Quality control of phage formulations during GMP manufacturing

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Biotechnology

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Preface

The work presented in this thesis was performed at the company Technophage (Lisbon, Portugal) and Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period February-July 2022, under the supervision of Cátia Peres. The thesis was co-supervised at Instituto Superior Técnico by Prof. Ana Azevedo.

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the *Universidade de Lisboa*.

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To all my friends, who have supported me through all our friendship years and heard me rant every time work was frustrating.

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Abstract

With the development of multi drug-resistant bacterial strains, there is a current need to explore alternative antibacterial strategies. Phages, viruses that infect exclusively bacteria as their specific host organisms, are a potentially interesting approach. Phage therapy has now resurfaced and consists in the use of bacteriophage as a therapeutic agent for human infections. With its developments, it is important to aim for fast and trustworthy methods of controlling the quality of phage formulations. The most used method to determine a sample's phage titre is the plaque assay, which is a slow method with reproducibility issues. This Master's Thesis aimed to find a method that could differentiate individual phages from aggregates and quantify them, to overcome the previous limitations and, thus, the dynamic light scattering technique was experimented with.

For this purpose, the T4 phage was amplified, and its phage lysate was purified with ion-exchange chromatography, which produced highly concentrated samples. These samples were subsequently analysed with DLS, and aggregation induction experiments were conducted. In the end, the samples were shown to have a low degree of purity and the DLS technique seemed to be unsuitable for highly complex samples such as tailed phage solutions. In terms of future work, working with multi-angle DLS with highly purified samples could provide better results.

Keywords:

Bacteriophage; Phage therapy; Downstream Processing; Dynamic Light Scattering

Resumo

Com o desenvolvimento de estirpes bacterianas multirresistentes aos antibióticos, há uma necessidade atual de explorar estratégias antibacterianas alternativas. Os fagos, vírus que infetam exclusivamente bactérias como organismos hospedeiros específicos, são uma abordagem potencialmente interessante. A terapia fágica reapareceu e consiste na utilização de bacteriófagos como agente terapêutico para infeções bacterianas humanas. Com os seus desenvolvimentos, é importante visar métodos rápidos e fiáveis de controlar a qualidade das formulações de fagos. O método mais utilizado para determinar o título de fago de uma amostra é o ensaio de placas, que é um método lento com problemas de reprodutibilidade. Esta tese de mestrado teve como objetivo encontrar um método que possa diferenciar fagos individuais de agregados e quantificá-los, para ultrapassar as limitações anteriores e, assim, a técnica de espalhamento dinâmico da luz foi escolhida.

Para este efeito, o fago T4 foi amplificado, e o seu lisado foi purificado com cromatografia de troca iónica, que produziu amostras altamente concentradas. Estas amostras foram subsequentemente analisadas com DLS, e foram realizadas experiências de indução de agregação. No final, as amostras mostraram ter um baixo grau de pureza e a técnica DLS mostrou-se não ser adequada para amostras altamente complexas, tais como as suspensões de fagos com cauda. Em termos de trabalho futuro, a utilização do DLS multi-ângulo em amostras altamente purificadas poderá proporcionar melhores resultados.

Palavras-chave

Bacteriófagos; Terapia fágica; Processamento a jusante; Espalhamento dinâmico de luz

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEC	Anion-exchange chromatography
AI	Aggregation index
ATP	Adenosin 5'-triphosphate
ATPS	Aqueous two-phase systems
BSA	Bovine serum albumin
CIP	Cleaning in Place
CV	Column volume
DAPI	4'6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DNA	Deoxyribonucelic acid
dsDNA	Double stranded deoxyribonucelic acid
dsRNA	Double stranded ribonucelic acid
EU	Endotoxing units
FDA	Food and Drug Administration
GMP	Good Manufacturing Practices
GST	Glutathione S-transferase
IEX	Ion-exchange chromatography
LLE	Octanol liquid-liquid extraction
LPS	Lipopolysaccharide
MADLS	Multi-angle dynamic light scattering
MOI	Multiplicity of infection
MPB	Master phage bank
MWCO	Molecular weight cut-off
OD	Optical density
PCR	Polymerase chain reaction
PDI	Polydispersity index
PEG	Polyethylene glycol
PFU	Plaque-forming units
pI	Isoeletric point
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
ssDNA	Single stranded deoxyribonucelic acid
ssRNA	Single stranded ribonucelic acid
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TFTC	Too Few to Count
TNTC	Too Numerous to Count

TSA	Tryptone soy agar
TSB	Trypticase soy broth
WPB	Working phage bank

1. Introduction

1.1 Background

Nowadays, the levels of bacterial resistance to traditional antibiotics are dangerously rising due to a variety of practices, including overuse, self-medication, and excessive prescription, which led to loss of effectiveness. This then caused the rise of bacterial strains resistant to most of these antibiotics. Additionally, no new class of antibiotics has been discovered and therefore, there is a focus on developing new, effective, and viable antibacterial agents. In this context, bacteriophage therapy is a potential alternative to combat infections caused by multidrug-resistant bacteria[1–5].

Phages, discovered in 1915, are viruses that infect exclusively bacteria, use them as hosts to replicate, and have a high level of specificity[6–8]. Although bacteriophages have several different applications in diverse areas, bacteriophage therapy has attracted the greatest interest[5].

Phage therapy refers to the clinical application of phages as antimicrobial agents to treat human infections, and it has been widely used since the early 20th century in eastern European countries. It presents several advantages that make it an appealing alternative to traditional antibiotics, such as effectiveness against multidrug-resistant pathogenic bacteria and the bacteriophages' high specificity, that keep the native human microbiome undisturbed, which is not the case with the broaden spectrum antibiotic therapy[5,6].

With the increase of compassionate use of bacteriophage suspension to treat patients with no alternative therapies, it is important to investigate and enhance the current production processes of these new medicines. When working with bacteriophages, the identification of the phage's presence throughout the manufacturing process and the determination of the concentration of the phage particles are key procedures. The standard technique is the double agar overlay plaque assay, where phage plaques are counted on agar plates that have been seeded with the bacteria in which the phages can propagate. Although it is simple, efficient and easy to implement, it has several disadvantages, such as high variability and poor reproducibility. It can be time consuming, and operator bias, pipetting errors, change in bacterial growth parameters and contaminations can severely impact the results[9–11]. As such, the bacteriophage industry would greatly benefit from investing in an improved method for particle monitoring through the manufacturing process.

1.2 Aim of Study

The main objective of this master's thesis was to find a method that can differentiate individual phages from phage aggregates and quantify them, to overcome the limitations of the double agar

overlay plaque assay method. Since dynamic light scattering has shown promising results in previous phage studies[12], this method was employed to study suspensions of the T4 model phage.

The initial stage of the experimental process consisted in streamlining the production process of the phage T4 by infection of the respective host bacterial cultures at a shake flask scale. With these results, a subsequent large-scale amplification in a bioreactor was performed, for a final lysate with the highest phage concentration possible.

Afterwards, the produced material was purified, in several trials, with ion-exchange chromatography, with the purpose of generating different samples for later analysis. To try to quantify and identify aggregated phages, these samples were measured in a dynamic light scattering equipment.

2. Literature Review

2.1 Phages

2.1.1 Phage Biology

Bacteriophages, or phages, are viruses that infect exclusively bacteria and use them as hosts to replicate[6]. They were first identified in 1896, by Ernest Hankin, and later described and characterized by Frederick Twort, in 1915 and again, in 1917, by Felix d'Herelle[7].

It is well known that phages are the most abundant living entities on earth and that, due to their long-term survivability, ability to replicate rapidly in appropriate hosts and high level of specificity, they play a very important role in maintaining and regulating the bacterial balance in natural ecosystems[8]. They exist in every ecosystem, for example seawater, forest floors and agricultural soils, and are present in a phage-to-bacteria ratio of 10:1. Most importantly, although bacteria can be destroyed by phages, they are harmless to other organisms, including humans[4,6].

Phages usually target particular bacteria species or a subgroup of this same species, which express specific membrane surface receptors. Infection cannot occur if the bacterial cell does not expose a specific surface receptor for the phage[4,6].

Phages can vary in length, usually from 24 to 200 nm and they exhibit a well-defined three-dimensional structure, the most common being an icosahedral protein capsid that encloses the genetic material in its core (DNA or RNA)[5]. Other elements that facilitate the phage/bacteria interaction can also be present, such as a spiral contractile tail or sheath and protruding tail fibers, which display proteins that recognize and bind specific receptors in the bacterial membrane surface (Figure 1)[6].

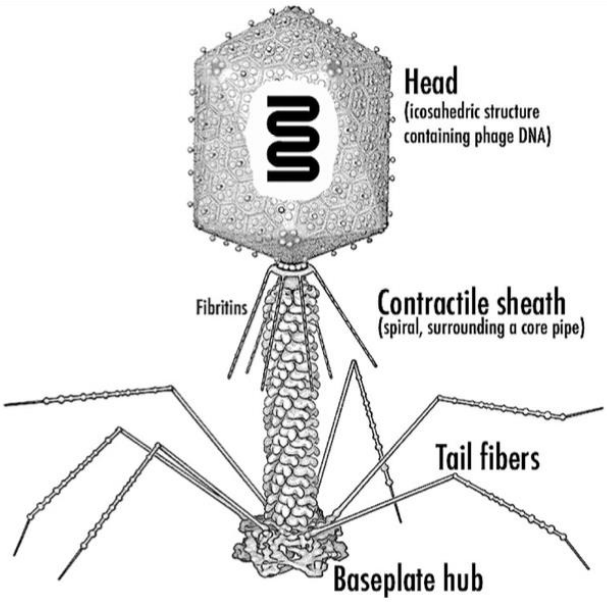

Figure 1 - Schematic representation of a bacteriophage. Adapted from Harada et al, 2018[5]

2.1.2 Phage Classification and Diversity

Phages are classified considering the phage morphology and genome characteristics[1]. Regarding the type of genetic material enclosed within the capsid's core: single stranded DNA phages (ssDNA), double stranded DNA phages (dsDNA), single stranded RNA phages (ssRNA) and double stranded RNA phages (dsRNA). Concerning the virion structure and morphology, phages are classified as tailed phages (binary symmetry), filamentous phages (helical symmetry), polyhedral phages (cubic symmetry) and pleomorphic phages. About 96% of all phages are documented to belong to the tailed phage Caudovirales order, which is then further divided into different families such as the Myoviridae with long contractible tails, the Siphoviridae with long noncontractable tails and Podoviridae with short noncontractible tails, among others[6,13].

2.1.3 Phage Infection Life Cycles

According to their mode of infection, phages can be divided into two categories. Virulent, or strictly lytic phages, can only replicate via lytic lifecycle. After adsorption to the bacterial cell surface, they inject their DNA, take over the host cell metabolism and induce the switch of its protein machinery to replicate their genome and produce new phages, through assembly of multiple copies of capsids and tails. This self-propagation leads to cell lysis and release of the newly produced virions, ready to initiate several other rounds of infection[5,14,15]. Some examples of virulent phages include the T4 phage, and less studied phages such as T1, T2 and T6[6].

On the other hand, temperate phages can display two different life cycles, lytic or lysogenic (Figure 2). In the lysogenic cycle, the viral genome is integrated in the bacterial genome and suppresses

expression of most of phage genes, except those needed to maintain the lysogenic state. The dormant phage, who is now called a prophage, replicates together with the bacterial DNA, or lysogeny DNA, for many generations as the cell goes through division[5,6,14,15].

While the prophage is integrated, the bacterium becomes immune to the infection by phage particles of the same strain, making it potentially more virulent and resistant to some phages. When the prophage is activated by a change of the host cell's metabolism, caused by stress or cellular damage, it can switch to a lytic life cycle by inducing the excision of its DNA from the bacterial genome[5,16]. If this excision is done incorrectly, neighbouring fragments of the hosts DNA can be removed together with the phage DNA and be packaged into the capsid, which can then be transferred to another host by transduction[17].

Furthermore, there are other possible phage life cycles associated with the previously described classes that can be considered separately, such as the pseudolysogenic and chronic infection lifecycles. The pseudolysogenic life cycle occurs when the viral nucleic acid remains viable but not as a prophage and no lytic infection is elicited. In this case, the phage nucleic acid resides within the host cell in a non-active form and there is insufficient energy available for the phage to initiate either a lytic or a temperate life cycle[18]. In a chronic infection life cycle, which is found in filamentous phages, there is a slow release of maturing virions from the cell, over a long period, without apparent bacterial cell death[19].

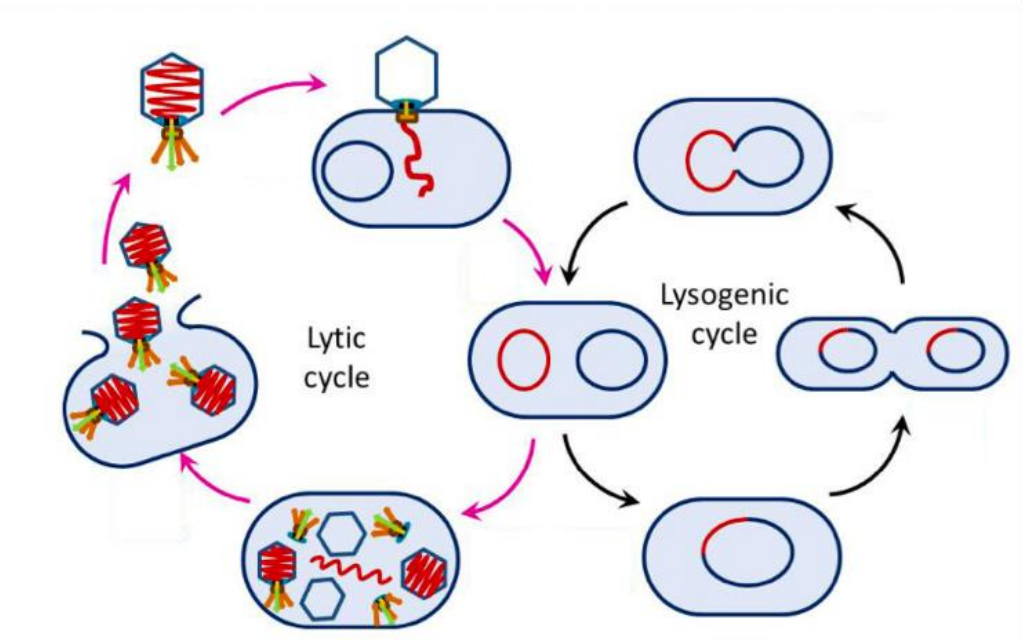

Figure 2 - The two main bacteriophage life cycles, lytic and lysogenic. Adapted from Orlova, 2012[14].

2.1.4 Phage Infection Mechanisms

The infection process of the host bacterial cells by virulent phages encompasses various tightly programmed steps, such as the phage adsorption on the host cell surface, the penetration of the phage

genome into the cell, the intracellular synthesis of the virus components and the assembly of phage virions and, finally, the lysis of the bacterial cell with the release of the newly produced phages. Several aspects of the process can be affected by the host metabolic state, like its efficiency and timing, all depending on the metabolic modifications the phage will induce[8].

The first step of the infection process is the adsorption, which is, by definition, the irreversible attachment of a virion to a host cell. It is described as a combination of virion movement, collision between the bacterial host cell and the virion, a reversible binding between the two, a transition to irreversible attachment to the bacterial surface and phage genome translocation into the bacterial cytoplasm[20].

The infection starts when the specialized adsorption structures bind to specific surface molecules on the target bacterial host. These binding sites are different between the two main types of bacteria, gram-negative and gram-positive bacteria. In gram-negative bacteria, structural and transmembrane proteins, oligosaccharides, and lipopolysaccharides (LPS) can act as receptors for a phage. In gram-positive bacteria, membrane components, like teichoic acids, peptidoglycan, and extracellular polysaccharides, can be found as phage binding sites[21].

After the irreversible attachment, the phage genome is introduced through the tail into the host cell, which involves mechanisms of DNA transfer specific for each phage. However, this process is hampered by the need to penetrate through a complex bacterial host cell envelope and phage virions must be equipped with tools that allow them to penetrate through the protective layers. Usually, the tail tip has an enzymatic mechanism for penetrating the peptidoglycan layer and the inner membrane to release the DNA directly into the cytoplasm[5,8].

The next step is the expression of the immediate early genes, which generally involves recognition by the host RNA polymerase of very strong phage promoters. These are usually involved in host takeover, like protection of the phage genome and restructure of the host for the needs of the phage[8,22]. Later, a set of middle genes is often transcribed, producing products that synthesize the new phage DNA, followed by a set of late genes, which tend to be relevant to the production of progeny virions and host lysis. These encode components of the phage particle, like packaging proteins that are involved in the introduction of newly replicated phage genomes[8,22].

Afterwards, the DNA is packaged into a preassembled icosahedral protein shell called procapsid. Normally, this assembly mechanism requires complex interactions between scaffolding proteins and major head structural proteins and, subsequently the proteolytic cleavage of both. The head expands and becomes stable, with increased internal volume for the nucleic acid. At one vertex of the phage head, a portal complex is formed, which will play an important role of being the starting point for head assembly and docking site for DNA packaging enzymes[8].

The final step of a virulent phage infection is the lysis of the host cell, where the mature phage progeny is released from the phage-infected bacteria. It is an event whose timing is tightly controlled. If it happens too quickly, too few new phages will have been made effectively to carry on the cycle, and if postponed for too long, it delays the initiation of subsequent infections and new cycles of replication[8,22].

Most phage lysis systems use a lysis from within, which involves a combination of two molecules: a holin, responsible for controlling the timing of the lysis, and an endolysin, which enzymatically degrades the cell wall of the infected bacterium. The holin assembles pores in the inner membrane at the appropriate time to allow the endolysin to reach the peptidoglycan layer, cleave one of its key bonds, and initiate lysis[8,22]. Additionally, lysis from without has been described, a process where lysis is induced by high multiplicities of phage adsorption to bacteria[22].

2.1.5 T4 phage

E. coli strains and their phages are the most commonly employed host-phage systems, due to the abundance of information about the T3, T4 and T7 phages[23]. For a long time, the T4 phage infection of *E. coli* has been one of the most extensively investigated model system in microbiology and molecular biology[24].

The T4 phage (Figure 3) and its relatives belong to the *Myoviridae* family[25]. It is one of the most complex and largest phages, with a length of about 200 nm, with an elongated icosahedron-shaped capsid, and a genome size of 174 kb[14,26,27]. The phage's inflexible tail is made up of two layers, the inner tail tube is wrapped by a contractile sheath that contracts when the bacterium is infected. A neck then separates the tail sheath from the head. *Myoviridae* phages have an extensive baseplate at the end of their tails that is covered in fibers. During infection, the tail tube penetrates an outer bacterial membrane to secure the channel for genome injection into the cell. In the tail, three types of fibrous proteins are found, long tail fibers, short tail fibers, and whiskers. Whiskers extending outwardly in the region of the tail connection to the capsid are joined to the baseplate and long and short tail fibres are attached to the baseplate. The principal reversible adsorption devices are the long tail fibres[14]. In terms of serology and DNA homology, tail fiber composition, and non-structural components such phage head internal proteins, the T4 phage group is extremely diverse[25].

T4’s total inhibition of host gene expression, which allowed researchers to distinguish between host and phage macromolecular syntheses, is one of T4's advantages as a model system. T4 biology, such as the T4 replisome, late gene transcription complex, and capsid assembly, gave rise to the present view of biological molecular machines[28].

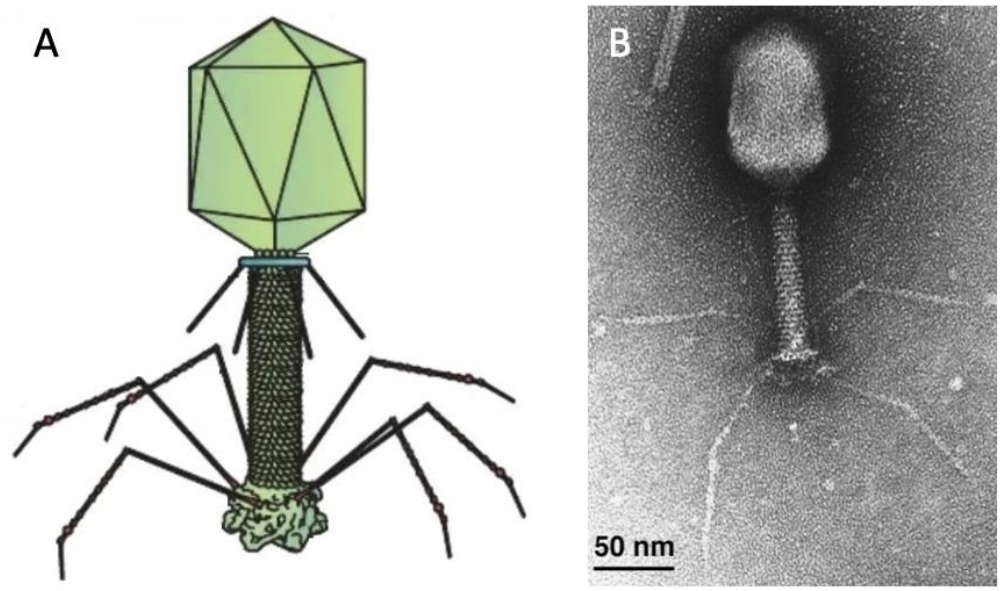

Figure 3 - A) Diagram representation of the T4 phage structure. Adapted from Norkin, 2010[26]. B) T4 phage obtained by transmission electron microscopy (TEM). Adapted from Miller *et al, 2003*[28].

2.1.6 Biotechnology Applications/Phage Therapy

Phage display is a method where phage particles are used to display exogenous peptides or proteins for epitope identification, antigen delivery, and drug discovery, among others, and it comes from the knowledge that phage particles exhibit a high level of organization and can be viewed as elaborate nanomachines that can be manipulated. This technique has now been widely used in antiviral research, targeted delivery, design of novel therapeutics and vaccines, cancer research or nanotechnology. Phage display vaccines and phage DNA vaccines have been developed for antigen delivery, which aim at producing immunogenic phage particles and incorporating foreign antigen genes in the phage genome under control of strong eukaryotic promoters, respectively[5]. Researchers have reported using phages like M13, Lambda and T7 for phage display techniques[4,5].

Phages can also be used to detect pathogenic bacteria. When the infection process reaches the end, and the host bacterial cell ruptures, in addition to the release of the virion progeny particles, there's also liberation of other intracellular components, which can be exploited as markers for biodetection purposes. Furthermore, since phages can bind to specific molecular motifs on the bacterium surface, it is possible to provide a rapid biodetection result, which is critical in case of emergencies, for example, in an outbreak situation. However, it hasn't been widely used for human samples. Only a few diagnostic kits for this purpose have reached the market, for example the FastPlaque TB assay (BIOTEC Laboratories Ltd, Mycobacterium tuberculosis) and the KeyPath test (MicroPhage Inc., Staphylococcus aureus MRSA/MSSA)[5].

The presence of bacterial biofilms and medical devices presents an important problem in healthcare and food industry environments, as bacteria embedded in biofilms are much less susceptible

to antibiotics and disinfectants, constituting a considerable health threat. As previously mentioned, endolysins are responsible for degrading the bacterial cell wall peptidoglycan during the last stages of lytic phage replication. These bacteriophage endolysins comprise a diverse range of hydrolytic enzymes, most of them are species specific, and their modular structure provides an opportunity to engineer enzymes with altered bacteriolytic activity to treat bacterial infections[5,29].

Exploitation of bacteriophage particles as vectors or matrices for new nanodevices has been growing in the last few years. Phages can be designed as nanocarriers for the targeted delivery of both therapeutic and diagnostic reporter molecules, as large amounts of drugs can be conjugated into the phage by chemical interaction or genetic manipulation. They can also be used for eradication of both pathogenic microorganisms and tumor cells[5,30].

Nowadays, we face a dangerous increase in the levels of bacterial resistance to the conventional antibiotics. They have been losing their effectiveness due to a combination of behaviors such as the overuse, the self-medication, and exaggerated prescription in human health, veterinary, and animal or agricultural areas. This led to the emergence of multidrug-resistant bacterial strains to most or all available antibiotics. Therefore, there is a need for developing new, effective, and viable antibacterial agents with different modes of action[3–5,31,32].

Phage therapy is the clinical use of natural or modified phages as antimicrobials for the treatment of human infections[6]. Its traditional concept is the administration of naturally isolated virulent phages directly to the patient with the purpose of lysing a bacterial pathogen that is responsible for a chronic or acute infection[3]. It has been extensively used since the early 20th century in Russia, the Republic of Georgia and Poland, however, only a limited number of clinical trials have been authorized by public health authorities and performed in humans in the European Union or the USA[3,5,32].

There are several advantages in phage therapy that make it an appealing alternative to traditional antibiotics. Phage therapy is effective against multidrug-resistant pathogenic bacteria, substitution of the normal microbial flora does not occur since phages are highly specific and it can respond quickly to the appearance of phage-resistant bacterial mutants, since the frequency of phage mutation is significantly higher than that of bacteria[5,31]. Bacteriophage treatment does not require continuous administrations of the agents, as the phage replicates on its own in the host and terminates its action when the host no longer exists. Additionally, the development costs for a phage treatment are cheaper than that of new antibiotics and side-effects are very rare, since the phages themselves are fundamentally non-toxic[5,31].

Despite all these advantages, there are some concerns and limitations that have been extensively discussed. One potential serious concern is the ability that some phages have to modify the host bacteria, making them more pathogenic, which is particularly relevant for temperate phages. When a prophage excises its genome from the bacterial host genome to enter the replicative lytic cycle, a part of the bacterial genome bacterium to another[31]. Additionally, temperate phages do not cause bacterial death and give the bacterial cell immunity against any attack by the corresponding lytic phages and leads to developed resistance. Although the possibility of added gene transfer events is very unlikely to bring any significant danger to patients using therapeutic phages, the presence of temperate phages

could increase the surfacing of new pathogenic bacteria and the overall risk of potentially dangerous genes being acquired by new bacterial strains. Therefore, their use should be avoided[2,33].

Moreover, a side effect of the action of lytic phages on gram-negative bacteria is the release of endotoxins and other bacterial toxins upon lysis. Endotoxins are constituents of their outer membrane and can provoke systemic immune responses in humans including fever, leukopenia and leukocytosis that can, ultimately lead to a fatal endotoxin shock[34,35]. The release of endotoxins places limits on phage treatment, as phage suspensions employed systematically have to undergo further purification steps to prevent carrying over endotoxins produced during the manufacturing [33].

In phage therapy, phage kinetics is dependent on critical parameters such as adsorption rate, latency period, initial phage dosage, clearance rate, ability of the phage to replicate *in situ*, animal or human anatomophysiology, environmental conditions and phage distribution in the human body. These can work differently in different routes of administration[5].

Pulmonary infections are among the most common antibiotic resistant infections and phages have showed to be able to battle these infections when administered via nebulization in aerosol form. They can be administered locally to lung tissues, which allows higher concentrations at the site of infection, avoiding dispersion and increasing their activity[5,36].

IntestiPhage, a cocktail of phages, was used in children from 1976 to 1982 for the treatment and prophylaxis of intestinal infections caused by various bacteria, like *Staphylococcus* sp., *E. coli* and *Pseudomonas* sp. Interestingly, the children treated with this cocktail presented a faster clinical improvement, compared to the groups treated with a phage-antibiotic combination or just antibiotics[37].

The route of administration is a challenge in phage therapy against gastrointestinal infections since the gastric acidity can destroy phages. In 2010, Stanford et al, proved that polymer microencapsulation is effective for the oral administration of phages[5,38].

Wounds can come from surgery, accident or burns. It does not always imply infection, however, breaches in the natural protective skin barrier make it more susceptible. Phages can be used for prophylaxis to prevent or to treat these infections[5,33]. Topical solutions with strictly lytic phage particles can be used to treat skin infections in cosmetic and pharmaceutical formulations. For purulent chronic wounds, they can be directly applied in the wound, via injection, soaked bandages or impregnation into biodegradable polymeric hydrogels[5]. Parenteral delivery is considered the most efficient method of administration of phages. However, it presents drawbacks such as the requirement of a health professional assistance and increased possibility of cross- contamination[5,39].

2.1.7 Phage Aggregation

Concentration and purification of the phage are the key for phage therapy. However, aggregation can occur when using high concentration viral suspensions[40]. Colloid science describes the well-known process of aggregation. It is influenced by a wide range of environmental factors, including

ionic strength, pH, temperature, and colloidal surface characteristics[41].

The idea of phage aggregation by salts with subsequent elimination by filtration has been mentioned before, for virion removal from water, where it was identified as a powerful inactivating agent that significantly decreased phage infectivity in RNA phage M2[42]. The aggregation process for the MS2 phage has also been shown to cause decrease in PFU (plaque-forming units) numbers[41].

In opposition to this, there have been isolated reports that phage aggregation is linked to phage survival. In comparison to their dispersed forms, MS2 bacteriophage aggregates have been found to be more resilient to microenvironmental alterations. It has been suggested that this aggregation process is significant as an evolutionary mechanism intended to reduce the effects of harsh environmental circumstances on virions. T4 phage aggregation has also been showed to only have a moderate inhibitory effect on infectivity.[42–44]. The presence of aggregates shows a stronger resistance to applied treatments, like UV or chlorine dioxide during water disinfection. At the same time, aggregation can greatly enhance viral clearance, as the aggregates can be more easily removed than individual particles, during membrane filtration[40,41].

It is well known that lowering the pH of the solution encourages viral suspension aggregation. In medium with pH considerably above the isoelectric point (pI), the absence of aggregation is frequently assumed. For example, MS2 phages do not aggregate for pH greater than its pI and high ionic strength, however, aggregation occurs at pH 4, which roughly correlates to its pI, when dispersed distributed in 1 and 100mM electrolyte concentrations. Qβ phage in low electrolyte solutions agglomerate at low pH values (pI close to 3) and at high ionic strengths[40,41,45].

Recently, Szermer-Olearnik et al. identified aggregation triggered by low ionic strength as a mechanism of viral regulation. In this study, it was discovered that reducing ionic strength below a specific threshold caused phage to aggregate dramatically and quickly, which simultaneously prompted all the virions present to respond in the same way. It was also seen that aggregation is not dependent on the pI of a whole T4 virion, as it was only observed at neutral and alkaline pH. The dynamics of phage aggregation depended on temperature, with bigger sized aggregates at higher temperatures[42].

Importantly, they also found that T4 phage aggregation caused by reduced salt is a reversible process. In their proposed mechanism, phage virions cluster when they leave the host bacteria and enter a low-salt environment, ensuring survival until they reach the next host. These aggregates can be stable for a long time, until they destabilize and disperse into single virions, upon increase of ionic strength[42].

These aggregation and dispersion cycles were also discovered in the recently developed purifying method oWILT, which effectively traps lipopolysaccharides. An unexpected result of this procedure was the discovery of this aggregation trigger. When the ionic strength is lowered to 20 mM, the phage particles quickly cluster into groups of 20-100 virions and, with the return of high ion concentrations, dispersion of an aggregate into single viable virions occurs[46].

These transitions allow the retention of phages on common microfilters, which can be seen as a breakthrough in biotechnology. The simple action of lowering the Na+ or K+ concentration below a

threshold appears to be sufficient to make small virions aggregate into larger aggregates that do not pass filter pores, which is a crucial step in concentrating phages for therapeutic uses and other applications[42,46].

2.2 GMP Phage Production and Manufacturing

2.2.1 Good Manufacturing Practice (GMP) for Phage Therapy

Current Good Manufacturing Practice regulations (cGMP) are, summarized by the American Food and Drug Administration (FDA), the "minimum requirements for the methods, facilities and controls used in manufacturing, processing and packaging of a drug product. The regulations make sure that a product is safe for use, and that is has the ingredients and strengths it claims to have."[47]

Good manufacturing practices represents the standard to guarantee quality, safety and efficacy to medicinal products, investigational or market approved. Resuming, CGMPs provide for systems that assure proper design, monitoring, and control of manufacturing processes and facilities. Since phages are classified as medicinal products, health agencies demand that the products given to patients are manufactured in compliance with GMP and follow the evaluation through clinical trials before obtaining market authorization[48].

The main goal is to control the manufacturing process through implementation of several duties, such as the qualification and monitoring of the facility and the equipment, the coaching and validation of the personnel, the qualification and control of raw material and consumables, the authentication of the process and quality control methods, and the inspection by the authorities[47,48].

2.2.2 Manufacturing Process

The establishment of phage therapy depends on the ability to efficiently manufacture phages across laboratory, pilot and industrial scales. Process development is the key to define the manufacturing process. This process refers to development, qualification, validation and scale-up of the process to manufacture, exploration, identification, development and scale-up of the product, any analytic development and product characterization[6].

The manufacturing process of a given phage involves several activities that are designed with the objective of producing a certain number of biologically active particles with specific qualities, which can be grouped into upstream and downstream processing stages[6]. The upstream process includes the phage and host selection, master and working phage and cell banks, and the host propagation and phage amplification processes, while the downstream operations include all the procedures needed to purify the phages, such as clarification, phage capture and purification to meet purity, potency, and safety requirements, while yielding the active drug substance. It provides the necessary transition from

the laboratory to a manufacturing environment where the target product is produced[6,49].

Understanding the biology of the phage infectious cycle is a crucial aspect in the design of upstream processes. The bacterial cell culture and infection are the center of the phage manufacturing process. The objective is to produce a great number of phages, measured as plaque-forming units (PFU). The production of phages for pre-clinical or clinical tests are based on lab-based protocols that were originally developed to prepare small amounts, and so low-volume batches, low cell densities, loosely controlled operating conditions and moderate phage titers are expected[6].

Before the GMP manufacturing process is initiated, the phage needs to be selected, where only strictly lytic phages are considered as possible therapeutic treatment, since lysogenic prophages are capable of carrying bacterial genes that can propagate in the patient. Other important factors to consider prior to the start of the manufacturing process are the phage host range, the usage of common bacterial hosts for production of several phages against a bacterial species, phage titer and stability. At the same time, the host bacteria should be selected very carefully while respecting several criteria, such as low level or absence of prophage and human or bacterial toxins[47].

Every phage batch should ensure reproducibility, process and product consistency and so, cGMP requires that the phage and cell source should always be the same. To fully comply with cGMP, all used materials should meet specific criteria, for example, no material should be derived from animal-components and a two-tiered system with a first level master bank and a second level working bank should be implemented[6]. The master phage bank (MPB) is essential and ensures the preservation of a uniform population of phages, their integrity, and a sufficient supply. The working phage bank (WPB) is then produced from a single vial of the MPB, and phages are to be expanded from the WPB for daily production[47].

Parameters like the adsorption rate (rate at which the phages attach to bacteria), the latency time (time between attachment to lysis), and the burst size (number of phages released from a bacterium) are crucial and define and characterize the phage population growth[6,50,51]. The rate of adsorption is associated with the first stage of the phage life cycle, and together with adsorption efficacy, it depends on the phage type. The phage latent period is influenced by parameters that define the physiological states of both bacteria and phages and the burst size relates to the bacteria-phage system and environmental factors. Additionally, multiplicity of infection (MOI), or commonly used as the number of phages added to start the infection per host cells, is also critical since it determines the dimensions the culture must have to generate the required number of phages[6,51].

The metabolic activity, specific growth rate and the density of phage receptors on the host will affect the previous parameters[50]. Process conditions such as temperature, pH, medium composition, agitation, aeration rate and presence of ions or cofactors can also impact the outcome of infections and, consequently, the yield of bacteriophage in the bioreactor operation[6,51]. The number of phages produced relatively to the amount of host cell debris generated is also a very significant aspect and can impact the downstream processing[6]. At the same time, it's important that the phage and host strains used should be free of virulence genes, since production at the industrial level requires large quantities, and the culture conditions should prevent coevolution of host and phage[23,50].

To optimize phage titers, the process should be designed to minimize the latent time and maximize adsorption rate and burst size[50]. This process optimization should also be directed towards infection conditions that deliver different quantities of phage and bacterial cells[6].

The type of bioreactor used is a very important aspect in establishing the fermentation conditions that will lead to optimal phage titers. These can be operated in batch, semi-continuous and continuous modes[6,50,51].

The most common operation for phage production schemes is the batch operation. Here, phage infection occurs in the same fermenter where bacterial growth occurs. The host cells are grown to a certain density and the infection starts at a given MOI. The host and phage population grow until the phage population takes over and cell lysis occurs, in a process highly dependent on momentary conditions and population dynamics[6,50,51].

When the infection is initiated at a low host infection load and low initial MOI, there is opportunity for the host population to grow to a high cell number before the population-wide lysis takes place. When a large infection load is used, together with a high initial MOI, the goal is to infect a large portion of the host population as fast as possible, to obtain a rapid population-wide lysis. The first approach leads to shorter batches while the second is favored for obtaining greater final titers and lower levels of cell debris[51].

It is the cheapest mode of manufacturing, very robust and easy to manage and control, and it is capable to achieve high titers. However, it has some downsides, such as low throughput, entailing a lot of manpower and requiring large equipment footprints and volumes. It also has a high cost of capital investment, and lot-to-lot product quality variability. Batch operations have a high downtime to production time ratio, since the bioreactor must be cleaned and sterilized prior to each run, if one is not using disposable systems[6,50,51].

Continuous processes, the most common being the chemostat and the turbidostat, provide significant benefits for bacteriophage production as it can overcome some of the limitations of batch operations[6,50,51]. Here, fresh culture medium is continuously supplied, while the depleted medium with cells and phages is continuously withdrawn from the bioreactor. It allows higher productivity, reduced costs, long runs, which reduces the downtime and increases equipment utilization, and easier control, which contributes to a better quality and consistency of the product. However, the setting up process is challenging and quite complex[6,50].

Single-state continuous bioreactors are not recommended for phage production at large scale since the dynamic nature of the host-phage interaction, like the rise of mutations and coevolution, makes it very difficult to operate the system at steady-state, a crucial criterion for reliable continuous production. Any variation in the addition rate of nutrients, rate of host cell proliferation and infection, and rate of cell lysis can lead to a different steady state, in terms of host cell concentrations and phage titer, or to the washout and loss of phage production[6,51].

To overcome these challenges, a multi-stage continuous system can be used. It consists of various connected continuous bioreactors with the objective of dissociating the growth of the host from

the infection process[51]. The bacterial cells are grown in a first bioreactor and infected in subsequent bioreactors, which allows continuous high-titer production, and minimal or no coevolution and mutations[6,50].

The two-stage continuous model consists of two continuous stirred tanks coupled with a final holding tank. The system enables control over the physiological state of the host to keep them in their exponential growth phase, optimal for phage production. It is then possible to create longer residence times for bacterial cells yielding higher bacterial concentrations. The two reactors can be operated at a different set of conditions, and can differ in volume, since the specific growth rate of the bacterial cells is significantly lower than the phages' and the second bioreactor is operated at a much higher dilution rate[6,50]. To sustain steady state, the rate of host cells fed to the second bioreactor should be proportional to the infection rate, or a significant number of phages will leave the fermenter without infecting a host cell[6].

Unfortunately, this process is not without its limitations. Since there is still a residence time distribution in both stages, and some host cells can be replicating in the second stage, there is a possible appearance of a resistant bacterial cell. At the same time, uninfected host cells are present in the outlet stream, which is not ideal[6,50].

Phages can also be produced in two-stage semi-continuous operations. It is similar to the two-stage continuous model but includes the addition of a self-cycling process[6]. The host is grown until a near-stationary phase in the first stage, which is operated as a sequential batch reactor, or as a self-cycling fermenter, and infection occurs in the second stage[6,50,51].

In the self-cycling fermenter, half of the bioreactor content is removed, when the cells reach the beginning of the stationary growth phase, and is replaced with fresh medium, starting a new growth cycle. This helps avoid the lag and stationary growth phases so that the cells are always in exponential growth and provides synchronization of the host cells with all cells dividing at the same time[6]. In the second bioreactor, operating under an automated self-cycling infection, a fraction of the previously produced phages is used to infect the host fed from the first stage[6,51].

This operation mode combines advantages of both batch and continuous systems, while improving the previously encountered issues. It includes the robustness and high phage titers obtained from the batch operation and the high volumetric throughput, reduced downtime and smaller equipment footprints from the continuous operation. Additionally, there are no residence time distribution issues and a significant decrease in the probability of coevolution[6,51].

Once the phage culture has produced the desired yield, and the phage is harvested, processing reagents, cell debris, host cell related molecules and potentially derived viral agents can be present[52]. The goal of the downstream process is to isolate, concentrate and purify the target phages to obtain the required product, since phages used for commercial purposes need to be free of process contaminants and impurities, which can present a health risk[6,15,50]. It should encompass a limited number of high-yield and short steps to minimize complexity, processing costs and residence time[6].

The major downstream steps are clarification, where the phage is separated from host bacteria

cells, capture, where the solution volume is reduced and the phage is isolated, subjected to purification and polishing, to remove impurities, prior to pre-formulation[47]. High phage titers upstream may result in higher levels of contamination in the crude lysate, which can have adverse effects in the subsequent downstream separation techniques[49]. In general, several unit operations are required to achieve the final specifications[6].

Following cell lysis, the first step is usually clarification, the removal of cells and cell debris from the phage-rich culture broth. Microfiltration, with 0.22 µm or 0.45 µm membranes, or low speed centrifugation can be used, or a combination of both. A common issue in this step is the high viscosity of the bacterial lysate, due to the release of nucleic acids. A nuclease treatment can be used to solve the problem[6,47].

The next step is often concentration or capture, to increase phage titers in the clarified supernatant stream, with tangential flow filtration (TFF) or precipitation. Volume reduction is the major goal, but some impurities can also be removed. The resulting stream will be comprised of variable amounts of genomic RNA, DNA, proteins and LPS that should be removed next. Several combinations of unit operations can be used in this step, such as additional TFF and precipitation steps, chromatography, and extraction with aqueous two-phase systems (ATPS)[6].

TFF is regularly used for separating, concentrating, and purifying biomolecules. When maintaining a constant transmembrane pressure and fluid flow along the membrane, the phages are concentrated and purified, while recirculating in the filtration system. However, a prolonged recirculation can have a negative impact in the phage stability and can damage the phage during the filtration process[47]. Ultrafiltration membranes are defined by their molecular weight cut off (MWCO) and, to guarantee a high retention of phages, diverse cut offs must be tested[6,47]. This method can also be used for the removal of endotoxins and is useful to condition the phage stream prior to chromatography, or even to introduce a more appropriate final buffer[6,50].

Precipitation is a simple and inexpensive technique that can be used for phage concentration and purification. It involves the addition of agents that promote phage precipitation, which can then be resuspended in a smaller buffer volume to obtain a concentration effect. A big advantage is the removal of un-precipitated low molecular weight impurities[6].

Polyethylene glycol (PEG), in combination with salts like sodium chloride, is the most common agent used for phage and virus precipitation. When adding PEG chains with an adequate concentration it is possible to sterically exclude phages from the solvent, promoting their precipitation. By adding salts, significant amounts of water molecules are seized for solvation, which will promote self-association and precipitation[6,15]. However, and although commonly used in research, this method is difficult to scale up with reproducible virus yields[47].

ATPS can also be used to concentrate and purify viruses. This operation is composed of two different water-soluble polymers, or of a polymer and a salt, that form two immiscible aqueous phases when mixed above a specific concentration. To promote a viable separation, phages and impurities should partition differently between these two phases, which is a function of their properties and system

parameters[6].

Several studies have been focusing on the use of ATP (adenosin 5'-triphosphate) for phage purification, like *Negrete et al*., who studied the partitioning of the T4 phage in PEG-8000-phosphate and PEG 600-sulphate ATPS. Unfortunately, although the phage partitioned preferably to the PEG 8000-phosphate system, the PEG 600-sulphate system presented a negative impact in the phage titer, probably due to the high concentration of the phase components used in this particular case, which could have led to the precipitation of phages at the interface[53]. Although ATPS can be interesting for phage purification, its implementation at process scale can have a number of obstacles such as the increased cost of goods, due to the large quantities of mass separating agents required, and the fact that increasing phage concentration in the target phase is difficult, meaning that large volumes are carried over to subsequent steps[6].

Chromatography separation techniques are based on the interaction between the target phage and the matrix column. It is the most commonly chosen method when high purity is required and it has four main points: physical structure and surface chemistry of the stationary phase, mobile phase composition, chromatography mode, and equipment. Different interaction modes can be used for phage purification, such as ion-exchange, size-exclusion, and affinity chromatography. Current methods focus on separation of phages from bacterial proteins, DNA, lipopolysaccharide, peptidoglycan, and other impurities. However, consideration should also be given to lytic phages that may also be contaminated with temperate phages[6,47,50].

Ion-exchange chromatography for phages is dependent on their charge. Ion-exchange refers to when the stationary phase is positively charged and negatively charged molecules are loaded and attracted to it. This type of chromatography requires individual experimental approaches for every phage, since their isoeletric points may differ. Most phages have an anionic nature with a pI < 6 and so anion-exchange chromatography (AEC) is used as a capture step. The separation is performed by selective elution, by changing the pH of the buffer or increasing its ionic strength. The phage solutions should be loaded on the column at an ionic strength high enough to avoid the binding of impurities with lower charge densities, however, many negatively charged impurities may still bind to the anion exchanger. Because of this, additional polishing steps may be mandatory to reach the required purity[6,47,50,52].

Other chromatographic methods can be used to purify phages. Monolithic chromatography, an alternative for resin-based methods, is based on large pore size monolithic anion exchangers, quaternary amine, and diethyl residues, and it can be combined with other purification methods. Chromatography monoliths are one-piece porous solids made of fused micrometer-sized globules of silica or an organic polymer that can be synthesized directly inside a chromatography tube. A necessary strategy for the success of this type of chromatography is to ensure the necessary contact of the materials, i.e. by connecting the materials covalently [6,47,50]. SEC, or size-exclusion chromatography, is a size-based separation technique that uses silica or agarose beads as a stationary matrix and can be used for separating phages from impurities, which are retained in the column[6,47].

In affinity chromatography, affinity-tags are used to purify phages from bacterial impurities and

similarly sized, which can be used to remove specific impurities like endotoxins[6]. For example, glutathione S-transferase (GST) and His-tag can be used as affinity tags to separate phages from other similar phages or to remove endotoxins[27,54]. However, when using affinity chromatography to remove endotoxins, some of the ligands used, like polymyxin and histamine, are not suitable for manufacturing of therapeutic products, due to their intrinsic biologic activity[6]. Affinity chromatography is one of the most effective methods for protein purification. This technique comprises a one-step procedure with a level of purification on the order of several thousand times, it is adaptable to various proteins, differentiating them in their size, shape, charge and other properties[27].

Multimodal chromatography is a recent technology that offers scalability, single use and other advantages. The pores of its matrix are modified with ionic charges, hydrophobic groups and affinity ligands, which makes it possible to combine size separation with chromatographic binding[6,47]. CaptoTM Core 700 is a promising multi modal resin that combines properties of SEC and adsorption chromatography by using beads with a functionalized core with an octyl-amine ligand attached and a nonfunctionalized outer layer. Here, viruses can flow through while impurities smaller than 700 kDa are captured[6,55].

The polishing step is critical to achieve the levels of purification of the phage drug substance potency and quality necessary. It should resolve impurities with similar properties to the phage, while minimizing phage losses. Endotoxin removal remains a challenge since no universal scalable purification method has been published. Although endotoxins are bound to the bacterial cell wall, they are continuously liberated into the environment, and are very stable molecules, able to resist to extreme temperatures and pH values. Besides phage dilutions, octanol liquid-liquid extraction (LLE), TFF and affinity chromatography have been used, although not without its limitations[49].

Formulation studies need to be conducted to test the phage stability during manufacturing and long-term storage. The development of phage formulations with improved stability with respect to physical and chemical stresses and for long-term storage will expand the therapeutic application of phages. Various parameters should be tested, such as pH, buffer composition, phage particles density, salt concentrations and types and stabilizing agents since formulation can severely change the shelf-life of a phage[49].

As a final task, filling, visual inspection, labelling and packaging are important, and any mistakes can lead to product misformulation, contamination or improper packaging of a whole produced batch[47].

2.3 Phage Detection

When working with phages, the determination of the concentration of the phage particles is an essential process[56]. The oldest, but still the most common technique is the double agar overlay plaque assay, or commonly referred just as plaque assay, where phage plaques are counted on agar plates seeded with the bacteria in which the phages can propagate[9,10]. However, there are other approaches

one can use to enumerate phages, such as transmission electron microscopy, or qPCR[11,57]. The advantages and disadvantages of each technique mentioned is presented on table 1.

2.3.1 Plaque Assay

The plaque assay was described shortly after d'Herelle discovered phages. A phage plaque is, by definition, a clearing in a bacterial lawn, formed via outward diffusion of phage virions fed by bacterial infection, and its development and size can be affected by anything that slows phage diffusion[9,58]. The bacterial cultures used to support plaque formation are described as an indicator strain or indicator bacteria, while the phages that are added can be either isolated virions or previously phage-infected bacteria[59].

In the double agar overlay plaque assay, serial dilutions of the phage preparation are mixed with the host bacterium that will support phage propagation, such as a dilute, mixed with molten agar, and dispersed evenly, to solidify, onto a standard agar plate. The uninfected bacteria will then resume their growth and form a lawn in the overlay when they eventually reach stationary phase. The phage particles will encounter the bacteria, infecting them, and the lytic cycle will be repeated numerous times, when the progeny phage from each infected bacterium infects neighbouring bacteria. After incubation, usually overnight, this will result into a growing zone of lysis, which becomes visible to the naked eye as a "plaque" in the smooth bacterial lawn[9,56]. Since the plaque only grows as long as the host cell metabolizes actively, when cell growth finishes, phage growth also halts. Each phage particle that results in a plaque is called a plaque-forming unit (PFU)[9].

PFUs can be described as both free phages and phage-infected bacteria. A single host cell, or a clump of cells, can be infected by a single virion, and all represent a single PFU[59].

Serial dilutions are necessary to enumerate a large number of phage particles encountered during culturing. Bacteriologic culture or phage diluent can be employed for this purpose, but it is important that this medium is isotonic, to prevent phage rupture from osmotic shock. If a phage stock has originally 10^8 PFU/mL, a final phage concentration of about 10^3 PFU/mL is desired, as 0.1 mL of this final dilution will have an average of 10^2 PFU/mL, which is appropriate for enumeration via plaque-count on one plate. The assay should always be done in duplicates or triplicates, as repetition of theismethod increases its robustness and precision[9].

In each plate, a sufficiently turbid bacterial lawn must be able to form, so that the plaque is visible. The plate needs to have sufficient plaque numbers for enumeration, but not so many that it is not possible to distinguish one from another. This issue of statistical importance is referred to as Too Few to Count (TFTC) or Too Numerous to Count (TNTC)[59]. The usual acceptable range is from 30 to 300 plaques[60].

The problem with high plaque counts is that plaque overlap leads to plaque undercounts, with multiple plaques being counted as an individual plaque, which signifies a measurement bias and results in incorrect titer determination. Smaller plaques are better distinguished from each other than larger plaques, but they can also be more difficult to distinguish from bubbles or other irregularities in the lawns,

and so more difficult to count. While larger plates can be used, there are limits to how large Petri dishes can be, in terms of time, handling, material costs and convenience. The issue of TNTC is most pertinent to determining which dilution, from a series of dilutions, is the most appropriately employed towards titer determinations, so that it does not provide either TNTC or TFTC counts[60].

On the other hand, too-low plaque counts lead to a problem of high between-plate unbiased error, making it an error of precision. Nonetheless, one should not avoid counting individual plates just because the plaque counts are too few to count, since it also introduces biases. It's possible to make up for too-low plaque counts by doing more platings per data point[60].

The clumping of phages into individual plaques has several outcomes, plaque visibility, secondary adsorption/infection, plaque variation across their diameters, and a potential to gain information from their appearance. The plaques can become macroscopic, making phage enumeration, isolation and characterization possible[59].

Plaques are three-dimensional entities that show variations in properties, as a response of location within a plaque and its periphery. They can be differentiated from inside outward, in three zones: (1) an area where no infected or phage-sensitive bacteria remain, (2) an area where infected bacteria persist but also where new phage infections are no longer initiated and (3) an area with a combination of reduced bacterial densities, phage-infected bacteria, phage virions and phage-uninfected bacteria, where new infections are beginning, and older infections are ending. In terms of turbidity, it is expected to be greater the nearest it is to the plaque's periphery, declining towards the plaque's center[58]. Additionally, it can be possible to observe halos outside of the plaque. These occur due to the diffusion of hydrolytic enzymes and, usually, can continue to grow even after plate/plaque refrigeration, since they are a consequence of partial chemical decomposition of uninfected bacteria[59].

Another consequence from phages displaying clumped dispersions is phage secondary adsorption. This refers to additional phages that adsorb to an already infected bacterium, and it is highly likely during plaque formation, since phage densities are higher within plaques. This can lead to lysis inhibition during plaque formation in some phages and induction of lysogenic infections for temperate phages. Lastly, different phage types can be distinguished by their plaque morphology, in terms of plaque size, or other subtler characteristics[59].

Plaque size is, usually, a conjunction of the duration of plaque growth, and the speed of plaque-size increase during plaque formation. The duration of plaque growth is affected by both phage and bacterial properties. A larger plaque growth is an outcome of early initial phage adsorption during lawn maturation, if all else held constant. Longer latent periods should result in slower plaque growth since virions will then spend less time diffusing, while faster and longer diffusion times should result in faster plaque spread. However, it is important to remember that physical and chemical aspects of the environment can affect these properties[59].

Richer media can provide shorter phage latent periods, making it so that lawns can mature earlier due to faster bacterial population growth, which can then lead to potentially faster plaque growth rates. At the same time, bacteria in richer media will probably grow to higher densities, which increases

the duration of plaque formation[59].

Additionally, there are two main ways by which phages can be applied to bacterial lawns. They can either be mixed with bacteria before lawn initiation or applied to lawns after lawn initiation. It's also possible to generate phage plaques by applying phages to already initiated lawns, where small volumes of liquid are dropped on top of already applied bacteria, allowing for multiple, singular phage enumeration processes per individual plate, called spots. These spots are a contained area where lawn turbidity is reduced, initiated by multiple bacterial growth inhibiting entities, and they can be produced by entities other than phages, present in phage lysates[59].

They should never be a certain description of a productive infection since false positives are common. One way to overcome this issue is the use of serial dilutions. In contrast, absence of a spot or plaque can be viewed as an indication that a bacterium, under the tested conditions, does not support phage replication, since the phage can kill the host, but not produce progeny[59].

However, while the plaque assay method is still considered the standard for determining phage concentrations, due to its simplicity, efficiency, and easy implementation in any laboratory with minimal investment costs, it is not without its drawbacks[10,11].

It has high variability and poor reproducibility, which is fundamental when manufacturing phage medicines. A small change in the nutrient agar can change a viable titer of phage preparations by more than 1000-fold, and the same phage against different bacterial host strains can yield different titers. This happens because the titer is dependent on the replication of the phage in a specific host, when we change the host, we are changing the phage replication and therefore, the phage titer[20].

At the same time, operator bias, pipetting errors, change in bacterial growth parameters and contaminations can severely impact the results. It can also be time consuming, as 18 to 24 hours are required to complete the assay, and it has to be optimized for every phage-bacterial host pair[10,11].

2.3.2 Electron Microscopy and Other Techniques

Electron microscopy has two main types, scanning and transmission where, in both, we have an electron beam aimed at a specimen. In scanning electron microscopy (SEM), images come from electrons that are reflected from the specimen while a small diameter beam is scanned across the area of interest and, in transmission electron microscopy (TEM), there's a large diameter beam that illuminates the area of interest. These can be combined as scanning transmission EM (STEM) in phage studies, but TEM is the predominant form used[57].

TEM can be used to quantify viral particles, although the sample must be highly concentrated (~10^6 particles/mL). It is highly accurate when determining the morphotype and total number, but it is considered expensive, time-consuming, and impractical for running several samples at once, and cannot be used for more complex samples. It requires a skilled operator, and the sample preparation can be tedious[11].

In EM, stains are used to improve contrast since, when used, it becomes the main contribution

to an image from the electrons scattered from the electron beam. Metals are more robust than the organic molecules of the biological specimens and so, by using them as a stain, we can avoid damage from the electron beam. In addition, the stained specimens last much longer[57].

Negative staining is the most used technique for examining phages by EM. Preparing the specimens is fast, easy, and inexpensive, while being the most dependable for quickly assessing phage samples, for sample purity, particle integrity and aggregation state. It involves coating the specimen with a heavy metal salt, which forms a cast around the biological object. If the object is porous, this salt will also infiltrate to internal regions. It also increases the density of the volume surrounding a phage particle, unlike other staining techniques[57].

Preparing the specimen can be done in less than five minutes. A solution of the phage is held by a grid support on a carbon support film, and the particles are adsorbed to it. It can then be washed to eliminate unbound materials. Lastly, the specimen is placed in a metal salt solution followed by air- or vacuum-drying, or blotting with filter paper, before imaging[57].

Unfortunately, it has limitations such as drying and chemical effects, limited resolution, and viewing of only stain-accessible surfaces. The obtained resolution is likely restricted by the chemical effects of the stain and the physical effects of drying. Particles can sometimes appear distorted, collapsed, or crushed. The phage structure may also be affected by the pH of the stain, as it is not physiological, and it changes as the specimen dries[57].

Flow cytometry is a technique that can be used to count whole particles. The phage particles are marked with a fluorescent dye and directed through a capillary. Its small diameter forces the particles through a single line, which enables the detection of light scatter caused by each particle. It is a fast and rigorous method. The fluorescent signal can be used to relate the number of target nucleic acid molecules to the number of viral particles. However, this is only possible if the samples are handled gently, no surfactants are used, negative controls are included to determine the auto-fluorescence of the medium, and the instrument and assay sensitivity is estimated prior to the experiment[11].

Epifluorescence microscopy can also be used, rapidly and accurately, to determine the abundance of phages. It has been shown to have higher accuracy and precision than the last previously mentioned techniques, TEM and flow cytometry, for counting virus particles. This method requires the viruses to be collected onto a small pore-sized filter and stained with a fluorescent dye, which then is excited with a specific light wavelength, and emits light a longer wavelength when bound to nucleic acids. This results in a glowing particle larger than the true size of the phage particles, making it possible to count the particles at a much lower magnification[61].

Originally, DAPI (4’6-diamidino-2-phenylindole) was used to count viruses in marine samples. The dye is excited with UV light and emits a blue light. Since the light yield from DAPI is relatively low, microscopes with high quality optics were mandatory. Other dyes can be applied to count viruses such as Yo-Pro-1, SYBR Green I and SYBR Gold, and all have been shown to provide accurate estimates of virus abundance[61].

A laser-based method, developed by NanoSight Limited, provides real time visualization and

allows enumeration of viral particles (viable and nonviable) in only a few minutes, based on dynamic light scattering by-laser illuminated optical microscopy[10,11]. This method has been found to be affected by background particle distributions and its results are difficult to interpret since various media for propagating bacteria elicited high levels of background noise. Although it offers results in a 5 or less minute timeframe and does not require any additional reagents, it has several drawbacks. It requires expensive equipment, it only provides reliable detection of phages larger than 40 to 50 nm, the phages must be suspended in a clear medium, and it requires phage concentrations between the 10^7-10^9 PFU/mL[10].

A quantitative real time PCR (qPCR) based approach has also recently been proposed for determining phage concentrations. PCR is a simple method, based on the detection of nucleic acids that verifies the presence of phages faster than plaque assays. qPCR has been shown to be a very accurate method for the quantification of phage particles, with high reproducibility and long-term stability[11,62]. Lysates quantified by qpCR have an excellent correlation with plaque assay titers when the standard and samples are treated similarly[62]. Additionally, it only requires a few reagents, provides high throughput, since many phage preparations can be examined at the same time, has a fast turn-around time of 3 to 4 hours, and has a potential for greater precision than the plaque assay method[10].

Although promising, qPCR also has its limitations. Besides the expensive equipment requirement, the PCR amplification conditions have to be optimized, an ongoing calibration with known standards, which is analyzed alongside with the test samples, is needed, and specific oligonucleotide primers and specific probes are critical[10]. Most importantly, it tends to report values higher than the real ones, since the number of phage genomes in a sample are always much higher than the number of actual intact phage particles, which is why it is not possible to distinguish viable from non-viable phages[63].

In 2019, mass spectrometry was proposed by Wang et al. as a way to enumerate M13 phages, via a large and a short peptide derived from a coat protein. The number of M13 phages was calculated using the Avogadro's number and the copy number of the protein per phage which was coincided well with the manufacturer provided number. It's important to note, however, that the size of the phage capsid will depend on the size of the encapsulated nucleic acid. As a consequence, its copy number can vary from wild type to mutant phages[63].

Table 1 – Advantages and Disadvantages of each phage enumeration method.

	Methods					
	Plaque Assay	Flow Cytometry	Epifluorescence Microscopy	TEM	NanoSight	qPCR
Advantages	Simple, no specialized equipment needed and allows direct	Fast and rigorous	Fast, high accuracy and precision	High accuracy	Very fast (<5 min), high reproducibility	Fast (3 to 4 h), high reproducibility

	enumeration of viable phage particles					
Disadvantages	Time consuming (18 to 24h), poor reproducibility and high variability	Expensive equipment, needs previous labelling of the sample	Expensive equipment, needs previous labelling of the sample	Expensive equipment, time consuming and requires high concentration of the sample	Expensive equipment and results are only accurate when phages are in clear solutions	Expensive equipment, requires synthesis of specific primers, can't distinguish between viable from non-viable phage particles

2.4 Dynamic Light Scattering

Dynamic light scattering (DLS) or photon correlation spectroscopy, is a technique that measures the size of particles. When the laser light encounters the particles, the incident light scatters in all directions and this scattering intensity is detected and documented by a detector. The modulation of the intensity of the scattered light is then analyzed as a function of time[64,65].

DLS is based on the Brownian motion theory, which explains the molecular motion of particles. Particles are subjected to random forces due to their continuous collision with solvent molecules, which results in random walk of particles, and, because of this motion, the mean squared displacement of particles is proportional to time[64]. DLS measures the Brownian motion of macromolecules in solution and relates it to their size. This motion will depend on the macromolecule size, temperature, and solvent viscosity. By monitoring their movement over a range of time, it is possible to get information on their size, since large particles diffuse slowly when compared to small particles[64].

Furthermore, scattered light waves combine to form a resultant wave where the amplitude is the sum of the individual waves. Interference can occur when light waves cancel out (destructive phases) or amplify each other (constructive phases), resulting in a measurable signal that is recorded and monitored by a detector[66].

The DLS instrument then generates a correlation function that is linked with particle size and its time-dependent light scattering capacity[65]. The intensity pattern is employed in this correlation function, which essentially indicates how long a particle stays in the same place within a solution. The function exponentially lowers as the particle moves away until it reaches the baseline, suggesting that the molecule is no longer in close vicinity to where it was originally. Since smaller molecules move faster than larger molecules, their correlation functions will differ, resulting in a slower decay for a larger particle[67].

DLS is a sensitive, non-intrusive, and powerful analytical tool, normally used for characterization

of macromolecules and colloids in solution[68]. It is important that samples for DLS analysis have already been purified by various forms of chromatography to achieve a high level of purity[64].

It has been previously used to study the stability of formulations, measure the particle size of dispersing colloidal samples and to detect the presence of aggregation. Since the molecular weight determines the scattering intensity of the particles, the technique is extremely sensitive to the presence of aggregates. Aggregated particles show a high polydispersity index (PDI), since they have a large variation in particle size. This polydispersity index describes the width of the particle size distribution, and it may vary from 0 to 1. Particles with a PDI less than 0.1 are monodispersed, and particles with values over 0.1 can imply polydisperse particle size distributions[65].

Depending on the chosen distribution, different weights can be assigned to a particle, i.e. signals can be amplified with variable scales. . The most common is the intensity-weighted distribution, where the contribution of each particle is proportional to the sixth power of its diameter. Two different size-related factors emerge from the size analysis: z-average and peak mean. When a sample is monodispersed, Z-average is the computed size of the entire sample and is considered the most reliable size parameter. Peak mean calculates the average size of each peak in a measurement, making it more reliable when a measurement has many peaks, and thus, the sample is polydispersed[67].

Although it is a very useful method, it does have its disadvantages. Its measurements are very sensitive to temperature and solvent viscosity, and so, the latter must be known, and the temperature should be kept constant. It is a low-resolution method that, oftentimes, cannot separate molecules that are closely related[64]. It is also restricted to transparent sample preparation. The cuvette used to hold the sample must be thoroughly cleaned, while the sample must be filtered prior to the experiment. Lastly, the signal depends on macromolecule's size and concentration, and so, to obtain trustworthy measurements, an optimization of the range of concentration is recommended[64].

Dual-angle DLS can also be used for detection of protein aggregation. As previously mentioned, DLS measures the size and size distribution of proteins in solution by measuring the intensity changes of scattered light. When measuring the sample with the backscatter angle, we are seeing a small sampling of our solution, that is, we are seeing scattering of particles near the cuvette surface. When the size is measured with the forward angle the laser has to completely cross the cuvette, and so, it is more likely that a larger particle, or an aggregate, will be detected[69,70]. If the particle size, when compared to the wavelength (λ) of the laser light, is very small, the scattered intensity is the same at all detecting angles. The scattered intensity begins to increase in the forward direction when the size of the protein or aggregate climbs to greater than λ /10 and approaches the wavelength of the laser. The beginning of protein aggregation can thus be monitored using measurements in both the backward and forward scattering directions. An aggregation index can then be calculated based on the mean z-average size measured for the two angles of scattering according to the equation below[71]:

$$Aggregation\ index = \frac{Z_{average\ forward}}{Z_{average\ backward}} - 1$$

More recently, MADLS (multi-angle dynamic light scattering) has become available. By

integrating scattering patterns from many angles, multi-angle DLS (MADLS) improves resolution over single-angle observations (13º, 90º, 173º). This can provide several benefits, such as improved component sensitivity within mixtures, improved resolution of components that are close in size and particle concentration can be calculated using the transformed number weighted size distribution[72].

MADLS has been showed to have applicability in the bacteriophage field. As presented in an FDA workshop for “Science and Regulation of Bacteriophage Therapy” by Malik, MADLS is believed to be able to differentiate individual phages from aggregates and quantify them in the different peaks obtained[12].

DLS has also been used as a fast complementary method for the characterization of proteins. The structural integrity of monoclonal antibodies can be monitored through DLS, by determining their hydrodynamic diameter, sample homogeneity and monomer-dimer equilibria. Since molecules are analyzed in their natural buffer environment, outside effects do not compromise how they behave. This technique, alone, in combination with size exclusion chromatography or analytical ultracentrifugation, is advantageous due to its speed, minimal volume demand, and simplicity[73].

Also, for example, subcutaneous injection of monoclonal antibodies can be an appealing and practical form of protein self-administration, especially for the treatment of autoimmune diseases and cancer. In this situation, because of the limited volume for subcutaneous injection, highly concentrated protein solutions are needed and, as a result, major issues with stability and formulation have been brought up, such as protein aggregation and viscosity, since an extended injection time can affect the patient's convenience and compliance when a solution has a high viscosity. Here, DLS has the ability to help in the creation of a highly concentrated protein formulation with an adequate level of conformational stability to reduce the high viscosity effect[74].

It is essential to identify and quantify sub-visible particles in protein medicinal products to ensure the quality of this class of medications. Protein aggregates are particularly dangerous because they may enhance the possibility of fatal immediate or delayed immunological reactions and diminish or even completely abolish the product's efficiency. DLS can then be used to analyze these particles in the sub-visible range for aggregation in protein therapeutic products[75]. It has also been used for the monitoring of protein unfolding for vaccine manufacturing, where a vaccine candidate, recombinant fusion protein SP1, was analyzed. Yu and team found that the unfolding of SP1 as a function of urea and the quantity of reducing agent is accurately reflected by the hydrodynamic radius determined by DLS and showed that this method can be utilized as a process analytical technology to supervise inclusion bodies solubilization, protein refolding and aggregation, which will help to reduce process variability and guarantee product quality[76].

3. Materials and Methods

A flowchart representing the general experimental process performed in this work can be observed in Figure 4.

Initially, T4 phage lysates were prepared in small-scale amplifications, mainly to determine the optimal conditions, such as the multiplicity of infection (MOI). Afterwards, a large-scale amplification was performed in a bioreactor, where the lysates were then filtered. The double-agar overlay plaque assay was performed after every amplification, for phage titre quantification. Then, chromatographic runs were conducted several times, until an optimized method was obtained. In each chromatography peak, the phage titre was quantified, as well as the endotoxins present. Lastly, each chromatography sample was measured for particle size in DLS and, later, aggregation induction experiments were performed and measured in DLS.

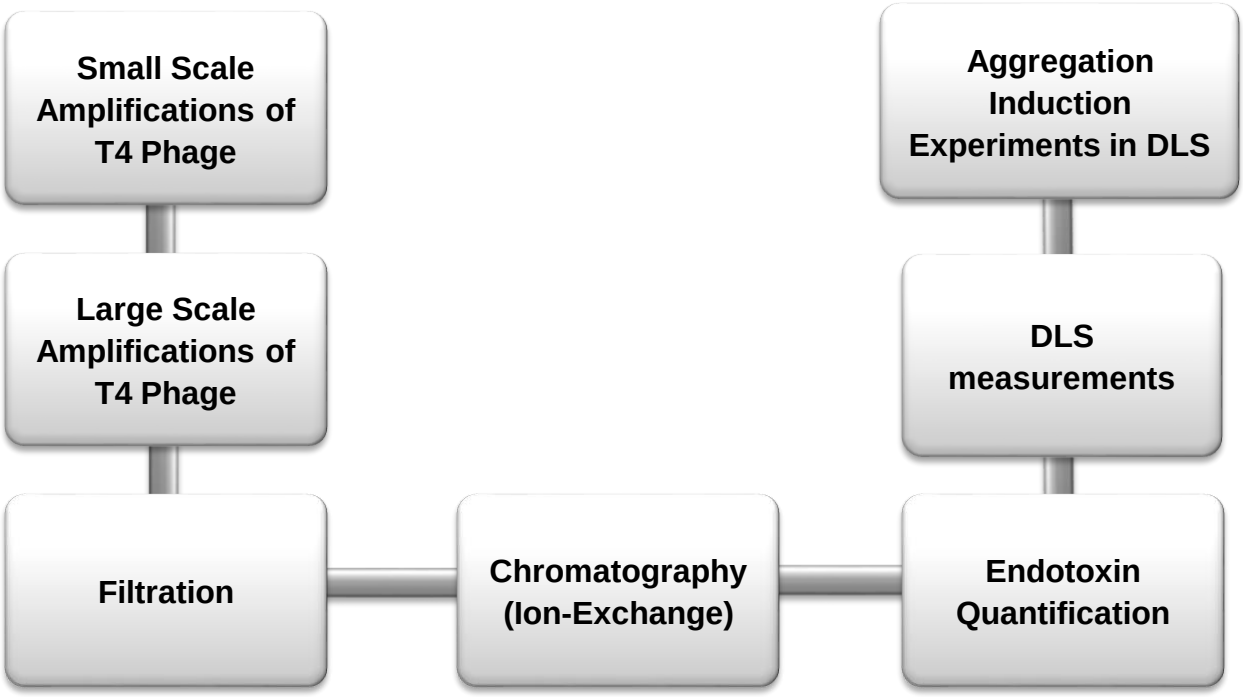

Figure 4 - Flowchart summarizing the experimental work developed in this Master's Thesis

4. Discussion and Conclusion

This Master's Thesis had the objective of assessing the DLS technique for differentiation of individual phages from aggregates and their quantification.

Initially the T4 phage had to be amplified to an acceptable concentration for subsequent purification, and so after optimizing the conditions, a bioreactor amplification was performed. Next, this lysate was purified by IEC, where chromatographic runs with different parameters were performed, with the aim of obtaining a pure sample with the highest possible concentration and with sufficient distinction between the peaks. After the optimized parameters were chosen, this same chromatography was repeated three times to generate more sample, where highly concentrated material was produced.

The endotoxins present in these samples were quantified and compared, however, in terms of quality control, these would not be accepted for pharmaceutical products, since the endotoxin concentration was above the tolerable limit for several delivery routes.

Next, all the samples generated from all the chromatographs were analyzed in DLS. Here, both backward (173°) and forward (12.8°) angles were used, and the aggregation index (AI) was calculated. It is important to remember that the Z-average calculated by DLS is most reliable when a sample is monodisperse, and that it is unwise to rely on samples with a high polydispersity index. The equipment also always calculated the quality of the results.

It is important to note that T4 phages are not spherical particles and, due to their morphology, their diameters are dependent on the angle used for the measurement. For the analysis of the phage samples, the maximum diameter described in the literature, of 200 nm, was assumed.

The chromatography samples were read at different times, with different storage times. Excluding the samples of the chromatography peaks with the highest phage concentration from each chromatography, every sample was shown to be outside the quality criteria. Due to their large polydispersity, they are not considered suitable samples for DLS. It's probable that these samples are highly polydisperse because they did not have a very high degree of purification. Different graph profiles were observed, depending on the samples, due to the variety of molecules present, such as different proteins, DNA or endotoxins.

To further investigate the ability of the DLS method to evaluate the presence of aggregates, aggregation was induced in chosen samples, with high phage concentrations. Initially, a trial was made with one of the samples where temperature was increased. Here, although the AI value somewhat increased, the difference in the graph profile was minimal.

In the end, the DLS technique available for this work did not meet the initial expectations. DLS seemed to not be very suitable for very complex biologic samples such as the phage suspensions. Since these suspensions did not have a high level of purification, other molecules probably caused a lot of

interferences. It was not possible to have or to calculate the specific phage concentration value of any sample, and this technique always showed very varied and inconsistent results in the aggregation studies.

In terms of future work, there are several experiments that could complement this thesis. Optimization of the test for phage aggregation induction, to understand if there would be a more significant sample change that would lead to different aggregation index values. Microscopy could also be an asset for higher reliability in the results, where the generated samples would be visualized, and their aggregation state confirmed. Microsphere suspensions with the same approximate theoretical diameter as our phage (since T4 is a *Caudovirales* phage and has an irregular morphology) could be experimented with and compared to the DLS results of our samples.

In the end, the most crucial step in future work would be, instead of the dual-angle DLS used in this thesis, to work with multi-angle DLS with phage suspensions with higher degrees of purification to eliminate any interference that could be caused by other molecules. This technique would provide better resolution and, most importantly, particle concentration could be calculated.

Regardless of tailed phages being a highly complex sample due to not having an easy structure for DLS size measurements, and the low degree of purification of the samples used resulting in several interferences, we expect better DLS results with samples with less complexity and a higher degree of purification, such as therapeutic suspensions. For the quantification of phages, however, other innovative strategies must be applied such as MADLS.

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