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Inline-tandem purification of viruses from cell lysate by agarose-based chromatography

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ABSTRACT

An efficient chromatography-based virus purification method has been developed and validated for the non-pathogenic infectious virus PRD1. Compared to the conventional method that consists of relatively time-consuming and labour-intensive precipitation and density gradient ultracentrifugation steps, the method developed here is performed in a single flow using tandem-coupled anion exchange and size exclusion chromatography (AIEX-SEC) columns. This inline approach helps to minimize the loss of virus in the process and streamlines time consumption, since no physical transfer of the sample is required between purification steps. In the development process, sample feed composition, dynamic binding capacity and elution conditions for the AIEX resin as well as different exclusion limits for SEC resins were optimized to achieve maximal yield of pure infectious viruses. Utilizing this new approach, a high-quality virus sample was produced from a lysate feed in 320 min with a total yield of 13 mg purified particles per litre of cell lysate, constituting a 3.5-fold yield increase as compared to the conventional method, without compromising the high specific infectivity of the product (6×10^{12} to 7×10^{12} pfu/mg of protein). The yield of infectious viruses of the lysate feed was 54%. The easy scalability of chromatography-based methods provide a direct route to industrial usage without any significant changes needed to be made to the purification regime. This is especially interesting as the method has high potential to be used for purification of various viruses and nanoparticles, including adenovirus.

1. Introduction

Technology developments in manipulating viral nanoparticles have led to several virus-based applications in various fields and especially in pharmacology, biomedicine, separation, and material science [1–3]. Viruses and virus-like particles (VLPs) are complex nanoparticles that vary in size, mass, density, shape, composition, hydrodynamic volume and physico-chemical surface properties e.g., charge distribution, hydrophobicity, and post-translational modifications. Typically, virus purification technologies exploit these properties and allow separation of the viruses from the host cell debris and process-derived impurities. Due to the diversity of virus shapes, sizes, and surface modifications, different approaches are needed to establish a purification method that is suitable for as wide range of applications as possible. New chromatographic matrixes and innovative purification strategies also facilitate e.g., the development of viral vaccines and gene therapy vectors. For clinical trials, the scalability of the purification process is an important parameter. In addition, the study of virus structures and the rapid development of structural biology methods, especially cryo-electron microscopy, enable the targeted design of antiviral drugs or analysis of potential virus candidates as nanocarriers of drugs or vaccines [4]. Here also, a high-quality and homogeneous virus sample plays a significant role.

The icosahedral membrane-containing bacteriophage PRD1 is a well-established model virus about which we have a wealth of knowledge and is one of the best studied viruses in the kingdom *Bamfordvirae* [5]. PRD1

Abbreviations: AIEX, anion exchange chromatography; DBC, dynamic binding capacity; pfu, plaque forming unit; SEC, size-exclusion chromatography; VLP, virus-like particle; CV, column volume.

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is a medium-sized virus with a diameter of 65 nm and a molar mass of 66 MDa, which coat structure is evolutionarily related to human pathogenic adenovirus [6]. PRD1 particles purified by conventional methods with limited scaling possibilities [7] have been utilized to obtain high-resolution virus structures and to understand the virus assembly, host infection mechanisms and virus evolution, which has only been possible with samples of high quality and purity [8–13]. In addition, PRD1 has been used to establish new separation methods for viruses and other large macromolecular complexes [14–16] and is capable to carry drugs [17].

Poor scalability of traditional, labour-intensive virus purification processes, such as density gradient ultra-centrifugations and precipitations, and sometimes inadequate purity and yield of the product, has established liquid chromatography as an important downstream process for virus purifications [18]. Agarose-based resins consisting of cross-linked porous beads have been used for decades in purifications of a diverse set of macromolecules [19,20] and they are currently widely used for isolation and purification of viruses and VLPs [21]. Agarose does not adsorb biomolecules non-specifically in significant extent, it has good flow properties, and it can tolerate extremes of pH and ionic strength, i.e. it is compatible with harsh cleaning and regeneration solutions [22,23]. The most frequently used chromatography technique for virus purifications is anion exchange chromatography (AIEX) that exploits the negatively charged viral surface proteins that adsorb to the positively charged ligands immobilized on the beads [24]. All positively charged molecules pass through the packed column and are removed from the feed. The adsorbed material is commonly eluted from the matrix by employing an increasing salt concentration. Another chromatography step often used is size-exclusion chromatography (SEC) [25,26], by which the molecules are separated based on their size. The macro-sized viruses, such as PRD1, are usually excluded from the pores of the resin. This makes them to migrate faster and elute in the void volume, while the smaller impurities enter the pores and are thus delayed in their passage through the column. Many resins have pore sizes in the range of 50–100 nm [27,28] indicating that smaller viruses, such as the 20–26 nm sized adeno-associated virus AAV [29], may partially occupy the inner surface volumes of the pores. Nowadays there are resins developed specifically for larger biomolecules with pore sizes of up to 1000 nm to promote virus entry into the pores [30]. However, the most common approach is to exploit the exclusion limit of the resins to achieve separation between impurities and target virus retaining in the void volume. AIEX and SEC are orthogonal chromatographic purification methods that, when combined, increase the efficiency of the virus purification. Here, we report an efficient scalable chromatographic inline-tandem virus purification method, where both agarose-based AIEX and SEC chromatographic purifications are conducted in one step (Fig. 1).

2. Materials and methods

2.1. Production of viruses and preparation of cell lysate

Wild-type bacteriophage PRD1 [31] was cultured on *Escherichia coli* HMS174(pLM2) [32] using LB broth at 37 °C. The virus stocks were made by using the double-layer agar method. The top-layer agar from the semi-confluent plates was collected and cultured in LB broth (3–4 mL per plate) for 3 h. Subsequently, the cell debris and top-layer agar were removed by centrifugation (Sorvall rotor F14, 8 000 rpm, 20 min, 4 °C). For virus production, the bacterial culture was infected at a cell density of 1×10^9 cfu/mL (optical density of 550 at 0.9) using a multiplicity of infection of 10. After virus induced cell lysis the culture was treated with DNase I (50 µg/mL; Sigma-Aldrich) for 30 min at 37 °C. Cell debris was removed by centrifugation (Sorvall rotor F12, 8 000 rpm, 20 min, 4 °C) and subsequent filtration (Polyethersulfone filter, 0.45 µm Thermo Fisher Scientific) to obtain the clarified cell lysate (from now on the cell lysate).

2.2. Precipitation and preparative ultracentrifugation of viruses

Viruses were precipitated from the cell lysate using 10% (w/v) polyethylene glycol (PEG) 6000 and 0.5 M NaCl by adding solid PEG and NaCl, which were dissolved by magnetic stirring at 4 °C for 30 min [33]. Particles were collected by centrifugation as above (30 min), and resuspended in 1/80 vol in the virus buffer (20 mM potassium phosphate, pH 7.2, 1 mM $MgCl_2$) to yield precipitated virus sample (Fig. 1B). The virus buffer was used in all subsequent purification steps.

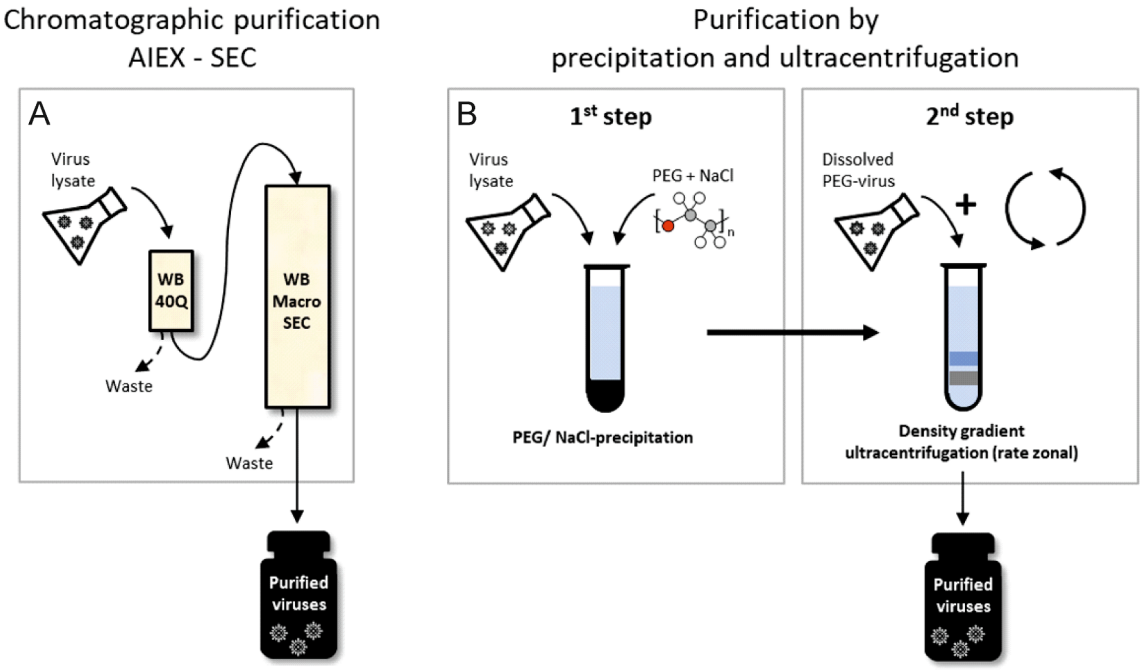

Fig. 1. Setup for the inline-tandem purification of viruses by agarose-based chromatographic resins (A) and conventional two-step virus purification method by PEG-NaCl precipitation and ultracentrifugation in density gradients (B).

Insoluble material from the PEG-virus sample was removed by centrifugation (Sorvall rotor F20, 7 000 rpm, 10 min, 4 °C). The supernatant was purified by sucrose density gradient centrifugation in a linear 5–20% (w/v) sucrose gradient (Sorvall rotor AH629, 24 000 rpm, 55 min, 15 °C)[7] and the zone containing mature infectious viruses was collected (Fig. 1B). The obtain purified virus was concentrated by differential centrifugation (Sorvall rotor T647.5, 32 000 rpm, 2 h, 5 °C) and resuspended in the virus buffer.

2.3. Optimization of individual chromatographic steps

Chromatographic steps were first performed individually to optimize the conditions. WorkBeads™ 40Q, WorkBeads Macro SEC, WorkBeads 40/1000 SEC, and WorkBeads 40/10 000 SEC, which all consist of 45 μm agarose beads (Bio-Works), were used. All chromatographic runs were conducted at ambient temperature. WorkBeads 40Q comprising agarose beads derivatized with quaternary amine was packed in 6.6 × 100 mm (column volume, CV: 3.4 mL) glass columns (Omnifit). WorkBeads SEC resins were packed in 10 × 300 mm (CV: 23.6 mL) glass columns (Omnifit) applying 5% bed compression according to the manufacturer's instruction. For the AIEX, virus buffer (as defined in Section 2.2) was used as binding buffer, and virus buffer supplemented with 1 M NaCl was used as elution buffer. Samples used were lysate supernatant and virus pre-purified by PEG-NaCl precipitation. All samples were stored at +4 °C before use.

WorkBeads 40Q was subjected to a cleaning-in-place (CIP) step consisting first of a wash with 3 CV of water, incubation with 0.5 M NaOH for 15 min, and a final wash with 3 CV of water prior to equilibration in binding buffer for 5 CV. The sample (40 mL or specified volume) was loaded onto WorkBeads 40Q at a flow rate of 0.9 min/mL (residence time of 4 min). Adsorbed viruses were eluted with a linear gradient of 0–100% elution buffer over 30 CV or a one-step gradient with 25% elution buffer for 10 CV followed by a 40-mL step at 100% elution buffer. The eluted material was collected in 0.5 mL fractions and analyzed for virus content.

The WorkBeads SEC resins were equilibrated with 2 CV of binding buffer (i.e. virus buffer) prior to sample loading. Virus feed eluted from WorkBeads 40Q (1 mL or specified volume) was loaded onto columns packed with either WorkBeads Macro SEC, WorkBeads 40/1000 SEC, or WorkBeads 40/10 000 SEC. Isocratic conditions were applied using virus buffer at a flow rate of 1 mL/min. Eluted viruses were collected in 0.5 mL fractions and analyzed for virus content. All eluted material as well as the different feeds were further analyzed for virus activity, yield and purity by plaque assays and sodium dodecyl sulphate polyacrylamide amide gel electrophoresis (SDS-PAGE)(Section 2.6).

2.4. Dynamic binding capacity

The dynamic binding capacity (DBC) of virus feed on WorkBeads 40Q, to get the maximal loading capacity for the resin, was determined at 10% breakthrough of viruses. The amount of viruses was defined by plaque forming units (pfu). Virus lysate was loaded onto a 2-mL column (6.6 × 60 mm) packed with WorkBeads 40Q at a residence time of 4 min (flow rate of 0.5 mL/min). Fractions (1 mL) were collected and analyzed by plaque assay. The maximal pfu was determined in the feed lysate which was loaded onto the column at time 0 (V_0; see the formula below) and when the column got saturated, i.e. reached its maximum loading capacity (10% of the maximal pfu), there was a breakthrough of the viruses at certain volume ($V_{10\%}$; see the formula below). A factor of 0.9 × CV compensates the delay volume in the column. The obtained DBC volume for the column is divided with CV to obtain $DBC_{10\%}$ (pfu/mL of resin). $DBC_{10\%}$ was calculated by using the following formula:

$$DBC_{10\%} = \frac{V_{10\%} - (V_0 + 0.9 \times CV)}{CV}$$

2.5. Virus purification by inline-tandem chromatography

WorkBeads 40Q and WorkBeads Macro SEC were packed as described above. The chromatographic experiments were conducted at ambient temperature and at a flow rate of 0.9 min/mL (AIEX: 150 cm/h; SEC: 65 cm/h). The two columns were connected in series using different valves in an ÄKTA™ system (Cytiva) which allowed column bypass or use in series depending on the flow path applied. See Fig. S2 for the inline setup in the Äkta system. The binding buffer and elution buffers were the same as above. After an initial CIP step, WorkBeads 40Q was equilibrated with binding buffer for 5 CV as a stand-alone column. WorkBeads Macro SEC was equilibrated with 2 CV of the same buffer. Samples were four biological replicates of the cell lysate. Forty milliliters of the sample were loaded onto WorkBeads 40Q with a subsequent wash with the binding buffer for 15 CV, followed by a step of 25% elution buffer for 10 CV to elute loosely adsorbed sample components. Elution of viruses adsorbed to WorkBeads 40Q was carried out using 12 CV (40 mL) of 100% elution buffer in one step. Upon elution of the WorkBeads 40Q column the flow-path was shifted towards WorkBeads Macro SEC via the column valve and the eluted virus fraction in 100% elution buffer was directly loaded onto the WorkBeads Macro SEC column that was pre-equilibrated with binding buffer (i.e. virus buffer) (Fig. 1A, Fig. S2). In end of run the SEC column was re-equilibrated.

2.6. Analytical methods

The number of infectious viruses (pfu) in the samples were determined by plaque assay using *E. coli* HMS174(pLM2) as a host. Protein concentrations were measured by the Bradford method [34] using a microplate reader (Thermo Scientific) and bovine serum albumin as a standard. Absorbance values at 260 and 280 nm were measured by Eppendorf Biophotometer. Homogeneity of samples was analyzed either (1) by SDS-PAGE (16% w/v acrylamide) [35] and Coomassie blue staining using PageRuler™ Unstained Protein Ladder (#26614; Thermo Scientific™) as a molecular weight standard or (2) by Bio-Rad™ Criterion™ TGX precast polyacrylamide gel 4–20% gels (567–1095, Bio-Rad) according to the manufacturer's instructions using Tris/Glycine/SDS buffer (161–0732, Bio-Rad), Laemmli sample buffer (#161-0747) including 200 mM dithiothreitol as a loading buffer, and precision plus protein standards (1610363, Bio-Rad) as a molecular weight standard.

3. Results and discussion

3.1. Optimization of the lysate sample feed

PRD1 releases its progeny from cells by host cell lysis resulting in the release of host chromosomal DNA of high molecular weight and viscous solution [16,36]. Removal of host DNA from the cell lysates can improve column loading, purification efficiency and yield by rendering the lysate less viscous. PRD1 production in *E. coli* HMS174(pLM2) yields typically ~1×10^{11} pfu/mL (Table 1). Treatment of the lysate with DNAse I, removal of the residual bacteria and other large complexes from the lysate by filtering (cut-off 0.45 μm), or their combination had no significant influence on the number of infectious viruses that was ~9.8×10^{10} pfu/mL. Further work was done with DNAse I-treated lysate which was clarified by combining high-speed centrifugation and microfiltration to improve the performance of chromatography in terms of purity and product yield.

3.2. Optimization of the AIEX chromatographic purification step

To improve the performance of PRD1 purification, two orthogonal chromatographic steps were combined: AIEX and SEC connected in series to minimize manual handling and time (Fig. 1A). For the development of the tandem method consisting of an AIEX capture step, followed by a SEC polishing step, the individual steps were first optimized.

Table 1
Virus purification by the optimized inline-tandem chromatography method and by the method based on PEG-NaCl precipitation and density gradient ultracentrifugation.[1]

Method	Sample	Volume (mL)	Virus titer (pfu/mL)	Infectious viruses in total (pfu)	Infectious viruses, yield (%)	A_{260}	A_{280}	Protein concentration (mg/mL)	Protein in total (mg)	Specific infectivity (pfu/A_{280})	Specific infectivity (pfu/mg of protein)	Purified particles, yield (mg / one liter of lysate)
Chromatography	Lysate feed	40.0[5]	$1.4 \times 10^{11} \pm 0.2 \times 10^{11}$	$5.5 \times 10^{12} \pm 0.8 \times 10^{12}$	100.0 ± 0.0	23.6 ± 1.2	14.0 ± 1.0	0.32 ± 0.02	12.6 ± 0.83	$9.8 \times 10^{9} \pm 1.0 \times 10^{9}$	$4.4 \times 10^{11} \pm 0.6 \times 10^{11}$	nd
	Purified virus	2.0	$1.5 \times 10^{12} \pm 0.3 \times 10^{11}$	$2.9 \times 10^{12} \pm 0.7 \times 10^{12}$	53.5 ± 14.3	5.0 ± 0.5	2.6 ± 0.4	0.26 ± 0.05	0.51 ± 0.11	$5.7 \times 10^{11} \pm 1.5 \times 10^{11}$	$5.8 \times 10^{12} \pm 1.5 \times 10^{12}$	12.8 ± 2.7
Precipitation	Lysate feed	40.0[5]	$1.1 \times 10^{11} \pm 0.1 \times 10^{11}$	$4.5 \times 10^{12} \pm 0.2 \times 10^{12}$	100.0 ± 0.0	28.8 ± 1.0	15.4 ± 0.5	0.33 ± 0.03	13.1 ± 1.00	$7.3 \times 10^{9} \pm 0.4 \times 10^{9}$	$3.4 \times 10^{11} \pm 0.3 \times 10^{11}$	nd
&	Precip. virus[2]	0.6 ± 0.2	$4.5 \times 10^{12} \pm 0.9 \times 10^{12}$	$2.6 \times 10^{12} \pm 0.3 \times 10^{12}$	57.6 ± 8.4	22.2 ± 6.0	17.5 ± 4.5	2.78 ± 0.88	1.81 ± 1.29	$2.6 \times 10^{11} \pm 0.4 \times 10^{11}$	$1.8 \times 10^{12} \pm 0.7 \times 10^{12}$	nd
Centrifugation	Purified virus[3]	2.2 ± 1.6	$4.5 \times 10^{11} \pm 1.4 \times 10^{11}$	$9.2 \times 10^{11} \pm 6.2 \times 10^{11}$	20.3 ± 13.8	0.8 ± 0.2	0.6 ± 0.2	0.07 ± 0.02	0.14 ± 0.09	$7.3 \times 10^{11} \pm 1.3 \times 10^{11}$	$6.6 \times 10^{12} \pm 0.9 \times 10^{12}$	3.5 ± 2.4
	Conc. virus[4]	0.02 ± 0.02	$4.5 \times 10^{13} \pm 2.8 \times 10^{13}$	$5.8 \times 10^{11} \pm 1.5 \times 10^{11}$	13.0 ± 3.5	108.8 ± 70.5	79.2 ± 51.4	6.78 ± 3.61	0.09 ± 0.01	$6.0 \times 10^{11} \pm 1.0 \times 10^{11}$	$6.6 \times 10^{12} \pm 1.9 \times 10^{12}$	2.2 ± 0.1

[1] Average and standard deviation (n = 4).
[2] After PEG-NaCl precipitation.
[3] After density gradient ultracentrifugation.
[4] Concentrated virus, a virus sample purified by precipitation and ultracentrifugation, and subsequently concentrated by differential centrifugation.
[5] Volumes have been normalized to 40 mL of original sample, allowing comparison of methods. The experimental sample volume of the lysate was 40 mL in chromatography and typically about 400 mL in precipitation-centrifugation.

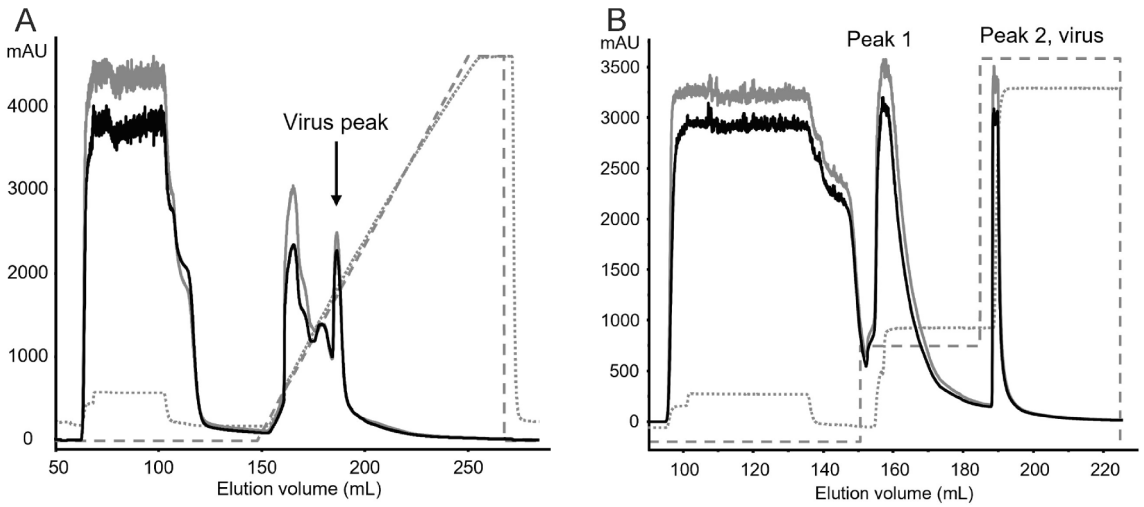

Fig. 2. Optimization of AIEX step of PRD1 purification using linear and step elution gradients. Elution profile during purification of PRD1 lysate on a 3.4 mL column packed with WorkBeads 40Q eluted with a linear 0–100% elution buffer gradient (A) or a step of 25% elution buffer followed by a 100% step (B). The virus containing peak is indicated in the chromatograms. The flow rate was 0.9 mL/min corresponding to a residence time of 4 min. Dashed line is the elution gradient, dotted line is the conductivity trace, and UV traces are shown as solid lines: absorbance at 260 nm (light grey) and absorbance at 280 nm (black).

The AIEX step was optimized for loading capacity and elution conditions. DBC using the lysate sample as feed was determined by frontal analysis at 10% breakthrough ($DBC_{10\%}$). The determined $DBC_{10\%}$ value was 23.6 mL lysate/mL of resin at a residence time of 4 min based on virus activity measured by plaque assay, where the virus lysate feed had activity of 1.2×10^{11} pfu/mL (Fig. S1). $DBC_{10\%}$ of 23.6 mL virus lysate/mL of resin corresponds to 2.8×10^{12} pfu (in a lysate)/mL of resin. The DBC value of the feed is largely dependent on the impurity profile since there will be a competition between all negatively charged molecules. For pure virus as feed, the DBC would be significantly higher but would not reflect our purification conditions.

In the next step, PRD1 behavior was analyzed on AIEX column. Virus lysate (40 mL i.e. 50% of the column's DBC) was loaded onto the AIEX column (3.4 mL) where the viruses as well as other negatively charged components adsorbed, while the positively charged components were eluted in the flow through. The elution was performed with a linear NaCl gradient to establish the salt concentration at which the target viruses elute. The viruses eluted in a single peak at ~0.4 M NaCl (~40% of elution buffer; Fig. 2A). By using the same loading conditions but by replacing the linear elution gradient with a pre-elution step with 25% elution buffer to remove weakly adsorbed material followed by a virus-elution step with the 100% elution buffer, a successful separation was obtained. There was no significant virus activity (0.12%) in the pre-elution step (peak 1), and the infectious viruses were found in the peak that eluted with the 100% elution buffer (peak 2 in Fig. 2B, Fig. S3, Table S1). All AIEX runs were conducted at a flow rate of 0.9 mL/min which equals 4 min residence time. This step-elution gradient might need optimization for each virus system with different lysate impurity profiles.

3.3. Optimization of the SEC chromatographic purification step

To determine the optimal exclusion limit of the resin for the SEC purification step, three different SEC resins all based on the same 45 μm agarose bead with increasing pore sizes were tested: WorkBeads 40/1000 SEC (cut-off: 1 000 kDa), WorkBeads 40/10 000 SEC (cut-off: 10 000 kDa) and WorkBeads Macro SEC (cut-off: 30 000 kDa). Forty milliliters of virus feed were pre-purified on AIEX, collected and applied (1 mL) onto a 10 × 300 mm column packed with the respective SEC resins at a flow rate of 1 mL/min (76.5 cm/h). There was a complete separation between the void peak and the remaining peaks for all SEC resins tested (Fig. 3A-C). Virus infectivity was present in the void (peak 1) and only minor virus activity was detected in the remaining impurity" peaks as determined by plaque assay (Table S1). The yields were high, and the protein profiles of the virus eluates were similar for all SEC resins (Table S1, Fig. S3). WorkBeads Macro SEC was chosen for the further optimization due to the high yield (98%) and large pore size. Next, a larger 5 mL sample of the AIEX-purified virus feed was loaded onto the Macro SEC resin to mimic the tandem setup runs (Fig. S4a) at a flow rate of 1 mL/min. A sample load of ~20% of the SEC columns' CV is a standard load for group separations. There was a baseline separation between the virus containing peak (peak 1 in Fig. S4a) and the second impurity peak. The homogeneity of the SEC-purified virus was confirmed by loading a sample to AIEX that resulted in a single peak (Fig. S4b).

Our purification method aimed to exclude the viruses from the resin pores and keep them in the void volume, while the impurities enter the pores. Thus, delayed elution of impurities would possibly increase the purity of the final product. Previous studies have been undertaken using WorkBeads 40/10 000 SEC for purification of the 28-nm sized adenoviral dodecahedron (Dd) VLPs with good separation [26]. WorkBeads Macro SEC resin has relatively high porosity (exclusion limit of 30 000 kDa) and the hydrodynamic diameter of an average pore size is less than the size of 65-nm diameter PRD1 particles [8]. Such resin is optimal for larger macromolecular complexes, such as PRD1, even though all SEC resins evaluated generated a good separation between PRD1 and smaller components (Fig. 3).

3.4. Testing of precipitation coupled with chromatographic purification

PEG precipitation of viruses is an efficient purification technique often used as the first step in virus purification, but it is not sufficient as a single purification step. To assess the benefit of conducting PEG precipitation prior to chromatographic purifications, we compared virus lysate versus PEG—precipitated virus as feeds. When PEG—precipitated PRD1 was purified on WorkBeads Macro SEC, the chromatogram indicated minor decrease in resolution as the baseline between the void peak (peak 1, virus) and the second impurity peak was shortened (Fig. 4A), when compared to the SEC purifications of virus lysate that was pre-purified with AIEX as a feed with a similar feed volume loaded (~20% of CV) (Fig. S4A). There was no increase in virus activity or purity, although the recovery was high (Table S1, Fig. 4D), which indicates the PEG precipitation followed by SEC purification was not enough to achieve desirable purity. When the PEG-precipitated SEC-purified virus

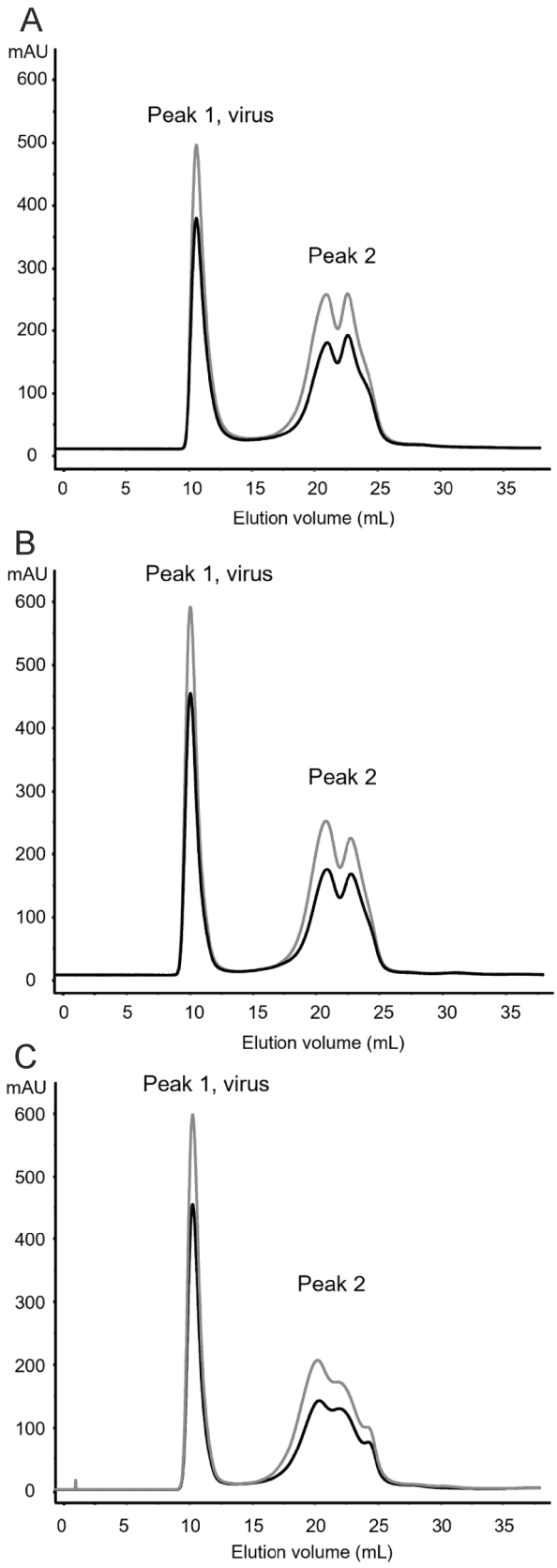

Fig. 3. Size-exclusion chromatograms using AIEX-purified PRD1 preparation and WorkBeads Macro SEC (A), WorkBeads 40/10000 SEC (B), or WorkBeads 40/1000 SEC (C). The size of the column was 23.6 mL. The flow rate was 1 mL/min. Virus-containing peaks (peak 1) and impurity-containing peaks (peak 2) are indicated in the chromatograms. UV traces are shown as solid lines: absorbance at 260 nm (light grey) and absorbance at 280 nm (black).

(peak 1 in Fig. 4A) was loaded onto AIEX column, it resulted in poor purity and separation between peaks (Fig. 4B, D) compared to AIEX-SEC purified virus feed (Fig. S4B).

The PEG-precipitated virus (4.5 mL; 2.5×10^{12} pfu/mL) was further tested with an inline-tandem chromatography setup, consisting of AIEX followed by Macro SEC at a flow rate of 0.9 mL/min. The final virus peak for the PEG precipitated feed was not well resolved and had low virus activity (Fig. 4C, D). The yield of infectious viruses (pfu) was 8.4% (Table S1). Thus, PEG seems to severely impact the effectiveness of the AIEX purification. This can be somewhat mitigated by first applying the sample to a SEC column/resin to remove salts, small molecules, and residual PEG.

3.5. Inline-tandem chromatographic purification of viruses by SEC and AIEX

Four biological replicates of cell lysate were purified by the optimized inline-tandem setup to verify the robustness and reproducibility of the method (Fig. 1A, Fig. S2). In parallel, the same biological replicates were purified by the method based on precipitation and ultracentrifugation in a density gradient for comparison (Fig. 1B). Forty milliliters of virus lysate were applied onto a 6.6 × 100 mm (CV: 23.6 mL) column packed with WorkBeads 40Q at a flow rate of 0.9 mL/min (residence time of 4 min). The virus titer in the lysate feed was 1.1×10^{11} pfu/mL with a specific infectivity of 4.4×10^{11} pfu/mg of protein (Table 1). The wash and elution steps were conducted as previously optimized (Section 3.2). During elution the flow path was shifted towards the WorkBeads Macro SEC via the column valve resulting in four identical chromatograms (Fig. 5A, Fig. S5A-C). The yield of the infectious viruses after inline-tandem purification of the lysate was 54% of the feed, yielding 12.8 mg of purified virus per liter of lysate (Table 1). Specific infectivity increased more than tenfold to 5.8×10^{11} pfu/mg of protein. In comparison, PRD1 purification using precipitation and density gradient ultracentrifugation (Section 2.2.) yielded 20% recovery of infectious viruses (pfu) and 3.5 mg of purified virus per liter of lysate. Thus, protein yield (purified virus) was almost four-fold higher using tandem purification compared to the conventional precipitation and centrifugation method, and the yield of infectious viruses was around three-fold. With both methods, the quality of the final samples was high with specific infectivities ranging from 5×10^{12} to 6×10^{12} pfu/mg of protein (Table 1). The concentration of the conventionally purified virus sample using differential centrifugation further reduced the yield (measured as protein and infectious viruses), but the high sample quality measured as specific infectivity was maintained (Table 1). Analysis of the inline-tandem purified virus samples by polyacrylamide gel electrophoresis revealed mainly PRD1-specific proteins confirming the good separation between the virus and the impurities (Fig. 5B). The inline-tandem chromatographic purification was completed in 320 min, as was the precipitation and density gradient ultracentrifugation-based purification method. Concentration of the virus sample by differential ultracentrifugation took additional 4–6 h, including dissolution of the sample.

3.6. Scaling-up and general applicability of the inline-tandem chromatographic purification method

For clinical studies and therapeutic uses, large amounts of homogeneous virus material are needed, which places demands on the scalability of the purification processes. This applies to many different viruses and VLPs. When scaling up from laboratory-scale to bioprocess-scale purification there are many parameters to consider. Contact time or residence time during the application of the virus—containing feed is the most important for the capacity and determines the actual flow rates in the bioprocess-scale columns [37]. The highly crosslinked agarose resin with a mean particle size of 45 μm generates only moderate back pressures. For example, a large diameter column with a bed height of 20

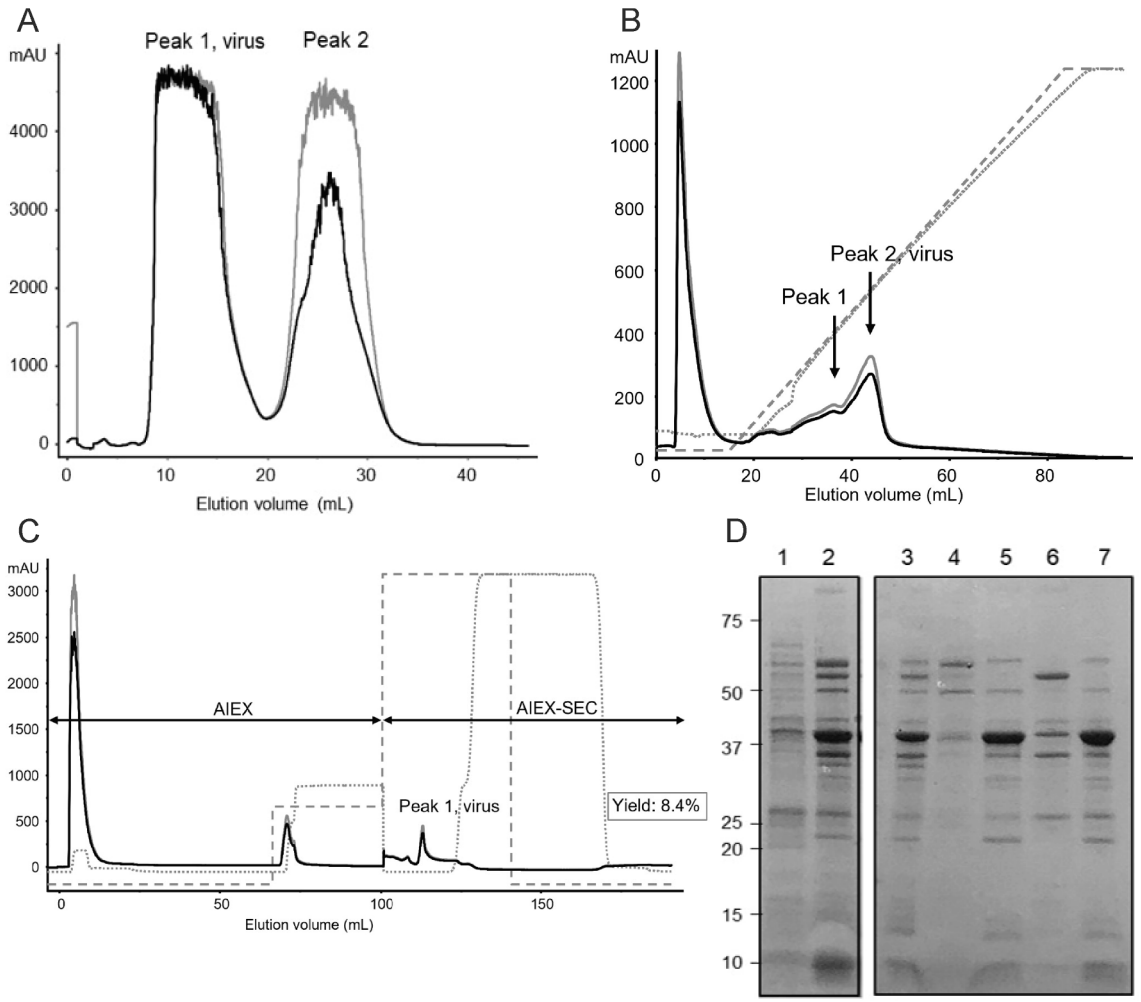

Fig. 4. SEC and AIEX of the PEG-precipitated virus feed. Purification of the PEG-precipitated virus by WorkBeads Macro SEC (A). SEC-purified virus (peak 1 in A) was applied onto WorkBeads 40Q (B). Peak 2 contained the virus and was collected for further analysis. PEG-precipitated virus feed was loaded onto an AIEX-SEC tandem setup, where peak 1 was collected for analysis (C). The flow path switch from AIEX as a stand-alone column to AIEX followed by SEC is indicated in the chromatogram with black solid double headed arrows. See Fig. S2 for a detailed setup scheme. Dashed line is the elution gradient in B and C, dotted line is the conductivity trace, and UV traces are shown as solid lines: absorbance at 260 nm (light grey) and absorbance at 280 nm (black). The flow rate was 1 mL/min in (A) and 0.9 mL/min in (B-C) and column sizes were 3.4 mL for WorkBeads 40Q and 23.6 mL for WorkBeads Macro SEC. Coomassie-stained SDS-PAGE gel of virus sample feeds and collected samples (see panels A-C) (D). Size marker (kDa) is indicated on the left. Lane 1: lysate feed, lane 2: PEG-virus feed, lane 3: SEC, peak 1/ virus peak (A), lane 4: SEC, peak 2 (A), lane 5: SEC-AIEX, peak 2/ virus peak (B), lane 6: AIEX-SEC, peak 1 (B) and lane 7: AIEX-SEC on PEG feed (C). Numeric data of the feeds and sample analysis are shown in Table S1.

cm results in 1.5 bar pressure drop over the bed when flow rate 150 cm/h was applied. This means that normal chromatographic equipment can be used [37]. All resins used in this study have bead sizes of 45 μm, which is a good compromise between resolution and back-pressure, thus they are highly compatible with scaling up parameters. The 45 μm-beads additionally exhibit a higher degree of cross-linking, rendering them more rigid, and thus avoiding packing issues in the bioprocess-scale columns that some agarose-based resins with high porosity may encounter since they tend to be relatively soft [38]. To scale up the developed virus purification chromatography method (Fig. 1A), the individual orthogonal steps can be performed as stand-alone steps making the process more generic and easier to implement. Robust and reliable scale-up designs minimize the risk of compromising the results, such as purity and yield. Viruses and VLPs are diverse complex nanoparticles with different physical, chemical, and physicochemical surface properties. In regard to one particular physicochemical surface property, viruses with an isoelectric point (pI) above 8.5 seem to be very rare [39]. Surface charge of viruses plays a major role in various adsorption processes, such as IEX, where viruses with acidic net surface charge are prone to interact with AIEX resins. This feature together with the large sizes of viruses, makes the proposed inline-tandem setup consisting of AIEX followed by WorkBeads Macro SEC very generic and versatile virus purification method. If a virus or VLP with a higher pI is to be purified, or a lower pH employed, the AIEX step can be exchanged for a cation exchange (CIEX) step. However, it is essential to remove all nucleic acids prior to the chromatography purification, since they will not be properly removed by CIEX inline with SEC. AIEX combined with CIEX can also be used as a virus purification method [40], but then only one feature is used to achieve the separation, i.e. charge. By exploiting two orthogonal features of the virus, i.e. charge and size, maximal resolution is ideally achieved.

Here, we studied PRD1 as the target virus. Our chromatography method introduced significant improvements in PRD1 purification practices, leading to considerable time savings and better yields. PRD1 has a coat structure evolutionarily related to human pathogenic adenovirus [6], a commonly used viral vector in gene therapy. Adenovirus purification with AIEX has been successful [41] and the application of AIEX and SEC for adenovirus purification has been previously employed using methacrylate-based weak AIEX resin [42]. The ultrafiltration step required between the AIEX and SEC steps alone led to

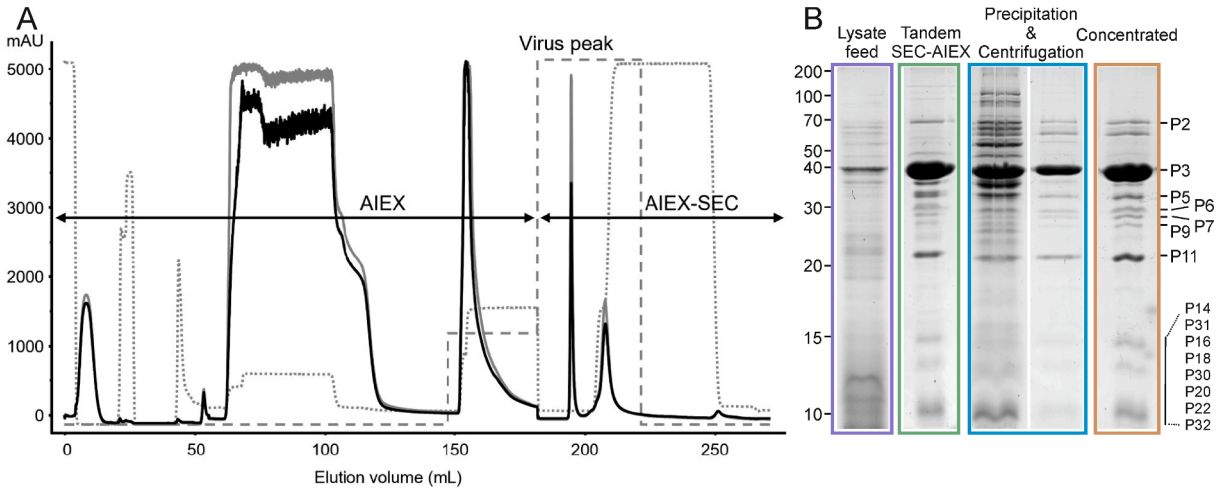

Fig. 5. Elution profile of optimized inline-tandem AIEX-SEC purification using virus-lysate feed. Four biological replicates of PRD1 lysate were purified using the tandem setup of AIEX and SEC. Dashed line is elution gradient, dotted line is the conductivity trace, and UV traces are shown as solid lines: absorbance at 260 nm (light grey) and absorbance at 280 nm (black). The flow rate was 0.9 mL/min and the column sizes were 3.4 mL for WorkBeads 40Q and 23.6 mL for WorkBeads Macro SEC. The flow path switch from AIEX as a stand-alone column to AIEX followed by SEC is indicated in the chromatogram with black solid double-headed arrows. See Fig. S2 for a detailed setup scheme. One of the replicates is shown here and other three replicates in Fig. S5A-C (A). Protein composition of the samples analyzed in an SDS-PAGE gel stained with Coomassie blue (B). Same lysate feed (purple border line) was used for the inline-tandem AIEX-SEC method (green border line) and the method based on PEG-NaCl precipitation and ultracentrifugation (blue border line). After ultracentrifugation, the sample was concentrated (brown border line). Molecular mass marker (kDa) is on the left and the positions of the PRD1 structural proteins are indicated on the right. Numeric data for the samples are shown in Table 1.

more than 50% loss of viral particles [42]. Thus, the inline-tandem AIEX-SEC method introduced here is also a potential method for adenovirus purification.

4. Conclusions

A typical virus purification may consist of several steps, for example precipitation followed by preparative ultracentrifugation using density gradients. This will ultimately generate a pure product but through several techniques and equipment and at the expense of manual labor. Moreover, the scaling-up is difficult to implement. PEG-NaCl-precipitation alone is an efficient precut purification technique (Fig. 5, Table 1) [33] but is not sufficient to achieve the required purity. By replacing the labor-consuming steps with two orthogonal chromatographic purification steps, the efficiency of the virus purification is increased. The setup using AIEX followed by SEC connected in series, still allowing the columns to be operated individually, resulted in a purification completed in only 320 min in total. Purifying the four biological replicates by AIEX-SEC method (Figs. 1A, 5, Table 1), almost 13 mg of purified particles were obtained per one liter of lysate, resulting in a significantly higher yield (over 300 %) than obtained with the conventional precipitation-ultracentrifugation based method (Fig. 1B, Table 1).

CRediT authorship contribution statement

Mikael Andersson Schönn: Methodology, Investigation. **Katri Eskelin:** Writing – review & editing. **Dennis H. Bamford:** Conceptualization, Resources, Writing – review & editing. **Minna M. Poranen:** Resources, Writing – review & editing, Funding acquisition. **Cecilia Unoson:** Validation, Resources, Writing – original draft, Visualization. **Hanna M. Oksanen:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123140.

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