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Asymmetric flow field flow fractionation methods for virus purification

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ABSTRACT

Detailed biochemical and biophysical characterization of viruses requires viral preparations of high quantity and purity. The optimization of virus production and purification is an essential, but laborious and time-consuming process. Asymmetric flow field flow fractionation (AF4) is an attractive alternative method for virus purification because it is a rapid and gentle separation method that should preserve viral infectivity. Here we optimized the AF4 conditions to be used for purification of a model virus, bacteriophage PRD1, from various types of starting materials. Our results show that AF4 is well suited for PRD1 purification as monitored by virus recovery and specific infectivity. Short analysis time and high sample loads enabled us to use AF4 for preparative scale purification of PRD1. Furthermore, we show that AF4 enables the rapid real-time analysis of progeny virus production in infected cells.

1. Introduction

Viruses that infect animals and plants generally receive more attention than do viruses of prokaryotes (bacteria and archaea) because of their medical, agricultural, and economic importance. However, prokaryotic viruses have an immense effect on global microbial communities and consequently on Earth's biogeochemical cycles and climate [1–5]. The current estimate for virus abundance in sea water is $\sim 10^{30}$ [1] and similar numbers have been proposed for soil [6]. Still relatively few prokaryotic viruses are known in molecular, structural, and biochemical detail. Such knowledge is essential for interpreting viral diversity at the genomic and structural level and for understanding viral roles in every ecosystem. It is worth mentioning here that our current knowledge of many cellular processes, including transcription, translation, DNA replication, protein sorting, etc., stems from research on prokaryotic viruses. In addition, many commercially available enzymes vital for contemporary molecular biology, including ligases, restriction enzymes and polymerases, originated from prokaryotic viruses.

Further basic and applied research on viruses requires samples of high purity in quantity. After optimised production, the first purification step typically involves precipitation or filtration [7,8]. Precipitation is applicable to high sample volumes where it can simultaneously concentrate and purify the viruses [8]. Downstream purification steps to remove impurities that co-precipitate with the virus particles due to similar biophysical and/or biochemical properties most commonly involve ultracentrifugation [9,10]. Depending on the ultracentrifugation method used, virus purification is achieved based on its sedimentation coefficient (rate zonal), buoyant density (isopycnic), or flotation [10]. A final purification step, such as differential ultracentrifugation or ultrafiltration, removes the gradient material and concentrates the viruses.

While ultracentrifugation methods often result in high purity virus preparations, recovery yields can be low [8] (see also Fig. S9). Therefore, preparative centrifugation of viruses requires expensive ultracentrifuge farms. Moreover, the viscous and hyperosmotic nature of some gradient media (e.g., sucrose, CsCl) combined with the strong shear forces generated during high speed centrifugation can damage viruses and lead to loss of infectivity [8]. Alternative methods have been developed as reviewed in [8,9]. Anion exchange chromatography using monolithic columns has proven efficient for purification of large biomolecules such as viruses [11–13]. The macroporous nature of these monoliths provides high surface accessibility for large molecules [13]. However, elution by increasing ionic strength can be harmful to some sensitive viruses and is unusable in the case of halophilic viruses that require high salt concentrations for infectivity [14].

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Asymmetric flow field flow fractionation (AF4) is a subtechnique developed from field flow fractionation (FFF) methods. Its principles and theory have been described in the original papers [15–18] and are summarized in many reviews [19–23]. In AF4, sample separation takes place in a trapezoidal flat channel under the influence of two flows: the channel flow (V_{out}) that has a parabolic profile and the cross-flow (V_c) that drives sample components towards the accumulation wall. This force is counteracted by the diffusion of sample components away from the wall. As a result, each sample component equilibrates at a distance from the accumulation wall that depends on its diffusion coefficient (D) and hydrodynamic molecular size [15,16]. In the normal separation mode smaller sample components elute before larger ones. Normal separation mode applies to sample components smaller than ~1 μm [20].

The omission of the stationary phase in AF4 decreases the pressure and shear forces during separation. In addition, the mobile phase composition can be readily modified to meet the demands of the sample components. As a result, AF4 is a gentle separation method that enables the analyzed molecules to retain their native conformation. AF4 has been successfully applied to various types of biological specimens [20,22,24] as well as in studies of particles and colloids of non-biological origin [25]. Viruses were among the first specimens analyzed when field flow fractionation was introduced in 1976 [15]. Already in 1977 symmetric field flow fractionation was applied to determine the diffusion coefficients of bacteriophages Qβ, f2, MS2, P22 and ϕX174 [26]. Nowadays, however, AF4 [16] has replaced symmetric flow field flow fractionation. It has been used to study the particle size, size distribution and particle counts of viruses [27,28] and virus-like particles (VLPs) [29–32]. AF4 has also shown its potential for determining the changes in the size distribution of VLPs upon assembly from purified modified viral protein components as well as the effect of encapsidation of heterologous DNA [30,31]. In addition, viruses have been utilized in experiments validating AF4 theory and performance [17,18]. However, although the potent of AF4 for purification of macromolecules, such as viruses, has been recognized, no published reports on the utilization of AF4 for large scale virus purification exists.

In this work, our goal was to develop a preparative scale fractionation procedure that would provide high purity accompanied by high yields of infectious viruses. We also compared AF4 to the established virus purification methods. We chose bacteriophage PRD1 [33] as our model virus as it already has an array of well-established purification methods [11,34,35] enabling comparisons on purification methods efficacy. PRD1 has an icosahedral, proteinaceous capsid with a diameter of ~66 nm and molecular mass of ~66 MDa. Virions are decorated with ~20 nm spikes at the five-fold symmetry axes. An internal membrane lies just inside the protein shell and encloses the double-stranded DNA genome [36–39]. Here we determined the optimal AF4 operation conditions for PRD1 and analyzed its purification using various types of starting materials. We also combined AF4 with monolithic anion chromatography. Both AF4 and monolithic anion chromatography were then compared to traditional ultracentrifugation methods. Our results demonstrate that AF4 has great potential for the purification of infectious viruses. Furthermore, we show that AF4 enables rapid real-time analysis of progeny virus production in infected cells.

2. Materials and methods

2.1. Sample preparation

Bacteriophage PRD1 was cultured and purified as previously described [35]. The host *Salmonella enterica* serovar *Typhimurium* LT2 DS88 [40] was grown in Luria-Bertani medium (LB) at 37 °C. Cells in logarithmic growth phase were infected using a multiplicity of infection (MOI) of 10. Cell lysis was detected by measuring culture turbidity at 550 nm (Chlormic, JP Selecta S.A., Barcelona, Spain). In specific cases, the culture was treated after lysis with DNase I (50 μg/mL; Sigma-Aldrich) or RNase A (30 μg/mL; Roche) for 1 h at 37 °C. Subsequent centrifugation (Sorval rotor SLA1500/3000, 8000 rpm, 20 min, 4 °C) removed cell debris to yield the cleared lysate. Viruses were precipitated from the lysate using 10% (w/v) polyethylene glycol (PEG) 6000 and 0.5 M NaCl, collected by centrifugation as above, and resuspended in a small amount of buffer (0.01 of the initial volume) to yield PEG-PRD1. Standard virus buffer (20 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$) was used in all purification steps. The resuspended viruses were purified by rate zonal centrifugation with a linear 5–20% (w/v) sucrose gradient (Sorvall rotor AH629, 24 000 rpm, 55 min, 5 °C). Zones containing mature infectious viruses were collected by differential centrifugation (Sorvall rotor T647.5, 32 000 rpm, 3 h, 5 °C) and resuspended in virus buffer yielding 1xPRD1. Alternatively, further purification of the zones by an additional buoyant density centrifugation in 20–70% sucrose gradients (Sorvall rotor AH629, 24 000 rpm, 20 h, 15 °C) followed by differential centrifugation as above yielded 2XPRD1 purified to homogeneity (see also Fig. S9A).

2.2. AF4 instrumentation and its operation

The AF4 experiments were carried out using an AF2000 MT instrument (Postnova Analytics, Landsberg, Germany) equipped with a solvent organizer (PN7140), a solvent degasser (PN7520), two isocratic high performance liquid chromatography (HPLC) pumps for generation of carrier flow (PN1130), a syringe pump (Kloehn v6) for controlling cross-flow, a purging port (PN1610) for rinsing, a manual injection valve (Rheodyne 9725i), a temperature controlled AF4 channel oven for sample fractionation (PN4020), preparative flow cell for UV (PN3211-003), and a fraction collector (PN8050). AF4 operation and data collection were carried out using Postnova AF2000 software. Separations were performed at 22 °C in a channel that contained a 350 μm or a 250 μm spacer. The channel had a tip-to-tip length of 27.5 cm, initial width 2.0 cm, and final width of 0.5 cm. A regenerated cellulose (RC) membrane with molecular weight cut-off (MWCO) value of 100 kDa (Z-MEM-AQU-529, Postnova) was used unless otherwise mentioned. The injection volume was 20–1000 μl. Prior to sample injection, aggregated material was removed by centrifugation (Eppendorf centrifuge 5415D, 10 000*g*, 5 min). The outlet flow was monitored at 260 or 280 nm using an inline variable wavelength detector (Shimadzu SPD-20A; Shimadzu, Kyoto, Japan) with detector range settings as appropriate for each input sample concentration.

Standard viral buffer (see above 2.1) served as the AF4 mobile phase. V_{out} was 0.2 ml/min unless otherwise mentioned. Focusing was performed applying the same cross-flow that was used for fractionation. Focusing time varied from 5 to 15 min depending on the amount and the expected polydispersity of the injected sample. Between successive AF4 experiments, material retained in the channel was removed by rinsing the channel without cross-flow until the UV signal reached the baseline. Repeatability was confirmed with at least three technical repetitions and using various specimen preparations (biological replicates). Fractions (0.5–1 ml) were collected from the start of the elution phase and stored at 4 °C.

Collection of UV-multi angle light scattering (MALS) data was performed with a second AF2000 MT instrument (Postnova Analytics, Landsberg, Germany) that was equipped with an analytical flow cell for UV (PN3211-003), a refractive index (RI) detector (PN3150) and MALS detector (PN3621) equipped with a green laser (532 nm emission wavelength). T_f was 5 min. Elution was performed with V_{out} of 0.5 ml/min. The 25 min linear elution gradient to 0.28 ml/min was followed by a 15 min exponential step to final elution at 0.08 ml/min. MALS data provided the radius of

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