QUALITY CONTROL OF PHAGE FORMULATIONS DURING GMP MANUFACTURING

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Abstract

With the development of multi drug-resistant bacterial strains, there is a current need to explore alternative antibacterial strategies. Bacteriophages, 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 titer 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 showed a low degree of purity and the DLS technique seemed to be unsuitable for highly complex samples such as tailed phages. In terms of future work, working with multi-angle DLS with highly purified samples could provide better results.

Keywords: Bacteriophage, Bacteriophage Therapy, Bacteriophage Production, Downstream Processing, Dynamic Light Scattering

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1. Introduction

Nowadays, mankind faces a dangerous increase in the levels of bacterial resistance to the conventional antibiotics. Bacteria have been losing their effectiveness due to a combination of overuse and self-medication by the patients and abusive prescription in human medicine, veterinary and animal or agricultural areas. This led to the emergence of multi-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. This shed some light on a promising alternative, phage therapy [1–5].

Bacteriophages, or phages, are viruses that infect exclusively bacteria and use them as hosts [6]. They are the most abundant living entities on earth, with population densities approximately ten-fold of prokaryotes in the same environments 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[7,8]. Usually, they target a particular bacteria species or a subgroup of this same species, which express specific membrane surface receptors[1,2,6].

According to their mode of infections, phages can be divided into two categories. Virulent, or strictly lytic phages, can only replicate via lytic lifecycle. They inject their DNA into the host, replicate their genome and produce new phages, which leads to cell lysis and release of newly produced virions[2,9,10]. On the other hand, temperate phages can display two different cycles, lytic of lysogenic. In the lysogenic, the viral genome is integrated into the bacterial genome and suppresses expression of most phage genes. The dormant phage, now a prophage, replicates together with the bacterial DNA for many generations as the cell goes through division[2,6,9,10].

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 goal of lysing a bacterial pathogen that is responsible for a chronic or acute infection[3]. Although it attracts the greatest interest, bacteriophages have several other applications in other areas, such as food safety, agriculture, wastewater treatment, among others[1]. 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[6], necessary for the GMP production of a medicine.

Concentration and purification of the phage are the key for phage therapy. However, aggregation can occur when using high concentration viral suspensions[11]. 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[12,13]. In opposition to this, there have been isolated reports that phage aggregation is linked to phage survival. 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, such as treatments like UV or chlorine dioxide during water disinfection[11–15].

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[11,13,16]. However, it has been recently reported that reducing ionic strength below a specific threshold caused the T4 phage to aggregate dramatically and quickly. While the dynamics of phage aggregation depended on temperature, the process was not dependent on the pI of a whole T4 virion, as it was only observed at neutral and alkaline pH. Importantly, it was also found that T4 phage aggregation caused by reduced salt is a reversible process[12]. These transitions allow the retention of phages on common microfilters, which can be seen as a breakthrough in biotechnology[12,17].

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 quality attributes, 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 operations 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[6,18].

When working with phages, the determination of the concentration of the phage particles is an essential process[19]. The oldest, but still the most common technique is the double agar overlay plaque assay, where serial dilutions of the phage preparation are mixed with the host bacterium that will support phage propagation, and a molten agar or agarose matrix, 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 infect the bacteria, and the lytic cycle will be repeated several times, while the progeny phage from each infected bacterium infects neighbouring bacteria. After incubation, this will result into a lysis halo, which becomes visible to the naked eye as a “plaque” in the smooth lawn[19,20]. Each phage particle that results in a plaque is called a plaque-forming unit (PFU)[20].

The plaque assay method is still considered the standard for determining phage concentrations. While it is simple, efficient and can be implemented in any laboratory with minimal investment costs, it is not without its drawbacks[21,22]. It has high variability and poor reproducibility, which is crucial when working in the development of a manufacturing process. A small change in the nutrient agar can change a viable titter of phage preparations by more than 1000-fold, and the same phage against different bacterial host strains can yield different titters. 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[21,22].

Dynamic light scattering (DLS) or photon correlation spectroscopy, is a technique that measures the size of particles. When the laser light meets 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[23,24]. It measures the Brownian motion of macromolecules in solution and relates it to their size. By monitoring their movement over a range time, it’s possible to get information on their size, since large particles diffuse slowly when compared to small particles[23].

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. Aggregated particles show a high polydispersity index (PDI), since they have a large variation in particle size. Depending on the chosen distribution, different weights can be assigned to a particle, i.e. signals can be amplified with variable scales. Two different size-related factors emerge from the size analysis: z-average and peak mean[24,25].

Dual-angle DLS can also be used for detection of protein aggregation. 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[26,27]. 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[28]:

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

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. Due to this, the method has been showed to have applicability in the bacteriophage field[29,30].

The main objective of this Master's Thesis was to find a method that can differentiate individual phages from aggregates and quantify them, to overcome the limitations of the previously mentioned plaque assay method. Since dynamic light scattering has shown promising results in previous phage studies, this method was employed and studied with T4 phage lysates.

The initial stage of the experimental process consisted in streamlining the production process of the model 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 titer possible.

Afterwards, the generated sample 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 DLS.

2. Experimental Plan

The experimental work developed in this master's Thesis can be described through Figure 1.

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.

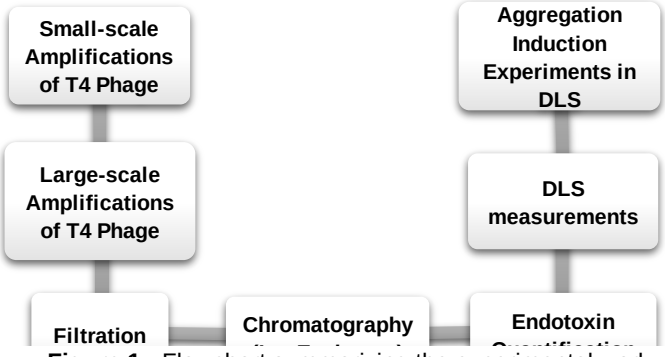

Figure 1 - Flowchart summarizing the experimental work developed in this master's Thesis.

3. Conclusions and Future Work

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 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. The samples showed a low level of purification and, due to their large polydispersity, they were not considered suitable samples for DLS. 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.

In the end, this was a preliminary study and the DLS technique available 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 induction of phage aggregation, 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.

4. References

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