Asymmetrical flow field-flow fractionation in purification of an enveloped bacteriophage phi6

Mirka Lampi[a], Hanna M. Oksanen[a], Florian Meier[b], Evelin Moldenhauer[b], Minna M. Poranen[a], Dennis H. Bamford[a], and Katri Eskelin[a]*

* Corresponding author: katri.eskelin@helsinki.fi, +358504486048

[a] Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Viikinkaari 9B, FI-00014 Helsinki, Finland

[b] Postnova Analytics, Max-Planck-Str. 14, 86899 Landsberg, Germany

Abstract

Basic and applied virus research requires specimens that are purified to high homogeneity. Thus, there is much interest in the efficient production and purification of viruses and their subassemblies. Advances in the production steps have shifted the bottle neck of the process to the purification. Nonetheless, the development of purification techniques for different viruses is challenging due to the complex biological nature of the infected cell cultures as well as the biophysical and -chemical differences in the virus particles. We used bacteriophage phi6 as a model virus in our attempts to provide a new purification method for enveloped viruses. We compared asymmetrical flow field-flow fractionation (AF4)-based virus purification method to the well-established ultracentrifugation-based purification of phi6. In addition, binding of phi6 virions to monolithic anion exchange columns was tested to evaluate their applicability in concentrating the AF4 purified specimens. Our results show that AF4 enables one-hour purification of infectious enveloped viruses with specific infectivity of ~1×10^{13} PFU / mg of protein and ~65-95 % yields. Obtained purity was comparable with that obtained using ultracentrifugation, but the yields from AF4 purification were 2-3 –fold higher. Importantly, high quality virus preparations could be obtained directly from crude cell lysates. Furthermore, when used in combination with in-line light scattering detectors, AF4 purification could be coupled to simultaneous quality control of obtained virus specimen.

Keywords: Field flow fractionation, macromolecular complex, virus purification

[1]Abbreviations

AF4, asymmetrical flow field-flow fractionation; BSA, bovine serum albumin; CIM, convective interaction media; D_{geo}, geometric diameter; DLS, dynamic light scattering; ds, double stranded; MALS, multi angle light scattering; MWCO, molecular weight cut-off; PEG, polyethylene glycol; PES, polyethersulfone; PFU, plaque forming unit; QA, quaternary amine; RC, regenerated cellulose; R_g, radius of gyration; R_h, radius of hydration; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ss, single stranded; TCA, trichloroacetic acid; V_c, cross-flow; V_f, focus flow; VLP, virus like particle; V_{out}, channel flow

1. Introduction

Several animal viruses have an outer lipid envelope surrounding the proteinaceous capsid. The envelope has a role in host recognition and cell penetration [1]. There is a group of bacteriophages (viruses of bacteria), cystoviruses, that have an outer lipid envelope [2][3]. Phi6, the type organism of the family *Cystoviridae*, infects *Pseudomonas syringae* and penetrates into cells by membrane fusion in a similar manner as animal viruses [1]. The virion of phi6 is spherical, ~85 nm in diameter and has a nucleocapsid composed of two proteinaceous layers beneath the lipid envelope [4]. Nucleocapsid shares similar structure with the double-layered particles of other double-stranded (ds) RNA viruses, such as rotavirus and bluetongue virus [5, 6]. Due to the similarities between important pathogenic viruses, phi6 is an ideal model organism to develop simpler, safer, and more cost-effective ways to purify animal viruses with an external lipid-protein envelope.

Traditional ultracentrifugation-based virus purification methods expose virions (i.e. mature infectious virus particles) to high forces potentially causing aggregation and/or otherwise damaging the virions and compromising their infectivity. Viruses with lipid envelopes are especially vulnerable to biophysical and biochemical stress (e.g. pH, temperature, acids, detergents, and shear forces), and are thus prone to lose their biological activity.

AF4 is a promising method for virus purification [7, 8]. It is a versatile method in which the sample components are separated by their hydrodynamic sizes [9]. No stationary phase is applied and separation takes place in a thin trapezoidal channel by using two perpendicularly acting flows: the laminar flow through the channel and the cross-flow, which pushes the sample components towards the accumulation wall. The pore size of an ultrafiltration membrane lying on the accumulation wall determines the size of molecules that pass through the membrane or stay in the channel for separation. Due to the parabolic profile of the laminar flow, flow rates in the middle of the channel are high and lower closer to the walls. Diffusion of sample components creates a counteracting motion against the cross-flow and the distance from the accumulation wall is determined by the hydrodynamic size of the sample components. In general, small particles with higher diffusion coefficients reach an equilibrium position in the higher flow rate and are carried to

the detectors before the bigger ones. Separated molecules are directed to the detectors by the laminar flow, which enables in-line analysis of measures such as concentration, shape, size, and size distribution, depending on the detectors available [10]. AF4 has been used to separate a wide variety of biomolecules and their complexes [10, 11]. In virus research, the focus has been mainly in analytical studies, i.e. in the analysis of the size, size distribution, and aggregation of viruses or in the quantitation of virus amounts. With virus like particles (VLPs), assembly conditions, stability, as well as the purity and homogeneity of VLP preparations have been analysed [12-16].

Here we present a new, rapid and gentle virus purification method for the enveloped phi6 using AF4. We demonstrate that AF4 purification of enveloped viruses straight from the cell lysate offers an attractive new alternative for virus purification leading to high yields of infective viruses with homogenous particle sizes. Previously we have shown the applicability of AF4 in purification of viruses with different morphologies, sizes, and buffer requirements [7, 8], yet this is the first attempt to utilize AF4 in purification of an enveloped phage that resembles animal viruses. To concentrate specimens after AF4 purification, we explored the applicability of monolithic columns that have been used in the separation and purification of a wide variety of biological materials, e.g. viral genomes, plasmids, as well as proteins and their complexes [18-20] that include viruses and VLPs [21]. Due to their high binding capacity towards phi6, monolithic anion exchange columns could be coupled to AF4 to concentrate and potentially further purify virus preparations.

2. Materials and methods

2.1. Virus production and precipitation by polyethylene glycol and NaCl

Bacteriophage phi6 [2] was cultured and purified as described previously [17]. The host bacterium *Pseudomonas syringae* HB10Y was grown in L-broth (LB) at 28 °C with aeration. Logarithmically growing cells were infected by using the multiplicity of infection (MOI) of 10. Cell culture turbidity was followed at the wavelength of 550 nm (Chlormic, JP Selecta S.A., Barcelona, Spain). After lysis, the infected culture was treated by DNase I (37 °C, 30 min, 50 µg/mL; Sigma-Aldrich). Cell

debris was removed by centrifugation (Sorvall rotor F12, 8000 rpm, 20 min, 4 °C). The resulting supernatant is referred as phi6 lysate. Viruses were precipitated from the lysate using 10% (w/v) polyethylene glycol (PEG) 6000 and 0.5 M NaCl and collected by centrifugation as above. Precipitated viruses were resuspended in 20 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$, in approximately 1/100 of the initial volume. The resulting virus sample is referred as PEG-phi6.

2.2. Virus purification by ultracentrifugation

Viruses were purified in 5−20% (w/v) sucrose gradient by rate zonal centrifugation (Sorvall rotor AH629, 24 000 rpm, 50 min, 15 °C). Sucrose solutions were prepared in a buffer containing 10 mM potassium phosphate [pH 7.2] and 1 mM $MgCl_2$. The light scattering virus zones were collected and concentrated by differential centrifugation (Sorvall rotor T647.5, 32 000 rpm, 3 h, 10 °C). The virus preparation resuspended in 20 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$ (~1/300-1/5000 of the initial lysate volume) is referred as 1×phi6. To obtain phi6 preparation purified to homogeneity (referred as 2×phi6), virus-containing zones collected from the rate zonal centrifugation were further purified by equilibrium centrifugation in 20–70% (w/v) sucrose gradient (Sorvall rotor AH629, 24 000 rpm, 16 h, 15°C). Viruses were collected and concentrated by differential centrifugation as described above.

2.3. AF4

AF4 experiments and data collection were performed by using the instrumentation and software (Postnova Analytics, Landsberg, Germany) described previously [7]. Separations were performed at 22 °C in a channel containing a 350 µm spacer. A regenerated cellulose (RC) membrane with a molecular weight cut-off (MWCO) value of 100 kDa (Z-MEM-AQU-529, Postnova) was used. The channel flow was monitored by UV-detection at 280 nm. The injected volume was adjusted to 1 mL with the virus-specific buffer (20 mM phosphate buffer [pH 7.2], 1 mM $MgCl_2$) that was also used as the mobile phase in AF4. The samples studied were the phi6 lysate, PEG-phi6, and 1×phi6.

AF4 was performed by utilizing the linear cross-flow (V_c) elution program as we previously used it successfully for the purification of other similar-sized viruses [7, 8]. The channel flow (V_{out}) was 0.2

mL/min. The focusing step of 10 min with 1 mL/min focus flow (V_f) and 1 mL/min V_c was used. In the elution phase, V_c decayed linearly from 1 mL/min to 0.1 mL/min in 25 minutes. V_c of 0.1 mL/min was continued until the UV-signal reached the baseline. Between runs, the channel was rinsed with the mobile phase without V_c. Fractions of 0.8 mL were collected starting from the beginning of the elution (10 or 15 min onwards), stored at 4 °C until the off-line analyses were conducted (see 2.5.).

Multi-angle light scattering (MALS) and dynamic light scattering (DLS) data was collected using a V_{out} of 0.5 ml/min for elution. The focusing step was 5 min. A 25 min linear V_c gradient was applied starting from 1.0 mL/min to 0.28 mL/min, followed by a 15 min exponential step to a final V_c rate of 0.05 mL/min. The radius of gyration (R_g) was calculated from the measured MALS signal intensities by applying an intensity distribution function P(ϑ) (sphere fit model) as previously described [7]. The geometric diameter (D_{geo}) was calculated from the obtained R_g-values using the formula $D_{geo}=2\times R_g / 0.775$ that is suitable for particles with spherical shape. The radius of hydration (R_h) was calculated from the measured DLS signal intensities using the cumulative fit method [18].

2.4. Monolithic chromatography

Chromatography using quaternary amine (QA), convective interaction media (CIM) 1 mL monolithic anion exchange column (BIA Separations) was performed at the flow rate of 1 mL/min. Column was washed, regenerated, and stored as recommended by the manufacturer. The buffer (20 mM phosphate [pH 7.4], 1 mM $MgCl_2$) and the virus sample (1×phi6) were filtered through 0.22 µm polyethersulfone (PES) membrane filters (Thermo Scientific) before use. After loading the sample, column was washed with 20 column volumes to remove unbound material. Elution was performed using 30 column volumes of buffer and a linear 0–1.5 M NaCl gradient. Fractions (1 mL) were collected starting from the beginning of the elution gradient.

2.5. Analytical methods

Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (w/v) acrylamide in the separation gel [19] and Coomassie staining. Nucleic

acids were visualized by ethidium bromide staining of the stacking gel. When necessary, proteins were concentrated by 10% (v/v) trichloroacetic acid (TCA) precipitation (30 min on ice) and centrifugation (Eppendorf centrifuge 5415D, 13,000 rpm, 30 min, 4 °C). Purified 1×phi6 or PageRuler Prestained Protein Ladder (#26616, Thermo Scientific, Fair Lawn, NJ, USA) were used as molecular mass markers. Gels were documented using ChemiDoc (Bio Rad, Hercules, USA).

Plaque assay was used to determine the number of infectious viruses (plaque forming unit, PFU) in the samples. Protein concentrations were measured using the Bradford assay [20] and a microplate reader (Thermo Scientific, Fair Lawn, NJ, USA), with bovine serum albumin (BSA) as a standard or by measuring A_{280} values by Eppendorf BioPhotometer (Hamburg, Germany). Specific infectivities of the virus samples (PFU/mg of protein or PFU/A_{280}) were calculated by using results from plaque assay, Bradford assay and photometry. Recovery of the protein amount or infectious viruses was calculated by formulas: $[100\%*(\sum A_{280,\ fractions})/A_{280,input}]$, $[100\%*(\sum \mu g\ protein_{fractions})/\mu g\ protein_{input}]$ or $[100\%*(\sum PFU_{fractions})/PFU_{input}]$.

3. Results

3.1. Precipitation and ultracentrifugation -based purification of phi6

Phi6 was purified with the established PEG-precipitation and ultracentrifugation-based method [35] to produce input samples for purification using AF4 and to provide reference materials for comparison of these two methods. As expected, the specific infectivity of the ultracentrifugation-purified samples increased as the purification proceeded and the amount of host and growth medium-derived impurities decreased (Table 1). In the starting material (phi6 lysate), the specific infectivity was ~9×10^{11} PFU/mg of protein, which was improved ~3-fold to ~3×10^{12} PFU/mg of protein after PEG-precipitation. The amount of infective PEG-phi6 viruses was ~80% of that of the lysate. PEG-phi6 was further purified by rate zonal and concentrated by differential ultracentrifugation. The recovered 1×phi6 had a specific infectivity of ~1×10^{13} PFU/mg of protein, i.e. the specific infectivity improved by ~3-fold from that of PEG-phi6. Because A_{280} values contain also the absorption derived from nucleic acid components, the A_{280}-based specific infectivity value for 1×phi6 was lower, ~1×10^{12} PFU/A_{280}, respectively (Table 1). For PEG-phi6 and 1×phi6 the corresponding specific infectivity values improved 25- and ~6-fold, respectively. The recovery of infective 1×phi6 viruses was ~30% of that of the lysate. Precipitation and preparative ultracentrifugation purification to near homogeneity (1×phi6) took ~1.5 working days and yielded ~7 mg of virus per liter of lysate (Table 1).

The analysis of the purification process by SDS-PAGE (see input samples in Fig.1) revealed that the amount of impurities declined after each purification step, whereas phi6 proteins were enriched. Phi6 virion contains 11 structural protein types with sizes ranging from 4.3 to 85 kDa [21].

3.2. Applicability of AF4 in the purification of phi6

Phi6 lysate, PEG-phi6, and 1×phi6 were next fractionated with AF4. For the lysate, the void peak (V_0) was followed by three peaks, whereas the fractograms of PEG-phi6 and 1×phi6 had two and one major peaks, respectively (Fig. 1 A). Phi6 eluted at the end of the cross-flow gradient as a single peak that contained the majority of infective viruses. For phi6, the recovery of infectious

viruses in the best fractions (No. 7; 35-39 min) were ~95% and ~65% of the input sample for lysate and PEG-phi6, respectively (Table 2). For 1×phi6, the corresponding best fraction contained ~35% of the infectious input virus, but comparable virus amounts were present in fraction eluting at 39-43 min (Fig.1 B). Thus, by pooling the virus-containing AF4 fractions, the yields could be further improved, yet the virus concentration might become more dilute (Fig. 1 B, Table 2). The corresponding yields of the best fraction for total protein were lower than in the input samples, indicating successful removal of impurities (Table 2).

The obtained specific infectivity was ~1×10^{12} PFU/A_{280} and ~$7\text{-}8\times10^{12}$ PFU/mg of protein, irrespectively of the input sample used (Table 2). Based on PFU/A_{280} values, the highest ~200-fold increase in specific infectivity was obtained for the AF4 purified lysate. The corresponding improvement in specific infectivity was ~5-fold for PEG-phi6 and ~2-fold for 1×phi6, respectively. Repeated experiments performed with a new biological batch of input samples resulted in comparable recovery of infectious viruses and improvement in purity (data not shown).

SDS-PAGE analysis of AF4 purified lysate revealed that the first two peaks contained a variety of host proteins (Fig. S1 A). Based on our previous studies, proteins ranging in size from 100 to 670 kDa cannot be separated with the applied flow settings and they elute as a single peak in the beginning of the cross-flow elution gradient [7] as also observed here (Fig. 1 A, Fig. S1 A, B). The virus peak was enriched with virus-specific proteins (Fig. 1 C, Fig. S1).

The size distribution of the components in the phi6 input samples was analyzed with an AF4 instrument that was coupled with MALS and DLS detectors and by applying V_{out} of 0.5 mL/min (Fig. 2, Fig. S2). Both the MALS and DLS signals indicated that the particles present in the virus peak were relatively homogenous in size (Fig. 2). The corresponding intensity distribution profiles showed good agreement with the applied spherical model (Fig. S3). The analysis of the purified 1×phi6 virus preparation resulted in a fractogram, where the system peak (~7 min) was followed by the virus peak (25-35 min) (Fig. 2B). The obtained estimates for R_g (MALS) and R_h (DLS) at the virus peak maxima were ~27 nm and ~81 nm, respectively (Table 3). The corresponding R_g and R_h estimates for the PEG-phi6 were ~28 nm and ~80 nm, and for the phi6 lysate ~30 nm and ~90 nm,

respectively (Fig. 2, Table 3). However, the DLS-signal intensities for phi6 lysate were low and consequently error-prone. Conversion of the obtained R_g into geometric diameter yielded estimates of ~71, 72, and 77 nm for the particles at virus peak maxima of 1×phi6, PEG-phi6 and phi6 lysate (Table 3), respectively. Previously reported 85 nm diameter for phi6 is based on analytical ultracentrifugation and ~70 nm when analyzed by cryo-electron microscopy [22].

Light scattering detectors revealed larger complexes eluting after the virus peak when phi6 lysate and PEG-phi6 were analysed (Fig. S2). These complexes were not obvious in the UV fractograms (Fig. 2). The obtained R_g range for such complexes was ~68-494 nm and ~50-180 nm, respectively (Fig. 2). Although the UV and MALS detectors only showed a single major peak for 1×phi6 in the fractograms (Fig. 2B, Fig. S2), the light scattering measurements revealed that some larger sample components with R_g range of ~40-85 nm eluted after the main virus peak as well.

3.3. Applicability of QA monolithic matrices in concentration of purified phi6.

Virus containing fractions may need to be combined when pursuing the maximal yield of viruses. This creates a need to concentrate the sample. AF4 purified phi6 lysate resulted in specimen with purity comparable to 1×phi6, but with relatively low virus concentration (Table 2). We analyzed the possibility to concentrate AF4 fractions by using QA monolithic columns. The overall applicability of QA monolithic column to bind phi6 was first evaluated with highly purified 2×phi6 preparation. The column bound phi6 efficiently as less than 0.001% of the infective particles (PFUs) and no protein were detected in the flow through (data not shown).

Next a 10 mL sample of phi6 containing 0.67 mg of protein and 1.6×10^{12} PFUs was used as an input. Two major peaks were observed (Fig. 3 A). Infective viruses and most protein eluted in the first peak at 0.35-0.55 M NaCl in 4 mL volume. This peak contained 0.34 mg of protein, 9.5×10^{11} PFUs, and ~60% of infective viruses. The specific infectivity (~3×10^{12} PFU/mg of protein) was comparable with that of the input, showing that the integrity of virions was not affected. However, only 1.5-fold concentration of the virus sample was obtained.

Based on SDS-PAGE analysis, the protein pattern typical for phi6 was observed in the first peak. The virus co-eluted with few impurities (Fig. 3 D), but some were also successfully removed (see fraction 5, Fig. 3 D). The second peak had high intensity (Fig. 3A) and low protein content (Fig. 3 B, D). Ethidium bromide staining of the SDS-PAGE stacking gel showed that it contained nucleic acids that presumably derived from phi6 genomic dsRNA (data not shown). Thus, QA columns successfully removed the free dsRNA and resulted in further purification of the sample.

4. Discussion

The increasing demand for highly purified viruses, VLPs, and their sub-complexes in basic research, as well as in applications such as development of virus vectors, vaccinations, and nanomaterials, has created a need for more efficient downstream processing of produced particles. The choice of purification method depends on the intended use of the purified viruses and on the properties of the virus of interest. Commonly used methods include precipitation, ultrafiltration, ultracentrifugation, dialysis, and various chromatography techniques [23]. In many cases, methods are combined to reach the best outcome. We compared two methods for purification of enveloped bacteriophage phi6.

Phi6 is traditionally purified from the infected culture supernatant through PEG-NaCl precipitation and rate zonal ultracentrifugation [17]. This method results in ~7 mg of highly purified infective viruses from 1 L of lysate with an average yield of ~30% in ~1.5 days. Specific infectivity is improved ~140-fold to ~1×10^{13} PFU/mg of protein. However, obtaining such samples is laborious, time consuming, and demands quantities of starting materials as well as simultaneous access to multiple ultracentrifuges. The size-based AF4 fractionation and passage of small components through the accumulation wall reduced impurities in phi6-containing fractions and led to increased specific infectivity that was similar, ~7-8×10^{12} PFU/mg of protein, for both the phi6 lysate and PEG-phi6 (Table 2), and comparable to the values obtained by PEG-precipitation and ultracentrifugation (Table 1) [35]. However, the obtained recoveries of infectious viruses from AF4 purification of phi6 lysate and PEG-phi6 were ~2-3-fold higher. Thus, our results support the previous reports indicating that the RC membranes are suitable for a wide repertoire of viruses with different

biophysical properties [7, 8, 12, 24]. When comparing the obtained results to our previous work on AF4 purification of bacteriophage PRD1 (~63 nm) having a proteinaceous outer surface [7], the yield of phi6 was approximately twice higher (~100%, ~40 µg/mL of lysate; assuming that 1 A_{280} unit of purified phi6 corresponds to 0.14 mg of protein, Table 1) than it was with PRD1 (~60%, ~24 µg/mL of lysate) [7]. Furthermore, no significant dilution of the phi6 lysate occurred contrary to the 7-fold dilution when PRD1 was purified by AF4 when the 350 µm spacer was used. In both cases, the virus purification straight from the crude cell lysate resulted in the highest improvement in specific infectivity of the three input samples analysed. For halophilic viruses, AF4 purification using high total ionic strength mobile phase yielded ~40-50% of infectious viruses when lysates or cleared cell supernatants were utilized [8].

A single 1h AF4 purification from 1 mL of the lysate yielded ~40 µg of phi6. Thus, obtaining milligram amounts of virus would require ~25 runs. We have previously used 5 mL injections to purify viruses from culture supernatants [8]. By using such injection volumes for phi6 lysate, milligram amounts of purified virus could be achieved in ~5 h. Since AF4 can be run in a semi-preparative mode with auto sampler and semi-preparative channel, larger scale purifications could be achieved by utilizing automatic, repeated injections and larger volumes of lysate. In general, the higher yields of infective viruses obtained from AF4 purification reduce the amount of starting materials needed. With phi6, an equal milligram amount of purified viruses can be reached by using six times less starting material than used for the ultracentrifugation-based purification.

AF4 coupled with MALS detector has been shown to provide an efficient method to separate and quantitate influenza virus particles within allantoic fluids of infected eggs and supernatants of infected cell cultures [24]. Here the light scattering data provided important information on the quality of purified enveloped viruses. MALS analysis indicated that the size range of particles in the virus peak of phi6 lysate and PEG-phi6 was larger than in the virus peak of 1×phi6. In addition, MALS revealed that the larger sample components were efficiently excluded from AF4 purified virus by performing fractionation at V_{out} of 0.5 mL/min. In general, virus purification is always

balancing between yield and purity, but careful fractionation leads in greater homogeneity of the final purified virus specimen.

Phi6 virions with negative surface charge [25] bound effectively to the strong QA anion exchange column. For other enveloped viruses and VLPs, QA columns have been used for purification of human immunodeficiency virus type 1 (HIV-1) gag VLPs and rubella viruses from infected culture supernatants with ~40% and ~90% yields, respectively [26, 27]. Chromatography of phi6 produced ~0.3 mg of highly infective viruses from 10 mL of input with ~60% yield in ~30 min. Also nucleic acids were removed efficiently. AF4 purification of bacteriophage PRD1 coupled with a monolithic column resulted in full recovery of ~5-fold concentrated sample with very high purity [7]. However, the obtained 1.5-fold improvement in the phi6 concentration was possibly due to the small amount, ~0.7 mg, of the input sample used, since the dynamic binding capacity of QA column is higher, ~5 mg of bacteriophage PRD1 [28] and according to manufacturer 20 mg of BSA.

Viruses can nowadays be produced in high quantities in bioreactors, but the removal of product- and process-derived impurities is the challenge. Therapeutic applications of viruses, including utilization of viral vectors and vaccine development, are rapidly growing fields of research that have strict safety standards and requirements of high level of sample homogeneity. Need for new efficient purification methods is obvious. We have previously shown the applicability of AF4 in purification of a membrane vesicle -like pleolipovirus HRPV-1 (~44×55 nm); a spindle-shaped virus His1 (~44×77 nm) that has a lipid-modified major capsid protein; two non-enveloped icosahedral viruses PRD1 and HCIV-1 (~63 and 70 nm), and a tailed virus HVTV-1 (head ~76×96 nm, tail ~73 nm) [7, 8]. This study added the enveloped virus phi6 (~70 nm) to the list of viruses that are amenable for AF4 purification. The benefits of AF4 are the following: i) one-step AF4 purification of viruses is rapid and highly repeatable, ii) the use of virus specific buffer and the omission of the stationary phase results in high yields of infectious viruses, iii) AF4 purification can be scaled up, iv) coupling AF4 with in-line detectors provides real-time information on the quality and quantity of the sample, and v) channel dimensions, membrane materials and the elution program can be tailored for different samples and purposes, making AF4 highly versatile. However, the elution of

the virus at the end of the cross-flow gradient may be problematic for input samples that contain aggregates or other large sample components. For such samples, a thinner spacer or higher V_{out} would promote their separation. For dilute input samples increased input sample volumes or pre-concentration steps might be necessary to obtain reliable detector reads and fractions with adequate virus concentrations. In conclusion, AF4 purification of viruses is an attractive alternative with great potential to meet high purity standards of virus research and applications. The similarities between phi6 and enveloped animal viruses, such as influenza virus, suggest that the results obtained here could be used as guidelines and a starting point in the purification of enveloped animal viruses as well.

Acknowledgements

The authors acknowledge the use of Instruct-HiLIFE Biocomplex unit (University of Helsinki and Instruct-FI) and the Academy of Finland support (grant 1306833) for the unit (Instruct Centre for Virus Production 2009-2017, ICVIR). This study was supported by Academy Professor (Academy of Finland) funding Grant 283072, Academy of Finland Research Program Grant 272507 (M.M.P.), by Sigrid Jusélius Foundation (M.M.P. and D.H.B), and the Finnish Concordia Fund (M.L.). The authors thank Helin Veskiväli and Tanja Westerholm for excellent technical assistance, and Sami Karja for his work with monolithic columns.

Compliance with Ethical Standards

Conflict of interests: The authors declare that they have no conflict of interest

References

[1] M.M. Poranen, R. Daugelavičius, D.H. Bamford, Common principles in viral entry, Annu Rev Microbiol, 56 (2002) 521-538.
[2] A.K. Vidaver, R.K. Koski, J.L. Van Etten, Bacteriophage ϕ6: a lipid-containing virus of Pseudomonas phaseolicola, J Virol, 11 (1973) 799-805.
[3] S. Mäntynen, L.R. Sundberg, M.M. Poranen, Recognition of six additional cystoviruses: Pseudomonas virus phi6 is no longer the sole species of the family Cystoviridae, Arch Virol, 163 (2018) 1117-1124.
[4] Z. Sun, K. El Omari, X. Sun, S.L. Ilca, A. Kotecha, D.I. Stuart, M.M. Poranen, J.T. Huiskonen, Double-stranded RNA virus outer shell assembly by bona fide domain-swapping, Nat Commun, 8 (2017) 14814.

[5] F.-W.C. Lanata CF, Olascoaga AC, Torres CX, Aryee MJ, Black RE; Child Health Epidemiology Reference Group of the World Health Organization and UNICEF., Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015, Lancet Infect Dis, 17 (2017) 909-948.
[6] N.J. Maclachlan, Bluetongue: history, global epidemiology, and pathogenesis, Prev Vet Med, 102 (2011) 107-111.
[7] K. Eskelin, M. Lampi, F. Meier, E. Moldenhauer, D.H. Bamford, H.M. Oksanen, Asymmetric flow field flow fractionation methods for virus purification, J Chromatogr A, 1469 (2016) 108-119.
[8] K. Eskelin, M. Lampi, F. Meier, E. Moldenhauer, D.H. Bamford, H.M. Oksanen, Halophilic viruses with varying biochemical and biophysical properties are amenable to purification with asymmetrical flow field-flow fractionation, Extremophiles, 21 (2017) 1119-1132.
[9] J.C. Giddings, Field-flow fractionation - analysis of macromolecular, colloidal, and particulate materials, Science, 260 (1993) 1456-1465.
[10] G. Yohannes, M. Jussila, K. Hartonen, M.L. Riekkola, Asymmetrical flow field-flow fractionation technique for separation and characterization of biopolymers and bioparticles, J Chromatogr A, 1218 (2011) 4104-4116.
[11] S.K. Williams, D. Lee, Field-flow fractionation of proteins, polysaccharides, synthetic polymers, and supramolecular assemblies, J Sep Sci, 29 (2006) 1720-1732.
[12] T. Bousse, D.A. Shore, C.S. Goldsmith, M.J. Hossain, Y. Jang, C.T. Davis, R.O. Donis, J. Stevens, Quantitation of influenza virus using field flow fractionation and multi-angle light scattering for quantifying influenza A particles, Journal of Virological Methods, 193 (2013) 589-596.
[13] Y. Chen, Y. Zhang, Y. Zhou, J. Luo, Z. Su, Asymmetrical flow field-flow fractionation coupled with multi-angle laser light scattering for stability comparison of virus-like particles in different solution environments, Vaccine, 34 (2016) 3164-3170.
[14] L.F. Pease, 3rd, D.I. Lipin, D.H. Tsai, M.R. Zachariah, L.H. Lua, M.J. Tarlov, A.P. Middelberg, Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy, Biotechnol Bioeng, 102 (2009) 845-855.
[15] B. Somasundaram, C. Chang, Y.Y. Fan, P.Y. Lim, J. Cardosa, L. Lua, Characterizing Enterovirus 71 and Coxsackievirus A16 virus-like particles production in insect cells, Methods, 95 (2016) 38-45.
[16] Z. Wei, M. Mcevoy, V. Razinkov, A. Polozova, E. Li, J. Casas-Finet, G.I. Tous, P. Balu, A.A. Pan, H. Mehta, Biophysical characterization of influenza virus subpopulations using field flow fractionation and multiangle light scattering: correlation of particle counts, size distribution and infectivity, Journal of Virological Methods, 144 (2007) 122-132.
[17] D.H. Bamford, P.M. Ojala, M. Frilander, L. Walin, J.K.H. Bamford, Isolation, purification, and function of assembly intermediates and subviral particles of bacteriophages PRD1 and ϕ6, in: K. Adolph (Ed.) Microbial Gene Techniques, Academic Press, San Diego (Ca), 1995, pp. 455-474.
[18] B.J. Frisken, Revisiting the method of cumulants for the analysis of dynamic light-scattering data, Appl Opt, 40 (2001) 4087-4091.
[19] V.M. Olkkonen, D.H. Bamford, Quantitation of the adsorption and penetration stages of bacteriophage ϕ6 infection, Virology, 171 (1989) 229-238.
[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem, 72 (1976) 248-254.
[21] M.M. Poranen, R. Tuma, Self-assembly of double-stranded RNA bacteriophages, Virus Res, 101 (2004) 93-100.
[22] L.A. Day, L. Mindich, The molecular weight of bacteriophage phi 6 and its nucleocapsid, Virology, 103 (1980) 376-385.
[23] P. Nestola, C. Peixoto, R.R. Silva, P.M. Alves, J.P. Mota, M.J. Carrondo, Improved virus purification processes for vaccines and gene therapy, Biotechnol Bioeng, 112 (2015) 843-857.
[24] R. Lang, L. Vogt, A. Zurcher, G. Winter, Asymmetrical Flow FFF as an Analytical Tool for the Investigation of the Physical Stability of Virus-Like Particles, Lc Gc N Am, 27 (2009) 844-852.

[25] K.A. Block, A. Trusiak, A. Katz, P. Gottlieb, A. Alimova, H. Wei, J. Morales, W.J. Rice, J.C. Steiner, Disassembly of the cystovirus ϕ6 envelope by montmorillonite clay, Microbiologyopen, 3 (2014) 42-51.
[26] P. Steppert, D. Burgstaller, M. Klausberger, E. Berger, P.P. Aguilar, T.A. Schneider, P. Kramberger, A. Tover, K. Nobauer, E. Razzazi-Fazeli, A. Jungbauer, Purification of HIV-1 gag virus-like particles and separation of other extracellular particles, J Chrom A, 1455 (2016) 93-101.
[27] D. Forcic, M. Brgles, J. Ivancic-Jelecki, M. Santak, B. Halassy, M. Barut, R. Jug, M. Markusic, A. Strancar, Concentration and purification of rubella virus using monolithic chromatographic support, J Chromatogr B Analyt Technol Biomed Life Sci, 879 (2011) 981-986.
[28] H.M. Oksanen, A. Domanska, D.H. Bamford, Monolithic ion exchange chromatographic methods for virus purification, Virology, 434 (2012) 271-277.

Table 1. Precipitation and preparative ultracentrifugation based purification of phi6[a]

	phi6 lysate	PEG-phi6	1×phi6
Virus amount (PFU)	4.9×10^{14}	4.0×10^{14}	1.3×10^{14}
Virus yield (%)	100	84	27
Total A_{280} units	48733	1590	93
A_{280} yield (%)	100	3.3	0.2
Specific infectivity (PFU/A_{280})	9.8×10^{9}	2.5×10^{11}	1.4×10^{12}
Protein (mg)	554	142	13.4
Protein yield (%)	100	26	2
Specific infectivity (PFU/mg protein)	8.8×10^{11}	2.8×10^{12}	9.6×10^{12}

[a] Data shown as an average of three biological batches obtained from 2 l of lysates per purification.

Table 2. AF4 purification of phi6 lysate, PEG-phi6 and 1×phi6[a].

	phi6 lysate			PEG-phi6			1×phi6		
	Input	AF4: peak fraction 7 (35-39 min)	AF4: pooled fractions 6-8 (31-43 min)	Input	AF4: peak fraction 7 (35-39 min)	AF4: pooled fractions 7-9 (35-47 min)	Input	AF4: peak fraction 7 (35-39 min)	AF4: pooled fractions 7-9 (35-47 min)
Volume (ml)	1.0	0.8	2.4	1.0[c]	0.8	2.4	1.0[c]	0.8	2.4
Virus amount (PFU)	$1.9x10^{11}$	$1.8x10^{11}$	$2.7×10^{11}$	$2.3x10^{12}$	$1.5x10^{12}$	$2.3×10^{12}$	$9.1x10^{12}$	$3.3x10^{12}$	$6.4×10^{12}$
Virus yield (%)	100	95	142	100	65	100	100	36	70
Total A_{280} units	32[b]	0.17	0.27	9.0	1.2	2.1	9.9	2.4	5
A_{280} yield (%)	100	0.53	0.8	100	13.3	16	100	24	51
Specific infectivity (PFU/A_{280})	$5.9x10^{9}$	$1.1x10^{12}$	$1.0×10^{12}$	$2.6x10^{11}$	$1.3x10^{12}$	$1.1×10^{12}$	$9.2x10^{11}$	$1.4x10^{12}$	$1.3×10^{12}$
Protein (mg)	0.3	n.d.	n.d.	1.25	0.18	0.31	1.43	0.48	0.98
Protein yield (%)	100	n.d.	n.d.	100	14	25	100	34	69
Specific infectivity (PFU/mg protein)	$6.3×10^{11}$	n.d.	n.d.	$1.8x10^{12}$	$8.3x10^{12}$	$7.4×10^{12}$	$6.4x10^{12}$	$6.9x10^{12}$	$6.5×10^{12}$

[a] Input samples, AF4 fractions having the highest number of infectious viruses and pooled fractions of virus peak were compared. Results are calculated from three technical repetitions.

[b] Buffer used as a blank. Total A_{280} unit value for the non-inoculated LB medium was 15. The corresponding protein concentration measured by Bradford was ~30 μg/ml.

[c] Input samples were diluted in buffer: PEG-phi6: 100 μl sample + 1000 μl buffer; 1×phi6: 50 μl sample + 1050 μl buffer.

n.d. not determined: protein concentration below detection limit.

Table 3. Radius of gyration, Radius of hydration and geometric diameter for AF4 fractionated phi6 samples.

Sample	Elution time (min)	R_g range (nm)[a]	R_g at peak maxima (nm)[a]	R_h range (nm)[a]	R_h at peak maxima (nm)[a]	Geometric diameter at peak maxima (nm)[b]
phi6 lysate	28-35.5	29-38	~30	15-344*	~90*	~77
PEG-phi6	28.7-37.5	27-39	~28	67-90	~80	~72
1×phi6	26.5-32.5	27-28	~27	47-240	~81	~71

[a] See Fig. 2 and Fig. S2 for UV, MALS and DLS data
[b] Geometric diameter converted form R_g
*Low signal intensity, might be inaccurate

Figures and figure legends: Lampi et al. 2018 J. Chrom B

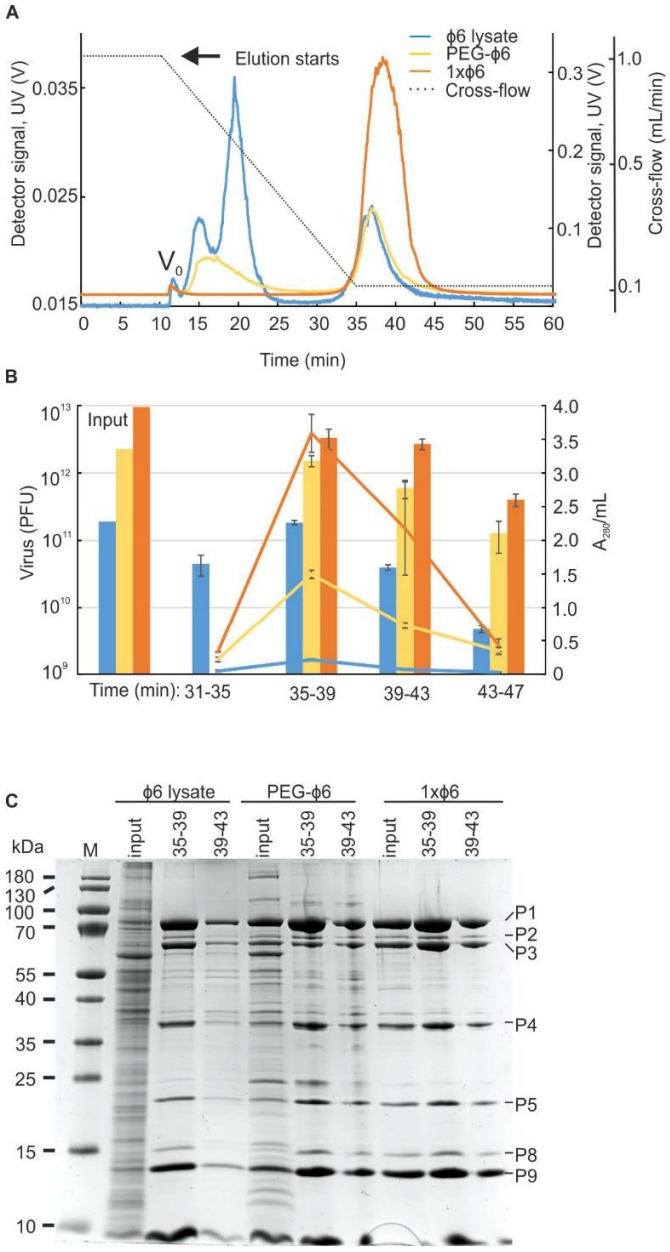


Figure 1. AF4 fractionation of phi6 lysate, PEG-phi6 and 1×phi6. (A) Fractograms obtained from the purification of different input samples. The UV-detector signal (solid lines) for phi6 lysate and PEG-phi6 is shown on the left y-axis and that of 1×phi6 on the right y-axis. Dashed line represents the cross-flow gradient (additional right y-axis). (B) Virus titers (bars) and protein concentrations (lines) measured from input samples and fractions eluting from 31 to 47 min. Coloring is the same as in panel (A). (C) SDS-PAGE analysis of input samples and fractions containing the highest number of infectious viruses. Proteins were precipitated from 540, 220, or 40 µl of fractions representing AF4

purified phi6 lysate, PEG-phi6, or 1×phi6. M stands for molecular weight marker (kDa). Major structural proteins P1-P9 of phi6 are indicated.

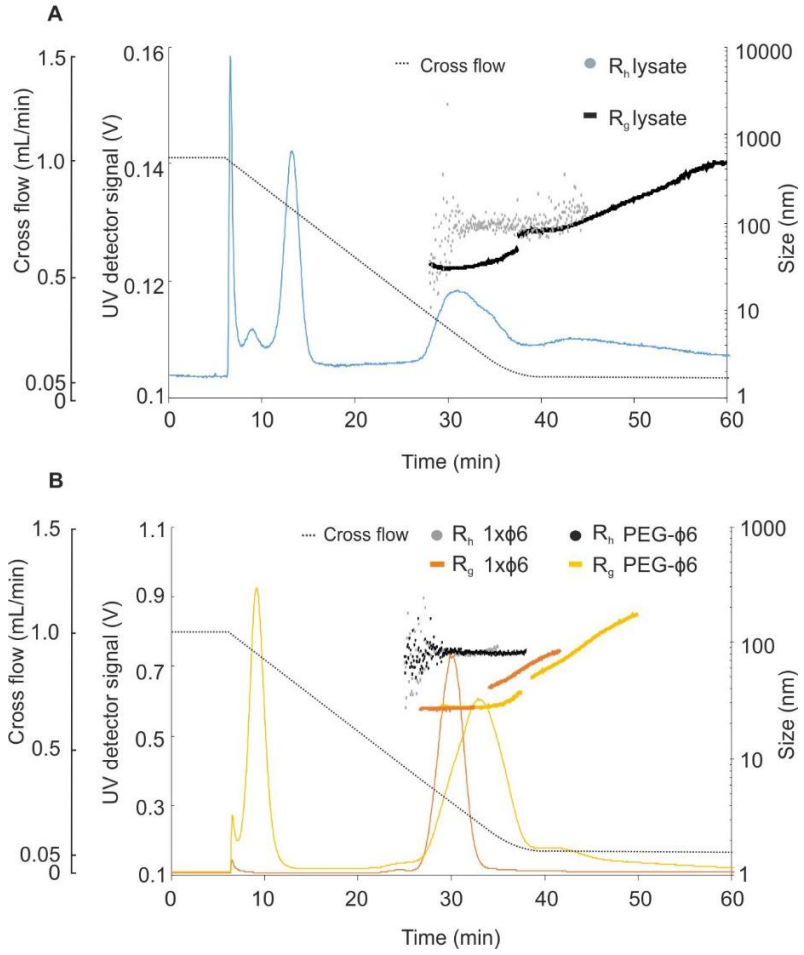


Figure 2. AF4-MALS-DLS analysis of phi6 samples. R_g was calculated from MALS data applying sphere fitting method and R_h from DLS data. (A) UV-fractogram for phi6 lysate (solid blue line, left y-axis), R_g (solid black line, right y-axis) and R_h (grey circles, right y-axis). (B) UV-fractograms for PEG-phi6 (thin yellow line) and 1×phi6 (thin orange line). R_g (right y-axis) is shown with thick solid lines in colors of the corresponding UV fractograms. R_h (right y-axis) for PEG-phi6 is shown with black circles and for 1×phi6 with grey circles. V_{out} applied in the analysis was 0.5 mL/min. Cross-flow gradient (additional left y-axis) is presented with dashed line in both panels.

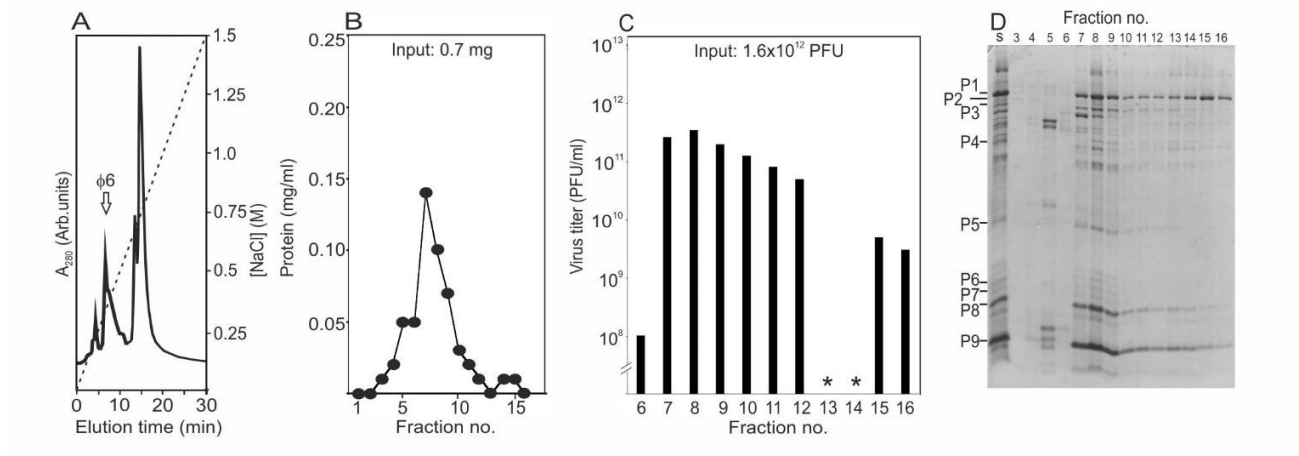


Figure 3. Concentration of phi6 by monolithic anion exchange. (A) Chromatogram for 1×phi6 using QA column. Solid line represents the absorbance at 280 nm and dashed line shows the NaCl gradient. (B) Protein concentrations in 1 mL fractions. (C) Virus titers of analysed fractions. Titers of fractions marked with asterisks were not determined, (D) Analysis of protein content of the input sample (marked with S) and fractions. Proteins were analysed from 250 µl of the corresponding fractions. Major structural proteins of phi6 are marked as P1-P9.

Appendix A

Supplementary data

Asymmetrical flow field-flow fractionation in purification of an enveloped bacteriophage **ϕ**6

Mirka Lampi[a], Hanna M. Oksanen[a], Florian Meier[b], Evelin Moldenhauer[b], Minna M. Poranen[a], Dennis H. Bamford[a], and Katri Eskelin[a]*

* Corresponding author: katri.eskelin@helsinki.fi, +358504486048

[a] Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki Viikinkaari 9B, FI-00014 Helsinki, Finland

[b] Postnova Analytics, Max-Planck-Str. 14, 86899 Landsberg, Germany

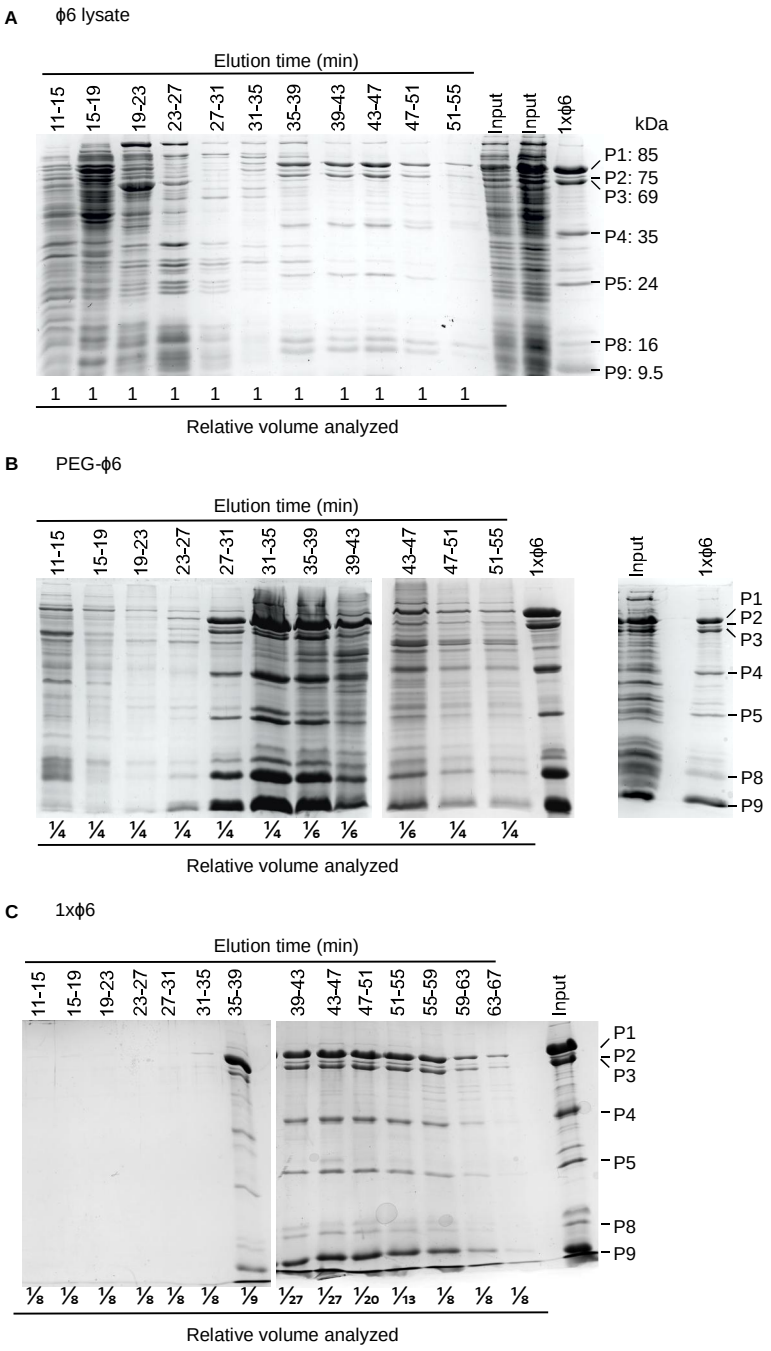


Fig. S1. Protein content of AF4 fractionated ϕ6 lysate (A), PEG-ϕ6 (B), and 1xϕ6 (C). Corresponding fractograms are shown in Fig. 1 A. Proteins from fractions and ϕ6 lysate input were concentrated by TCA precipitation prior SDS-PAGE analysis as described below. 1xϕ6 served as a control. Positions and sizes of the ϕ6 major structural proteins P1-P9 are indicated.

ϕ6 lysate: Protein samples (25 µl) resulted from the precipitation of pooled fractions (V_{tot} 1800 µl) from three AF4 purifications.

PEG-ϕ6: Protein samples (25 µl) resulted from the precipitation of pooled fractions (V_{tot} 1350 µl) from two AF4 purifications.

1xϕ6: Protein samples (80 µl) resulted from the precipitation of pooled fractions (V_{tot} 850 µl) from two AF4 purifications.

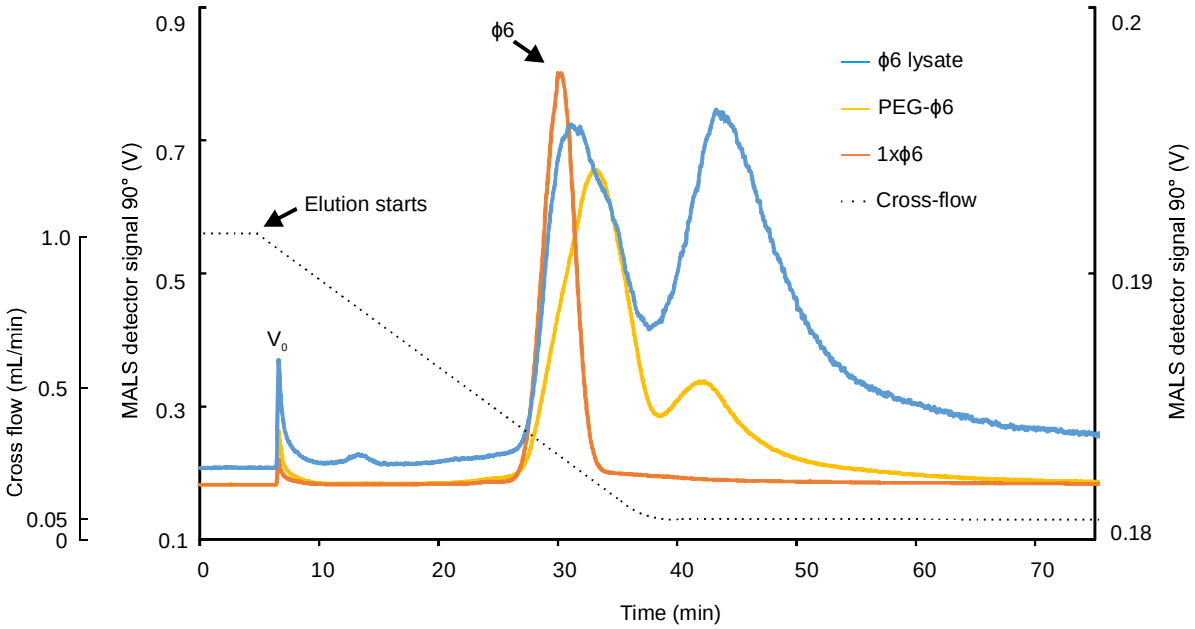
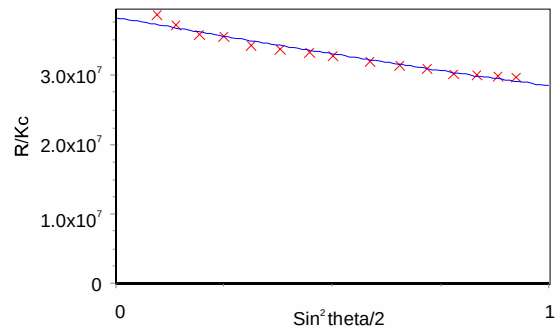
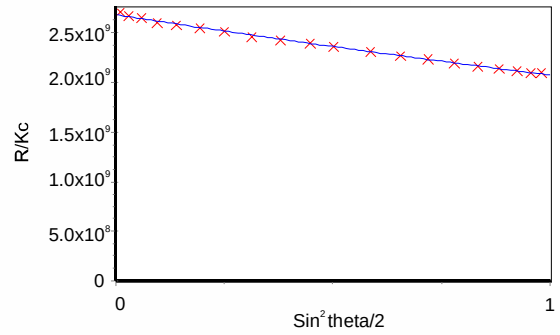


Fig. S2. MALS measurement during AF4 reveals the presence of large sample components. MALS signal at 90° for ϕ6 lysate (blue line, right y-axis), PEG-ϕ6 (yellow line, left y-axis) and 1xϕ6 (orange line, left y-axis). V_{out} applied in the analysis was 0.5 mL/min. Cross-flow gradient is shown with dashed line (additional left y-axis). See Fig. 1A and Fig. 2 for the corresponding UV

A



B



C

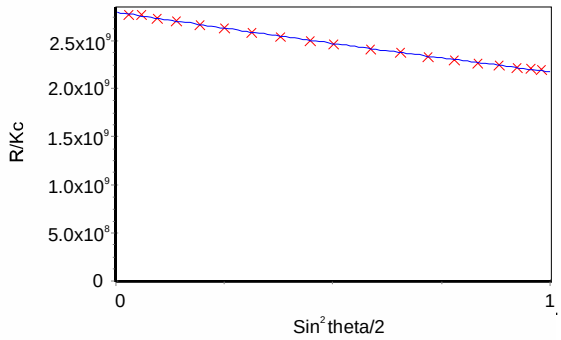


Fig. S3. Fitting the MALS-signal to the sphere model: (A) ϕ6 lysate, (B) PEG-ϕ6, and (C) 1xϕ6.