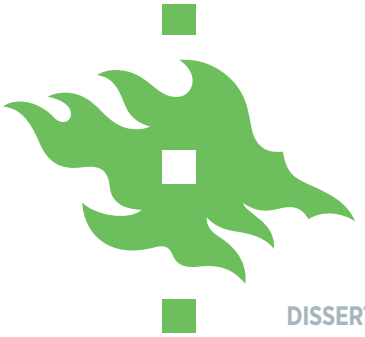

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MIRKA LAMPI

ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION IN VIRUS PURIFICATION

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Asymmetrical flow field-flow fractionation in virus purification

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ACADEMIC DISSERTATION

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No. Try not. Do.. or do not. There is no try.

Grand Master of the Jedi Order, Yoda

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Eskelin K, Lampi M, Meier F, Moldenhauer E, Bamford DH, and Oksanen HM (2016). **Asymmetric flow field flow fractionation methods for virus purification**. Journal of Chromatography A **1469**, 108-119.

II Eskelin K, Lampi M, Meier F, Moldenhauer E, Bamford DH, and Oksanen HM (2017). **Halophilic viruses with varying biochemical and biophysical properties are amenable to purification with asymmetrical flow field-flow fractionation.** Extremophiles **21**, 1119-1132.

III Lampi M, Oksanen HM, Meier F, Moldenhauer E, Poranen MM, Bamford DH, and Eskelin K (2018). **Asymmetrical flow field-flow fractionation in purification of an enveloped bacteriophage ϕ6.** Journal of Chromatography B **1095**, 251-257.

The doctoral candidate's contribution to the articles included in this thesis:

I The doctoral candidate ML performed the life cycle measurements of PRD1 with AF4. ML participated in analysis of the data obtained from AF4 purification of different PRD1 input samples. ML participated in the preparation of virus samples used in the study. ML participated in the writing of the manuscript and preparation of the figures.

II The doctoral candidate ML performed AF4 experiments conducted with HVTV1 and HRPV1, except for the light scattering measurements, and performed the life cycle measurements of His1. The doctoral candidate ML participated in the preparation of virus samples and analysis of the experimental data obtained for all four studied halophilic viruses. ML also performed the analysis on the effect of ionic strength on elution of proteins and PRD1. ML participated in the writing of the manuscript and preparation of figures.

III ML participated in the planning of the research and the experiments, prepared virus samples used in the study, performed all AF4 experiments except for the light scattering measurements, analysed the data, and wrote the manuscript, on which all authors commented.

ABBREVIATIONS

1×virus	input virus material purified from culture supernatant through PEG-NaCl precipitation and ultracentrifugation
AF4	asymmetrical flow field-flow fractionation
CRISPR	clustered regularly interspaced short palindromic repeats
CsCl	cesium chloride
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
EM	electron microscopy
His1	Haloarcula hispanica virus 1
HRPV1	Halorubrum pleomorphic virus 1
HVTV1	Haloarcula vallismortis tailed virus 1
ICTV	International Committee on Taxonomy of Viruses
MALS	multi-angle light scattering
MWCO	molecular weight cut off value
NCIB	National Centre of Biotechnology Information
PEG	polyethylene glycol
PEG-virus	input virus material precipitated from the culture supernatant with PEG and NaCl
PFU	plaque forming unit
RC	regenerated cellulose
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
TEM	transmission electron microscopy
VLP	virus-like particle
WHO	World Health Organization

SUMMARY

Viruses are the most abundant entities in the biosphere, the estimated amount of viruses is more than 10^{30}. The number is incomprehensible and exceeds the amount of host cells at least by one order of magnitude. Viruses are extremely diverse entities by means of morphologies, sizes, genomes and biochemical and biophysical properties. As obligate parasites, viruses can only be propagated in living cells. This sets challenges for the virus purification, since the starting material contains host and growth media derived impurities. Medical applications such as phage therapy, vaccine development, and gene therapy require large amounts of highly purified viruses and virus-like particles (VLPs). Nanotechnology utilizes viruses and VLPs as building blocks for nanoscale materials and devices and also requires virus purification methods which maintain the biophysical and biochemical properties of the particles.

Viruses are often purified with combinations of different methods. The most common ones are precipitation and ultracentrifugation. Precipitation does not lead into high purities and is generally applied as a pre-step for purification. Ultracentrifugation leads to high purity but it exposes viruses to high shear forces possibly leading to losses of infectivity. The large size of many viruses may restrict utilization of traditional chromatography. However, monolithic matrices are applicable for virus purification.

In this work asymmetrical flow field-flow fractionationation (AF4) method was developed for virus purification. AF4 is a highly versatile size-based separation method applicable for samples with sizes ranging between ~1–500 nm. The separation in AF4 is conducted with the aid of liquid flows. Solid stationary phase is not applied at all, thus no strong interactions during the separation occur making the method gentle. Several parameters in the AF4 system are adjustable, making the method highly versatile and an attractive alternative for virus purification.

In this study, AF4 conditions were optimized for purification of six prokaryotic viruses, having different morphologies and properties. Analytical sample channel and preparative UV-detector were utilized. Yields of infective viruses were high and purity levels comparable to the ones obtained with a method based on precipitation and ultracentrifugation. AF4 was proven to be applicable for all tested viruses, also the ones requiring high ionic strength conditions were amenable for AF4 purification. The AF4-method is fast and obtained virus preparations were homogenous. As the system is highly versatile, it is expected that it can be tailored for other viruses as well, to meet the further needs of virus purification.

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1. Introduction

1.1. Viruses

Viruses are contradictory entities in the field of microbes. They are obligate parasites of cells, yet they do not fill all the classical hallmark features set for living organisms. Their replication occurs only in living cells, with the aid of host factors, but when released to cell surroundings, viruses are inert vessels protecting the genome and carrying it to the next host cell. This contrast between intracellular and extracellular stages of virus replication cycle has led scientists to an everlasting debate of viruses being living entities or not (Forterre 2016). All cellular organisms have double-stranded DNA genomes, whereas viruses may contain genomes comprised of double- or single-stranded DNA or RNA. Additionally, the genome can be circular, linear or segmented and RNA genomes can be either positive- or negative-sense (Baltimore 1971).

To date the number of complete virus genomes in the database of National Centre for Biotechnology Information (NCBI) is about 8,000, even though the number of viruses at our planet is assumed to be astronomical. Oceans are estimated to contain more than ~10^{30} viruses (Suttle 2007) and the estimates for virus amounts in soils are comparable or even higher (Srinivasiah et al. 2008). Thus the number of viruses seems to exceed the number of host cells by at least 10 times (Bamford 2003; Srinivasiah et al. 2008; Suttle 2007). Based on these numbers, and the observation that many viral genes have no homologs, it could be stated that viral genomes are still the most unexplored nature reservoir of genetic information (Yin and Fischer 2008). Additionally, viruses come in tremendous variations of size, shape and other biochemical and biophysical properties (chapter 1.2.).

The role of viruses in the biosphere is inevitable. They control population dynamics, as more than 90% of the living biomass in seas is comprised of microbes, and ~20% of them are killed daily by viruses (Suttle 2007). Viruses in soils have not yet been thoroughly explored. Nevertheless, it is estimated that majority of them are bacteriophages, i.e. viruses of bacteria (Williamson et al. 2017). Virus-induced host lysis plays a major role in the nutrition cycles by releasing contents of infected cells to their surroundings. Indeed, in both aqueous as well as soil environment, top-down control of microbial population, and occurrence of virus shunt has been proposed (Suttle 2005). Virus shunt refers to cycling of the organic matter, especially carbon, stemming from the viral lysis of microbes to environment as pools of dissolved and particulate organic material, free for consumption (Wilhelm and Suttle 1999).

As viruses are significant in ecological processes, they also are the driving force for evolution. Genetic material is often horizontally transferred from the host cell to another via transduction by progeny viruses (Canchaya et al. 2003). Additionally, the ability of hosts to restrict virus infections and viruses evolving ways to counteract the restriction gives rise to coevolution of viruses and their hosts (Forterre and Prangishvili 2009). Overall, the role of viruses in maintaining diversity of host population as well as modulating the environment is highly important but due the complex nature of ecosystems, not yet completely understood.

Viruses are causative agents of diseases in animals and plants, having major effects in lives of people as well as in crop yields and well-being of livestock. Viral diseases in human range from mild to severe, have a huge load on the healthcare system, and enormous economic effects. The estimated burden of influenza in U.S. during the season 2017–2018 reveals high numbers: 23,000,000 medical visits, 960,000 hospitalizations and 79,000 deaths (Center for Disease Control and Prevention). Viruses pose threats also to the agriculture and food production. Corn is amongst the most cultivated crops globally (Food and Agriculture Organization of United Nations). It is cultivated and consumed at all continents and considered as a major staple food in Africa (Ranum et al. 2014). The most important pathogenic virus of corn is maize dwarf mosaic virus. It is epidemic globally and has caused 70% losses in corn yields since 1960 (Kannan et al. 2018). Virus infections decrease food production but also compromises food safety in the case of zoonotic diseases. Foot-and-mouth disease is one of the most devastating virus diseases of cloven-hoofed animals (Grubman et al. 2008). During outbreaks, foot-and-mouth disease forces to mass slaughters of animals, restrictions in animal transportation and exportation of live animals as well as meat products, thus leading to enormous economic losses (Prempeh et al. 2001).

Even though the headlines about viruses focus on life-threatening disease outbreaks, viruses are also beneficial, thus playing a dual role in science and society. Infection with certain viruses may prevent simultaneous infection with pathogenic bacteria. Mice infected with herpesvirus are protected against *Listeria monocytogenes* and *Yersinia pestis* that are pathogens behind food borne illness and plaque, respectively (Barton et al. 2007). In a complex three-way mutualistic symbiosis between a grass, fungus and virus, the virus offers thermotolerance for the plant allowing it to grow at soil temperatures higher than 50 °C (Redman et al. 2002). In addition, intestinal viruses are able to substitute some of the beneficial functions of intestinal bacteria in cases when they are absent (Kernbauer et al. 2014).

Since current knowledge of viruses is still only a tip of an ice berg of all what is out there, one can only imagine what kind of findings will be unveiled when scientists

continuously dig deeper to the universe of viruses. Cumulating knowledge of viruses, their properties and different roles are enabling scientists to harness viruses to work for us. Several fields of science are already utilizing viruses in one way or another and after a short introduction to virus sizes, properties and life cycles, I will present few of these application (chapter 1.6).

1.2. Properties of virus particles

Martin Beijerinck found viruses when he repeated experiments of Adolf Mayer and filtrated fluids from diseased tobacco plants in 1898. He observed that filtered fluid was infectious and the infectious agent multiplied in living cells. Beijerinck defined that viruses are entities that are filterable through pores smaller than bacterial cells (Bos 2000) that typically range between 0.5–5 µm. In recent years, however, the definition of virus has been challenged and virus diversity has been expanded by characterization of gigantic viruses. The first giant virus, mimivirus (for 'mimicking microbes'), was isolated in 2003 from ameba culture (La Scola et al. 2003). It is ~400 nm in size with ~80 nm fibrils extending from the capsid. Since that the size record holder of giant viruses to date is pithovirus sibericum having length of ~1.5 µm and diameter of ~0.5 µm (Legendre et al. 2014). The other extreme, the smallest known virus which replicates autonomously in cells is porcine circovirus type 1 (Mankertz et al. 1997; Tischer et al. 1974). The capsid of this virus is only ~17 nm in diameter, thus almost 90,000 copies of the virus would be needed to form a line with a length corresponding to the pithovirus size.

The mature infectious virus is referred as virion. Virions are macromolecular assemblies that are found in wide variety of sizes, shapes and properties. However, in the simplest form, virion contains only protein and nucleic acid: proteinaceous capsid encloses and protects the genome composed of DNA or RNA. Capsid proteins mediate their self-assembly. Besides of protecting the genome, capsid also mediates attachment and entry to the host cell (Bhella 2018). Viral capsid has a metastable structure to be robust enough to encounter extracellular phase, but also labile enough to allow the transfer of the genome to the host cell to begin the new infection cycle.

Most of the known virion capsid morphologies so far fall into categories of icosahedral- or helical viruses or to combination of these two (Caston and Carrascosa 2013)(Fig. 1). Icosahedral capsid is formed from twenty facets assembled from capsid proteins (Caston and Carrascosa 2013). Several icosahedral virions also carry spike or fibril structures protruding from the virion surface. Helical viruses can be either filamentous or rod shaped cylinders. They are formed, when capsid proteins are arranged side by side in helical array to surround the genome, and the genome size determines the

length of a helical virion (Stubbs and Kendall 2012). Variations of icosahedral and helical viruses are abundant, but the underlying basic structure is the same (Fig. 1).

Combination of these two architectures is formed, when icosahedral head, enclosing the genome, is attached to a helical tail. Head-tailed viruses may also include several additional structures, such as a portal in the icosahedral head, collar connecting the tail to the head as well as contractile structures in the tail (San Martin 2013). Icosahedral as well as helical viruses are found to infect host cells from all three domains of life, whereas head-tailed architecture is only met in bacteriophages and viruses of archaea. There are also another diverse virus morphologies that do not fit into these categories. They include, for example, droplet, bottle and bullet shaped virions. Especially viruses infecting archaea represent a variety of morphotypes (Pietilä et al. 2014).

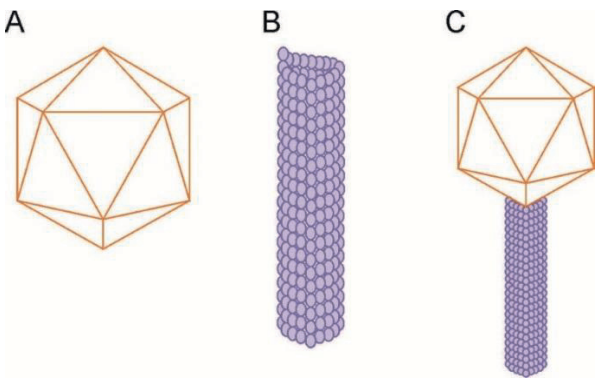

Figure 1. Schematic drawings of principal architectures of viruses. A) Icosahedral virus structure, B) Helically symmetrical structure and C) Head-tailed virus, combination of icosahedral and helical symmetry.

In addition to the basic architecture of the proteinaceous capsid and nucleic acid, several viruses contain lipid bilayers either as an outermost envelope or inside the capsid (Mäntynen et al. 2019). Yet another lipid bilayer containing virus morphotype is pleomorphic virion. Instead of having a proteinaceous capsid, pleomorphic viruses resemble lipid vesicles with randomly organized protein spikes embedded to the envelope (Bamford et al. 2017). Lipid containing viruses acquire the lipid envelope from host cell membranes (Welsch et al. 2007).

All components of the capsid are typically required for virions to be infective and the integrity may be compromised in many ways, depending on the properties of the virion. The proteinaceous capsid might be relatively robust, but to be infective, possible tails, spike structures and lipid layers must be intact. Viruses are affected by changes in pH

and ionic strength of the environment, which may lead into reversible or irreversible changes in protein conformations and their interactions. Changes in the capsid proteins can be detrimental. With several viruses, acidic pH in endocytic pathway induces conformational changes leading to alterations in envelope fusion proteins. Thus, applying low pH may induce premature changes in particles leading to alterations in infectivity (Stegmann et al. 1987). In addition, aggregation of viruses would lead into losses of infective viruses. Since lipids of the virion are typically involved in entry processes (Poranen et al. 2002), distorted envelopes lead into loss of infectivity. Lipid containing viruses are often inactivated by detergents and chemicals such as chloroform, which was before extensively utilized to avoid bacterial contamination in viral preparates (Mäntynen et al. 2019). In addition, lipid bilayer is not as robust structure as protein capsid, thus it is more vulnerable for shear forces.

1.3. Prokaryotic viruses used in this study

In this work two viruses of bacteria and four archaeal viruses were utilized (Fig. 2): bacteriophages PRD1 and ϕ6 and archaeal viruses HVTV1, His1, HRPV1 and HCIV1.

1.3.1 Bacteriophages

Enterobacteria phage PRD1 is an icosahedral bacteriophage of Gram-negative bacteria such as *Salmonella* (Olsen et al. 1974). PRD1 virion consists of proteinaceous icosahedral capsid with diameter of ~66 nm that has ~20 nm spike structures at the icosahedron vertexes. The capsid encloses internal lipid bilayer with embedded membrane proteins (Fig. 2) (Abrescia et al. 2004; Cockburn et al. 2004). The replication cycle of PRD1 is relatively short and progeny viruses are released via lysis in about 35 minutes post infection (Olsen et al. 1974). PRD1 has been extensively studied during decades and has well established production and purification procedures (Bamford and Bamford 1991).

Pseudomonas phage ϕ6, belonging to cystoviruses, is a virus of plant pathogenic *Pseudomonas* (Vidaver et al. 1973). It has spherical shape with complex layered structure including lipid envelope as an outer layer (Fig. 2). The envelope has proteins embedded and spike structures protruding from it. The diameter of ϕ6 virion is ~85 nm (Poranen et al. 2017). The next layer beneath the envelope is icosahedral nucleocapsid composed of two concentric protein shells enclosing the genome (Sun et al. 2017). ϕ6 enters the host cell via membrane fusion in a similar manner to animal viruses (Bamford et al. 1987) and exits the cell by lysis of the host cell in ~85 min post infection (Vidaver et al. 1973).

1.3.2 Viruses of archaea

Haloarcula vallismortis tailed virus 1 (HVTV1) is a head-tailed virus of an archaea *Haloarcula vallismortis.* HVTV1 has ~96 nm sized icosahedral head (vertex to vertex) and ~73 nm long and flexible, non-contractile tail (Atanasova et al. 2012; Pietilä et al. 2013b). Virions of HVTV1 are released via host lysis after ~12 h post infection (Pietilä et al. 2013b).

Haloarchaeal virus His1 is a spindle-shaped virus of *Haloarcula hispanica* (Bath and Dyall-Smith 1998). Viruses with this morphotype are unique to archaeal hosts. His1 virion is ~44×77 nm in size with ~7 nm tails in one end. The capsid of His1 is elastic and the capsid proteins have lipid modifications (Hong et al. 2015). The size of tails may vary, thus the virus population might not always be homogenous (Hong et al. 2015; Pietilä et al. 2013a). His1 virions are released without host cell lysis, but it causes retardation of host growth (Bath et al. 2006).

Halorubrum pleomorphic virus 1 (HRPV1) was the first pleomorphic virus described for archaea and to date the only archaeal virus carrying single-stranded DNA genome. The size of HRPV1 is ~44×55 nm with randomly embedded glycosylated spikes that cause heterogeneity to virus population (Pietilä et al. 2012; Pietilä et al. 2009). HRPV1 has a non-lytic replication cycle (Pietilä et al. 2012).

Haloarcula californiae icosahedral virus 1 (HCIV1) has an icosahedral capsid enclosing a lipid envelope. The capsid has horn-like spike structures at vertexes of the icosahedron and the diameter of the capsid is ~70 nm. Virions are released via cell lysis ~12 h post infection (Demina et al. 2016; Demina et al. 2017).

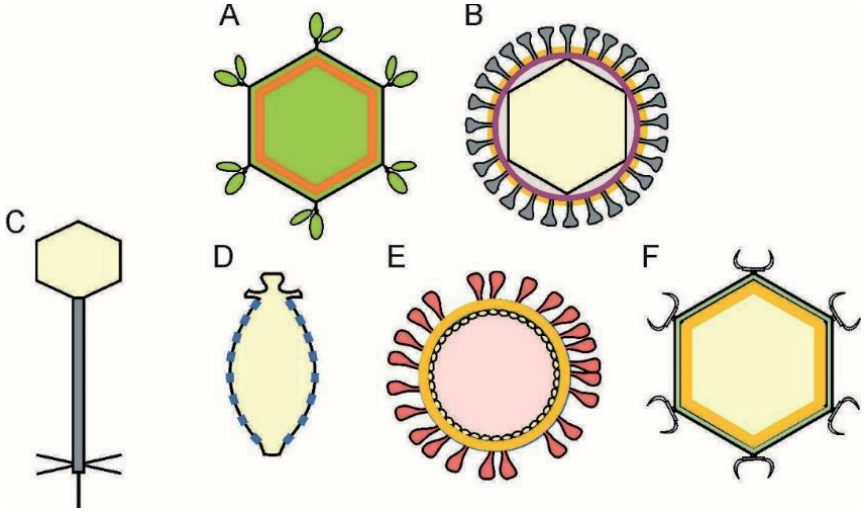

Figure 2. Schematic morphologies of viruses utilized in this work. A: PRD1, B: ϕ6, C: HVTV1, D: His1, E: HRPV1, F: HCIV1. Not in scale.

1.4. Prokaryotic virus life cycles

Typically the life cycle of prokaryotic viruses includes seven steps: attachment to host cell surface, binding to the receptor, genome delivery, replication of viral genome, synthesis of virion components, virion assembly and host cell lysis (Fig. 3). Binding to the receptor is crucial and limiting step for the infection, since only cells displaying receptor specific for certain virus, are susceptible for infection.

Binding of the virion to the receptor initiates series of events that lead to genome delivery to the host cell. Host cell barriers that bacteriophages encounter are peptidoglycan cell wall and one or two membrane bilayers. Several bacteriophages deliver their genome to the bacterial cell through the helical tail structure (Fig. 2, Fig. 3) or through a special portal vertex of the icosahedral capsid (Poranen et al. 2002). Cystoviruses are the only group of bacteriophages that have a lipid bilayer as an outer envelope. In the case of cystovirus ϕ6, viral lipid envelope fuses with the host outer membrane, peptidoglycan wall is then locally degraded by viral enzymes, and the virus particle is finally internalized through the invagination of cytoplasmic membrane (Bamford et al. 1987; Mindich and Lehman 1979; Poranen et al. 1999; Romantschuk et al. 1988). The entry mechanisms of archaeal viruses are not well characterized yet, but the apparent similarities to the counterparts in bacteriophages and viruses of eukaryotes suggests similar strategies in their entry mechanisms (El Omari et al. 2019; Pietilä et al. 2009). No matter how it is implemented, getting the viral genome inside the cell is the essential step for every virus infection.

When the genome is in the cell, virus begins to replicate the genome with the aid of host factors or using viral polymerases. For successful replication, viruses need to hide from host cells defense mechanisms such as restriction-modification and CRISPR/Cas-system (Mojica et al. 2005; Seed 2015) and be able to hijack the control of the cell. This is a delicate process where many functions of the cell are shut down, cellular factors needed for replication and assembly are recruited, and cell is converted to a virus factory (Goodwin et al. 2015). After the genome replication or simultaneously to it, virus begins to produce virion components which are then assembled. Virion capsid may be assembled on top of the genome or the genome may be packed to the preformed procapsid after it has been assembled. Details of these steps are highly variable between different viruses (Aksyuk and Rossmann 2011).

Replication cycle is finished when the new progeny viruses are released from the host cell (Fig. 3). The release of virions may be lytic or non-lytic. In case of lytic release, cell bursts and releases virions which were assembled in the cytosol. The release via lysis is highly regulated, so that the lysis occurs only when the majority of virions are assembled and matured to encounter the cell exterior (Young 2014). Lytic cycle is typical amongst bacteriophages. However, non-lytic cycle has been described for pleomorphic viruses (Pietilä et al. 2009). In non-lytic cycle, virions are released from the cell in continuous manner without bursting the host cell.

Instead of exiting the host, the life cycle of prokaryotic virus may turn to lysogenic cycle (Fig. 3). In lysogenic life cycle virus establishes more or less permanent association with the host cell. Thus, virus genome is inserted into host genome as a prophage or it exists in the cell as an episome (Strömsten et al. 2003). Prophage replicates as a part of host genome and is distributed to next cell generations (Ramisetty and Sudhakari 2019). The lysogenic cycle may be reverted back to lytic one, when beneficial for virus survival (Bobay et al. 2013; Nanda et al. 2015).

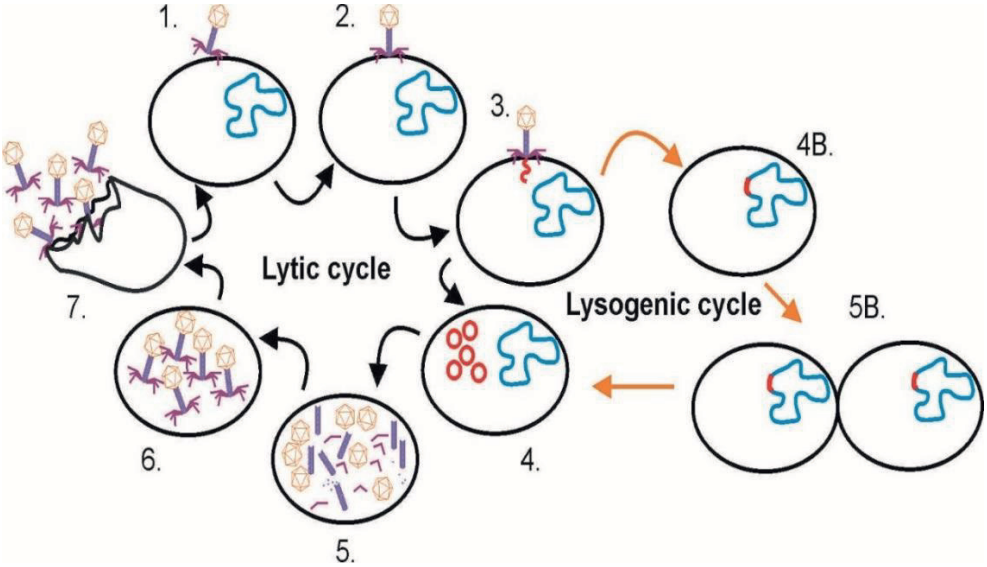

Figure 3. Schematic presentation of lytic and lysogenic life cycle of a prokaryotic virus. The infection may result in degradation of the host chromosome. Lytic cycle: 1. attachment, 2. receptor binding, 3. genome delivery, 4. genome replication, 5. synthesis of virion components, 6. virion assembly and 7. host cell lysis. Lysogenic cycle proceeds from genome delivery to 4B. prophage insertion and to 5B. cell division.

1.5. Virus purification

Virus research and different virus-based applications require highly purified viruses and virus-like particles (VLPs). Since viruses can nowadays be propagated to large quantities in bioreactors, the bottleneck step in providing viruses and VLPs for applications, has shifted from production to purification processes (Nestola et al. 2015). Due to the fact that viruses can only be propagated in living cells, the starting material for virus purification is biologically complex. Cell lysate or the supernatant of infected cell culture contains impurities derived from the host cells and growth media. Virus infections are difficult to synchronize. Thus, the starting material may vary between propagated batches depending on the growth conditions, the stage of infection, and the growth phase of the cells. In most cases, all components of the virus particle need to be intact to have infectious particle. Additional challenges to the virus purification rises from the great variety of morphologies, sizes and other properties of viruses. Several methods are typically needed and often used in different combinations to reach the best outcome (Morenweiser 2005). However, the applied methods depend on the intended use of purified viruses: is the highest possible yield or only extreme homogeneity of virus preparation desired? Separation techniques may be based on

different virus properties such as, size, shape, charge, density or sedimentation (Nestola et al. 2015). Several methods are currently applied routinely in laboratories and here I present the ones most important in context of this work.

1.5.1 Precipitation

Purification often begins with precipitation of viruses from the lysate, infected cell culture or for example from dilute environmental sample. One of the most common precipitation method used is precipitation with polyethylene glycol (PEG) and sodium chloride (NaCl). PEG is a polymer that precipitates molecular complexes based on their solubility. Precipitates are then easily removed from the original sample by centrifugation (Fahie-Wilson and Halsall 2008; Yamamoto et al. 1970). Since the precipitate can be dissolved in significantly smaller volume than the original sample, this step is also important in concentrating the sample before next purification steps. Indeed, PEG-precipitation in virus purification is typically considered as a concentrative pre-step of the purification process. PEG precipitation is gentle, thus loss of biological activity or protein denaturation does not typically occur (Asenjo and Andrews 2012). Virus size and morphology determine the PEG concentration required for optimal precipitation. However, precipitation with 10% PEG and 0.5 M NaCl is relatively universal (Yamamoto et al. 1970). As a downfall of this method, PEG concentrates also nucleic acids and other large complexes present in the starting material (Hagen et al. 1996).

1.5.2 Ultracentrifugation

Ultracentrifugation method is based on differences in sedimentation properties and utilizes high speed and centrifugation force. In virus purification three methods of ultracentrifugation are typically applied: rate zonal-, equilibrium-, and differential ultracentrifugation. Rate zonal ultracentrifugation utilizes low density gradients and separates viruses based on their shapes and sizes. In equilibrium centrifugation, higher density gradient is applied and the separation is based on the differences in densities of particles (Mohr and Völkl 2017). The gradients are typically comprised of sucrose or cesium chloride (CsCl) which are hyperosmotic and viscose. This can be detrimental for virions or to further applications. Gradient material may be removed with further purification steps e.g. by differential centrifugation or by using buffer exchange devices (Segura et al. 2006). Differential ultracentrifugation is utilized without gradient, to harvest and concentrate viruses.

In ultracentrifugation viruses are exposed to high shear forces potentially leading into aggregations and losses of biological activity. However, ultracentrifugation is routinely utilized in virus purification. Acquiring larger amounts of viruses with ultracentrifugation-based purification requires simultaneous access to several

expensive ultracentrifuges. Furthermore, preparing the gradients as well as loading and unloading samples is laborious and time consuming.

1.5.3 Chromatography

Chromatography is defined as a separation technique where the sample is applied to a stationary phase and eluted from it with the aid of mobile phase. Sample components are stalled in the system by the stationary phase according to the characters of the sample components and the stationary phase itself. Variety of different chromatography techniques and sub techniques have been developed and are widely used in the separation of biological and non-biological components. Even though viruses are nanoscale entities, in the field of chromatography they are considered as large macromolecular complexes leaving many traditional chromatography resins inapplicable for the purification of viruses.

In virus purification, liquid chromatography utilizing size exclusion or ion exchange are commonly applied methods. Size exclusion chromatography (SEC), as the name refers, is a chromatographic separation method based on size. In SEC, the stationary phase column is packed with porous beads. As the sample is applied on the column, small sample components are retained, since they diffuse into the porous beads (Fekete et al. 2014). Larger components, such as viruses, do not fit into the pores. Thus they elute in the void volume before smaller ones. However, other large complexes present in the sample are difficult to separate form viruses or VLPs with SEC (Andreadis et al. 1999).

Stationary phases more suitable for virus purification have been recently developed. Monolithic convection interaction media matrices have continuous, sponge-like structure, with cavities wide enough to allow viruses to enter. Monolithic stationary phases in virus purification are mainly utilized in ion exchange chromatography (Krajacic et al. 2017; Oksanen et al. 2012). Ion exchange chromatography is based on electrostatic interactions, thus the sample components bind to the opposite charge displayed in the monolith cavities. Elution may be achieved by changing the pH or by increasing the ionic strength of the carrier solution. The elution with pH change is based on the interplay with isoelectric point of the bound capsid proteins, thus by altering the net charge and matrix binding capacity of the virus. Elution by increasing the ionic strength of the carrier solution is based on addition of ions which compete of binding and eventually displace the bound viruses (Cummins et al. 2011). Monolithic columns have been applied in successful purification of viruses with different morphologies and properties (Kramberger et al. 2010; Oksanen et al. 2012; Smrekar et al. 2011) and led to high yields of purified, infective particles in short time. The elution with high ionic strength carrier solution or by changing the pH may, however, compromise the infectivity of labile virions. As the elution requires changes in ionic strength of the

carrier solution, the infectivity of viruses requiring high salinity may be compromised. However, viruses that tolerate lowered ionic strength for short periods of time may be amenable for anion exchange chromatography.

1.5.4 Asymmetrical flow field-flow fractionation

In flow field-flow fractionation techniques, the separation is based on differences of hydrodynamic sizes of the sample components. The separation is conducted with the power of liquid flows without applying solid stationary phase. Flow field-flow fractionation was introduced by Giddings in 1976 and a sub technique derived from it, asymmetrical flow field-flow fractionation (AF4) was developed in 1987 (Giddings et al. 1976; Wahlund and Giddings 1987). The size-based separation in AF4 occurs in a thin sample channel, which has sandwich-like composition (Fig. 4). The bottom wall of the channel is a permeable frit which has an ultrafiltration membrane placed on top of it forming the accumulation wall. The pore size of the membrane defines the molecular weight cut of limit (MWCO) for the sample components – components smaller than membrane pores flow through the channel bottom and only the larger ones are retained for separation. Thin spacer positioned on top of the membrane determines the thickness and the trapezoidal shape of the channel. The top layer of the channel is a solid impermeable wall.

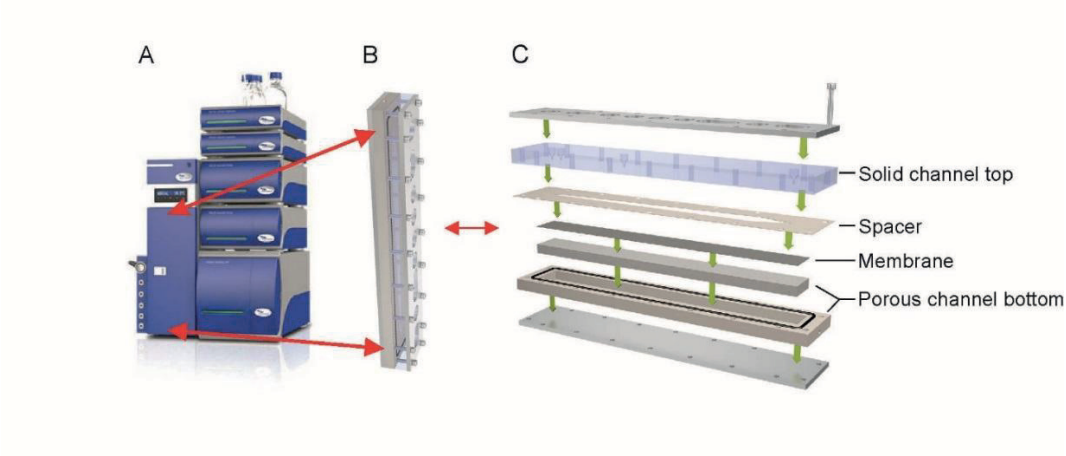

Figure 4. AF4 machinery, and the channel composition. A) AF4 machinery. The channel is positioned in the temperature controlled oven (indicated with arrows). B) Assembled AF4 channel, C) The sandwich-like structure of the channel. Modified from figures of Postnova.

In AF4 analysis, the sample is injected to the channel along the incoming flow (flow in, Fig. 5A). In the channel, there is a constant detector flow with parabolic profile that

transports the sample components towards the outlet (flow out, Fig. 5A). However, during the focusing step, the sample encounters an opposing focus flow which concentrates the sample to a thin zone (Fig. 5B). Cross flow, perpendicular to detector flow, is also applied and it drives the sample components towards the accumulation wall that has the ultrafiltration membrane. Cross flow creates the flow field that generates the separation power of AF4 (Wahlund and Giddings 1987). During the focusing step, sample components are equilibrated to the channel according to their hydrodynamic sizes. Due to diffusion, components migrate back against the cross flow and eventually equilibrate to a certain distance from the membrane (Fig 5B). After this focusing step, focus flow is switched off and the sample is let to elute from the channel to detectors and fractionation, respectively (Fig. 5C). Detector flow has parabolic profile, meaning that the flow rates are higher in the middle of the channel and lower close to the walls. The smaller components diffusing further from the channel bottom, reach higher flow rates and elute before bigger components (Wahlund and Giddings 1987).

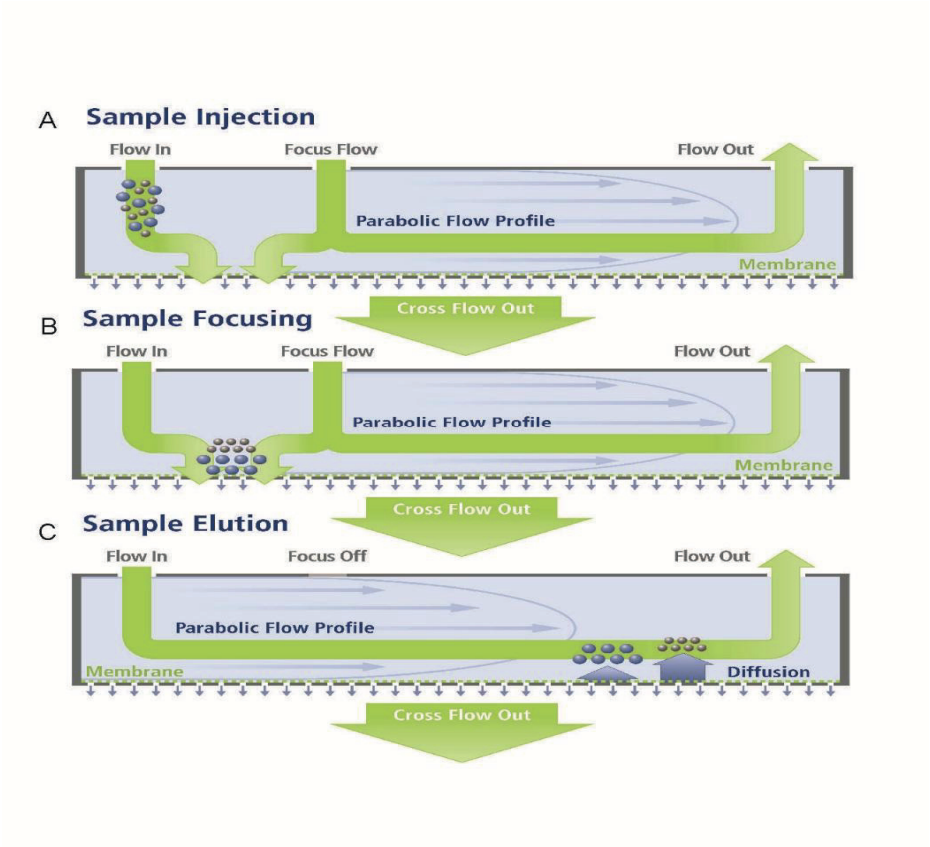

Figure 5. Schematic presentation of AF4 separation. A) Injected sample is carried to the channel by the incoming flow (flow in). B) During focusing, the focus flow concentrates the sample to a narrow zone C) Elution is achieved, when the focus flow is switched off and sample components elute according to their hydrodynamic sizes, smaller ones first and larger ones later. AF4 channel can be coupled to various detectors (at flow out) that enable data collection on concentration, size and size distribution or fluorescence, depending on the detector(s). Photo courtesy of Postnova.

In general, higher cross flow leads to later elution, as it hinders the diffusion of sample components into higher detector flow velocities. Thus, cross flow is often programmed to decline during the elution step to enhance the elution of larger components. The elution program in AF4 is readily tailorable. One can choose to apply constant cross flow or a gradient can be programmed to decay in step-wise, linear or exponential manner (Fig. 6). Also the rate of cross-flow reduction can be adjusted.

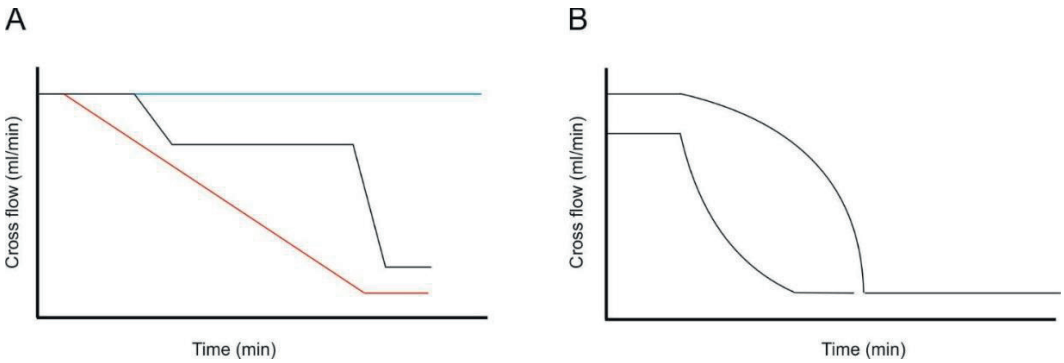

Figure 6. Possible elution programs in AF4 analysis. A) The linear reduction of cross-flow is shown in red, step-wise in black and constant cross-flow in blue. B) The cross-flow rate can also be altered exponentially.

The omission of the solid stationary phase in AF4 allows gentle separation to be conducted without strong interaction or shear forces between sample and matrix. The carrier solution may be selected according to the requirements of the sample. However, great versatility due to the possibility to adjust several parameters might lead to time consuming optimization of the conditions and requires understanding of the theory behind the method (Gimbert et al. 2003). The MWCO and the material of the membrane can be adjusted as well. The sample channel can be temperature controlled and the flow rates and elution gradient is easily altered to reach optimal separation. Several detectors such as UV-, multi angle light scattering (MALS)-, dynamic light scattering (DLS)- and fluorescence detectors can be connected to AF4, allowing real-time data collection. In the normal mode AF4 is suitable for sample components with sizes ranging between ~1–500nm. AF4 analysis is typically fast. Even though the AF4 theory is rather complicated, operating the AF4-interface is relatively simple.

AF4 has been widely applied in the studies of biological and non-biological macromolecules as well as nanoparticles, their size distribution, aggregation as well as interactions (Leeman et al. 2015). Viruses were one of the first objects studied with AF4. They were applied to demonstrate the power of the technique in the determination of the diffusion coefficients of particles and to study links between the theory and practice of the method (Litzen 1993). Size, size distribution, dissociation, stability and aggregations of viruses and VLPs have also been analyzed (Bousse et al. 2013; Chen et al. 2016; Eskelin and Poranen 2018; Lipin et al. 2008; Pease et al. 2009; Wei et al. 2007). The purification in more preparative manner, however, has not been studied before.

1.6. Virus applications

Viruses are often called to be molecular machineries. Indeed, their natural properties are routinely exploited in basic research, industry as well as in medicine and several types of applications. Here I present examples of virus applications in medicine and nanotechnology to showcase the innovations derived from virus research and to highlight the motivation for production and purification of viruses and VLPs.

1.6.1 Medical applications

Emerging of multiresistant bacteria has led to intensive search for alternative medicine and phage therapy has witnessed a renaissance. Antibiotics are no more effective against all pathogenic bacteria and it has raised concerns of the possible epidemics with pathogens that are currently held back by effective antibiotics. Indeed, the present time is already referred as an advent of post antibiotic era (Lin et al. 2017). In phage therapy, pathogenic bacteria are killed by applying strictly lytic bacteriophages specific to the bacteria of interest (Gordillo Altamirano and Barr 2019). Since bacteriophages are host specific, no damage to normal microbiota should occur and after host cells are killed, no more phages are produced. The emergence of commercially available broad spectrum antibiotics during the Second World War collapsed the interest towards phage therapy. However, Eliava institute in Georgia continued the phage therapy studies and they have reported wide variety of successful case studies and collected phage libraries during decades (Kutateladze and Adamia 2008). As the World Health Organization (WHO) listed the fight against multiresistant bacteria as one of the most important topics in research, also the rest of the world has re-adopted the phage therapy in their research interests. Since current regulations in western countries are heavily restricting the utilization of phage therapy, most of the data cumulates from case-studies and from few clinical trials (Furfaro et al. 2018).

Due to the natural properties of viruses enclosing their genome in capsid and delivering it to host cell in cell specific manner, viruses are of a high interest to be used as vectors in gene therapy. In gene therapy, the goal is to replace malfunctioning gene of the patient or to deliver a new gene which would be translated into therapeutic product by the cell (Edelstein et al. 2004). Viral vector-based gene therapy is studied the most in cancer treatment, where cancer cells can be addressed as targets for the vector. Also monogenetic diseases are of a high interest. Delivering a functioning counterpart to restore the function of impaired gene requires delivery to dividing stem cells (Ginn et al. 2013). The research around viral gene vectors is blooming – around 2,600 clinical trials have been approved between years 1989–2017, of which more than ~65% are cancer related therapy and ~11% are for the treatment of monogenetic diseases (Ginn et al. 2018). Viral vector based gene therapy against pathogenic diseases are also

developed. The first viral vector-based gene therapy drug was accepted to the markets in China in 2003. The vector is based on oncolytic adenovirus carrying human tumor suppressor *p53* gene (Peng 2005). Another viral vector-based gene therapy drug exploiting human oncolytic herpes simplex virus 1 against melanoma, was released to markets in U.S. and Europe in 2015 (Pol et al. 2015). In addition to viruses enclosing genetic material, their capsid surface properties have been studied to be used in medical purposes: bacteriophages have been applied to carry medical substances by conjugating them to the capsid proteins via genetically or chemically engineered linker (Hemminga et al. 2010). This technique could carry high therapeutic loads and release it to high local concentrations. Viruses have also been proposed to be used as drug enclosing nano carriers (Sunderland et al. 2017).

Vaccination against diseases is based on inducing protective immunity for the vaccinated individual so that the adaptive immune system would recognize and eliminate the same invading pathogen before the disease develops. Thus the vaccination with whole pathogen or pathogen-derived antigens mimics the invading, pathogen and activates immune system in a safe, pathogen-specific manner. The first vaccination procedure was conducted against small pox by Edward Jenners in 1796. The first real vaccines included whole killed pathogens and soon after attenuated living viruses such as polio and rabies. Viruses were attenuated by passing them through generations in non-human cells, until they lost the ability to cause infection in human (Zepp 2010). Nowadays also VLPs that are self-assembled from viral capsid protein are utilized in vaccines. VLPs typically lack the viral genome, yet their structure and antigens resemble native viruses (Azevedo et al. 2013). Even though development of vaccinations has been a huge triumph of medicine, the fast variability of virus antigens causes continuous problems to keep the vaccines up to date. In the case of influenza viruses, the prediction of circulating viruses has to be made a half year before the upcoming influenza season to provide enough time for production and purification of sufficient amount of vaccine components. Thus, more efficient processes would be needed to accurately target the specific circulating viruses.

1.6.2 Nanotechnology

Nanotechnology is relatively young field of science, nevertheless, it has already led into substantial discoveries of how to turn viruses and VLPs into a good use. The nanoscale size and highly precisely arranged capsid structures have made viruses attractive objects for nanomaterial development. As in gene therapy, the natural property of a virus, to encapsulate cargo to the protein capsid, or cage, as nanotechnology often refers to it, offers a possibility to encapsulate desired artificial cargo such as proteins, inorganic nano particles as well as polymers into symmetric protein structures (Koudelka et al. 2015; Uchida et al. 2018). VLPs are also utilized in biotemplating which

applies metallization or mineralization of desired material on the inner or outer surface of the capsid. Encapsulation and biotemplating has been utilized in a wide variety of applications such as nanowires, imaging applications and in enzyme studies using virus-derived nanoparticles as nanoreactors (Pokorski and Steinmetz 2011; Zhou et al. 2012). In addition, nanotechnology has applied VLPs for tissue scaffolding, where they form a biofilm enhancing proliferation and adhesion of the cells (Lin et al. 2008).

2. AIMS OF THE STUDY

In this work, our goal was to explore the applicability of AF4 in virus purification. We aimed also to evaluate the performance of AF4 in elevated salinities to extend the method also for viruses requiring high ionic strength conditions to remain infective. Our ultimate objective was to provide a novel virus purification method applicable for different types of viruses and resulting in high yields and purity.

Another aim was set to analytical studies of viruses. Since the purification can be conducted after virus propagation, we wanted to develop AF4 method to monitor virus production. The objective was to provide a rapid tool for optimization of virus production.

The main aims of the study are summarized below:

1. To optimize AF4 conditions for efficient and rapid virus purification.

2. To expand the method to be applicable for different types of viruses.

3. To provide a tool for rapid monitoring of virus production and optimization of conditions for virus propagation.

3. MATERIALS AND METHODS

Table 1. Summary of methods used in this work.

Method	**used in**
Cultivation of prokaryote hosts	I, II, III
Preparation of agar stocks and virus propagation in liquid culture	I, II, III
Plaque assay	I, II, III
Virus life cycle analysis	I, II
Bradford assay for measuring protein concentration	I, III
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	I, II, III
Coomassie blue staining of proteins	I,II,III
Negative staining and transmission electron microscopy (TEM)	II
Proteinase treatment of samples	II
Nuclease treatment of samples	I, II, II
Dynamic light scattering analysis (DLS)	III
Multi angle light scattering analysis (MALS)	I, II, III
Monolithic anion exchange chromatography	I, III
Ultracentrifugation	I,II,III
AF4	I,II,III

Table 2. Parameters adjusted or evaluated in AF4 method development

AF4 parameter
Membrane molecular weight cut off limit (MWCO)
Spacer thickness
Focusing step duration and flow rate
Cross flow rate and elution program
Detector flow rate
Linear working range

4. RESULTS AND DISCUSSION

4.1 AF4 method development for virus purification

AF4 allows gentle separation of sample components of wide size range. However, there are several parameters which can be adjusted for optimal separation. The effects of these parameters are interconnected, having an effect on each other. Thus they all need to be carefully considered and experimentally verified when developing a novel purification method. In this work, samples applied as starting materials for purification were infected culture supernatants, viruses precipitated from culture supernatant with PEG and NaCl (PEG-virus) and viruses purified through PEG-NaCl precipitation, subsequent rate-zonal and differential ultracentrifugation (1×virus).

4.1.1 Membrane

Several membrane materials for AF4 are available with different MWCOs. Membranes are the potential sites for the interactions between sample components. Strong attractive interactions between the virus and membrane might reduce the infectivity of virions and yields of purified viruses as virions would be retained in the channel. On the other hand, in case of repulsive interactions viruses might have access to higher channel flow rates than expected by their sizes. This could lead to unexpected elution behavior and to the early co-elution of viruses with smaller sample components present in the input sample. Thus, the choice of membrane has to be carefully considered.

Based on previous studies, bacteriophages ϕ6 and PRD1 were expected to have negative surface charge (Block et al. 2014; Oksanen et al. 2012). As the regenerated cellulose (RC) membrane has negative charge in neutral pH conditions (Bendixen et al. 2014) no attractive interactions were expected. In AF4, channel interactions are often tested by repeated injections with and without cross-flow. The intensity, retention time and shape of the peaks are compared to see any differences in the elution manner. When developing the purification method for PRD1, repeated injections were conducted without cross flow, and no difference in the intensity or peak shape was observed (I). The A_{280} values of the peak regions were almost identical between repetitions and similar when compared to the input sample. These results verified the absence of attractive interactions. RC membrane has also previously been successfully applied in studies of biological samples (Lang et al. 2009). The fact that high yields of infective virions were obtained for all studied viruses indicate that no significant interaction between the membrane and viruses occurred (Table 6; I, II, III).

Table 3. Cross-flow rates in the beginning of the observed elution of studied viruses.

Virus sample*	Cross flow rate (ml/min)**	Reference
PRD1	~0.2	I
ϕ6	0	III
HVTV1	~0.4	II
His1	~0.1	II
HRPV1	~0.4	II
HCIV1* **	~0.3	II

*Results are shown for purified lysates/culture supernatants when 350 µm spacer was used.
**Detector flow of 0.2 ml/min was applied for all viruses.
***HCIV1 was purified from agar stock.

Membranes with pore sizes of 10 kDa have been applied in analytical studies of viruses and VLPs (Chuan et al. 2008; Citkowicz et al. 2008; Lang et al. 2009; Wei et al. 2007), but in the context of purification, we predicted that 100 kDa MWCO would be applicable. Pore size of 100 kDa would allow larger fraction of small components to flow through the membrane. This would improve the separation especially with highly complex samples. RC membranes with smaller pore size have higher pore density that could possibly accommodate higher amount of the sample in the pores leading to reduced yields (Ashby et al. 2014; Singh et al. 1998). In this work the absorbance (A_{260} or A_{280}) values of the input culture supernatant and AF4 purified virus fractions were compared and significant decrease was observed (I, II, III). This, in addition to high yields, ~60–100%, of infective viruses obtained from the fractionation, showed that most of host-derived impurities were removed, yet viruses were retained in the channel for separation. Thus, the applicability of 100 kDa MWCO in virus purification was confirmed.

4.1.2 Spacer

The spacer determining the channel shape, size, and volume affects the elution of sample components. With thicker spacer, retention time increases, thus the duration of the analysis is longer. Spacer of 350 µm has been utilized in most of the previous AF4 studies of VLPs and viruses (Chuan et al. 2008; Citkowicz et al. 2008; Lang et al. 2009; Wei et al. 2007). In this study 350 µm spacer was also successfully applied to separate viruses in the end of the cross flow gradient and smaller sample components in the beginning the gradient (Table 3; I,II,III). This late elution of viruses was suitable in context of this work, since viruses were expected to be the largest components present in the starting material.

When the channel thickness is small, less diffusion is required to reach flow rates high enough for elution. Thus, larger sample components elute during the cross flow

gradient, which empowers their separation. Consequently, thinner spacer allows faster analysis because of reduction in the retention time. In this work spacer of 250 µm was applied in the life cycle analysis of PRD1 and His1 (I, II). As expected, the thinner spacer resulted in decreased retention time of the virus and the elution occurred during higher cross-flow rates than when purification was conducted with the 350 µm spacer and same elution program was applied. The retention time of PRD1 decreased by ~13 min and elution shifted from ~0.2 to ~0.7 ml/min cross flow (table 3; I). In the case of His1, virus elution begin at ~0.6 ml/min, whereas it was ~0.1 ml/min when virus was purified applying the 350 µm spacer (Table 3; II). Applying thinner spacer also decreased the consumption of carrier solution. Spacer of 250 µm has recently been applied in controlled dissociation studies of ϕ6, also resulting in virus elution during the cross flow and enabling the separation from larger complexes if present in the samples (Eskelin and Poranen 2018).

Dilution is a known drawback of AF4 that can be tackled with using thin spacer. When the 350 µm spacer was applied, ~seven-fold dilution occurred in the purification of PRD1 lysate, ~2-fold with HVTV1, His1 and HCIV1, but no dilution with bacteriophage ϕ6 lysate. PEG- and 1×viruses diluted more than low purity input samples (I, II, III). When the 250 µm spacer was applied in life cycle studies of PRD1 and His1, less dilution occurred (I, II). There is another ways to reduce dilution. Majority of the channel volume is free of sample components, as they accommodate only ~1 µm layer above the membrane. Thus, an additional pump separating the sample-free flow can be applied (Prestel et al. 2006). Also careful fractionation reduces sample dilution.

4.1.3 Carrier solution and the effect of high ionic strength

Carrier solution (mobile phase) affects the interactions between sample components as well as between sample and the membrane. In low ionic strength conditions, electrostatic interactions are dominant, whereas higher ionic strength shields electrostatic repulsion, enhances hydrophobic interactions and thus allows sample components to equilibrate closer to the membrane resulting in later elution (Mudalige et al. 2017; Wagner et al. 2014b).

The data available on the carrier solution effects in AF4 is limited to relatively low, 75–150 mM, ionic strengths (Benincasa and Caldwell 2001; Neubauer et al. 2011; Wagner et al. 2014b). In this work neutral pH carrier solutions of low (2 mM), medium (0.6 M) and high (1.6 M) ionic strength were applied to evaluate the elution of standard proteins with acidic pI values and sizes ranging from 150 to 669 kDa as well as PRD1 with size of 66 MDa (II). Ionic strengths were tested with two combinations of membrane and spacer (Table 4).

Table 4. Combinations of carrier solutions, membrane MWCO and spacer tested.

Total ionic strength of the carrier solution	**Membrane and spacer combination**	
	10 kDa + 250 µm	100 kDa + 350 µm
Low (2 mM)	not tested	x
Medium (0.6 M)	x	x
High (1.6 M)	x	not tested

x = Ionic strength tested with the membrane and spacer combination in question.

With both membrane-spacer combinations and with all tested samples, higher ionic strength carrier solution increased the retention time as was expected. In case of the 100 kDa membrane and the 350 µm spacer, the change in the retention time was larger and peaks were slightly broader with the higher ionic strength buffer. Interestingly, when the combination of 100 kDa membrane and 350 µm spacer was applied with medium 0.6 M carrier solution, no signal for alcohol dehydrogenase of 150 kDa was detected. Nevertheless, with 2 mM carrier solution the protein was observed (II). Since the size of alcohol dehydrogenase is somewhat close to the 100 kDa pore size, it is plausible that higher ionic strength environment induced conformational changes in the protein, thus allowed it to pass through the membrane. Indeed, with the membrane and spacer combination of 10 kDa and 250 µm, alcohol dehydrogenase was observed also with medium and high ionic strength carrier solutions. Therefore, MWCO of the membrane should be carefully considered if high ionic strength is applied in the analysis of sample that contains components with sizes close to the MWCO. In addition to standard proteins, we also tested these carrier solutions with PRD1 and the combination of 10 kDa and 250 µm spacer. Similarly to standard proteins, the retention time of PRD1 increased when the ionic strength was higher.

In conclusion, also higher ionic strength carrier solutions are applicable for AF4. The results with PRD1 and halophilic viruses showed no disadvantages in applying elevated salinity for virus purification with AF4 (I, II). This is especially important when viruses, which are adapted to high ionic strength environments are purified, since their infectivity may be lost in low salinity. It is also noteworthy, that viruses tested in this work, did not elute solely according to their sizes, for example icosahedral PRD1 with diameter of 66 nm elutes with lower cross flow than HVTV1 with ~76×96 nm head and 73 nm tail. This indicates that the sample nature and salinity of the carrier solution affected the elution (Table 3). This furthermore highlights that the ionic strength of the carrier solution should be optimized.

4.1.4 Elution program design

Besides membrane and spacer, the elution program for viruses was optimized. This included adjusting focusing step, detector- and cross flow rates as well as elution gradients. Also the linear working range was determined.

4.1.4.1. Focusing step

Before elution begins, sample components need to be equilibrated in the channel to enable them to elute according to their sizes. This is conducted by applying the focusing step to the AF4 method (Wahlund and Giddings 1987). As sample components are concentrated to a narrow zone during focusing and pushed towards the membrane, interactions between sample components and between the sample and the membrane may be enhanced. Consequently, extensive focusing may cause increased retention time, reduced yields and induce aggregation (Wahlund 2013). On the other hand, proceeding to the elution program before properly equilibrating the sample, leads to increased elution of the sample in the void and to reduced separation power (Wagner et al. 2014a).

To optimize the focusing step for virus purification, PEG-precipitated PRD1 sample (PEG-PRD1) was analyzed by applying focusing step of 5, 10 and 15 min with cross flow of 1 ml/min. Elution was performed with linearly decaying cross flow gradient and resulted in a fractogram with a low intensity peak and more intense second peak. No difference in the intensity or separation of these two peaks was observed between different focusing times. However, the void peak was smaller and better separated when 10 or 15 minutes of focusing was applied (I). As this method was intended to be applied for more complex input samples and large sample amounts, the void needed to be as small as possible without compromising the yields and purity. In general, the obtained high yields of infective viruses and the homogeneity of virus particles indicated that no irreversible sample retention in the channel or aggregation due to 10 or 15 min focusing occurred (I, II, III). In previous studies, focusing time of 15 minutes has been shown to be suitable for influenza virus (Wei et al. 2007).

4.1.4.2. Flow rates and gradients

Cross flow has the major effect on retention and elution of the sample components. Cross flow rates of 0.5, 0.75, 1.0 and 1.5 ml/min with 15 minutes linearly decaying gradient to 0.1 ml/min were compared when developing the method with PEG-PRD1 (I). The applied detector flow was 0.2 ml/min. The intensity of the virus peak was low and the shape wide with 0.5 ml/min cross flow rates, suggesting that 0.5 ml/ml cross flow was not high enough for PRD1 to be sufficiently retained in the channel. High cross flow drives the sample components to close proximity of the membrane and may enhance membrane-sample and sample-sample interactions. In case of 0.75, 1.0 and

1.5 ml/min flow rates, peaks were similar in shape, thinner and more intense. The difference in the retention time between the lowest and the highest cross flow was ~3 min as measured from the top of the peak. Too high cross flow is connected to dilution of the sample as well as to peak broadening (Wei et al. 2007). Large components are especially prone for dilution (Kok and Qureshi 2010). Molecules with different shapes require different cross flows to be retained in the channel for sufficient separation. Based on the intensities of peaks the highest applied cross flow did not induce significant dilution. Globular molecules, due to their dense conformation, require rather high cross flows, whereas more expanded conformations have lower diffusion coeffiences and are retained in the channel with lower cross flows (Wittgren and Wahlund 1997).

Two combinations of detector- and cross flows were compared with PEG-PRD1: 0.2 and 0.5 ml/min as well as 1.0 and 1.5 ml/min, respectively (I). All combinations resulted in high yields of infective viruses (61–82 %). Of all combinations tested, detector flow of 0.2 ml/min combined to cross flow of 1.0 ml/min led to the best purity of ~5×10^{11} plaque forming units PFU/A_{280}. The corresponding value of the input sample was ~1×10^{11} PFU/A_{280}. With these experiments we defined that detector flow of 0.2 ml/min and cross flow of 1.0 ml/min were suitable for virus purification and successfully applied them to other five viruses as well (I, II, III) (chapter 4.2).

In this work we tested elution programs with step wise or linearly decaying cross flow (I, II, III). Lysates and culture supernatants were known to contain sample components of a wide size range. Thus the elution program needed to be designed so that the resolution between other sample components and large viruses would be high enough. The highest purity level, according to specific infectivity, was obtained with linearly decaying cross-flow gradient (I). Due to the relatively large size of viruses, they are well retained in the channel and elute at low cross-flows. PRD1 eluted at low cross flow rate (0.2 ml/min) and resulted in high yields of infective viruses (Table 3, Table 5). Similar results were observed with all viruses studied in this work (Table 3, Table 5).

4.1.4.3 Working range

One main questions when a purification method is developed is the efficacy and one important parameter defining it is the amount of sample that can be purified at the time. In AF4, the channel overloading occurs if sample components cannot be accommodated to the correct equilibrium position in the channel because of high concentration of similarly sized components. Sample ends up in higher channel velocities and elutes early. Overloading is typically observed as broadening of the peaks (Caldwell et al. 1988; Hupfeld et al. 2009; Wiedmer and Yohannes 2012).

In this work, analytical AF4 channel and preparative UV-detector were applied. The linear working range of AF4 with PRD1 was explored by applying the 350 µm spacer, 100 kDa RC membrane and pre-purified PRD1 as a series of ten-fold dilutions from 0.3 µg to 3,300 µg. UV signal from 0.3 µg injection was poorly separated from the background signal. When 3,300 µg of virus was injected to the channel, the peak showed slight distortion, thus the upper limit with these elution conditions were reached. When 8800 µg of virus was applied, the peak broadening and spiky appearance was observed. The linear working range was defined to be wide: ~1 µg –~3 mg of virus which equals 1.4×10^{9}–1.4×10^{13} PFU (I).

4.2 Virus purification results

When the performance of AF4 in virus purification was explored, infectivity of obtained viruses as well as purity level and the yield of purified viruses were evaluated. AF4 purification was compared to the PEG-NaCl and ultracentrifugation -based purification method (Fig. 7).

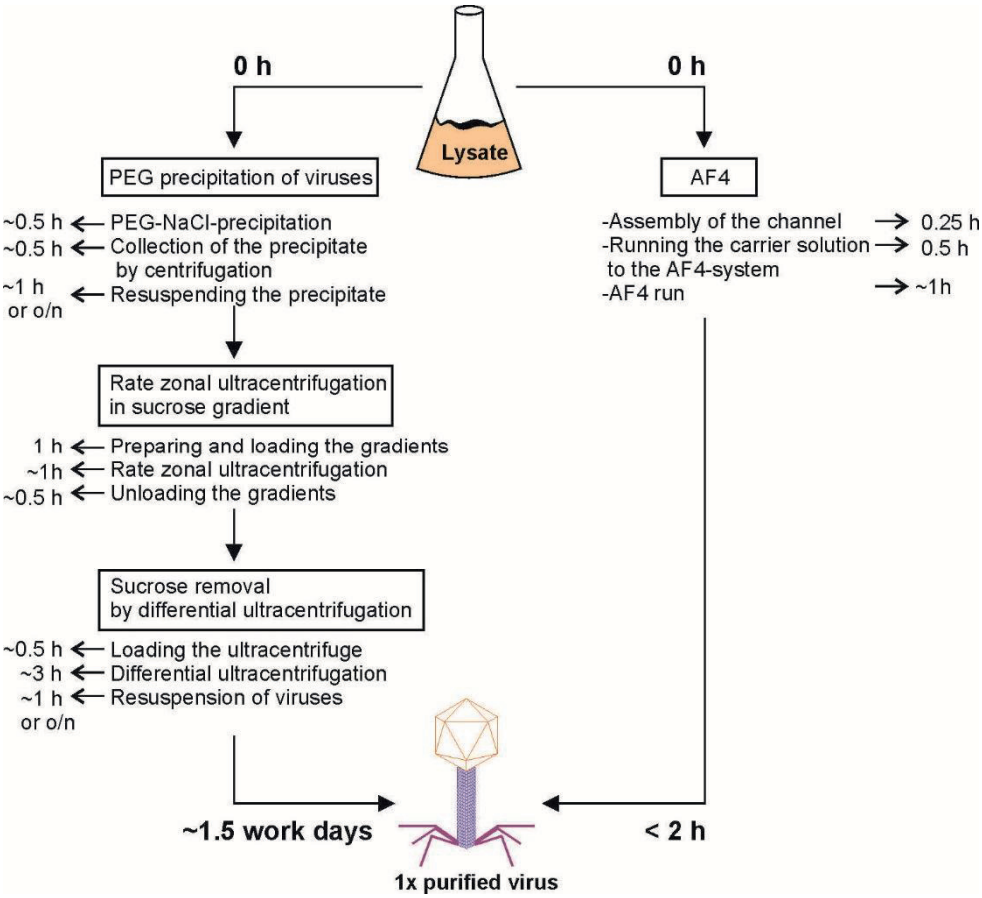

Figure 7. Workflow of PEG-NaCl precipitation and ultracentrifugation -based virus purification compared to virus purification with AF4. Presented duration estimates may slightly vary depending on a virus.

4.2.1. Virus integrity

In this work, integrity of AF4 purified viruses was assessed through infectivity. Since AF4 does not have the solid stationary phase and applied elution conditions were optimized for viruses using virus specific buffers (see previous chapters), no strong interactions

were expected to occur and virus integrity was predicted to be maintained. Indeed all six viruses maintained their infectivity throughout the AF4 fractionation. Importantly, high specific infectivities were observed for all viruses, independently of the purity level of the input sample (I, II, III). Thus, high purity viruses can be obtained from crude cell lysates or culture supernatants. Furthermore, analyses of the AF4 purified virus samples by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the presence of all the structural protein subunits in correct rations (I, II, III).

4.2.2. Purity of AF4 fractionated viruses

The purity of AF4 purified viruses was mainly assessed through the specific infectivities of collected virus preparates. The specific infectivity values were formed as a ratio of plaque forming units (PFU) to milligrams of protein or to an absorbance value (A_{260} or A_{280}) of the preparates. Due to size based AF4 fractioning and flow of components smaller than 100 kDa through the membrane the total amount of protein or the absorbance value of fractions was lower in AF4 fractionated virus preparates than in the input sample. The reduction in total protein amount was significant especially with when lower purity level inputs were fractionated.

When the pre-purified 1×virus was subjected to AF4 purification, the improvement of the specific infectivity was insignificant for most of the viruses. Further purification from 1×virus input was observed only for HVTV1, revealing ~5-fold higher specific infectivity after AF4 purification (II). When PEG-precipitated viruses were purified with AF4, improvement in specific infectivities ranged between 3 to 8-fold. The highest improvement in purity, 10 to 140-fold, was observed for the least purified starting materials, lysates and culture supernatants (Table 5). Purification of HCIV1 utilizing agar stock as starting material resulted in ~8-fold improvement in purity level (II; Table 5). The important observation here was that regardless of the starting material used for purification, the specific infectivity in AF4 purified preparates were similar. Thus crude virus samples are highly applicable for AF4 purification. When AF4 purification of PRD1 (I), ϕ6 (III) and HRPV1 (II) was conducted with in-line MALS and DLS detectors, particles in the peak fractions were observed to be homogenous in size, thus no aggregations co-eluted with AF4 purified virus preparates (I, II, III).

SDS-PAGE analysis of protein contents in purified virus fractions verified the numeric data. Vast majority of protein impurities present in input samples were observed in fractions eluting in the beginning of the elution gradient. Protein patterns typical for each virus were dominant in virus fractions where only few protein impurities were present. Purity level of HRPV1 was also visually monitored through electron microscope (II). Virus shape and size correlated with published data (Pietilä et al. 2009) but filamentous material was observed to co-purify with viruses

Table 5. Relative purity of AF4 fractionated virus samples compared to the input material

	Improvement in purity*		
	lysate/culture supernatant	PEG-virus	1×virus
PRD1	10	7	0
ϕ6	140	5	0
HVTV1	24	3	0
His1	60	8	5
HRPV1	60	3	0
HCIV1	8**	not tested	not tested

*Purity measured as specific infectivity

**HCIV1 was purified from agar stock.

4.2.3 Yields of infective viruses

Precipitation and ultracentrifugation based purification of viruses with well-established methods used in this project, led into yields varying between ~3 –~27% depending on the virus (Table 6). The total yield of infective viruses from AF4 fractionated 1×viruses ranged between 70–100%, and with PEG-viruses 40–100 % (Table 6). In the case of lysate or culture supernatants, the yields of infective viruses were between ~60 and ~100 %. Thus in all cases the obtained yield was higher than with the traditional method (Table 6). The yield of HCIV1 purified from agar stock was ~35%. That was lower than the typical ~44% yield obtained from lysate by utilizing precipitation-ultracentrifugation (Table 6). Agar stock may need pre purification to reduce viscosity before AF4 fractionation.

The microgram amount of virus-specific proteins obtained from the AF4 purification were determined for PRD1 and ϕ6. Virus fractions of halophilic viruses contained such high salinity that Bradford protein concentration measurement was not applicable. In the case of PRD1, ~12 µg of virus protein was obtained from 0.5 ml of lysate, whereas with ϕ6 ~40 µg of virus protein was collected from 1 ml of lysate. In contrast, ~1 mg of PRD1 and ~7 mg of ϕ6 virus protein was obtained from 1 l of lysate with precipitation and ultracentrifugation based purification method (Table 7, Fig.7, I, III). Even though protein amounts obtained from a single ~1h AF4 purification were low, the yield can be increased by repeated fractionations and by applying larger sample volumes. If 5 ml injections would be applied on PRD1 and ϕ6, equal milligram amounts would be obtained with 9 and 35 AF4 runs corresponding to 45 and 175 ml of lysate, respectively (Table 7). According to the working range determined for PRD1 (see chapter 4.1.3.3.), the low protein concentrations in PRD1 and ϕ6 lysates suggests, that 5 ml injections

could be conducted (I, III). HRPV1 was purified from 5 ml of culture supernatant (II). AF4 can also be run in semi-preparative mode utilizing repeated, automatic injections, and semi-preparative channel allowing fractionation of larger lysate volumes and upscaling the method. This will be tested in near future.

Table 6. Precentual yields of infective viruses from the purification with traditional precipitation-ultracentrifugation and AF4.

Virus	Precipitation-ultracentrifugation	AF4*			Reference
		lysate/culture supernatant	PEG precipitated virus	1×virus	
PRD1	~18	~60	~40	~70	(I)
ϕ6	~27	~100	~100	~70	(III)
HVTV1	~3	~60	~65	~80	(II)
His1	~7	~85	~90	~100	(II)
HRPV1	~11	~70	~100	~70	(II)
HCIV1	~44**	~35	N.D.**	N.D.**	(II)

*Yields calculated from combined fractions of the virus peak area.

**N.D = not determined

***Demina et al., 2016

Table 7. Comparison of predicted virus yields from different volumes of starting material applying precipitation-ultracentrifugation method and AF4-purification.

Purified Sample	PRD1 lysate		ϕ6 lysate	
Method applied	PEG-UC*	AF4	PEG-UC*	AF4
Yield of viruses (mg)	~1	~1	~7	~7
Volume of the starting material (ml)	1000	45	1000	175

*PEG-UC = precipitation-ultracentrifugation method

4.3 Life cycle analysis

Before any detailed research on virus structure or function can be done, they need to be produced and purified. Optimization of production conditions of new viruses takes time and effort. The infection cycle is conventionally studied by one step growth experiment where the turbidity of the infected culture is monitored (Ellis and Delbrück 1939). If viruses are released via lysis, the turbidity of the culture collapses dramatically, whereas in case of non-lytic infection cycle, virus release goes unnoted since cells remain intact and turbidity does not decrease. One step growth experiment is often combined with the plaque assay to see when the amount of free phages is the highest.

However, this approach is requires lots of sampling. This is especially time consuming when working with slowly growing hosts, such as halophilic archaea, which often need ~two days for a single one-step growth assay and 3–6 days for the plaque assay.

We monitored samples from the one step growth assays of a lytic bacteriophage PRD1 and non-lytic His1 (I, II) by AF4 to see if it could be utilized to optimize virus growth conditions. Samples from the infected cultures were analysed from different time points to observe virus release to the growth media (I, II). In both cases the uninfected host culture were also analysed to distinguish any changes derived from the virus infection. The release of new virions appeared as a new peak appeared in the fractograms. This was verified by the plaque assay. Since PRD1 is a lytic virus, the release of cell contents was seen in the AF4 fractogram concurrently to virus appearance as wide and intense peak in the beginning of the elution gradient. As the infection proceeded the peak for contaminants was reduced. When the infection cycle of His1 was analyzed, the virus release was observed only 24 h after infection. Since His1 is a non-lytic virus, release of cell contents was not seen in the fractogram as clearly as it was with PRD1.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this work, a novel virus purification method based on AF4 was developed and compared to traditional method based on precipitation and ultracentrifugation. To our knowledge this is the first attempt to utilize AF4 in virus purification, even though AF4 has been applied in virus analysis before. Six viruses with different morphologies, biochemical and biophysical properties were applied for AF4 purification. Four of the viruses required elevated salinity to maintain their infectivity. All viruses remained intact and infective throughout the AF4 fractionation and yields of infective viruses were high.

The purity of AF4 fractionated viruses, measured as specific infectivity was comparable to that obtained with the traditional method. Importantly, the obtained specific infectivity values were similar irrespectively of the starting material. Crude samples such as lysates and infected culture supernatants can be applied as starting materials in AF4 purification, streamlining the multi-step purification process (Fig. 7). Applying dilute, low purity level starting material is also encouraged by the fact that no dilution occurred with these samples. Since lysates and culture supernatants are dilute, higher injection volumes would be applicable. Utilizing larger volumes, automatic reinjections and thicker spacer or semi-preparative sample channel, would allow faster purification of milligram amounts of viruses. High virus yields from AF4 purification would significantly reduce the amounts of required starting material, thus leading to lower costs of virus production (Table 7). For example, in case of the enveloped bacteriophage ϕ6 purification, 6-times less starting material would be needed to obtain as much viruses as was obtained with traditional method.

AF4 was also proven to be suitable for life cycle analysis of lytic and non-lytic viruses. The release of progeny viruses to the growth media was observed by applying rapid ~45 min elution program. Thus, AF4 could be applied in the optimization of virus growth conditions. This would omit the need for massive plaque assays on the samples collected from different conditions and phases of infection.

Now that the step towards semi-preparative scale virus purification with AF4 has been taken and the starting point is established, the method can be further explored and developed for other viruses. Since the developed method is highly tailorable, it can also be applied in virus analytics.

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