Purification Of *Minute Virus Of Mice* Using High Performance Tangential Flow Filtration

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Abstract

Membrane technology has proven to be a mainstay separation technology over the past two decades. Some major advantages of membrane technology are application without the addition of chemicals and a comparatively low energy use. With its current applications, membrane technology has been widely used in biotechnology processes. Cell harvesting and virus purification/removal are important processes in many downstream purifications of biopharmaceutical products. For this project, ultrafiltration (UF) for virus purification from cell culture broth was used. Recently, it has been demonstrated that UF is a powerful tool for purification of other viruses such as *Aedes aegypti* and virus-like particles. More precisely, high-performance tangential flow filtration (HPTFF) will be used, which was first introduced by Robert van Reis in 1997. To date HPTFF has been used in other projects, as for protein concentration, purification, and buffer exchange as a single unit operation. The virus used in this study was the parvovirus *Minute Virus of Mice* (MVM); characterized by an average diameter of 22-26 nm and icosahedral symmetry. Experiments were conducted with 300, 100 and 50 kDa Sartorius membranes. Results obtained indicate that using the 50 or 100 kDa membrane, viral particles get excluded, whereas the 300 kDa membrane allows the passage of the virus particles into the permeate. In HPTFF mode the permeate flux decline of the 300 kDa ultrafiltration membrane is much greater than for the other membranes used. One possible explanation for this decay could have to do with the virus particles' access to the membrane pores (gradual pore narrowing). Additionally the permeate flux and level of protein rejection as well, are strongly affected by the cell culture medium.

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1. Introduction

Membrane technology has proven to be a mainstay separation technology over the past two decades. Some major advantages of membrane technology are application without the addition of chemicals and a comparatively low energy use. Cell harvesting and virus purification/removal are important processes in many downstream purifications of biopharmaceutical products [1]. In order to avoid membrane fouling ultrafiltration with tangential flow is emplyed because high density cell cultures and the corresponding increase in the level of cell debris cause fouling of membrane systems. Thus membrane fouling by cell debris and or cell-derived proteins presents a serious problem. For this project, ultrafiltration (UF) for virus purification from cell culture broth was used. Recently it has been demonstrated that UF is a powerful tool for purification of other viruses such as *Aedes aegypti* and virus-like particles [4, 5, 6]. More precisely, high-performance tangential flow filtration (HPTFF) will be used, which was first introduced by Robert van Reis in 1997 [2, 3]. The virus used in this study was the parvovirus *Minute Virus of Mice* (MVM); characterized by an average diameter of 22-26 nm and icosahedral symmetry. In general, members of *parvoviridae* are among the smallest known DNA viruses. They replicate in the nucleus of actively dividing cells. The genome of MVM is linear, single stranded, and approximately 5kb long [7, 9]. Environmental extremes, like pH or temperature do not critically damage the virus [8]. MVM has a broad in vitro host range such as NBK324 cells, A9 cells, and T-cell lymphomas producing cytopathic effects. As part of this project, A9 mouse cells were infected with MVM.
The experiments were conducted with 50, 100 and 300 kDa Sartorius membranes. The analysis methods included flux measurement, real-time PCR and protein concentration determination (data not shown).

2. Materials and Methods

Cell culture and virus production

A9 mouse Fibroblast cells (ATCC® No. CCL-1.4, Manassas, VA) were grown at 37°C and 10% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM High Glucose, with 4.5g/L Glucose, Fisher Scientific-Hyclone, Cat. No. SH3002201, Pittsburgh, PA) in plastic cell culture T-75 flasks (TPP®, Product No. 90076, Trasadingen, Switzerland). The medium was supplemented with 1% penicillin (Invitrogen, 50 units/ml, Carlsbad, CA) and streptomycin (Invitrogen, 50 µg/ml) and 10% heat inactivated fetal bovine serum (Atlas Biologicals, Catalog No. F-0500-A, Fort Collins, CO). After formation of an adherent cell monolayer, sub-cultivation was carried out by removing old medium and adding fresh Trypsin- EDTA solution (Gibco-Invitrogen, 0.25% Trypsin-EDTA, Product No. 25200). For initial infection of A9 cells with *Minute Virus of Mice* (MVM), approximately 200 µl MVM stock solution (ATCC® No. VR-1346) was added to a confluent monolayer of cells in a T-75 flask. The infected cells were incubated for 6-8 days under the same conditions as uninfected cells. After incubation the cells (Corning Incorporate, Corning tubes, 50ml, Corning, NY) cells were lysed using three freeze/thaw cycles with freezing at -80°C and thawing in a 37°C water bath. The cell lysate was then centrifuged using a Beckman GS-6R centrifuge (Beckman, Fullerton, CA) at 1500 rpm at 4 °C for 15 minutes to remove large cell debris. The supernatant, containing the virus, was filtered using 0.22 µm bottle top sterilization filters (Nalgene Company, Rochester NY) and stored at -80 °C. Before using the virus solution for experiments, a 1:100 dilution with pure media is carried out, meaning that the amount of FBS in samples collected is low.

Membrane Filtration

Filtration experiments were conducted using flat sheet Sartocon® Slice 200 cassettes (Sartorius AG, Göttingen, Germany). Three ultrafiltration membranes, Sartorius polyethersufone 308 1465002E SG, 308 1466802E SG and 308 1467902E SG, with molecular weight cut offs (MWCO) of 50, 100 and 300 kD were tested in this study. All experiments were run at a feed flow rate of 150 mL/min controlled by a peristaltic pump. Figure 1 shows the experimental set up for high performance tangential flow filtration (HPTFF) whereas the experimental set up for tangential flow filtration (TFF) is not shown. Then 500 mL of clarified medium containing virus was added to a feed reservoir. Additionlly, throughout the entire experiment 1ml samples of the feed, retentate and permeate were collected at fixed mass points of intervals of 25g of the permeate, for analysis of virus titer and protein concentration. All samples were analyzed in triplicate and average results reported.

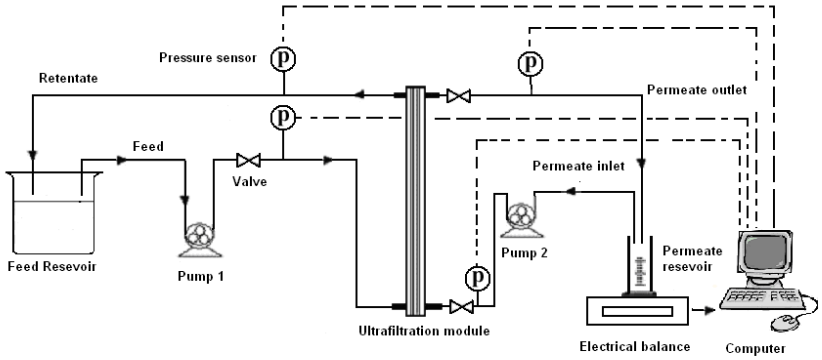

Figure 1: High performance tangential flow filtration system 50kDa, 100kDa and 300kDa PES membranes were used. Experiments were run at a flow rate of 150 ml/min

Protein assay

Protein concentration was measured using a bicinchoninic acid assay (BCA, Protein Assay Kit, Pierce, Rockford, IL) following the manufacturer's instructions. As described by the manufacturer, the protein concentration is determined and reported with reference to a standard albumin solution provided by the manufacturer. All samples were analyzed in triplicate and average values reported.

Quantitative Polymerase Chain Reaction (QPCR, rt-PCR)

The quantitative QPCR assay is a rapid, sensitive and efficient way to compare samples. The QPCR assay will detect both viral genomic and naked DNA. In order to prevent detection of naked DNA, samples are DNAse (RQ1 RNA- free DNAse, Category No. M6101, Promega, Madison, WI) treated for 45minutes. Reverse and forward primers were designed for quantification of *Minute Virus of Mice* (MVM). Primers for MVM DNA amplification were as follows:forward,5'-GAC GCA CAG AAA GAG AGT AAC CAA-3' and reverse, 5'-CCA ACC ATC TGC TCC AGT AAA CAT-3'. Further melting curve analysis was conducted in order to receive information about the length of the fragment and of the specificity of the primers. Amplification and real-time detection of PCR products were performed on the DNA samples using the iCycler system (Bio-Rad Laboratories, iQTM 5 iCycler, Multicolor Real time PCR Detection System) with

SYBR Green Mastermix (Bio-Rad Laboratories iQ™ SYBR® Green Supermix). At the end of the extension step of every cycle, the fluorescence was measured. Cycling conditions consisted of an initial step at 95 °C for 10 minutes, which is vital for breaking up viral capsids and it also activates the polymerase enzyme. This step was followed by 40 cycles with the following thermal profile: 95°C and 15s, 57°C and 10s, and 72°C and 45s, and 72°C and 10s for real time detection.

3. Results

Virus titer analysis

The variation of virus titer in retentate and permeate over permeate volume collected for HPTFF and TFF mode is displayed in figures 2 and 3. Permeate virus titers in HPTFF and TFF are only shown when they were detectable.

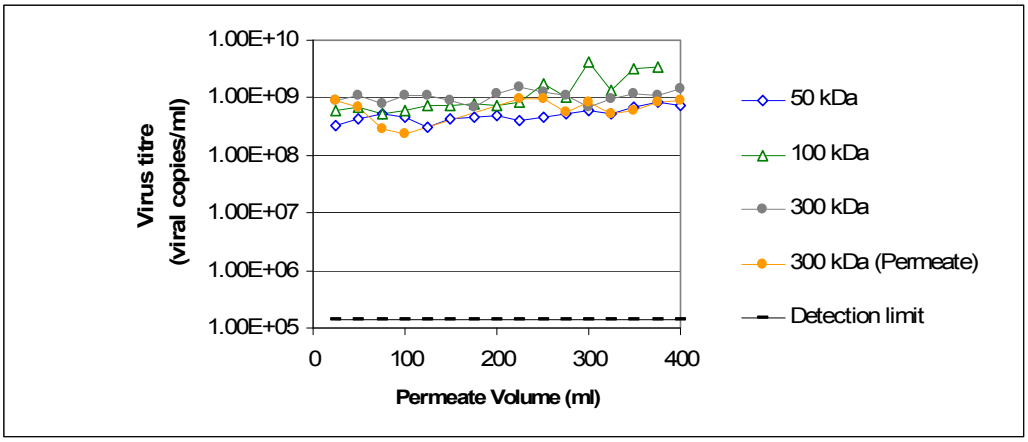

Figure 2: Variation of the virus titer in the retentate and permeate with cumulative permeate volume during HPTFF. Samples were diluted 1000 fold for rt - PCR

For the 50 and 100 kDa membranes, no virus particles were detected in permeate samples, whether for TFF nor for HPTFF mode; whereas the 300 kDa membrane showed no retention of virus particles. To be precise, it was possible to detect virus particles in retentate and permeate samples of the 300 kDa membrane in both modes. Further, complete passage is shown by the fact, that the same viral titers were measured, using rt-PCR, in both retentate and permeate samples. Consequently, exclusion of virus particles is only feasible with 50 kDa and 100 kDa membrane cassettes. Pore size of 300 kDa is nominal, which means that the greatest percentages of pores are around 300 kDa. Potentially, virus particles “squeeze” through membrane pores, meaning the virus takes advantage of the pore size distribution. The determined detection limit, using Sybr Green I dye for the detection of M*inute Virus of Mice*, was determined to be 14 viral copies/µl. Due to an extra 10 fold dilution, which has to be added because of DNase treatment of samples, the final detection limit was set to $1.4*10^5$ viral copies/ml. We showed that with a 95% confidence less than $1.4*10^5$ viral copies/ml are present in permeate samples (data not shown). Furthermore, the retention percentage is less than 0.01%, as determined by dividing the limit of detection by the starting viral titer (approximately 10^9).

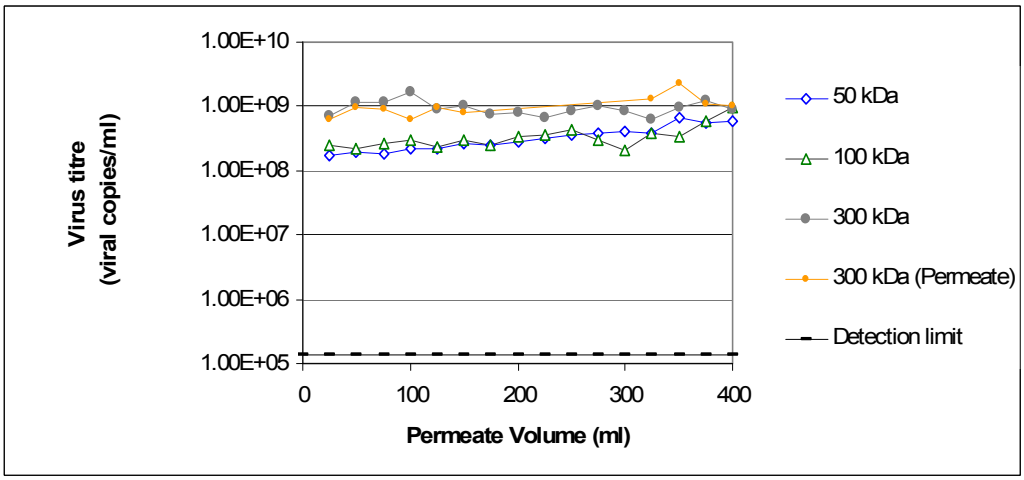

Figure 3: Variation of the virus titer in the retentate and permeate with cumulative permeate volume during TFF. Samples were diluted 1000 fold for rt - PCR

Tangential Filtration

The variation of permeate flux with permeate volume in HPTFF mode for 50, 100, 300 kDa membranes is presented in figure 4. The graph demonstrates, that all flux regimes are relatively constant during the experiments, except for drop in 300 kDa curve. In addition, the 300 kDa curve shows a decline in flux throughout the entire experiment. One possible explanation for the decay could have to do with the virus particles' access to the membrane pores. In the case of the 50 and 100 kDa membranes, the virus particles have little to no access to the pores (particles are too large) and therefore could not non-specifically bind to the internal pore structure of the membrane. Gradual increases in virus particle binding to the inside of the pores (and therefore gradual pore narrowing) could account for the observed flux decrease. The regular interval drops observed in all flux curves correspond to points where samples were taken from the permeate outlet.

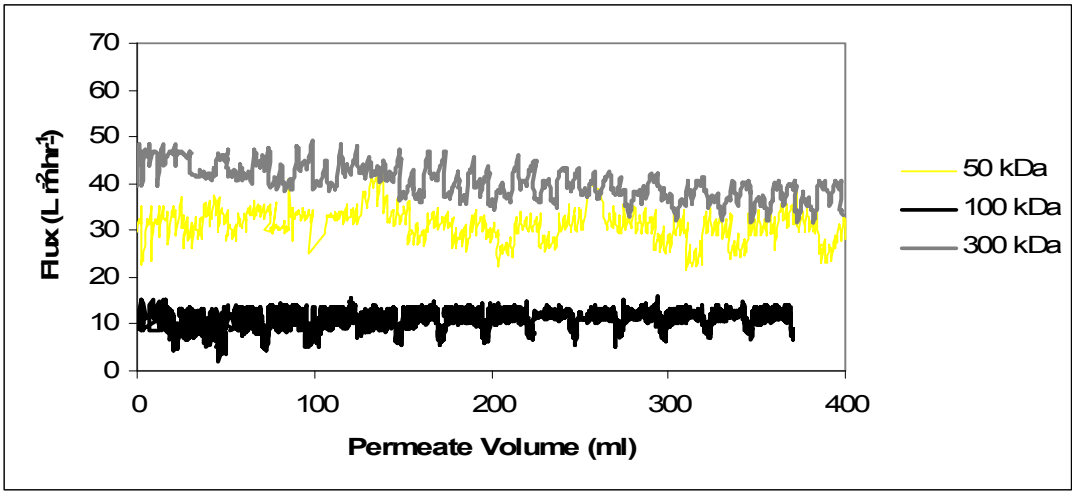

Figure 4: Variation of the permeate flux with cumulative permeate volume in HPTFF mode. Virus in DMEM medium was pumped through PES 50, 100 and 300 kDa membranes at a flow rate of 150 ml/min (1:100 dilution of initial virus solution with pure media was carried out before each experiment)

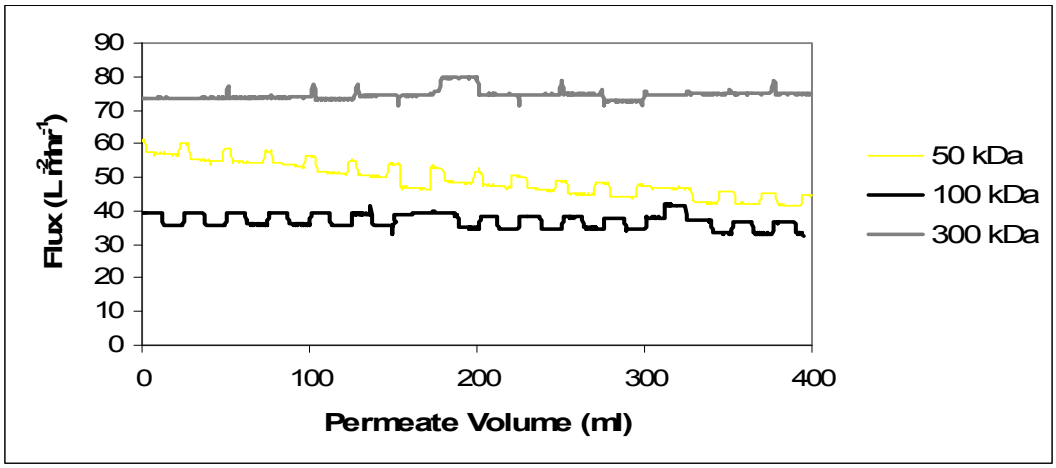

Figure 5: Variation of the permeate flux with cumulative permeate volume in TFF mode. Virus in DMEM medium was pumped through PES 50, 100 and 300 kDa membranes at a flow rate of 150 ml/min (1:100 dilution of initial virus solution with pure media was carried out before each experiment)

Figure 5 shows as expected, the highest permeate flux with the 300 kDa membrane cassette. Comparing the permeate flux of 50 and 100 kDa membranes, it is significant that those two fluxes differ from each other. At first, this seems to be an expected result, but the fact that the 50 kDa flux is higher than the 100 kDa flux leads to the question, why do the larger pores of the 100 kDa membrane not give a higher flux compared to the smaller pores of the 50 kDa membrane. Occurrence of higher flux using the 50 kDa membrane can be explained by an irregularity in pores sizes (larger pore-size distribution). Pores are never identical or uniform due to production steps. This hypothetical assumption was discussed and agreed upon by Sartorius (email contact with Sartorius). This high flux decay for the 50 kDa membrane can be explained by a gradual blocking of the small pores of the membrane, which then results in a flux decrease. During this type of process, tangential flow filtration, a thin cake layer of retained viral particles and other particles builds up on the membrane surface. The thickness and especially particle size distribution of this layer controls the passage of most soluble components [9].

4. Conclusions

Results obtained indicate that 50, and 100 kDa membranes are able to retain the Parvovirus *Minute Virus of Mice*, whereas the 300 kDa membrane is not capable of excluding viral particles of MVM, due to the fact that the same amount of viral particles is found in the 300 kDa permeate and retentate for TFF and HPTFF mode (measured by rt-PCR). The decrease in permeate flux for the 300 kDa ultrafiltration membrane is much greater than for the 50, and 100 kDa membranes for HPTFF, indicating possible entrapment of virus particles in membrane pores. The permeate flux and level of protein rejection is strongly affected by the cell culture growth medium.Feed fluxes for all membranes were always lower than initial water fluxes due to the higher viscosity of DMEM media supplemented with FBS compared to water viscosity (data not shown). Real-time PCR was used for virus titer determination as well as for evaluation of the ultrafiltration membranes ability to exclude viral particles. By using the Sybr Green assay we developed, it was possible to detect the amount of viral particles in samples collected with a determined detection limit of 14 viral copies/µl.

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