Development of a scalable process for the purification of bacteriophages

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Abstract

The recent development of antibiotic resistant bacterial strains has led to a need of devising new therapies for severe infections. The use of bacteriophages has resurfaced in recent years as a potential alternative. As phages are produced in bacterial cultures, purification of the lysate is an important step, in order to remove bacterial contaminants such as proteins, DNA and endotoxins. Most traditional purification methods are optimized for use in scientific research and it is necessary to develop new methods for phage purification suitable to large-scale production, using, for example chromatography.

In this work, the purification of a phage solution from bacterial contaminants was studied and processes were identified for further research. Phage stocks were amplified and clarified through centrifugation or microfiltration, before concentration and diafiltration. The phage stocks were then purified though different chromatographic modes including anion-exchange, multimodal, size-exclusion and a combined anion-exchange/multimodal strategy. After each chromatographic trial, the peaks were assayed for their phage, protein and DNA content. During anion-exchange optimization, an optimized stepwise gradient mode allowed the recovery of most injected phages, although the elution profile of the phages appeared to have changed, possibly due to aggregation during storage. When a multimodal column was used, satisfactory recoveries were obtained, and total recovery was attained in multimodal chromatography following anion-exchange, with removal of bacterial proteins and DNA. A combined anion-exchange-multimodal chromatography purification process allows good recoveries, and the elimination of some key impurities.

Keywords

Bacteriophage; Phage therapy; Downstream processing; Purification; Chromatography.

Resumo

O desenvolvimento recente de estirpes bacterianas resistentes a antibióticos levou à necessidade de criar terapias contra infeções severas. O uso de bacteriófagos reemergiu nos últimos anos como uma possível alternativa. Como os fagos são produzidos em culturas bacterianas, a purificação do lisado é um passo importante, de modo a remover contaminantes bacterianos, como proteínas, DNA e endotoxinas. Os métodos de purificação tradicionais estão otimizados para a investigação e é necessário desenvolver novos métodos para a purificação de fagos em larga escala, usando, por exemplo, cromatografia.

Neste trabalho, a purificação de uma solução de fagos de contaminantes bacterianos foi estudada, e processos foram identificados para investigação futura. *Stocks* de fagos foram amplificados e clarificados através de centrifugação ou microfiltração, antes de ser concentrada e diafiltrada. Os *stocks* foram depois purificados através de diversas abordagens cromatográficas, incluindo troca aniónica, multimodal, exclusão de tamanho e uma estratégia combinada de troca aniónica-multimodal. Após cada ensaio cromatográfico, analisou-se o conteúdo em fagos, proteínas e DNA dos picos. Durante otimização da troca aniónica, o perfil de eluição dos fagos aparenta ter mudado, possivelmente devido a agregação. Contudo, um método otimizado permitiu a recuperação da maior parte dos fagos injetados. Quando uma coluna multimodal foi utilizada, recuperações satisfatórias foram alcançadas, e recuperações totais foram obtidas em cromatografia multimodal após troca aniónica, com remoção de proteínas e DNA bacteriano. O processo combinado permite uma recuperação aceitável e simultaneamente a remoção de impurezas chaves, sendo de interesse para estudos futuros.

Keywords

Bacteriófagos; Terapia fágica; Purificação; Processamento a jusante; Cromatografia.

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List of Abbreviations

EU- Endotoxin Unit

PEG- Polyethylene glycol

CIM- Convective interaction media

QA- Quaternary amine

DEAE- Diethylamine

AEC- Anion-exchange chromatography

MMC- Multimodal chromatography

SEC- Size-exclusion chromatography

DNA- Deoxyribonucleic acid

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

PCR- Polymerase chain reaction

qPCR- Quantitative polymerase chain reaction

RNA- Ribonucleic acid

dsDNA- Double stranded deoxyribonucleic acid

MRSA- Meticillin-resistant *Staphylococcus aureus*

LPS- Lipopolysaccharides

TNF-α- Tumor necrosis factor alpha

IL- Interleukin

NK cell- Natural killer cell

IFN-α- Interferon alpha

ATPS- Aqueous two-phase systems

IEC- Ion-exchange chromatography

pfu- Plaque-forming units

1- Introduction

1.1- Motivation

Ever since World War II, the use of antibiotics has led to the decline of deaths resultant from bacterial infections, being considered a great advance in medical history[1]. However, due to the overuse of antibiotics, resistance to these compounds has become widespread among many different pathogenic strains, leading to a challenging problem in medicine[2]. Due to the development of these strains, there has been a focus in discovering and developing novel strategies for the treatment of bacterial infections. Among the many methods currently being investigated[3], bacteriophage therapy is a potential viable alternative to antibiotic treatment[4].

Discovered in 1915, bacteriophages or phages are viruses that infect and destroy bacteria. Since their discovery, phage therapy has been proposed and attempted. Despite early successes, the lack of knowledge on viral and general phage biology at the time, and the eventual discovery and development of antibiotics, led to the decline of phage therapy investigation in the Western countries[4]. Even though the Western World abandon phage therapy, in the Eastern Bloc research continued until today, leading to an increased knowledge regarding this treatment. With this knowledge, and in order to find substitutes for antibiotic treatment there has been a surge in interest towards phage therapy

Phage therapy presents many advantages when compared to traditional antibiotic treatment. The mode of lysis of phages is different from any antibiotic allowing them to bypass antibiotic resistance, the stricter spectrum of action prevents collateral damage on the gut microbiota, and phage therapy has shown better efficacy compared to antibiotic treatment[4]. However, phage treatment also shows drawbacks in comparison to antibiotics. Phage choice is important, considering the two possible life cycles - lytic or temperate - and the narrower range of action, and it is necessary to precisely identify the bacterial agent causing the disease, potentially delaying treatment. It should also be taken into consideration that phage production requires the use of bacterial strains similar to the ones being treated[4]. After fermentation the culture broth containing the phages also contains several bacterial components and cellular debris that need to be removed[5]. Of note is lipopolysaccharide, also designated as endotoxin, which is a molecule found on the outer membrane of gram-negative bacteria, and can induce adverse effects such as endotoxin shock, tissue damage or even death, when administered to mammals. As such there is a maximum level of 5 endotoxins units (EU) per kg of body mass per hour allowed for pharmaceutical products[6]. Other key impurities include genomic DNA and bacterial proteins. Purification of phage preparations is thus a critical step in its production.

Several methods have been used in research for phage purification, including centrifugation in CsCl density gradients, ultracentrifugation, ultrafiltration and precipitation with polyethylene glycol (PEG). However most of these methods are not suitable for industrial-scale production, mostly by the difficulty in scaling-up, and, in addition, they were not optimized for endotoxin removal[7]. As such, there is a growing need of developing novel strategies for phage purification.

Among the methods currently in study, chromatography has been the most preferred, as it is an easily scalable process for use in industrial context, is not time-consuming[8] and has already been shown to reach high yields while being fast, consistent and automatable[9]. Among the different chromatographic method, ion-exchange is the one most commonly employed mode[9], with traditional packed-bed[10], expanded-bed[11] and monolithic media[12–14] having been used for phage purification Methods such as size-exclusion[15,16] and affinity chromatography[8,17] have also been used. However, for all methods the results can vary, both in endotoxin removal and phage recuperation, across different phages. Phage purification strategies might be needed to be developed individually for each phage.

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1.1- Aim of Study

Current phage purification methods for research are not be suitable for large-scale production of highly pure phage preparations. Since modern-day phage therapy is still a relatively new concept, the development of novel strategies for phage purifications is required. Among the different methods being studied, chromatography is a promising candidate, due to its easy scalability, versatility and effectiveness. The main goal of this work is to develop a scalable purification process for phages suitable for phage therapy.

To this end, phage stocks were prepared by inoculating a *Pseudomonas aeruginosa* culture with a phage stock solution supplied by the Portuguese biopharmaceutic company TechnoPhage (henceforth designated as TP-PA4). The resulting *P. aeruginosa* lysates were afterwards processed either by centrifugation or microfiltration for broth clarification, and further concentrated and diafiltrated by ultrafiltration for buffer exchange and removal of small molecular weight impurities. The resulting diafiltered stocks were then purified through different chromatographic methods such as anion-exchange, multimodal and size exclusion, and the resulting samples were analysed for their phage titre, as well as their content in bacterial proteins, DNA and endotoxins.

The phages were first purified through anion-exchange chromatography (AEC). The process was optimized in order to determine the preferable elution conditions for the phage, using linear and stepwise gradients for elution, and to find the ideal sample load. Afterwards, the purification of phages was tested using multimodal chromatography (MMC), as well as using a combined AEC/multimodal strategy. Finally, the use of size-exclusion chromatography (SEC) was attempted. For confirmation a stock containing another phage (TP-PA3) was also prepared and processed through AEC and multimodal chromatography.

In each chromatographic separation the peaks obtained in the chromatogram were kept and pooled in order to analyse their phage titre through a double-layer plaque assay, their protein content through SDS-PAGE and Bradford assay and their bacterial genomic DNA content, through PCR and qPCR.

2- Literature Review

2.1- Bacteriophages

Bacteriophages, or phages, are viruses that have the ability to invade and destroy bacteria. These viruses are among the most common biological entities known and were described independently, in 1915, by Frederik Twort and, in 1917, by Félix d'Herelle, who characterized bacteriophages as bacteria pathogens.

Like most viruses, bacteriophages contain a protein coat, the capsid that encapsulates the DNA or RNA genome, which might be composed from one or more nucleic acid molecules. Their size ranges from 20 to 825 nm in length. Genome size might vary from a few thousand base-pairs to 480 000 base pairs in phage G[7]. As of 2011, around 4500 viruses (96% of all bacteriophages) belong to the *Caudovirales* order[18]. This tailed-phage order is further divided into 3 families: the *myoviridae*, characterized by phages containing a long contractible tails, and which accounts for 24% of the order; the *siphoviridae*, with long non-contractible tails, which make up 62% of the order; and the *podoviridae*, composed of viruses with short non-contractible tails and accounting for 14% of *Caudovirales.*

The *Caudovirales* virion is composed of an icosahedral protein shell, containing a single linear dsDNA molecule, and a protein tail which recognizes and binds to bacterial surface receptors, and through which the DNA travels when the phage infects a bacterial cell[18] (Figure 1[19]). Most phages are capable of only infecting a narrow range of closely related bacteria, showing a high degree of specificity, while some are able to infect a wide range of bacterial cells[20].

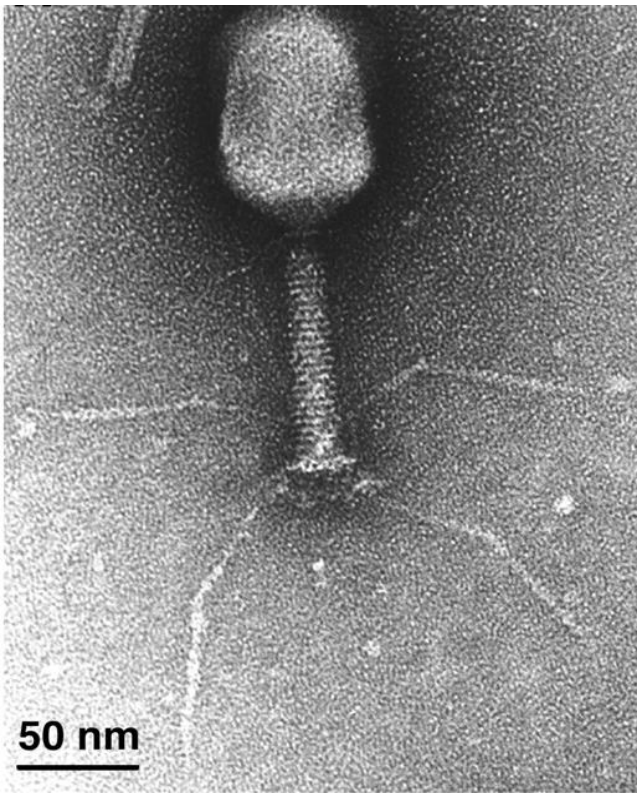

Figure 1- Electron micrographs of bacteriophage T4.

2.1.1- Bacteriophage live cycle

Bacteriophages can be divided into two different groups, according to the life cycle they display. Virulent phages can only display a lytic life cycle, in which self-propagation causes lysis of the bacterial cell. After adsorption of the phage to the bacterial cell's surface, and injection of the genome onto the host, expression of early viral genes will start taking over the host metabolism. Expression of late viral genes will then lead to the replication of the viral genome, and synthesis of capsid proteins, which will be later assembled into new viral particles. After the production of a high number of viral particles, the bacterial cell is lysed, leading to the release of the newly-produced virions[5,7].

Phages can be considered temperate if they display two different life cycles. After infection, they can display either a lytic life cycle, leading to production of a high quantity of viral particles, lysis of the bacterial cell, and release of the virions, or a lysogenic life cycle. When undergoing a lysogenic life cycle the viral DNA integrates into the bacterial genome and supresses expression of most of its genes. The viral genome under this dormant state is designated as a prophage, and replicates together with the bacterial DNA as the cell goes through division, remaining in this state and being maintained in the hosts progeny for many generations[5].

While integrated a prophage confers resistance to the bacteria against infections by phages genetically related to the prophage. Prophages can later switch to a lytic life cycle, inducing the excision of the viral DNA from the hosts genome and the production of virions, under stressful conditions[21]. If a prophage is excised incorrectly from the hosts genome, nearby fragments of bacterial DNA can be removed together with the viral DNA and be packaged into the capsid. These fragments of DNA, which can include bacterial genes, can then be transferred to another host, in a process denominated transduction[22].

2.2- Antibiotic resistance and phage therapy

In previous decades, research on bacteriophages was conducted with a focus on molecular biology. Phages were used as model organism in the mid-twentieth century and were involved in major breakthroughs in viral biology and in molecular biology in general[23]. In the following years, phages became research tools[24]. Nowadays, there is a renewed interest by the modern biotechnological industry in several potential applications of phages, including their use in phage display techniques, in which a desired protein or peptide is genetically engineered into the viral genome for display on the phage surface, in phage-delivered vaccines and, of note, in phage therapy (the use of lytic phages to kill specific bacterial pathogens in cases of infection)[24].

2.2.1- The problem of antibiotic resistance

Since the adoption of antibiotics in the mid-twentieth century, their use allowed a considerable reduction of morbidity and mortality associated with bacterial infections[2]. However, the overuse of antibiotics in clinical, agricultural and animal settings has led to the development of antibiotic-resistant bacterial strains[25] . Some of these strains have become resistant to multiple classes of antibiotics, such as the meticillin-resistant *Staphylococcus aureus* or MRSA, vancomycin-resistant enterococci, and

opportunistic pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Burkholderia cepacia*. These pathogens, often called superbugs, have become one of biggest challenges faced today by medicine[2]. Antibiotic-resistant *P.aeruginosa* in particular is a major opportunistic pathogen of immunocompromised patients, being a leading cause of chronic pulmonary infections and mortality in cystic fibrosis patients, pneumonia, and sepsis in burn patients[26].

While many antibiotics classes, still in use today, were quickly discovered in the decades following the introduction of penicillin, nowadays, few novel antibiotics are being produced[2,27]. Nevertheless, any new antibiotic class created today runs the risk of being rendered ineffective in a few decades due to the development of resistant strains.

As such, due to the prevalence and increase in antibiotic resistance in pathogenic strains not only is it necessary to discover and synthetize new antibiotics but it is also important to develop and implement novel therapies for the treatment of bacterial infections, as a complement and even as a replacement to antibiotic therapy. Several approaches have been identified as potential alternatives for antibiotics, such as antibodies, probiotics, lysins and the previously mentioned phage therapy[3] .

2.2.2- The history of phage therapy

After their discovery in the early 20th century, the use of phages has been proposed as a therapy to treat acute and chronic bacterial infections, considering their specificity towards bacterial cells and inability to attack human cells and cells from other organisms[4].

Félix d'Herelle, who first discovered bacteriophages during a severe outbreak of haemorrhagic dysentery among French soldiers in 1915, used these viruses, in 1919, to treat dysentery patients[28] in what was probably the first therapeutic application of phages. Phage therapy would afterwards be used in trials for the treatment of staphylococcal skin disease[28], bubonic plague[29] and cholera[30].

Despite these early successes, due to a lack of proper controls and inconsistent results, the use of phages as a therapy was controversial. Due to a lack of knowledge regarding the biological nature of bacteriophages, there was a decrease in phage therapy popularity. With the advent of World War II and the discovery of penicillin, phage therapy was forgotten in the Western world[30]. However, the therapeutic application of phages continued on the Soviet Union, with many studies being performed in the USSR[28,31]. Many of these studies were carried out in the Eliava Institute and in the Hirszfeld Institute, in present-day Georgia and Poland respectively, which are among the most important institutes dedicated towards phage therapy today[32].

Nowadays due to the previously mentioned problems of increasing antibiotic resistance and decrease in the rate of discovery of new antibiotics, alternative treatments are required. Phage therapy has been regarded as a complement or replacement to traditional antibiotics, reviving interest in western countries towards this area[33].

With the continued research into phage therapy in the Soviet Union, today most of the knowledge regarding the therapeutic application of phages comes from ex-USSR countries, particularly

Georgia and Poland. In both countries, phage therapy is a component of standard medical practice. In Georgia wide-range phage cocktails are used in many hospitals and clinics, while in Poland individual phage preparations have been used by physicians when antibiotics fail[32,34].

In Western countries, despite the abandonment of phage research after the 40s, in the 80s, Smith *et al.* undertook several experiments in animals[35], showing the potential of phage therapy in treating bacterial infections and reopening this field to the Western world. In 2009, clinical trials were conducted in the US[36] with phage cocktails specific towards *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, showcasing the safety in the administration of these cocktails to patients. Clinical trials realized throughout 2007 in the UK[37] in patients with chronic otitis, showed that there was a statistically significant improvement in patients who received a phage cocktail against *P. aeruginosa* over those in the control group.

Today, the *Phagoburn* study is the first large scale clinical trial to be carried out, resulting from a collaboration of French companies Pherecydes Pharma and Clean Cells with the French Ministry of Defense, supported by the national drug regulatory agencies of France, Belgium and Switzerland, and financed by the European Union[38]. It aims to evaluate the effectiveness of a phage cocktail in treating burn wound infections caused by *P. aeruginosa*, and *E. coli,* in Belgium, France and Switzerland.

2.2.3- Advantages of phage therapy

There are multiple advantages to phage therapy that make it an attractive alternative to traditional antibiotics therapy. Smith *et al.*[35] demonstrated that phage therapy has the potential to be more efficient that antibiotic treatment. In this work, mice were infected with a lethal strain of *E. coli*. A single dose of anti-K1 phage was more effective in preventing death than multiple doses of different antibiotics like ampicillin, chloramphenicol or trimethoprim, possibly due to the self-replicating nature of the phage. Since bacteriophages are able to destroy bacterial cells though mechanisms unrelated to the ones used by antibiotics, drug resistance has no effect on phage efficacy. As such, phage preparations can be used to treat infections caused by antibiotic resistant strains[4] like *S. aureus* infections.

Since most antibiotics have a broad spectrum of activity, antibiotic treatment can affect multiple bacterial strains, besides the pathogenic strain causing the infection, which can cause long-term imbalances in the normal gut microbiota, and facilitates the development and increase in antibiotic resistance[39]. On the other hand, because of its specificity, phage therapy has a narrow spectrum of activity, only affecting few closely-related bacterial strains. As such, phage therapy targeting a pathogenic strain is unlikely to cause collateral damage to non-target bacterial strains in the gut microbiota[40,41], avoiding long-term disturbances in the gut microbiota and reducing of likelihood of development of phage resistance. Bacteriophages themselves are also inherently non-toxic and no harmful immune responses were ever detected, indicating that negative side effect are not a reason for concern in phage therapy[42].

Bacteria can become phage resistant by several mechanisms for example, by changing surface receptors. However, not only is induced phage-resistance relatively low during therapy, but overcoming resistance is also relatively easy and fast. Isolation of novel bacteriophages from environmental sources is possible, and phages are able to evolve simultaneously with resistant bacterial strains, unlike antibiotics[43]. In some cases phage-resistance can be associated with a cost for the bacteria, resulting in a decrease in the virulence[44].

Besides their direct use as an antibacterial agent, phages can also be modified in order to treat bacterial infection through other mechanisms. Phages can be modified in order the increase the antibiotic activity of a certain drug, by, for example, expressing genes that increase the sensitivity of bacteria to antibiotics, interfere with quorum sensing or, in the case of biofilms, genes that degrade the biofilm matrix. Phages can be engineered in order to expand the host range or to target bacteria that contain a specific DNA sequence. Phages could also be used as a tool for bacterial detection and diagnostics or as a drug delivery vehicle, using phage display technology[45].

Apart from these advantages, phage therapy also has additional benefits over antibiotic treatment[42]: i) potential for a single, low dose, as phages are able to multiply *in situ*, and reach the required dose, according to bacterial density; ii) phages are inherently non-toxic, and although they can interact with the immune system, there is no evidence that harmful immune responses are a cause of concern in phage therapy; iii) as phages are mainly composed of nucleic acids and proteins, and due to their biological method of production there is little environmental impact associated with phage therapy, unlike with antibiotic treatment; and iv) the cost of development and production of phage preparation are relatively low.

2.2.4- Limitations of phage therapy

Despite all of the advantages of phage therapy, there are still several limitations and aspects to consider, before phage therapy can become a widespread treatment.

The first thing to consider is phage selection. Due to a narrower range of action, when compared with antibiotics, the main disadvantage of phage therapy is that the pathogenic agent causing the infection must be determined, through standard microbiology detection methods. This problem can be mitigated by using cocktails of phages with different host ranges, which collectively are likely to infect the bacterial strains that are thought to be the cause of infection[46]. These cocktails might be composed of multiple narrow range phages, fewer broad phages or phages that display the ability of expanding their host range[46].

The first phage preparations for clinical use in the West are likely to be based on phage cocktails. For more specific preparations the pathogenic bacteria should be isolated from infection and tested against a wide range of well characterized phages, to determine the most effective treatment. This custom-designed treatment is often more effective that generalized treatments, and is the most commonly used approach in Poland[31].

Phages that display a lytic life cycle are preferred over those displaying a lysogenic one, for several reasons. First of all, temperate phages display delayed bacterial lysis, which is not desirable when the aim of the treatment is to treat a severe infection. Temperate phages often contain certain genes that alter the phenotype of the infected bacteria. These genes might include virulence factors like the cholera toxin, which would increase the virulence of the target bacteria[5]. Finally, temperate phages are capable of transduction, leading to the transfer of genetic material from one bacterial cell to another, when the prophage is excised incorrectly. This transduction has the potential of carrying virulence factors, or resistance genes from host to host, leading to the development of more pathogenic or resistant strains[5,47].

A side effect of the action of lytic phages on gram-negative bacteria is the release of endotoxins (lipopolysaccharides or LPS) and other bacterial toxins upon lysis. In order to prevent this release of toxins, that could be deleterious for the patient, lysis genes could be eliminated from lytic phages, with the side effect of preventing phage replication. Alternatively, lysis deficient phages, such as the filamentous M13, could be used, that extrude themselves from the bacterial cell wall, without causing lysis. These phages can be engineered to express holin, an enzyme that causes lesions on the cytoplasmic membrane, promoting depolarization of the membrane and causing cell death, without release of toxins[48].

2.2.5- Bacterial impurities from phage production

Since phages are obligate parasites, bacterial cells are needed to produce the doses required for clinical application. After the fermentation process, the resulting lysate contains, beside phages, living bacterial cells, cell debris, media components and bacterial metabolites[5]. The major pyrogen found in the culture is the cell wall component LPS, also known as endotoxin, which is present in Gram-negative bacteria.

These compounds, with a molecular size ranging from 10-30 kDa consist of three distinct regions: the hydrophobic lipid A region, responsible for the toxicity of LPS, the core oligosaccharide and the hydrophilic O-specific polysaccharide, with this region being the most variable between different Gram-negative bacteria. The core is partially phosphorylated, and thus endotoxins exhibit a negative charge in common solution. Their amphipathic nature leads to the formation of aggregates and micelles in aqueous solutions[6].

Endotoxins can elicit systemic immune responses in humans, including fever, leukopenia and leucocytosis that may ultimately lead to a lethal endotoxin shock[49]. The toxic effects of endotoxins are not caused directly by the molecule itself, but due to its interaction with the immune system, activating certain components, such as macrophages which release pro-inflammatory mediators like TNF-α, IL-6 and IL-1[50,6]. While oral or dermal administration of endotoxins is considered safe[50], injections of low doses of endotoxins (1 ng/mL) is enough to cause immune reactions[6]. As such, its removal is required in all therapeutic biological products, with all pharmacopeia establishing an upper threshold limit of endotoxins for intravenous applications of 5 Endotoxin Unit (EU)/Kg. The term EU refers to the biological activity of the endotoxin and corresponds roughly to 100 pg of this molecule[50].

Bacterial DNA, most notably unmethylated CpG motifs, also stimulate the hosts immune system by promoting the activation of NK cells, B cells and inducing the production of IFN-α[51]. The recognition of DNA motifs, prevalent in prokaryotic but not eukaryotic cells, by the immune system, can be considered a defense mechanism, increasing the resistance of the host towards bacterial infection[51]. No autoimmune diseases or other adverse health effects have been noted with the administration of these motifs in humans, indicating their relative safety[51]. However, under severe infection conditions, the increased production of IFN-α by the presence of bacterial DNA can augment the production of TNF-α and IL-6 in response to LPS, leading to increased toxicity of this molecule[52].

While the oral administration of phages appears to be safe[40,41], intravenous application of non-purified phage preparations is not ideal, mainly due to the strong inflammatory proprieties of endotoxins, which may cause severe side effects[5,53]. In phage therapy, preparations should thus be sufficiently purified and decontaminated to prevent such effects. As such, measures should be taken during the downstream processing of phage preparations, in order to reduce contaminant load in the final product. Early purifications steps are essential for clarification of the crude lysate, while following steps will be focused in removing most bacterial impurities.

2.3- Production and early purification steps

As mentioned previously, viable bacterial host cells are required, in order to produce bacteriophages. Any culture medium suitable for the growth of the host bacterium, should also allow the propagation of bacteriophages[7]. For the maximum production of phages, the pathogenic host that is to be targeted should be used. However, the preparation should be carefully purified and decontaminated of any living bacterial cell before therapeutic applications, since as previously described the use of non-purified phage preparations containing cell debris and high concentrations of toxins can be dangerous[53]. An alternative would be use non-virulent strains or closely related hosts, as a way to decrease the purification requirements[5,54]. Additionally, the use of a propagation media, free of possible contaminants and animal products should also help reduce the contamination load during downstream purification.

The downstream processing of bacteriophages preparation is usually composed of several purification steps, with different objectives. The first step is usually for broth clarification, removing cells and cellular debris from the media in order to facilitate further purifications steps. It can be accomplished either by low-speed centrifugation or depth filtration[7,55]. During this initial step some phages can remain inside non-lysed cells. Chloroform can be added to the lysate before purification, in order to induce lysis of the cells and release the phages, as long as the phages do not contain lipids. The clarified lysate can be directly used in many applications but for clinical applications further purification is required.

2.4- Phage purification methods

2.4.1- General endotoxin removal

Endotoxin removal is complex, due to the wide range of sizes and the presence of a hydrophobic region, leading to a high tendency of micelle formation, complicating removal. Endotoxins also tend to aggregate to proteins and are very stable molecules, resisting to extreme ranges of temperature and pH [6,56,57]. Due to these characteristics, endotoxin removal from phage preparations can be difficult.

Many methods for the removal of endotoxin from biotherapeutics have been developed but are product-specific[57]. As such, for each biomolecule, there is a need to assess which method is more efficient, depending on the characteristics of the target molecule. These methods include LPS affinity resins, aqueous two-phase extraction processes, ultrafiltration and chromatographic methods like hydrophobic interaction, ion-exchange and size exclusion[6]. Anion-exchange exchange can be used to purify positively charged proteins but is cumbersome in decontaminating negative proteins or biomolecules like bacteriophages, as both bind to the media[56]. Affinity chromatography with histidine or polymyxin B, which display a high binding affinity towards the lipid A region, can also be effective[6]. Addition of a detergent, such as Triton X-100, can also facilitate removal of LPS with certain methods, by releasing LPS monomers from micelles, decreasing their size[57].

2.4.2- Traditional and emerging methods for phage purification

The downstream processing steps that follow broth clarification have the objective of concentrating and purifying phage preparations, removing most bacterial impurities and toxins from the lysate. A second step in downstream purification can be used in order to concentrate the phage titre, using precipitation or ultrafiltration by tangential flow. A diafiltration step can also be incorporated in order to exchange the phage particle from the lysate media into an appropriate buffer, especially if a chromatographic step is desired. For the removal of impurities, a variety of methods have been traditionally used, including ultracentrifugation, centrifugation with a density gradient (caesium chloride being the most used), precipitation by PEG[7] and ultrafiltration.

These methods, while useful for the purification of phages, particularly at laboratory scale, show certain disadvantages that complicate the process scale-up for use in industrial settings, such as the high costs of materials and equipment, being time consuming or affecting phage recovery and infectability[5,7,16,55,58]. The main difference between the more common protein biopharmaceuticals, and the emerging viral biopharmaceuticals, when purification is concerned, is their size, as viruses can be several orders of magnitude larger than therapeutic molecules and proteins[55]. This difference presents a challenge in phage therapy and in therapy with viral particles in general, leading to developments in downstream purification methods for these large particles. As such, different methods for the purification of phage preparations are being currently considered and researched, which could be more easily used in an industrial context, such as extraction in aqueous two-phase systems or chromatography.

Extraction in aqueous two-phase systems (ATPS) have been used for a long time in the purification of biomolecules, including viruses[58] and might present a viable alternative to phage

purification. These systems are composed of two different polymers, soluble in water, or a polymer and a salt, which are present at such high concentrations, that two different, immiscible aqueous layers are formed, where the light phase, or top phase is rich in one polymer, and the heavy phase, or bottom phase is rich in the second polymer or salt, with an interface separating them[59]. These systems can then be used for bioseparations, as biomolecules partition to different phases, according to many factors such as the type, size and concentration of the phase forming components, the ionic strength of the salt used, the working pH and the properties of the biomolecule being separated (size, charge and hydrophobicity), as well as its affinity towards the phase forming components[60].

Many recent studies have looked at the use of ATPS for purification of phage preparations. González-Mora *et al.*[59] tested several PEG-salt and ionic-liquid-salt systems in purifying phage M13. Extraction using organic solvents as also been attempted. Szermer-Olearnik *et al.*[61] used 1-octanol and 1-butanol, water-immiscible, easily removed organic solvents to purify T4 phage. While the phages remain in the aqueous phage, endotoxins were transferred to the organic phage.

2.4.3- Phage lysate purification with chromatography

Chromatography is a method that is commonly chosen when high purity of a product is required and is scalable to industrial settings. Different modes of chromatography have been used in phage purification including ion-exchange chromatography, based on the charge of the particles being separated, size-exclusion chromatography, which is based on size, and affinity chromatography, which is based on the high binding potential between the molecule of interest and a specific ligand present in the chromatographic media.

Among of the different modes of interactions that can be explored in a chromatographic process, ion-exchange chromatography (IEC) is one of the most promising as typically bacteriophages exhibit a negative charge, with an isoelectric point normally bellow 6, and can consequently be retained in a positively charged, anion-exchange resin. Anion-exchange chromatography (AEC) can achieve purities similar to CsCl centrifugation, while being faster, consistent and capable of being automatized[9]. It is also easily scaled-up, allowing for industrial applications[8]. However, many impurities can also bind to anion exchange media under the same conditions as phages. Elution of the particles by increasing the ionic strength of the elution buffer can selectively elute the phage particles and further remove bound impurities[55]. AEC is commonly used as a capture step, and further polishing steps need to be performed, such as size-exclusion chromatography (SEC)[9].

Monjezi *et al.*[10] used a traditional packed-bed strong anion-exchange column SepFast™ Super Q, with a $N^{+}(CH_3)_3$ functional group to elute a clarified bacteriophage M13 lysate, recovering 74% of the original phage titre when using 1.5 M NaCl as elution buffer. However, the purity of the phage was not tested, regarding the presence of bacterial host contaminants. Smith *et al.* used a hydroxtapatite column to elute filamentous bacteriophages (49% original titre) and remove most host cell proteins, with a combined NaCl-phosphate elution buffer[62].

In chromatography, pore diameter of the media is an important parameter. Larger molecules might not be able to access the media's pores, and only adsorb in the outer surface, resulting in a decrease of the binding capacity of the column[63]. For purification of phages, the chromatographic support should have a high pore diameter, in order to increase accessibility of the particles to the media, increasing the binding surface and binding capacity of the column[13].

The more traditional packed bed chromatographic columns are challenged by the size of phages, with regards to their capacity. Monolithic media is a suitable alternative for purifications of phages[12]. Monoliths can be considered a chromatographic material composed of a single block, which features large pores and interconnected channels, providing a larger binding surface and increasing the binding capacity. Monoliths have also been successfully scaled up, with columns of up to 8 L available for use in preparative processes[12,13]. For purification of large biomolecules such as phages, monolithic media are superior over packed-bed media both in resolution and binding capacity, leading to increased productivity.

Many recent studies have focused on using methacrylate monoliths, also referred to as Convective Interaction Media (CIM) disks. Smrekar *et al.*[63] successfully recovered T4 phages using a strong anion-exchange quaternary amine (QA) CIM column and NaCl elution buffer. Most of the phage particles eluted at an elution buffer concentration of 20%, equivalent to around 0.3-0.5 M of NaCl, with around 70% of the original titre recovered. The authors then used stepwise gradient in order to obtain narrower peaks, leading to highly concentrated phage. While phages eluted at 0.5 M NaCl, bacterial DNA was eluted at 1 M NaCl and only 1% of the host proteins in the feed were detected in the phage fraction. However, the presence of endotoxins was not tested.

S. aureus and *Mycobacterium smegmatis* phages were also purified on a QA CIM and diethylamine (DEAE) columns, with successful scaling-up up to 8 mL[13,14]. For the purification of *S. aureus* phages in a CIM QA disk with a 100 mM phosphate solution as equilibration buffer, a higher conductivity was required (0.6 M NaCl) in order to recover 60% of phage particles while removing most bacterial DNA and proteins[13]. For elution of *M. smegmatis* phages, a stepwise gradient strategy of 0.6-1-2 M NaCl was employed, with around 40& of phage particles eluting at 1 M NaCl. It is possible to observe that there is a considerable difference in elution conditions between the different phages[14].

None of these studies, however, determined the effectiveness of endotoxin removal from phage preparations using this chromatographic media. Van Belleghen *et al.*[64] used QA and DEAE CIM columns, along with other methods, to remove endotoxins from phage preparations previously purified using an Endotrap HD column. Endotoxins removal efficacies varied according to the phage being purified, from 98% to 40%, and large losses of two orders of magnitude in phage titre were registered. As the endotoxins are eluted at similar conditions as the phages, endotoxin removal through DEAE CIM media might be cumbersome. However, the authors obtained varied results, both in endotoxin removal and phage recuperation, for all purification methods tested, failing to obtain a general protocol for purification of phage preparations from endotoxins, indicating that these strategies need to be developed individually for each phage.

Expanded bed anion-exchange chromatography has also been attempted. While the traditional packed bed chromatography requires a previous filtration step in order to remove particles such as cellular debris, expanded bed forgoes the need for particulate removal. This is accomplished by increasing the distance between adsorbent particles, allowing the flow of larger particles from the lysate and combining clarification and purification in a singular step.

In Ling *et al.*[11], while traditional PEG precipitation followed by centrifugation resulted in a yield of 37% of the phage titre, expanded bed chromatography, with a DEAE matrix, resulted in a recovery yield of 83%. While this method is less time-consuming than the more traditional protocol (3.8 h compared to 18.5h), the authors indicate that this method might give rise to a lower purity phage preparation, although no date regarding the purity was obtained. Nevertheless, this method might be suitable for the industrial production of phages, as it is less time-consuming, and more easily scalable.

Chromatographic methods besides ion-exchange chromatography have also been attempted. SEC is commonly used for desalting, fractionalization of different size proteins, and molecular size determination[65]. Separation by SEC is based on the size differences between different molecules, leading to their elution at different fractions. SEC is commonly employed in combination with other chromatographic methods[66]. On its own, however, SEC is not able to remove large impurities[9].

Zhakarova *et al.*[15] used SEC to purify filamentous phage M13, in order to reduce the impact of high concentrations of salt on the bacteriophage. The elution profile of the phage consisted of two peaks, with most of the phage particles elution in the first. This procedure successfully removed most contaminants however the phage preparation went through PEG precipitation beforehand.

Borantinsky *et al.*[16] also used SEC to purify different *E. coli* and *P. aeruginosa* phages. However, despite concentration of the phage particles and partial removal of some contaminants, endotoxins remained in the lysate. In spite of their small size, the formation of large structures like micelles prevented removal of these impurities. Endotoxins were only removed after chromatography in a Matrex Cellufine Sulfate matrix which mimics the affinity of heparin, and thus combining ion exchange and affinity interactions to the phage particles. At low ionic conditions, the phages were bound to the media, while endotoxins and other contaminants were removed. However large titre losses were registered.

Ceglarek *et al.*[8] used affinity chromatography not only to purify bacteriophages from bacterial contaminants, but also to remove contaminant bacteriophages. As these bacteriophages are similar in size and zeta potential, affinity chromatography can be an alternative. To do so, the authors transformed the producing bacterial cells with a plasmid, expressing a recombinant phage capsid protein, containing GST or His-Tag. During the capsid assembly the wild-type capsid proteins, expressed from the viral genome compete with the recombinant capsid proteins, and virions containing the affinity tags are produced, without previous genetic modification on the phage.

Using this method, the authors managed to purify a recombinant *E. coli* T4 phage from similar phages. On a previous study[17], which used genetically modified phages expressing the recombinant capsid protein, affinity chromatography using glutathione or Ni-NTA agarose was shown to be also able to remove endotoxins from phage preparations.

2.4.4- Current purification methods for phage therapy

Currently, there is few information on the downstream purification process of bacteriophages, in the context of phage therapy, where a scalable and efficient process is required, and much less on the use of chromatography in such a process, taking into account that phage therapy is still in its infancy with the first clinical trials having just recently started, and few case studies were published. Merabishvili *et al.*[67] in order to evaluate the safety of a phage preparation in burn wound infections, used centrifugation followed by filtration for clarification of the lysate. Endotoxin purification was accomplished using a commercially available kit (EndoTrap^{tm} Blue, Cambrex BioScience). Bruttin *et al.*[40] and Sarker *et al.*[41] tested the safety of oral administration of *E. Coli.* bacteriophages to volunteers. In both cases, the phage preparations were purified using a combination of differential centrifugation and sterile filtration. It should be noted however, that the oral administration of endotoxin-contaminated therapeutics does not constitute as much of a danger. No information regarding the downstream processing of the phage preparations used by Wright *et al.*[37] and by Rhoads *et al.*[36] is available.

3- Workflow

A flowchart representing the general experimental process performed in this work can be observed in Figure 2. Briefly, phage lysates were prepared by amplification in bacterial cultures. These lysates were then filtered, clarified through centrifugation or microfiltration. The clarified lysates were then processed for concentration and diafiltration. After stock preparation, different chromatographic trials were conducted. In each peaks were identified and recovered in order to analyse their phage titre through a plaque assay, bacterial protein content through SDS-PAGE and Bradford assay and bacterial DNA content though PCR and qPCR.

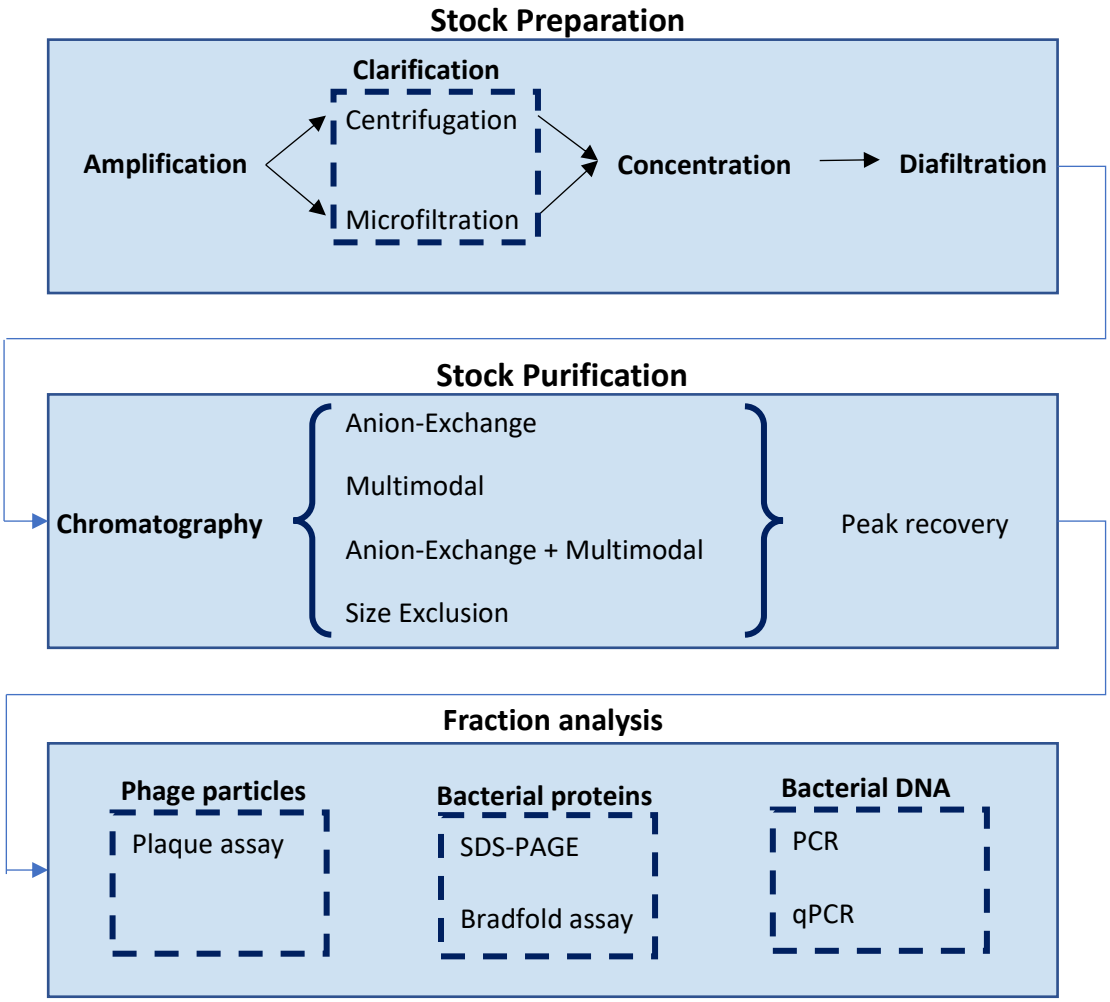

Figure 2- Flowchart indicating the experimental process employed in this work

4- Discussion and Conclusion

The main objective of the work here presented was to study the purification of a preparation of a phage solution from bacterial contaminants and identify processes that might be interesting for further research. To this end, phage stocks were prepared, clarified and, for purification, several chromatographic approaches and combined strategies were attempted.

For clarification, both microfiltration and centrifugation were attempted. Although it was not possible to compare the phage losses between both methods, microfiltration was time-consuming. For protein removal, microfiltration appears to be more efficient, however, acceptable removal was obtained for both methods.

For concentration and diafiltration, only small losses in the phage titre were detected. These steps appear to be important for phage stability, since the first stock, after centrifugation, remained stored for one month in the culture media, leading to phage precipitation and large losses in the titre. In both processes, loss of bacterial proteins to the permeate was also observed. In the first two stock, as the initial protein load was low, the removal of proteins by these processes led to a very low concentration, which prevented a correct appraisal of the protein removal efficiency of the chromatographic processes by Bradford assay and SDS-PAGE. These processes also led to removal of a portion of the DNA present in the clarified lysate.

For chromatography AEC, MMC and SEC were tested. In AEC, during initial optimization, phage particles seemed to elute preferably at a particular elution buffer concentration. Optimization of the injection load was also carried out, and larger injection volumes provided more intense peaks and concentrated fractions. However, when injection load optimization was being carried out, the elution profile of the particles seemed to change. While the reason for this change is unknown, it might be due to small-scale aggregation between phage particles, leading to less interaction between each particle and the media. During stock preparation, large-scale aggregation caused the loss of a significant portion of phages in the clarified lysate. While the phages are stable under the stock buffer, prolonged storage time might have led to some particle aggregation. In response, an optimized stepwise gradient mode was created, which allowed for satisfactory recovery of phage particles at different steps. This optimized strategy was thus employed in following AEC trials.

Regarding bacterial protein and DNA removal, it was seen that the filtration processes removed a large portion of the proteins in the lysate, leading to low levels in the stock. Although absolute values could not be obtained, it was possible to see that most of the remaining contaminants were removed from the phage fraction, although a residual portion still remained. Some small proteins were detected in different elution fractions, but it was not possible to confirm if these proteins were in association with the phage particles or were phage proteins. Additionally, the identity of some of the obtained peaks could not be confirmed, but could be caused by other, untested bacterial contaminants.

When MMC was attempted, very good phage recoveries were obtained. Due to the characteristics of the column, smaller impurities, like endotoxins and proteins will remain in the column,

while phages are able to pass through, allowing purification. In the peaks obtained, only residual quantities of proteins, that were likely bound to the viral capsid, and bacterial DNA were detected. When a combined AEC-MMC strategy was attempted, although a low titre was obtained in AEC, which was not expected, full recovery was obtained in MMC, with elimination of most residual proteins, and bacterial DNA. While the endotoxin content was not detected, in theory the column used for MMC should result in removal. As such, out of the different approaches tested in this work, a combined AEC-MMC strategy seems to be the most promising for further studies.

A SEC strategy was also tested. However, due to time restrains, only two trials, with different sample loads, were performed. Due to the differing results obtained in these trials, no conclusions could be drawn. Finally, the combined AEC-MMC approach was also tested with other type of bacteriophage. Although a significant amount of pfu was recovered from AEC, large losses were detected in MMC.

For the large-scale production of phages for therapeutic purposes, three conditions are required for the downstream process. It should be able to quickly process large volume of lysate, the phage recovery should be high enough for economic feasibility, and it should be able to remove most bacterial impurities such as endotoxins. While in this work identified a potential strategy for the purification of phage particles, several questions arose, that required additional research. Despite the importance of the removal of endotoxins from the phage preparation, it was not possible, unfortunately, to measure the efficiency of the methods here presented in their removal. As such, further tests, with this objective in mind, should be performed. Other tests should also be performed, such as determining the binding capacities of the columns to the phages.

Additionally, while AEC led to good recoveries and contaminant removal, phage elution was observed at two different conductivities. While phage aggregation could explain this change, it was not proved in this work. Electron microscopy could be used in order to clarify whether aggregation did actually occur, and if it interferes with chromatography. If the hypothesis is confirmed, the possible impact of phage aggregation on large-scale production should be studied. While it should be expected that downstream processing of the lysate would occur immediately following amplification, not allowing time for the agglomeration of the phage particles, the high titres necessary could induce this aggregation. Finally, the trials for SEC shown here were not conclusive.

In conclusion, in this work strategies for the downstream processing of bacteriophages has been studied and a combination consisting of centrifugation, filtration, and purification through AEC and MMC has been proposed. Further research is required in order to determine the endotoxin removal efficacy, and to test a possible integration of SEC into the process. Due to the growing need to develop new alternatives to antibiotics, work into this topic is sure to continue in the future.

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