clean cells
SOLUTION FOR BIOLOGICS

PURIFICATION OF THERAPEUTIC BACTERIOPHAGES: ENDOTOXIN (LPS) REMOVAL

ABSTRACT

The emergence of antibiotic-resistant bacteria is one of the most prominent concerns of the World Health Organization (WHO). Administering therapeutic bacteriophages is a potential solution to mitigate the threat posed by antibiotic resistance. Implementing new systems for the manufacture and purification of therapeutic bacteriophages in compliance with Good Manufacturing Practices (GMP) will prove essential if we are to use these viruses as medicinal products.

As with viruses produced in mammalian cells, lysis cycles follow a production of bacteriophages in bacteria. Bacterial lysates contain bacteriophages and various impurities requiring removal prior to administration to allow for safe conditions, devoid of undesirable side effects such as immune response or septic shock. Bacterial lysates are subject to purification steps to ensure that optimal yields are reached while maintaining quality and potency of bacteriophages, in particular through residual contaminant removal. Specific attention should be devoted to endotoxins (EU for Endotoxin Unit), generated from gram-negative bacteria, whose concentration should not exceed 5 EU/Kg/h for parenteral medicinal products. Endotoxin removal is often challenging as yield loss tends to occur when separating bacteriophages from endotoxins.

The Process Development department of Clean Cells has recently worked at identifying an efficient method to remove LPS from bacteriophage bulks. The main objectives to be achieved for the purification of therapeutic bacteriophages were as follows:

- Efficiency and performance
- Simple and quick setup
- Applicability to various industrial scales and compatibility to GMP

In this work, three systems were tested, two in chromatography flowthrough mode and one in chromatography positive mode. An efficient purification method was then developed using one of the best systems with the final goal of achieving the pre-defined criteria. This method allows for a 4 log reduction of EU, achieving concentration below 1 EU/10^9 PFU while maintaining a very high yield, close to 100%.

INTRODUCTION & OBJECTIVES

The emergence of antibiotic-resistant bacteria is currently becoming worldwide prominent, limiting our capacities to cure infectious diseases. It is one of the major concerns of the World Health Organization (WHO).

To that end, administering therapeutic bacteriophages which are targeting specifically bacteria seems to be one of the most promising solutions as an alternative to antibiotics. Numerous scientific medical teams are investigating this remedy to cure multi-resistant bacterial infections. Cases of bacteriophage treatments in compassionate use allowed spectacular remission (N. Dufour et al, 2017). Therefore, EMA and FDA agencies are asking companies developing bacteriophages-based drug products to conduct clinical trials essential to obtain marketing authorization as requested for all medicinal products.

To perform such clinical trials, it is crucial to ensure that bacteriophage products are

consistently produced and controlled according to high quality standards meeting Good Manufacturing Practices (GMP) requirements. Consequently, there is a need to implement new production systems for the manufacture and purification of therapeutic bacteriophages.

One of the major challenges in bacteriophages purification is the removal of various impurities. As with viruses produced in mammalian cells, bacteriophages production is obtained in bacteria after several infection-lysis cycles. Cellular membrane rupturing leads to the release of a large number of cell wall components and other impurities. The outer layer of gram-negative bacteria, which are widely used to produce therapeutic biomolecules (Van Belleghem et al, 2017; Overton et al, 2014; Terpe, K. et al, 2006), is composed of lipid A, the hydrophobic anchor of lipopolysaccharides (LPS) also known as endotoxins.

LPS are highly immunogenic and if present in large quantities may, via cytokine signaling, cause septic shock leading to intravascular coagulation, multiple organ failure, and even death (Raetz and Whitfield, 2002). LPS concentration should not exceed 5.0 Endotoxin Unit (EU)/Kg/h for intravenous medicinal products (Council of Europe, 2005). Removing undesirable LPS is a crucial issue to ensure the safety of pharmaceutical products.

Unfortunately, LPS removal is often challenging as yield loss tends to occur when separating bacteriophages from endotoxins. Conventional purification treatments such as membrane filtration are adequate for removing bacteria cells and debris but not effective for removing dissolved endotoxin to a significant extent (Razdan et al, 2019).

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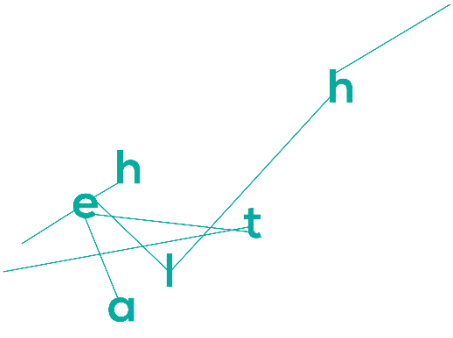
METHODS

Purification systems were tested on lysates of *Escherichia coli* culture infected with the bacteriophage T7. Prior to the purification step, the fresh harvest was treated with DNAse to hydrolyze DNA impurities then clarified using depth filter to remove cells debris and other macro impurities. The clarified harvest was then concentrated and buffer exchanged by TFF ultrafiltration. Finally, the bacteriophage bulk was 0.2 µm filtered before injection in each chromatographic system.

Three different technologies provided by three acknowledged suppliers were tested and identified as System A, B and C:

- System A is a ready-to-use kit based on affinity chromatography technology targeting LPS using pre-formulated elution and regeneration buffers.
- System B is a membrane technology based on ion exchange chromatography using in-house formulated elution and regeneration buffers.
- System C a monolith technology also based on ion exchange chromatography using in-house buffers.

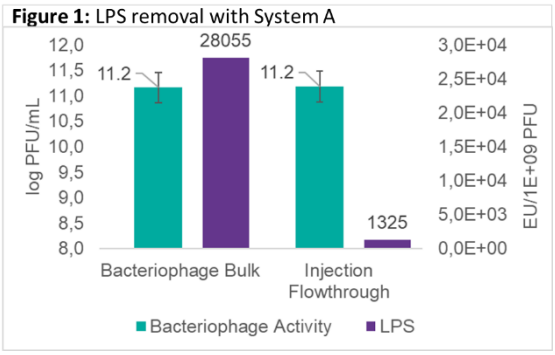
System A is a standalone device working with gravity. System B and C were used with an FLPC system as classical chromatography column. For this study, non-GMP version of these systems were tested according to suppliers' recommendations.



RESULTS

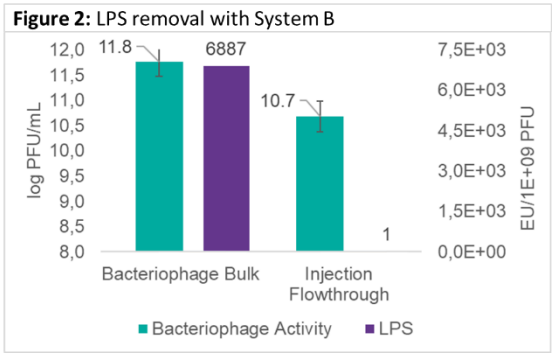
Purification system comparison:

System A



System A allowed a 1.33 log reduction factor of the LPS concentration with no loss of bacteriophage activity (Figure 1). It seems that bacteriophages were neither adsorbed nor damaged by the chromatography support of System A.

System B

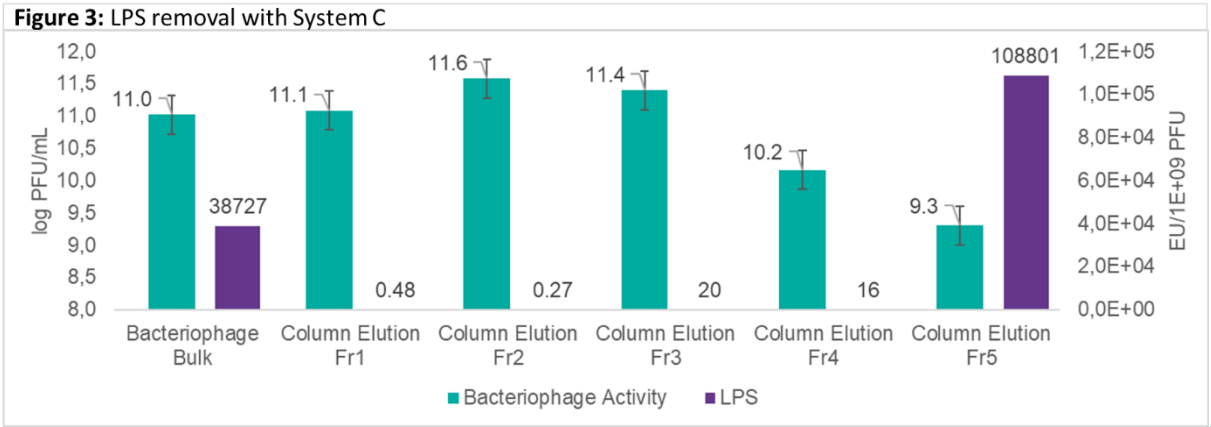


System B allowed a massive LPS concentration decrease with a 3.83 log reduction factor (Figure 2). However, the bacteriophage activity seemed to suffer a major decrease (from 11.8 to 10.7 log PFU/mL). Hardly any phage activity was detected in column elution and column regeneration peaks. This would mean that bacteriophages might have been damaged by the injection conditions used with this system.

System C

No phage nor LPS were detected in the injection flowthrough of System C. However, the elution phase presented several peaks with different retention times. These elution peaks were collected in 5 different fractions (Figure 3). Fractions 1-2-3 showed high functional bacteriophage concentrations (resp. 11.1, 11.6, 11.4 log PFU/mL) combined with very low LPS concentrations (resp. 4.82E-01, 2.66E-01, 1.99E+01 EU/10^9 PFU). Fraction 5 corresponded to the LPS elution showing a high concentration of endotoxin at 1.09E+05 EU/10^9 PFU. Fraction 4 presented a less interesting composition with a low bacteriophage activity of 10.2 log PFU/mL and an LPS concentration of 1.65E+01 EU/10^9 PFU.

To conclude, this study showed that System A allowed to keep high bacteriophage activity but presented the least interesting LPS removal. Furthermore, the pre-formulated elution and regeneration buffers did not let the phage/LPS separation to be optimized.



This system was not selected to carry on this study.

System B showed a very good LPS concentration reduction but coupled with an important phage activity loss. System C presented interesting phage/LPS separation potential with high functional bacteriophage recovery yields and low LPS concentrations.

According to these results, System C appeared to be the best option to separate LPS from bacteriophages. Nevertheless, adsorption chromatography performance was strongly linked to the characteristics of the product to purify (structure, charge, hydrophobicity…). On the opposite, System B allowed to perform chromatography purification in flow through mode which specifically adsorbs and removes LPS impurities. This purification mode might overcome the large bacteriophage diversity and be applicable to other bacteriophage families. Accordingly, System B was selected to pursue this study. Optimizing elution and phage solubilization conditions might enhance the bacteriophage recovery yield. System C will be investigated in another study.

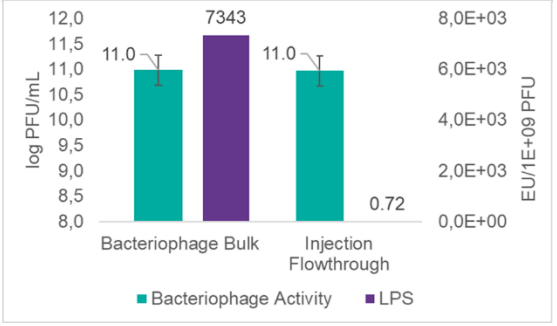
System B elution optimization:

Different elution buffers and bacteriophage bulk conditionings were tested to increase the bacteriophage recovery yield. The optimization step was carried out varying buffers pH and conductivity levels (Table 1).

One optimal pH/conductivity couple was identified allowing to reach a bacteriophage recovery yield of 96.6 % while having a residual LPS concentration of 0.72 EU/10^9 PFU.

This optimal condition allowed a massive LPS concentration reduction showing a log reduction factor of 4 while maintaining a high bacteriophage activity of 11.0 log PFU/mL (Figure 4). The LPS binding capacity of System B was assessed via complementary tests to be about 10^7 LPS/mL of membrane.

Figure 4: LPS removal in optimized conditions



CONCLUSION

In this study, three purification systems were tested and their efficiency to remove endotoxin from bacteriophage suspension were compared. One of the most efficient purification methods was further explored. This method afforded an important LPS charge reduction reaching the concentration of 0.72 EU/10^9 PFU while preserving a high bacteriophage activity with a recovery yield 96.6 %.

Table 1: Bacteriophage recovery and LPS concentration of optimization trials

pH	Ionic Strength (mS.cm^{-1})	Bacteriophage Recovery Yield (%)	LPS concentration of the flowthrough (EU/10^9 PFU)
6.0	20.2	8.1	1.01
	26.1	22.4	37.9
8.0	24.3	96.6	0.72
	26.7	44.7	0.18

To complete this work, this purification method has now to be challenged with other bacteriophage families specific from other gram-negative bacteria.

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