.7.2558.

[84] Delbruck M. The growth of bacteriophage and lysis of the host. J Gen Physiol 1940;23:643–60.

[85] Abedon ST. Lysis from without. Bacteriophage 2011;1:46–9. http://dx.doi.org/10.4161/bact.1.1.13980.

[86] Denou E, Bruttin A, Barretto C, Ngom-Bru C, Brüssow H, Zuber S. T4 phages against *Escherichia coli* diarrhea: potential and problems. Virology 2009;388:21–30. http://dx.doi.org/10.1016/j.virol.2009.03.009.

[87] Schmerer M, Molineux IJ, Bull JJ. Synergy as a rationale for phage therapy using phage cocktails. PeerJ 2014;2:e590. http://dx.doi.org/10.7717/peerj.590.

[88] Vandenheuvel D, Lavigne R, Brussow H. Bacteriophage therapy: advances in formulation strategies and human clinical trials. Annu Rev Virol 2015;2:599–618.

[89] Kucharewicz-Krukowska A, Slopek S. Immunogenic effect of bactriophage in patients subjected to phage therapy. Arch Immunol Ther Exp (Warsz) 1987;35:553–61.

[90] Huff WE, Huff GR, Rath NC, Balog JM, Donoghue AM. Prevention of *Escherichia coli* infection in broiler chickens with a bacteriophage aerosol spray. Poult Sci 2002;81:1486–91. http://dx.doi.org/10.1093/ps/81.10.1486.

[91] Semler DD, Goudie AD, Finlay WH, Dennis JJ. Aerosol phage therapy efficacy in *Burkholderia cepacia* complex respiratory infections. Antimicrob Agents Chemother 2014;58:4005–13. http://dx.doi.org/10.1128/AAC.02388-13.

[92] Oliveira A, Sereno R, Nicolau A, Azeredo J. The influence of the mode of administration in the dissemination of three coliphages in chickens. Poult Sci 2009;88:728–33. http://dx.doi.org/10.3382/ps.2008-00378.

[93] Cano V, March C, Insua JL, Aguiló N, Llobet E, Moranta D, et al. *Klebsiella pneumoniae* survives within macrophages by avoiding delivery to lysosomes. Cell Microbiol 2015;17:1537–60. http://dx.doi.org/10.1111/cmi.12466.

[94] Fumagalli O, Tall BD, Schipper C, Oelschlaeger TA. N-glycosylated proteins are involved in efficient internalization of *Klebsiella pneumoniae* by cultured human epithelial cells. Infect Immun 1997;65:4445–51.

[95] Singla S, Harjai K, Katare OP, Chhibber S. Encapsulation of bacteriophage in liposome accentuates its entry in to macrophage and shields it from neutralizing antibodies. PLoS One 2016;11:e0153777. http://dx.doi.org/10.1371/journal.pone.0153777.

[96] Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Bacteriophage treatment of intransigent diabetic toe ulcers: a case series. J Wound Care 2016;25:S27–33.

[97] Wright A, Hawkins CH, Änggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. Clin Otolaryngol 2009;34:349–57. http://dx.doi.org/10.1111/j.1749-4486.2009.01973.x.

[98] Merabishvili M, Pirnay J, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One 2009;4:1–10.

[99] Rose T, Verbeken G, De Vos D, Merabishvili M, Vaneechoutte M, Jennes S, et al. Experimental phage therapy of burn wound infection: difficult first steps. Int J Burn Trauma 2014;4:66–73.

[100] Puapermpoonsiri U, Spencer J, van der Walle CF. A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. Eur J Pharm Biopharm 2009;72:26–33. http://dx.doi.org/10.1016/j.ejpb.2008.12.001.

[101] Lee SW, Belcher AM. Virus-based fabrication of micro- and nanofibers using electrospinning. Nano Lett 2004;4:387–90. http://dx.doi.org/10.1021/nl034911t.

[102] Briers Y, Miroshnikov K, Chertkov O, Nekrasov A, Mesyanzhinov V, Volckaert G, et al. The structural peptidoglycan hydrolase gp181 of bacteriophage phiKZ. Biochem Biophys Res Commun 2008;374:747–51. http://dx.doi.org/10.1016/j.bbrc.2008.07.102.

[103] Knezevic P, Obreht D, Curcin S, Petrusic M, Aleksic V, Kostanjsek R, et al. Phages of *Pseudomonas aeruginosa*: response to environmental factors and in vitro ability to inhibit bacterial growth and biofilm formation. J Appl Microbiol 2011;111:245–54. http://dx.doi.org/10.1111/j.1365-2672.2011.05043.x.

[104] Leung SSY, Parumasivam T, Gao FG, Carrigy NB, Vehring R, Finlay WH, et al. Production of inhalation phage powders using spray freeze drying and spray drying techniques for treatment of respiratory infections. Pharm Res 2016;33:1486–96. http://dx.doi.org/10.1007/s11095-016-1892-6.

[105] Dini C, de Urraza PJ. Effect of buffer systems and disaccharides concentration on *Podoviridae coliphage* stability during freeze drying and storage. Cryobiology 2013;66:339–42. http://dx.doi.org/10.1016/j.cryobiol.2013.03.007.

[106] Korehei R, Kadla J. Incorporation of T4 bacteriophage in electrospun fibres. J Appl Microbiol 2013;114:1425–34. http://dx.doi.org/10.1111/jam.12158.

[107] Clark WA. Comparison of several methods for preserving bacteriophages. Appl Environ Microbiol 1962;10:466–71.

[108] Davies JD, Kelly MJ. The preservation of bacteriophage H1 of *Corynebacterium ulcerans* U103 by freeze-drying. J Hyg (Lond) 1969;67:573–83. http://dx.doi.org/10.1017/S0022172400042030.

[109] Ackermann H-W, Tremblay D, Moineau S. Long-term bacteriophage preservation. WFCC Newsl 2004;38:35–40.

[110] Wang W. Lyophilization and development of solid protein pharmaceuticals. vol. 203. 2000; 2000. http://dx.doi.org/10.1016/S0378-5173(00)00423-3.

[111] Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev 2006;58:1688–713. http://dx.doi.org/10.1016/j.addr.2006.09.017.

[112] Telko MJ, Hickey AJ. Dry powder inhaler formulation. Respir Care 2005;50:1209–27. http://dx.doi.org/10.2165/00128413-200615470-00016.

[113] Engel HWB, Smith D, Berwald LG. The preservation of mycobacteriophages by means of freeze drying. Am Rev Respir Dis 1974;109:561–6.

[114] Merabishvili M, Vervaet C, Pirnay JP, de Vos D, Verbeken G, Mast J, et al. Stability

of *Staphylococcus aureus* phage ISP after freeze-drying (lyophilization). PLoS One 2013;8:1–7. http://dx.doi.org/10.1371/journal.pone.0068797.
[115] Malenovská H. The influence of stabilizers and rates of freezing on preserving of structurally different animal viruses during lyophilization and subsequent storage. J Appl Microbiol 2014;117:1810–9. http://dx.doi.org/10.1111/jam.12654.
[116] Golshahi L, Lynch KH, Dennis JJ, Finlay WH. In vitro lung delivery of bacteriophages KS4-M and φKZ using dry powder inhalers for treatment of *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* infections in cystic fibrosis. J Appl Microbiol 2010;110:106–17. http://dx.doi.org/10.1111/j.1365-2672.2010.04863.x.
[117] Puapermpoonsiri U, Ford SJ, van der Walle CF. Stabilization of bacteriophage during freeze drying. Int J Pharm 2010;389:168–75. http://dx.doi.org/10.1016/j.ijpharm.2010.01.034.
[118] Dai M, Senecal A, Nugen SR. Electrospun water-soluble polymer nanofibers for the dehydration and storage of sensitive reagents. Nanotechnology 2014;25:225101. http://dx.doi.org/10.1088/0957-4484/25/22/225101.
[119] Cox CS, Harris WJ, Lee J. Viability and electron microscope studies of phages T3 and T7 subjected to freeze-drying, freeze-thawing and aerosolization. J Gen Microbiol 1974;81:207–15.
[120] Leung SSY, Parumasivam T, Gao FG, Carrigy NB, Vehring R, Finlay WH, et al. Effects of storage conditions on the stability of spray dried, inhalable bacteriophage powders. Int J Pharm 2017. http://dx.doi.org/10.1016/j.ijpharm.2017.01.060.
[121] Markoishvili K, Tsitlanadze G, Katsarava R, Morris JG, Sulakvelidze A. A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. Int J Dermatol 2002;41:453–8.
[122] Dini C, Islan GA, de Urraza PJ, Castro GR. Novel biopolymer matrices for microencapsulation of phages: enhanced protection against acidity and protease activity. Macromol Biosci 2012;12:1200–8. http://dx.doi.org/10.1002/mabi.201200109.
[123] Alfadhel M, Puapermpoonsiri U, Ford SJ, McInnes FJ, Van Der Walle CF. Lyophilized inserts for nasal administration harboring bacteriophage selective for *Staphylococcus aureus*: in vitro evaluation. Int J Pharm 2011;416:280–7. http://dx.doi.org/10.1016/j.ijpharm.2011.07.006.
[124] Hoe S, Ivey JW, Boraey MA, Shamsaddini-Shahrbabak A, Javaheri E, Matinkhoo S, et al. Use of a fundamental approach to spray-drying formulation design to facilitate the development of multi-component dry powder aerosols for respiratory drug delivery. Pharm Res 2014;31:449–65. http://dx.doi.org/10.1007/s11095-013-1174-5.
[125] Chew NYK, Chan HK. The role of particle properties in pharmaceutical powder inhalation formulations. J Aerosol Med 2002;15:325–30.
[126] Klingler C, Müller BW, Steckel H. Insulin-micro- and nanoparticles for pulmonary delivery. Int J Pharm 2009;377:173–9. http://dx.doi.org/10.1016/j.ijpharm.2009.05.008.
[127] Weers J. Inhaled antimicrobial therapy - barriers to effective treatment. Adv Drug Deliv Rev 2015;85:24–43. http://dx.doi.org/10.1016/j.addr.2014.08.013.
[128] Vandenheuvel D, Singh A, Vandersteegen K, Klumpp J, Lavigne R, Van Den Mooter G. Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. Eur J Pharm Biopharm 2013;84:578–82. http://dx.doi.org/10.1016/j.ejpb.2012.12.022.
[129] Jończyk E, Kłak M, Międzybrodzki R, Górski A. The influence of external factors on bacteriophages—review. Folia Microbiol (Praha) 2011;56:191–200. http://dx.doi.org/10.1007/s12223-011-0039-8.
[130] Walbeck AK. US20138501453B2: methods for drying bacteriophage and bacteriophage-containing compositions, the resulting dry compositions, and methods of use. 2013; 2013.
[131] Matinkhoo S, Lynch KH, Dennis JJ, Finlay WH, Vehring R. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. J Pharm Sci 2011;100:5197–205.
[132] Vandenheuvel D, Meeus J, Lavigne R, Van Den Mooter G. Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. Int J Pharm 2014;472:202–5. http://dx.doi.org/10.1016/j.ijpharm.2014.06.026.
[133] Crowe LM, Reid DS, Crowe JH. Is trehalose special for preserving dry biomaterials? Biophys J 1996;71:2087–93. http://dx.doi.org/10.1016/S0006-3495(96)79407-9.
[134] Steckel H, Bolzen N. Alternative sugars as potential carriers for dry powder inhalations. Int J Pharm 2004;270:297–306. http://dx.doi.org/10.1016/j.ijpharm.2003.10.039.
[135] Walbeck AK. US 2009/0093041 A1. Methods for drying bacteriophage and bacteriophage containing compositions, the resulting dry compositions, and methods of use. 2009; 2009.
[136] Wong Y-L, Sampson S, Germishuizen WA, Goonesekera S, Caponetti G, Sadoff J, et al. Drying a tuberculosis vaccine without freezing. Proc Natl Acad Sci U S A 2007;104:2591–5. http://dx.doi.org/10.1073/pnas.0611430104.
[137] Sahota JS, Smith CM, Radhakrishnan P, Winstanley C, Goderdzishvili M, Chanishvili N, et al. Bacteriophage delivery by nebulization and efficacy against phenotypically diverse *Pseudomonas aeruginosa* from cystic fibrosis patients. J Aerosol Med Pulm Drug Deliv 2015;28:353–60. http://dx.doi.org/10.1089/jamp.2014.1172.
[138] Golshahi L, Seed KD, Dennis JJ, Finlay WH. Toward modern inhalational bacteriophage therapy: nebulization of bacteriophages of *Burkholderia cepacia* complex. J Aerosol Med Pulm Drug Deliv 2008;21:351–60. http://dx.doi.org/10.1089/jamp.2008.0701.
[139] Prouty CC. Storage of the bacteriophage of the lactic acid streptococci in the desiccated state with observations on longevity. Appl Microbiol 1953;1:250–1.
[140] Ibekwe VC, Fadda HM, McConnell EL, Khela MK, Evans DF, Basit AW. Interplay between intestinal pH, transit time and feed status on the in vivo performance of pH responsive ileo-colonic release systems. Pharm Res 2008;25:1828–35. http://dx.doi.org/10.1007/s11095-008-9580-9.
[141] McConnell EL, Fadda HM, Basit AW. Gut instincts: explorations in intestinal physiology and drug delivery. Int J Pharm 2008;364:213–26. http://dx.doi.org/10.1016/j.ijpharm.2008.05.012.
[142] Esteban PP, Alves DR, Enright MC, Bean JE, Gaudion A, Jenkins ATA, et al. Enhancement of the antimicrobial properties of bacteriophage-K via stabilization using oil-in-water nano-emulsions. Biotechnol Prog 2014;30:932–44. http://dx.doi.org/10.1002/btpr.1898.
[143] Ma Y, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A, et al. Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. Appl Environ Microbiol 2008;74:4799–805. http://dx.doi.org/10.1128/AEM.00246-08.
[144] Gul O, Dervisoglu M. Application of multicriteria decision technique to determine optimum sodium alginate concentration for microencapsulation of Lactobacillus casei Shirota by extrusion and emulsification. J Food Process Eng 2017;40:1–10. http://dx.doi.org/10.1111/jfpe.12481.
[145] Ma Y, Pacan JC, Wang Q, Sabour PM, Huang X, Xu Y. Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing *Staphylococcus aureus* intestinal carriage. Food Hydrocoll 2012;26:434–40. http://dx.doi.org/10.1016/j.foodhyd.2010.11.017.
[146] Lotfipour F, Mirzaeei S, Maghsoodi M. Preparation and characterization of alginate and psyllium beads containing *Lactobacillus acidophilus*. ScientificWorldJournal 2012;2012:680108. http://dx.doi.org/10.1100/2012/680108.
[147] Hathaway H, Alves DR, Bean J, Esteban PP, Ouadi K, Sutton JM, et al. Poly (N-isopropylacrylamide-co-allylamine) (PNIPAM-co-ALA) nanospheres for the thermally triggered release of Bacteriophage K. Eur J Pharm Biopharm 2015;96:437–41. http://dx.doi.org/10.1016/j.ejpb.2015.09.013.
[148] Bean JE, Alves DR, Laabei M, Esteban PP, Thet NT, Enright MC, et al. Triggered release of bacteriophage K from agarose/hyaluronan hydrogel matrixes by *Staphylococcus aureus* virulence factors. Chem Mater 2014;26:7201–8.
[149] Samtlebe M, Ergin F, Wagner N, Neve H, Küçükçetin A, Franz CMAP, et al. Carrier systems for bacteriophages to supplement food systems: encapsulation and controlled release to modulate the human gut microbiota. LWT- Food Sci Technol 2016;68:334–40. http://dx.doi.org/10.1016/j.lwt.2015.12.039.
[150] Nieth A, Verseux C, Barnert S, Süss R, Römer W, Nieth A, et al. A first step toward liposome-mediated intracellular bacteriophage therapy. Expert Opin Drug Deliv 2015;12:1411–24. http://dx.doi.org/10.1517/17425247.2015.1043125.
[151] Kuhn JC, Zussman E. US 2009/0061496A1. Encapsulation of bacteria and viruses in electrospun fibers. 2009; 2009.
[152] Salalha W, Kuhn J, Dror Y, Zussman E. Encapsulation of bacteria and viruses in electrospun nanofibres. Nanotechnology 2006;17:4657–81. http://dx.doi.org/10.1088/0957-4484/17/18/025.
[153] Korehei R, Kadla JF. Encapsulation of T4 bacteriophage in electrospun poly (ethylene oxide)/cellulose diacetate fibers. Carbohydr Polym 2014;100:150–7. http://dx.doi.org/10.1016/j.carbpol.2013.03.079.
[154] Vonasek E, Le P, Nitin N. Encapsulation of bacteriophages in whey protein films for extended storage and release. Food Hydrocoll 2014;37:7–13. http://dx.doi.org/10.1016/j.foodhyd.2013.09.017.
[155] Lone A, Anany H, Hakeem M, Aguis L, Avdjian AC, Bouget M, et al. Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. Int J Food Microbiol 2016;217:49–58. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.10.011.
[156] Kaikabo AA, AbdulKarim SM, Abas F. Evaluation of the efficacy of chitosan nanoparticles loaded FKAZ14 bacteriophage in the biological control of colibacillosis in chickens. Poult Sci 2017;96:295–302. http://dx.doi.org/10.3382/ps/pew255.
[157] Tang Z, Huang X, Baxi S, Chambers JR, Sabour PM, Wang Q. Whey protein improves survival and release characteristics of bacteriophage Felix O1 encapsulated in alginate microspheres. Food Res Int 2013;52:460–6. http://dx.doi.org/10.1016/j.foodres.2012.12.037.
[158] Kim S, Jo A, Ahn J. Application of chitosan–alginate microspheres for the sustained release of bacteriophage in simulated gastrointestinal conditions. Int J Food Sci Technol 2015;50:913–8. http://dx.doi.org/10.1111/ijfs.12736.
[159] Tang Z, Huang X, Sabour PM, Chambers JR, Wang Q. Preparation and characterization of dry powder bacteriophage K for intestinal delivery through oral administration. LWT- Food Sci Technol 2015;60:263–70. http://dx.doi.org/10.1016/j.lwt.2014.08.012.
[160] Katsarava R, Alavidze Z. US6703040B2: polymer blends as boidegradable matrices for preparing biocomposites. 2004; 2004.
[161] Govender M, Choonara YE, Van Vuuren S, Kumar P, Du Toit LC, Pillay VA. A gastro-resistant ovalbumin bi-layered mini-tablet-in-tablet system for the delivery of Lactobacillus acidophilus probiotic to simulated human intestinal and colon conditions. J Pharm Pharmacol 2015;67:939–50. http://dx.doi.org/10.1111/jphp.12389.
[162] Katsarava R, Beridze V, Arabuli N, Kharadze D, Chu CC, Won CY. Amino Acid-Based Bioanalogous Polymers. Synthesis, and Study of Regular Poly(ester amide)s Based on Bis(alpha-amino acid) alpha,omega-Alkylene Diesters, and Aliphatic Dicarboxylic Acids. J Polym Sci: Part A: Polym Chem 1999;37:391–407.
[163] Davis SS, Hardy JG, Fara JW. Transit of pharmaceutical dosage forms through the small intestine. Gut 1986;27:886–92. http://dx.doi.org/10.1136/gut.27.8.886.

[164] Davis SS, Illum L, Hinchcliffe M. Gastrointestinal transit of dosage forms in the pig. J Pharm Pharmacol 2001;53:33–9. http://dx.doi.org/10.1211/0022357011775163.
[165] Uriot O, Galia W, Awussi AA, Perrin C, Denis S, Chalancon S, et al. Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. Food Microbiol 2016;53:18–29. http://dx.doi.org/10.1016/j.fm.2015.05.007.
[166] Philip AK, Philip B. Colon targeted drug delivery systems: a review on primary and novel approaches. Oman Med J 2010;25:79–87. http://dx.doi.org/10.5001/omj.2010.24.
[167] Choonara BF, Choonara YE, Kumar P, Bijukumar D, du Toit LC, Pillay V. A review of advanced oral drug delivery technologies facilitating the protection and absorption of protein and peptide molecules. Biotechnol Adv 2014;32:1269–82. http://dx.doi.org/10.1016/j.biotechadv.2014.07.006.
[168] Amidon S, Brown JE, Dave VS. Colon-targeted oral drug delivery systems: design trends and approaches. AAPS PharmSciTech 2015;16:731–41. http://dx.doi.org/10.1208/s12249-015-0350-9.
[169] Van Citters GW, Lin HC. Ileal brake: neuropeptidergic control of intestinal transit. Curr Gastroenterol Rep 2006;8:367–73. http://dx.doi.org/10.1007/s11894-006-0021-9.
[170] Hua S, Marks E, Schneider JJ, Keely S. Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. Nanomed Nanotechnol Biol Med 2015;11:1117–32. http://dx.doi.org/10.1016/j.nano.2015.02.018.
[171] Brayden DJ, Jepson MA, Baird AW. Intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 2005;10:1145–57. http://dx.doi.org/10.1016/S1359-6446(05)03536-1.
[172] Inman LR, Cantey JR. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. J Clin Invest 1983;71:1–8. http://dx.doi.org/10.1172/JCI110737.
[173] Pappo J, Ermak TH, Steger HJ. Monoclonal antibody-directed targeting of fluorescent polystyrene microspheres to Peyer's patch M cells. Immunology 1991;73:277–80.
[174] Shakweh M, Ponchel G, Fattal E. Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery. Expert Opin Drug Deliv 2005;1:141–63.
[175] George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan - a review. J Control Release 2006;114:1–14. http://dx.doi.org/10.1016/j.jconrel.2006.04.017.
[176] Ensign LM, Cone R, Hanes J. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. Adv Drug Deliv Rev 2012;64:557–70. http://dx.doi.org/10.1016/j.addr.2011.12.009.
[177] Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev 2009;61:158–71. http://dx.doi.org/10.1016/j.addr.2008.11.002.
[178] Jain D, Panda AK, Majumdar DK. Eudragit S100 entrapped insulin microspheres for oral delivery. AAPS PharmSciTech 2005;6:E100–7. http://dx.doi.org/10.1208/pt060116.
[179] Silva CM, Ribeiro AJ, Ferreira D, Veiga F. Insulin encapsulation in reinforced alginate microspheres prepared by internal gelation. Eur J Pharm Sci 2006;29:148–59. http://dx.doi.org/10.1016/j.ejps.2006.06.008.
[180] Kietzmann D, Moulari B, Béduneau A, Pellequer Y, Lamprecht A. Colonic delivery of carboxyfluorescein by pH-sensitive microspheres in experimental colitis. Eur J Pharm Biopharm 2010;76:290–5. http://dx.doi.org/10.1016/j.ejpb.2010.06.013.
[181] Chawla A, Sharma P, Pawar P. Eudragit S-100 coated sodium alginate microspheres of naproxen sodium: formulation, optimization and in vitro evaluation. Acta Pharm 2012;62:529–45. http://dx.doi.org/10.2478/v10007-012-0034-x.
[182] De Arce Velasquez A, Ferreira LM, Stangarlin MFL, Da Silva CDB, Rolim CMB, Cruz L. Novel Pullulan-Eudragit S100 blend microparticles for oral delivery of risedronate: formulation, in vitro evaluation and tableting of blend microparticles. Mater Sci Eng C 2014;38:212–7. http://dx.doi.org/10.1016/j.msec.2014.02.003.
[183] Stuart M a C, WTS Huck, Genzer J, Müller M, Ober C, Stamm M, et al. Emerging applications of stimuli-responsive polymer materials. Nat Mater 2010;9:101–13. http://dx.doi.org/10.1038/nmat2614.
[184] Lautenschläger C, Schmidt C, Fischer D, Stallmach A. Drug delivery strategies in the therapy of inflammatory bowel disease. Adv Drug Deliv Rev 2014;71:58–76. http://dx.doi.org/10.1016/j.addr.2013.10.001.
[185] de Souza DF, Goebel K, Andreazza IF. Development of enteric coated sustained release minitablets containing mesalamine. Braz J Pharm Sci 2013;49:529–36. http://dx.doi.org/10.1590/S1984-82502013000300014.
[186] Sekirov I, Russell S, Antunes L. Gut microbiota in health and disease. Physiol Rev 2010;90:859–904. http://dx.doi.org/10.1152/physrev.00045.2009.
[187] Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-Gut microbiota metabolic interactions. Science 2012;336:1262–8.
[188] Mountzouris KC, Tsirtsikos P, Kalamara E, Nitsch S, Schatzmayr G, Fegeros K. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult Sci 2007;86:309–17.
[189] Hovgaard L, Brondsted H. Current applications of polysaccharides in colon targeting. Crit Rev Ther Drug Carrier Syst 1996;13:185–223.
[190] Vandamme TF, Lenourry A, Charrueau C, Chaumeil JC. The use of polysaccharides to target drugs to the colon. Carbohydr Polym 2002;48:219–31. http://dx.doi.org/10.1016/S0144-8617(01)00263-6.
[191] Lorenzo-Lamosa ML, Remuñán-López C, Vila-Jato JL, Alonso MJ. Design of microencapsulated chitosan microspheres for colonic drug delivery. J Control Release 1998;52:109–18. http://dx.doi.org/10.1016/S0168-3659(97)00203-4.
[192] Calinescu C, Mateescu MA. Carboxymethyl high amylose starch: chitosan self-stabilized matrix for probiotic colon delivery. Eur J Pharm Biopharm 2008;70:582–9. http://dx.doi.org/10.1016/j.ejpb.2008.06.006.
[193] Bernkop-Schnürch A, Humenberger C, Valenta C. Basic studies on bioadhesive delivery systems for peptide and protein drugs. Int J Pharm 1998;165:217–25. http://dx.doi.org/10.1016/S0378-5173(98)00017-9.
[194] Thanou M, Verhoef JC, Junginger HE. Chitosan and its derivatives as intestinal absorption enhancers. Adv Drug Deliv Rev 2001;50:91–101. http://dx.doi.org/10.1016/S0169-409X(01)00180-6.
[195] Van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan and its derivatives in mucosal drug and vaccine delivery. Eur J Pharm Sci 2001;14:201–7. http://dx.doi.org/10.1016/S0928-0987(01)00172-5.
[196] Lameiro MH, Malpique R, Silva AC, Alves PM, Melo E. Encapsulation of adenoviral vectors into chitosan-bile salt microparticles for mucosal vaccination. J Biotechnol 2006;126:152–62. http://dx.doi.org/10.1016/j.jbiotec.2006.04.030.
[197] Takeuchi H, Matsui Y, Sugihara H, Yamamoto H, Kawashima Y. Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs. Int J Pharm 2005;303:160–70. http://dx.doi.org/10.1016/j.ijpharm.2005.06.028.
[198] Thirawong N, Thongborisute J, Takeuchi H, Sriamornsak P. Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin-liposome nanocomplexes. J Control Release 2008;125:236–45. http://dx.doi.org/10.1016/j.jconrel.2007.10.023.
[199] Shi L, Li Z, Li D, Xu M, Chen H, Zhang Z, et al. Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. J Food Eng 2013;117:99–104. http://dx.doi.org/10.1016/j.jfoodeng.2013.02.012.
[200] Matsubara T, Emoto W, Kawashiro K. A simple two-transition model for loss of infectivity of phages on exposure to organic solvent. Biomol Eng 2007;24:269–71. http://dx.doi.org/10.1016/j.bioeng.2007.02.002.
[201] Olofsson L, Ankarloo J, Andersson PO, Nicholls IA. Filamentous bacteriophage stability in non-aqueous media. Chem Biol 2001;8:661–71. http://dx.doi.org/10.1016/S1074-5521(01)00041-2.
[202] Waddell TE, Johnson R, Mazzocco A. US7601347 B2: methods and compositions for controlled release of bioactive compounds. 2009; 2009.
[203] Milo S, Thet NT, Liu D, Nzakizwanayo J, Jones BV, Jenkins ATA. An in-situ infection detection sensor coating for urinary catheters. Biosens Bioelectron 2016;81:166–72. http://dx.doi.org/10.1016/j.bios.2016.02.059.
[204] Weinberger A, Tsai FC, Koenderink GH, Schmidt TF, Itri R, Meier W, et al. Gel-assisted formation of giant unilamellar vesicles. Biophys J 2013;105:154–64. http://dx.doi.org/10.1016/j.bpj.2013.05.024.
[205] Pautot S, Frisken BJ, Weitz DA. Engineering asymmetric vesicles. Proc Natl Acad Sci 2003;100:10718–21. http://dx.doi.org/10.1073/pnas.1931005100.
[206] Balcao VM, Glasser CA, Chaud MV, del Fiol FS, Tubino M, Vila MMDC. Biomimetic aqueous-core lipid nanoballoons integrating a multiple emulsion formulation: a suitable housing system for viable lytic bacteriophages. Colloids Surf B Biointerfaces 2014;123:478–85. http://dx.doi.org/10.1016/j.colsurfb.2014.09.045.
[207] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4:145–60. http://dx.doi.org/10.1038/nrd1632.
[208] Xiao B, Laroui H, Ayyadurai S, Viennois E, Charania MA, Zhang Y, et al. Mannosylated bioreducible nanoparticle-mediated macrophage-specific TNF-alpha RNA interference for IBD therapy. Biomaterials 2013;34:7471–82. http://dx.doi.org/10.1016/j.biomaterials.2013.06.008.
[209] Pichai MVA, Ferguson LR. Potential prospects of nanomedicine for targeted therapeutics in inflammatory bowel diseases. World J Gastroenterol 2012;18:2895–901. http://dx.doi.org/10.3748/wjg.v18.i23.2895.
[210] Schmidt C, Lautenschlaeger C, Collnot EM, Schumann M, Bojarski C, Schulzke JD, et al. Nano- and microscaled particles for drug targeting to inflamed intestinal mucosa - a first in vivo study in human patients. J Control Release 2013;165:139–45. http://dx.doi.org/10.1016/j.jconrel.2012.10.019.
[211] Coco R, Plapied L, Pourcelle V, Jérôme C, Brayden DJ, Schneider YJ, et al. Drug delivery to inflamed colon by nanoparticles: comparison of different strategies. Int J Pharm 2013;440:3–12. http://dx.doi.org/10.1016/j.ijpharm.2012.07.017.
[212] Cu Y, Saltzman WM. Stealth particles give mucus the slip. Nat Mater 2009;8:11–3. http://dx.doi.org/10.1016/j.immuni.2010.12.017.Two-stage.
[213] Cu Y, Saltzman WM. Controlled surface modification with poly(ethylene)glycol enhances diffusion of PLGA nanoparticles in human cervical mucus. Mol Pharm 2008;6:173–81. http://dx.doi.org/10.1021/mp8001254.
[214] Saltzman WM, Radomsky ML, Whaley KJ, Cone RA. Antibody diffusion in human cervical mucus. Biophys J 1994;66:508–15. http://dx.doi.org/10.1016/S0006-3495(94)80802-1.
[215] Agrawal AK, Gupta CM. Tuftsin-bearing liposomes in treatment of macrophage-based infections. Adv Drug Deliv Rev 2000;41:135–46. http://dx.doi.org/10.1016/S0169-409X(99)00061-7.
[216] Broxmeyer L, Sosnowska D, Miltner E, Chacón O, Wagner D, McGarvey J, et al. Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent *Mycobacterium*: a model for phage therapy of intracellular bacterial pathogens. J Infect Dis 2002;186:1155–60. http://dx.doi.org/10.1086/343812.
[217] Danelishvili L, Young LS, Bermudez LE. In vivo efficacy of phage therapy for *Mycobacterium avium* infection as delivered by a nonvirulent *Mycobacterium*. Microb Drug Resist 2006;12:1–6.
[218] Owais M, Gupta CM. Targeted drug delivery to macrophages in parasitic infections. Curr Drug Deliv 2005;2:311–8.

[219] Orozco LC, Quintana FO, Beltran RM, Moreno ID, Wasserman M, Rodriguez G. The use of rifampicin and isoniazid entrapped in liposomes for the treatment of murine tuberculosis. Tubercle 1986;67:91–7.
[220] Roesken F, Uhl E, Curri SB, Menger MD, Messmer K. Acceleration of wound healing by topical drug delivery via liposomes. Langenbecks Arch Surg 2000;385:42–9. http://dx.doi.org/10.1007/s004230050010.
[221] Ferreira H, Matamá T, Silva R, Silva C, Gomes AC, Cavaco-Paulo A. Functionalization of gauzes with liposomes entrapping an anti-inflammatory drug: a strategy to improve wound healing. React Funct Polym 2013;73:1328–34. http://dx.doi.org/10.1016/j.reactfunctpolym.2013.05.012.
[222] Yerushalmi N, Arad A, Margalit R. Molecular and cellular studies of hyaluronic acid-modified liposomes as bioadhesive carriers for topical drug delivery in wound healing. Arch Biochem Biophys 1994;313:267–73. http://dx.doi.org/10.1006/abbi.1994.1387.
[223] Pearson HA, Sahukhal GS, Elasri MO, Urban MW. Phage-bacterium war on polymeric surfaces: can surface-anchored bacteriophages eliminate microbial infections? Biomacromolecules 2013;14:1257–61.
[224] Bennett AR, Davids FG, Vlahodimou S, Banks JG, Betts RP. The use of bacteriophage-based systems for the separation and concentration of *Salmonella*. J Appl Microbiol 1997;83:259–65.
[225] Sun W, Brovko L, Griffiths M. Food-borne pathogens. Use of bioluminescent *Salmonella* for assessing the efficiency of constructed phage-based biosorbent y,z. Int J Food Microbiol 2001:273–5.
[226] Tolba M, Minikh O, Brovko LY, Evoy S, Griffiths MW. Oriented immobilization of bacteriophages for biosensor applications. Appl Environ Microbiol 2010;76:528–35. http://dx.doi.org/10.1128/AEM.02294-09.
[227] Anany H, Chen W, Pelton R, Griffiths MW. Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat by using phages immobilized on modified cellulose membranes. Appl Environ Microbiol 2011;77:6379–87. http://dx.doi.org/10.1128/AEM.05493-11.
[228] Donlan RM, Lehman SM, Garcia AJ. US9457132B2: controlled covalent attachment of bioactive bacteriophage for regulating biofilm development. 2016; 2016.
[229] Scott H, Mattey M. WO2003093462A2: immobilisation and stabilisation of virus. 2003; 2003.
[230] Cooper IR, Illsley M, Korobeinyk AV, Whitby RLD. Bacteriophage-nanocomposites: an easy and reproducible method for the construction, handling, storage and transport of conjugates for deployment of bacteriophages active against *Pseudomonas aeruginosa*. J Microbiol Methods 2015;111:111–8. http://dx.doi.org/10.1016/j.mimet.2015.02.005.
[231] Handa H, Gurczynski S, Jackson MP, Auner G, Walker J, Mao G. Recognition of *Salmonella typhimurium* by immobilized phage P22 monolayers. Surf Sci 2008;602:1392–400. http://dx.doi.org/10.1016/j.susc.2008.01.036.
[232] Singh A, Glass N, Tolba M, Brovko L, Griffiths M, Evoy S. Immobilization of bacteriophages on gold surfaces for the specific capture of pathogens. Biosens Bioelectron 2009;24:3645–51. http://dx.doi.org/10.1016/j.bios.2009.05.028.
[233] Yang LC, Tam PY, Murray BJ, Mcintire TM, Overstreet CM, Weiss GA, et al. Virus electrodes for universal biodetection. Anal Chem 2006;78:3265–70.
[234] Richter Å, Matuła K, Lesniewski A, Kwasnicka K, Łos J, Łos M, et al. Ordering of bacteriophages in the electric field: application for bacteria detection. Sens Actuators B 2016;224:233–40. http://dx.doi.org/10.1002/9780470015902.a0024053.
[235] Tawil N, Sacher E, Rioux D, Mandeville R, Meunier M. Surface chemistry of bacteriophage and laser ablated nanoparticle complexes for pathogen detection. J Phys Chem C 2015;119:14375–82. http://dx.doi.org/10.1021/acs.jpcc.5b02169.
[236] Vladisavljevic GT, Williams RA. Recent developments in manufacturing emulsions and particulate products using membranes. Adv Colloid Interface Sci 2005;113:1–20. http://dx.doi.org/10.1016/j.cis.2004.10.002.
[237] Kukizaki M. Shirasu porous glass (SPG) membrane emulsification in the absence of shear flow at the membrane surface: influence of surfactant type and concentration, viscosities of dispersed and continuous phases, and transmembrane pressure. J Membr Sci 2009;327:234–43. http://dx.doi.org/10.1016/j.memsci.2008.11.026.
[238] Vladisavljević GT, Kobayashi I, Nakajima M. Production of uniform droplets using membrane, microchannel and microfluidic emulsification devices. Microfluid Nanofluid 2012;13:151–78. http://dx.doi.org/10.1007/s10404-012-0948-0.
[239] Kosvintsev SR, Gasparini G, Holdich RG, Cumming IW, Stillwell MT. Liquid-liquid membrane dispersion in a stirred cell with and without controlled shear. Ind Eng Chem Res 2005;44:9323–30.
[240] Vladisavljević GT, Williams RA. Manufacture of large uniform droplets using rotating membrane emulsification. J Colloid Interface Sci 2006;299:396–402. http://dx.doi.org/10.1016/j.jcis.2006.01.061.
[241] Holdich RG, Dragosavac MM, Vladisavljević GT, Kosvintsev SR. Membrane emulsification with oscillating and stationary membranes. Ind. Eng. Chem. Res. 2010;49:3810–7. http://dx.doi.org/10.1021/ie900531n.
[242] Silva PS, Dragosavac MM, Vladisavljevic GT, Bandulasena HCH, Holdich RG, Stillwell M, et al. Azimuthally oscillating membrane emulsification for controlled droplet production. AIChE J 2015;61:3607–15. http://dx.doi.org/10.1002/aic.
[243] Anna SL. Droplets and bubbles in microfluidic devices. Annu Rev Fluid Mech 2016;48:285–309. http://dx.doi.org/10.1146/annurev-fluid-122414-034425.
[244] Engl W, Backov R, Panizza P. Controlled production of emulsions and particles by milli- and microfluidic techniques. Curr Opin Colloid Interface Sci 2008;13:206–16. http://dx.doi.org/10.1016/j.cocis.2007.09.003.
[245] Yobas L, Martens S, Ong W-L, Ranganathan N. High-performance flow-focusing geometry for spontaneous generation of monodispersed droplets. Lab Chip 2006;6:1073–9. http://dx.doi.org/10.1039/b602240e.
[246] Chu LY, Utada AS, Shah RK, Kim JW, Weitz DA. Controllable monodisperse multiple emulsions. Angew Chem Int Ed 2007;46:8970–4. http://dx.doi.org/10.1002/anie.200701358.
[247] Wang W, Xie R, Ju X-J, Luo T, Liu L, Weitz DA, et al. Controllable microfluidic production of multicomponent multiple emulsions. Lab Chip 2011;11:1587–92. http://dx.doi.org/10.1039/c1lc20065h.
[248] Abate AR, Weitz DA. High-order multiple emulsions formed in poly(dimethylsiloxane) microfluidics. Small 2009;5:2030–2. http://dx.doi.org/10.1002/smll.200900569.
[249] Kim SH, Weitz DA. One-step emulsification of multiple concentric shells with capillary microfluidic devices. Angew Chem Int Ed 2011;50:8731–4. http://dx.doi.org/10.1002/anie.201102946.
[250] Yang S, Guo F, Kiraly B, Mao X, Lu M, Leong KW, et al. Microfluidic synthesis of multifunctional Janus particles for biomedical applications. Lab Chip 2012;12:2097. http://dx.doi.org/10.1039/c2lc90046g.
[251] Nie Z, Li W, Seo M, Xu S, Kumacheva E. Janus and ternary particles generated by microfluidic synthesis: design, synthesis, and self-assembly. J Am Chem Soc 2006;128:9408–12. http://dx.doi.org/10.1021/ja060882n.