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Effects of Filtration Conditions on Clearance of Bacteriophage

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Effects of Filtration Conditions on Clearance of Bacteriophage

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Abstract

Virus Clearance is one of the major challenges in biopharmaceutical industry, especially in the manufacturing of drugs. U.S. Food and Drug Administration (FDA) recommends using several model bacteriophages as surrogates of pathogenic mammalian virus for process validation. In this work, two bacteriophages were tested against 30 kDa, 100 kDa, 300 kDa polyethersulfone (PES) membranes to investigate the effects of pore size on virus clearance. Virus particles were spiked into the protein feed solutions containing bovine serum albumin (BSA) or lysozyme at different concentrations. Besides protein concentration, the effects of feed pH on the filtration performance and virus rejection were also investigated.

The results indicate that 100 kDa PES membrane can remove 4 logs of virus from the feed stream with and without protein while maintaining a moderate flux performance. pH does not appear to significantly affect the virus rejection. However, flux is somewhat higher when the pH of the feed is close to neutral. These results indicate that electrostatic interaction between the protein, virus particle and the membrane has a strong effect on flux and virus rejection.

Acknowledgments

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1. Introduction

1.1. Bioprocess Overview

Since the first therapeutic murine monoclonal antibody (mAb) was introduced into clinical studies in 1980s, major pharmaceutical companies started to invest on developing therapeutic mAbs and mAb fragments because of their higher Likelihood of Approval (LOA) over small molecule therapeutics and the diversification they bring to the therapeutic pipeline (Reichert et al. 2005). By end of 2016, a total of 67 mAbs have been approved by FDA and this number is continuously growing with approximately four new mAbs per year (Ecker et al. 2015). The global market of mAbs production has reached 74.5 billion USD and is projected to grow at a compound annual growth rate (CAGR) of 12.2% during the next four years (BCC Research, 2013).

The manufacturing of monoclonal antibodies can be divided into two processes (Shuler et al. 2002): 1) an upstream bioprocess using genetically modified cell lines to produce raw materials from the upstream bioreactors; 2) a downstream bioprocess using a validated mAb purification platform that consists of different purification steps to meet the regulatory requirements. Depends on the actual purification platform, the number and types of purification steps may vary (Sommerfeld, et al., 2005).

The main goal of downstream bioprocessing is to remove impurities and any potential contaminants i.e. nucleic acids, host cell proteins(HCP), product variants, endotoxins, viruses and other small molecule impurities to a certain level (Serabian et al., 1999). For example, the acceptable range of host cell protein in the mAb products is 1-100 ng/mg (nanogram HCP per gram mAb) (Wang et al., 2015).

1.2. Virus Clearance

Viral contamination can be introduced by endogenous viruses that comes from the specific cell lines being used for mAb production, and/or by adventitious viruses that comes from contaminated reagents, buffers or unappropriated product handling during the manufacturing process (Brorson et al., 2004). Considering the potential risk of viral contamination during mAb manufacturing process, virus clearance has become one of the key performance indicators (KPI) of the quality assurance steps (Wu et al., 2008).

Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER) provides several process validation regulatory guidelines for viral clearance during biopharmaceutical manufacturing. In the document entitled *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* (1996), CBER recommended that each purification protocol for mAb production must include at least two orthogonal (based on different mechanisms) robust virus removal steps. Robust virus removal/ inactivation steps are defined as the ones that have been proven to perform well under various operation conditions, such as different pH and ionic strength across different mAbs. According to the aforementioned FDA guidelines, inactivation of virus with low pH, heat inactivation and virus filtration are all considered to be robust steps. CBER also emphasizes the importance of virus clearance studies and implies using several model viruses of different types: large and small viruses, DNA and RNA viruses, chemically sensitive non-enveloped viruses and resistant lipid enveloped viruses. In another document entitled *Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A* (1999), ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) elaborates three principles and complementary approaches to control potential viral

contaminations during manufacturing process including 1) encompass selecting/testing of the cell lines and raw materials, 2) quality inspection on end products for absence of infectious viruses 3) validating virus clearance steps in the manufacturing process.

There have been tremendous efforts dedicated to developing robust methods that can inactivate and remove viruses from pharmaceutical products during the downstream bioprocessing. To evaluate different methods for virus clearance, log removal value (LRV) is used. It is considered effective if LRV of a method is above four, moderately effective if the LRV is between 1-4 and ineffective if LRV is less than one (Smith, 2014). Generally, virus inactivation by breaking down the viral genetic materials using gamma radiation and ultraviolet radiation that can result in a LRV of 2 to 6. Virus inactivation by dissolving lipid envelope of enveloped viruses using solvent/detergent can achieve a LRV of more than 6 (Shukla et al. 2006). In addition to the abovementioned virus clearance methods, many other processes in capturing and purification sections, such as protein A chromatography, ion exchange chromatography, membrane chromatography and flocculation et al. can also remove viruses even though they are not generally considered as dedicated virus clearance steps (Curtis et al. 2003, Parkkinen et al. 2006).

Virus inactivation can cause damages to the virus particles and render the virus particles inactive. However, the majority of virus particles remain in the final product instead of being removed from the process stream. In order to physically remove both intact virus particles and damaged virus particles, more robust virus clearance steps will need to be incorporated into the mAb manufacturing platform in addition to the virus inactivation steps. Virus filtration is among the most robust methods that is based on size exclusion of porous membranes and can

consistently result in a LRV of 3-6 with very low batch to batch variations (Kuriyel et al. 2000, Shukla et al. 2006, Rathore et al. 2011).

ICH guidelines (1999) also specifies the type of viruses that can be used in virus clearance studies as shown in Table 1. Given the size of small parvovirus is very close to some of the large mAb molecules, size selectivity is critical for virus filtration. Among all the well-studied viruses, parvoviruses such as minute virus of mice (MVM) are normally chosen.

Table 1. Examples of Viruses that are being used in virus clearance studies

Virus	**Genome**	**Envelope**	**Size (nm)**	**Resistance to physical/chemical treatment**
MuLV	RNA	Yes	80-110	Low
BVDV	RNA	Yes	50-70	Low
SV40	DNA	No	40-50	Very high
Polivirus sabin type I	RNA	No	25-30	Very high
Parvovirus	DNA	No	18-24	Very high

According to the definition by ICH guidelines (1999), viral clearance studies is to validate 1) the effectiveness of a designated inactivation or a removal step as well as 2) the overall virus reduction level during the entire process. A complete virus clearance study begins with preparing challenge solution by spiking certain number of virus particles into the feed, which in most cases being the mAb solution. Different fractions are then collected during the virus inactivation/removal step. In the end, virus clearance performance will be assessed with Process Analytical Techniques (PAT) such as qPCR, Plaque assay and $TCID_{50}$ (Wang et al. 2005, LaBarre et al. 2001). Because many virus filters are scalable (Huang et al., 2001), researchers are able to challenge the small scale virus filters with much higher load of virus while obtaining meaningful data that can be used to understand the large scale processes.

1.3. Virus Filtration

1.3.1. Virus Filtration Membranes

Virus Filtration (VF) membranes are normally used to separate proteins of 1-10 nm from viruses of 18-110 nm (Van et al. 2007). Virus filtration should fall into the category of ultrafiltration (UF) with membrane pore size of μm to nm in diameter. Most VF membranes are single-use and are operated in normal flow (NFF or dead-end filtration) mode (Van et al. 2001). Because NFF VF membranes are economically efficient and easy to scale up, they are more favored by the biopharmaceutical manufacturers. Table 2 shows some examples of commercially available virus filtration membranes.

Table 2. Commercially available virus filtration membranes
(Shukla et al. 2006; Bakhshayeshirad, 2011)

Vendor	Brand	Target Virus	Operation Mode	Material	LRV
Millipore	Viresolve® 70	Parvovirus	TFF	Hydrophilic PVDF	>4 polivirus >7 retrovirus
	Viresolve® 180	Retrovirus	TFF	Hydrophilic PVDF	>3 polivirus >6 retrovirus
	Viresolve® NFP	Parvovirus	NFF	Hydrophilic PVDF	>4 Phi-X174 bacteriophage
	Viresolve® NFR	Retrovirus	NFF	Hydrophilic PES	>6 retrovirus
	Viresolve® Pro	Parvovirus	NFF	Hydrophilic PES	>4 parvovirus
Pall	Ultipor® DV20	Parvovirus	NFF	Hydrophilic PVDF	>3 PP-7 bacteriophage >6 PR-772 bacteriophage
	Ultipor® DV50	Retrovirus	NFF	Hydrophilic PVDF	>6 PR-772 bacteriophage
	Pegasus® LV6	Retrovirus	NFF	Hydrophilic PVDF	>6 retrovirus
	Pegasus® SV4	Retrovirus	NFF	Hydrophilic PVDF	>4 PP-7 bacteriophage
Asahi Kasei	Planova® 15N	Parvovirus	TFF/NFF	Hydrophilic CRC	>6.2 parvovirus >6.7 polivirus
	Planova® 20N	Parvovirus	TFF/NFF	Hydrophilic CRC	>4.3 parvovirus >5.4 encephalomyocardits
	Planova® 35N	Retrovirus	TFF/NFF	Hydrophilic CRC	>5.9 BVD >7.3 HIV
	Planova® BioEX	Parvovirus	TFF/NFF	Modified PVDF	>4 parvovirus
Sartorius	Virosart® CPV	Parvovirus	NFF	Hydrophilic PES	>4 PP-7 bacteriophage, >6 retrovirus

The materials that VF membranes are made of are also very important. Different materials have different membrane pore sizes and pore structures which will result in different sieving properties for the VF membranes. Also, different VF membrane materials can have different chemical and thermal stability which allows for different applications. The most commonly used VF membranes are made from polyethersulfone (PES), polyvinylidene fluoride (PVDF) and cuprammonium regenerated cellulose (CRC).

Most asymmetric membranes were operated with the side of small pore size facing the feed. However, it has been shown that VF membranes perform better when the orientation is reversed (Bakhshayeshi et al. 2008). This is because the support structure can function as a pre-filter, remove aggregated proteins thus protects the skin side from fouling (Brown et al. 2010).

Membrane fouling is one of the biggest challenges in almost any types of filtration. In virus filtration process, the major foulants are usually host-cell proteins, DNAs and protein aggregates. Buffer condition plays an important role in filtration performance because the conformation and properties of a mAb is pH and ionic strength dependent. Buffer also mediates the electrostatic interactions among the mAb molecules, virus particles and virus filter (Ireland et al, 2004). Previous studies also have indicated that high protein concentrations can result in high possibility for the products to form aggregates and clog the membrane pores (Parkkinen et al., 2005). Therefore, a number of lab scale filtration conditions will have to be tested in order to find the optimal operation conditions that are suitable for the large scale processing.

In this thesis research, polyethersulfone (PES) membranes with different molecular weight cut-off (MWCO) were challenged with two different protein solutions (Bovine Serum Albumin, BSA and Lysozyme) at various concentrations. Flux and virus titer from each fraction

collected were measured to evaluate the effects of pH, membrane pore size and challenging protein solutions on virus filtration performance.

1.3.2. Virus

Being the smallest microorganisms on the earth, a virus consists of a genetic material (DNA or RNA, can be either single stranded or double stranded) encapsulated by a protein capsid, and sometimes surrounded by an additional spikey lipid coat called an envelope. Virus can only replicate in the living cells and can only attack certain types of host organisms (van et al., 2000). For example, human immunodeficiency virus (HIV) only infects mammalian cells while bacteriophage only infects bacteria only. As shown in Table 1, FDA recommends different size viruses as model viruses for clearance studies. Because mAbs are typically produced in Chinese Hamster Ovary (CHO) cells (Trill et al. 1995), which are the targets of certain types of mammalian viruses including minute virus of mice (MVM) and murine leukemia virus (MuLV) (Mascarenhas et al. 2016, Strauss et al. 2009). Biopharmaceutical manufacturers tend to use MuLV (a model retrovirus, enveloped, 80-110 nm in diameter) representing large viruses and MVM (a parvovirus, non-enveloped, Φ=18-24 nm) representing smallest viruses for clearance studies (Han et al. 2005, Gruvegard et al., 2009).

While using closely related mammalian virus for virus clearance studies is intuitively appropriate, these viruses typically require a long period of time for cell culture and virus titer analysis, and require higher biosafety levels. Bacteriophages, on the other hand, have some advantages over mammalian viruses for the virus clearance studies. For example, bacteriophage requires a lower biosafety level and is generally considered less of a biological threat to the laboratory personnel.

In our studies, a non-enveloped 25-27 nm single-stranded DNA bacteriophage Phi-X174 (Chrysikopoulos et al., 2011) and an 82 nm double-stranded DNA bacteriophage PR-772 (Lute et al., 2004) were used as the surrogates for the virus filtration experiments. The icosahedral bacteriophage Phi-X174 has a spherical shape (Chrysikopoulos et al., 2011, Sun et al., 2014). The burst of a single host cell is approximately 16 min after infection yielding 150 to 200 infectious virions (Sinsheimer et al., 1968). PR-772 is a double strand DNA bacteriophage with a lipid membrane beneath its icosahedral shell (Lute et al., 2004), Parenteral Drug Association has developed a standard large-virus filter based on the retention of bacteriophage PR-772 (Aranha-Creado et al., 1997).

2. Material and Methods

2.1. Materials

Bovine Serum Albumin (> 99%) were purchased from Sigma Aldrich (St Louis, MO). Lysozyme (egg white, > 95%) was purchased from OmniPur (Gibbstown, NJ). Sodium chloride (ACS grade) was purchased from Macron Fine Chemicals (Center Valley, PA). 2XYT medium broth were purchased from AMRESCO. Host cells and viruses were purchased from ATCC (Manassas, Virginia). The two bacterial hosts were Escherichia coli (Migula) Castellani and Chalmers (ATCC #13706) and Escherichia coli strain K12 J53-1 (ATCC #BAA-769). Upon arrival, viruses were further propagated via their corresponding host cells: Escherichia coli bacteriophage Phi-X174 (ATCC 13706-B1) and Escherichia coli bacteriophage PR-772 (ATCC BAA-769-B1). Polyethersulfone (PES) membranes of three pore sizes (30 kDa, 100 kDa, 300 kDa) were obtained from EMD Millipore Corporation.

2.2. Virus Stock Preparation

Host cells were grown in a flask containing 2XYT medium broth under aerobic condition with a preset shaking speed of 200 rpm at 37^{o}C following the ATCC cell culture protocols. When the bacterium culture reached an absorbance value of 0.600 at the wavelength of 600 nm, the cell culture medium was then spiked with 1% (v/v) of PFU=10^{8} virus stock. The virus culture was then under constant shaking for 24 hours. Host cells were then lysed by three freeze–thaw cycles. Lysate was centrifuged at 9,500 g / 8,874 rpm under room temperature using Beckman Coulter Avant J-25 High Performance Centrifuge and JA-25.50 fixed angle rotor for 30 minutes to isolate free virus particles from host cells lysate. The supernatant containing high titer of viruses was collected and further purified by filtering through a 0.2 μm Thermo Fisher Disposable Vacuum Filter System. When not in use, the purified virus stocks were either kept at 4^{o}C for short term storage or frozen at -80^{o}C for long-term storage.

2.3. Flux Measurement

The filtration experimental design was derived based on the method described by Wickramasinghe et al. (2010). Filtration experiments were performed by three PES membranes with MWCO of 30 kDa, 100 kDa and 300 kDa respectively. These are asymmetric membranes with a barrier layer and a porous support structure. All membranes were initially filtered with 20 mL DI water and 20 mL broth before filtration of any virus feed solutions. All filtration experiments were performed in a laminar flow cabinet with an 8050 Amicon stirred cell. The 8050 Amicon stirred cell has a reported filter diameter of 44.5 mm and was set up in the normal flow filtration (NFF) mode at a constant pressure of 2 psi.

A DI water flush and a broth media flush were performed for each membrane before the filtration of the feed solutions. A total volume of 100 mL of feed solution was used for each

filtration experiment. A total of 10 fractions about 10 mL each were taken from the permeate. Fraction weight was calculated by subtracting the tube weight from the measured weight using mass balance. Fraction density was calculated by measuring the weight difference before and after taken 1 mL fraction out of fraction tube. Fraction volume was calculated by dividing fraction weight by fraction density. Flux data were then plotted based on the volume from each fraction and filtration time.

2.4. Titer Analysis

Each fraction was analyzed using Clokie and Kropinski's Bacteriophage plaque counting method (Kropinski et al., 2008) to determine the virus titer. Upper agar media solution (31g/L 2XYT medium broth and 7g/L agar) was prepared and incubated in a warm water bath at 50^{o}C for 15 min. Base agar media solution (31g/L 2XYT medium broth and 15g/L agar) was poured into base cell culture plates and sit in the laminar flow cabinet for 20 min to allow solidification. Host cells were grown till absorbance reached 0.600 at the wavelength of 600 nm. A total of 190 μL aliquots of the host cells along with their cell culture media were spiked with 10 μL of either an original fraction or a sequential 10x diluted fraction. For each filtration, sample plates were used to determine appropriate dilution fold. The 200 μL solution containing logarithmic growth phase host cells and virus filtration fraction (seed solution) were briefly vortexed and allowed to sit at 37^{o}C for 15 min for sufficient pre-infection. A total of 200 μL seed solution was then quickly mixed with pre-warmed 3 mL of upper agar media solution and was poured onto the top of a base plate containing 15 mL of pre-warmed and solidified base agar and allowed solidification in the laminar flow cabinet. When solidified, the double agar plates were transferred into a 37^{o}C incubator overnight to allow plaque forming. Plaque counting was performed the next day and

were done on each plate. Each fraction was repeated three times. Plaque Forming Units (PFU) and Log Removal of Virus (LRV) for each fraction are calculated using the formulas below:

$$PFU = \frac{Average\ Plaque\ Counts\ for\ each\ fraction}{Dilution\ Factor\ \times\ Volumne\ of\ Sample\ to\ be\ tested}$$

$$LRV = log_{10}\frac{Feed\ PFU}{Fration\ PFU}$$

3. Results and Analysis

A series of filtration experiments with different feed solutions were performed to explore their effects on flux and virus rejection with different pore size membranes.

3.1. Pore Size Effect

PES membranes of three different pore sizes (PES 30 kDa, PES 100 kDa and PES 300 kDa) were tested to investigate the effect of pore size on flux performance and bacteriophage clearance during the filtration challenge. The 8050 Amicon stirred cell was used in the dead-end mode for the filtration experiments. Harvested high titer bacteriophage stock solutions were used as the feed solution in this study. The feed solution was loaded into the Amicon stirred cell immediately after 20 min DI water flush and broth media flush respectively. A total of 10 fractions were collected from the permeate for flux measurement and titer analysis.

Figures 1 and 2 show the flux and log reduction value (LRV) of Phi-X174 respectively for the 10 fractions collected. As is shown in Figure 1, PES 300 kDa membrane exhibits highest initial flux at 48.5 LMH whereas both 100 kDa and 30 kDa membranes show a much reduced flux at around 12 LMH only. However, significant flux decay was observed for 300 kDa membrane during the subsequent measurements reaching 14 LMH during the last fraction. On the other hand, the other two narrower pore size membranes did not demonstrate much flux decay during the entire filtration period. The high flux decay for the 300 kDa membrane could

be attributed to severer membrane fouling resulting from the plugging of the membrane pores. As can be seen from Figure 2, the LRV for the 300 kDa membrane is below 1 during the first 80 mL of the filtration. Viruses can easily go through the membrane plugging the membrane pores. As more pores are being plugged, the rejection of the virus increases with LRV reaching 1.3 at the end of the filtration. For the 100 kDa and 30 kDa membranes, membrane pores are much smaller than the Phi-X174 virus particle leading to the high rejection of the virus at the start of the filtration experiments. The LRV for both membranes are at around 3.5 indicating that over 99% of the virus particles are rejected. Moreover, starting from the third fraction, the LRV increases steadily to 4 and above indicating that plugging of the pores by viruses and cell debris leading to an enhanced rejection of the virus particles and slightly reduced fluxes. The cake layer formation on the membrane surface may also contributed to the improved rejection and somewhat reduced fluxes. After 8 fractions, the LRV further increases reaching 5 during the next several fractions. The flux decay for the 100 kDa and 30 kDa membranes is less than 30% during the entire filtration period indicating that cake layer formation on membrane surface and pore plugging do not affect the flux as much as the rejection of the virus particles.

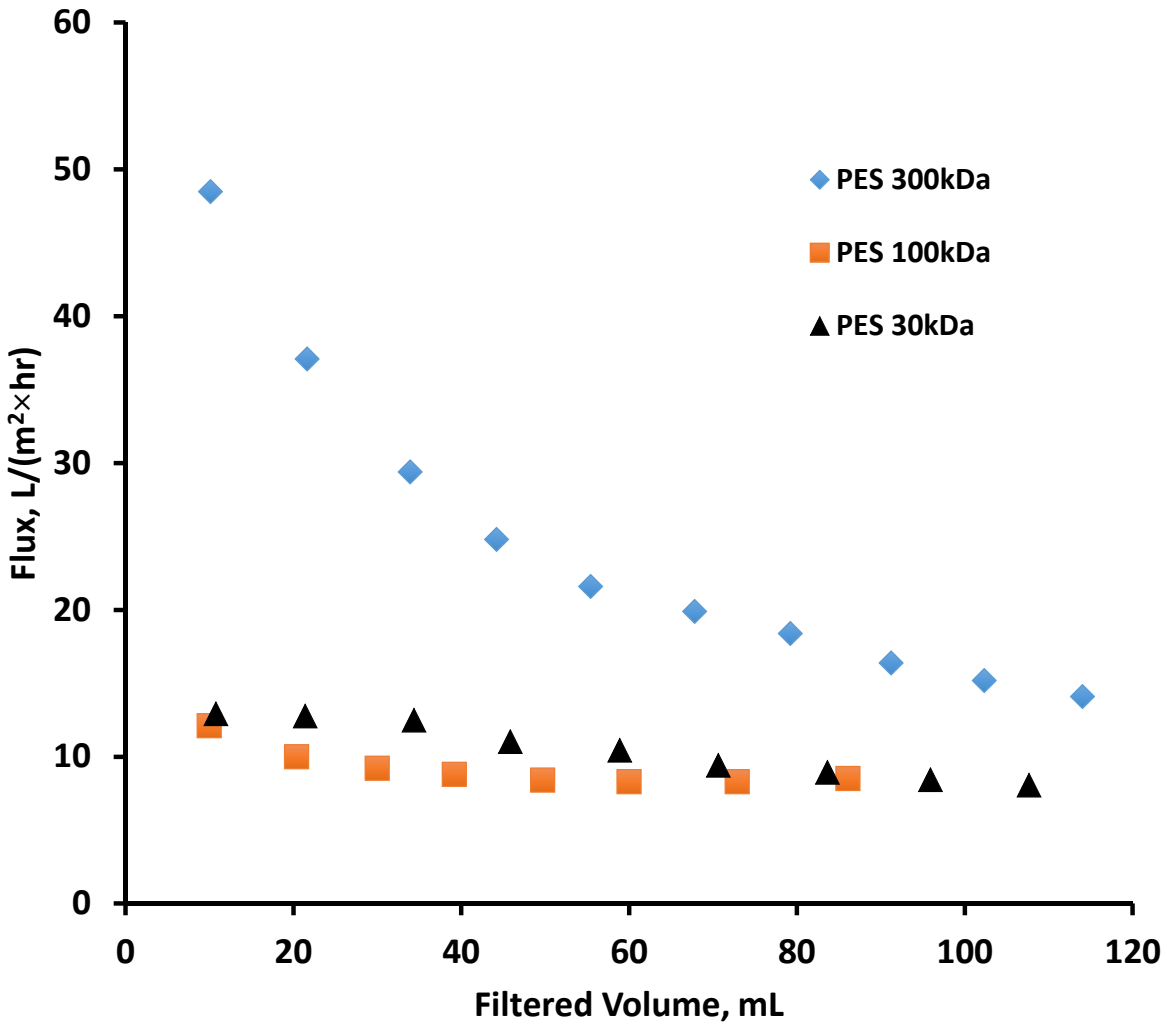

Figure 1. Flux measurement during Phi-X174 virus filtration with PES 30, 100 and 300 kDa membranes.

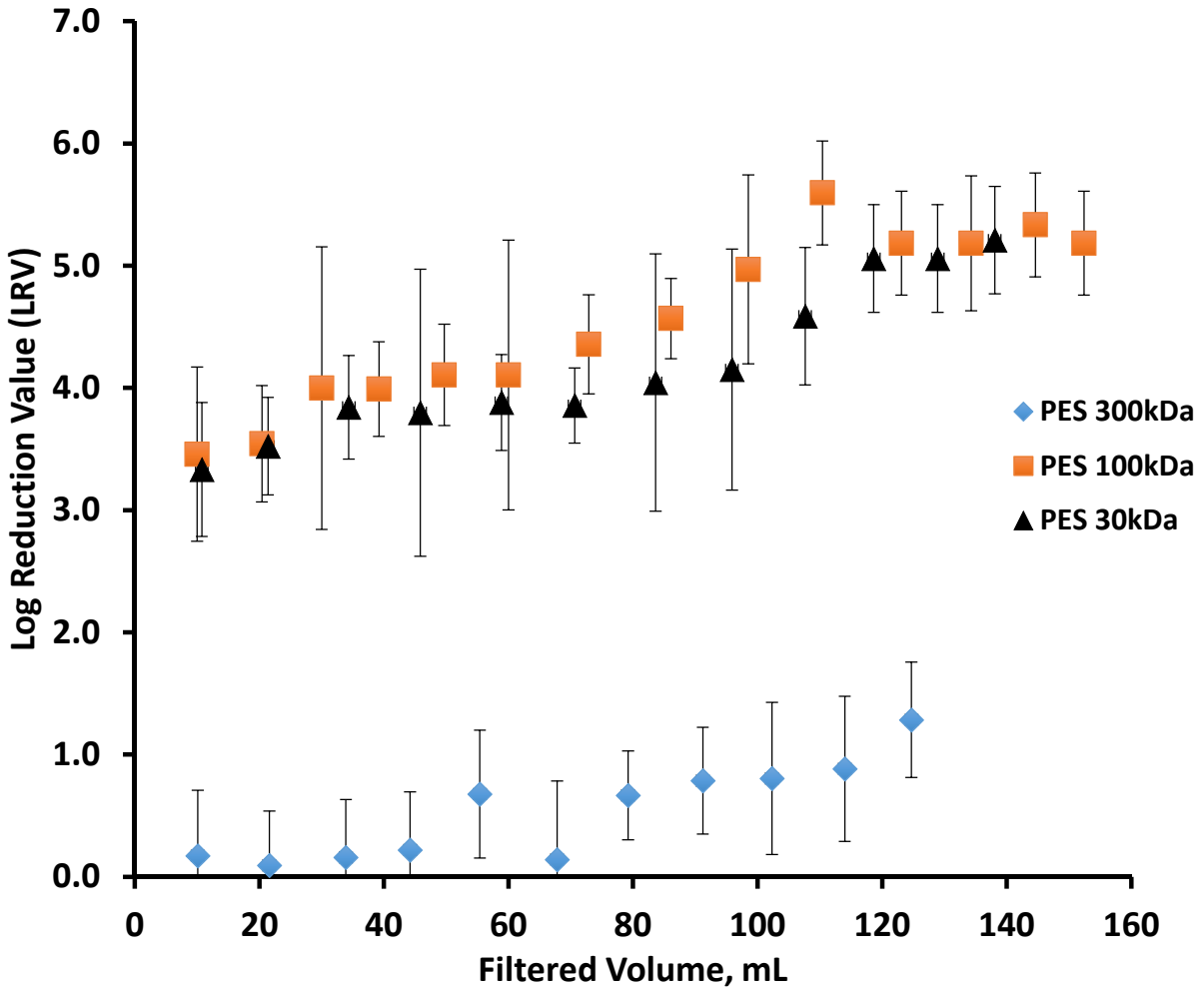

Figure 2. Titer analysis for Phi-X174 filtration for each fraction collected with PES 30, 100 and 300 kDa membranes.

3.2. Protein Spiking Effect

A total of 4 feed solutions were prepared by dissolving protein powders in harvested high titer virus stock solutions to reach final concentrations of 0.5% [w/v] BSA and 1% [w/v] BSA, 0.5% [w/v] lysozyme and 1% [w/v] lysozyme respectively. After dissolving BSA and lysozyme powder in the virus stocks, the solutions were stirred for 30 minutes and filtered through a 0.2 µm Thermo Disposable Vacuum Filter System. The pH of the feed solutions was measured to be 6.5. The feed solution was loaded into the Amicon stirred cell immediately after a DI water flush

and a broth media flush. Nearly 10 fractions were collected from the permeate for flux calculation and titer analysis. The number of fractions collected depends on the flux towards the end of filtration.

With the presence of proteins in the process stream, the flux decreases significantly (Figure 3, 5 7). This effect is more evident when using a smaller MWCO membrane for virus filtration (Figure 4). BSA appears to be more likely to cause membrane fouling when spiked into the feed solutions compared to lysozyme and the flux decline was more significant as shown in Figures 4 and 6. The titer analysis results indicate that the presence of proteins in the feed streams can lead to a higher LRV as plotted in Figures 5 and 7. The concentration of the spiked protein also plays an important role in the dramatic flux decay during virus filtration. In both scenarios, flux decay was much faster when the feed stream was spiked with higher concentration protein. However, titer analysis indicate that protein concentration does not have a significant impact on the LRV as seen from Figures 5 and 7. The presence of lysozyme in the feed stream showed a higher LRV and lower flux than those in the presence of BSA. Zeta potential measurement (Salinas-Rodriguez et al, 2015) show that PES membrane is negatively charged at close to neutral pH. Lysozyme and BSA have pI values of 11.35 and 4.7 respectively leading to the positively charged state for the former and negatively charged state for the latter at pH= 6.5. The positively charged lysozyme will likely form a cake layer on the negatively charged PES membrane surface. This leads to a strong initial reduction of the flux in the presence of lysozyme. In the case of feed solutions spiked with BSA protein, the electrostatic repulsion leads to a low probability of a cake layer formation. The 66 kDa BSA protein is more likely to cause pore plugging of the PES membrane compared to much smaller lysozyme. As a result, the initial fluxes are lower for feed solutions spiked with lysozyme but a smaller flux

decay over time. On the other hand, the initial fluxes are higher for the feed solutions spiked with BSA but a higher flux decay over time as shown in Figure 4 with 100 kDa PES membrane. Consequently, the initial LRV of ~5 is higher in the presence of lysozyme compared to that of BSA at ~4. As more pores are plugged by BSA, the LRV increases steadily for the subsequent fractions collected reaching a steady value of 5.8 similar to the LRV with lysozyme. For the feed solution without any spiked protein, the flux decays rapidly at the beginning but a steady state is established quickly. The LRV is significantly lower without spiked protein, but increases steadily reaching 5.6 at the final fraction collected due to membrane fouling. For PES 300 kDa as shown in Figures 4 and 6, similar trends are found but the overall LRV is much smaller.

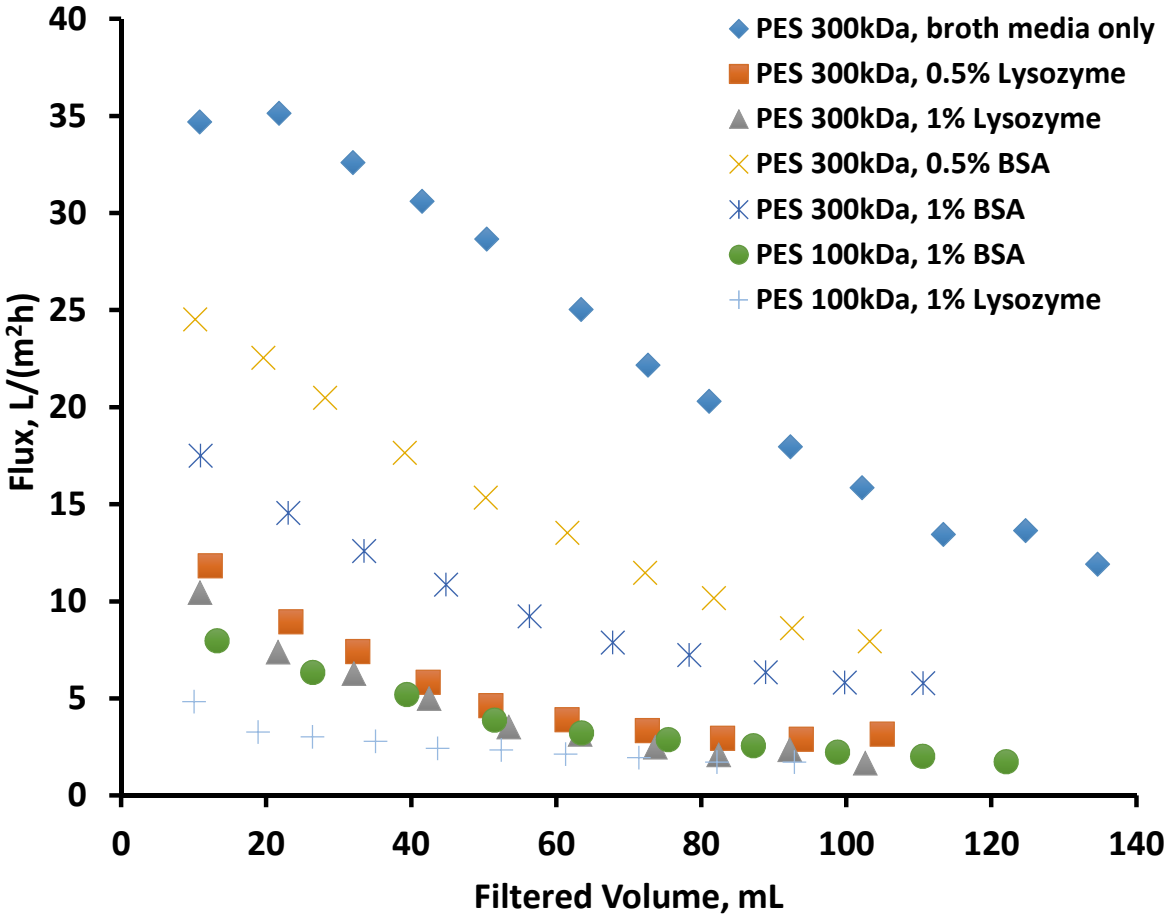

Figure 3. Flux measurement under different protein spiking conditions with PES 100 and 300 kDa membranes.

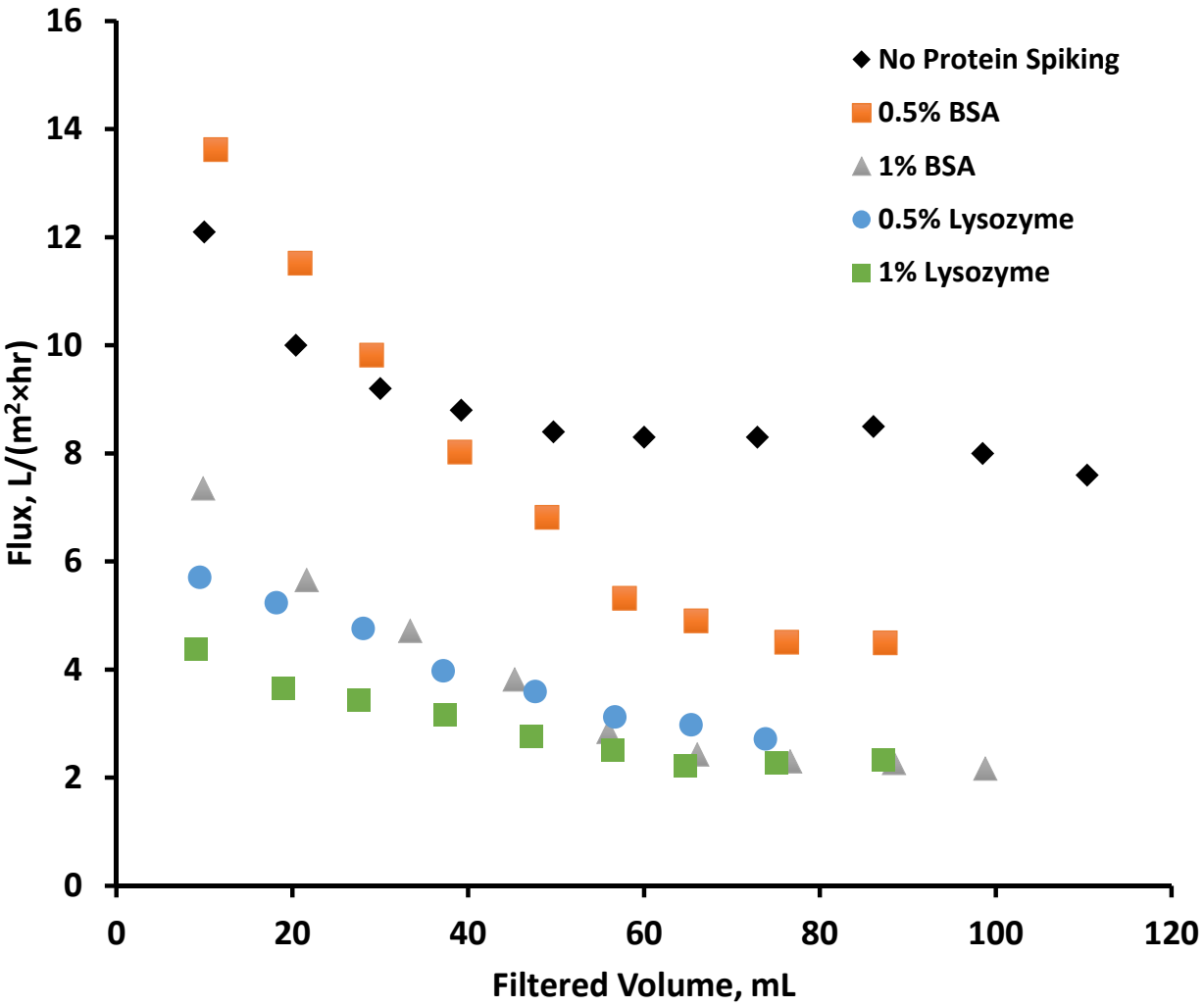

Figure 4. Flux measurement during Phi-X174 filtration using PES 100 kDa membrane in the absence and presence of lysozyme and BSA proteins at different concentrations.

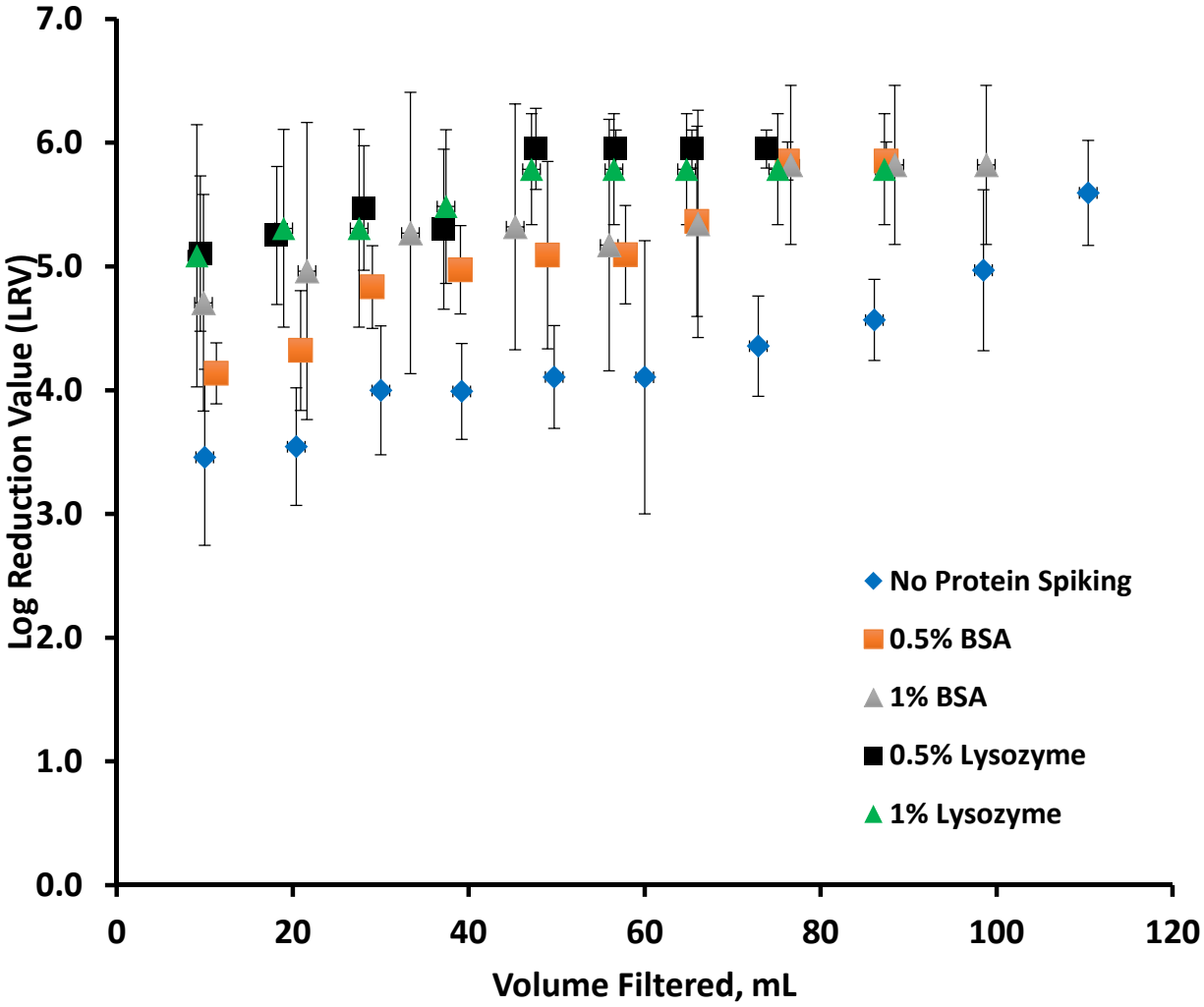

Figure 5. Titer analysis on each fraction collected from Phi-X174 filtration using PES 100 kDa membrane in the absence and presence of lysozyme and BSA proteins at different concentrations.

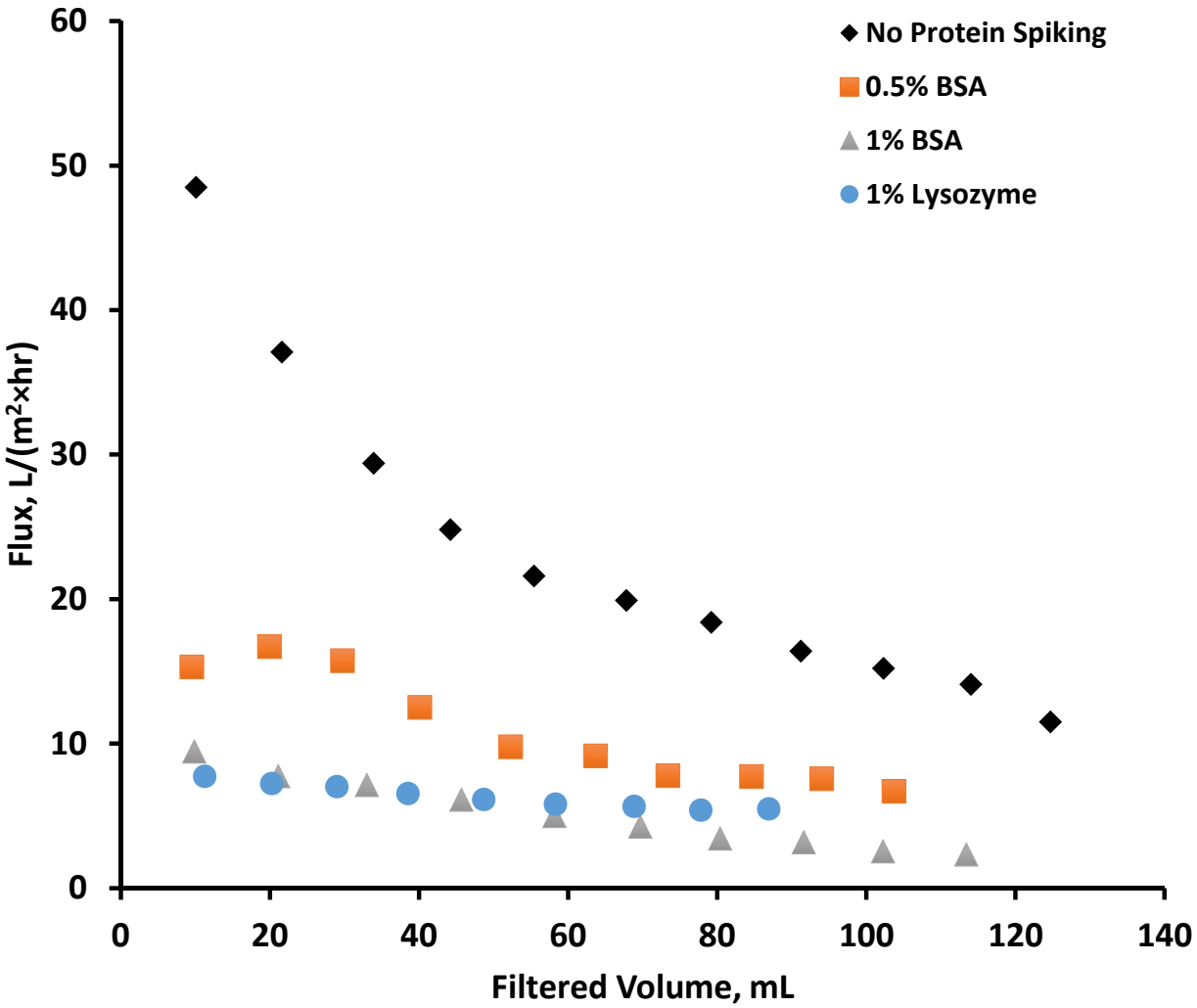

Figure 6. Flux measurement during Phi-X174 virus filtration using PES 300 kDa membrane in the absence and presence of lysozyme and BSA proteins at different concentrations.

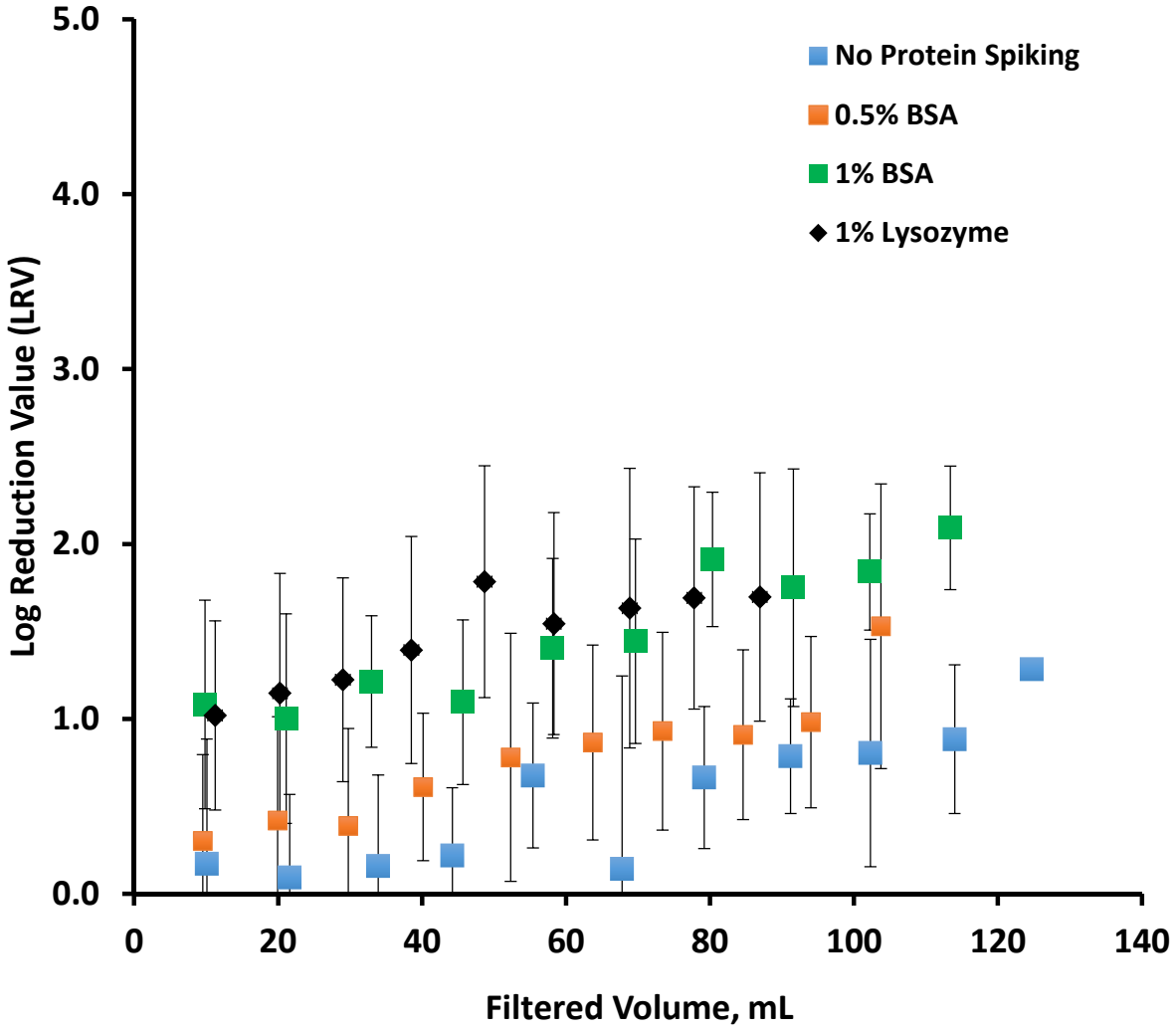

Figure 7. Titer analysis on each fraction collected from Phi-X174 filtration using PES 300 kDa membrane in the absence and presence of lysozyme and BSA proteins at different concentrations.

3.3. pH Effect

Challenge solutions of 5 different pH conditions ranging from 5.50 to 6.94 were tested against PES 100 kDa membrane to investigate the effects of pH on flux performance and bacteriophage clearance. The solutions were prepared by dissolving BSA protein powders in harvested high titer virus stock solutions to reach final concentrations of 1% [w/v]. The solutions were then stirred for 30 minutes and filtered through a 0.2 μm filter. pH adjustment was made right after the protein spiking but prior to the sterile filtration. The feed solution was loaded into the

Amicon stirred cell immediately after DI water flush and a broth media flush. A total of 10 fractions were collected from the permeate for flux calculation and titer analysis.

As shown in the Figure 8, there is no significant influence of pH on flux in the pH range tested except a higher flux was observed for pH = 6.97. Moreover, there is no significant difference in bacteriophage clearance under different filtration pH conditions as shown in Figure 9. The results indicate that at the pH values investigated, the virus, protein and membrane are all negatively charged. The effects of pH on flux and LRV are similar. However, at pH close to 7, the electrostatic repulsion is even stronger, thus a higher initial flux was found. As more proteins are deposited on the membranes due to fouling, flux decay were observed for all the pH conditions, but more pronounced at pH close to 7.

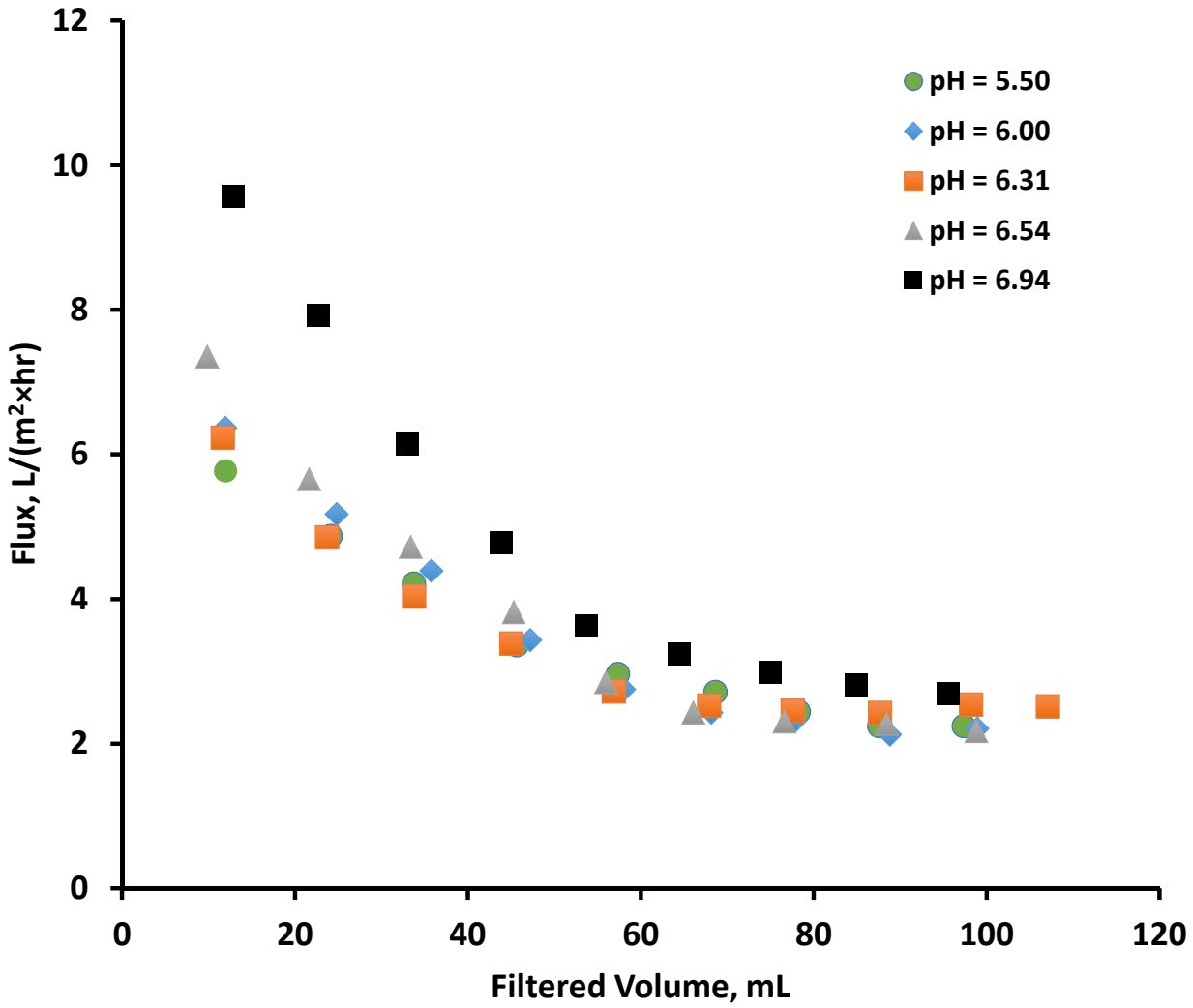

Figure 8. Flux measurement during Phi-X174 filtration using PES 100 kDa membrane spiked with 1% w/v BSA under multiple solution pH conditions.

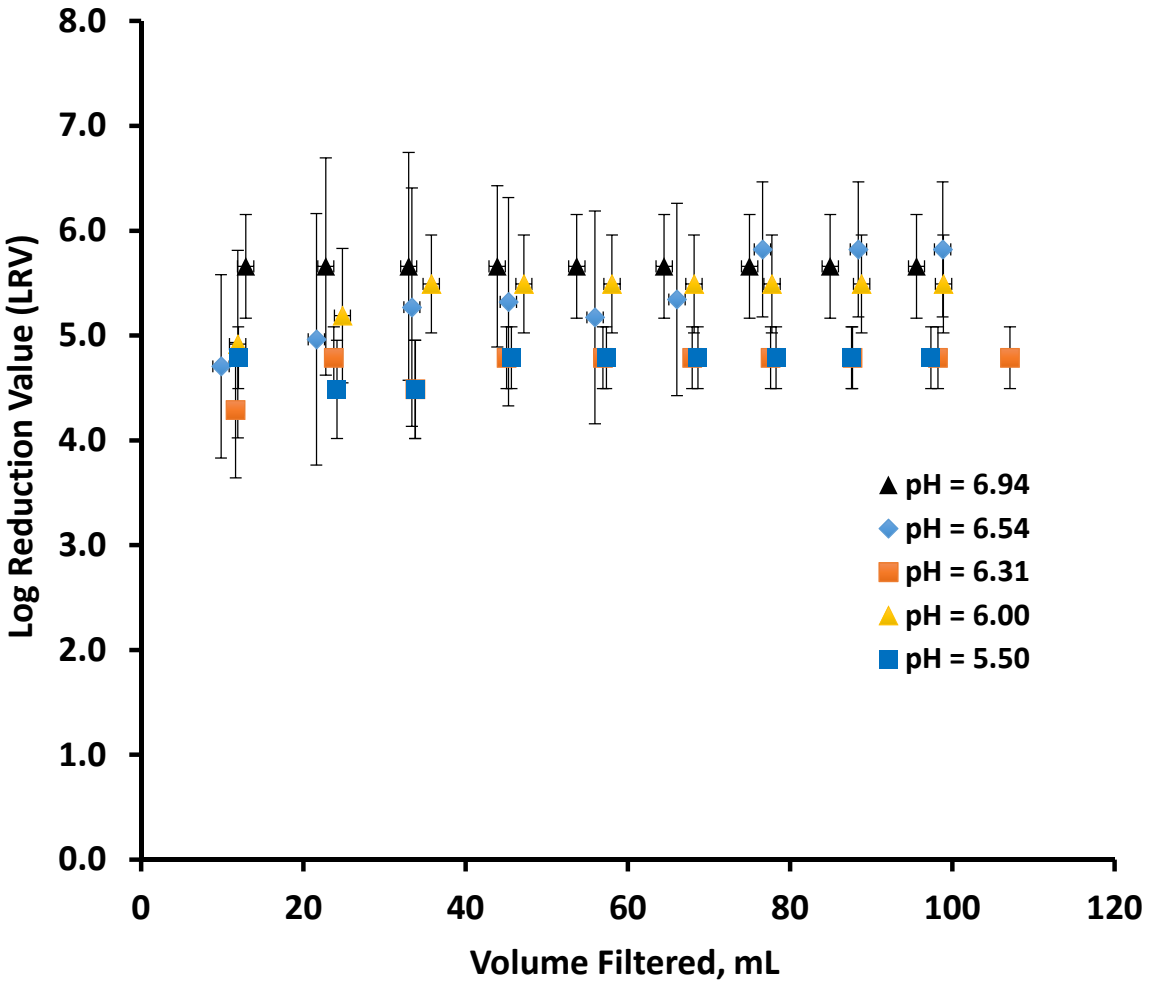

Figure 9. Titer analysis on each fraction collected from Phi-X174 filtration using PES 100 kDa membrane and spiked with 1% w/v BSA under various pH conditions.

3.4. Phage spiking Effect

Harvested high titer PR-772 and Phi-X174 phage stock solutions were tested against PES 300 kDa membrane to investigate phage spiking effect on flux performance and bacteriophage clearance. The feed solution was loaded into the Amicon stirred cell immediately after DI water flush and broth media flush. Nearly 10 fractions were collected from the permeate for flux calculation and titer analysis.

As shown in Figure 10, PR-772 filtration has a much lower flux compared to that of Phi-X174. PES 300 kDa membrane can reject many more PR-772 particles than Phi-X174 virus under the same operating condition as shown in Figure 11. This phenomenon can be explained by the fact that PR-772 has a diameter of 82 nm (Lute et al., 2004) which is larger than the MWCO of PES 300 kDa membrane and much larger than the 26 nm Phi-X174.

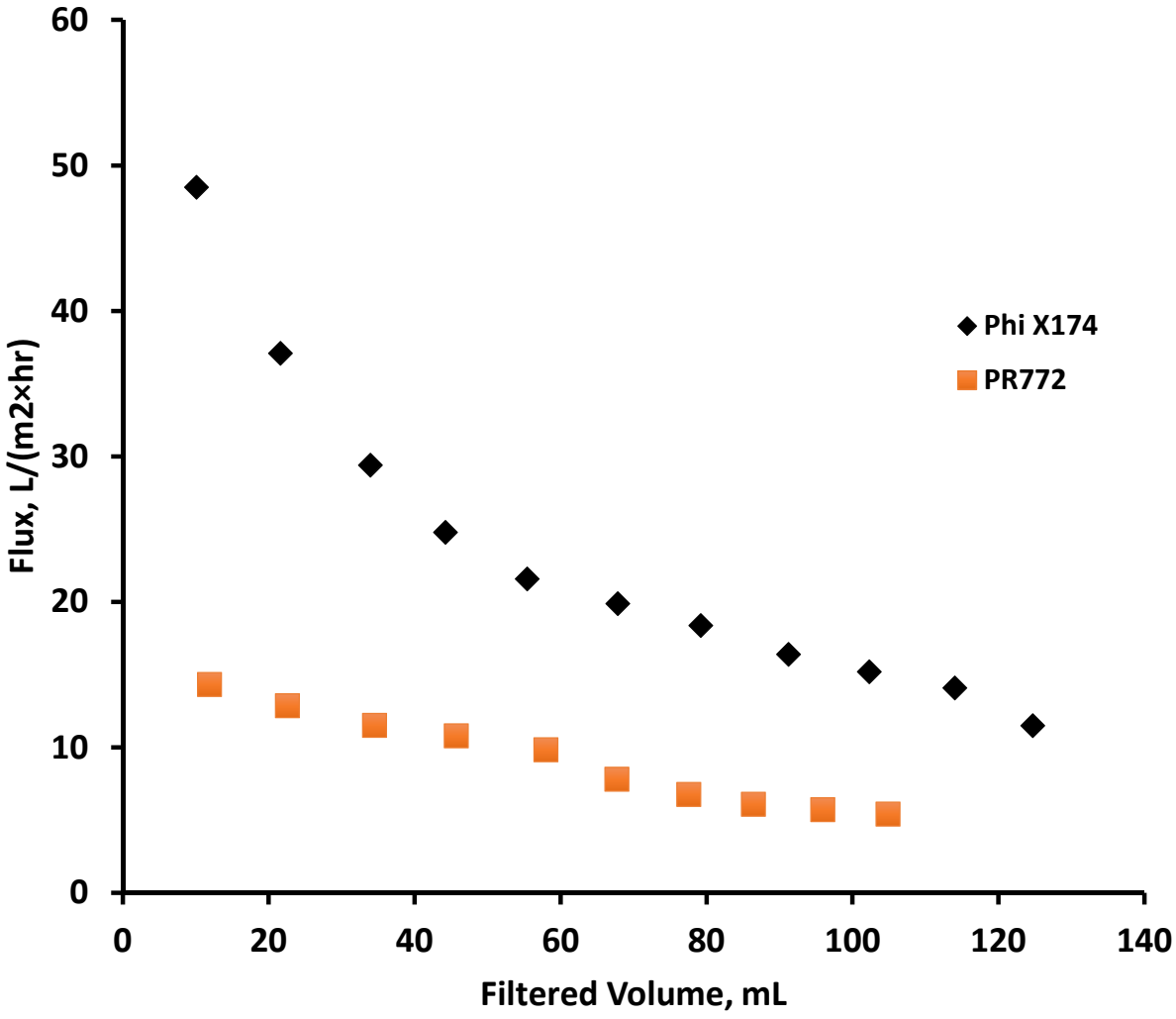

Figure 10. Flux measurement during Phi-X174 filtration and PR-772 filtration using PES 300 kDa membrane.

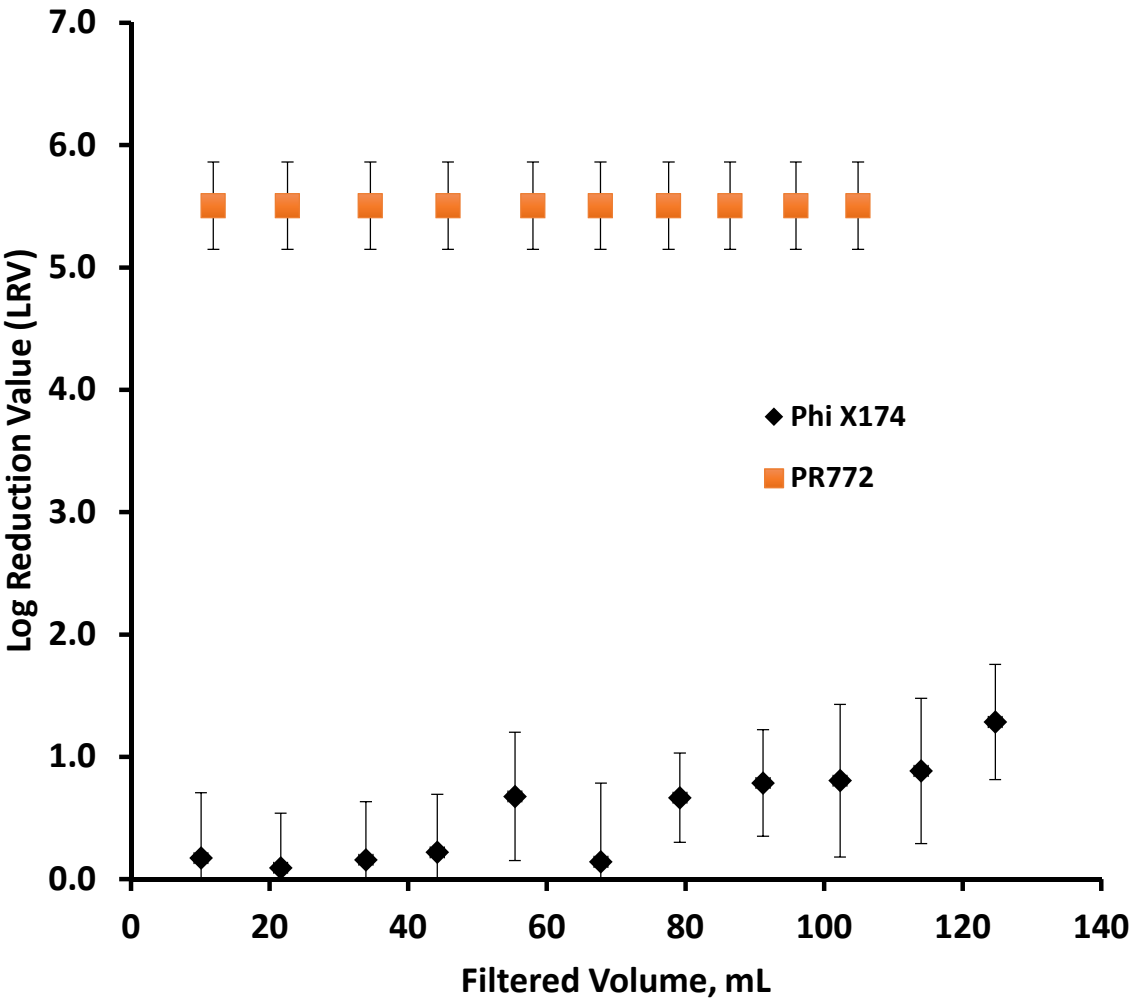

Figure 11. Titer analysis on each fraction collected from Phi-X174 filtration and PR-772 filtration using PES 300 kDa membrane.

4. Discussion

Although virus filtration is typically thought to be a size exclusion-based process, previous studies have shown that virus removal is a function of solution pH, ionic strength of the filtration buffer, isoelectric point of the testing proteins, isoelectric point of virus and the zeta potential of testing VF filter (Schaldach et al., 2006, Bakhshayeshi et al., 2008). These abovementioned effects result in virus adsorption, virus aggregation and protein-virus complexes formation etc. (Zerda et al., 1985)

The results of protein spiking effect as shown in Figures 3 demonstrate that flux decreases as protein concentration increases in the feed streams. The LRV is more affected by the protein type rather than by the concentration as is shown in Figure 5 and Figure 7. Lysozyme can lead to a higher LRV and a lower filtration flux than BSA at same feed concentration as is shown in Figure 4-7.

The size of BSA ranges between 9 to 25nm (Reichert et al. 2005) which is close to the MWCO of the PES 100 kDa membrane as well as the size of Phi-X174 bacteriophage. The size of lysozyme is approximately 1.9 nm (Ecker et al., 2015). However, these two proteins are oppositely charged at the filtration pH conditions. Therefore, when spiked with BSA, the membrane will most likely undergo a cake filtration process with partial pore blocking caused by electrostatic repulsion between BSA and PES membrane (Stump et al., 2007). However, when spiked with lysozyme, the PES membrane will most likely undergo a cake filtration process with standard pore blocking, caused by electrostatic attraction between lysozyme and PES membrane (Stump et al., 2007).

5. Future Directions

Future work will be focus on assessing the virus clearance and flux performance using a wider range of filtration conditions to enhance the process understanding and unveil the filtration mechanism. Various titer analysis methods will be used to measure the titer of both intact virus particles as well as partial damaged virus particles. Virus of different types including PPV and mammalian viruses will also be used as validation model in the filtration studies. Also given that the virus clearance and flux performance can be both product specific and process specific. We will be also considering to use proteins of different types and a broader spiking concentration ranges in the future virus filtration studies.

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