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PURIFICATION AND RECOVERY OF INFECTIOUS VIRUS PARTICLES USING OSMOLYTE FLOCCULATION

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PURIFICATION AND RECOVERY OF INFECTIOUS VIRUS PARTICLES USING OSMOLYTE FLOCCULATION

By

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Table of Contents

List of Figures

List of Tables

List of Abbreviation

WHO: World Health Organization

CDC: Center for Disease Control and Prevention

UNICEF: United Nations International Children's Emergency Fund

GAVI: Global Alliance for Vaccine and Immunization

DCVMN: Developing Countries Vaccine Manufacturers Network

NIAID: National Institute of Allergy and Infectious Diseases

HIV: Human immunodeficiency virus

AIDS: Acquired immune deficiency syndrome

FDA: Food and Drug Administration

NRA: National regulatory authority

HSA: Health sciences authority

PPV: Porcine Parvovirus

SINV: Sindbis virus

MMR: Measles, mumps and rubella

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

VLPs: Virus like particles

IPV: Inactivated polio vaccine

HCP: Host cell proteins

HPLC: High pressure liquid chromatography

AEC/ AEX: Anion exchange chromatography

PRRS: Porcine reproductive and respiratory syndrome

TFF: Tangential flow filtration

NFF: Normal flow filtration

PEG: Polyethylene glycol

AAV: Adenoassociated virus

AdV: Adenovirus

CsCl: Cesium Chloride

PCR: Polymerase Chain Reaction

ICH: The International Conference on Harmonisation

MEM: Minimum essential medium

SDS: Sodium dodecyl sulphate

FBS: Fetal bovine serum

MTT: Thiazolyl blue tetrazolium bromide

MTT_{50}: The 50% infectious dose

TEM: Transmission Electron Microscope

PK-13: Porcine Kidney cells

BHK: Baby hamster kidney cells

RP-HPLC: Reverse phase high pressure liquid chromatography

TFA: Trifluoroacetic acid

PES: polyethersulfone

MWCO: Molecular weight cut off

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Abstract

Viral infectious diseases cause millions of deaths and severe illness all around the world affecting public health and economics. Viral vaccines are helping to fight against viral diseases, but current vaccines are expensive and unavailable, especially in poor and developing countries. When measuring the entire manufacturing processing, the downstream processing of vaccines is the major cost of production. Our goal for this research is to develop a low-cost alternative downstream processing platform for new vaccine manufacturing infrastructures.

We have developed a novel osmolyte flocculation method for viral particles. To create a platform purification for several types of viral particles, we used two model viruses: porcine parvovirus (PPV) and Sindbis virus (SINV). PPV is a non-enveloped virus, one of smallest known mammalian viruses with a diameter of approximately 20 nm. The enveloped virus, SINV, has a size of 48-52 nm. Using mannitol osmolyte flocculation we demonstrated recovery for both viruses by diafiltration using a micropore membrane. This will allow easy scale-up to production scale and creates a low cost platform. Our lab's previous study showed that osmolyte flocculation was specific to viruses as compared to proteins which are present as the contaminants in the process. This preferential flocculation is due to the active hydrophobic surface differences on viruses and protein surfaces. We studied the effect of membrane pore size on the recovery of viruses and were able to achieve 60% recovery of infectious PPV using a 0.1 μm and 500 kDa pore size filters. Recovery of infectious SINV was 79% using 0.1 μm and 96% using 500 kDa pore size membrane filter. Increasing the concentration of virus results in enhanced recovery of infectious particles, but at high concentration, membrane pores can get blocked, causing membrane fouling. We also examined the purity of the recovered virus samples for DNA and protein contaminants. In conclusion, we have developed a novel purification process that was able to purify and recover infectious viral particles using large pore size filters, which can decrease overall processing costs.

1. Introduction

Infectious diseases have caused an enormous death toll worldwide over the last century. Viral pathogens cause many of the infectious and severe diseases. HIV/ AIDS causes 1.6 million deaths every year, and about 36.9 million people are living with the illness according to the World Health Organization [1]. Vaccines are the best tool to fight against many viral diseases and can help prevent millions of deaths. According to the WHO, by 2020 the Global Vaccine Action Plan (GVAP) [2] calls for more equitable coverage of basic existing vaccines to all communities in the world. These plans are focusing on increasing current vaccine manufacturing capacity and creating new manufacturing units in developing countries like India and China. This will be done in partnerships with the Developing Countries Vaccine Manufacturers Network (DCVMN) and Global Alliance for Vaccines and Immunization (GAVI) [3]. They will be focused on finding cost-effective vaccine manufacturing process. The findings from this work will be able to contribute in research and development for alternative platforms in downstream processing of vaccine manufacturing. A platform approach for vaccine production can be a fixed setup and technique for downstream processing that can be applicable to various types of vaccines with minimal changes in the process and protocol. Downstream processing deals with the recovery and purification of target viral product for vaccine formulation.

Recent techniques used in vaccine downstream processing are using traditional methods such as the combination of several chromatographic steps and ultrafiltration for the purification of viral products [4]. Chromatographic methods have been used for proteins and other biological products and are not properly optimized for large biological molecule purification, like viral products. Ultrafiltration method uses small pore size membranes that often foul and has a significant pressure drop, which increases processing cost. In a typical purification process, more than 75% of total production cost is from downstream processing [5]. Therefore, developing a low-cost platform downstream process could substantially reduce the cost of a vaccine product. We propose an alternative approach to chromatography or ultrafiltration, using microfiltration to

reduce the membrane cost and the required pressure for filtration. We also plan to apply this novel process to many different virus types so that this platform approach can be applied to multiple viral products.

In this work, we have used a novel osmolyte flocculation method, which was demonstrated in our lab's previous research publications [6, 7]. This flocculation study has shown that we can flocculate viruses using naturally occurring compounds known as osmolytes, which are found naturally in many organisms. After screening several osmolytes, Gencoglu et.al showed that the osmolyte mannitol was able to flocculate the model viruses PPV and SINV and was able to remove >80% of the infectious virus particles with a 0.2 μm filter [6, 7]. The osmolyte mannitol has been FDA approved to be used in human therapeutics or as the therapeutic agent. Mannitol 20% injection USP drug is being used as osmotic diuretic for certain kidney conditions, brain damage for reducing swelling and pressure in brain and eyes [8, 9]. Therefore, as it is naturally occurring and FDA approved, complete removal of mannitol in the downstream processing is not a concern. In continuation to this previous study from our lab, we are developing a downstream processing platform for purification and recovery of viruses using a diafiltration method.

This thesis begins with a brief background and motivation for these studies. Chapter 2 is the literature review, giving background for viral diseases and vaccines, current vaccine manufacturing method and motivation and rationale as to why we are looking for new approaches. In chapter 2, we demonstrated how this scientific study is needed and can contribute to the need for faster and less expensive vaccine manufacturing platforms.

Chapter 3, contains experimental details and materials used for all experiments. Chapter 4 shows the results for all experiments with different parameters for PPV and SINV virus removal, stating results, findings and a discussion of this research. Chapter 5 has the concluding comments about the overall research and includes proposed future work, which can shed more light on the osmolyte flocculation mechanism.

In this research, we have shown recovery and purification of PPV and SINV. First, by studying the effect of different membrane pore size for recovery of viruses, we found that using larger pore size membrane of 0.1 μm can give 60% recovery of infectious PPV while decreasing the pore size results in increased recovery. Using diafiltration for PPV we demonstrated purification of viruses in the retentate from host proteins using HPLC analysis. We also explored the effect of flocculation time and initial virus concentration. We have found that using high concentrations of viruses can optimize the recovery of infectious virus particles, but the high concentration of viruses can lead to membrane fouling and can increase the pressure requirements for filtration. We analyzed recovered PPV samples for purity, and we are able to remove 85% contaminant proteins for purification and only 45% DNA content removal was observed.

For SINV recovery, we compared all parameters listed above for their effect on recovery of infectious virus particles. Using a large pore size of 0.2 μm gives a 65% recovery and using a 0.1 μm filter gives 79% recovery. For the purification of SINV, our current analysis showed only 37% removal of protein contaminant and 49% DNA removal. Future work will focus on increasing removal of DNA using additional treatments. All presented results show flocculation of virus particles and recovery of infectious virus particles using large pore size membranes. These findings show promise that mannitol flocculation can be applied within the industry to provide low-cost downstream processing which is safe and scalable.

2. Literature Review

2.1. Viral Diseases, Viruses, and Vaccines

Infectious diseases have, and continue to be, a major threat to public health. Emerging infectious diseases has threatened human health throughout the history of humankind. Infectious diseases are caused by the bacteria, viruses, parasites or various fungi [10]. Viruses cause many diseases that are fatal to their host. Some of the common viral human diseases and their global effect are shown in **Table 2-1**.

Table 2-1 Infectious diseases and effects worldwide, [1]

Disease	**Virus**	**Effect**
Measles	Paramyxovirus	114,900 measles deaths globally about 314 deaths every day or 13 deaths every hour (2014)
AIDS	Human immunodeficiency virus	1.2 –1.6 million deaths every year (2014)
Hepatitis B	Hepatitis B virus	780,000 people die every year
Ebola haemorrhagic fever	Ebola virus	Worldwide 28,646 cases of Ebola virus disease and 11,323 deaths
Dengue and severe dengue fever	Dengue virus and serotypes	390 million dengue infections per year
Influenza A(H1N1) and A(H3N2) subtypes	Influenza virus and serotypes	3 to 5 million cases of severe illness, and about 250,000 to 500,000 deaths annually

Viruses are pathogens which can replicate only inside the cells of living host organisms [11]. Viruses can infect all types of organisms such as human, animals, plants and bacteria. Reports show that there are about 5000 different types of viruses that have been described at current times, and there could be millions more that we are not yet aware of [12]. Viruses are typically very tiny, although they range from a few nanometers

to a micron in size. The smallest known virus is porcine circovirus, at around 17 nm in diameter and porcine parvovirus about 20-24nm [13-15]. Other viruses are larger than some bacteria such as vaccinia virus (230 nm), mimivirus (500 nm) or Pandora virus (about 1 μm) [12, 15].

Viruses are categorized mainly by their outer layer structure and nucleic acid content. For the nucleic acid content, viruses encapsulate either DNA or RNA genetic material [12, 16]. Depending on the genome content of viruses, viruses are classified as RNA viruses and DNA viruses. RNA viruses comprise about 70% of all the viruses and can contain single-stranded (ssRNA) or double-stranded (dsRNA) viral content [17].

Depending on the outer layer structure of viruses can be divided into enveloped and non-enveloped viruses. The encapsulated nucleic acid genome contains a protective protein layer which forms the capsid [16]. If the virus contains an outer lipid bilayer membrane around the virus protein capsid, they are called enveloped viruses. This outer lipid layer around enveloped viruses contains viral proteins which help in binding to the host cells. Whereas viruses which do not have outer lipid layer are called non-enveloped viruses. In the case of non-enveloped viruses, the function of binding to the cells is carried out by the capsid proteins [18]. Due to the large chemical differences in the outer layer of the virus, developing a processing method which can be applied to different types of viruses is an important aspect of developing a platform downstream processing approach.

One of the most efficient methods to date to combat viral diseases is through vaccines [19], and vaccines are best accomplishments through science for the benefit of public health [20]. Viruses which causes infectious diseases can be used to fight against diseases in the form of viral vaccines. The goal of the vaccines is to help the human body to form immunity against a viral infection by producing an antibody response [21]. This allows the body to fight against the virus when the body encounters it, before the infection taking hold in the body. During 2000-2014, measles vaccination prevented 17.1 million deaths. Polio cases from the world have been decreased by 99% because of

vaccines. Influenza vaccines save millions of people from high influenza complications and can provide reasonable protection in adults upon vaccination worldwide [1].

The first vaccine by Edward Jenner for smallpox was able to eradicate smallpox [22, 23]. Also, the first attenuated vaccine developed by Louis Pasteur for rabies by attenuating rabies virus in the laboratory [24]. Discovery of the first vaccine was the landmark achievement as it shows that using the virulent virus as a vaccine can help to fight against the disease. Since Jenner's discovery and Pasteur's vaccine, the advances in immunological studies have helped us to understand how the immune system can help to fight against diseases using vaccines [22, 23]. There has been continuous research going on to find improved technology for improving quality and quantity of vaccine production. Even with all the recent advancement in immunology and vaccine manufacturing, there is no equality in distribution and availability of vaccines in industrialized developed countries as compared to developing and poor countries [25]. The current global vaccination plans focus on the public health within developing and poor countries which requires wide and easy access to large quantities of vaccines at affordable cost. One of the major strategic goals of the WHO includes more focus on the research and development in vaccine manufacturing in developing and middle-income countries [2]. There are about 22 millions of children in poor and developing counties which are still not protected against viral diseases due to lack of vaccination availability [26]. Global coverage for basic vaccines shows that there is need of a major supply of vaccines needed in south-east Asia, Western Pacific region and African region. **Figure 2-1** shows the global coverage of current basic vaccines. Out of this total global coverage for vaccines, **Table 2-2** shows the areas affected most by low coverage of immunization.

Table 2-2 Global vaccine coverage by April 2015, [1]

Vaccine	Coverage
Measles vaccine	Global coverage at 85%, only 11% in the African region
Hepatitis B vaccine	Global coverage at 38%, only 10% in the African Region
Haemophilus influenzae type b (Hib) vaccine	Global coverage at 56%, only 21% and 30% in the Western Pacific Region and in the South-East Asia Region, respectively
Pneumococcal vaccine	Global coverage at 31%, about 50% in the African Region but is only at 2% in the Western Pacific Region
Rotavirus vaccine	Global coverage at 19%, but 0% and 1% in the South-East Asia Region and in the Western Pacific Region, respectively

WHO along with DCVMN and GAVI are working together to provide access to vaccines in poor and developing countries like India, China, and the African Union. So, emerging and developing countries are looking for affordable vaccine manufacturing processes so they can start their own vaccine production units [27].

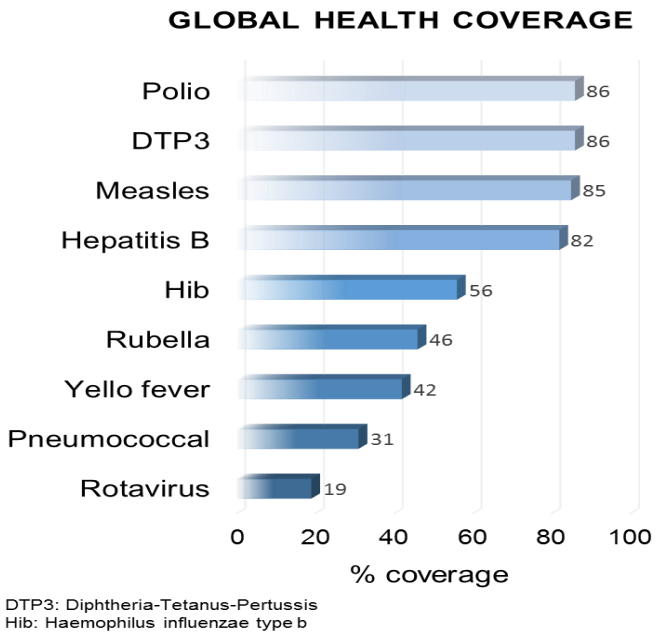

Figure 2-1 Global immunization coverage WHO [1]

Unlike other chemical drugs, vaccines are made from biological agents which are susceptible and can be compromised during various manufacturing processing stages. At

every stage of vaccine processing, it needs strict regulations for safety and quality monitoring in the manufacturing. There are various regulatory authorities for maintaining regulations for vaccines such as Food and Drug Administration's (FDA) Center for Biologics Evaluation and Research (CBER) in the United States, the European Medicines Evaluation Agency on behalf of WHO, and the National regulatory authority (NRA). The regulatory agencies focus on safety, efficacy and purity. In the exploration of new virus purification methods, the regulatory restrictions on safety, efficacy, and purity must be kept in mind.

We are pursuing methods to reduce the cost of viral vaccine manufacturing so that distribution to GAVI countries can be greatly increased. The focus of our research is to create an economical vaccine manufacturing processes using FDA approved osmolytes as preferential flocculants while keeping the regulatory requirements of safety, efficacy and purity in perspective as a platform approach. Also, the approach should not be limited to lab scale and should be easily scalable for large scale production. Our goal is to establish a low-cost vaccine manufacturing process so that vaccines can be available at reduced rates in poor and developing countries.

2.2. Types of Vaccines

Traditional vaccines contain part or all of a disease-causing agent. Many of the vaccines are based on viruses. There are three main types of vaccines, live/attenuated, inactivated and subunit vaccines. There is a sense of balance between protection and efficacy when choosing which vaccine type to pursue.

Live attenuated vaccines contain the whole viruses in an attenuated form which are weakened by passing through multiple cell cultures so that it cannot cause a serious disease in humans. They provide a strong immune response against the disease and can provide lifelong immunity with one or two doses. Live attenuated vaccines are successful against human viral diseases, however as this vaccine type contains live viruses that are very similar to the natural infectious virus, there can be some safety issues. Live

attenuated vaccines can become virulent or mutate at some point and cause the disease [3, 28]. There have been cases reported about live attenuated polio vaccine which was found to contain wild-type polioviruses [28, 29]. Report from 2009 showed that the polio vaccine paralyzed several children in 2007, 2008 and 2009 [1].Therefore, the quality and purification processing during live vaccine manufacturing is critical to minimize the downsides of live vaccines. The examples of live attenuated vaccines are measles, mumps, rubella (MMR combined vaccine), rotavirus, varicella (chickenpox), influenza (live attenuated influenza vaccine) [30]. Also one of major limitation with the live attenuated vaccine is storage, as these vaccines need to be refrigerated to keep the potency and effectiveness. This leads to a higher cost vaccine. Developing countries and poor countries lack widespread refrigeration. So, considering these are important factors, manufacturers from developing countries are looking to manufacture vaccines in their own region so it will minimize the cost of vaccines and avoid shipping overseas from other countries.

Inactivated vaccines are produced by using viruses that cannot replicate due to chemical, heat or radiation inactivation methods [31]. Inactivated vaccines typically create a weaker immune response than live attenuated vaccines, so inactivated vaccines are given in several dosages or booster shots may be required to keep immunity against the disease [32]. The high number of dosages leads to high immunization costs in areas where there are no health care facilities available, specifically in poor and developing countries. Inactivated polio (IPV) and hepatitis A (HAV) are examples of inactivated vaccines [33].

Instead of using the whole virus for vaccines, subunit vaccines contain only part of an antigen from the virus, which activates an immune response against it. Subunit vaccines are typically expressed recombinantly in bacterial cells, decreasing the cost and complexity of manufacturing, as compared to mammalian or insect cells that are needed for live-attenuated and inactivated vaccines [32]. Hepatitis B, influenza (injection), and haemophilus influenza type b (Hib) are examples of subunit-conjugate vaccines [33, 34]. As inactivated vaccines may need several dosages, it may be challenging to provide

vaccination in the areas where people do not have continuous access to health care services and are unable to get all the dosage or booster shot in a timely fashion [35].

2.3. Model Viruses

To study viral purification and virus recovery and propose a universal platform that can be applied to all types of viruses, we need to select suitable types of viruses which can be a good model for viruses used as human therapeutics. There are two major types of viruses, enveloped and non-enveloped viruses. Non-enveloped viruses do not contain an outer phospholipid coating; there is only a protein capsid that surrounds the viral nucleic acids. Enveloped viruses contain an outer envelope around the viral capsid which is made of phospholipids and proteins or glycoproteins [36]. We wanted to study both types of viruses, i.e. enveloped and non-enveloped virus as presence or absence of envelope can affect the flocculation studies significantly.

For our virus purification study, two viruses were selected as models; the non-enveloped PPV and the enveloped SINV. Parvoviruses cause the variety of diseases in vertebrates and arthropods and have been isolated from mammals like humans, dogs, cats, rodents, cows, and pigs [37]. Parvoviruses are the second smallest known mammalian virus at 18-26 nm in diameter and contain single-stranded DNA [13, 14]. PPV is a common cause of reproductive failures in swine [38]. PPV is a good model virus for human B-19 parvovirus, hepatitis A, and poliovirus. B-19 parvovirus is widespread in human and causes erythema infectiosum skin rash illness and it is more common in children than adults (CDC) [39]. We use PPV as a model virus because it is small, making it difficult to separate by using size-based methods and represent non-enveloped, DNA virus type. PPV is resistance to heat and is chemically inert as it shows resistance to physical or chemical treatment [40-42].

Our enveloped virus model, SINV, is an arthropod-borne virus from the Togaviridae family [15]. It is a single-stranded RNA virus with an icosahedral capsid and contains a protein envelope made of glycoproteins. SINV causes epidemic polyarthritis and rash sickness commonly known as Pogosta diseases in humans which may lead to prolonged

arthritis [43]. SINV is one of smallest enveloped virus with size about 50-60nm [14, 44]. SINV is a model virus for eastern and western equine encephalitis viruses which causes infection in human and horses and are widespread deadly diseases. As it contains an outer envelope layer, surface properties of SINV such as hydrophobicity and charge play a major role in different purification methods.

As successive research in virus purification with various viruses has provided the detailed understanding of the viral structures and how properties of viruses such as size, isoelectric potential (pI), virus hydrophobicity, and the presence of envelope can alter purification processes [4, 45]. Viruses possess a pH dependent surface charge in polar media such as water or buffer. This surface charge determines the absorption of virus particles on surfaces, and it can govern the colloidal behavior of viral particles [46, 47]. One measure of colloidal charge is the isoelectric point (pI). The pI is the pH at which surface charge changes its sign or acts as a neutral molecule. The isoelectric point (pI) for PPV is about 5.5 [48], and the pI for SINV is about 4.4 [49].

For our studies for flocculation with osmolytes, the hydrophobicity of viruses is an important property as flocculation is hypothesized to be based on the preferential hydration and hydrophobicity difference between viruses and proteins [6, 7]. Hydrophobic interactions are the forces between two non-polar groups which attract two particles together. Non-polar molecules tend to form intermolecular contacts to reduce their surface contact with polar molecules, such as water. In the case of our hypothesis, viruses are hydrophobic in nature and contain water shell around outer surface in an aqueous solution. Upon removing the water surrounding the virus particles with osmolyte, viruses tend to attract each other and forms aggregate. This effect is explained on proteins by C.J. VanOss, 1995 through precipitation of proteins by dehydration method. By removing water around particles, their surface at the interface becomes more hydrophobic. This changes the normal repulsive forces between particles and turns them into the net attraction forces causing particle aggregation [50]. Studies in the literature have shown the presence of hydrophobic areas on the virus surfaces.

Both enveloped and non-enveloped viruses contain sites for fusion proteins on their outer surface which helps the attachment to the host cell membranes. In the study by Badani et al. 2014, it was shown that viral fusion proteins or viral membranes contain hydrophobic segments. It has shown virus attachment to the host cell is related and driven by the hydrophobicity of viral binding proteins or viral membrane surface [51]. As per their hypothesis, the virus entry inhibition is explained by the physical chemistry of hydrophobicity i.e. active hydrophobic sites and patches on different sites of virus surface (e.g. class I, class II and class III fusion proteins on viruses) on the viruses as well as on the cell membranes. The Wimley–White interfacial hydrophobicity scale is used to determine the hydrophobicity range based on the interfacial hydrophobic interactions [52]. This method determines the transfer free energy of the amino acids along with peptide sequence. The transfer free energy is a scale of the propensity for amino acids to transfer from water to phosphatidylcholine interface [51]. Badani et al. used this Wimley-White interfacial scale to score hydrophobicity based on interfacial hydrophobic interactions of viruses. On this scale, a positive score indicates the presence of hydrophobic interaction based on peptide sequence and hydrophobic interactions. Zero point on scale divides the peptide sequence regarding free energy into hydrophobic or not. They have shown positive scores for Dengue virus, West Nile virus, murine hepatitis virus, respiratory syndrome coronavirus, influenza virus, hepatitis C virus [51]. This demonstrates that viruses have active hydrophobic sites present on their surface. This study was showed for all enveloped viruses. Another study has shown the presence of hydrophobic sites on non-enveloped Reovirus membrane [53].

Our past work on PPV concluded that both hydrophobicity and charge play a major role in the binding of porcine parvovirus, and we have shown that viruses are more hydrophobic than proteins thus hydrophobicity of viruses plays an important role in flocculation of viruses using osmolyte [6, 7].

2.4. Vaccine Manufacturing

Current manufacturing methods for vaccines, related therapeutics, bioproducts are very product specific. Traditionally, vaccines were prepared using embryonated chicken eggs or animals. For example, Edwards Jenner's first vaccinia was inoculated in cows [22, 23]. More recently, the seasonal flu vaccine is prepared using fertilized embryonic eggs, which usually takes a long time to prepare [54]. Egg-based methods are a labor consuming method, requires a significant amount of fertilized eggs at one time and is highly susceptible to bacterial contamination. Additionally, individuals that are allergic to eggs may not receive this type of vaccine.

Since the development of new methods and research in vaccine manufacturing, cell-based vaccine manufacturing processes are being used to produce vaccines. Cell-based vaccine manufacturing was first done by using in vitro cultivation with non-neural human cells by Enders in 1949 for poliovirus and then in 1955 by Salk for inactivated polio vaccine using monkey kidney cells [55]. From that time, industries are significantly looking to use cell culture-based vaccine production using mammalian cells. Cell culture based vaccine manufacturing methods can be much safer, as processing takes place in closed systems, the chances of contamination are reduced [56]. The vaccine regulatory authorities encourage cell culture based vaccine manufacturing and this method can be scaled up for emergency large production requirements. The production time for cell culture-based vaccine manufacturing is reduced to half as compared to traditional vaccine manufacturing using embryonic eggs based method [54]. However, using cell culture-based vaccine manufacturing produces less virus, and the volumetric yield is low [54]. This requires large volume bioreactors to achieve the desired yield of viruses, which adds up to higher capital investment for the production plant. The relatively higher manufacturing cost may translate to more expensive vaccines. New research and development teams are focusing towards developing current vaccine manufacturing processes so that the cost of manufacturing and vaccines will be more economical. Our focus is to develop alternate low-cost vaccine manufacturing platforms to create an economic vaccine manufacturing process.

2.4.1. Upstream and Downstream Processing

Vaccine manufacturing process is classified into two different stages, upstream and downstream processing, as shown in **Figure 2-2**. Upstream processing comprises of growing and cultivation of cells, infecting the cells with the virus of interest and harvesting of cell lysates. Once cells are infected completely, and viruses are reproduced, cell lysis is typically performed through homogenization or adding non-ionic detergents, like Triton X [57, 58]. Next, cell debris is removed through clarification, which can be carried out with centrifugation, or filtration methods [59-61]. Once the clarified solution is obtained, it proceeds to downstream processing, involving purification, concentration, and polishing.

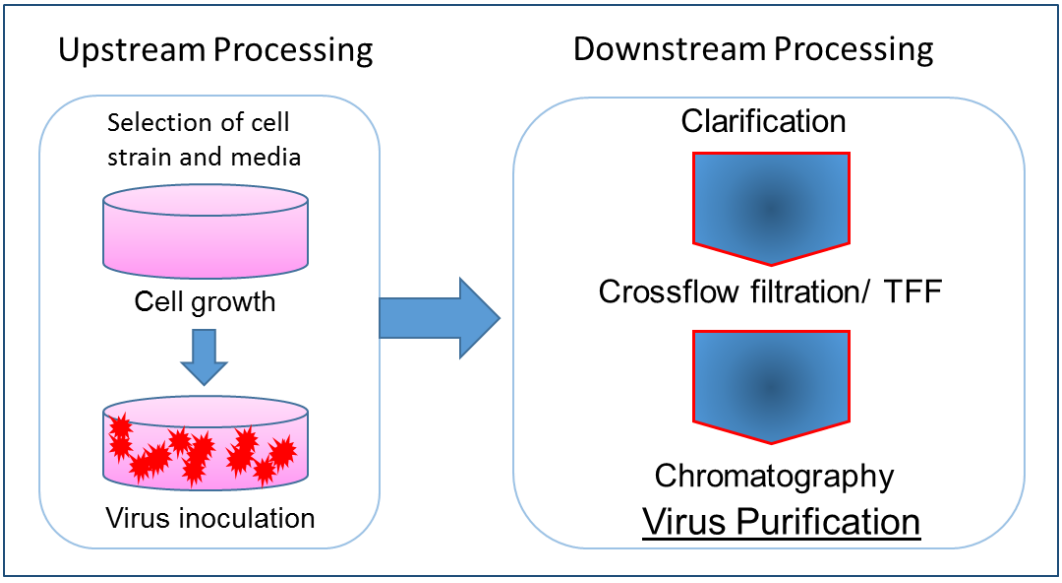

Figure 2-2 General outline for Vaccine manufacturing with upstream and downstream processing, upstream processing includes cell growth, virus production, and downstream processing deals with clarification, purification, and recovery of viruses.

After harvesting viruses from the host cells, downstream processing deals with the purification and concentration of the viral products. Purification is aimed at removing contaminating host cell proteins, DNA, and impurities introduced during the purification

process. Using a variety of physical properties as size, isoelectric potential (pI), and the hydrophobicity different methods are selected for purification [4, 45]. There are several methods for virus purification. Commonly used methods for virus purification are ultrafiltration and chromatography [4, 62-65]. Membrane-based ultrafiltration is typically used for partial purification and chromatography is usually used for final product purification. Other purification methods are depending on the viral size and properties such as ultracentrifugation which uses density based separation, precipitation by chemicals, PEG, flocculation using salts, and membrane absorption which uses charge based properties of viruses [60, 66-69].

2.4.2. Ultracentrifugation

One of the methods employed for virus purification is ultracentrifugation. Density gradient centrifugation using cesium chloride or iodixanol gradient is one of the well-known and established method for the virus in preclinical studies [4, 66, 70]. Also, there are concerns over CsCl toxicity and its complete removal after viral purification can be challenging. While ultracentrifugation leads to very pure products, the lack of scale-up options makes it undesirable for manufacturing. This method can be used for limited sample separation by using ultracentrifugation, which uses very high rotational speeds. The purification is based on the different buoyant densities of virus particles and contaminants. Ultracentrifugation will even separate full from empty viral particles [71, 72]. This method is mostly used in a lab due to the lack of industrial scale ultracentrifuges. A CsCl density gradient was used for the purification of adenovirus (Ad) and adeno associate virus (AAV) [66, 73], showing lab-scale purification for Ad and AAV. A recent study showed a 60% yield for CsCl and a 65% infectious units recovery for an iodixanol based AAV purification method [74]. While yield and purity can be high with ultracentrifugation, the lack of scale-up options makes it unfeasible for vaccine production.

2.4.3. Chromatography

In the biopharmaceutical industry, chromatography is the most commonly used separation and purification technology due to its easy applications to all products and its

high resolution. Chromatography separation is based on the different interactions between viruses and the surface of chromatographic beads such as size based (size exclusion chromatography), charge based (ion exchange chromatography), and hydrophobicity based (hydrophobic interaction chromatography HIC, reverse phase chromatography). Ion exchange chromatography is charge based separation depending on the interactions between charged particles and ionic ligands on the chromatography beads. Therapeutic molecules bind to the column under low salt condition and elution is done using high salt gradient because the ionic interaction is disrobed by the high ionic strength solution. Hydrophobic interaction chromatography is based on binding of particles to the hydrophobic surfaces depending on the hydrophobicity of particles. In HIC, particles are loaded in the high salt buffer which promotes the hydrophobic effect and drives adsorption with solid support. The separation works with low salt gradient based on the difference in hydrophobicity as a result of desorption from resin.

Several studies have shown that various chromatography methods such as ion exchange, size exclusion chromatography, affinity chromatography in combination with multiple chromatographic steps in series or with membrane filtration can be used for virus purification and recovery [64, 75, 76]. Bead chromatography methods have a high surface area in the internal pores of the beads, and the capacity of the resin subject to the diffusion of molecules into the pores of the beads. This poses a big limitation for the purification of large molecules that either cannot enter the pores or quickly plug the pores upon binding. One newer method to overcome the problem of bead chromatography is membrane chromatography. In membrane chromatography, all of the surface area is accessible by convective flow and does not rely on diffusion into pores [77]. This allows for the much more accessible surface area by large biomolecules. However, there is still low overall surface area per volume with membrane chromatography. It has therefore not been implemented as much as originally thought. In Table 2-3, we have shown some of the work which uses the chromatographic technique as primary purification method with or without the combination of other methods.

Table 2-3 Literature review for some of the chromatographic techniques used in purification of viruses

Study	Method	Results	Reference
3 step chromatographic process for Influenza type A & B purification	Anion exchange chromatography, benzonase treatment and final size exclusion chromatography	68% virus yield and 98% DNA removal	[78]
Purification of Rotavirus-like particles (RLP's) using chromatography	Anion-exchange membrane chromatography	46% global yield, 100% DNA removal, 98% HCP removal	[75]
Purification and characterization (immunogenicity) of norovirus (NoV) VLPs.	Anion exchange chromatography Retained immunogenicity (immunoblotting)	Final yield is not mentioned, 90% purification w.r.t. DNA content and	[79]
Combined ultrafiltration and chromatography process for Porcine reproductive and respiratory syndrome virus (PRRSV)	Ultrafiltration using 300,000 nominal molecular weight limit (NMWL) membrane followed by heparin affinity chromatography	54% final PRRSV recovery, 96% cellular and medium proteins removal	[80]
	Recovery of porcine reproductive and respiratory syndrome (PRRS) virus using ultrafiltration and anion exchange chromatography	50% recovery of PRRSV	[65]
Purification of Adenovirus using 2 step anion exchange chromatography	Set of 2 anion exchange chromatography (1st Q Sepharose, 2nd Source 15Q)	40% infectious unit recovery of Adenovirus and 99% purity	[81]
Purification of Adenovirus using combined chromatography technique	Primary purification using anion exchange chromatography and final polishing using size exclusion chromatography	80% virus particles recovery, 99% purity	[82]

Influenza A virus recovery and purification using 2 chromatography steps	Size-exclusion chromatography (SEC) and anion-exchange chromatography	Final product yield of 52%, and 19-fold HCP reduction, 500-fold DNA content reduction	[83]

As discussed earlier chromatography offers advantages for high purification and product yield but it has several disadvantages. Chromatography is mainly used for protein purification and has been adapted for virus purification. There is no platform chromatography method which can be used for viruses because of different structure, size, and charges of viruses. Also, the concentration of viruses cannot be done solely using chromatographic technique [84]. So there is a need for downstream processing to look for new platform approach other than using chromatographic techniques for viral vaccine processing.

2.4.4. Filtration

Filtration has been an integral part of biotechnology, and it is used as both a concentration and separation method for viral products [68]. Membrane based separation and purification methods are typically applied as size-based separations. This approach can provide good separation and purification and is easy for scaling up at commercial scale with high throughput. Current membrane based separation methods typically use ultrafiltration membranes. Diafiltration using ultrafiltration membranes has been widely used in virus DSP for concentration and separation as described in the literature [68, 85, 86]. Ultrafiltration membrane range in pore size from 0.5 to 1000 kDa MWCO [87]. Filtration methods can be operated in two types of flow, normal flow filtration (NFF) (also called dead end filtration) and tangential flow filtration (TFF). In NFF, flow is perpendicular to the membrane and particles larger than the membrane pore size are typically withheld by the membrane, while smaller particulates pass through the membrane. This mode is usually used when the product of interest passes through the

membrane. This is because the larger size particles are retained on the top, causing a buildup of particles on top of the membrane, which leads to cake formation on the membrane, increased sieving and increased transmembrane pressure [88, 89]. To avoid this, TFF uses the tangential flow of fluid with applied pressure. As in NFF particles larger than pore sizes are retained and smaller particulate pass through the membrane, while tangential flow sweeps the retained particles to avoid buildup on the membrane. In operation of TFF, once a steady-state is reached, the transmembrane pressure stays relatively stable. Membrane-based TFF is applicable in downstream processing of many biologics and viruses. Low pressure and cross-flow pattern of TFF with hollow fiber membranes provide less stress on particles than dead end filtration and promotes gentle treatment for virus particles and proteins to retain their structural activity [5]. However, there is still possibly a flux decline due to fouling and shearing of the particles, which could cause virus inactivation. To avoid this disadvantages using membrane-based separation method, we are looking at the combination of flocculation and membrane filtration methods so that we can use large pore size membranes for virus purification and recovery.

TFF using the ultrafiltration membrane for concentration and purification is one of the most common purification steps before or as a final polishing step [90-93]. It was shown that the membranes with smaller pore sizes than the virus particles can be used for concentration and purification of viruses [92]. Using small pore size ultrafiltration membranes for virus/ viral vector recovery was performed in several studies as shown in **Table 2-4.**

Table 2-4 Tangential flow filtration studies for various virus/ VLP/ vectors from the literature

Study Virus	**Size**	**Method, Results**	**Reference**
HIV-1-derived lentiviral vector	80-130 nm	TFF (300 kDa), 100% recovery TFF (100 kDa), 100% recovery TFF 100 kDa+ 5 hour centrifugation 26000g, 18% recovery* TFF 100 kDa+ 2 hour centrifugation 76000g, 94% recovery* * transducing units recovery after centrifugation	[90, 94]
Aedes aegypti densonucleo-Sisvirus	20-30 nm	TFF using ultrafiltration membranes, 30 kDa and 50 kDa able to retain virus particles while using 100 kDa and 300 kDa particles can be seen in permeate	[91, 95]
Influenza A virus (Human virus Type A)	80-120 nm	Screening of ultrafiltration membranes100 kDa, 300 kDa, and 0.1 μm, 0.2 μm MWCO membranes, results showed 300 kDa membrane gives optimal recovery and 84% protein removal	[92]
Flavivirus pseudoinfectious virus	40-60 nm	2 step purification method with TFF (using 100 kDa and 500 kDa TFF cassette) followed by anion exchange chromatography. TFF with 100 kDa gives recovery 80% followed by 54% recovery in AEX	[96, 97]
Parvovirus Minute Virus of Mice	22-26 nm	Purification and recovery using high-performance tangential flow filtration, optimal virus exclusion observed with 50 kDa and 100 kDa membranes, 300 kDa membrane not effective in retaining virus particles	[98]
HIV VLP	120 nm	Recovery and concentration of HIV VLPs using 500 kDa hollow fiber on automated TFF system	[99]
Viral adventitious agents (AAs)	Adenovirus 70-90 nm, Parvovirus 18-26 nm, Herpesvirus 110 nm, Simian virus 45 nm	Large scale (500 liters) recovery and concentration of 4 viruses using 100 kDa MWCO hollow-fiber filters: Human adenovirus type 5, 84 % recovery Bovine parvovirus, 93 % recovery Bovine herpesvirus 4, 85 % recovery Simian virus 40, 88 % recovery	[100]

These studies have shown using ultrafiltration membranes of size ranging 30kDa to 100kDa can be utilized for purifying viral particles based on the size of particles. Optimum recovery was shown in the retentate while removing host cell proteins. Pore plugging due to pore size variability in membranes, plugging of proteins, small viral fragments reducing the flow rate, declining membrane reflux, and requirements of high throughput pressure system is shown in many studies [91, 96, 98, 99]. As described above regarding the current ultrafiltration methods, to overcome challenges microfiltration can be a better option. As disadvantages of ultrafiltration add up to high processing cost as the high-pressure system is required. Also, it can increase the processing time and membrane washing steps are required, and there will be reduced flow rate [101]. We are using large pore size membranes which will eliminate this challenges and will be able to provide a cost effective method.

2.4.5. Precipitation and Flocculation

Precipitation is commonly used method for protein concentration [102]. It is typically performed by the salting out of proteins with the high concentration of salts such as ammonium sulphate ($(NH_4)_2SO_4$) or sodium sulphate (Na_2SO_4) [102, 103]. Precipitation by polyethylene glycol (PEG) has been used for virus concentration and purification for viruses such as murine leukemia virus [104] and influenza virus at a low concentration of PEG [105]. Several PEG-based precipitation experiments have shown that PEG precipitates proteins [106-108] when used at greater than 5% concentration. Therefore, higher PEG concentrations can concentrate, but not purify virus. While lower concentrations of PEG can purify virus by precipitating virus and not proteins, salt precipitation can precipitate proteins and can affect the virus integrity [102]. Flocculation using polyaluminum chloride has shown flocculation of bacteriophages due to influence on the surface characteristics of phage particles, but this method also affects viral activity and shows inactivation of bacteriophages [109]. This flocculation in the presence of polyaluminium chloride happens because at that pH the surface charge of phages becomes neutral and electrostatic forces becomes insignificant which drives particles to

aggregates. The study for selective precipitation of Immunoglobulin G (IgG) and proteins showed that different concentration of ammonium sulfate for precipitation. The Concentration of 2.5 M ammonium sulfate found to precipitate bovine serum albumin but not IgG [110]. **Table 2-5** shows some methods from literature for precipitation of viruses.

Table 2-5 Various precipitation method used for purification and recovery of viruses

Virus	**Precipitation method**	**Reference**
Ocean Viruses	Recovery using $FeCl_3$ flocculation method with tangential flow filtration, 90% recovery.	[111]
Enterovirus & MS2 bacteriophage	Recovery using ammonium sulfate (low pH 3.5) precipitation method with centrifugation, 70% enterovirus recovery, 84% bacteriophage recovery.	[112]
Recombinant VLP from yeast homogenate	Recovery and purification PEG precipitation (PEG 6000) using centrifugation, 90% recovery in sediment	[113]
Bacteriophage	Purification using salt precipitation with $MgSO_4$, NaCl, and PEG PEG 6000+ NaCl: 92% recovery PEG 6000+ $MgSO_4$: 91% recovery	[102]
PPV	Virus removal using osmolyte mannitol and alanine flocculation method followed by microfiltration, 80% removal of PPV	[6]
SINV	Virus removal in the presence of osmolyte mannitol as flocculating agent and removal using microfiltration, 96% removal of SINV with mannitol	[7]

Precipitation or flocculation is typically followed by centrifugation or filtration to either remove or recover the flocculated/precipitated species. Flocculation prior to filtration is highly desirable as it can reduce the membrane fouling, increase product recovery and decrease the number of steps for final product purification [114, 115]. While in **Table 2-5**, it is shown that $FeCl_3$, $MgSO_4$, NaCl, and PEG have been used for

the flocculation of viral products, previous work in our lab has demonstrated that a class of natural compounds called osmolytes can flocculate virus particles without affecting proteins.

Our lab's previous studies have explored a variety of osmolytes for flocculating the model non-enveloped and enveloped viruses, PPV and SINV [6, 7]. Different osmolytes and concentrations were screened for their ability to flocculate and remove the virus with a 0.2 µm filter. The osmolytes mannitol, alanine, glycine, trehalose showed the removal of PPV of greater than 80% (**Figure 2-3-A**). Comparing the size of the virus, which is about 20 nm, to the 0.2 µm filter, this results supports the hypothesis that the osmolytes can flocculate virus particles. For the enveloped virus SINV, mannitol, glycine, betaine, and proline were able to remove >80% of the virus with a 0.2 µm filter **(Figure 2-3-B)**. Mannitol was able to remove 98% of the SINV in solution.

Studies were carried out to conclude the effect of ionic strength and pH on the removal of PPV and SINV. Increasing the ionic strength of solution by addition of salt 0.2 to 0.6 M NaCl to 1M mannitol decreased the PPV removal, possibly because the addition of salt affected the viscosity of the solution, decreasing flocculation. Increasing the ionic strength of SINV solution did not change the removal. While in the case of the control studies using water and PBS [6, 7], increasing the ionic strength increased removal, likely due to the salting out effect which also affects proteins. Effect of pH on the virus interaction for osmolyte flocculation has been shown for PPV. The interaction between zwitterionic osmolyte compound and PPV and SINV is favorable near the virus pI [6, 7]. When the virus is negatively charged above its pI, there are ionic repulsive forces which likely decrease virus aggregation.

Shear stress studies were carried out which includes incorporation of high stress during virus flocculation [6, 7]. Results showed that applying high shear to the flocculated samples reduced the removal of viruses as stress is breaking the flocs. Keeping the disturbances to the samples to the minimum extent during filtration can help to maintain the flocculation and optimize virus yield, demonstrating that pump choice

and filtration configuration will be important to the industrial implementation of this method.

Looking at the data for both PPV and SINV, it was determined that mannitol was the flocculant that had high removal for both viruses. So, we decided to use mannitol as our flocculating osmolyte for further studies to demonstrate recovery and purification. Since we were unable to recover the virus from the membrane surface using dead end filtration, we also changed the filter configuration to leave virus in solution and relieve the need to recover the virus from the membrane filter.

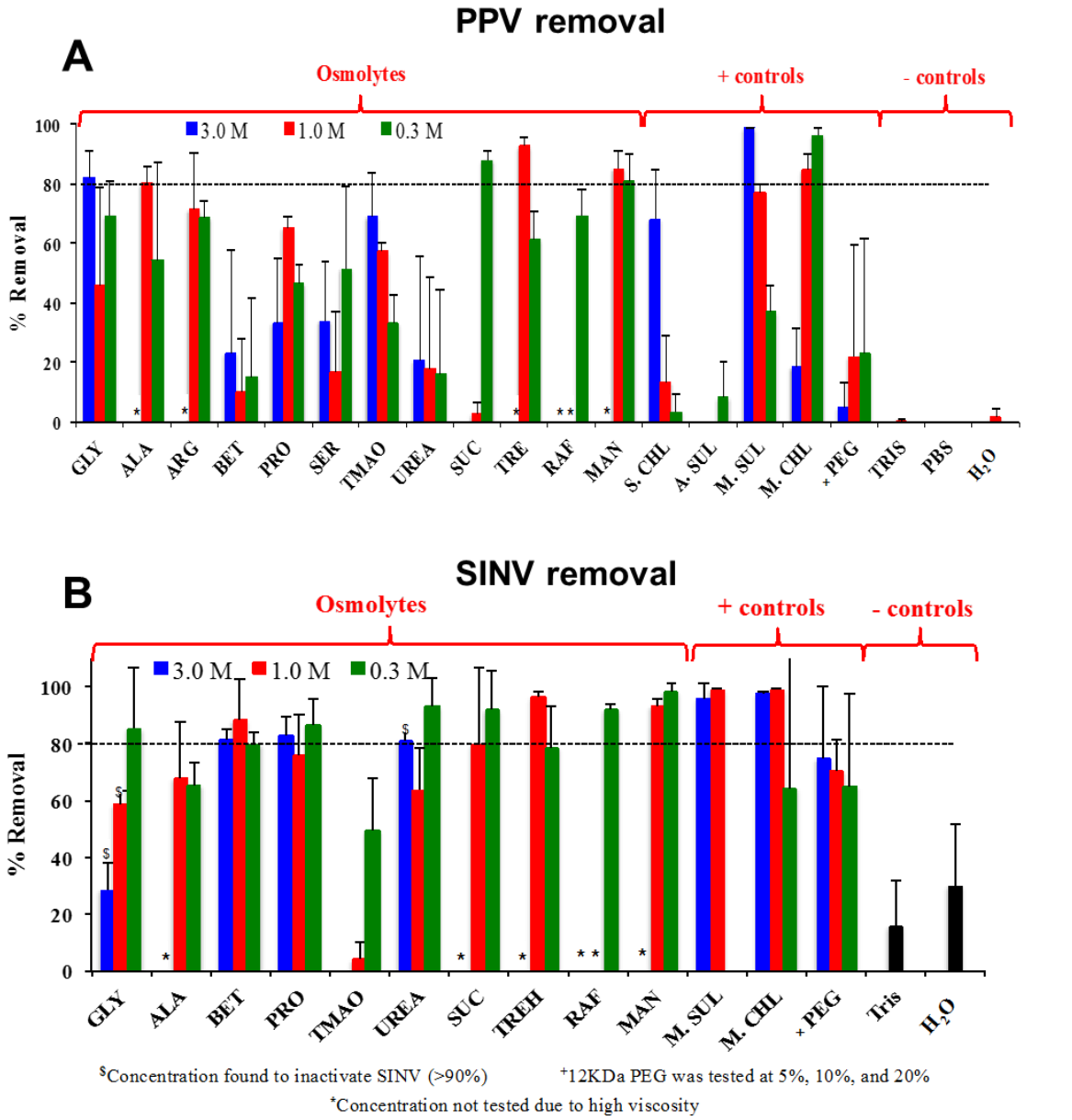

Figure 2-3 Screening of osmolytes, salt, PEG, control tris buffer and water for removal of PPV and SINV, virus removal was calculated using flocculated virus titers before filtration and after filtration. All Experiments performed using 0.2 µm 96-plate filtration, flocculation time allowed was 2 hours [6, 7, 116]. Images taken with permission from publisher and author, permission attached in the appendix.

2.5. Osmolytes

Osmolytes are naturally occurring compounds found in the cells of many organisms, including mammalians and marine animals. As the name osmolyte suggest, they help in maintaining osmotic pressure in cells by pulling water towards them, especially marine animals who live in high salt environments [117]. This mechanism of osmolytes helps to control the cell volume by changing the water content of the cells. Osmolytes are also known to stabilize the proteins. There are two types of osmolytes, depending upon their action with the proteins, protecting and denaturing. The categories of osmolytes are N-oxides, amino acids, sugars and polyols, and denaturing, which includes urea and guanidine hydrochloride [118]. Protecting osmolytes force protein folding by excluding the protein backbone from water molecules as they do not bind directly to the proteins while denaturing osmolytes causes the proteins to unfold by binding to the protein backbone. Naturally occurring osmolytes help in stabilizing proteins against denaturing stresses by disruption of unfolded state of proteins in the presence of osmolyte [117, 119]

Although there is no universal theory behind the mechanism of osmolytes interaction, work by Street and Bolen has shown that the strength of osmolytes to interact with the protein backbone may explain osmolytes ability to stabilize proteins [117]. In their study, the ΔG, or Gibbs transfer energy of transfer, was measured for the protein backbone being transferred from water to a 1M osmolyte solution. Protecting osmolytes have a positive ΔG free energy of transfer, demonstrating that osmolytes interact unfavorably with the protein backbone. This suggests that the osmolytes do not bind to the protein backbone. Instead, they are binding to the water around the protein surfaces resulting in a depletion of water around the protein backbone. In contrast, denaturing osmolytes have a negative ΔG transfer energy, demonstrating a favorable interaction between osmolytes and protein backbone. This suggests that the mechanism of protein denaturation by denaturing osmolytes is due to direct binding of the osmolyte to the protein backbone. We hypothesize that the ability of protecting osmolytes to control the water structure around the proteins can be used as a potential flocculant for the viruses as

virus capsids are made of proteins. Our hypothesis is based on the preferential hydration mechanism of osmolytes. As due to active hydrophobic surface on virus particles, when water is removed around the viruses they tend to aggregate with each other. The addition of osmolyte to virus solution will structure water around the viruses, removing water around can help the viruses come together to form the bigger flocs due to the hydrophobicity of viruses [6, 7, 116].

3. Materials and Methods

3.1. Materials

Mannitol ($C_6H_{14}O_6$), thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS) hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Reagents for cell culture, minimum essential medium (MEM), phosphate-buffered saline (PBS, pH 7.2), 0.25% trypsin/EDTA, and penicillin/streptomycin (pen/strep) and gentamicin (gentamicin sulfate) were purchased from Life Technologies (Carlsbad, CA). Tryptose phosphate broth (TPB) was purchased from VWR supplier (Radnor, PA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). We used NanoPure water with a resistivity of >18 MΩ.cm (Thermo Scientific, Waltham, MA) for all the solution preparation and were sterile filtered using 0.2µm syringe filter (Nalgene, Rochester, NY) or with a 0.22 µm bottle top filter (Millipore, Billerica MA). For the HPLC study, HPLC grade acetonitrile 99.93% and trifluoroacetic acid (TFA) ≥99.0% were purchased from Sigma-Aldrich (St. Louis, MO). For TEM work, propylene oxide, glutaraldehyde (Grade I, 70% in H2O), sodium cacodylate (BioXtra, ≥98%), lead citrate (purum, for electron microscopy) and agarose gel (Type I, low EEO) were acquired from Sigma-Aldrich (St. Louis, MO). The embedding kit for preparing samples for electron microscopy EMBed-812 embedding kit, osmium tetraoxide 2% aqueous solution and uranyl acetate were obtained from Electron Microscopy Sciences (Hatfield, PA).

3.2. Cells and Viruses

3.2.1. Cell culture

Porcine kidney (PK-13) cells are grown and cultured in (MEM) completed with 10% FBS and 1% pen/strep. Baby hamster kidney (BHK) cells are grown and cultured in MEM completed with 10% FBS, 5% tryptose phosphate broth (TPB) and 1% gentamicin. To maintain the cells, they were washed in PBS, followed by addition of 3 ml of trypsin for removing the attached cells from the flask wall. PK-13 cells are more adherent to the

flask wall, so after waiting 10 minutes we hit the flask manually to remove cells from wall. BHK cells are easily detached from the flask wall in 3-5 minutes. After cells detach from the wall, trypsin was neutralized using equal quantity of completed media. Cells were separated from trypsin by centrifugation in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) at 1500rpm for 3 minutes. PK-13 cells were propagated every 3 days when the confluency reached >80% and a split ratio of 1:5. BHK cells were propagated every day with a split ratio of 1:3. All cells were maintained at 37°C, 5% CO_2 and 100% humidity.

3.2.2. Virus preparation and titration

PPV virus was propagated in PK-13 cells. PK-13 cells were seeded at a density of 6 x 10^5 cells/flask and incubated for about 24 hours, with the goal of 90% cell confluency. The media was removed, and the flasks were inoculated with 1 ml of PPV at a concentration of 10^3 MTT_{50}/ml diluted in PBS, 3% FBS and 1% pen/strep. After 1.5 hours, 9 ml of fresh media was added to the flasks and the infected cells were incubated for five days. Flasks were frozen at -20°C, scraped and clarified using centrifugation at 5000 rpm for 15 minutes at 4°C using Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA). The clarified virus solution was stored at −80°C.

SINV was propagated on BHK-21 cells. Similar procedures were followed for preparation of SINV except where noted. After cells were inoculated with SINV, infected cells were incubated for two days until the cells lysed. The cells were scraped without freezing and clarified. Clarified SINV solution was stored using 10% glycerol and kept in -80^0C until further use for experiments [120].

Virus quantification was done using the colorimetric cell viability assay, the MTT assay, as described earlier [120]. This assay determines the concentration of infectious virus needed to maintain a 50% cell viability. For PPV, PK-13 cells were seeded in a 96-well plate at a cell density of 8×10^5 cell per well and for SINV, BHK cells were seeded at a cell density of 5×10^5 cells per well. Plates kept in an incubator for 24 hours and infected with samples to be analyzed. Typically, the samples were diluted 5-fold across the 96-

well plate. After incubation (5 days for PPV, 2 days for SINV) 10 µl/well of 5 mg/ml MTT solution (tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in PBS was added to the plates. After 4 hours the solubilizing agent (0.01 M HCl and 10% SDS in water) was added and plates were incubated overnight. The ddition of MTT solution forms purple color formazan inside the mitochondria of metabolically active cells and the addition of solubilizing agent solubilize this formazan. Upon dissolving the formazan crystals, the cell viability was quantified by measuring the absorbance of the solution at 550 nm using a Biotek Synergy Mx plate reader (Winooski, VT). The virus infectivity was calculated using the MTT_{50} 50% infectious dose value which can be determined based on absorbance values of infected cells, as described earlier by Heldt et.al [120]. So for this analysis, we grow cells in 96 well plates and infect cells in serial dilution manner with the collected virus sample from experiments. After infection and incubation MTT solutions mentioned above are added. Based on 50% dose, the absorbance values are recorded for average uninfected cell wells. This reflects the infectivity in terms of logarithmic factor based on dilution factor. The majority of analysis was performed using 5-fold series dilution in 96- well plates, and for high titer values 10- fold series dilutions were used.

3.3. Virus Flocculation and Diafiltration

1M mannitol in NanoPure water was prepared fresh for every experiment to avoid dissolution. For flocculation of virus particles with osmolytes, 9720 µl of mannitol and 405 µl of virus (PPV and Sindbis at 6 log MTT_{50}/ml in PBS, unless stated otherwise) were mixed and kept for 2 hours at room temperature with manual rotation every 15 minutes. This 1M concentration and ratio of osmolyte to the virus was adapted from our previous studies in which we used 720 µl osmolyte with 30 µl of the virus [6, 7]. As a control, a water and virus mixture were also prepared and kept for 2 hours. The ratio of solutions was kept the same for all experiments with enveloped and non-enveloped virus.

Flocculated virus with osmolyte solution was filtered using a batch diafiltration method as shown in **Figure 3-1**. A 10 ml Amicon filtration cell filter (Model 8010), a gift

from EMD Millipore, was equipped with different pore size membranes. The membranes were Durapore Membrane Filter PVDF 0.2 μm and 0.1 μm and BioMax's polyethersulfone (PES) 500 kDa and 300 kDa MWCO membranes, also gifts from EMD Millipore. All membrane size were 25 mm and fits in filter holder of Amicon cell filter model 8010. Filtration cells were operated at 10 psi pressure, unless otherwise stated, from a compressed nitrogen tank and without stirring as stirring could break the flocculated particles. Initially, 10ml of mannitol and virus solution was added to the filtration cell, and pressure was applied until 2-3 ml of filtrate was collected, with 2 ml being held up in the outlet tubing. This was the first fraction collected for filtrate (Filtrate 1 or F1). A 300 μl sample was removed from the retentate and labeled retentate 1 (R1). After collection of the first fraction, 5 ml of 1M mannitol was added to make a total of 10 ml solution in the filter unit. The pressure was applied to the filtration cell unit and the second fraction was collected. Similarly, for the third fraction, 5 ml of mannitol was added and filtrate (F3) and retentate (R3) were collected immediately. The diafiltration method is illustrated in **Figure** 3-1. As a control, the same procedure was applied using water instead of mannitol and all samples were analyzed by titration using an MTT assay mentioned earlier in section 3.2.

As we are adding more diavolumes to the diafiltration for collecting second and third fractions, the addition of more osmolyte solution may be causing breaking of flocs, to solve this problem we decided to perform flocculation time studies in between the fractions. In flocculation time studies, after addition of osmolyte, we allowed settling the virus and osmolyte solution more time prior to filtration. All samples collected were analyzed using the MTT assay.

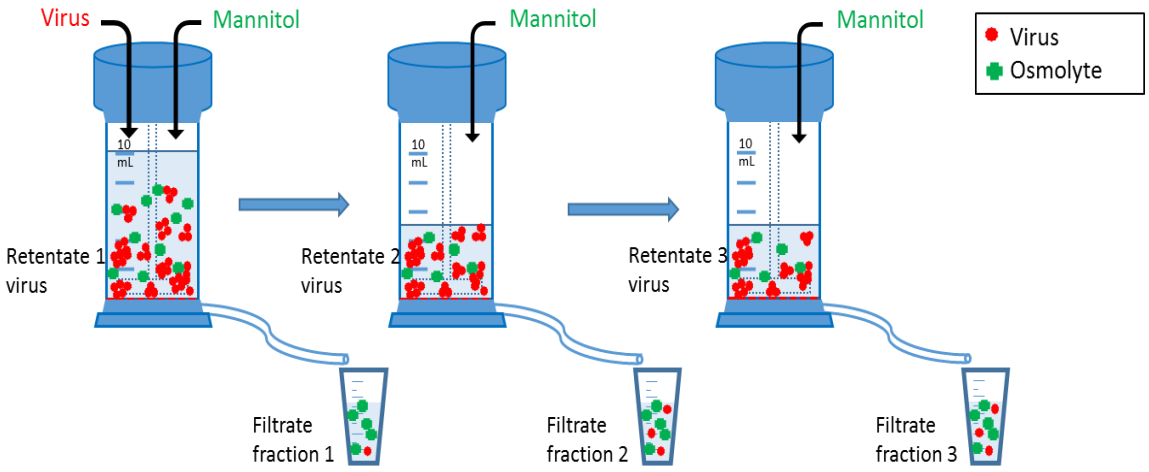

Figure 3-1 Batch diafiltration setup for recovery of infectious virus particles using filtration cell with manual addition of virus and mannitol, samples collected in three fractions with 5ml addition of mannitol at 2nd and 3rd fraction

We also explored different concentrations of infectious virus particles as a starting material for flocculation. A variety of starting concentrations were analyzed using the same parameters as stated earlier with diafiltration. Mannitol solution for all samples was prepared in water at 1M concentration unless otherwise stated. For the high concentration titer solutions (log 11 MTT_{50}/ml for PPV and log 10 MTT_{50}/ml for SINV), 1M mannitol was prepared in virus solution instead of water to avoid dilution of virus concentration taking measured quantity of mannitol powder dissolved into virus solution.

All the results obtained through MTT assay gave the titer values for each collected samples. The MTT_{50}/ml values were converted into mass per MTT based on the volume of samples. The step yield of infectious virus particles was calculated at each fraction of retentate with respect to input at each fraction as shown following equations i.

$$Y_i = \left(\frac{Mass\ per\ MTT\ for\ retentate\ i}{Mass\ per\ MTT\ for\ input\ in\ i^{th}\ fraction} \right) \times 100 \qquad \text{(i)}$$

Where Y_i represents step yield in the fractions, where i starting from 1. Step yield calculations were performed considering input at each fraction as we are removing some

quaintly from retentate for the analysis thus reducing the number of particles in each input. All percent recovery values for each experiment were averaged over repeated experiment to get more accurate data. After calculation of step yield at each fraction, overall yield was calculated based on step yield for 2nd and 3rd fraction as described earlier in equation (i), where for 2nd fraction it will be i+1, and for 3rd fraction i+2. Recovery for first remains the same for unit yield calculations, and overall yield calculations were performed using equation (ii).

$$Overall\ Y_{i+1} = Y_{i+1} \times Y_i \qquad \text{(ii)}$$

3.4. HPLC C-18 Chromatography

The samples collected from diafiltration using the 0.1 µm filters for PPV were analyzed for purification using reverse phase chromatography (RP-HPLC). A Waters XBridge BEH C18 Column 4.6 mm × 150 mm was used for samples analysis on a Waters® e2695 HPLC equipped with a Waters 2998 Photodiode array detector UV/Vis spectrophotometer detector. A sample volume of 10 µl was injected onto the column, and all the samples were run at the same conditions. The mobile phase used for started at 100% buffer A, comprised of 0.1% TFA in water, and an increasing buffer B, comprised of 0.1% TFA in acetonitrile. The flow rate for all samples was maintained at 0.500 mL/min, a sample temperature of 15°C and a column temperature of 25°C. The column was washed in between samples using 100% acetonitrile. The area under the peak was analyzed using Empower software which uses the numerical integration method using trapezoidal rule equation (iii).

$$Area\ under\ the\ curve = (t_2 - t_1)\left[\frac{f(t_1)+f(t_2)}{2}\right] \qquad \text{(iii)}$$

Where t_1 and t_2 are the time range for chosen peaks with respect to UV absorbance value at that times. Using the area under the curve, we further calculated percent removal of peak area in different peaks using equations **(iv) and (v)** where A_{BF} represents area

under the curve of before filtration sample, A_{F1} for filtrate F1, and A_{R1} for retentate R1 sample.

$$Percent\ removal\ in\ Filtrate\ F1 = \left(\frac{A_{BF} - A_{F1}}{A_{BF}}\right) * 100 \quad \text{(iv)}$$

$$Percent\ removal\ in\ Retentate\ R1 = \left(\frac{A_{BF} - A_{R1}}{A_{BF}}\right) * 100 \quad \text{(v)}$$

3.5. DNA Quantification

DNA quantification was done using the Quant-iT PicoGreen (Invitrogen, Eugene, OR) dsDNA reagent. All the samples collected from diafiltration were analyzed for dsDNA contents. For PPV and SINV infectious particles recovery, diafiltration was done using 0.1 µm pore size filter, and concentration of PPV and SINV used at log 8 MTT_{50}/ml. At lower concentration of log 6 MTT_{50}/ml for PPV and SINV, we were unable to detect DNA content as it was below detectable threshold levels. For preparing all PicoGreen reagent 10mM Tris-HCL1 1mM EDTA buffer (TE Buffer) was used at pH 7.5. A standard curve was obtained using Lambda DNA standard at 2 different range of standard 1 ng/mL to 1 µg/mL and 25 pg/mL to 25 ng/mL so as to detect even low concentration of DNA contents. PicoGreen reagent and DNA standard are diluted using TE buffer. All reagents prepared in a plastic container rather than glass as reagent may absorb to a glass surface and also protected from light using aluminum foil as reagents are light sensitive. The lambda DNA standard, given at 100 µg/mL concentration in the kit, dilute it to 50-fold in TE buffer to make the 2 µg/mL as base solution. This 2 µg/mL is diluted in series and used for standard curves. Then added aqueous working solution of Quant-iT PicoGreen reagent to each well of 96-well plate along with standard DNA samples and unknown samples from diafiltration. After mixing well and incubating for 2-5 minutes at room temperature protected from light, 96-well plates were read at fluorescein wavelengths (excitation ~480 nm, emission ~520 nm) with a fluorescence microplate reader Synergy Mx plate reader. Calculations for our samples DNA quantification was performed using slope equation of standard curve for both standard

range and low concentration range also. Removal of DNA content per step in retentate samples was calculated using equation (vi).

$$Percent\ removal\ of\ DNA\ = \left(1 - \left(\frac{DNA\ content\ in\ retentate}{DNA\ content\ in\ initial\ input}\right)\right) \times 100 \quad \text{(vi)}$$

DNA removal at each fraction was calculated using equation (vi) and input at each fraction was taken into consideration the amount of sample removed for analysis to get more accurate results over each fraction.

3.6. Transmission Electron Microscopy

For studying flocculation of PPV with mannitol, TEM imaging of flocculated and non-flocculated samples were taken. Samples prepared as a mixture of 1M mannitol with PPV (720 µl mannitol and 30 µl PPV at log 6 MTT_{50}/ml and similarly with water with PPV (720 µl water and 30 µl PPV at log 6 MTT_{50}/ml) and allowed for flocculation for 2 hours. Flocculated virus samples were then inactivated before imaging for safety purpose using 0.5% glutaraldehyde for 1 hour [121]. Samples were mounted directly on copper grids (EMS200-Cu, Electron Microscopy Sciences) using 10 µl sample with micro-pipette and air dried overnight. Grids were washed in nanopure water and then stained in droplets of 2% uranyl acetate on the parafilm wax paper for 2 minutes. Grids were washed again after staining with pure water to prevent contamination. TEM images were captured on a JEOL JEM-2010 (Peabody, MA) and imaging was done at 80 kV and 30000x magnification and 40000x magnification.

4. Results and Discussion

To develop a platform approach, we have selected non-enveloped PPV and enveloped SINV as model viruses to explore the potential of flocculation as a universal platform virus purification process. Our previous work has demonstrated that a range of osmolytes such as alanine, glycine, trehalose, mannitol successfully flocculate viruses and not model proteins. This is likely due to preferential hydration that causing virus aggregation. From the screened osmolytes, 3 M glycine was able to remove 96% PPV particles, and 1 M mannitol showed >80% PPV removal [6]. We also showed mannitol flocculation for enveloped SINV particles was able to achieve 96% removal at a mannitol concentration of 0.3 M [7]. From this work, in an effort to develop a novel virus recovery and purification process we decided to pursue flocculation with mannitol as it worked for both model enveloped and non-enveloped virus systems. We are using mannitol at optimal 1M concentration to work with both PPV and SINV virus purification and recovery.

4.1. Recovery of Non-Enveloped Porcine Parvovirus (PPV)

4.1.1. Overall recovery of PPV using different filter pore size

The first parameter studied was with different filter pore size. PPV is a small mammalian virus with a diameter of 18-24nm [13, 14]. The screening work demonstrated that PPV could be withheld with a 0.2 μm filter. However, since we wanted high recoveries for such a small virus, we decided to start with the largest pore size of 0.1 μm. The results are shown in **Figure 4-1-A.** The largest pore size was able to obtain a recovery of 58% and the smallest pore size, a 300 kDa MWCO membrane, was able to recover 85% of the infectious particles, demonstrating that as the pore size was reduced, the recovery increased. Both pore sizes showed a significant difference from the water negative control as smaller virus particles of PPV in water were able to pass through the membrane pores, but mannitol caused flocculation to allow the virus to be withheld by the filter. For the 500 kDa MWCO membrane, the negative control recovery was not

significantly different from the mannitol recovery. This could be due to self-aggregation of viruses in that batch during virus production based on the contaminant concentration in specific batches. High contaminants in virus stock may cause HCP aggregates and pore plugging in control studies. We also measured the amount of virus in the filtrate, and the mass balanced closed. Therefore, there was no indication that the virus was absorbing to the membranes or the filter housing.

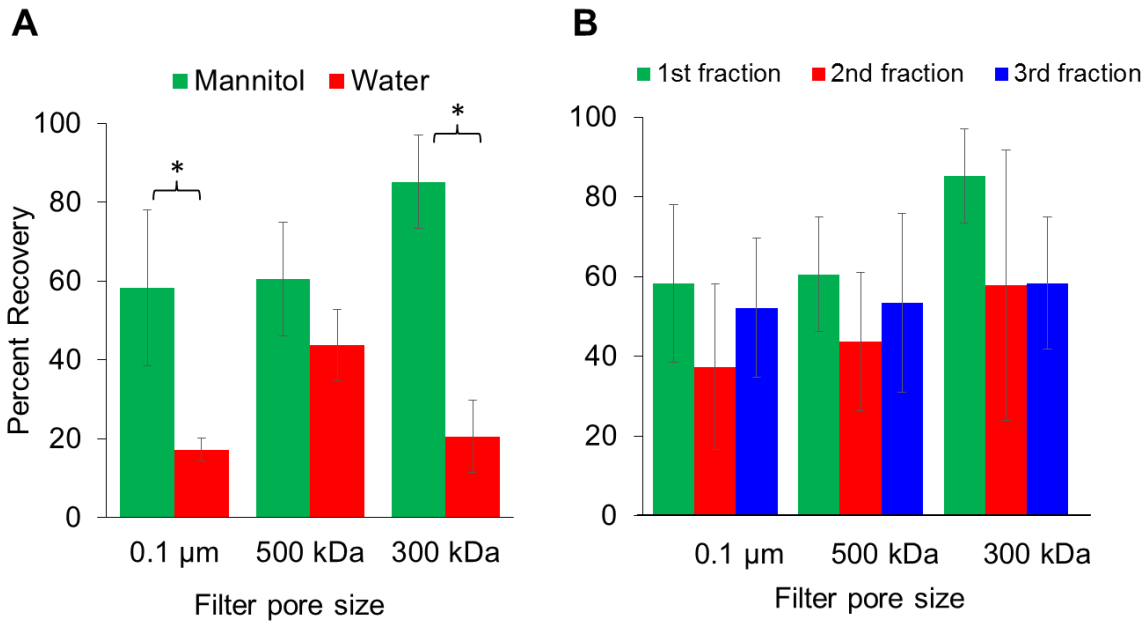

Figure 4-1 Recovery of porcine parvovirus using different pore size filters. A: Comparison of the recovery of PPV using 1M mannitol and the negative control water. B: Step yield of retentate samples for flocculation with 1M mannitol after each batch diafiltration fraction. Details are shown in Figure 3-1. Error bars are the standard deviation of three separate trials *$p<0.05$

To explore the ability of this system to recovery infectious in diafiltration mode, we did additional batch fractions by adding pure mannitol to replace the filtrate volume. This is shown in **Figure 4-1-B.** Each fraction remained at a consistent recovery except for the smallest pore size, where the 2nd and 3rd fraction dropped due to disturbances in the filtration unit while adding more mannitol. As we are adding more mannitol to system, we are filtering immediately, this allows very less time for osmolyte flocculation for interaction. As the aggregates are temporary manual disturbances in system breaks aggregates and smaller virus particles are passing through the membrane.

4.1.2. Flocculation time effect

To increase the recovery of the 2nd and 3rd fractions, we increased the incubation time. The flocculation time of the first fraction was set at 2 hours due to the 2 hours quiescent time that was used in the original screening work [6, 7], but the subsequent fractions were only given 5 minutes from the addition of mannitol until the filtration. Our previous work also demonstrated that the flocs are sensitive to shear stress [6, 7]. Therefore, it was hypothesized that the filtration immediately after mannitol addition could have broken the flocs from the shear stress of mixing. We increased the filtration time as shown in **Table 4-1**. The times were randomly chosen to minimize waiting time and to optimize experimental duration as no previous studies had been performed on the time effect of osmolyte flocculation. The results of increasing the time can be found in **Figure 4-2**. There was not a significant difference between the recovery of PPV at the 0.1 μm filter size. However, the averages vary due to the large error that is found in the MTT assay used to measure these values.

Table 4-1 Flocculation time effect between fractions of diafiltration for PPV

Fraction	**Original diafiltration time[a]**	**Increased diafiltration time**
1st fraction	2 Hours	2 Hours
2nd fraction	Immediate fraction collection after addition of mannitol	45 minutes flocculation time
3rd fraction	Immediate fraction collection after addition of mannitol	30 minutes flocculation time

[a]Diafiltration time in **Figure 4-1**.

As a control study, the PPV solution was flocculated with water instead of mannitol (data not shown). Control study results show that there is no difference in the recovery with normal flocculation and timed flocculation study and the overall recovery is 16% in the first fraction with water as flocculation agent. In conclusion, we did not observe a significant difference in PPV recovery with increased hold time between fractions. We saw in the figure 4-1 that addition of more mannitol gives very little improved recovery

(no statistical difference) and allowing time between fraction shows less difference in significant flocculation effect. In future allowing higher time for flocculation in between fraction can be tested to see does it help in aggregation.

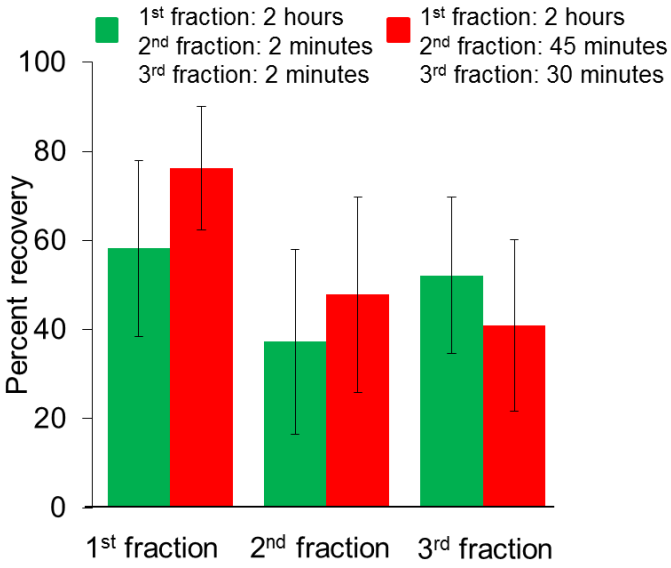

Figure 4-2 Flocculation time effect in between the fractions during diafiltration using a 0.1 μm pore size membrane for PPV, Comparative study of step yield of PPV with original diafiltration time (green columns) and increased flocculation time (red columns) using 1M mannitol flocculation. Error bars are the standard deviation of three separate trials.

4.1.3. Recovery of PPV with different starting concentration

To study the effect of the starting material concentration on flocculation, we explored different initial PPV concentrations, and the results can be seen in **Figure 4-3**. All the diafiltration experiments were carried out at a back pressure of 10 psi, however, for the log 11 MTT_{50}/ml, the pressure required for a system for flow-through was 30 psi. This indicates that there was membrane fouling at the higher PPV concentrations as flux is decreased. Results from this study show that using a starting material concentration of log 9 MTT_{50}/ml increases the recovery of PPV in the retentate as compared to previous experiments with log 6 MTT_{50}/ml starting concentration.

We also explored the recovery of different diafiltration fractions, shown in **Figure 4-3-B**. Even though no wait time was given for the flocculation, the recoveries remained similar to the first fraction.

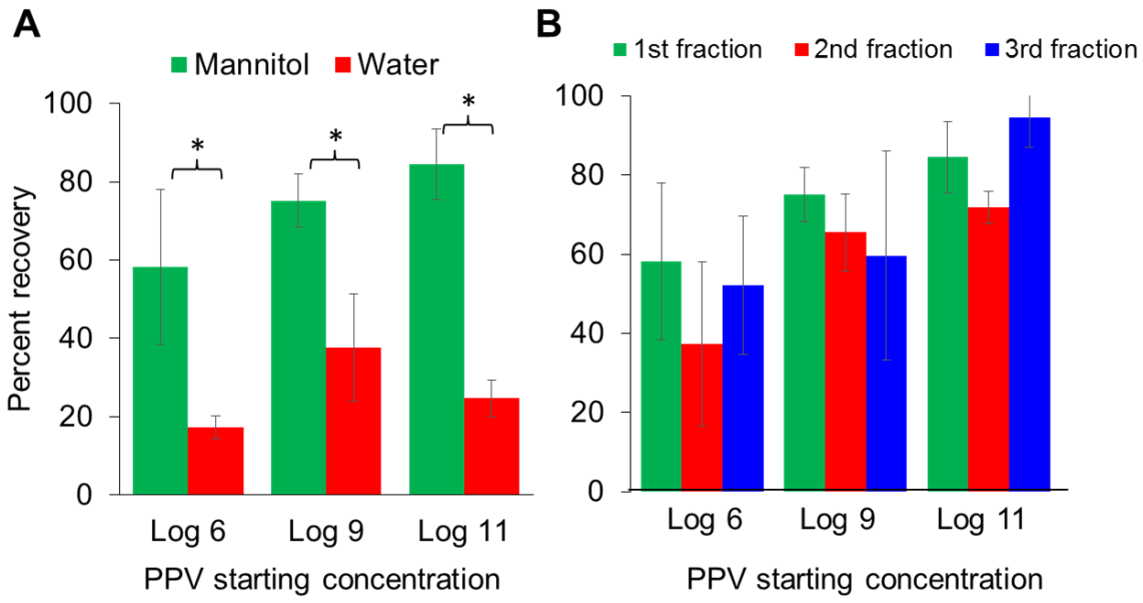

Figure 4-3 Recovery of porcine parvovirus with different starting PPV concentrations as log 6, log 9 and log 11 MTT_{50}/ml with diafiltration experiments, the filter used for all experiments is 0.1 µm. A: Comparative percent recovery with mannitol and control water for 1st fractions. B: The step yield using 1M mannitol and PPV with different concentration for all collected fractions during diafiltration. Statistical difference is calculated using Avona factor between mannitol and water 1st fractions, *$p<0.05$. Error bars are the standard deviation of three separate trials.

4.1.4. Purification of PPV

The protein content of samples after diafiltration was analyzed using HPLC reverse phase chromatography on the C18 column. As we perform the diafiltration, the impurities from serum proteins present in the cell culture media are hypothesized not to be affected by mannitol flocculation. Thus after filtration, the impurities will pass through the filter and the retentate should contain the virus and a lower concentration of serum proteins. Since the total serum protein content was low to begin with, we used reverse phase chromatography to measure the protein content. In **Figure 4-4** we can see the chromatographs for PPV flocculated with mannitol before filtration, after filtration, and the PPV retentate. We performed this study in duplicates for each sample to make sure to

minimize any error in sample eluting. From the chromatographs, we can see that before filtration and filtrate are showing 2 major peaks while retentate graph has no major peaks. The analysis shows that with diafiltration, we are able to purify the retentate from the bovine serum albumin which is coming from the fetal calf serum in the completed media.

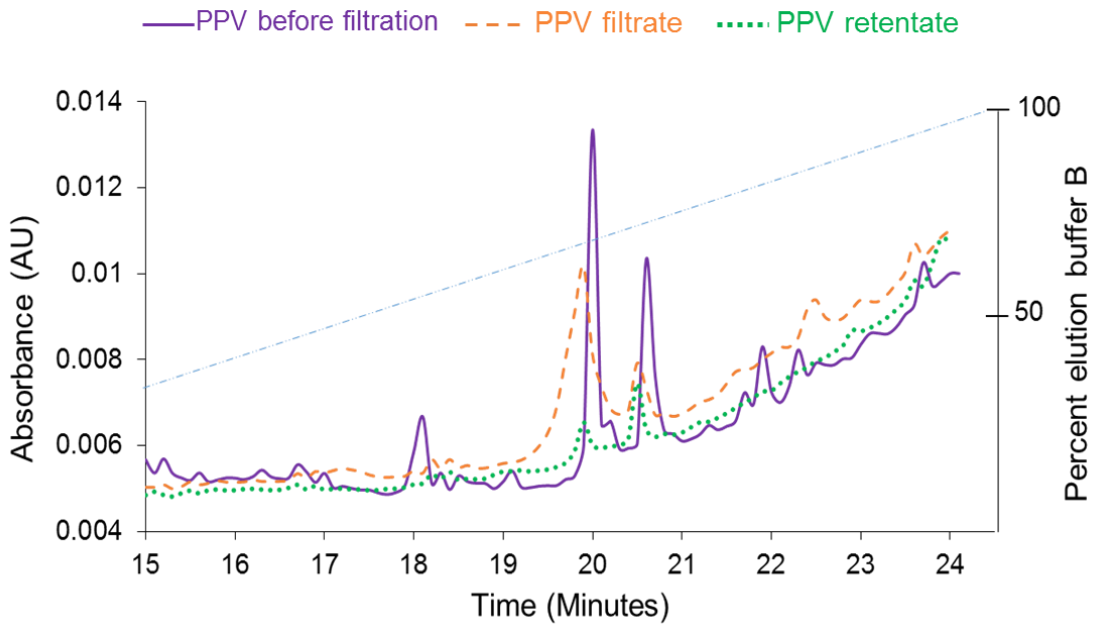

Figure 4-4 Purification of PPV using diafiltration. Reverse phase chromatography using C-18 column of the diafiltration samples collected with 0.1 µm pore size membrane before filtration(purple), after filtration (dashed orange), and retentate (dotted green) with buffer A as 0.1% TFA in water and eluting buffer B as 0.1% TFA in acetonitrile (dotted blue line).

Further, we also determined the area under the curves of the peaks in the chromatograms to calculate the percent removal of impurities using equation 3.8. The area under the curve was taken from area integration calculation from the Empower software that controls the HPLC. With our calculations, the percent removal of impurities in the retentate PPV sample was found out to be 85% as compared to the before filtration sample. This is an acceptable result for a single diafiltration pass. We are able to get good recovery and high purification as recovery above 30% is acceptable standard in the industries [101]. More purification may be possible with the additional diafiltration fractions. With the successful recovery of infectious PPV particles and purification from

HCP, this is a good ground for future work to setup a prominent downstream processing using osmolyte flocculation and microfiltration.

4.1.5. DNA quantification for PPV

DNA quantification was performed on all the diafiltration collected samples before filtration and after filtration samples were analyzed to see whether flocculation with osmolytes helps to remove the host cell DNA contents from virus particles. Results show in Figure 4-5 that the negative water control is able to remove more DNA than mannitol flocculation. Therefore, the osmolytes likely flocculate the DNA. DNA removal for PPV was about 45% in the first fraction and less than 7% DNA removal in next fractions due to the addition of mannitol flocculated DNA remaining from the previous step. While in the case of water, as there is no flocculation mechanism occurring it was not affecting the DNA content and it was showing DNA content removal >60% in 1st fraction and about 40% in each next fractions. This was due to flushing out the DNA from the retentate.

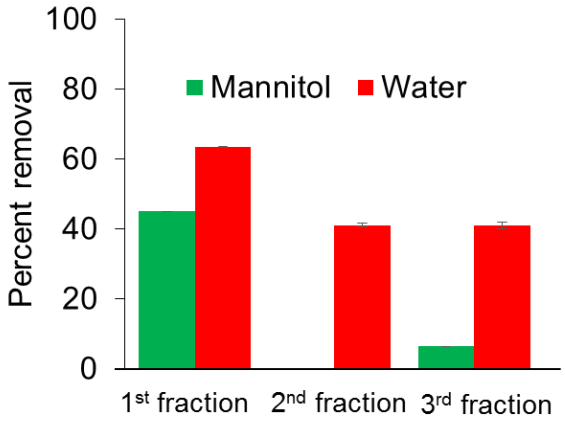

Figure 4-5 DNA quantification for PPV. Percent removal of DNA in retentate samples for PPV by mannitol flocculation using Picogreen DNA quantification. All samples collected using 0.1 µm filter pore size membrane and titer log 9 starting material for diafiltration. Error bars are the standard deviation of two separate trials

4.1.6. Overall PPV Recovery

The overall summary for the purification and recovery of PPV is shown in **Table 4-2**. This data shows that in the retentate samples, we were able to retain high PPV titers, however, we did not concentrate the virus particles. Concentration would have been expected since the volume of each retentate samples is reduced by half. Recovery increases with each fraction for the water control because the starting titer of each unit drops. The overall recovery includes all fractions earlier. It can be seen that the overall PPV recovery after 3 fractions with the 0.1 um filter is not statistically different from the water control, but the titer is a little higher. For the smaller pore size filters, it is even more pronounced. More work is needed to improve the recovery of the virus in later fractions. As shown previously, the DNA removal with mannitol flocculation found was low because mannitol is likely flocculating DNA during diafiltration. As DNA are charged particles, reasonable explanation for DNA being aggregated in presence of mannitol can be the reduction of electrostatic forces due to mannitol. This leads to DNA being aggregated and retained above membranes in retentate. Protein recovery was calculated from reverse phase chromatography and shows an 85% protein removal which comes from the cell culture media used in the virus preparation.

Table 4-2 Overall recovery and purification of PPV,. Comparison of concentrations before and after diafiltration, step yield, overall recovery with different filter pore size using diafiltration for PPV recovery along with DNA removal and protein removal for mannitol

PPV			**Flocculation with mannitol**				
Filter pore size	DNA removal[a] (%)	Protein removal[b] (%)	Starting concentration (MTT_{50}/ml)	Final retentate concentration (MTT_{50}/ml) by fraction		Step yield[c] (%) by fractions	Overall recovery[d] (%) by fraction
0.1 µm	45	85	6.63 ± 0.1	1st	6.35 ± 0.2	58 ± 19	58 ± 19
				2nd	5.75 ± 0.3	37 ± 20	21 ± 15
				3rd	5.47 ± 0.4	52 ± 17	10 ± 9
500 kDa	-	-	6.93 ± 0.5	1st	6.70 ± 0.5	60 ± 14	60 ± 14
				2nd	5.29 ± 0.4	43 ± 17	25 ± 8
				3rd	5.94 ± 0.6	53 ± 22	11 ± 1
300 kDa	-	-	6.47 ± 0.2	1st	6.55 ± 0.2	85 ± 11	85 ± 11
				2nd	6.06 ± 0.1	57 ± 33	53 ± 31
				3rd	5.86 ± 0.1	58 ± 16	36 ± 22
			Negative control flocculation with water				
0.1 µm	63	0	6.60 ± 0.2	1st	5.84 ± 0.2	17 ± 2	17 ± 2
				2nd	5.43 ± 0.3	44 ± 22	7 ± 2
				3rd	5.29 ± 0.3	77 ± 27	7 ± 4
500 kDa	-	-	7.04 ± 0.7	1st	6.68 ± 0.8	43 ± 9	43 ± 9
				2nd	6.01 ± 0.7	23 ± 14	9 ± 3
				3rd	5.09 ± 0.5	29 ± 3	2 ± 1
300 kDa	-	-	6.61 ± 0.4	1st	5.96 ± 0.3	20 ± 9	20 ± 9
				2nd	5.64 ± 0.3	55 ± 31	12 ± 11
				3rd	5.44 ± 0.3	81 ± 24	8 ± 3

Error bar are standard deviation from three trials.

a: Based on Quant-iT PicoGreen dsDNA quantification method

b: Based on area under the curve calculations from reverse phase chromatography (RPC)

c: Based on infectious particles titer calculations using MTT50 assay for step yield calculations from equation 3.1-3.3

d: Based on infectious particles titer calculations using MTT50 assay overall recovery calculation from equation 3.1, 3.4 & 3.5.

4.2. Recovery of Enveloped Sindbis Virus

4.2.1. Recovery of SINV using different filter pore size

For the recovery of enveloped SINV, experiments were carried out using 1M mannitol flocculation followed by diafiltration. We started the diafiltration study with a 0.2 µm pore size filter and then studied smaller pore size filters. As the size of SINV is about 50nm [14, 44], we want to accomplish high recovery using as large pore size filter as possible. While in the case of PPV, we started with 0.1 µm pore size filter because of the small size of PPV. **Figure 4-6-A** showing percent recovery for SINV in the first fractions of retentate using different pore size membrane filters with mannitol flocculation and water as control. Recovery of infectious SINV particles using 0.2 µm filter shows 65% with mannitol. Using 0.1 µm membrane for the same experiments shows increased recovery of 77%. Further using smaller membrane of 500 kDa achieved 96% recovery of infectious virus particles in the retentate. A control study for all pore size shows no significant recovery. When a 300kDa membrane was used, SINV was withheld with the control water. Comparative study of different pore size shows an increase in the recovery as we use smaller pore size filter, while control sample does not show increased recovery. This demonstrates that recovery of particles is based on the bigger flocs of viruses formed due to mannitol flocculation.

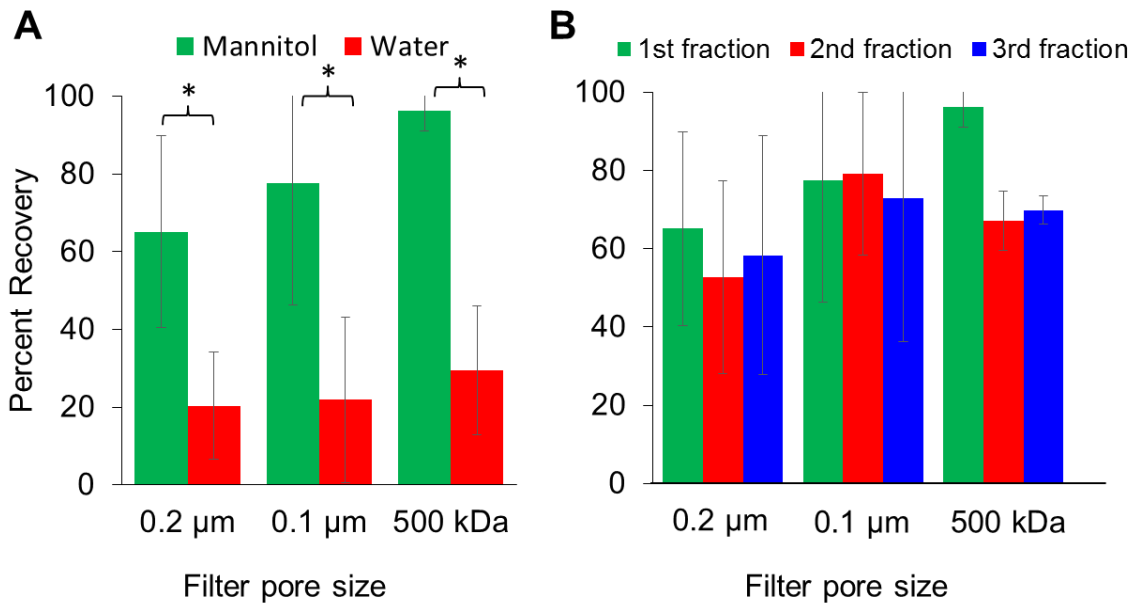

Figure 4-6 Recovery of SINV using different pore size, using 0.2 µm, 0.1 µm, 500 kDa pore size filter with diafiltration method, A: Percent recovery in 1st fraction of diafiltration retentate samples for flocculation with 1M mannitol and control water for different membrane pore size. B: The step yield in all fractions using mannitol flocculation for each filter membrane pore size. Error bars are the standard deviation of three separate trials *p<0.05

Looking further into each fractions of diafiltration, **Figure 4-6-B** shows results for all fractions along with different pore size membranes. Studies with 0.2 µm and 0.1 µm show consistent recovery in all fractions with mannitol flocculation method. Smaller pore size filter 500 kDa was used for diafiltration to achieve a high recovery of infectious virus particles. With 500 kDa pore size filter, we performed diafiltration, and the recovery of SINV particles was about 96% in the first fraction using 1M mannitol flocculation. This is the highest recovery of SINV achieved using 3 different pore size filters as 0.2 µm, 0.1 µm, and 500 kDa filters. After addition of more diavolumes 1M mannitol to the retentate from the first fraction and performing filtration, in the second fraction of retentate it is showing 67% recovery and 69% recovery in the 3rd fraction of retentate as shown in **Figure 4-6-B**. As a control for study with all the three different pore size filter, water is showing low recovery of infectious SINV, which suggesting that water is not affecting the size or flocculation of virus particles at all. Comparing results from **Figure 4.6-A** and **4.6-B**, we can see that we are able to get high virus recovery with mannitol flocculation as compared to water flocculation.

4.2.2. Flocculation Time Effect

Similar studies as mentioned in previous section 4.1.2 for the non-enveloped virus for flocculation time studies was carried out with enveloped SINV. Keeping all the parameters for the study similar except the flocculation time in between the fraction. We performed flocculation time studies using 0.2 µm filter pore size and compared results with 0.2 µm diafiltration without additional waiting time during fractions. Time allowed in between the 1st and 2nd fraction was 45 minutes and for 2nd and 3rd fraction time allowed 30 minutes as shown in **Table 4-3**.

The ratio of the virus with mannitol or water and the initial flocculation time were kept the same for both studies. The recovery of infectious SINV was 66 % in the 1st fraction of retentate which is same as we have seen in normal flocculation study as shown in **Figure 4-7**. After allowing more flocculation time before filtering the 2nd fraction, a significant increase in recovery was observed. Improved recovery can also be seen in 3rd fraction after allowing more flocculation time, however, there was no statistical difference shown. While looking at **the water** control, it showed no effect of flocculation time between fractions there was no significant rise when the flocculation before the 2nd and 3rd fractions was increased (data not shown).

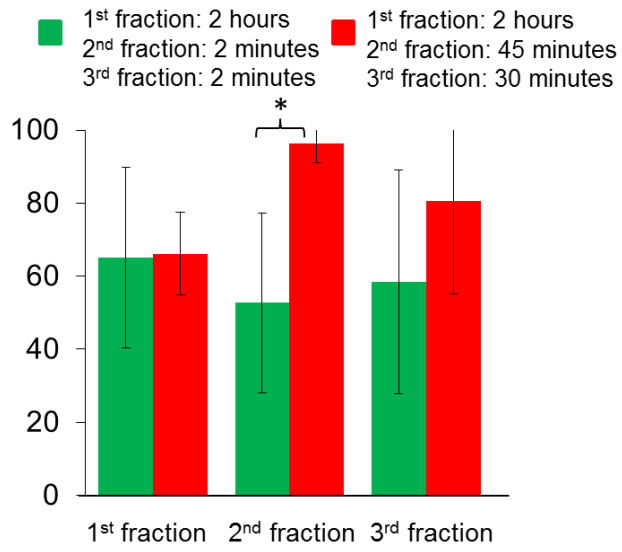

Figure 4-7 Flocculation Time Effect in between fractions during diafiltration using 0.1 µm MWCO for SINV with 1M mannitol. Comparative study of the step yield of SINV with normal diafiltration (green) and allowing flocculation time in between the fractions (red), Error bars are the standard deviation of three separate trials *p<0.05

4.2.3. Recovery of SINV with different starting concentration

Different starting material concentration log 6, log 9, log 10 MTT_{50}/ml of SINV was added for the flocculation experiments to study the effect of concentration for recovery of virus particles. All experiments were performed using a 0.1 µm membrane filter to have the same comparison across all concentration range and with PPV. Using a higher concentration of SINV for log 10 MTT_{50}/ml shows there is significant membrane fouling with low throughput at the membranes and pressure required for the system is about 30 psi, while for the lower concentration of log 6 and log 9 MTT_{50}/ml and SINV pressure required for the system is 10 psi.

Figure 4-8-A shows the percent recovery of SINV using different initial concentration for mannitol and the negative water control. First fractions with mannitol using log 9 MTT_{50}/ml SINV concentration showed 79%. Increasing concentration to log 10 MTT_{50}/ml we are able to get 90% recovery of SINV. No significant high recovery was observed with the negative control, except for the log 11 MTT_{50}/ml. Note that the

membrane fouling that occurred at high concentration of virus, causing partial recovery with the water control.

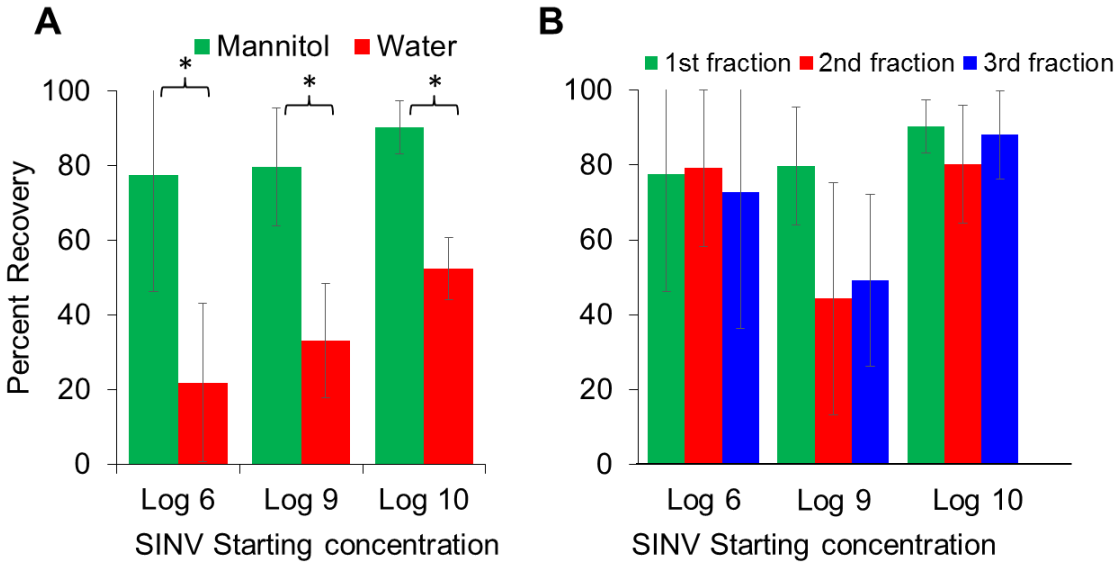

Figure 4-8 Recovery of SINV showing different starting concentration as log 6, log 9 and log 10 MTT_{50}/ml with diafiltration experiments, A: Overall yield of SINV in presence of mannitol and control, B: Step yield for SINV showing all 3 fractions recovery in presence of mannitol only. Membrane filter used for all experiments is 0.1 µm, Error bars are the standard deviation of three separate trials *p<0.05

Further details into each fraction of mannitol flocculation samples are shown in **Figure 4-8-B**. Results with log 6 MTT_{50}/ml SINV concentration were explained previously in section 4.2.1. Using log 9 MTT_{50}/ml of SINV shows 79% recovery in first fraction which is about the same for log 6 MTT_{50}/ml concentration for SINV. In the 2nd and 3rd fraction recovery is 44% and 49% respectively due to braking of aggregates in manual addition. Increasing the initial concentration of SINV to log 10 MTT_{50}/ml and 1M mannitol without diluting in water, higher recovery was recorded. In the 1st fraction recovery of 90% achieved followed by consistent recovery in each subsequent fractions.

4.2.4. Purification of SINV

For the diafiltration of SINV, we collected fractions of filtrate, retentate and compared with before filtration samples for the purification validation using reverse phase chromatography (RPC) using C18 column. As we hypothesis, we expected HCP

will pass through the membrane after flocculation and microfiltration and purified SINV should be retained. Several repeated HPLC analysis for the SINV diafiltration experiment, it was not showing high purification. The total protein concentration in the SINV preparations was very low because the virus was stored in glycerol during freezing. We had to remove the glycerol with dialysis prior to HPLC, but it also removed many of the contaminating proteins. The graphs in **Figure 4-9** showing the chromatographs of before filtration sample, filtrate sample and retentate. The before filtration sample shows contaminant BSA peak at time 20-21 minutes (second peak on the continuous line curve), filtrate is showing reduced contaminant while retentate has the contaminant BSA peak (green dotted line curve). Area under the curve calculations shows that we were able to remove about 37% of contaminant in the retentate sample, but starting concentration of protein content was very low. To overcome low detectable values for HCP, we prepared samples with added impurities (conditioned media as representative for HCP) and still analysis was unable to get proper purification data. While in case of PPV purification, we demonstrated 80% contaminant protein removal using same diafiltration experiment with 1M mannitol.

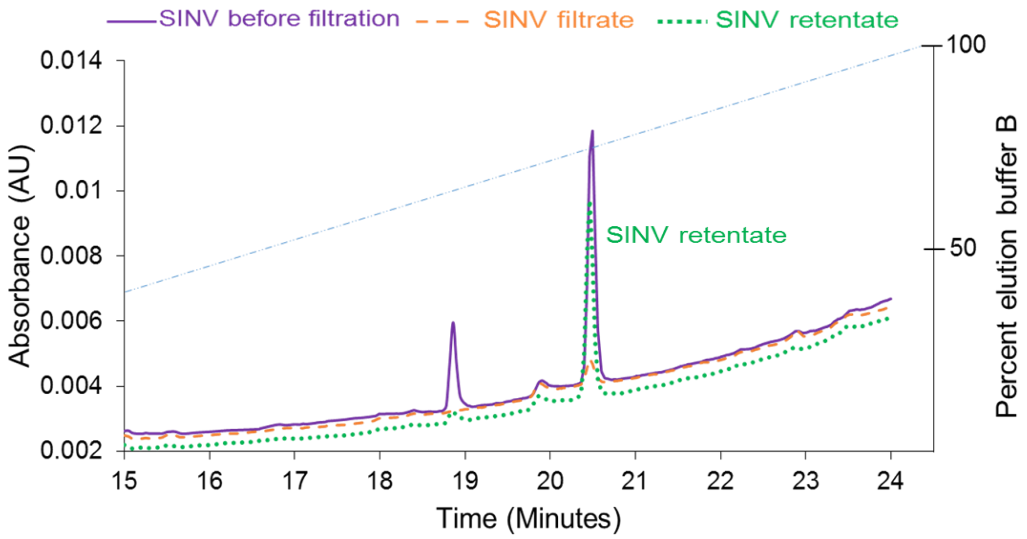

Figure 4-9 Purification of SINV analysis using Reverse phase chromatograph using C-18 column of the diafiltration samples before filtration(purple), after filtration (dashed orange), and retentate (dotted green) with buffer A as 0.1% TFA in water and eluting buffer B as 0.1% TFA in acetonitrile (dotted blue line)

4.2.5. DNA Quantification

In case of SINV diafiltration experiments, all collected fractions were analyzed for DNA content for DNA removal. Samples collected from mannitol flocculation demonstrate partial flocculation of DNA in first fraction as removal is about 50%, as shown in Figure 4-10 but further addition of mannitol decreased removal of DNA. Samples with water as control showing higher removal than with mannitol. So addition of water is not affecting DNA removal, while addition of mannitol has opposing effect on DNA removal. Mannitol flocculation was flocculating DNA also so we were unable to get high removal of DNA in retentate. As DNA are charged particles, possible explanation for DNA being aggregated in presence of mannitol can be the reduction of electrostatic forces due to mannitol. To solve this problem and achieve better DNA removal during diafiltration we would like to further study using benzonase endonuclease treatment as several literatures have shown DNA removal using benzonase endonuclease [86, 122]. We can use this endonuclease for removing DNA content from our virus samples prior to flocculation and filtration which is effective method for removing DNA as a future work.

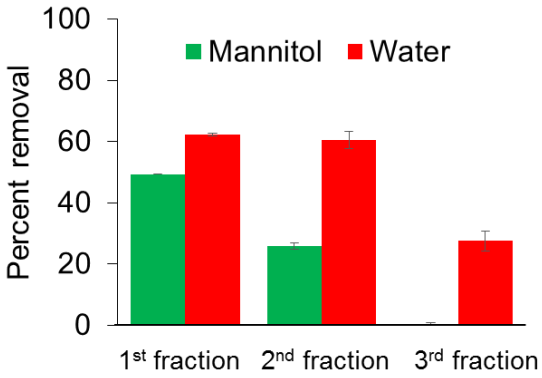

Figure 4-10 DNA quantification for SINV . Percent removal of DNA in retentate samples for SINV by mannitol flocculation using Picogreen DNA quantification. Diafiltration performed using 0.1 μm membrane with log 9 SINV concentration. Error bars are the standard deviation of two separate trials

4.2.6. Overall Summary for enveloped SINV

Looking at the overall recovery and purification for SINV, **Table 4-3** shows the comparative data for DNA removal, protein removal, concentrations and recovery by fractions. DNA quantification and protein removal study by reverse phase chromatography was performed on samples collected by 0.1 µm membrane based diafiltration. Concentrations in terms of MTT_{50}/ml are showing the infectious virus titers in each fractions of retentate samples. For samples from diafiltration using 0.1 µm, 500 kDa and 300 kDa membrane, with starting concentration of SINV, we can see in retentate we are able to get high titer values close to starting concentration in first fraction and then decreasing titer in next fractions. While our negative control shows drop in the concentration of retentate after diafiltration.

Table 4-3 Overall recovery and purification of SINV, Comparison of concentrations before and after diafiltration, step yield, overall recovery with different filter pore size using diafiltration for SINV recovery along with DNA removal and protein removal for mannitol

SINV	**Flocculation with mannitol**						
Filter pore size	DNA removal[a] (%)	Protein removal[b] (%)	Starting concentration (MTT_{50}/ml)	Final retentate concentration (MTT_{50}/ml) by fraction		Step yield[c] (%) by fractions	Overall recovery[d] (%) by fraction
0.2 µm	49	37	6.43 ± 0.2	1st	6.33 ± 0.5	65 ± 24	65 ± 24
				2nd	5.95 ± 0.5	52 ± 24	45 ± 25
				3rd	5.77 ± 0.5	58 ± 30	35 ± 20
0.1 µm	-	-	7.03 ± 0.8	1st	7.10 ± 0.5	77 ± 31	77 ± 31
				2nd	6.96 ± 01	79 ± 20	62 ± 24
				3rd	6.64 ± 0.6	72 ± 36	45 ± 18
500 kDa	-	-	6.07 ± 0.7	1st	6.10 ± 0.7	96 ± 5	96 ± 5
				2nd	5.89 ± 0.7	67 ± 7	71 ± 8
				3rd	5.39 ± 0.7	69 ± 3	46 ± 2
	Negative control flocculation with water						
0.2 µm	62	35	6.69 ± 0.2	1st	6.03 ± 0.5	20 ± 13	20 ± 13
				2nd	6.20 ± 0.5	86 ± 22	32 ± 24
				3rd	5.77 ± 0.8	48 ± 27	20 ± 18
0.1 µm	-	-	7.55 ± 1	1st	6.63 ± 1	21 ± 31	21 ± 21
				2nd	6.50 ± 1	65 ± 26	22 ± 18
				3rd	5.26 ± 1	76 ± 39	21 ± 24
500 kDa	-	-	6.95 ± 1	1st	6.35 ± 1	29 ± 16	29 ± 16
				2nd	5.66 ± 0.7	36 ± 28	11 ± 7
				3rd	5.60 ± 0.9	74 ± 23	7 ± 4

Error bar are standard deviation from three trials.

a: Based on Quant-iT PicoGreen dsDNA quantification method

b: Based on area under the curve calculations from reverse phase chromatography (RPC)

c: Based on infectious particles titer calculations using MTT50 assay for step yield calculations from equation 3.1-3.3

d: Based on infectious particles titer calculations using MTT50 assay overall yield calculation from equation 3.1, 3.4 & 3.5.

4.3. Transmission Electron Microscopy

To support the flocculation hypothesis along with all the results shown earlier, we performed imaging of the flocculated virus particles in osmolyte solution. In **Figure 4-11** it is showing images for flocculated PPV in mannitol and in water as a control. **Figure 4-11 A and B** are showing that PPV particles in mannitol at two different resolutions. Aggregates are easily found. The aggregate size around 100 nm is also supported by the filtration results that below a 0.1 um filter, there is a rise in virus found in the retentate. In comparison, images of PPV in water (shown in **Figure 4-11 C and D**) showed separated and individual particles. This further supports the theory that mannitol is causing virus flocculation.

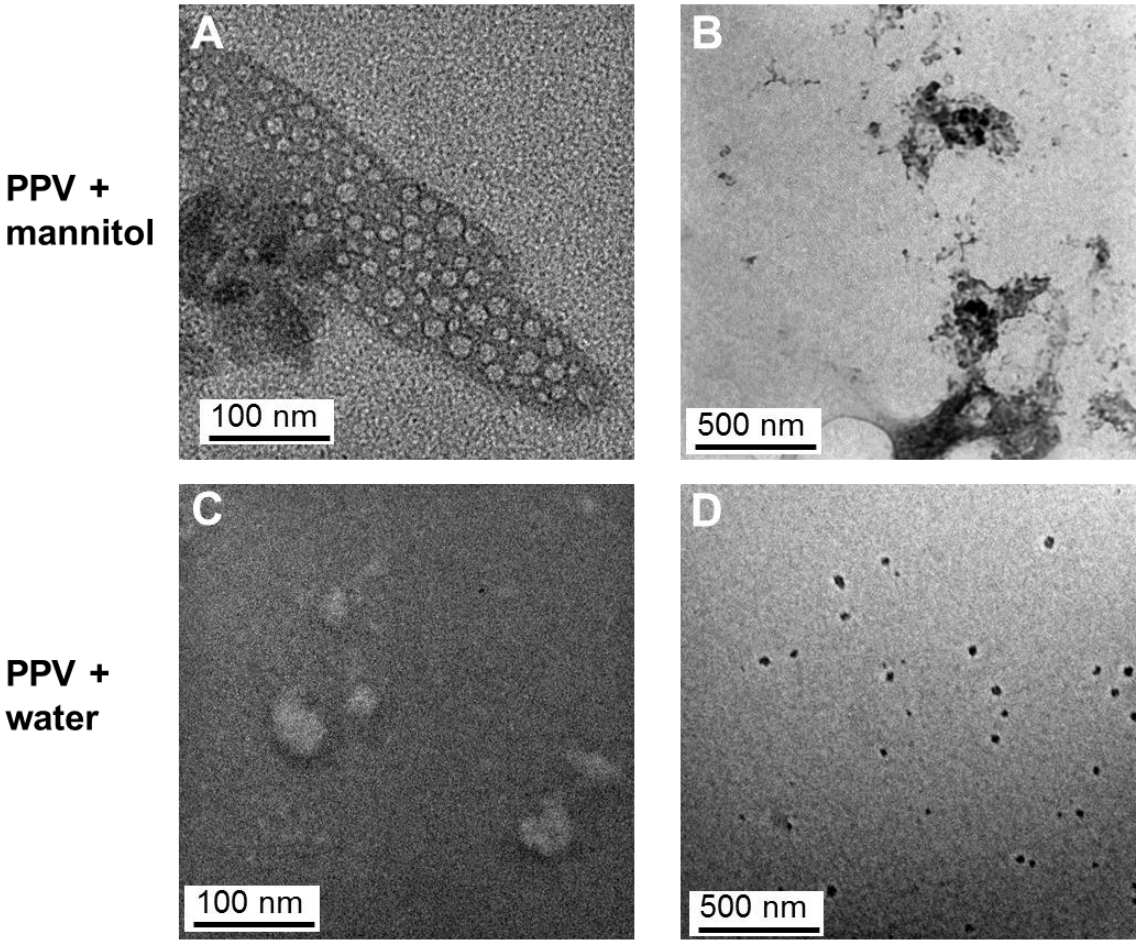

Figure 4-11 Transmission electron microscope images for PPV sample in mannitol and water. Figure A (100 nm) & B (500 nm) shows PPV with mannitol, Figure C (100 nm) & D (500 nm) showing PPV with water

4.4. Discussion

To prove our hypothesis that mannitol flocculation works for all types of viruses and can be applied as a novel platform approach with minimal changes, we studied the non-enveloped PPV and enveloped virus, SINV. For both viruses, osmolyte mannitol was able to flocculation based on our results of virus retentions using large pore sixe filters. Addition of mannitol, removes the bound water around the viruses due to preferential hydration of osmolyte. Section 4.1, shows recovery and purification data for PPV. We got 58% recovery using 0.1 µm filter for the PPV and highest recovery of 85% was achieved using 300 kDa filter. In Section 4.2, we have shown all the results for recovery

of SINV using different pore size filter such as 0.2 µm, 0.1 µm and 500 kDa filters. Using 0.2 µm pore size filter we were able to obtain a 65% recovery in the first fraction and 79% recovery using a 0.1 µm pore size filter. Highest recovery was achieved using a 500 kDa pore size filter which was 96%. As we are using different pore size filter, recovery of infectious SINV particles was increasing with decreasing filter pore size. Considering the size of SINV, which is about 50 nm, we are able to recover SINV particles even with large pore size filter as 0.2 µm. As a control study and to show mannitol is responsible for forming the flocs of virus. We also performed control studies with water. As seen in our previous results from section 4.1 and 4.2, it is showing that the percent recovery of virus particles is low or virus particles are passing though the filter while performed with water.

This recovery using microfiltration is significantly promising as compared to the size of virus. In literature review, we have seen studies for recovery of viruses using small MCWO membranes such as 30 kDa, 50 kDa and 100 kDa for parvovirus MVM (22-26 nm size) [98] and Aedes aegypti densonucleosisvirus (20-30 nm) [91, 95]. While many studies have used multiple step purifications using two chromatography techniques and series or combination of chromatography and ultrafiltration as we saw in Table 2-3 and Table 2-4. So comparing size of membranes used for retaining virus, we are able to retain small virus using micro-filters. This can reduce the cost related to ultrafiltration in terms of cost of membranes, pressure requirements for the system. Also using large pore size membranes can reduce pore plugging due to small debris, protein contaminants blocking the small pores of ultrafiltration membranes. In section 2.4, we have seen recovery and purification of viruses using multiple chromatography steps. Using set of chromatography was able to get high purity and product recovery was still moderate as 40% to 70% [65, 75,78, 80]. With ultrafiltration using small pore sized membranes literature have shown moderate recoveries and used final polishing step such as chromatography to get high purity [90, 92, 96, 98]. With our approach, we were able to get moderate recovery using large pore size filters and high recovery with 300 kDa for PPV. For SINV moderate recovery is seen with 0.2 µm filter, which is usually used for larger particle filtration. In normal conditions, without virus flocculation 0.2 µm filters

will not be able to retain small viral particles of size 50nm. High recovery was obtained using 0.1 μm and 500 kDa filter. From our purification data, PPV purification was good and acceptable as per industrial requirements [101]. While in industry or in literature review we saw that high purification was achieved using multiple steps or combination of different steps. Using osmolyte flocculation can be developed and used as a platform approach. In terms of DNA removal, we can improve purity by using endonuclease treatment to remove more DNA content from the virus retentate product [86, 122].

As we study and compare the results for no flocculation time between fraction with increased flocculation time, we only observe an improvement in the recovery in the 2^{nd} fraction for SINV, while with PPV recovery, we are not getting any improvement. Comparing the size of both viruses, PPV virus is a non-enveloped virus and has smaller capsid while SINV is an enveloped and contains outer lipid layer. SINV is bigger in size about 48-52 nm while PPV is the smallest parvovirus of size about 18-24 nm. The outer layer of viruses could be the decisive element in effective flocculation with osmolyte as the interactions for aggregations may change with the presence or absence of enveloped bilayer on virus particles. As we are performing recovery of virus particles using larger pore size filters the size of aggregated virus plays an important role.

While we are able to show advantage of using flocculation and microfiltration, we anticipate some disadvantages to our process. We have seen that virus flocs are not permanent and can be broken with disturbances or agitation in the mixture. It is both good and bad for the system. It is good because after product recovery, we don't have to worry about breaking flocs with other treatment. Flocculated particles can be easily dispersed in buffer such as PBS. It is not good for the recovery of viruses, as breaking of flocs allows particles to pass through the membrane and reduces overall yield. We have seen addition of mannitol for 2^{nd} and 3^{rd} fractions created disturbances in system and recovery was reduced. This is an important factor for consideration while designing large scale configuration for this approach it is important factor. Using large scale tank reactor similar to Amicon filtration cells we have used and allowing quiescent time after addition of virus and osmolyte for flocculation without disturbances in the system is

recommended. Allowing dormant time at ambient temperature for flocculation can be disadvantageous for some sensitive products which are susceptible to reduced activity at room temperature.

5. Conclusions and future work

5.1. Conclusion

This work describes the purification of two viruses using a common, novel flocculant, mannitol. As mannitol is currently used as therapeutic substance for treatment in diuretic conditions, brain swelling conditions it is acceptable as biological drug and is FDA approved [8, 9]. PPV being smallest parvovirus of size about 20-24 nm [13, 14], is difficult to recover using filtration methods. However, with our novel approach of flocculation using 1M mannitol, we were able to form flocs that could be removed with larger pore size filters. Using a diafiltration method, we were able to recover infectious particles in the retentate. We explored the effect of different pore size filters, ranging from 0.2 µm to a 300 kDa MWCO filter. We have successfully shown that 58% of infectious PPV can be recovered with a 0.1 µm filter and 65% of infectious SINV. The PPV had an 85% reduction in contaminating proteins, while SINV had low protein removal of 37%. We were able to get no significant improvement for the non-enveloped PPV, and only a significant improvement can be seen in case of enveloped SINV for the 2nd fraction. Time is still a variable that may need further study. The flocculation process was not able to get high removal of DNA content as it was also retained in the retentate with virus particles. Compared to control studies, we can see that mannitol was aggregating DNA content as well while in case of control study DNA content was removed. We therefore propose to use benzonase to reduce the size of the DNA prior to flocculation in order to inhibit DNA flocculation with mannitol. TEM images for PPV flocculation provide good support to our theory and all work shown in this research.

Our recovery results show that mannitol flocculation followed by diafiltration is able to recover an enveloped and non-enveloped virus and purify a non-enveloped virus. Our platform approach can be used as a potential method to replace chromatography and nanofiltration. This results can be used for future work and can be applied to large to medium scale to develop an industrial processing protocol. Adding advantages over replacing multiple traditional and expensive methods as chromatography or

ultrafiltration, new platform approach can be an effective new method in new vaccine production setups.

5.2. Future Work:

Currently, we have shown high recovery for an enveloped and non-enveloped virus and purification for a non-enveloped virus using batch diafiltration. The traditional batch diafiltration approach is applicable in lab scale models, but is not a good choice for industrial scale. We would like to develop this diafiltration method into a continuous process by incorporating continuous method with recycling of osmolyte solution. Currently our setup for diafiltration includes manual addition of mannitol solution into the filter unit. By using mannitol reservoir tank incorporated with pressure application, it can be used in a continuous diafiltration mode. For improved recovery of viruses, we would like to explore if we can change the hydrophobicity while performing the flocculation step. Increasing the hydrophobic interactions by addition of ethanol to the flocculation solution may affect the flocculation of viruses. Presence of polar group on ethanol enhances the hydrophobic nature of ethanol and when added to virus solution it should add effective hydrophobic forces on virus particles. Studies have shown increase in hydrophobicity with increasing concentration of ethanol on membranes [123, 124]. To overcome the current challenge for DNA removal, we can use benzonase treatment for efficiently remove DNA content while performing diafiltration method for higher purification. Benzonase nuclease is endonuclease enzyme which can degrade DNA and RNA which are considered as contaminant from host cells [86, 122]. Our preliminary data using benzonase nuclease treatment with PPV prior to flocculation and diafiltration decreased the initial DNA content and showed high DNA removal (data not shown) as compared to given results in section 4.1.5.

We are interested in trying both enveloped and non-enveloped virus flocculation using another osmolyte, glycine, which has been demonstrated for virus removal in previous studies in our lab [6] and show promise as a flocculant for both viruses. In addition, we would like to perform flocculation experiments with different viruses such as minute virus of mice (MVM) and phage MS2. In the future, we will focus on improved purification and recovery of viruses by capture with charged anion membranes for vaccine manufacturing. Using charged anion membranes can help for recovery based on size of virus particles as well as surface charges of viruses. These membranes can be used

in bind and elute mode, similar to standard chromatography, and would allow for better clearance of impurities.

To develop this method as an acceptable platform approach, I would like to work on creating large scale module for this method. Demonstrating that osmolyte flocculation is easily scalable, this experiments can be performed at large scale of 400 ml. Using continuous diafiltration method, removing the need to manual addition of osmolyte system can work in continuous manner and more likely decrease the disturbances in system which are responsible for breaking up the aggregates. More detailed results can be obtained regarding membrane permeability, reflux effect, flow rate by using pressure transducers, flow rate controllers for monitoring inlet to outlet pressure ratios, flow rate changes in the system. This setup would be similar to the current filtration systems as commercially available TFF or NFF systems with tank reactor operated in normal flow direction or dead end filtration. Tank reactor for holding flocculation mixture with replaceable filter membranes holder and standard tubing. The inlet and outlet will be connected with inline pressure sensors to determine the change in efficiency of flow across the membrane by monitoring pressure changes. Such systems are currently common in industry for virus purification with TFF which are scalable from pilot plant to large scale production. Instead of using multiple ultrafiltration filtration steps, we can introduce flocculation and microfiltration setup.

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