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RESEARCH ARTICLE

Clinical-grade Oncolytic Adenovirus Purification using Polysorbate 20 as an Alternative for Cell Lysis

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Abstract: ***Introduction*:** Oncolytic virus therapy is currently considered as a promising therapeutic approach for cancer treatment. Adenovirus is well-known and extensively characterized as an oncolytic agent. The increasing number of clinical trials using this virus generates the demand for the development of a well-established purification approach. Triton X-100 is commonly used in cell lysis buffer preparations. The addition of this surfactant in the list of substances with the very high concern of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation promoted the research for effective alternatives.

***Methods*:** In this work, a purification strategy for oncolytic adenovirus compatible with phase I clinical trials, using an approved surfactant – Polysorbate 20 was developed. The proposed downstream train, composed by clarification, concentration using tangential flow filtration, intermediate purification with anion exchange chromatography, followed by a second concentration and a final polishing step was evaluated for both Triton X-100 and Polysorbate 20 processes. The impact of cell lysis with Polysorbate20 and Triton X-100 for each downstream step was evaluated in terms of product recovery and impurities removal. Overall, 61 ± 4% of infectious viral particles were recovered. Depletion of host cell proteins and ds-DNA was 99.9% and 97.1%, respectively.

***Results & Conclusion*:** The results indicated that Polysorbate 20 can be used as a replacement for Triton X-100 during cell lysis with no impact on product recovery, potency, and purity. Moreover, the developed process is scalable and able to provide a highly purified product to be used in phase I and II clinical trials.

Keywords: Adenovirus, cell lysis, downstream processing, GMP process development, oncolytic virus, polysorbate 20.

1. INTRODUCTION

Oncolytic viruses are a naturally occurring virus that can target cancer cells, replicate within them and successfully kill these cells, saving the non-tumoral ones [1-3]. Moreover, the proteins that are released during cell lysis stimulate a response of the immune system, making them promising cancer gene therapy agents [1]. Although oncolytic viruses are naturally occurring, they have been engineered to be more specific for cancer cells, in order to create new cancer therapeutics - Oncolytic virotherapy - that can be used in addition to traditional treatments [4]. In October 2015, US FDA approved the first oncolytic virus therapy, T-VEC (Imlygic®; Amgen, CA), which consists of a genetically modified herpes virus for the treatment of melanoma [5].

A diversity of oncolytic virus, often genetically modified, has advanced to clinical studies. Among them are the Herpesvirus, Vaccinia virus, Reovirus, Adenovirus, Measles virus and Parvovirus, where the Adenovirus is one of the most extensively characterized oncolytic agents [6-9]. This non-enveloped virus is constituted by a capsid of approximately 90 nm and a genome ranging from 30 to 38 kb. Its ability to infect cells, as well as the ability to manipulate its genome easily, makes this virus a desired agent for oncolytic therapy. Oncolytic adenovirus was one of the firsts to be used in clinical trials and represents the vehicle of choice for gene therapy treatment [10]. Since then, several efforts have been made to improve its efficacy and safety to be used in the clinic. However, to move the therapies from the laboratory into the clinic it is necessary to have not only a highly-defined virus characterization but also a robust and scalable manufacturing process.

The laboratorial scale purification process consists firstly of a cell lysis step using freeze and thaw methods, followed by a density gradient ultracentrifugation and finally a desalt-

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ing step [11, 12]. This process allows achieving a high purity level since most of the host cell proteins and DNA are removed, while maintaining a low viral/infectious particles ratio (bellow 30). This process, however, presents some drawbacks regarding the processing time and the scalability. Cell lysis for large-scale processes is usually achieved by the addition of detergents [13]. The use of a mild non-ionic detergent known as Triton X-100 in this step is popular, as it solubilizes the cell membranes, allowing the release of the viruses [14-16]. In addition, the use of this detergent is widely described for protein solubilization, protein-lipid, and lipid-lipid aggregation, and enveloped virus clearance [17]. However, evidences were found that the use of Triton X-100 can have serious effects on the environment due to endocrine disrupting properties during degradation. Triton X-100 is on the authorization list (Annex XIV) of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation, prohibiting its use and placement on the market from 4 January 2021, unless an authorization is granted to the user [18]. Thus, there is a need for new alternative methodologies for cell lysis that can be used in large-scale manufacturing. Today, most of the described adenovirus purification operations are composed of several processes including cell lysis, clarification, concentration, intermediate chromatography, polishing and sterile filtration [11, 19]. With these processes, we must achieve the required specifications for impurity levels, such as host cell DNA and proteins, as well as for virus potency, identity, and sterility, set by the European Pharmacopoeia World Health organization [20].

To replace Triton X-100, an approved surfactant Polysorbate 20 is evaluated here. This surfactant is a stable, nontoxic and non-ionic surfactant commonly used in domestic and pharmacological applications. The evidence of its ability to reduce protein aggregation [21] and surface adsorption [22], allows this surfactant to be widely used for therapeutic protein formulations, present in almost all licensed mAb formulations [23].

On top of that, we evaluated the effect of replacing Triton X-100 by Polysorbate 20 in the different downstream steps from the cell harvest to the final sterile filtration step. The results showed that Polysorbate 20 has no negative effect on the steps of the process and, can increase the virus recoveries and removal of impurities. Finally, a scalable process using this alternative surfactant, while maintaining the product recovery, potency and purity, was also performed and shown to work favorably.

2. MATERIALS AND METHODS

2.1. Virus Production

A549 cells purchased from ECACC (86012804, ECACC) were used for virus production. The cells were amplified in T-flasks with 150 cm^2 using Ham's F12 medium, Kaighn 's modification (SH30526.01, GE Life Sciences) supplemented with 10% FBS (SH30071.01, GE Life Sciences) and then amplified in HYPERflask® (10020, Corning), in a humidified atmosphere of 5% CO_2 in air at 37ºC. When cells achieved 90% of confluence, the harvest was performed by trypsinization and centrifugated at 200g, to remove the serum-containing medium. The cell pellet was resuspended in CFM4HEK293 medium (SH30858.02, GE Healthcare Life Sciences) supplemented with 4 mM GlutaMAX (35050-038, ThermoFisher) and transferred to a ReadyToProcess WAVE bioreactor. The cells were inoculated at a concentration of 0.3×10^6 cells/mL to 0.6×10^6 cell/mL. Samples were taken every 24 hours for cell viability and concentration assessment. After 72 hours in culture, the A549 cells were infected with a Multiplicity of Infection (MOI) of 10 infectious particles per cell. The harvest was performed 48 hours post-infection (hpi). Cell concentration and viability were determined using the trypan blue (Invitrogen) dye exclusion method.

2.2. Cell Lysis and Nuclease Digestion

Two detergents were evaluated for cell lysis, Triton X-100 and Polysorbate 20. Triton cell lysis was performed by the addition of a stock solution of 10% (v/v) Triton X-100 in 10 mM Tris-HCl, pH 8, to a final Triton concentration of 0.1% (v/v). Cell lysis of the harvested material was performed with Polysorbate 20 at 5% (v/v) concentration to achieve a final concentration of 0.5% (v/v). For DNA digestion, 100 U/mL of Benzonase (1.01656.0001, Merck) was used. The digestion proceeded for 4 hours at 37ºC in the bioreactor with agitation (16 rpm). The nuclease activity was monitored by Picogreen assay every hour.

2.3. Clarification

Clarification was performed using an ULTA Prime™ GF filter followed by an ULTA Prime™ CG filter (DGF-A-02-470, DMP-CG92-470, GE HealthCare Life Sciences). The filters were previously washed with Milli-Q water and equilibrated with 50 mM Hepes, pH 7.5. The clarification was performed at a constant flux of 600LMH using a Tandem 1081 Pump (Sartorius Stedim Biotech) and the pressure was monitored using a pressure transducer in-line (080-699PSX-5, SciLog). The filter capacity (Vmax) as determined using ULTA™Prime GF and CG 47 mm discs by recording the cumulative weight of filter effluent at a constant pressure of 2 bar.

2.4. Tangential Flow Filtration

A 26 cm^2 750 000 NMWC hollow fiber (UFP-750- C-MMO1A, GE Healthcare Life Sciences) was evaluated in this step. The tangential flow filtration was performed using a Tandem 1081 Pump (Sartorius Stedim Biotech) and the pressures were monitored using the pressure transducers in-line (080-699PSX-5, SciLog). A constant feed flux of 900 LMH was set-up. The Transmembrane Pressure (TMP) was kept at 1 bar by a pinch valve on the retentate side. The hollow fiber was previously flushed with Milli-Q water to remove the preservatives and equilibrated with 50 mM Hepes, 150mM NaCl, pH 6.5. 80 mL of the clarified bulks were concentrated 2 times and diafiltrated 4 times.

2.5. Intermediate Purification

The viruses were further purified by anion exchange chromatography using a 5 mL Hiscreen CaptoQ impRes (17547051, GE Healthcare Life Sciences) column pre-equilibrated with 50 mM Hepes, 150 mM NaCl, pH 6.5. The chromatography was performed at a constant flow rate of 300 cm/h and an elution step was performed with 50 mM Hepes, 1 M NaCl, pH 6.5. Viruses' corresponding fractions

were pooled and diluted 1:4 with 20 mM Tris-HCl, 25 mM NaCl, pH8, to avoid losses in virus infectivity. An AKTA Avant system (GE Healthcare Life Sciences) equipped with UV and conductivity/pH sensors was used for this chromatographic step.

2.6. Polishing

The pooled virus fractions were concentrated and then polished using size exclusion chromatography. For concentration, a 16 cm^2 300 000 NMWC hollow fiber was used (UFP-300-C-MM01A, GE Healthcare Life Sciences). The tangential flow filtration system was set up as previously described. The viruses were concentrated 9 times and diafiltrated 2 times with 20 mM Tris-HCl, 25 mM NaCl, pH 8. The concentrated sample was then loaded into a 35 mL Sepharose 4 FF resin (17014901, GE Healthcare Life Sciences) packed in an XK 26/20 column (28988948, GE Healthcare Life Sciences) at a flow rate of 12 cm/h. The column was packed according to manufacturer's recommendation compression factor and the height equivalent to a theoretical plate (HETP) and peak asymmetry were determined. Before loading the viruses, the column was equilibrated with 20 mM Tris-HCI, 25 mM NaCl, pH 8. The viruses' corresponding fraction was pooled for further sterile filtration.

2.7. Sterile Filtration

Before filtration the viral samples were formulated by the addition of a 25% (v/v) stock solution of glycerol in 20 mM Tris-HCI, 25 mM NaCl, pH 8, to a final concentration of 2.5% (v/v). The viral preparation was then filtered using a 0.22 μm filter. In this step, an ULTA™Prime CG disc (DMP- CG92-470, GE Healthcare Life Sciences) was used. The filtration was set at a constant flux of 600 LMH. The disc was previously flushed with Milli-Q water and equilibrated with 20 mM Tris-HCI, 25 mM NaCl, 2.5% glycerol, pH 8.

2.8. Scale-up

A scale-up of the purification process was performed at a 2L scale. Virus was harvested as previously described. For the clarification, an ULTRA PRIME GF 5 μm 6" (KGF-A-0506GG, GE Healthcare Life Sciences) was used, followed by an ULTA PRIME CG 4" (KMP-CG9204GG, GE Healthcare Life Sciences). The clarification was performed at a constant flux of 600 LMH. Before clarification, the filters were washed with Milli-Q water and equilibrated with 50 mM Hepes, 150 mM NaCl, pH 6.5. The first tangential flow filtration (TFF) was performed using a 750 kDa hollow fiber with a membrane area of 290 cm^2 (UFP-750-C-3X2MA, GE Healthcare Life Sciences). The TFF was performed at a constant feed flux of 620 LMH and a TMP of 1 bar. The virus bulk was concentrated 2 times and diafiltrated 4 times with 50 mM Hepes, 150 mM NaCl, pH 6.5. For the intermediate purification 120 mL of Capto Q impRes resin (17547301, GE Healthcare Life Sciences) was packed in an Hiscale XK50/20 column (28988952, GE Healthcare Life Sciences). The chromatographic run was performed at a constant flow rate of 300 cm/h. The column was equilibrated with 50 mM Hepes, 150 mM NaCl, pH 6.5. An elution step was performed with 50 mM Hepes, 1M NaCl, pH 6.5. The eluted virus fractions were pooled and diluted 1 to 4 with 20 mM Tris, 25 mM NaCl, pH 8. Before polishing, a second TFF was performed using a 300 kDa hollow fiber with 140 cm^2 (UFP-300-C-3MA, GE Healthcare Life Sciences) at a constant feed flux of 860 LMH and a TMP of 1 bar. The sample was concentrated 9 times and diafiltrated 2 times with 20 mM Tris, 25 mM NaCl, pH 8. An XK50/60 column (28988951, GE Healthcare Life Sciences) was packed with 300 mL of Sepharose 4 FF (17014901, GE Life Sciences) for the polishing step. The run was performed at a flow rate of 12 cm/h and a buffer containing 20 mM Tris, 25 mM NaCl, pH 8 was used. The samples containing the target were pooled and formulated with 20 mM Tris, 25 mM NaCl, 25% glycerol (v/v), pH 8 to a final glycerol concentration of 2.5% (v/v). The sterile filtration was accomplished using an ULTA Prime CG disc (DMP-CG92-470, GE Life Sciences) in an in-line filter holder (XX4304700, Merck). The membranes were pre-equilibrated with 20 mM Tris-HCI, 25 mM NaCl, 2.5% glycerol, pH 8. The filtration was performed at a flux of 600 LMH.

2.9. Characterization Assays

2.9.1. Infectious Particles

Infectious particles were quantified using the 50% Tissue Culture Infective Dose ($TCID_{50}$) assay. A549 cells Ham's F12 medium, Kaighn 's modification (SH30526.01, GE Life Sciences) supplemented with 5% FBS were incubated in 96-well plates in a humidified atmosphere of 5% CO_2 in air at 37ºC for 24 hours. The cells were then infected with successive viral dilutions, from 10^{-1} to 10^{-11} and incubated under the same conditions, for 10 days. After that time, the Cytopathic Effect (CPE) was evaluated and the concentration of infectious particles was determined using the Spearman-Karber statistical method.

2.9.2. Genome Particles Quantification

For the quantification of viral genome copies, quantitative real-time Polymerase Chain Reaction (qPCR) was used. To ensure only the quantification of viral particles and the removal of the free viral genome, 20 μL of viral samples were incubated with 10 U of DNAse (04716728001, Roche) for 30 minutes at 37°C. The reaction was then stopped by the addition of EDTA to a concentration of 8 mM and heated at 75ºC for 10 min. The viruses' genomes were then extracted using High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (11858874001, Roche). The viral genome copies were quantified by qPCR using the LightCycler® 480 Probes Master (0470749001, Roche) in the LightCycler® 480 instrument (Roche).

2.9.3. Total dsDNA Quantification

Total dsDNA was assessed using the Quant-iT™ Picogreen™ ds-DNA Assay Kit (P7589, Invitrogen), according to the manufacturer's instructions. The assay was performed in a black 96 well microplate and the fluorescence was measured on the Infinite 200 Pro plate reader (Tecan). Lambda DNA was used as a standard curve with concentrations between 1 and 0.008 μg/mL.

2.9.4. Total Protein Quantification

Total protein concentration was determined by BCA protein assay kit (23225, ThermoFisher). The analysis was performed according to the manufacturer's instructions. The assay was performed in transparent 96 well plates and the

absorbance was measured at 562 nm, using the Infinite 200 Pro plate reader (Tecan). Bovine Serum Albumin (BSA) was used as a standard curve with a concentration of 2000 to 25 µg/mL.

2.9.5. Host Cell Protein Quantification

A549 host cell proteins were measured using an enzyme-linked immunoassay (ELISA) (F230, Cygnus Technologies). The assay was performed according to the manufacturer's instructions and the samples' absorbance was measured at 450 nm using the Infinite 200 Pro plate reader (Tecan). A549HCP was used as a standard curve with concentrations between 200-0 ng/mL.

2.9.6. Benzonase Quantification

To ensure that the endonuclease added in the process was removed, the Benzonase® concentration in the final sample was determined using the Benzonase® ELISA kit II (1016810001, Merck). The assay was performed according to the manufacturer's instructions and the absorbance was measured at 450 nm using the Infinite 200 Pro plate reader (Tecan). Benzonase® was used as a standard, with concentrations of 200 to 0.1 ng/mL.

2.9.7. Transmission Electron Microscopy

The presence of adenovirus was also confirmed by electron microscopy. Briefly, a drop of sample was adsorbed onto a formvar-coated 150 mesh copper grid from Veco (Science Services) for 2 minutes. The grid was washed 5 times with sterile filtered H_20 then soaked in 2% of uranyl acetate for 2 minutes and dried in the air at room temperature. The grids were analyzed using a Hitachi H-7650 120 Kv electron microscope (Hitachi High-Technologies).

2.9.8. Polysorbate 20 Quantification

Polysorbate 20 was quantified in the initial and final sample of the scale-up process using an ultra high-performance liquid chromatography technique as described elsewhere [24]. Briefly, the chromatographic analysis was performed on an UltiMate 3000 UHPLC (Thermo Scientific). The separation was performed using a Water Oasis Max column (2.1x20 mm, 30 µm particle size, P/N 186002052). Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B was 0.1 % (v/v) formic acid in acetonitrile (Optima TM LC/MS Grade, Fisher Scientific). The column temperature was maintained at 30ºC, and a flow rate of 500 µl/min was used. The calibration curve was obtained with Polysorbate 20 standard (10 – 500 ng) and a linear correlation was observed between the peak area and the amount of Polysorbate 20 injected.

3. RESULTS AND DISCUSSION

3.1. Cell Harvest and Clarification

The first purification steps of oncolytic adenovirus are the cell harvest and the lysis to release the virus. The cell lysate contains cell debris that should be removed. For this purpose, a clarification is necessary at this stage to protect the next downstream steps. For cell lysis, we propose the use of Polysorbate 20 as a replacement for Triton X-100 [25], since this detergent fits the demands for a new cell lysis agent. Polysorbate ability to lyse the cells while maintaining virus infectivity was evaluated. Firstly, a comparison of infectious particles released by the traditional freeze-thaw method, and both detergents, 0.1% Triton X-100 and 0.5% Polysorbate 20, was performed using the same concentration of cells producing oncolytic adenovirus. The concentration of infectious viral particles released by infected cells using the different lysis methods is shown in Fig. (**1**). The results indicated that the use of 0.5% Polysorbate 20 is comparable to Triton regarding virus release by infected cells.

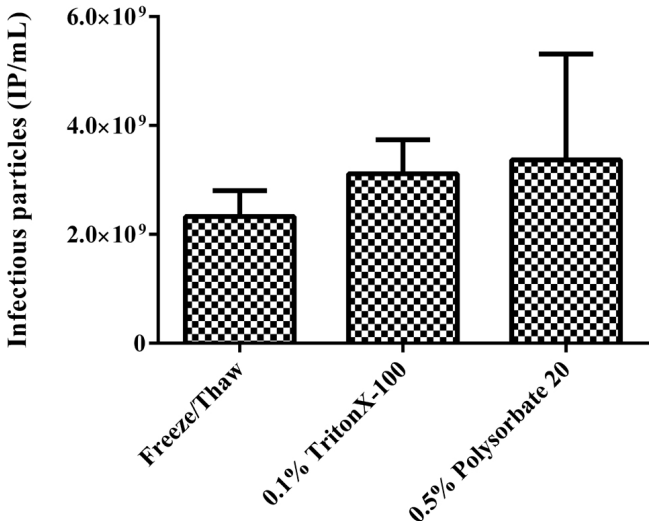

Fig. (1). Infectious viral particles (IP/mL) obtained after cell lysis using freeze/thaw method, addition of 0.1% Triton X-100 or 0.5% Polysorbate 20 detergents. Infectious viral particles were determined using 50% tissue culture infective dose ($TCID_{50}$) assay. Data are expressed as mean ± standard deviation (n=3).

Due to cell lysis, a substantial amount of DNA is present which must be eliminated early in the process. The clearance of host cell DNA is one of the main challenges in adenovirus purification. Adenovirus can associate with DNA, creating complexes that complicate the downstream processing [26]. To overcome this challenge, a nuclease treatment was added to the process. Benzonase® is used to reduce the host cell DNA content and its activity can be affected by the addition of detergents. For this reason, we evaluated the effect of addition of both detergents in the enzyme activity. The enzyme was added in a concentration of 100 U/mL to each sample and it was incubated for 4 hours at 37ºC. DNA removal was evaluated along time (Fig. **2**). After the 4 hours of incubation, both detergents gave similar results for the decrease of DNA. This result shows that Polysorbate 20 has a positive influence in the nuclease digestion, as described for higher concentrations of Triton X-100 [27]. Clarification of the supernatant containing virus is performed after the nuclease incubation. This step will ensure the removal of cell debris and large aggregates and will protect the following purification steps. The clarification train consisted in two normal flow filters with 2 µm and 0.2 µm. The comparison of both detergents in the clarification performance was done using several parameters: turbidity; protein and dsDNA removal; genome particles recovery and filter capacity (Table **1**). Clarification results show a lower turbidity removal during the second filtration when using the Polysorbate 20 as cell lysis agent. In addition, a reduction in the first filter capacity was

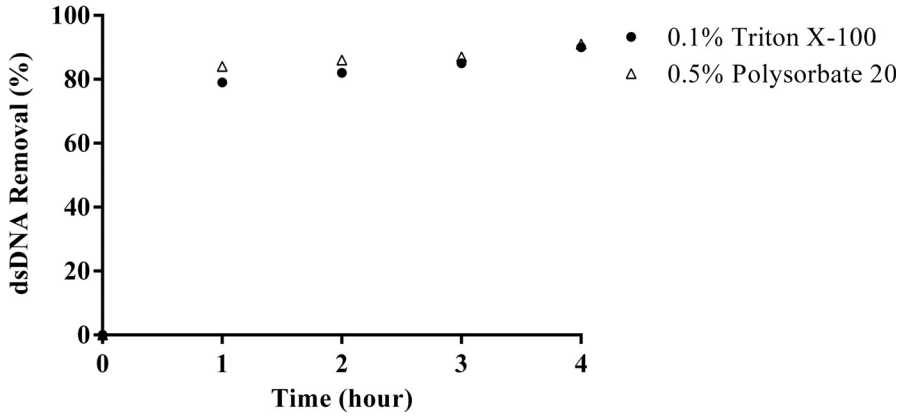

Fig. (2). Effect of nuclease addition after cell lysis on the removal of DNA impurities in both processes (Polysorbate 20 and Triton X-100). DNA removal was accessed by Picogreen assay, during 4 hours of incubation with nuclease.

Table 1. Comparison of the clarification train results regarding turbidity (NTU), protein removal (%), dsDNA removal (%), total particles recovery (%) and filter capacity (L/m^2) of both processes (Polysorbate 20 and Triton X-100), using the 2 µm ULTA prime GF and 0.2 µm ULTA prime CG filters. Total viral particles (TP) recovery was determined by qPCR.

-	Filters	Turbidity (NTU)	Protein Removal (%)	dsDNA Removal (%)	TP Recovery (%)	Filter Capacity (L/m^2)
0.1% Triton	GF- 2 µm	10	28	22	58	61
	CG-0.2 µm	4	40	36	99	48
0.5% Polysorbate 20	GF-2 µm	18	23	20	60	44
	CG-0.2 µm	10	28	32	94	55

also noticed. Nevertheless, there was no significant difference in DNA and protein removal neither in virus particles recovery yields. This indicates that Polysorbate 20 does not have a negative impact on clarification; however, it decreases the filter capacity, so a larger filter area will be required when using this detergent.

3.2. Concentration by Tangential Flow Filtration

After clarification of virus bulk, a concentration step was performed allowing the removal of small size molecules, such as low molecular weight proteins and DNA fragments [16]. For tangential flow filtration of adenovirus, hollow-fiber devices with a membrane cut-off of 750 kDa were used. Fig. **3** depicts the removal of impurities (DNA and proteins) during the four diafiltrations performed after the two-fold concentration has been achieved; no significant differences in DNA removal was observed between the two detergents. However, for protein removal, Polysorbate 20 is more efficient during the first diafiltrations, converging to similar values of removal in the end (72% for Triton comparing with 83% for Polysorbate).

This might suggest that, as described for other proteins [28], Polysorbate improves protein stabilization and reduces aggregation, leading to higher protein removal. In contrast, the recovery of virus particles was significantly different for both detergents. Only 55 ± 28% of genomic particles were recovered when using Triton X-100 for concentration, while 92 ± 13% was recovered when using Polysorbate 20. The fact that this detergent is known by the non-specific binding to the PES membranes [29], might contribute to it having the highest recovery yield when it is used in the tangential flow filtration. The detergent binds to the membrane surface, reducing the membrane area available for unspecific binding of viral particles, thus improving the recovery yield.

3.3. Intermediate Purification

Adenovirus has been successfully purified through chromatographic processes, providing higher virus concentrations while maintaining their biological activity [30]. Among several chromatographic techniques, anion exchange chromatography has been already used to purify adenovirus [31, 32]. After the concentration step, 45 mL and 38 mL of viruses, from both Polysorbate 20 and Triton X-100 processes, were loaded into a strong anion exchange resin - Capto Q impRes. It was possible to achieve a concentration of 2.1×10^{12} TP/mL and 2.2×10^{12} TP/ml for Polysorbate 20 and Triton X-100, respectively, after this chromatographic step. However, in Table **2** it can be observed that the recoveries and the DNA removal for Polysorbate are slightly lower, compared with Triton.

3.4. Polishing and Sterile Filtration

Samples derived from AEX were further purified using Size Exclusion Chromatography (SEC) as a polishing step.

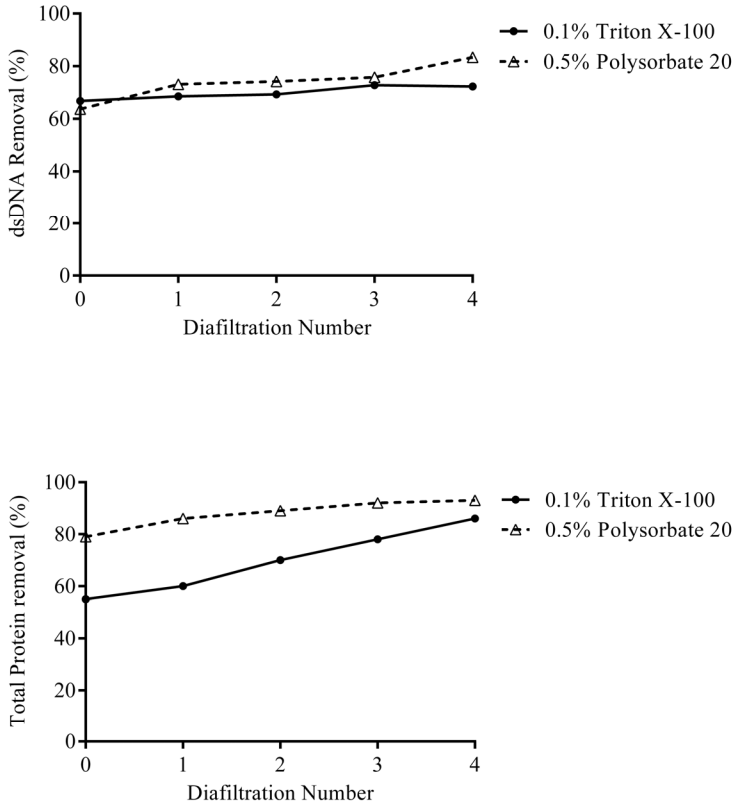

Fig. (3). Evaluation of impurities removal as a function of the performed number of diafiltrations. Analysis of dsDNA removal (top graph) and total protein removal (bottom graph) for both Triton X-100 and Polysorbate lysis detergents processes. Both figures display the clearance of impurities after a 2 fold concentration.

Table 2. Comparison of recoveries and impurities removal during AEX chromatography, tangential flow filtration, size exclusion chromatography and final sterile filtration steps, for both processes (Polysorbate 20 and Triton X-100). Total viral particles (TP) recovery was determined by qPCR.

-	DSP Step	dsDNA Removal (%)	Total Protein Removal (%)	TP Recovery (%)
0.1% Triton X-100	AEX	72	92	80
	TFF2	54	12	69
	SEC	92	78	44
	Sterile filtration	78	37	100
0.5% Polysorbate 20	AEX	96	59	72
	TFF2	61	40	79
	SEC	85	73	56
	Sterile filtration	81	41	100

Before performing this step the adenoviruses must be concentrated. For that, a tangential flow filtration was performed using a hollow fiber with a lower cut-off - 300 kDa. At this stage of the process, the majority of the impurities were already removed in the AEX, decreasing the chance of membrane blocking. Size exclusion chromatography was already successfully implemented as a polishing step for adenovirus purification [12, 31]. The selected size-exclusion medium was Sepharose 4 Fast Flow, a highly cross-linked 4% agarose matrix that was already proven to completely exclude adenovirus from its pores and eluting them in the column void volume [33]. The use of SEC allows to change the buffer to the one used in the formulation (2.5% Glycerol, 20 mM Tris, 25 mM NaCl, pH 8) which is known to confer cryopro-

tection on the adenovirus preparations during freeze-thaw and long-term storage [34]. In the TFF2 step, a higher genome particle recovery was achieved for Polysorbate process (Table **2**). This can be explained by the presence of Polysorbate 20, that even in low amounts, binds to the membrane, avoiding the virus being entrapped. Regarding the SEC step, the recoveries were lower for both samples comparing with other DSP steps, although these values are close to the ones previously described for the same size-exclusion media [33]. Finally, and to ensure a completely compatible GMP process, a sterilizing step before filling and storage of the final product is mandatory [35]. However, for purified and concentrated adenovirus, aggregation is affected by different parameters including virus concentration [36] indicating that this sterile filtration can be critical. In our study, we used a ULTA Prime CG containing a PES 0.2 μm filter and a 0.6 μm prefilter. In this stage, the concentration of detergents present in the samples should be minimal and should not affect the filter performance. In this case, similar results were obtained for recovery and impurities removal. In fact, no virus losses were obtained in this final DSP step, which makes this filter a recommended candidate for manufacturing processes.

3.5. Scale-up

Having successfully purified oncolytic adenoviruses using the different downstream steps presented before, we next developed an approach for scale-up experiments. Scale-up runs of 2 L of oncolytic adenovirus were performed using the Polysorbate 20 as the cell lysis agent, and both downstream materials and conditions described before. Fig. (**4**) shows genome particles recoveries for each step of the scale-up with Polysorbate 20, compared with the previous yields obtained for Triton X-100 in each step. An improvement in particles recovery is observed throughout the purification process. Additionally, the use of a hollow fiber with a larger membrane cut-off (750 kDa) when compared with those described in the literature [37] was successfully implemented, without compromising recovery. Moreover, intermediate purification using the AEX Capto Q impRes offered a high binding capacity, without virus losses in the flowthrough. Comparing the scale-up process with small-scale experiments, the recovery yields of size exclusion chromatography are also higher for large-scale process. The use of ULTA Prime CG for the final sterile filtration step demonstrated to be robust as we were able to load up to 8×10^{12} particles per cm^2 of the membrane without particles loss.

The final sample was evaluated according to its purity, quantity and potency (Table **3**). It was possible to achieve a final concentration of $9.0 \times 10^{11} \pm 4.6 \times 10^{11}$ TP/ml and $1.7 \times 10^{11} \pm 1.5 \times 10^{10}$ IP/ml, with a global recovery yield of 52 ± 18 % and 61 ± 4 % for total and infectious particles, respectively. The removal of impurities was determined for DNA, total protein and host cell protein achieving over 97.1%, 99.5% and 99.9% removal, respectively. Taking into consideration the specifications for the concentration of host cell DNA from the European Pharmacopeia and the World Health Organization [38], and assuming a dose of 1×10^{10} total viral particles [39] we are still below the limit of 10 ng/dose, using the Picogreen assay (Table **3**). Nevertheless, the DNA in the sample was analyzed for all double strand DNA, by the Picogreen assay. In Table **3**, it is also possible to observe that the concentration of host cell proteins and residual DNA in the final sample are also below the limits.

The scale-up process was performed with Polysorbate 20 as cell lysis agent; in order to confirm the removal of the detergent, an UHPLC quantification was assessed for the final process sample. No detergent was detected in the final sample indicating that the downstream process totally removed the detergent. In Fig. (**5**) it is also possible to observe that the oncolytic adenovirus particles are present in the final product and maintain their integrity.

The ratio between total/infectious particles is a critical parameter of quality of the final product and should not exceed the value of 30 according to the proposed FDA guidelines (FDA Gene Therapy Letter, 2000). Limitations on TP/IP ratios are established to minimize the exposure of patients to high concentrations of inactive virus particles. The ratio achieved with this process was 6, meaning that the virus has high quality, being a potential candidate for clinical trials.

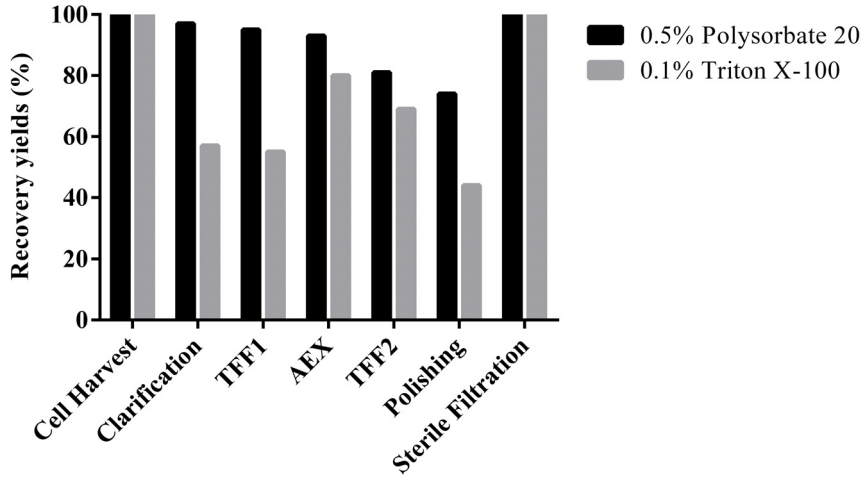

Fig. (4). Evaluation of the different steps in the downstream processing of oncolytic adenovirus. Comparative virus recovery yields of each downstream step using two different detergents for cell lysis - Triton X-100 and Polysorbate 20.

Table 3. Purification results for the scale-up process of oncolytic adenovirus using Polysorbate 20 as cell lysis detergent. Evaluation of purity and the required targets for phase I clinical trials, assuming a dose of 1×10^{10} total viral particles. Values are shown as the mean ± standard deviation (n=2).

-	Scale-up	Purity Targets
TP/IP Ratio	6 ± 3	<30
DNA (ng/dose)	3 ± 0.5	10
HCP (µg/mL)	7.9 ± 3.4	1-100
Benzonase (ng/mL)	BLD*	<5

*BLD: Below detection limit.

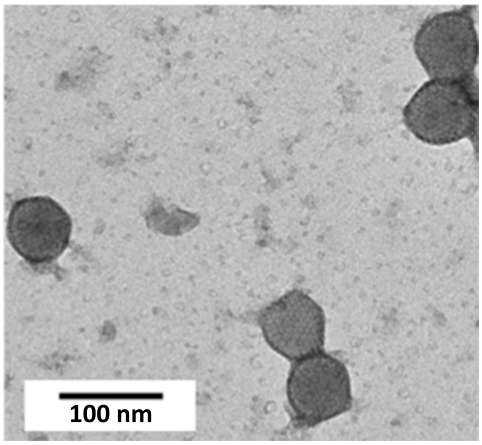

Fig. (5). Transmission electron microscopy of purified oncolytic adenovirus from the scale-up experimenys to confirm their presence, morphology and integrity. Magnification 20000 x.

CONCLUSION

The rise of oncolytic virotherapy field requires the development of downstream purification platforms that are scalable for use in clinical grade. However, as the traditional cell lysis detergent, Triton X-100, has been included in the REACH list, alternatives are needed. In this work, we assess a downstream process platform for clinical grade production of oncolytic virotherapy, using Polysorbate 20 instead of Triton X-100 for cell lysis. The results indicate that Polysorbate 20 can improve the recovery at critical stages - such as tangential flow filtration - and does not interfere with impurities removal throughout the process. A 2 L scale of oncolytic adenovirus was purified and the developed strategy was successfully implemented with a virus particle recovery of 65%. The virus obtained presented high purity (low levels of dsDNA and HCP), and quality (low TP/IP ratio) necessary to be implemented for clinical applications. The process that was established offers significant advantages over the laboratory freeze-thaw method for cell lysis and the traditional CsCl gradients for adenovirus purification. Thus, this purification process can be used for clinical batches biomanufacturing. Nevertheless, a further study could assess different holding points on the process to have a better understanding of the virus behavior using the alternative detergent.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors Mafalda G. Moleirinho, Sara Rosa, Ricardo Silva, Paula Alves, Cristina Peixoto and Manuel Carrondo declare no competing financial interests. The authors Åsa Hagner-McWhirter, Gustaf Ahlén, Mats Lundgren are employees of GE Healthcare.

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