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Virus purification, detection and removal

Khrupa Saagar Vijayaragavan
Michigan Technological University

VIRUS PURIFICATION, DETECTION AND REMOVAL

By

Khrupa Saagar Vijayaragavan

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Department of Chemical Engineering

Dissertation Advisor: *Dr. Caryn L. Heldt*

Committee Member: *Dr. Adrienne R. Minerick*

Committee Member: *Dr. Megan C. Frost*

Committee Member: *Dr. Timothy C. Eisele*

Department Chair: *Dr. Komar S. Kawatra*

Dedications

To my parents,

Mr. K. Vijayaragavan & Mrs. Subhashree Vijayaragavan

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Preface

My PhD work and dissertation is aimed at making significant contribution in the field of biopharmaceuticals and particularly in the downstream processing of biotherapeutics. Downstream processing is the recovery and purification of the target biomolecules as antibodies, enzymes, viruses etc. from animal or plant protein contaminants following the fermentation step (upstream processing). Downstream processing also holds the responsibility of maintaining the functionality of the molecule. My work is focused on improving virus purification for vaccine development and virus removal for water purification by understanding viruses and specially their surface characteristics. Additionally I have also performed work on virus detection mechanism through mammalian cells for creation of antiviral compounds.

Chapter 2 is the literature review detailing the current practices available for viral vaccines recovery and water purification from viruses and the need to improve them. The figures 2.9, 2.10, 2.11, 2.12, 2.13 and 2.14 have been used with permission from relevant journals.

Chapter 3 is virus purification using chromatography strategies and characterization of virus using a panel of standard proteins. The chapter comprises a total of 18 figures. Reverse phase chromatography and the experimental work for figures 3.11, 3.12 and 3.14 was performed by an undergraduate student Ms. Amna Zahid (Chemical Engineering, Michigan Technological University). My advisor Dr. Caryn Heldt analyzed and plotted figure 3.12. All the figures in chapter 3 except figure 3.12 was analyzed and plotted by me. I also collected data for figures where Ms. Zahid was not involved. A part of the chapter is planned for submission and the paper will be written by my advisor.

Chapter 4 is virus trafficking and using immunohistochemistry for identifying antivirals (osmolytes) compounds. The first author in this work is a graduate student Ms. Maria Tafur (Chemical Engineering, Michigan Technological University). She collected data, analyzed and plotted figures to show the antiviral activity using osmolytes and wrote the peer reviewed journal paper. The immunohistochemistry work was crucial to show the action or function of osmolytes against infection. The immunohistochemistry work was performed, analyzed and plotted by me and as shown in the figures 4.9, 4.10 and 4.11. The permission for these figures have been obtained from Tafur et al. (2013). The third and corresponding author was my advisor. She guided in the paper writing process.

Chapter 5 is improving vaccine production systems for non-enveloped porcine parvovirus virus using aqueous two-phase system. The complete chapter has been used with permission from Vijayaragavan et al. (2014). I am the first author of the publication and the article was written by me. Co-author and undergraduate student Ms. Amna Zahid (Chemical Engineering, Michigan Technological University) collected data for figure 5.7 and 5.8. Co-author and undergraduate student Mr. Jonathan Young (Chemical Engineering, Michigan Technological University) was an invaluable support in collecting data for figure 5.3 and 5.4. Transfer student from the MICUP program and a current undergraduate student Ms. Sarah Corrion (Chemical Engineering, Michigan Technological University) was an invaluable support in collecting data for figure 5.1 (A). All the figures were analyzed and plotted by me. My advisor Dr. Caryn Heldt was the corresponding author.

Chapter 6 is virus removal using chitosan membrane matrix for potable water. The chapter is planned for submission and all of the work including figures, texts was performed, analyzed and plotted by me.

Acknowledgements

I would like to thank my advisor Dr. Caryn Heldt for providing me the enriched opportunity of working and contributing to the field of Bioseparations. I consider myself very fortunate to have her as my advisor for I have learnt through her the importance of hardwork, creativity, scientific thinking and perfection in a professional environment. I would like to express gratitude to her for constant support and advice throughout my Ph.D helping me meet my research goals. I would like to thank the Department of Chemical Engineering for the strong foundation, timely support and funding. I would like to thank the NSF for funding and Novo Nordisk A/S, Denmark for the human insulin samples as a generous gift.

I would like to thank Dr. Minerick for teaching me transport phenomena and being in my dissertation committee. I express my gratitude to her for being gracious in providing lab space and trusting us with room access for using our microscope. I would like to thank Dr. Megan Frost and Dr. Timothy Eisele for being in my dissertation committee. I would like to thank all my committee members for their ideas, discussions, invaluable support and spending time reading my Ph.D defense proposal in 2013 and dissertation. I sincerely thank Dr. Chin-An Peng for sharing spectrophotometer and refrigerator, Dr. Patricia Heiden for electrospinning apparatus, Dr. Wenzhen Li for vacuum drying oven, Dr. Faith Morrison for sharing lab space, Mr. Owen Mills for guidance and discussions on electron microscope, Dr. Martin Thompson for his guidance on conjugate chemistry and Dr. Komar Kawatra for career guidance and acting as a mentor.

I thank my all my lab colleagues for a great working ambience in the laboratory. I special thank Ms. Maria Gencoglu for her support and assistance in lab duties and also for her friendship. My sincere thanks to Ms. Amna Zahid, Mr. Jonathan Young and Ms. Sarah Corrion for helping me with experiments for my publication. My sincere gratitude

to Ms. Maria Gencoglu and Ms. Xue Mi for collaboration on osmolytes and virus removal project respectively.

I thank International Programs and Services for their guidance and support, student organizations Indian Students Associations, Graduate Student Government and International Club for giving me leadership opportunities and teaching me the importance of multi-tasking and self-management.

Finally I would like to thank friends and family. My friends for a joyous, fun loving environment at Houghton and to listen to my practice talks. I would like to thank my uncle Mr. Damo Srinivas and my cousins for their encouragement and acting guide away from home. I would like to thank Rasika Gawde for her unconditional love and support. My sincere and deep gratitude to my parents for their love and belief in me.

Abstract

The biopharmaceutical industry has a growing demand and an increasing need to improve the current virus purification technologies, especially as more and more vaccines are produced from cell-culture derived virus particles. Downstream purification strategies can be expensive and account for 70% of the overall manufacturing costs. The economic pressure and purification processes can be particularly challenging when the virus to be purified is small, as in our model virus, porcine parvovirus (PPV). Our efforts are focused on designing an easy, economical, scalable and efficient system for virus purification, and we focused on aqueous two-phase systems. Industry acceptable standards for virus vaccine recovery can be as low as 30% due to demand of high final titer, virus transduction inhibitors and presence of empty or defective virus capsids as impurities. We have overcome these shortcomings by recovering a high 64% of infectious virus using an aqueous two-phase system. We used high molecular weight polymer and citrate salt to achieve a good yield and eliminated the major contaminant bovine serum albumin.

Viruses are also studied for ensuring pure and safe drinking water. Low pressure microfiltration are continuously being investigated for water filters as they allow high permeate flux and low fouling. Viruses such as PPV are small enough to pass through the microporous membranes. Control of viruses in water is crucial for public health and we have designed an affinity based membrane filter to capture virus. Nanofibers have a high surface to volume ratio providing a highly accessible surface area for virus adsorption. Chitosan an insoluble, biocompatible and biodegradable polymer was used for adsorbing trimer peptide WRW. About 0.2 μmoles of cysteine terminal WRW peptide was conjugated to amine terminal chitosan using maleimide conjugation chemistry. We achieved 90-99% virus removal from water adjusted to a neutral pH. The virus removal

from affinity based chitosan was attributed to electrostatic and hydrophobic driven binding effect.

Chapter 1

Introduction and overview

1.1 Introduction

Viruses can either cause disease and death, or they can be manipulated into lifesaving vaccines and gene therapy vectors. This dissertation focuses on methods to purify and remove viruses for vaccines or water purification, respectively. The overarching theme is that the study of virus surface chemistry allows us to create operations that will improve a host of applications, including clean water, biotherapeutics, disease detection, and reduce disease transmission.

1.2 Overview

The dissertation starts with exploring different chromatography modes to achieve PPV purification from cell or media protein contaminants. Conventional downstream processing faces a challenge to effectively purify virus particles for vaccine therapeutics due to issues pertaining to purity, potency and quality. To overcome this issue we have investigated and designed an optimal aqueous two-phase system achieving a high virus recovery compared to industry standards. The dissertation also involves work on virus removal studies. Millions of people die every year due to bacterial and viral diseases from contaminated water especially in developing countries. In a quest to create economical point-of-care water filtration devices we have designed peptide-functionalized chitosan membranes for virus removal. Apart from virus purification and removal we also attempt to physically characterize the virus. Virus structural properties can help aid the design of virus surface adsorption and virus-cell interactions which would be useful for the development of separation strategies and antiviral drugs.

In this dissertation, Chapter 2 is the literature review of the downstream processes available for purifying virus particles. We discuss the challenges and drawbacks encountered for each unit operation and propose ideas on improving virus recovery. In the second half of literature review chapter we discuss current water purification strategies used for human water consumption. Viruses can be extremely small, highly resistant to chemicals and cause illness even if consumed in a parts per million dose. Keeping this in perspective, we have identified many purification methods lack of ability to remove all of viruses. Towards the end of the chapter, we discuss techniques and instrumentation available to characterize viruses by their surface properties and size.

In chapter 3 we explore different methods to purify and characterize virus particles. We describe chromatography techniques for purifying virus based on charge and size. We used ion exchange chromatography to detect and purify virus from protein impurities. Next, we used size exclusion chromatography for different virus concentrations samples to examine if the technique can be an effective virus quantification tool. The predominantly used virus quantification method in our lab is the MTT cytotoxicity assay. The MTT assay can be a laborious and time consuming technique, requiring abundant consumables. Later in the chapter, we investigated the experimental hydrophobicity of virus. The surface hydrophobic residues of virus was examined using reverse phase chromatography and ANS fluorescent probe.

Our pursuit on identifying a tool for virus quantification also featured observing individual virus particle trafficking through cells. In chapter 4 we developed a protocol to tag the virus with a dye label and observe it under fluorescent microscope as it

makes its way through the cellular machinery. In the second half of the chapter, the protocol was used towards studying osmolytes behavior in the presence of virus using immunohistochemistry. The protecting nature of osmolytes was determined to reduce the virus infection by 4 LRV by my colleague Ms. Maria Tafur. IHC determined that the capsid proteins were still produced, even in the presence of protecting osmolytes. We hypothesize that the osmolytes reduce the ability of capsid proteins to assemble. The immunohistochemistry work has been published in Antiviral Research.

Chapter 5 is dedicated to virus recovery work for vaccine therapeutic production. Aqueous two-phase system (ATPS), a polymer-salt precipitation technique, is often evaded for biomanufacturing process due to co-precipitation of impurities and low yield. In the chapter we design a robust ATPS by manipulating the biomolecular charge, virus surface hydrophobicity, and surface tension of the system to achieve a high recovery in the polymer phase. The work has been published in the Journal of Chromatography B.

Chapter 6 deals with the creation of microfiltration membrane for virus removal from potable water. Microfiltration membranes have a low back pressure, which prevents fouling and allows for a high water flux. To adsorb virus, we have synthesized cysteine regulated trimer WRW peptide ligands on the electrospun chitosan nanofibers. We discuss the conjugation chemistry to attach peptide on the fibers and report results of filtration from virus contaminated water.

Chapter 7 reviews the results from all the previous chapters and provides ideas for future work.

Chapter 2

Virus characterization and its separation from aqueous medium

2.1 Introduction

Viruses are known to cause diseases and many times even leading to death. On the contrary viruses can also be purified and used for creating therapeutic vaccines. The goal of this dissertation is to study virus surface characteristics and manipulate their mechanism to a) improve virus recovery for vaccine production and b) remove virus from solution for creating potable drinking water.

Vaccines are biological components which provide immunity from infectious diseases. Vaccines are administered by introducing foreign antigens or a weakened virus strain that is incapable of triggering disease but induces the production of antibodies. The antibodies are capable of remembering the antigen as a foreign material and destroy the invader when it is later encountered. Vaccines continue to save millions of lives. The current global decade vaccine action plan is to avert an additional 24-26 million deaths and a hundred million illnesses using a total of 10 vaccines [1]. Five strategic objectives have been proposed by the WHO to accomplish the vaccine action plan. A crucial strategic objective among the five is to improve research and development in low and middle income countries and to enable multidisciplinary technology for reducing the financial burden on vaccine production [1]. For several decades, the egg-based technique has been the predominant technique for the production of vaccines [2]. However the pandemic over the lack of preparedness for the Influenza vaccine in the recent years, frequent bottlenecks in production and financial burden has highlighted the fragile nature of egg-based system [3]. The main drawbacks include a six to nine month production lead time and requirement of a pre-planned choice of the virus strain.

These shortcomings has demanded an innovative modern technology alongside the traditional egg-based vaccine [4]. Cell-based vaccines have become an alternative to the embryonated eggs but the downstream operations or purification steps remain unchanged. Research on the purification mechanisms for cell-based techniques is required to decrease the large financial burden that is placed on the downstream operations of vaccine manufacturing [5].

In addition to vaccine purification, many of the techniques we study can be applied to the removal of viruses from drinking water. Clean drinking water is a basic human right. Lack of access to safe potable water jeopardizes the social and physical well-being of an individual and his or her human dignity [6]. In spite of the importance of water, 884 million people lack access to safe clean water and 2.6 billion people are denied proper sanitation and toilets [7]. In 2001 it was estimated that 26% of deaths worldwide were caused by pathogen containing water supplies, and the pathogens included virus, bacteria and protozoans [7]. Even today the burden of infectious diseases due to the microbial organisms remains quite high. Although several thousand species of pathogens have been recorded in the past, new species seems to be continuously emerging causing infectious diseases such as middle east respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS). Among the main classification of pathogens into bacteria, protozoa, fungi and viruses, it is the viruses which are responsible for 44% of the emerging diseases [6]. A traditional technique for providing safe drinking water from virus has been to use a multiple barrier system. The multiple barrier system has included pretreatment systems, chemical treatment, membrane filtration and inactivation using disinfectants [8, 9]. Despite these measures, it has been difficult to

adopt these techniques universally for all virus families, especially in the developing and underdeveloped countries. Our effort is focused on providing a working solution to reject virus from water using a specialized functional filtration mechanism with a model virus.

2.2 Improving virus recovery for cell-based vaccines

Cell culture based vaccines are produced by introducing a virus strain into susceptible cells in a bioreactor, followed by cell lysis and finally the removal of virus from the aqueous medium by a series of unit operations. Vaccines created in cell culture are reliable and robust due to faster production, shorter lead times, higher purity, reduced contamination and ability to combine upstream and downstream processes [10, 11]. With the current advances in upstream operations, which have increased yields, it is the downstream processing that requires innovative and optimized controls to reduce the 70% financial burden on the manufacturing process [5]. In vaccine manufacturing, the cell culture is commonly accompanied with cellular debris, unwanted media proteins, adventitious agents, residual DNA, nucleic acid and many process related leachable contaminants. As per the FDA vaccine approval requires the freedom from extraneous material whether or not harmful to the recipient [12]. Viruses have a unique size, shape and surface chemistry, i.e. hydrophobicity and charge. Most often a series of unit operations are required to increase the yield of virus particles. The currently used operations involve a combination of precipitation, centrifugation, filtration and chromatography [13, 14].

2.2.1 Precipitation

Precipitation of virus is generally achieved using polyethylene glycol (PEG), ammonium sulfate, or calcium phosphate [15-17]. Among all precipitation techniques PEG has been most frequently used. PEG has shown an enhanced virus yield up to 64% in the case of bovine rotavirus compared to 7% from the ultracentrifugation process [18]. Some other examples include 8% PEG 8000 has been used as a preliminary step to improve the transduction efficiency and optimize the sequential CsCl gradient ultrafiltration [19]. PEG and salts can purify virus by altering its solubility causing precipitation or salting out effect [16, 17]. An alternate concept has been to precipitate the impurities from the virus while leaving the virus in solution. A 750 kDa polyethyleneimine (PEI) of 0.0045 w/v% solution was able to precipitate 85% of the DNA after centrifugation [20]. Polysorbate 80 or sodium chloride has also been useful during precipitation protocol by breaking up the aggregates between DNA and viruses which are held together by hydrophobic and electrostatic charge interactions [21]. Dissolution of aggregates is followed up with chromatography to achieve a final DNA concentration of 5 pg/dose [21]. The residual DNA is well below the expected standards of EP (European Pharmacopoeia) or FDA (Food Drug and Administration) of 10 ng/dose [12, 22]. There is tremendous potential for PEG or precipitation in general, however a high recovery yield and reduced immunogenicity needs to be addressed for improved application of this downstream operation in large scale manufacturing.

2.2.2 Centrifugation and density gradient

Centrifugation is an easy to use, large scale separation method based on density differences. Biotherapeutic manufacturing uses centrifugation often in monoclonal antibody production; however, when it comes to virus particles, the high speed and strong centrifugal force can render the particles non-infectious [23]. An alternate mechanism is a density gradient using sucrose, cesium chloride (CsCl) or iodixanol. In the case of sucrose, the solution is highly viscous and hyper osmotic, reducing the overall yield of the virus stock [24]. CsCl gradient has known to increase the virus particle to infectious virus ratio up to 1600:1, whereas the requirement is in a much lower range around 20:1 [25]. This shows the reduced efficiency of CsCl in terms of maintaining liability of virus particle. Iodixanol density gradient is a low viscous system which can form an iso-osmotic solution and maintain the functionality of the virus structure [26]. Retrovirus recovery of 37% and a promising 95% purity has been reported using Iodixanol gradient [24]. Gradient centrifugation has yielded satisfactory result in laboratory scale, but it continues to remain a time consuming and laborious process which is impractical in large scale manufacturing.

2.2.3 Tangential flow filtration

Tangential flow filtration (TFF) is the commonly used size based filtration technique for virus purification from cell culture medium. A two stop process was created to purify influenza virus particles. A large pore size (0.45 μm) was used to allow the passage of virus while holding cell debris. This was followed by a 100 kDa filter that retained the virus and allow host cell proteins to pass through the filter [27]. For smaller viruses, such as the minute virus of mice (MVM) which range in the 20

nm size, a smaller filter pore size is required to retain viruses [28]. Such a small pore size often leads to membrane fouling and pore plugging from the virus particles, protein aggregates and the media contaminants such as bovine serum albumin (BSA) [29]. Another disadvantage encountered to small pore size membranes is the osmotic pressure gradient that can lead to concentration polarization. It causes unwanted protein contaminant retention on the feed side, therefore reducing virus yield [30]. All of the discussed factors also lead to a major issue of high transmembrane pressure (TMP) and low permeate flux causing reduced filter throughput and poor membrane performance. To try and reduce the concentration polarization that occurs with small pore sized membranes, research has been conducted with the polymeric skin, or the functional part of the nanofiltration membrane, is placed away from the feed (i.e. loading the filter backwards from manufacturers recommended configuration). Viresolve 180 filter (membrane nominal pore size ~ 18 nm) using cysteinylated BSA as a model protein with skin-surface away from the feed showed a higher flux at 240 L/m^2 compared to skin-surface facing the feed at 13 L/m^2 [31]. A higher virus removal can be expected with skin-side away due to reduced osmotic effects from the better control over the membrane supporting structure leading to the skin. In summary of TFF, researchers have recommended a narrow pore size distribution to retain maximum virus and a good pore interconnectivity allowing the liquid media to flow through easily. Such measures will control the TMP and increase the permeate flux with no fouling. TFF continues to be used frequently for large scale manufacturing but many troubleshooting issues due to the cell media proteins, pore size distribution,

permeate flux continue to be experienced especially for the small pore size filters less than 100 nm.

2.2.4 Chromatography

Chromatography is a separation technique based on the interaction between a target virus and a stationery column matrix. The separation functionality can be broadly categorized into (1) charge, (2) size, (3) hydrophobic and (4) affinity. Conventional chromatography is comprised of porous resin beads that have a high internal surface area (**Figure 2.1 (A)**). Large virus particles have diffusion limitations that often preclude penetration into the pores and low dynamic binding capacity due to small pore sizes as compared to virus diameters. A kinetic and convective mass transfer limitation was desired to overcome this drawback and this led the way for membrane chromatography. A stacked membrane is used instead of packed resin beads. The virus is forced through the pores, reducing the process time and pressure drop, as shown in the **Figure 2.1 (B)**.

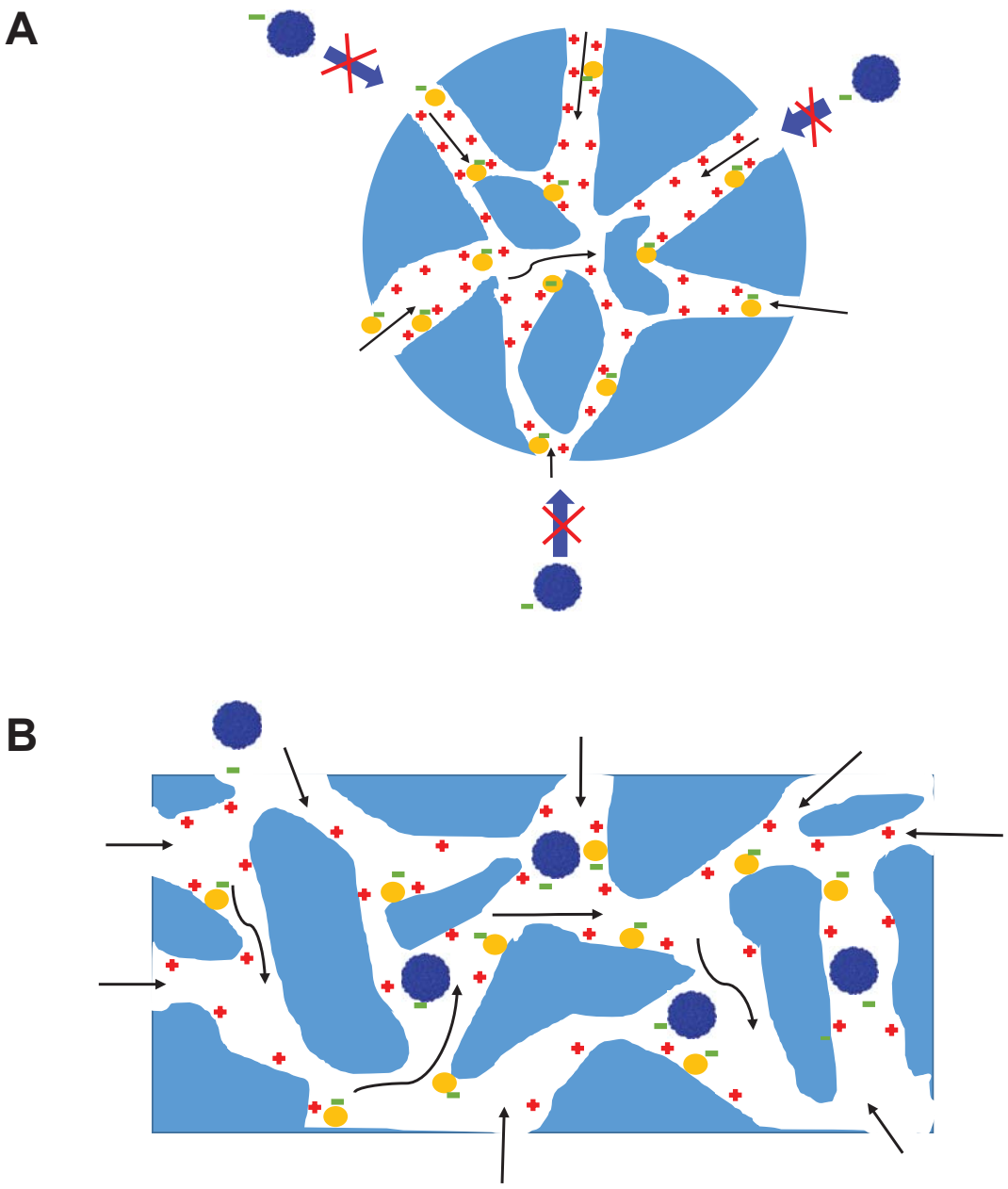

Figure 2.1 Chromatography column matrix. (A) conventional porous bead where large virus diameter is diffusion limited and restricted from entering internal pore surface area necessary for adsorption. (B) membrane adsorbers where diffusion limitation in conventional resin beads is overcome by convection based interaction allowing larger biomolecules.

2.2.4.1 Ion exchange chromatography

Ion exchange is a widely used mechanism for separation of biomolecules, where the opposing charge of the matrix and the virus particle dictates the adsorption effect, as shown in the **Figure 2.2** with regard to a particular section of resin surface.

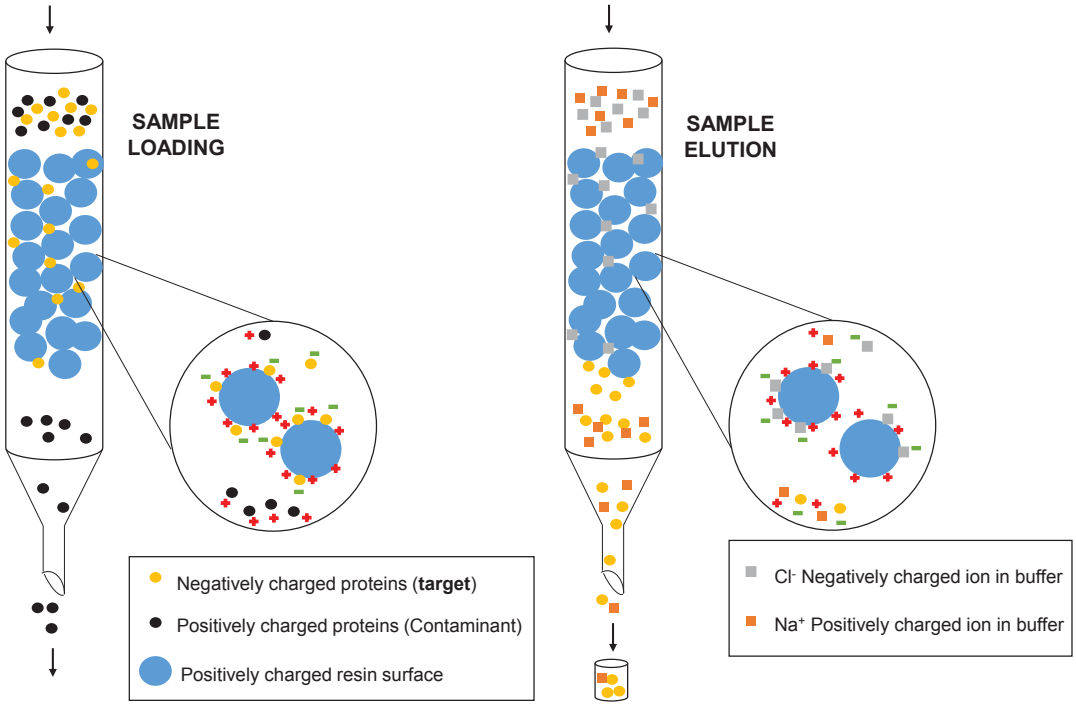

Figure 2.2 Ion exchange chromatography. Oppositely charged resins and target biomolecule causes adsorption of molecule which can be eluted upon subjecting the column matrix to strong salt ions which replace the biomolecule. The figure above is a representation of anion exchange chromatography.

All charge based virus interactions are dependent on the difference between the isoelectric point (pI) of the virus and the charge on the surface matrix. The pH of the solution is chosen such that the viruses are negatively charged and the base matrix is positively charged or vice versa creating electrostatic difference. Aedes densonucleosis virus (AeDNV) purification was conducted on Sartobind strong quaternary amine (Q) and Sartobind weak secondary amine (D) anion exchange

membranes **(see Figure 2.3)** [32] to study the virus concentration based on the strength of electrostatic interaction. The neutral pH of the buffer solution with respect to the virus pI of 3.5 was crucial to capture virus on the membranes effectively. However for the same neutral pH the weak D anion exchange membrane experienced a lack of pH buffering capacity due to the hydroxyl ions (OH^-) that are released during the virus capture step on the membrane surface. This causes pH fluctuations and hence a lower dynamic binding capacity [32]. In addition to hydroxyls, the limited capacity on weak anion exchange was also noticed due to the competition from amino acids, host cell DNA (same size of virus) and extraneous proteins (similar pI of virus) causing a significant reduction in binding capacity by several orders [32, 33].

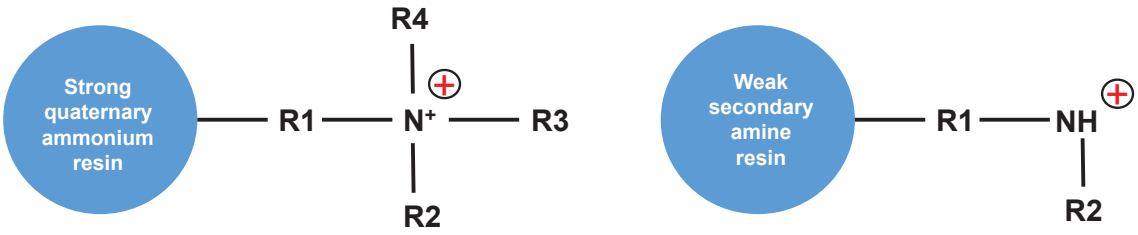

Figure 2.3 Anion exchange chromatography. Strong (Q) and weak (D) amine resins.

Limitations due to weak anion exchange chromatography (AEC) has also been reported for the purification of influenza virus on monolithic chromatographic support. Monolithic chromatography is a convection controlled separation process on membrane monoliths, similar to membrane chromatography. The recovery yield of influenza virus (pI 4-4.5) for a strong anion exchange monolith with a Q functionality was 70-90% and was reduced to 30-50% when using the weak D ligand [34]. In this case, the reason for low yield was again due to the lack of pH buffering capacity.

The pH rise up to 9.5 during NaCl elution caused loss of immunogenicity where influenza is susceptible to inactivation [34]. The authors hypothesize that strong ion exchange ligands can adsorb virus effectively, however they too have had issues of maintaining virus surface conformation during elution [33]. Protein conformation on the virus surface is very crucial since the proteins attach to cell receptors during vaccine therapy. Some other drawbacks that have been reported for ion exchange membranes is the steric hindrance effects [35]. In pretext to design strong electrostatic attraction between the stationery phase and virus, the first inlet of virus can display excessive covalent binding. This excessive binding sterically hinders other virus particles from binding to the membrane and reduces the virus particle recovery. The steric hindrance issues can prevent the continuous flow use and can reduce the working life of the ion exchange membranes [35].

2.2.4.2 Size exclusion chromatography

Size exclusion chromatography (SEC) is a separation technique using tightly packed stationery matrix of silica or agarose gel beads [36]. In SEC, the large molecules elute first because they only pass through the interparticle void volume. The small molecules elute with longer residence time after passing through the pore volume and the void volume as shown in the **Figure 2.4**. SEC can be used for virus concentration by collecting virus in the void volume and separating it from the small protein contaminants. Studies reported on recombinant baculovirus and the turkey coronavirus have shown a high purity but very moderate recovery in the range of 30-40% [14, 37]. The main advantages of SEC are the low cost of resins and ease in operation due to isocratic mode. However the technique severely lacks selectivity,

needs low flow rate operation and also suffers from low productivity. A scale up operation using SEC is restricted because the column can easily get saturated with host cell proteins, preventing the separation of host cell proteins and large viruses. These drawbacks question the application of SEC as a key process in vaccine manufacturing.

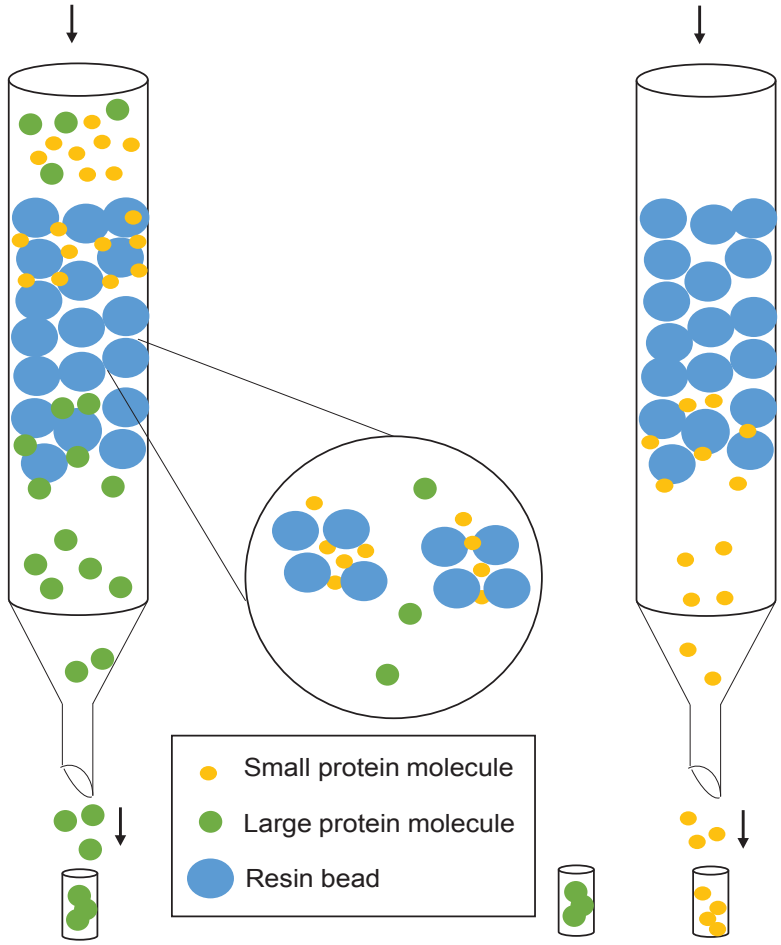

Figure 2.4 Size exclusion chromatography. Large biomolecules elute earlier in the void volume compared to the small molecules which use a longer path between resin pores and narrow spaces in the column.

2.2.4.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is governed by the interaction of hydrophobic patches on the target molecule with hydrophobic ligands on the stationary matrix. At high salt concentration the kosmotropic salt structures the water around itself and strips the hydrophobic biomolecule of their solvation water as shown in **Figure 2.5** [38]. This phenomena exposes the hydrophobic patches on the molecule and causing it to nucleate on the surface of ligand. The recovery and purification of target molecule is acquired by reducing the salt conditions and annulling the hydrophobic interaction mechanism on ligand surface.

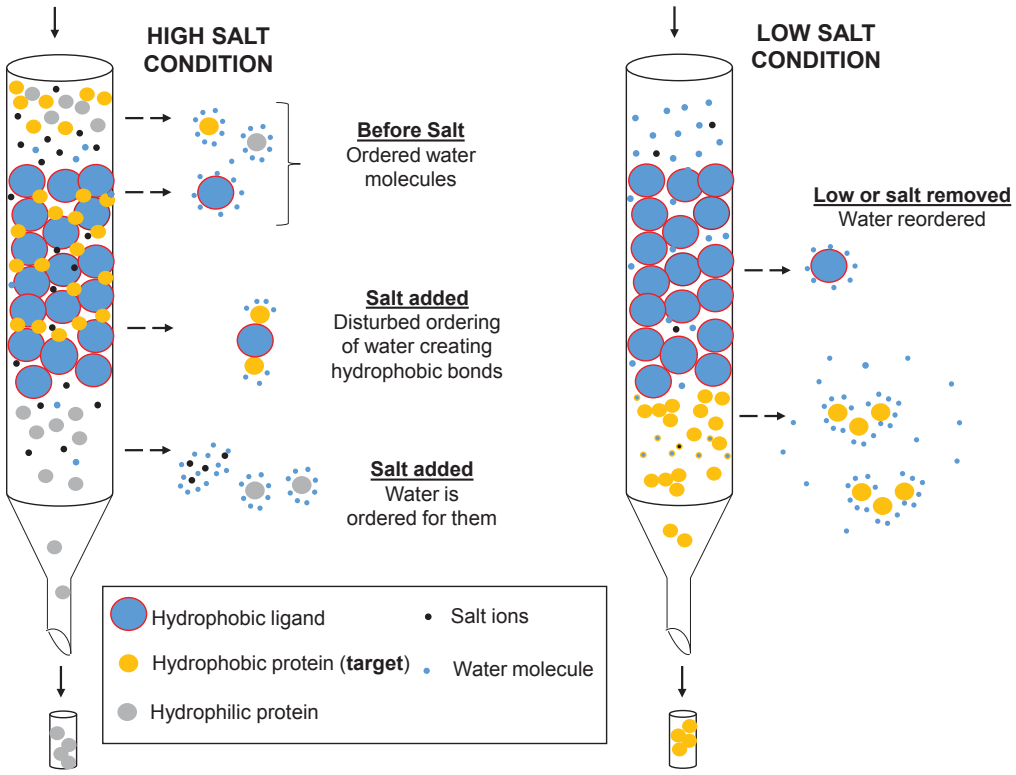

Figure 2.5. Hydrophobic interaction chromatography. At high salt conditions, the water structuring salt reduces the solvation of protein facilitating the hydrophobic interaction between the biomolecule and ligand. At low salt, the molecule-ligand hydrophobic interaction is minimized eluting the molecule by restructuring of water and restoration of protein solvation phenomena.

HIC is used frequently in biotherapeutics for purifying proteins or impurity removal but seldom for virus purification [39, 40]. For producing high virus purities, large amounts of DNA removal has been achieved using the various HIC resins with butyl, phenyl and hexyl ligands [41]. The hydrocarbon side chains, as shown in the **Figure 2.6,** form hydrophobic interactions with the non-polar amino acids on proteins and virus surfaces [42]. Purification of cell culture derived vaccinia ankara virus using the three mentioned HIC resins removed DNA easily between 48-64% from the flow through peak while the virus eluted using a gradient elution from 1.7 M to 0 M ammonium salt $(NH_4)_2SO_4$ [41]. Although successful results were noticed for DNA removal, the virus recovery was reduced to 34-37% mainly due to the high salt conditions of 1.7M $(NH_4)_2SO_4$ [41] caused by reduced immunogenicity. In another study related to immunogenicity, Adeno associated virus (AAV) was subjected to the first capture step using IEC accompanied by HIC with a butyl resin. The AAV was eluted by a step gradient from 1.5 M to 0.6 M $(NH_4)_2SO_4$. An overall recovery of 75% was obtained however the virus particle to infectious virus ratio was an average 17500:1 making it a less than optimal system [43]. Canine adenovirus was recovered up to a high 88% on Fractogel propyl resin with a step gradient from 0.85 M to 0 M $(NH_4)_2SO_4$, followed by a diafiltration and ultrafiltration step. The virus recovery was measured with real time-polymerase chain reaction (RT-PCR) which fails to provide adequate information on immunogenicity of the virus [44]. Not only can the high salt reduce the immunogenicity of the virus, but the high salt concentration can also aggregate the virus particles [45].

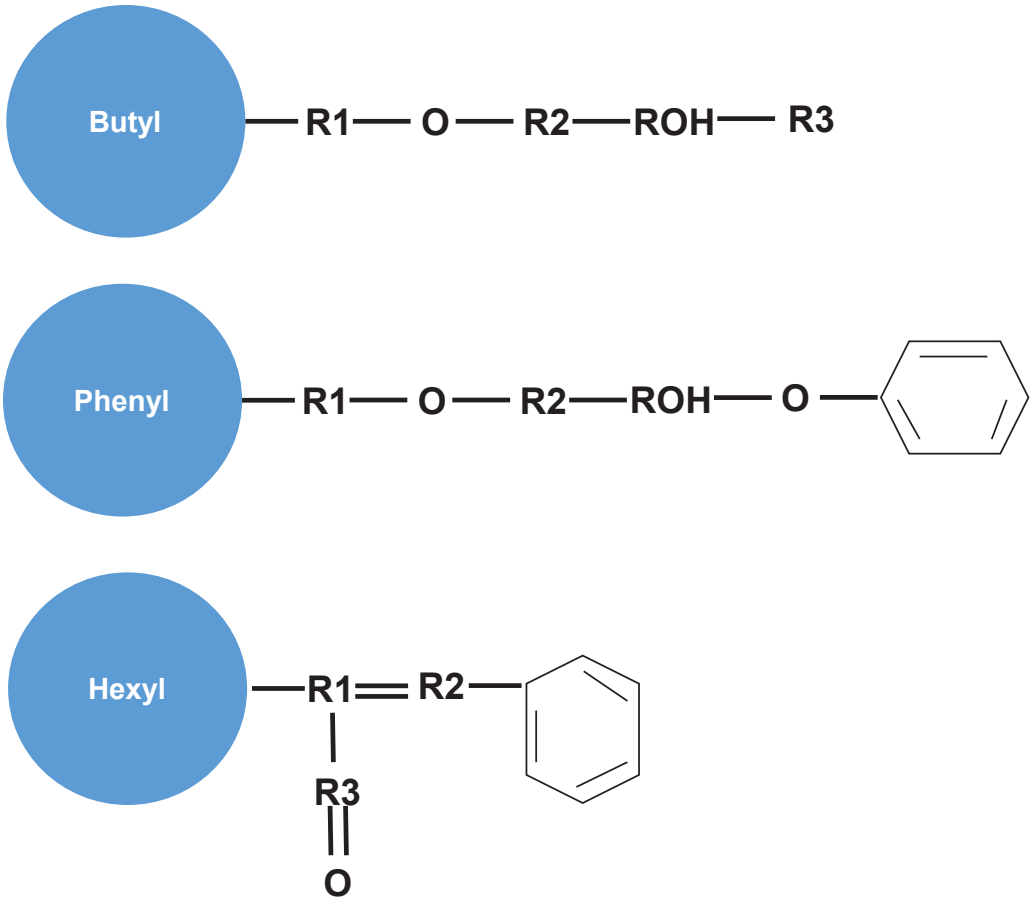

Figure 2.6 Hydrophobic resins.

2.2.4.5 Affinity chromatography

Affinity chromatography or pseudo affinity chromatography is based on creating reversible interaction sites between an immobilized ligand and a biomolecule as shown in **Figure 2.7**. The technique can offer high selectivity, resolution and capacity for adsorbing the molecule of interest. Affinity chromatography interactions often resemble the interaction of a molecule with its natural substrates. For recovering the biomolecule the ligand matrix can be treated with a competing molecule or by changing the ionic strength or pH. A few of the important ligand types

that have been applied for virus purification are metal affinity, heparin sulfate and a lectin ligand.

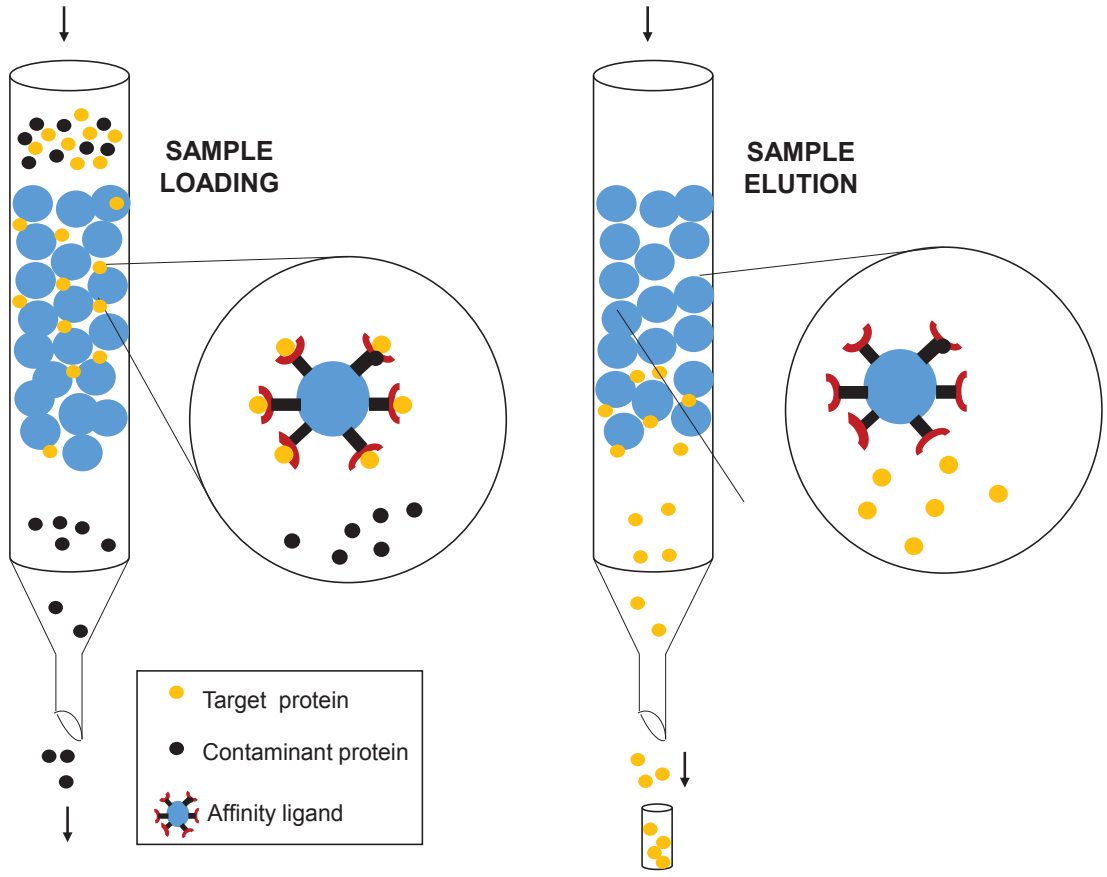

Figure 2.7 Affinity chromatography. The target biomolecule is adsorbed on the specifically designed ligand using a unique interaction mechanism and desorbed by treating the ligand with competitive molecule, change in pH or ionic strength.

Immobilized metal affinity chromatography (IMAC) consists of a matrix containing a chelating agent and a metal ion. The chelating agent (electron donor) is covalently bound to the metal ion (electron acceptor) on the matrix [46]. The virus particles are purified on the IMAC setup due to histidine, cysteine or tryptophan affinity to the available metal ion active residues. Metal ion sites for virus affinity have also been coupled with electrostatic and hydrophobic interactions in affinity systems requiring a

thorough investigation of ion strength and pH to optimize the purification. Influenza virus purification on a zinc-modified Sartobind iminodiacetic acid membrane at 1M NaCl and pH between 7-8 showed 93% recovery [47]. The process achieved removal of 93% DNA and 74% protein contaminant in the final sample [47]. Some other studies performed using IMAC have shown the use of cobalt for 78% recovery of herpes simplex virus and removal of 96% of the contaminant proteins [48]. Although IMAC is able to achieve good virus recovery, it must be noted that the binding is often obtained from multiple covalent sites. The elution of viruses require strong ionic strength or pH conditions, or by addition of imidazole or glycine, which compete for active sites [23]. Harsh desorption conditions for viruses from ionic strength or pH can change the virus conformation, reducing its transfection properties or even causing virus degradation [23]. Imidazole and glycine, if used, would need to be removed from the final product with additional removal steps. IMAC is also known for leaching of the metal ions as in the case of nickel ions for retroviruses [49]. This would again require additional steps to remove ions which can increase the operating procedures and reduction in virus yield.

Lectin affinity chromatography (LAC) is based on lectin ligands on porous beads or membrane adsorbers. The ligands have an affinity towards glycan proteins or carbohydrate residues on the virus surfaces. For example, the Euonymus Europaeus lectin (EEL) and Erythrina Christagalli lectin (ECL) were used for the purification of influenza A/B virus from Vero and MDCK cell line. For the MDCK cell line, the EEL recovered 80-86% of virus by binding to the (α1,3) galactose receptor, DNA removal of 64-84% and the final cell protein content removal of 98% [50]. For Vero cells, the difference in glycosylation patterns from MDCK yielded poor binding

affinity for both ECL and EEL [50]. Where EEL on MDCK yielded >80% recovery, ECL on MDCK did not yield good recovery due to slow and weak binding which would require low flow rates or buffer recirculation for efficient recovery, and these conditions are not practical for scale up operations [50]. LAC are often designed for specific virus progenies. LAC severely lacks process robustness and hence it cannot be used as a universal platform approach for a range of viruses.

Heparin sulfate, another type of pseudo-affinity chromatography, uses heparin, a heavily sulfated glycosaminoglycan consisting of hexuronic acid and D-glucosamine residues, which act as receptors for virus attachment [51]. Downstream operation of Moloney Murine leukemia virus (MoMLV), porcine reproductive respiratory syndrome (PRRS) and influenza virus have been studied using heparin ligands. The recoveries achieved were 43% for MoMLV [52], 53% for PRRS [53] and 82% for influenza [54]. The operation also achieved >90% removal of cellular proteins but the DNA was between 75-1725 ng/dose [54] which is much higher than 10 ng/dose standard set forth by the EU . Heparin sulfate, similar to LAC, is specific to virus epitopes that can cause lower yield and restrict its use as a platform technology. Additionally, the branched structure of the heparin ligand and the hydrogen bond between a virus and heparin make it difficult to elute the virus without effecting its immunogenicity [55].

In summary affinity chromatography is a highly selective process with a high dynamic binding capacity due to the multivalent interactions (charge, hydrophobic and hydrogen) between the ligand and the virus. It has immense potential to be incorporated after a clarification step, achieving high recoveries and contaminant removal. However, for large scale vaccine applications, a more detailed analysis is still required as the performance remains to be unpredictable due to the virus

subtypes, post translational modification and glycosylation changes. In addition there remains a limited knowledge on the cell receptor binding capacity for vaccine products due to the expected conformational changes on the virus surface after the salt elution process on affinity systems. Affinity chromatography is specific to each virus subtype, making it a specialized process per vaccine product. A more universal approach to vaccine purification would lead to lower costs and faster time to market for desperately needed vaccine products.

2.2.5 Aqueous two-phase extraction

Currently, the downstream processing of viral products combines several of the previously mentioned unit operations. The biopharmaceutical industry currently considers 30% an acceptable virus recovery for vaccine products [56]. Our goal is to produce a high infectious yield with an alternate robust technique that has the potential to be applied as a platform technology. To fulfill this goal, we have been exploring aqueous two-phase systems (ATPS) as an optional mechanism to purify virus. ATPS combines clarification, concentration and purification into a single, integrated step to obtain a high yield with a low financial burden [57, 58]. ATPS is formed by mixing water soluble polymers, or a water soluble polymer and a salt, above a critical concentration that results in two immiscible aqueous phases [58]. It is a versatile method used for the separation and purification of biological molecule [59, 60]. For virus-like particle separation, a 54% recovery was noted for human papillomavirus [61] and 37% for a DNA plasmid vaccine [62] in a PEG-phosphate system. A high recovery for infectious virus is more difficult and sought after since the ratio of infectious particles to non-infectious particles can be as high as 1:1000. A

30-38% recovery was obtained for infectious bacteriophage T4 using a PEG-phosphate system [63]. A recovery yield of < 55% demonstrates the need to improve the virus purification using ATPS. Other shortcomings with ATPS include reduced transduction from chemicals, co-purification of proteins, and difficulty of polymer recycling. We have successfully tackled a majority of the concerns and this will be discussed in detail in Chapter 5.

In the last few years, there has been extensive research on the development of cell-based vaccines alongside egg-based vaccines. Egg-based vaccines face several drawbacks, including lengthy process time, requirement of a large inventory, frequent bottlenecks etc. Cell-based vaccines are a faster way of producing vaccines, however it too can be burdened with financial constraints. The majority of the financial burden is experienced by downstream processes and creating an easily scaled and uniform platform approach for virus purification could greatly improve vaccine production systems.

2.3 Virus removal for water purification

Modern methods of virus removal for the purification of drinking water focus on sized-based removal with membranes, disinfection with UV or ozone treatment and affinity adsorption to surfaces. While many of these methods have been shown to successfully remove viruses from drinking water, a widely accepted technology to curb worldwide deaths from lack of purified water has yet to emerge. While many of these methods have been shown to successfully remove viruses from drinking water, a widely accepted technology to curb worldwide deaths from lack of purified water has yet to emerge. Here I will discuss many of the many drinking water

purification methods, as related to viruses. Many methods are similar to those used in virus purification, but cost and lack of other proteins in the system make some of the methods distinct for water purification.

2.3.1 Size based

Membrane filtration is a separation process using semipermeable membrane. It works on the principle of differential hydraulic pressure by passing water from one side of the membrane to another. Methods used for the membrane filtration include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). **Figure 2.8** represents the size range for each of the filtration type and **Table 2.1** provides the operating pressure of the filters.

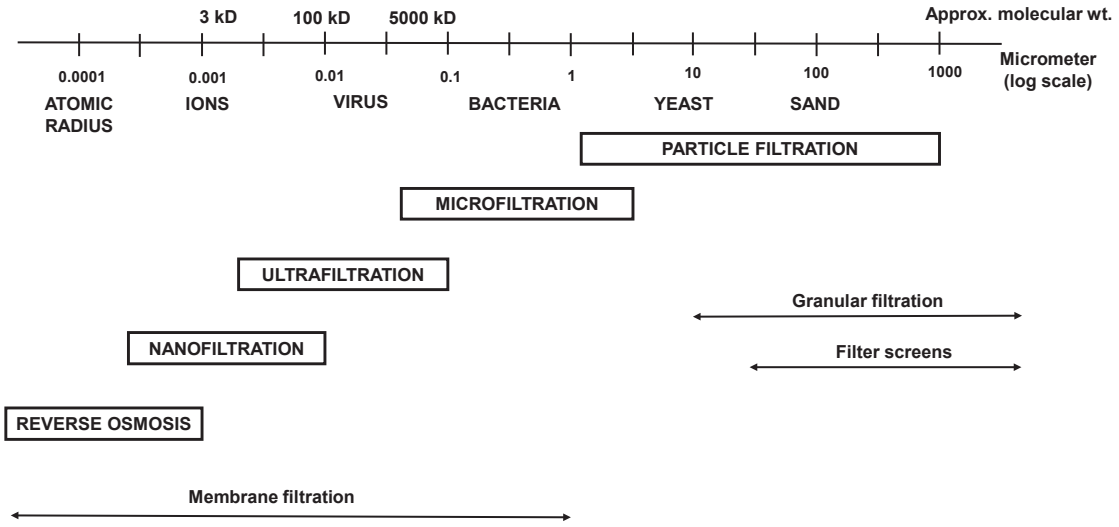

Figure 2.8 Size range of different filtration methods

Table 2.1 Membrane pore size and applied pressure

Filter type	Size (μm)	Operating Pressure (kPa)
Microfiltration	0.1 – 1	30-50
Ultrafiltration	0.1 - 0.01	30-50
Nanofiltration	0.001 - 0.001	500-1000
Reverse Osmosis	0.001- 0.0001	1000-5000

The virus removal mechanism is analyzed and quantified using the log reduction value (LRV). LRV is a mathematical term used to show the relative number of virus particles eliminated after treatment as highlighted in **Equation 2.1.** The Environmental Protection Agency (EPA) demands greater than 99.99% removal or 4 LRV as a drinking water standard and to make it available for safe consumption [64].

$$LRV = -log_{10} \frac{permeate\ virus\ concentration}{feed\ water\ concentration} \quad (2.1)$$

2.3.1.1 Ultrafiltration

In 2006, it was reported that globally about 3 billion gallons of water per day was processed through low pressure filters, of which 60% catered to drinking water needs and 22% for wastewater facilities [65]. The low pressure filters were MF and UF. UF has a pore size of 0.1 - 0.01 μm and it can remove some but not all viruses. In the United States 43 out of 50 states have not credited UF to achieve satisfactory log removal of viruses [66]. Regardless, the UF mechanism has often been stated to achieve moderate removal between 1 to 6 LRV for MS-2 and GA bacteriophage (a

virus that infects bacteria) [67] and 3 to 4 LRV for influenza viruses [68]. The reason for the moderate removal in the majority of reports was not only attributed to the size exclusion effect, but other interactions, such as hydrophobicity, electrostatic charge and presence of colloids, contributed towards the virus removal. A virus removal study for drinking water using MS2 bacteriophage, which is 27 nm in diameter, and Φ174 bacteriophage, which is 33 nm in diameter, has shown that MS2 produces higher removal. Although MS2 is smaller in size, the electrostatic repulsion effect increases its removal [69]. At pH 7.4, the differences in the pI of the viruses (MS2 - 3.5, Φ174 - 6.6) causes changes in the electrostatic charge, playing a key role in the high LRV for MS2. In a study involving organic matter, MS2 was studied at neutral pH conditions with a 0.1 μm nominal pore size filter. Five different factors of virus removal were identified, namely adsorption, sieving, charge, small organic matter concentration and large organic matter concentration [70]. The sorption and sieving could only achieve 1 LRV [70]. The maximum recovery of 3.4 LRV was witnessed in the presence of the organic matter due to the ability of colloids to cause pore constriction and cake formation [70]. A cartoon of each removal mechanism is shown in **Figure 2.9**.The other factor which can influence the virus concentration in UF is the transmembrane pressure (TMP). Increasing TMP has caused a decrease in virus retention due to abnormal enlargement of pores under pressurized conditions. For Φ174, a LRV was reduced from 3.15 to 2.68 with an increase of TMP from 100 kPa to 500kPa [71]. On the contrary, another study for capture of Φ174 on commerical Ultipour DV20 membrane filter has shown that pressure release from 210 to 100 kPa can cause a virus to penetatrate the membrane for a loss of 1 LRV [72]. Ultrafiltration has been shown to achieve good virus removal but a lack of

consistent separation makes this form of filtration very susceptible to virus breakthrough.

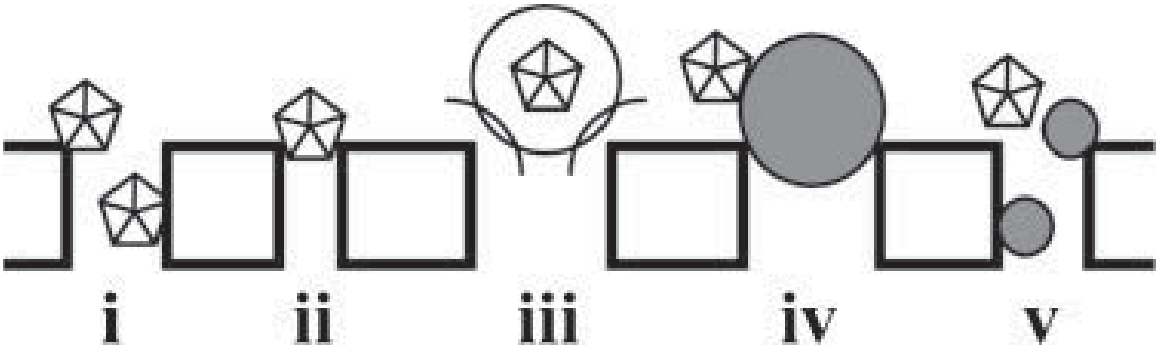

Figure 2.9 Five potential mechanisms for virus removal. (i) Sorption to the membrane, (ii) physical sieving, (iii) electrostatic repulsion, (iv) large organic matter induced removal, v small organic matter induced removal. Reprint permission granted from [70].

2.3.1.2 Nanofiltration and reverse osmosis

NF is a pressure driven process and has a smaller pore size than MF and UF. The MWCO of 200 to 1000 Da is likely to remove the majority of viruses. In a Minnesota field study, a cellulose acetate membrane CA2PF (MWCO 2000 Da) and polyamide membrane AFC30 (MWCO 350 Da) achieved 4.1 - 4.2 LRV for MS2 bacteriophage [73]. A study involving NF membranes showed 4.3 – 6.7 LRV of MS2 [74]. However imperfection in the membranes, such as the presence of an abnormal pinhole, faulty barrier coating and glue line discontinuity have been witnessed [75] to reduce the performance drastically to a low 1 LRV of MS2 [74]. NF membrane are also highly prone to boundary layer fouling from multivalent ions and organics, reducing the permeate flux [76].

RO works on the principle of diffusion unlike MF, UF and NF which operate based on sieving. RO works on the principle opposite to osmosis where high pressure is exerted for water movement from low concentration (high salt) to high concentration (low salt). Studies have shown varying viral removal efficacy from 2 LRV to 5.9 LRV for the MS2 virus [77, 78]. This inconsistency is believed to be due to the imperfections in the membranes that are correlated with a loss of salt rejection [79]. RO membranes are also known to undergo membrane compaction resulting in reduced permeability [80].

In summary NF and RO are seldom used for virus removal in water purification due to their high cost compared to previous filtration steps [81]. NF techniques are frequently used in biotherapeutics industry to achieve virus removal as the costs of NF is small in comparison to the higher prices of therapeutic drugs. One of the major disadvantages with NF is also its membrane fouling due to the increased pressure and the small pore size.

2.3.1.3 Flocculation

Membrane filters are commonly prone to fouling due to blockage of membrane pores by adsorption of the particulate compounds. In the case of virus in water containing natural organic matter (NOM), precoagulation or flocculation is necessary to prevent blockage and to remove viruses. Coagulation is a hybrid system followed by microfiltration to remove the flocs. MS2 bacteriophage removal in water containing NOM was able to achieve a 6 LRV by addition of ferric chloride (FeCl) and polyaluminium chloride (PACl) followed by hybrid ceramic microfiltration [82]. Without the FeCl or PACl only 1 LRV of MS2 (pI 3.5) was achieved. To accomplish a

high 6 LRV, a pH 5.0 for FeCl and 5.5-6.5 for PACl was required to maintain the negative charge on the virus particles. A high dose of 8 mg Fe/L and 4 mg Al/L was used to enable efficient charge neutralization since NOM are anionic competitors to the virus [82]. For Qβ bacteriophage, a PACl concentration of 1.08 mg/L and pH 6.8 for negative charge was able to achieve > 6 LRV [83]. The addition of coagulants for flocculation shows that a highly regulated coagulant dose and pH were important to achieve a high removal of pathogens. The major drawback of flocculation is the presence of residual metal ion content in the potable water may be higher than the legal regulation limit [82].

2.3.2 Inactivation process using UV and ozone treatment

Filtration may leave traces of microbial pathogen, which can be removed by the follow up technique of inactivation. Inactivation is a process of using disinfectants such as chlorine and ozone which bring about chemical oxidation of organic microbial species. The factors involved in this process are disinfectant concentration, contact time, temperature and pH. Chlorine is theorized to destroy microorganisms by combining with proteins to form N-chloro compounds and by interacting with sulfhydryl compounds of proteins [84]. Chlorine causes physiological damage to the cellular membrane along with causing decreased glucose, nutrient transport and energy level of cells [85]. Primary sewage effluents were treated with 8, 6 and 30 mg/L of chlorine to remove poliovirus by 2.8 LRV and MS2 for significantly lower value of 0.1-0.2 LRV [86]. Murine norovirus and poliovirus have also shown > 4 LRV at 0.1 mg/L for contact time of 120 minutes and 0.5 mg/L for 0.5 minutes [87].

However the major drawback of using chlorine is the formation of halogenated compounds with NOM which may be carcinogenic in nature [88].

Ozone is known to inactivate resistant microorganisms more effectively than free chlorine. It requires reduced concentration and shorter contact time than free chlorine to achieve similar inactivation [89]. Ozone inactivates virus by attacking protein capsid, liberating the nucleic acid and attacking them [89]. Adenovirus type 40 AD40 was inactivated > 3 logs with ozone concentration between 0.30-0.49 mg/L with a contact time of 2 minutes or more [90]. Murine norovirus was ozone disinfected at 0.3 mg/L and 1.2 mg/L to achieve 2 and 3 LRV respectively [91]. Ozone is also known to be carcinogenic in nature when treated with bromide ions in water [92].

2.3.3 Adsorptive virus removal from water

Virus removal by size is the most common in water purification applications. Membranes that are tuned to have a specific charge and hydrophobicity have shown promise for the purification of water and hence continue to be developed. A growing area of research is exploring the adsorption of viruses to functionalized surfaces using affinity interactions and multimodal binding for water purification applications.

Carbon nanotubes, incorporated as the pores of a PTFE membrane, are capable of removing pathogens from water [93]. The charge of the virus and the matrix can be manipulated with the knowledge of the pI for each and strict control over the pH of the solution. At a pH of 3, 8.13 LRV was achieved since the pI of MS2 is 3.5 where MS2 had a slight positive charge and MWCNT was negative. As the pH increased, making both the bacteriophage and the surface negatively charged, the

LRV dropped to 5.38 at pH 5.5 and a lower 4 LRV at pH of 9 [93]. Anodic multiwalled carbon nanotubes (MWCNT) act as electrochemical filters have also been used for MS2 removal. The positively charged anodic filter and negative virus particles can facilitate pathogen attachment, following which the virus undergoes inactivation by oxidation [94]. The electrical conductivity of the carbon nanotube was able to showcase 5.2 to 7.9 LRV or inactivation of MS2 bacteriophage under the application of a small DC potential between 2- 3 V [94]. Charge based filtration was also investigated using magnesium oxyhydroxides on ceramic depth filters. When the diatomaceous silica sand was coated with magnesium oxide (MgO), the outer surface gained $Mg(OH)_2$ or oxyhydroxide nature allowed a 4 LRV of MS2 and Φ174 particles [95]. The pI of MgO of 12 exhibited increased virus removal compared to Fe, Al, Zr and Y which had lower pI between 8-10 [95]. These studies clearly demonstrate the importance of pH for virus removal studies that focus on ionic interactions.

Virus particles tend to aggregate when the pH is equal to the pI of the virus, reducing the electronic repulsion amidst the virus particles. Studies involving bivalent cations such as Ca^{+2} and Mg^{+2} have shown that apart from the electrostatic interactions, steric interactions and specific binding between the cation charge and carboxylate moieties on the virus capsids also play a role in determining virus adsorption on membrane surfaces [96, 97]. Virus removal studies from water for MS2, Φ174 and hepatitis A virus (HAV) on colloidal alumina particles functionalized with amino ($-NH_2$), carboxyl (-COOH), phosphate (PO_4), chloropropyl ($-PO_3H_2-$) and sulfonate groups further emphasized that virus particle interactions cannot be determined by simple charge repulsions and attractions. The acidic groups COOH

and H_2PO_3 showed 1-2 LRV and HSO_3 demonstrated 4 LRV despite the negative zeta potential on all of the mentioned functionalized substrates. The molecular composition and the localized virus surface characteristics can be vital and in fact are likely more important than the net zeta potential or hydrophobicity [98].

Addition of salt at high pH and minimal electrostatic charge difference has shown increased LRV due to secondary interactions, such as hydrogen bonding and hydrophobic interactions. The ability to improve adsorption has been feasible due to the use of kosomotropes. Kosmotropes such as citrate anion are water structuring agent capable of inducing secondary interactions [99]. The water structuring phenomena and hydrophobic associations was confirmed by exhibiting high LRV of poliovirus in microfilters with an pI of 7 at pH conditions on both sides of the pI of 3.5 and 9.5 [100]. Hydrophobic and hydrophilic MF membrane with pore size of 0.22 μm were used to test the hydrophobic interaction pattern on the MS2 removal from water. The hydrophobic MF showed a 5.9 LRV at pH 3.9, close to pI of MS2, and a 4.3 LRV at pH 7 [101]. The hydrophilic MF showed 0.3 LRV at pH 3.9 and 0.04 LRV at pH 7 [101].

Many size, charge and hydrophobic-based filtration and removal mechanisms have been studied on an individual basis. However, there is still a need to improve current water purification methods. We plan to combine multiple binding modes into a single system, known as multimodal binding for virus capture. Multimodal binding should provide a high LRV with flexible operating conditions. In our investigation, we propose to remove porcine parvovirus (PPV), one of the smallest known mammalian viruses, using multimodal binding of charge and hydrophobicity from water by using peptides on nanofiber membrane filters. For porcine parvovirus (PPV) removal, it

was noted that trimeric peptide WRW and hexameric peptide YKLKYY achieved 4.5-5.5 LRV in human blood plasma [102] on a resin chromatographic column. The trimer WRW exhibited binding affinity to the virus due to the charge and hydrophobic interaction and in case of hexamer YKLKYY it is believed to be due to the secondary structure of the peptide [102, 103]. Our current research study will add to the current knowledge of creating superior water filtration systems using affinity and multimodal-based adsorption.

2.4 Virus characterization

2.4.1 Virus and protein structure

Viruses are macromolecular biological entities that cannot replicate by themselves and undergo assisted multiplication within its host cell organism [104]. Commonly, virus particles introduce a genome into the cell which is capable of producing infectious virions that can rupture the cell [104]. The virus is made of nucleic acid and a protective coating of proteins called a capsid. Many viruses also have a lipid bilayer envelope composed of receptor binding glycoproteins and are known as enveloped viruses. Viruses that do not contain a lipid bilayer are called naked or non-enveloped viruses (see **Figure 2.10**). The two types of nucleic acid which form the genetic material are RNA and DNA.

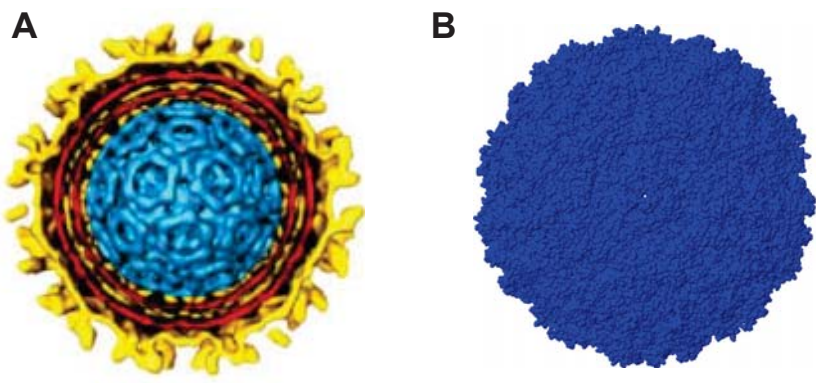

Figure 2.10 Virus structure (A) enveloped. Reprint permission granted from [105] and, (B) non-enveloped virus.

A thorough understanding of the viral surface can be useful for managing the purification of vaccine biopharmaceuticals or enabling the virus removal from water. A viral surface is made up of a variety of functional groups. **Figure 2.11 (A)** shows a slightly negative charge due to the carboxyl groups whereas **Figure 2.11 (B)** shows a neutral charge since the carboxyl groups are replaced by amine groups [106]. Due to the multiple functional groups on viral coat proteins, differences in strains and lack of purification processes to obtain an absolute pure sample make it difficult to quantify the virus pI and other surface properties [106].

The hydrophobic interaction of virus is another relevant property which can be used for designing virus separation strategies. In a study involving removal of GA, MS2 and Qβ bacteriophage from water using ultrafiltration, the GA was removed up to 2.2 LRV compared to > 4 LRV for MS2. It was hypothesized that this was due to the hydrophobic difference of the phages, which was determined to be GA > Qβ > MS2 by testing the binding of the phages to the hydrophobic substrate 1-dodecanethiol gold-coated surface and polypropylene. For viruses, the non-enveloped PPV was removed using hydrophobic peptides attached to a

chromatography resin [102, 103]. Influenza virus affinity to gold surface again highlighted hydrophobic effects of virus since gold is hydrophobic in nature [107].

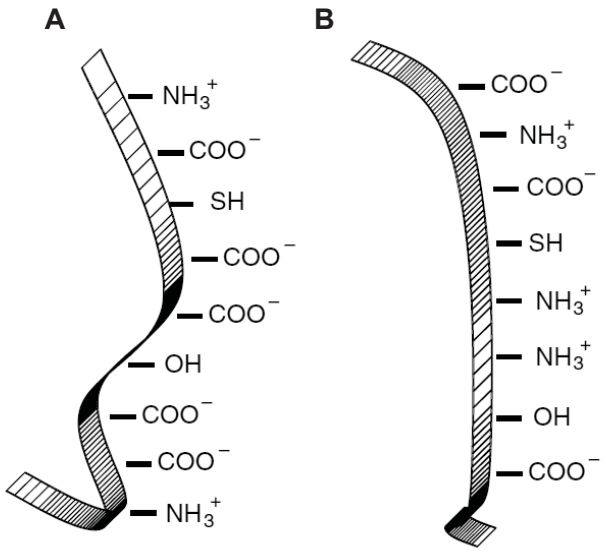

Figure 2.11 Coat proteins of virus strains. The strand (A) is negatively charged due to excessive carboxyl groups and strand (B) is positive due to excess amine groups. Reprint permission granted from [106]

The study on the morphology and functionality of viruses has grown substantially over the last few years. Information on surface characterization is still lacking and an improvement here will help the bioprocessing and water purification industries improve their pathogen removal systems.

2.4.2 Surface antigen characterization

Physical characterization of viruses is severely limited in the literature. It can be vital for recognizing the specific molecular recognition sites on the surface of the virus which help to purify large titer virus and achieve efficient separations. Analytical tools that quantify the size and adsorption characteristic of viruses will be discussed in detail in the following sections.

2.4.2.1 Size characterization

Knowledge of the size of virus particles can enhance our design of virus purification or removal processes. The particle size will then dictate the pore size needed in chromatography or membrane removal process. For aqueous two-phase system (ATPS), an increase in virus particle size can be used to determine if PEG is interacting with the virus particle and interfering with the purification. Understanding the size of virus particles can also provide in depth knowledge on the aggregation or flocculation of virus. Similar to purification studies, virus removal systems can be improved with the knowledge of virus morphology so that the permeate flux can be maintained and the available surface area for virus adsorption can be optimized.

2.4.2.1.1 Dynamic light scattering

Dynamic light scattering (DLS) or photon correlation spectroscopy is an optical analytical method used to measure the hydrodynamic size and its accompanying size distribution of particles [108]. It works on the principle of the Brownian motion of the particles and their ability to scatter a laser light at different fluctuations or intensities [109]. Analysis of these fluctuations yields the size distribution of particles.

During the purification process, virus particles are subjected to elevated pressure, change in pH and ionic salt strength. In addition they are burdened by the presence of cell protein contaminants, which may cause the virus to lose conformational stability and form aggregates, therefore reducing the immunogenic properties of the virus. The human influenza virus [110], parvovirus [111], adenovirus [112], baculovirus [113] after either a TFF or IEC were investigated for particle volume distribution, monodispersivity, aggregation or conformational changes of the

virus for sample characterization using DLS. Determination of virus structure with DLS from biomolecular separation involving ATPS is limited in literature but the technique has been applied for immunoglobulins which are in the size range of 10 nm, close to the virus size [114]. Virus structural validation after purification has also been performed using an antigen-antibody system as in the case of separation of VLP's from the yeast cell homogenate after each step of cell lysis, fermentation and chromatography [108].

For virus removal systems, virus or proteins aggregation can cause membrane fouling, flux decay and permeability effects in filters [115]. The hydrodynamic diameter of virus acquired using DLS is useful in process validation to improve filter performance and govern the virus filter spacing. Filter performance using DLS have been assessed for parvoviruses [111] and bacteriophages Φ 147 [116], pp7 [116], PR772 [117], MS2 and Qβ [118] on commercial virus filters. The bacteriophages are most often considered as surrogates for mammalian viruses especially the parvovirus in the range of 20 nm. In these studies the virus sample to be passed through filter systems are analyzed for non-aggregated particles and a low polydispersity index. The biggest challenge in DLS technique for viruses is the heterogeneous sample especially if contaminants are in similar sized components as virus themselves.

2.4.2.1.2 Electron microscopy

An image or picture of a biomolecular structure can speak a thousand words and capturing ultrastructure dimensions and conformations using electron microscopy remains an important technique for virus detection. The electron microscopy is an

acceleration of monochromatic beam of electrons from gun source on a thin specimen under high vacuum and voltage [119]. The scattered electrons are imaged using optical microscopy. Transmission electron microscopy (TEM) visualizes the internal and external virus structure in 2D and scanning electron microscopy (SEM) visualizes the 3D surface topology [120]. However among the two techniques the TEM has been used more predominantly for determining the morphology of virus capsids.

As discussed in the DLS section the virus purification procedures often run the risk of debilitating or aggregating the virus particles during to operating parameters. TEM is a significant tool to identify intact viruses or report structural changes encountered during downstream steps. Virus size and shape for a variety of the purification strategies namely the Tick bone encephilatis virus from SEC [121], Nervous necrosis viruses from heparin chromatography [122], herpesvirus from TFF and centrifugation [123] and White spot syndrome virus from differential centrifugation [124] have been shown using the TEM. An example of virus structure acquired on TEM is shown in **Figure 2.12**. For the investigation of purification of PPV from ATPS, TEM can be used after removal of virus from the PEG phase. For virus removal, the TEM is a potential tool to assess the virus morphology before the filtration and after it is washed and collected post filtration. The framework of the pathogen will be valuable to evaluate the filter performance and in accordance with assay and chromatography studies it can be crucial to investigate mechanism of adsorption. The sample preparation for TEM will importantly involve the staining procedure using uranyl acetate or phosphotungstic acid. The stain scatters the

electrons better, creating a well-defined image but also needs careful administration from virus aggregation or drying.

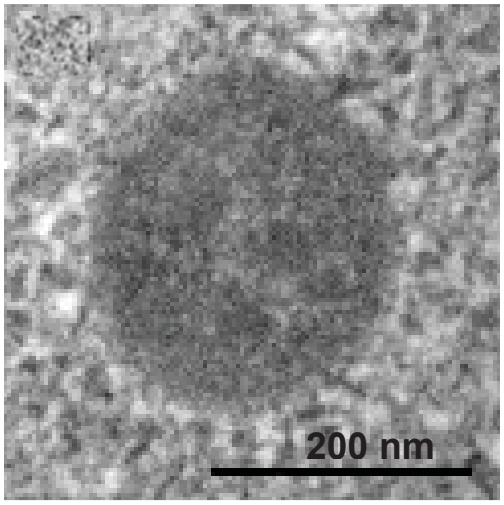

Figure 2.12 TEM image of virus. A negative stained vaccine virus providing size ~ 200 nm and spherical shape features. Reprint permission granted from [125].

2.4.2.1.3 Atomic force microscopy

Atomic force microscopy (AFM) is a versatile technique for obtaining three dimensional topographical images of a sample in the order of nanometers [126]. To form a well-defined image, AFM uses a cantilever with a sharp tip that measures the interfacial atomic forces when in close proximity to a sample. These forces can be interpreted as a distance (Hooke's law of elasticity) and the data are communicated to the user as an image [127] The apparatus and working principle has been shown in **Figure 2.13 (A)**. The greatest advantage of AFM over other methods is it can be performed in aqueous medium without disturbing its natural state. Although liquid state AFM is available, many virus studies have been performed in the dry state, thus negating one of the most powerful aspects of AFM. Other advantages of AFM

include a reduced sample preparation time and that there is no need for staining, fixing or synthetic preparation [126].

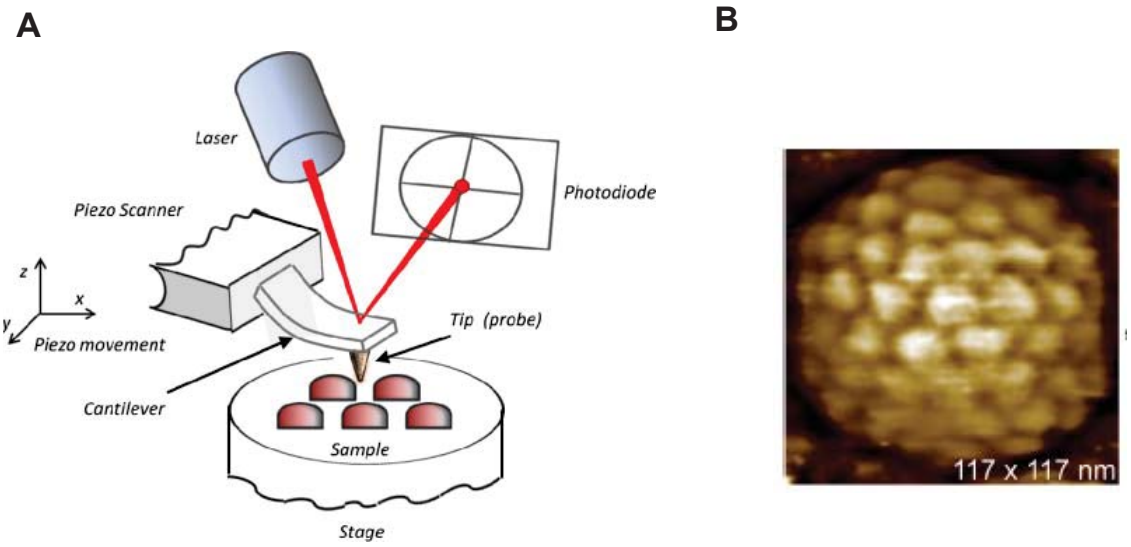

Figure 2.13 AFM apparatus (A) A microscope stage with x,y,z scanner is attached to a cantilever with a probe tip. The tip moves over the biomolecule providing deflections that are traced by laser and converted into a digital image for the observer [128] (B) An icosahedral symmetry of protoretrovirus that infects yeast showing capsid structure and capsomeres. Reprint permission granted from [129].

AFM can image fine details of virus capsid architecture as well as protein subunit, capsomeres on the virus as seen in **Figure 2.13 (B)** [129]. Topographical images in a lateral and vertical resolution at a nanometer range have been procured for Tick bone encephilatis virus after SEC purification [121], hepatitis B after salt precipitation [130], hepatitis B after ultracentrifugation and SEC [131], and orthopoxvirus after sucrose gradient [126]. For virus purification and removal systems, the AFM is a useful method to obtain molecular structural definition similar to TEM. It is suitable to assess the shape and size framework along with its aggregation behavior. The information helps evaluate the purification performance

from the perspective of contaminant proteins and virus denaturation. The virus morphology can help evaluate and improve filter performance.

2.4.2.2 Adsorption mechanism

Virus adsorption to a functionalized substrate surface can provide knowledge on molecular binding interactions and bonding mechanisms (covalent or secondary interactions) which can be key to designing effective virus removal systems. Based on the adsorption mechanism used, one can also obtain kinetic and thermodynamic variables which can provide details for identifying adsorption isotherms as a function of concentration and temperature.

Surface plasma resonance (SPR) is a label-free, real-time detection of biomolecular interactions between a receptor and target analyte. Bioreceptors in SPR for virus are usually an antibody, but investigations have also been conducted on peptides [132], and nucleic acids [133]. In SPR, a polarized light is incident upon an electrically conducting thin film on a sensor chip, creating total internal reflection of the incident light which is detected by a photodetector as shown in **Figure 2.14 A** [134]. In the presence of an antibody-analyte interaction on the sensor chip, a reduced intensity of reflected light is observed from the propagation of a plasmon wave at a resonance angle. The change in SPR angle and band shift can be then used to determine the mass of the antibody-analyte on the film interface [134, 135].

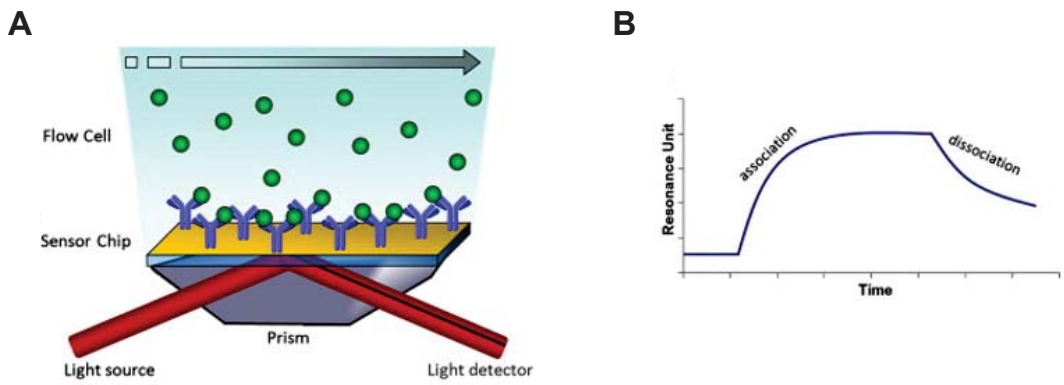

Figure 2.14 SPR working principle. (A) A shift in SPR band from red to black position is noted when antigen (green circles) is bound to antibody (blue Y-shaped objects) on sensor chips. (B) Response curve showing association of antigen-antibody after injection and dissociation after injection completion. Reprint permission granted from [134].

Biospecific response from feline calcine virus [134], baculovirus [136], influenza virus [137, 138], hepatitis B [139] and tobacco mosaic virus [136] were obtained against specific antibodies. The above examples used SPR for virus detection [134], epitope mapping [137], infection cycle [137] and interaction mechanism for therapeutic drugs [137, 138]. In these studies the change in response due to antigen-antibody was noted and plotted as resonance units vs. angle or time (see **Figure 2.14 (B)**). Kinetic association and dissociation can be obtained from the response units, which can help deduce binding interaction useful for virus structural conformation. Additionally, if the binding interactions are measured over a temperature range they can determine thermodynamic variables of change in enthalpy ΔH or entropy ΔS [140, 141]. SPR can be a valuable tool for examining the hydrophobic surface residues and charge species using PPV antibodies. The binding response can be useful to make valid conclusions on results procured from

ATPS and water purification project. Peptides synthesized on SPR surface can help understand the binding kinetics of PPV to the ligand.

2.5 Conclusions

Vaccine production in therapeutic industry is currently performed on a case-by-case basis for each virus and a single platform system to purify viruses is still lacking. A traditional ATPS system was used for virus precipitation but lack of selectivity for virus against protein contaminants and difficulty in separation from polymer phase inhibited ATPS progress towards vaccine development systems. In our study we have borrowed the traditional ATPS and customized it with new considerations that can help improve the infectious virus yield and help prevent co-precipitation of host cell contaminant proteins. The second goal of my Ph.D work is on designing membrane filters for removal of pathogen from water. Water is an important source for maintaining public health. Current filter systems for removing viruses use small pore size by size exclusion but this reduces permeate flux and cause membrane fouling. Our study uses peptides adsorbed on woven chitosan polymer with large micropores to remove viruses by chemical adsorption interactions and not size. Virus characterization by size and adsorption mechanism as discussed in the later section are some of the many ways that will help fulfill our objectives of better inexpensive vaccines and easy-to-use point of care water filters devices.

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Chapter 3

Virus characterization and purification using chromatography

A part of this chapter is planned for submission in a research journal.

3.1 Introduction

Animal cell cultures are grown in sterile conditions with specialized media and cellular nutrients for proliferation. Viruses are grown *in vitro* by inoculating cells with virus. The cells are lysed to produce a virus solution which contains media components, host cell DNA, endotoxins and cellular components. In this chapter we will focus on porcine parvovirus (PPV) purification using downstream processing techniques such as ion exchange chromatography (IEC) and size exclusion chromatography (SEC). The chapter will also describe the characterization of the virus, with a focus on hydrophobicity. Understanding virus surfaces can be important to the development of therapeutic vaccine and designing viral vector for gene therapy. Our work here will also explore the surface hydrophobicity characteristic of PPV and compare it to a range of different proteins using C18 reverse phase chromatography (RP-HPLC) and a fluorescent probe assay.

3.1.1 Model viruses

PPV is a non-enveloped virus of the *parvoviridae* family and known to cause reproductive failure in swine. PPV is a small, icosahedral, single-stranded DNA virus around 16-28 nm in diameter [1]. It is a model for the B19 human parvovirus [2, 3]. B19 parvovirus is pathogenic, causing a common childhood rash and in adults it has known to cause chronic anemia or mimic rheumatoid arthritis [3]. In addition to B19's hazardous nature, it is also difficult to propagate in cell culture. Hence we use PPV for laboratory experimentation. PPV is negatively charged at neutral pH and has a pI of 5 [4]. PPV has a high resistance to inactivation from heat, pH and chemical

treatment [5]. The robustness provides a broad scope for experimental working conditions.

Sindbis virus is an enveloped virus from the *Togaviridae* family and a member of alphavirus subfamily [6]. It is known to cause flu-like symptoms in humans. Sindbis virus is 40-70 nm in diameter with icosahedral symmetry. It is a single stranded positive-sense RNA virus [6, 7]. Sindbis virus is negatively charged at neutral pH and has a pI of 6 [8]. The heat resistant strain of the virus has a healthy virus particle to infectious virus ratio of close to unity, which makes it an excellent model for infectious virus studies [9].

3.1.2 Chromatographic purification of PPV

Traditionally, virus is purified by ultracentrifugation on cesium chloride (CsCl) or sucrose gradients [10, 11]. However, the shear force has been known to reduce virus infectivity and moreover, it is time consuming, labor intensive and difficult to scale up [12]. Virus precipitation with modest results have been obtained using salts ammonium sulfate [13] or calcium phosphate [14]. Polyethylene glycol (PEG)/aqueous two-phase system has also been studied for virus purification [15], but the technique lacks the ability to purify virus from many cell culture contaminants. Chromatography a popular technique for biomolecular purification has been seldom used for virus recovery due to diffusional limitations and large virus size, restricting access to internal surface area of beads. The technique has not provided high virus yield but it is known to work well for protein biomolecules < 5 nm in size [16]. Chromatography has gained wide spread attention due to its ability to create specificity for a biomolecule based on several variables as charge, size and

hydrophobicity [17-19]. The virus produced in the lab is heavily contaminated with cell media proteins and our goal is to consider each variable individually (charge, size and hydrophobic) for maximum protein removal with best possible recoverable virus. The purified virus can be very useful to analyze cell protein and contaminant interference during lab scale experimentation.

In this chapter we will show the results found from accomplishing the following objectives:

Objective 1: Develop a chromatography method to purify virus

Objective 2: Develop a chromatography method to quantify virus

Objective 3: Characterize the hydrophobicity of virus with chromatography and ANS dye fluorescence.

3.2 Materials and Method

3.2.1 Materials

For the phosphate buffer solution, sodium hypophosphite ($NaH_2PO_4.H_2O$) was purchased from VWR (Radnor, PA) and sodium triphosphate (Na_3PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride was purchased from Macron Chemicals (Center Valley, PA). Guanidine hydrochloride was purchased from Sigma (St. Louis, MO). Sodium hydroxide was purchased from Acros Organics (New Jersey, NY). Hydrochloric acid was purchased from EMD Chemicals (Billerica, MA). Solutions were made with water that was purified with a NanoPure water system (Thermo Scientific, Waltham, MA) to a resistance of >18 MΩ and filtered with a 0.22 μm bottle top filter (Millipore, Billerica, MA) or a 0.2 μm syringe filter (Nalgene, Rochester, NY) prior to use.

Phosphate-buffered saline (PBS) pH 7.2, 0.25% trypsin/EDTA, penicillin/streptomycin (pen/strep) and minimum essential medium (MEM) for cell propagation were purchased from Life Technologies (Grand Island, NY). For virus titration, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO).

The proteins in this study, bovine serum albumin, BSA, (Sigma, St. Louis, MO), chicken egg white lysozyme, LYS (CalBioChem, Billerica, MA), bovine fibrinogen, FIB (Sigma, St. Louis, MO), bovine hemoglobin, HEM (Sigma, St. Louis, MO), and human insulin, INS (a generous gift from Novo Nordisk A/S, Denmark) were used as received. 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA), respectively.

3.2.2 Cells, virus and titer assay

Porcine kidney cells (PK-13) were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% pen/strep at 37°C, 5% CO_2 and 100% humidity. The cells were propagated every 3-4 days at a split ratio of 1:5. Conditioned media was withdrawn from the cells and centrifuged prior to use.

Baby hamster kidney cells (BHK-21) cells were grown in EMEM supplement (Hyclone, Logan, UT) with 10% FBS (Atlanta Biologicals, Oakwood, GA), 10 µg/ml gentamicin (Gibco,Frederick, MD), and 5% tryptose phosphate broth (TPB) (Gibco,

Frederick, MD) at 37°C, 5% CO_2 and 100% humidity. The cells were propagated every 1-2 days at a split ratio of 1:3. Conditioned media was withdrawn from the cells and centrifuged prior to use.

PPV strain NADL-2 and Sindbis virus heat resistant strain (SVHR) were also gifts from Dr.Ruben Carbonell and Dr. Raquel Hernandez, respectively, at North Carolina State University. PPV and SVHR were propagated in PK-13 cells and BHK-21 cells respectively, as described previously [20] and clarified with centrifugation prior to use.

PPV and SRHV were titrated with a cell viability assay, the colorimetric MTT assay, as described previously [20, 21]. Briefly the PK-13 and BHK-21 cells were seeded in 96-well plates. Plates were infected with 25 µl of the virus and serially diluted across the 96-well plate. After five days of incubation for PPV and two days incubation for SRHV, the MTT salt solution was added. Four hours later, solubilizing agent was added. Plates were read on a Synergy Mx microplate reader (BioTek, Winoski, VT) at 550 nm between 4-24 hours after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that corresponded to an absorbance of 50% of the uninfected cell absorbance. The value was converted to a per millimeter basis and stated as the MTT_{50}/ml titer [20].

3.2.3 Ion exchange chromatography

Ion exchange chromatography was performed using the quaternary amine Q Sepharose XL virus (GE healthcare, Pittsburgh, PA) on a Waters Alliance HPLC equipped with a photo diode UV-Vis detector. The resins had an average particle size of 90 nm and mean pore size of 20 nm [22]. They were manually packed into an

Omega 2 mm * 5 cm (small scale size) and an Omega 4.6 mm * 25 cm (large scale size) chromatography column, purchased from Idex Health Science (Oak Harbor, WA). Buffer A was 10 mM phosphate in water and Buffer B was 10 mM phosphate, 150 mM NaCl in water and both at pH 7.2. 6M guanidine hydrochloride was used for cleaning after each run. A linear gradient of increasing Buffer B was performed and the % of NaCl needed to elute the protein was taken as the %B elution on IEC. PPV NADL-2 strain was filtered through a 0.2 µm syringe filter and injected at a volume of 10 µl and a flow rate of 0.25 ml/min for the small scale column. For a large scale column we used an injection volume of 264 µl and a flow rate of 1.32 ml/min. The optimized method was a gradient of 5 to 55% Buffer B accomplished from 2.1 min to 24.35 min. The method and the column were validated for a cleaning procedure after every single run with 6M guanidine hydrochloride to maintain the accuracy in peak area and height.

Samples were collected for all the chromatogram peaks at different retention times and the virus recovery using IEC was calculated by conducting the MTT assay. The cumulative virus titer for all samples against the starting stock value provided the % recovery value for the process. **Equation 3.1** provides the mathematical term to calculate the recovery. The term V_f and V_i represents the volume of each fraction and the injection volumes respectively. For a small scale column V_f was 0.25 ml and for a large scale column it was 1.0 ml.

$$\%\ virus\ recovery = \frac{\sum (10^{Log\left(\frac{MTT_{50}}{ml}\right)f_1}.V_f + 10^{Log\left(\frac{MTT_{50}}{ml}\right)f_2}.V_f + \cdots + 10^{Log\left(\frac{MTT_{50}}{ml}\right)f_{final}}.V_f)}{Injected\ material\ (10^{Log\left(\frac{MTT_{50}}{ml}\right)f_1}.V_i)}\ X\ 100 \quad \text{..... (3.1)}$$

3.2.4 Size exclusion chromatography

Size exclusion chromatography was performed on a Waters Alliance HPLC equipped with a photo diode UV-Vis detector using Sephacryl S-300 HR purchased from GE healthcare (Pittsburgh, PA). Sephacryl was manually packed into an Omega 4.6 mm * 25 cm column. Buffer was 50 mM phosphate and 150 mM NaCl in water at pH 7.2. PPV was injected at a volume between 50 µl and 200 µl at a flow rate of 1.0 ml/min.

3.2.5 Reverse phase chromatography

Reverse phase chromatography (RP-HPLC) was performed with a Waters XBridge BEH 130 C18 column on a Waters Alliance HPLC equipped with a photo diode UV-Vis detector. Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. Proteins at 5 mg/ml were injected at a volume of 20 µl and a flow rate of 1.0 ml/min. The conditioned media had an injection volume of 75 µl and the PPV injection volume was 150 µl. All samples were individually injected onto a C18 column. A linear gradient of increasing Buffer B was performed and the % of acetonitrile needed to elute the protein was used to estimate the experimental hydrophobicity values.

3.2.6 SDS-PAGE

SDS-PAGE was run using a 4-12% Bis-Tris NuPage gel (Life Technologies, Grand Island, NY) in NuPage MES running buffer (Life Technologies, Grand Island, NY). Samples were reduced in DTT (Life Technologies, Grand Island, NY) and

heated to 90°C for 10 min prior to loading onto the gel. SDS-PAGE was stained with the SilverXpress kit (Life Technologies, Grand Island, NY).

3.2.7 ANS Fluorescence

ANS was dissolved to a concentration of 20 µM in 50 mM H_2PO_4 (Sigma, St. Louis, MO). The ANS and protein were mixed at a ratio of 95 µl to 5 µl, respectively and read on a Synergy Mx microplate reader (BioTek, Winooski, VT) at an excitation of 350 nm and an emission of 482 nm. A plot was made of ANS fluorescence versus protein concentration and the slope of the line was expressed as the ANS fluorescence/µM protein. Samples that required desalting step was carried out using a 5 ml Zebra spin desalting column from Thermo scientific (Waltham, MA). The column eluent was collected by spinning the contents at 1000 *xg* for 2 minutes in a Sorvall ST16R Centrifuge (Thermo Scientific, Waltham, MA).

3.3 Results and Discussion

3.3.1 Virus purification using IEX chromatography

In biomolecular separation, the ability of a technique to distinguish between a target analyte and protein contaminants and to increase the specificity of the target molecule requires an understanding of the properties of all the components present in a given sample. Among the properties of size, polarity and charge, which can be used to distinguish the virus from cell media contaminants, this section will address the charge specificity. Charge-based separations can be performed using electrophoresis, isoelectric focusing and ion exchange chromatography. Electrophoresis and isoelectric focusing can be used for purification but they are

primarily used for confirming the purity qualitatively. IEC is a highly robust and accurate technique which is more commonly used for obtaining purified samples.

The miniaturized scale of anion exchange chromatography Q-sepharose column was initially used to establish the capacity to purify the PPV. The IEC on Q-sepharose yielded sharp peaks which were recognized using the UV absorbance at 280 nm as shown in the **Figure 3.1.** The first peak at 0-2 mins was the flow through peak. The flow through represents the PPV and protein contaminants which were unable to adsorb to the column resin beads based on charge or due to overloading of the column. The remaining well-defined peaks eluted at 11 min and 14 min. The likely proteins were the cell media protein contaminant of bovine serum albumin (BSA) and PPV respectively. The proteins were present in bulk in the stock and close proximity in peaks bodes well with the close range in pI 4.7 for BSA and pI ~ 5 for PPV. The peak at 11 min and 14 min were analyzed for virus concentration using the MTT assay and analyzed for proteins using the silica matrix SEC and SDS-PAGE.

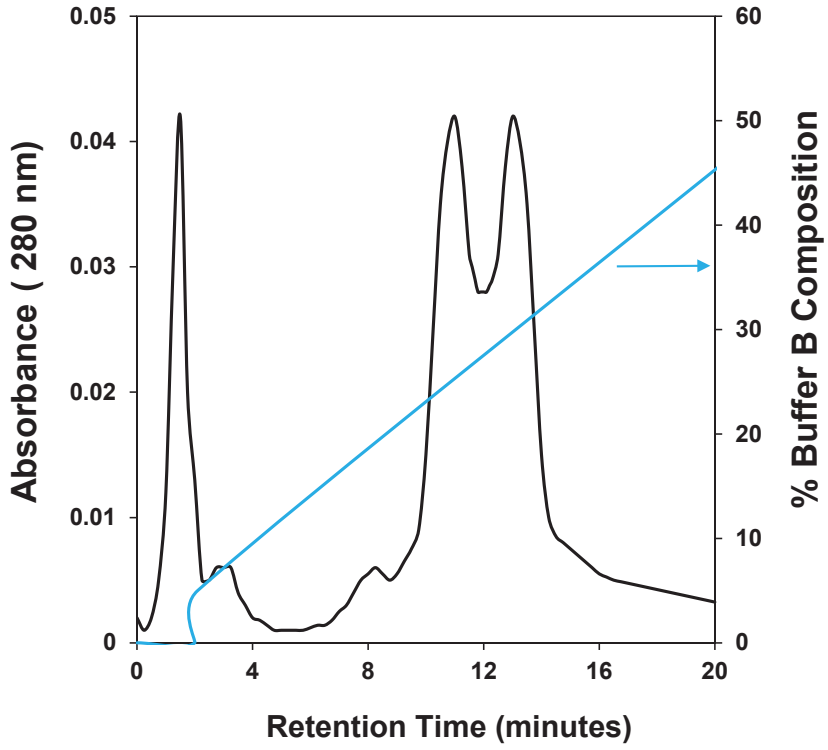

Figure 3.1 Ion exchange chromatography for PPV purification on a small IEC column packed with Q-sepharose resin (the figure is a representation and not an actual chromatogram)**.** 10 µl PPV was injected through on small column manually packed with Q-sepharose beads. The Buffer A was 10 mm phosphate at pH 7.2 and elution Buffer B was 10 mM phosphate and 150 mM NaCl at pH 7.2. The gradient used was 5% to 55% of Buffer B from 2.1 min to 24.35 min.

MTT assay results for the samples collected in the interval of 10 min to 16 min are displayed in **Figure 3.2.** The fractions at 10 and 11 min show a limit of detection of 1.6 $\log_{10}$ MTT_{50}/ml and 12, 13, 14, 15 min exhibit the 3-4 $\log_{10}$ MTT_{50}/ml infectivity. The data supports the virus elution between 12 and 15 min. The overall recovery was calculated at an average low of 0.2 %. The recovery was calculated by the **Equation 3.1**.

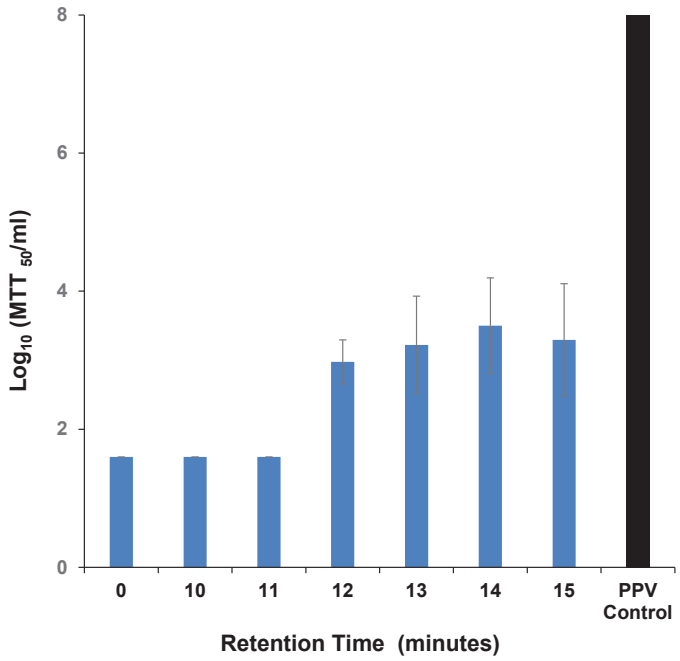

Figure 3.2 PPV concentration of samples from a small IEC column. Titer results of 0.25 ml fractions collected at different time points from the small column after 10 µl PPV injection. The limit of detection is 1.6 $\log_{10}$ MTT_{50}/ml. All data points are the average of two separate tests and error bars represent the standard deviation.

SDS-PAGE was performed on the fractions as it is considered the gold standard for protein validation. All the fraction samples shown in **Figure 3.3**, including controls of media, BSA and PPV, show bands in the range of 66 kDa. The main protein in the PPV capsid is VP2, and it has a molecular weight (MW) of 64 kDa [23] and the MW of BSA is 66 kDa. Over 80% of the PPV capsid is VP2 and hence the PPV and BSA are seen to run closely together in SDS-PAGE. The fractions at 11, 12 and 13 min show a thick protein band. The PPV recovery and concentration as obtained from the MTT assay is low to obtain thick protein bands hence the bands are likely BSA. The fraction at 15 min appears to have removed the majority of the contaminants, but the data presented so far is not substantial enough to prove an increase in purity.

The experimental investigation using MTT titer was often very close to the limit of detection, which prevents virus quantification and possibly causing a low recovery. To circumvent the low recoveries and the overlapping bands on SDS, the process was scaled up to the large-scale IEC.

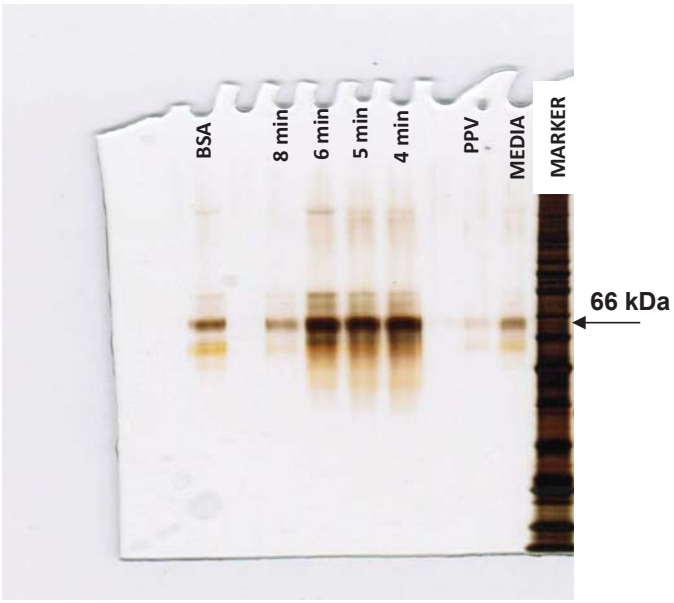

Figure 3.3 SDS-PAGE of PPV samples from a small IEC. Proteins bands of 0.25 ml sample fractions collected at different time points from the 10 µl PPV injection on small column.

A large IEC column of 4.6 mm x 25 cm was manually packed with the Q-sepharose resin. The larger column was scaled-up to maintain the linear flow rate (cm/min) of the smaller column, a common practice in chromatography scale-up. This residence time was same for both small and large column. The injection capacity of the instrument could not be increased past 264 µl, so the inject volume per volume of packing was reduced. The gradient elution pattern was maintained the same as the small scale column. The chromatogram for the large column is shown in

Figure 3.4. Samples were collected with intervals of 1 min and the fractions were validated using the MTT assay.

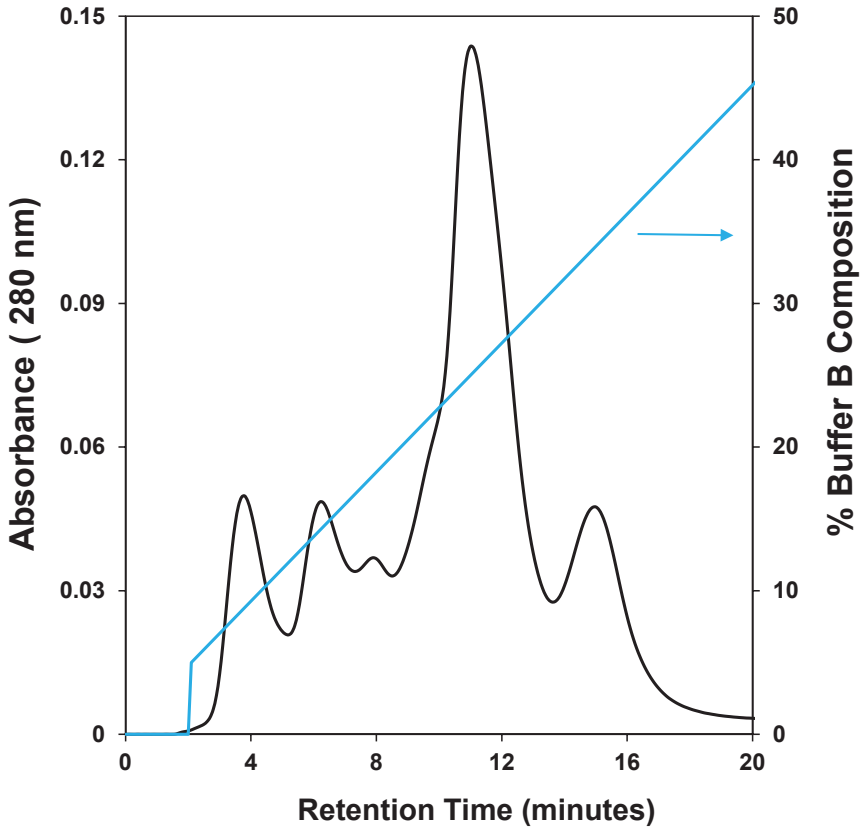

Figure 3.4 Ion exchange chromatography for PPV purification on large IEC column packed with Q-sepharose resin. 264 µl PPV was injected through a large column manually packed with Q-sepharose beads. The Buffer A was 10 mm phosphate at pH 7.2 and elution Buffer B 10 mM phosphate and 150 mM NaCl at pH 7.2. The gradient used was 5% to 55% of Buffer B from 2.1 min to 24.35 min.

The MTT assay results showed an increase in log values for all fractions (see **Figure 3.5**), overcoming the issue of being near the limit of detection. All the fractions from 10 min to 16 min had PPV at a titer of 5-6 Log_{10} MTT_{50}/ml. The PPV in the large column started eluting out earlier as compared to the small column. Scale up of a column can have issues as lower bead stability due to lack of wall

support and increase in bed pressure due to a larger amount of resin [24]. These variables can influence operating conditions and sample loading causing change in peak width and shape [25]. Nonetheless an improvement with the virus recovery at 0.8% was noted compared to 0.2% of small scale IEC. The protein bands on the SDS-PAGE (not shown) had similar inferences as observed on the small scale due to the close MW of PPV and BSA. Without further confirmation that BSA was being removed from the virus preparation, we did not continue to pursue purification of PPV with IEC.

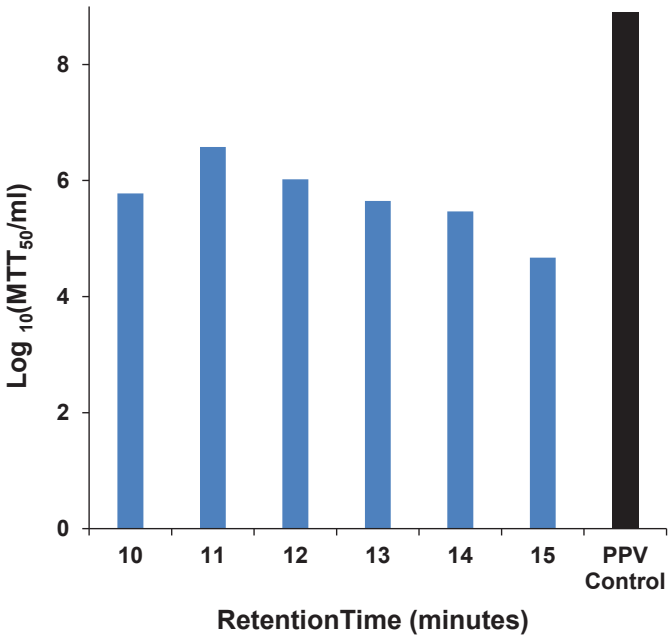

Figure 3.5 PPV concentration of samples from a large IEC column. Titer results of 1 ml fractions collected at different time points from the large column after 264 µl PPV injection. The data points are plotted using results from a single test.

Virus purification and recovery analysis was also performed on Sindbis virus. A 264 µl Sindbis virus injection into the large IEC column was performed with the same gradient protocol as PPV. The elution pattern for Sindbis virus was similar to the PPV as shown in the **Figure 3.6**.

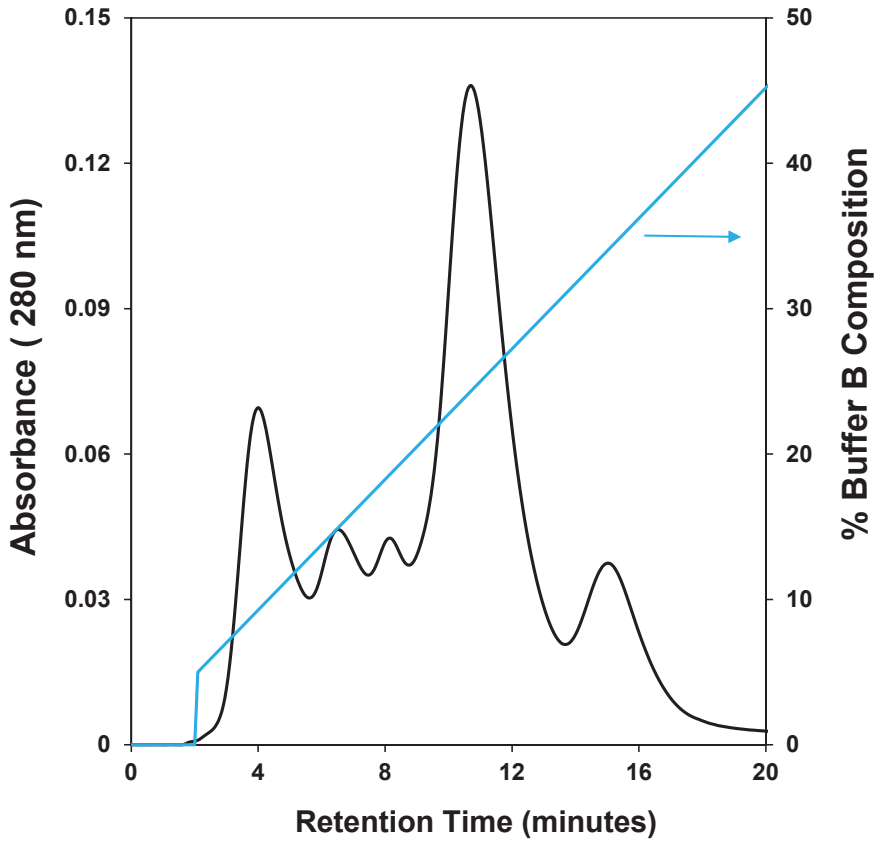

Figure 3.6 Ion exchange chromatography for Sindbis purification on a large IEC column packed with Q-sepharose resin. 264 µl Sindbis was injected through a large column manually packed with Q-sepharose beads. The Buffer A was 10 mm phosphate at pH 7.2 and elution Buffer B 10 mM phosphate and 150 mM NaCl at pH 7.2. The gradient pattern used was 5% to 55% of Buffer B from 2.1 min to 24.35 min.

The samples collected from the fraction collector were analyzed with the MTT assay and SDS-PAGE as shown in **Figure 3.7 and 3.8**. A higher recovery of 2.2% on the MTT assay was obtained. For the SDS-PAGE, the BSA (66 kDa) presence was seen from the media contents but low concentration of Sindbis virus showed no trace of protein bands preventing us from making valid conclusions.

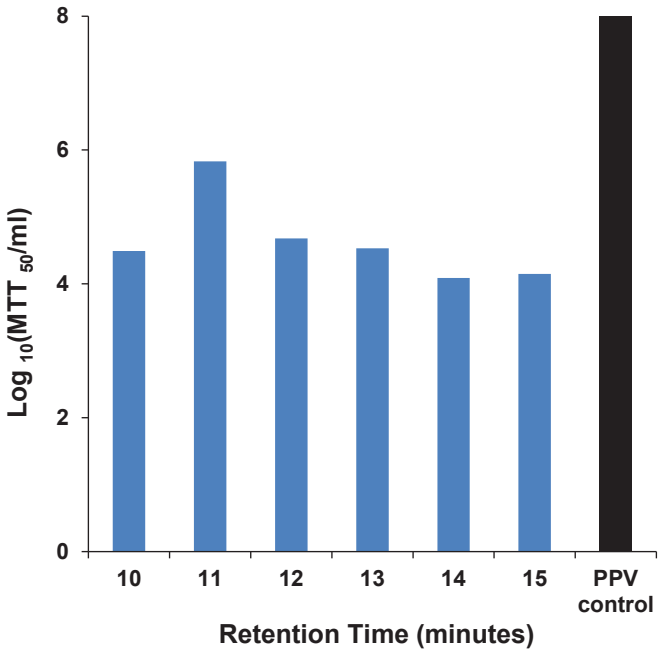

Figure 3.7 Sindbis virus concentration of samples from a large IEC column. Titer results of 1 ml fractions collected at different time points from the large column after 264 µl Sindbis injection. The data points are plotted using results from a single test.

Figure 3.8 SDS-PAGE of Sindbis virus samples from a large IEC. Proteins bands of 1 ml sample fractions collected at different time points from the 264 µl Sindbis injection on a large column.

3.3.2 Size exclusion chromatography

Measurement of infectious virus is an expensive and time consuming process. Techniques commonly employed for measuring viruses from mammalian cells are reverse transcriptase polymerase chain reaction (RT-PCR), plaque assay or $TCID_{50}$. PCR can measure the DNA/RNA of the virus but lacks the ability to distinguish between infectious and non-infectious particles. Plaque assay is a gold standard approach but the number of samples to be processed in a given time is limited because it is a labor intensive and time consuming process. $TCID_{50}$ is also a time consuming process and requires an experienced operator to obtain values consistently. The MTT assay, a cell viability assay, was used in our lab for PPV and Sindbis quantification. The tetrazolium salt has the ability to cleave to the mitochondria of live cells changing the yellow salt into purple formazan crystals [26]. The crystals are solubilized and the absorbance was measured. MTT assay for parvovirus takes 5 days for a full cycle of infection in addition to a lot of supplies [20]. To try and reduce the time needed for virus quantification, we considered developing a virus quantification procedure with chromatography. Successful results with chromatography can be very productive and efficient since it is a rapid method for the detection of proteins. In this section we will be discussing the work on applying SEC for purifying and quantifying virus from cell culture media.

Sephacryl S-300 high resolution resin with a fractionation capacity for globular proteins between $1x10^4 – 5 x 10^6$ Da was packed in a 4.6 mm x 25 cm column. PPV stock from virus production was repeatedly shown to be 8 $\log_{10}$ MTT_{50}/ml in our lab. The stock was diluted to prepare samples from 8 $\log_{10}$ MTT_{50}/ml to 3 $\log_{10}$ MTT_{50}/ml.

The values were confirmed using the MTT assay. Samples ranging from 8 to 6 log_{10} MTT_{50}/ml are superimposed and shown in **Figure 3.9**. For a 50 µl injection a limit of detection was noted at 6 log_{10} MTT_{50}/ml and hence the chromatograms from 5 log_{10} MTT_{50}/ml to 3 log_{10} MTT_{50}/ml have not been shown in the figure. The peak area for the retention time of 2 min on all samples was plotted against the MTT assay values to obtain a linear plot (see **Figure 3.10).**

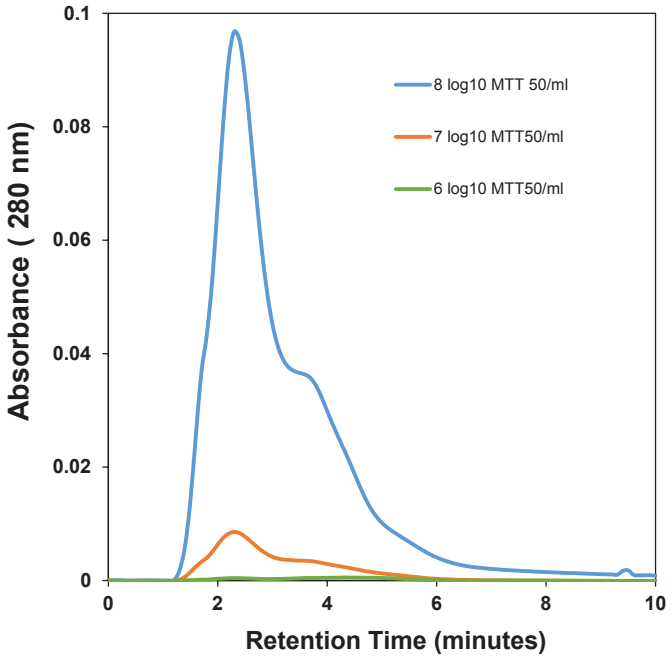

Figure 3.9 SEC for PPV purification on large IEC column packed with sephacryl resin. 50 µl PPV samples of 8 log_{10} MTT_{50}/ml to 6 log_{10} MTT_{50}/ml was injected through a large column manually packed with sephacryl beads. The Buffer was 10 mm phosphate and 150 mM NaCl at pH 7.2 at rate of 1 ml/min.

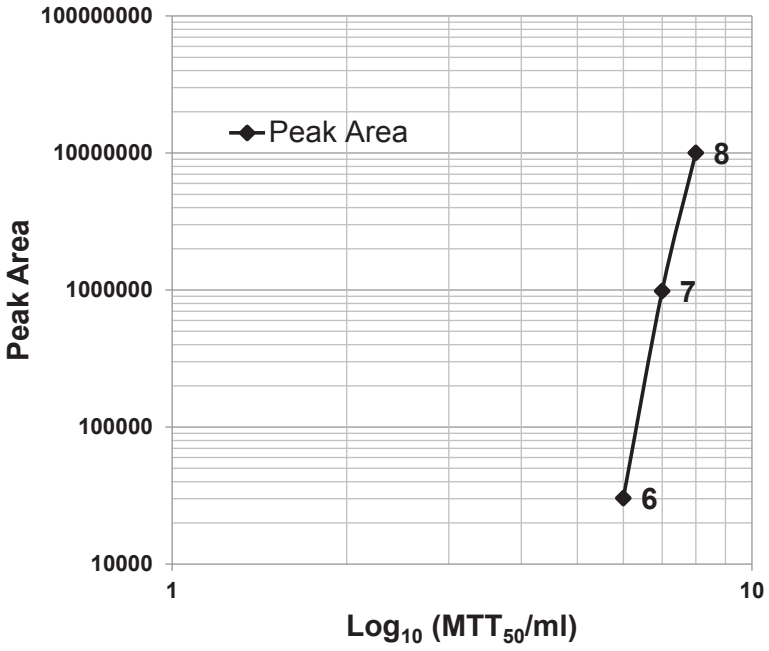

Figure 3.10 SEC vs. MTT assay. The chromatogram peak obtained from injection of 50 µl PPV samples 8 $\log_{10}$ MTT_{50}/ml to 6 $\log_{10}$ MTT_{50}/ml on a SEC sephacryl column was measured for peak area and compared against corresponding MTT assay values.

Injection volumes were steadily increased from 50 µl to 200 µl for each of the six samples of 8 $\log_{10}$ MTT_{50}/ml to 3 $\log_{10}$ MTT_{50}/ml to improve the detection limit. A limit of detection 5 $\log_{10}$ MTT_{50}/ml was noted for 200 µl injection close to injection capacity and any more improvement in terms of detection was not expected.

In summary, the SEC experiment has the potential to be used for samples greater than 5 $\log_{10}$ MTT_{50}/ml. However the detection limit is inconvenient for investigations involving virus in our lab and hence the idea of using SEC was withdrawn. If the technique is used for virus quantification in the future, then additional experimentation towards detecting any protein contaminants in the peak should be conducted. BSA is a major contaminant in the cell media during virus

production and it is likely present along with the PPV in the chromatogram peak discussed above.

3.3.3 Validating the hydrophobicity on viruses

Virus surface characteristics information can be important in virus purification during attachment to a chromatographic matrix, membrane operations and in developing gene vectors for specific cell receptor attachment. The knowledge on the surface hydrophobicity of viruses is limited. Phages (viruses that infect bacteria) were shown to have a varying degree of hydrophobicity, as demonstrated by carbon adsorption [27]. B19 human parvovirus [28] and our work of PPV [29] being precipitated with glycine concluded that the action was likely due to the highly hydrophobic surface of the virus. A considerable scope is available to improve the literature on the nature of the hydrophobic residues on viruses. The work in this section highlights the hydrophobicity measurement using reverse phase chromatography and ANS (1-anilino-8-naphthalene sulfonate), a fluorescent dye that attaches to hydrophobic patches on proteins [30]. The investigation of virus surface hydrophobicity can be used to better understand virus-cell interactions, as well as create improved methods to detect, remove, and purify viruses.

3.3.3.1 Reverse-phase chromatography

The virus hydrophobicity was measured and compared against the hydrophobicity of a panel of proteins. The proteins used for the experiment are shown in the **Table 3.1.** The RP-HPLC hydrophobicity was measured by determining the percentage of acetonitrile required to elute the sample from a C18 column. Higher acetonitrile and protein residence time meant a higher hydrophobicity. The

investigation of the elution of proteins on RP-HPLC was performed by an undergraduate colleague in our lab group, Amna Zahid. It was simple to determine the location of the protein peak for pure proteins since they only had one major peak. The peaks obtained for proteins were sharp and well defined peaks. The order of hydrophobicity based on the residence time was INS< LYS< BSA< FIB< HEM as seen in the **Figure 3.11.**

Table 3.1 Panel of proteins

Protein/Virus	**Abbreviation**	**Molecular weight (MW)**
Bovine Hemoglobin	HEM	67
Bovine Serum Albumin	BSA	66
Fibrinogen	FIB	340
Insulin	INS	6
Lysozyme	LYS	14.3

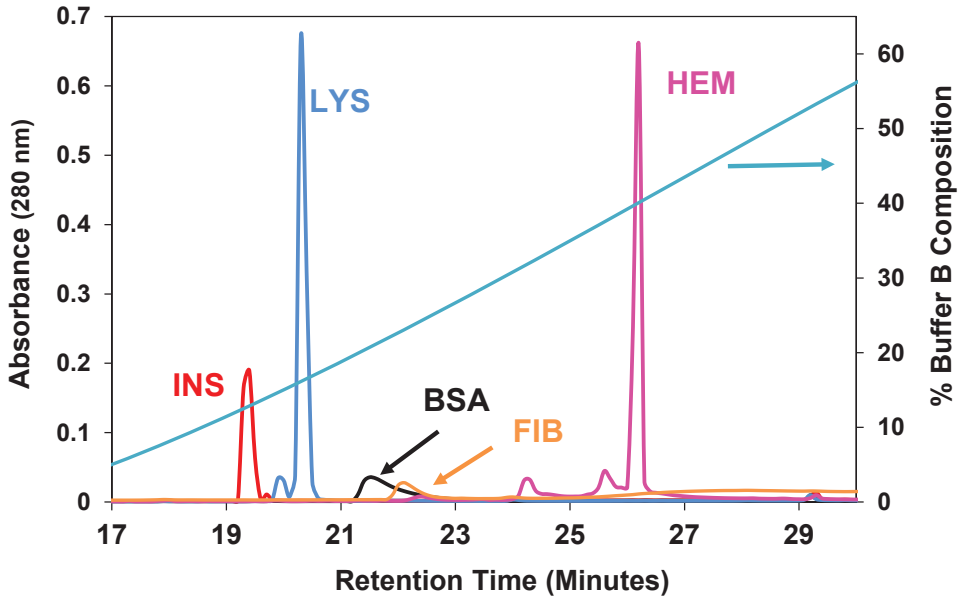

Figure 3.11 Reverse-phase chromatogram of pure proteins. 20 µl of 5 mg/ml of each protein was injected into a C18 RP-HPLC with a buffer flow rate of 1 ml/min. Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. The percentage of acetonitrile needed to remove the protein from the column was used as the experimental hydrophobicity.

It was more challenging to identify the virus peaks. Virus is produced by the process of cell lysis and hence it is accompanied with cell media and additional proteins formed during the cell growth together known as conditioned media. It is also at a low molar concentrations ~ 4 µM. Hence we first compared crude virus solutions to conditioned media as shown in **Figure 3.12**. The large peak at 21 min was BSA from the fetal calf serum in the crude PPV and conditioned media, as confirmed by the pure BSA peak in **Figure 3.11**. To confirm that the earlier peaks that had different absorbance values were associated with the conditioned media, we desalted the PPV and the conditioned media with a spin column. Most of the peaks prior to 20 min in the conditioned media were reduced or eliminated as shown

in **Figure 3.13**. The concentration of PPV was the same before and after desalting, as measured by the MTT assay (data not shown).

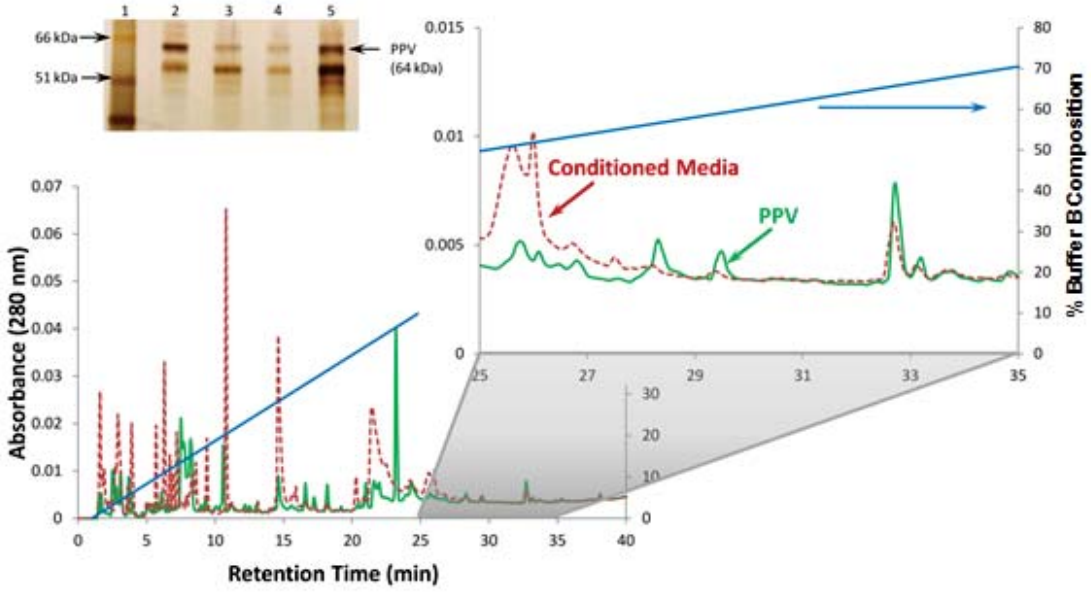

Figure 3.12 Reverse-phase chromatography of PPV and conditioned media. PPV (solid line) was compared to conditioned media (dotted line) to determine the acetonitrile needed to elute PPV. Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. The insert shows the range of 50 – 70% acetonitrile. The SDS-PAGE has lane 1 – marker, lane 2 – stock PPV, lane 3 – 27.5-28.5 min, lane 4 – 28.5-29.5 min, and lane 5 – 29.5-30.5 min.

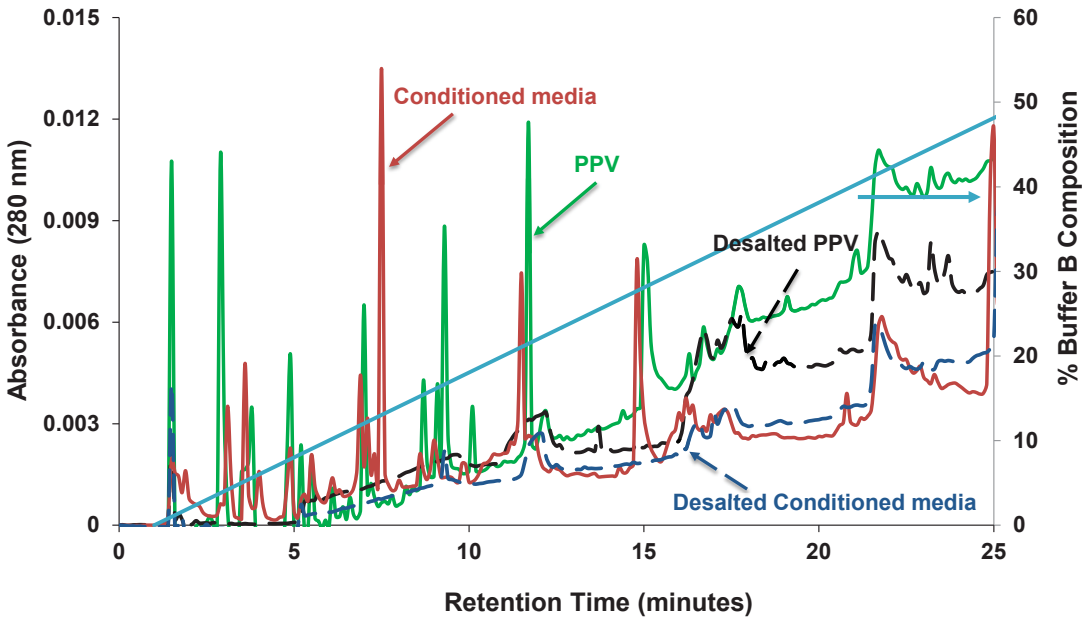

Figure 3.13 Reverse-phase chromatography of PPV and conditioned media: stock and desalted. PPV and the conditioned media (solid line) was compared to desalted PPV and desalted conditioned media (dotted line) to determine the contaminants 0 to 20 min belong to conditioned media. Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile.

Now in order to identify the PPV peak in the **Figure 3.12** the PPV trace was compared with conditioned media for different times and the unique peaks were subjected to SDS-PAGE for PPV validation. Three peaks were found in the PPV sample that were not in the conditioned media. These peaks eluted at 23, 28.5 and 29.5 min. The peak at 23 min was disregarded as being too large for the concentration of PPV that was in the sample. The SDS-PAGE shown in **Figure 3.12** was run for fractions collected at 27.5, 28.5 and 29.5 min and labelled as lane 2, 3 and 4. The peak 29.5 min had the highest amount of PPV, even showed a protein band at MW range of PPV at 66 kDa. We therefore labeled the 29.5 min as the PPV peak.

The retention time of PPV on RP-HPLC confirmed the hydrophobicity of PPV was stronger than the panel of proteins. A schematic for retention time of proteins is shown in **Figure 3.14**. Hence the hydrophobicity evaluation and the hypothesis of viruses having a strong hydrophobic surface using RP-HPLC was successful but another study comparison of PPV against panel of proteins would improve this investigation.

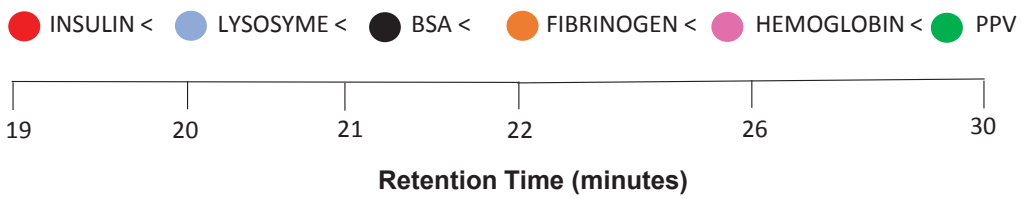

Figure 3.14 Order of protein hydrophobicity using reverse phase chromatography. The hydrophobicity sequence based on protein retention time was experimentally determined using the C18 column and the % acetonitrile for elution.

3.3.3.2 Surface hydrophobicity using ANS

ANS fluorescent probe is an organic compound with a high affinity for the hydrophobic patches on a protein surface. ANS is a non-florescent probe in water, but in the presence of non-polar surfaces, ANS undergoes a blue shift and high florescence emission. ANS measurements have been used to determine the hydrophobicity of serum albumins [30].

Graphs were created for the ANS fluorescence vs. protein concentration as shown in **Figure 3.15**. The slope determined from the plots was used for calculating the average surface hydrophobicity values for the proteins tested [30]. All proteins, except for insulin and lysozyme, produced a good linear correlation fit. The slope

equations are presented in the graphs. The insulin and lysozyme gave inconsistent data including large error bars (not shown). Insulin has given us inconclusive results in other studies performed in our lab on hydrophobicity. Insulin is known to have many different conformations with minor changes in pH [31]. It is likely that the different pHs of our experiments (low pH for RP-HPLC and neutral pH for ANS) changed the conformation of insulin.

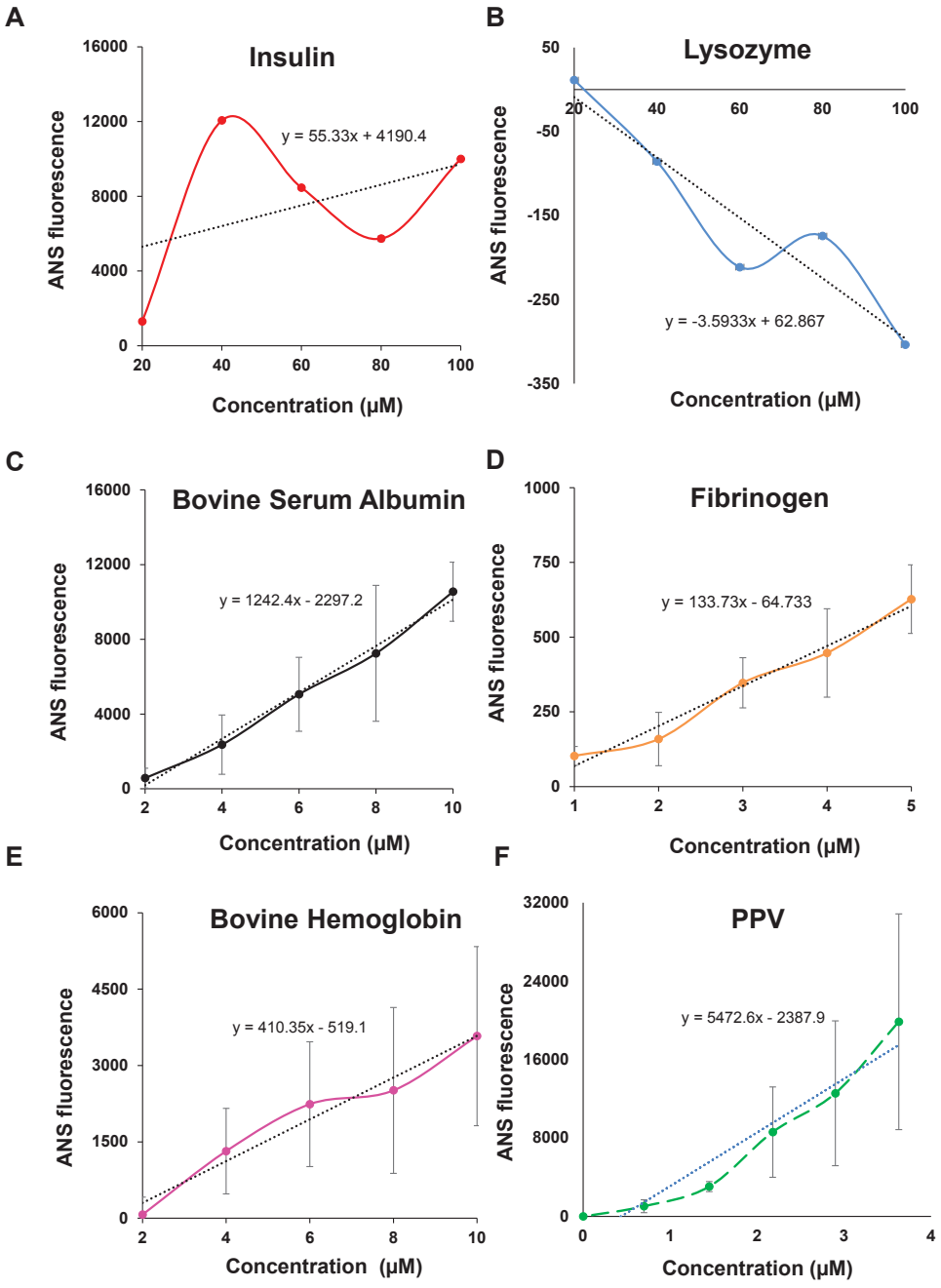

Figure 3.15 Average surface hydrophobicity measurement using ANS fluorescence. Proteins A) Insulin B) Lysozyme C) BSA D) Fibrinogen E) HEM F) PPV of volume 5 μl at different concentrations (μM) and ANS of volume 95 μl at 20μM concentration were mixed to measure fluorescence at Ex/Em 350/482 nm. Slopes were measured by plotting fluorescence against protein concentration (μM) to determine average surface hydrophobicity. All data points are the average of three separate tests and error bars represent the standard deviation.

From the average measured values of fluorescence/μM, the order of hydrophobicity strength was gauged as shown in the **Figure 3.16.** The sequence was not as observed as per the C18 RP-HPLC and this comparison between the two techniques is shown in **Figure 3.17**.

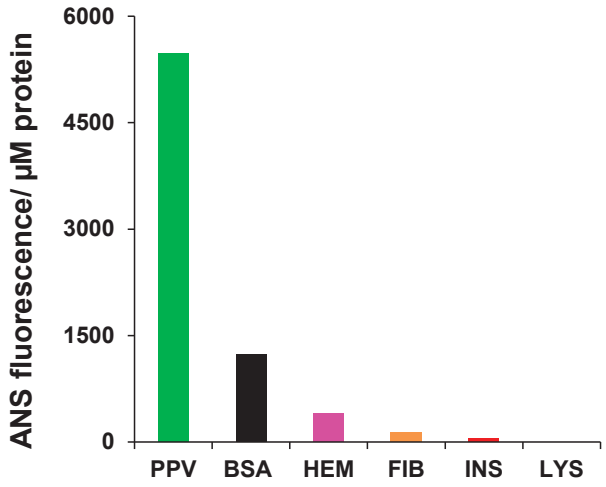

Figure 3.16 Order of protein surface hydrophobicity using ANS fluorescence. The decreasing sequence of hydrophobic protein affinity to fluorescent probe was experimentally determined using the ANS assay and fluorescence measurement. All data points are the average of three separate tests and error bars represent the standard deviation.

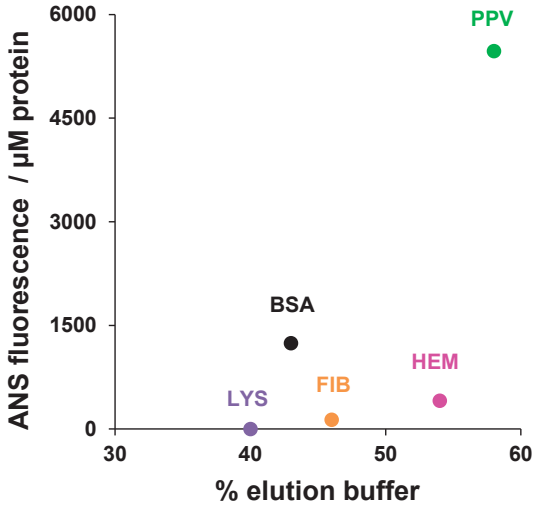

Figure 3.17 ANS surface hydrophobicity vs. % elution buffer from C18 RP-HPLC.

The PPV fluorescence was extremely high and this may be due to the media and conditioned cell proteins procured during the virus preparation. Virus production in the lab is performed by inoculating the virus in mammalian cells. Hence the conditioned media comes along with the PPV stock. The presence of conditioned media causing ANS fluorescence in PPV sample was proven by measuring ANS for desalted conditioned cell media which is shown in **Figure 3.18**. The PPV curve in the figure is similar to the conditioned media especially considering the error bars. The decrease in PPV fluorescence units can be due to additional clarification step involved during virus production. However it is clear that PPV may be obtaining a large ANS value due to conditioned media.

Removal of the extraneous proteins was attempted for bettering the surface hydrophobicity measurement from the virus itself. The ANS fluorescence was measured for desalted PPV that gave a slope of 3800 ANS / μM protein and a 30% reduction in slope value from PPV. The value of 3800 ANS / μM protein is extremely high compared to second highest BSA at 1242 ANS / μM protein. It is likely from the evidence that the ANS is from media proteins rather than a measurement of virus hydrophobicity.

The hydrophobic evaluation from ANS was not successful as the evaluation of the hydrophobicity by C18 RP-HPLC. However the foundation for the technique has been laid out with ANS values for a panel of proteins. When a highly pure strain of PPV has been obtained from contaminant media proteins the ANS for PPV determination is likely to fit the trend of surface hydrophobicity as noted for RP-HPLC.

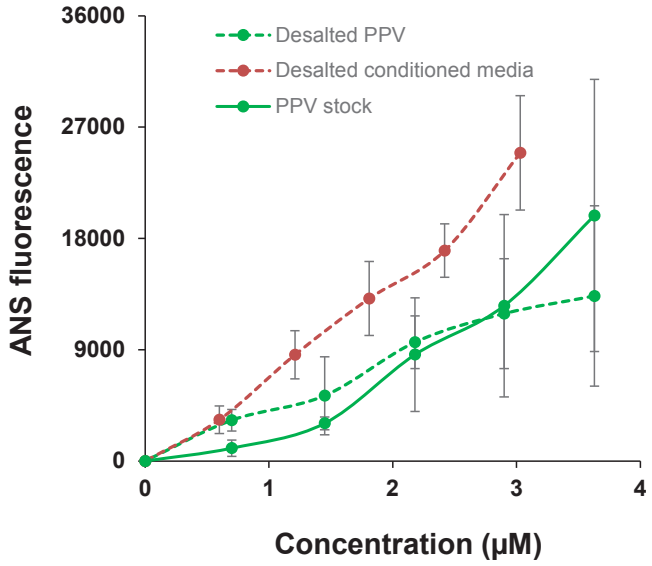

Figure 3.18 ANS fluorescence measurement for virus and conditioned media. The comparison of slopes of ANS fluorescence vs. concentration for stock sample (dotted line) and desalted samples (solid lines). All data points are the average of three separate tests and error bars represent the standard deviation.

3.4 Conclusions

Purification of virus is usually performed from density gradient centrifugation or salt or polymer precipitation techniques. The methods are difficult to scale up or lack specificity from co-precipitating impurities. Filtration is another technique for virus purification but this may cause virus degradation from shear stress, especially in tangential flow filtration conditions. An efficient and fast paced system to achieve high purity virus is required. Chromatography is the preferred method of purification due to its easy scale up, robust and repeatability. We studied virus purification on IEC and SEC for PPV without successful conclusions.

The IEC was performed on a small scale column, which identified multiple peaks. Virus peaks were selected by virus infectious titer however, the overall yield was

only a low 0.2%. To improve the yield we scaled up the column and increased the virus input. The large scale column provided a similar chromatogram, which was encouraging, since scale up operations are often hindered by a change in resin bead pressure and wall stress conditions. The overall yield for PPV in the large column was 0.8%. Gel electrophoresis was used to visualize the purity of the virus preparations. However the result from the gel was inconclusive since the MW of the VP2 protein in PPV is 66 kDa and for BSA it is 65 kDa. These run very closely in the gel and cannot be separately identified.

Our learning on virus purification for PPV using IEC was worthwhile since our preliminary results identified simple well-defined peaks. The MTT assay was a good virus quantification tool for eluted virus from the IEC columns. The study lacks the validation of media proteins since the MTT assay only measures virus concentration. In the future, a Western blot technique where an affinity bind capture step can help distinguish the PPV from BSA on the gel would be helpful. However, antibodies with enough specificity to PPV are not currently available.

Surface hydrophobicity of viruses is an important feature that can help build viral vectors or develop therapeutic vaccines or drugs. The knowledge of surface hydrophobicity of viruses is limited and hence we were interested in comparing the virus hydrophobic strength to other proteins. We used C18 RP-HPLC for calculating the acetonitrile elution strength of proteins, which is one method to determine the hydrophobicity of a protein. The result obtained was promising and the PPV eluted at a higher acetonitrile concentration as compared to the proteins tested. In a quest to strengthen the hypothesis, we used ANS fluorescence as a hydrophobic measurement. Our results indicated the PPV to have a higher surface hydrophobic

value compared to standard proteins. However further examination revealed to us the high ANS value for PPV could have been due to cell media proteins, including BSA. We proved the exaggerated fluorescent values were coming from the cell proteins when we read ANS values for conditioned media obtained during cell culture.

The surface hydrophobicity investigation was useful to prove the higher hydrophobic values for PPV compared to standard proteins. The ANS was useful to validate surface residues for all proteins however the analysis on pure virus strain is pending. When the PPV is available in its pure form without media proteins the ANS study should be repeated to make a strong case for virus hydrophobicity.

3.5 References

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Chapter 4

Single virus tracking in cells and immunohistochemistry to study the action of osmolytes in infected cells

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A part of this chapter has been accepted for publication in the *Journal of Antiviral Research*.

4.1 Introduction

Virus and cellular structures and their interactions are highly dynamic in nature. Virus infection is an intriguing process and a thorough understanding of the infection pathway of a virus particle through a cellular organism has tremendous potential for the development of antiviral drugs and gene therapy vectors. Every virus follows a unique mechanism of cell recognition, internalization, cellular transport, genome release, replication, and new virus particle release [1]. The subject is extremely vast and hence we will be limiting our discussion to porcine parvovirus (PPV) that will be used for trafficking investigation in this chapter.

PPV belongs to the family *Parvoviridae*. PPV is known to cause reproductive failure in swine [2, 3]. Parvoviruses are small non-enveloped virus that are ~26 nm in diameter. The genome is negative single-stranded DNA of about 5 kb in length [4]. The PPV capsid is made up of three proteins VP1, VP2 and VP3, of which 90% can comprise of the VP2 protein [5].

The PPV infection process starts with the virus binding to the surface cell receptors. Entry into the cell happens through receptor-mediated endocytosis. During endocytosis, viruses uses glycan, glycolipid, glycoproteins to attach on the cell surface followed by rapid uptake into endosomes [6, 7]. Entry of PPV is typically found to be through clathrin-mediated endocytosis [5, 7, 8]. Once the viruses are within the endosome, they undergo acidification, which creates conformational changes that release VP1 capsids into the cytoplasm [5-7]. The endosomal acidification is also responsible for the release of phospholipase A2 domain, which creates early steps of gene expression [7, 9]. PPV have been known to reside within the endosome for several hours and release into cytoplasm appears to be a very

slow process [10]. PPV in the cytoplasm conjugates to ubiquitin which causes capsid transformation allowing the virus to transition into nucleus [8, 11]. The virus pathway from the cytoplasm to the nucleus itself is poorly understood. The minute virus of mice (MVM) parvovirus has been known to infect a cell by degrading the nuclear membrane [12]. Once the virus is in the nucleus transcription is initiated when the cell enters the S-phase. Here the single strand DNA is replicated along with synthesis of mRNA. The mRNA are transported into cytoplasm where the ribosome translates the mRNA into capsid proteins. Mature viruses formed here cause cell apoptosis and infection of remaining cells.

4.1.1 Single virus tracking using fluorescence microscopy

Virus quantification for PPV has been performed by reverse transcriptase polymerase chain reaction (RT-PCR) [13], plaque assay [14], MTT assay [15] and $TCID_{50}$ [15, 16]. However each of these techniques have their shortcomings. RT-PCR can quantify nucleic acids but they cannot distinguish infectious from non-infectious particles. For PPV this can be a major disadvantage since the particle to infectious ratio can be as large as 1000:1 [7, 8]. Methods such as plaque assay, MTT assay and $TCID_{50}$ are more accurate for infectious virus but they are time consuming, laborious and have high errors. Our goal is to establish a fast-paced effective technique for quantification for infectious PPV. Here we are proposing to fluorescently tag virus particles and observe particles trafficking through the cellular machinery under the microscope. The technique has immense potential to quantify virus immediately upon its intrusion into cellular compartments.

One of the widely used technique to unravel the infection cycle of a single virus particle is real time imaging using fluorescence microscopy [17]. The common microscopes used for tracking fluorescently-labeled virus particles are confocal microscopy, total internal reflection microscopy (TIRM) and fluorescence imaging microscopy [18]. The confocal microscope gives a 3D imaging but the large focal area can cause signal loss. TIRM has minimal signal loss but the lack of imaging depth restricts the tracking study to only the cell surface. The fluorescence microscope has the largest imaging depth and is often preferred when a long-range and detailed study of the virus-cell interaction is desired [18].

The virus and cell must be sufficiently labelled with dye molecules and analyzed by procuring high temporal and spatial resolution images. The most common ye label is fluorescent labels. Commonly used chemical fluorophores for attachment to proteins are fluorescein, rhodamine and Alexa fluor dyes [19]. Of the three dyes, Alexa fluor is known to have good photostability and less pH sensitive than the other proteins based dyes and this can be useful for visualizing virus particles during the acidification process of the infection cycle. In virus labeling, it is important that the fluorophore does not hinder virus activity [20]. Zhang et al. studied the labelling and internalization of Dengue virus with Alexa Fluor 597, maintaining a balance between the dye brightness and pathogen function [21].

In our investigation we will be tracking the PPV capsid through a mammalian cell line using an Alexa fluor dye. We optimized the labelling efficiency from reducing virus immunogenicity, prevented impurity labeling and obtained a time study of virus trafficking through the cellular machinery.

4.1.2 Immunohistochemistry

Immunohistochemistry (IHC) is an integral research tool to diagnose infectious disease in tissue culture systems by creating an antigen-antibody interaction and then identifying the antibody (or immunoglobulins) via direct or indirect labeling [22, 23]. For direct labeling, an antigen is detected by binding with a labeled antibody [22, 24] as shown in **Figure 4.1 (A)**. The method is quick, but lacks the sensitivity to detect many antigens due to the lack of obtaining pure, conjugated antibodies. In indirect labeling, the antigen is detected by using two antibodies. The primary antibody is bound to the antigen and then the secondary labeled (or second layer) antibody is bound to the primary antibody [22, 24] as shown in **Figure 4.1 (B).**

Antibodies are of two kinds monoclonal and polyclonal. Monoclonal antibodies are highly specific to an epitope on an antigen [25] and polyclonal antibodies can bind to multiple epitopes on an antigen. Polyclonal antibodies generate an effective antigen signal but this can be disadvantageous since there is a high likihood of non-specific binding that would produce false positive signals [25]. Indirect labeling often uses a monoclonal antibody (unlabeled) as the first antibody and a polyclonal antibody as the second antibody (labeled). By doing this, the specificity for an antigen target is increased and then the label is amplified by binding the secondary antibody to more than one site on the primary antibody. IHC has often been accompanied with the use of chemical inhibitors to block the specific pathways giving valuable information on the virus dissemination through a living cell [26]. Specific chemical antivirals against porcine reproductive respiratory syndrome virus (PRRV) replication was identified by IHC [26]. For our investigation we will be using

the IHC technique to study the action and effect of antiviral compounds during the PPV infectivity of PK-13 cells.

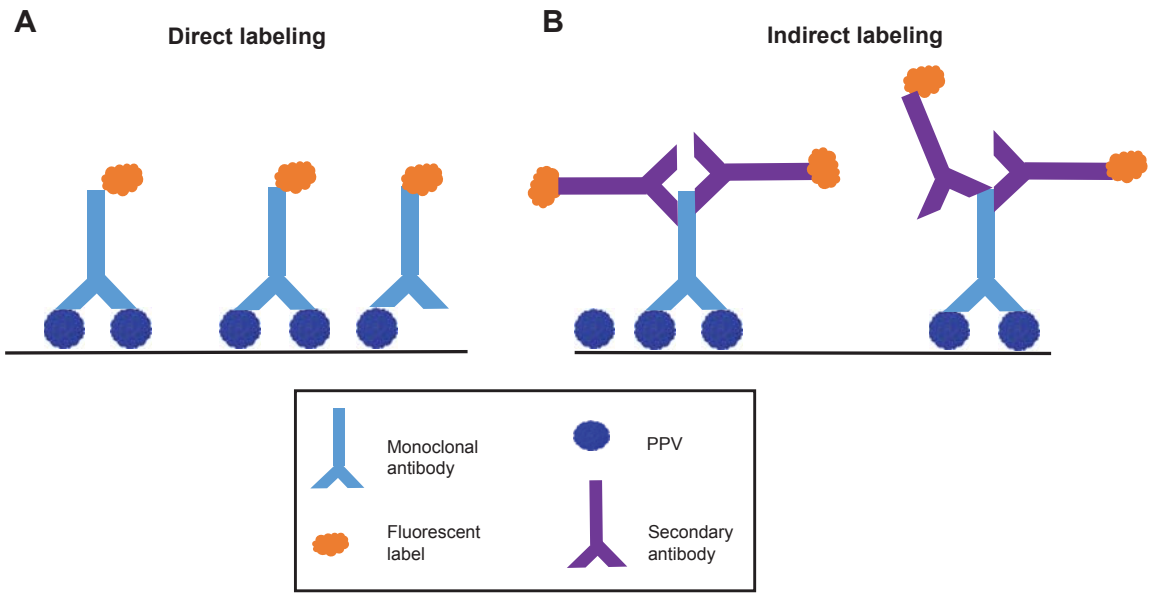

Figure 4.1 Immunohistochemistry techniques (A) direct labeling and (B) indirect labeling.

In this chapter we will show the results found from accomplishing the following objectives:

Objective 1: Explore the PPV infection by tracking the movement of fluorescently labeled viral particles

Objective 2: Determine if PPV capsids are formed when cells are in contact with antiviral osmolytes.

4.2 Materials and methods

4.2.1 Materials

Sodium bicarbonate ($NaHCO_3$) for buffer was purchased from Sigma–Aldrich (St. Louis, MO). For pH control the 12.1 M hydrochloric acid (HCl) was purchased from VWR (Radnor, PA). The osmolytes trimethylamine N-oxide (TMAO) dehydrate and glycine were purchased from Sigma–Aldrich (St. Louis, MO). The bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS, pH 7.2) and 0.25% trypsin/EDTA for cell propagation were purchased from Life Technologies (Grand Island, NY). Poly-L-lysine for cell attachment on glass slide and 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclei staining were purchased from Sigma–Aldrich (St. Louis, MO). 3.7% formaldehyde for cell permeabilization and fixation was purchased from VWR (Radnor, PA). The Alexa fluor 488 NHS ester (succinimidyl ester) for antigen tag was purchased from Life Technologies (Grand Island, NY). The monoclonal mouse anti-PPV primary antibody was purchased from VMRD (Cat no. 3C9D11H11, Pullman, WA) and the polyclonal Alexa fluor 546-conjugated rabbit anti-mouse secondary antibody was purchased from Life Technologies (Cat no. A11060, Grand Island, NY). All solutions were made with Nano-Pure water (Thermo Scientific, Waltham, MA, resistance >18 MΩ) and filtered with either a 0.2 μm syringe filter (Nalgene, Rochester, NY) or a Millipore 0.2 μm bottle top filter (Billerica, MA) prior to use.

4.2.2 Cells, titer and assay

Porcine kidney cells (PK-13) were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% pen/strep at 37°C, 5% CO_2 and 100% humidity. The cells were propagated every 3-4 days at a split ratio of 1:5. PPV strain NADL-2, also a gift from Dr.Ruben Carbonell at North Carolina State University, was propagated in PK-13 cells, as described previously [15]. PPV was clarified with centrifugation prior to use.

PPV was titrated with a cell viability assay, the colorimetric MTT assay, as described previously. Briefly, PK-13 cells were seeded in 96-well plates. Plates were infected with 25µl of PPV and serially diluted across the 96-well plate. After five days of incubation, the MTT salt solution was added. Four hours later, solubilizing agent was added. Plates were read on a Synergy Mx microplate reader (BioTek, Winoski, VT) at 550 nm between 18-24 hours after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that corresponded to an absorbance of 50% of the uninfected cell absorbance. The value was converted to a per millimeter basis and stated as the MTT_{50}/ml titer [15].

4.2.3 Virus trafficking in cells

The Pierce bicinchoninic acid (BCA) protein assay kit, from Thermo scientific (Waltham, MA), was used for determining the protein content of PPV solutions. For cellular trafficking 500 µl PPV (2 mg/ml) was mixed with 50 µl of 1M $NaHCO_3$ and different volumes of Alexa flour dye in a Rotoshake Genie for 2.5 hours. The tagged PPV sample was added to a 5 ml Zebra spin desalting column from Thermo

scientific (Waltham, MA). The column eluent was collected by spinning the contents at 1000 *xg* for 2 minutes in a Sorvall ST16R Centrifuge (Thermo Scientific, Waltham, MA). 1 ml PBS was added to the column followed by centrifuging to collect additional factions. A UV lamp was used to observe sample fluorescence after each collection. All samples were stored at -20°C in micro centrifuge tube wrapped in foil to prevent fluorescence loss. Size exclusion chromatography (SEC) was performed on a Waters Alliance HPLC equipped with a fluorescent detector using Sephacryl S-300 HR purchased from GE healthcare (Pittsburgh, PA). Sephacryl was manually packed into an Omega 4.6 mm * 25 cm column. The elution buffer was 50 mM phosphate and 150 mM NaCl at pH 7.2. Alexa tagged PPV was injected at a volume of 10 µl at a flow rate of 1.0 ml/min.

To prepare glass slides for cellular attachment, the slides were soaked in 2 M HCl for 1 hour to etch and remove any grease. Then, 200 µl of poly-L-lysine was added to the area of cell growth. After five minutes, the slides were washed with water and dried for 2 hours under UV light. Cells were seeded on the slides at a density of 5 x 10^4 cells/slide with a total volume of 50 µl/slide. Cells were incubated for 6 mins at 37 °C, and 5% CO_2, then 10 ml of fresh media was added to the petri dish that contained the slides. After 24 hours of incubation, the media was removed and cells were washed with PBS. Each slide was infected with 15 µl/slide of Alexa fluor 488 tagged PPV (10^8 MTT_{50}/ml) for 30 minutes. The slide was then washed twice with PBS to remove any unattached PPV and stored in media. At different time intervals post-infection, the media was removed, washed with PBS and the infected cells were fixed with 200 µl of 3.7% formaldehyde for 20 min at room temperature. The slide was washed twice with PBS and the cells were blocked with 200 µl of 5

mg/ml BSA for 10 minutes. The slide was washed with PBS twice. Finally, 50 µl of 150 µM DAPI was added for 5 min at room temperature and washed with PBS. Images of the cells were taken with an Olympus IX51 microscope with a DP72 camera (Olympus, Center Valley, PA).

4.2.4 Immunohistochemistry

Glass slides were prepared as described in Section 4.2.3. Cells were seeded on the slides at a density of 5 x10^4 cells/slide with a total volume of 50 µl/slide. Cells were incubated for 6 mins at 37 °C, and 5% CO2. Ten ml of fresh media was added to the petri dish that contained the slides. After 48 hours of incubation, the media was removed and cells were washed with PBS. Each slide was infected with 50 µl/slide of either PPV (10^8 MTT_{50}/ml), PPV containing either 0.20 M TMAO or glycine (10^8 MTT_{50}/ml), PBS, or media containing 0.20 M TMAO or glycine. After 30 min, the cells were washed twice with PBS to remove any unattached PPV, and 10 ml of fresh media with or without osmolytes were added. The cells were placed at 37 °C, 100% humidity, and 5% CO_2 for different times. The media was removed and the cells were washed once with PBS. At different time intervals post-infection, the infected cells were fixed with 200 µl of 3.7% formaldehyde for 20 min at room temperature, and then washed twice with PBS. The cells were blocked with 200 µl of 0.3% low-fat milk in PBS. After 1 hour, 50 µl of 1:100 v/v anti-PPV antibody were added and incubated for 1 hour at 37 °C followed by two PBS washes. Then, 50 µl of Alexa fluor 546-conjugated rabbit anti-mouse IgG (1:500 v/v) were added and incubated for 1 hour at 37 °C followed by two PBS washes. Slides were washed again with PBS. To fix the antibodies, 200 µl of 3.7% formaldehyde were added for

20 mins at room temperature, and then the cells were washed twice with PBS. Finally, 50 µl of 150 µM of dye was added for cell nuclei staining for 5 mins followed by a PBS wash and cell mounting procedure. Images of the cells were taken with an Olympus IX51 microscope with a DP72 camera.

For virus quantification we convert the procured image into binary image using imageJ software. ImageJ analysis was performed by gray scaling 100 ms exposure PPV images and 5 ms exposure DAPI images. The obtained multi-color images were converted to single color images by converting to 16 bit or a gray scale image. The threshold was adjusted and consistent values were maintained to analyze the entire set of images. A binary version of the image was created with pixel intensity of 0 (white) and 255 (black). Any noise levels less or equal to 2 pixel density was removed and each image was analyzed for particle count and area.

4.3 Results and Discussion

4.3.1 Cellular trafficking of virus

When a virus infects a cell, it goes through a range of processes, including endocytosis, internalization through organelles, nucleus entry, genome replication, assembly and finally cellular release. Understanding the disease pathogenies and virus mechanism can be very useful for antiviral drug and therapeutic vaccine development. We have considered a non-enveloped virus, tagged it with a fluorescent label and investigated to breakdown the various stages of infection cycle by using single particle time study.

4.3.1.1 Optimization of fluorescence label

Prior to Alexa fluor label of virus particles, it was first important to determine the concentration of the virus and the total protein concentration. The protein concentration was determined with a microBCA kit. A calibration curve with BSA was created as shown in **Figure 4.2** and then used to determine the protein concentration of the PPV solutions. The final PPV concentration was 2.4 mg/ml. The total protein concentration in the PPV solution was diluted to 2 mg/ml for the following Alexa fluor tagging experiments.

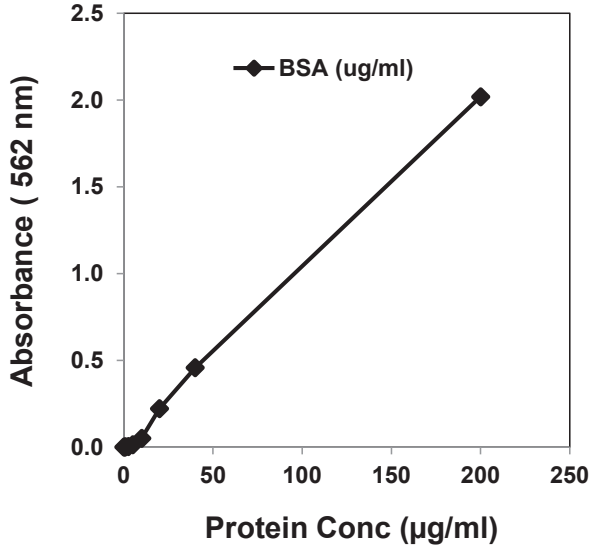

Figure 4.2 BSA Calibration curve. A standard curve was plotted by measuring absorbance at 562 nm for different known BSA concentrations. The data points shown are results from a single test only.

We used a conjugated succinimide ester dye in order to label the proteins in the PPV solution. Experiments were conducted for 1 µl, 3 µl, 5 µl and 9 µl of dye with a consistent virus concentration. It was not known what dye concentration would give a high fluorescence signal without lowering the virus infectivity. After tagging, 1 ml

samples were collected from the desalting column until all the fluorescent dye was eluted by visible observation. A total of 7-8 samples were collected and each of the samples were subjected to the MTT assay. An example of samples collected and titer for the 5 µl dye on PPV and buffer is shown in the **Figure 4.3**.

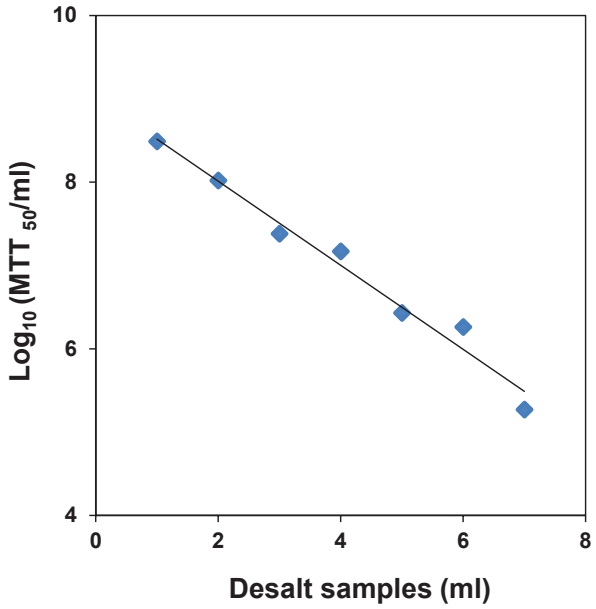

Figure 4.3 MTT assay of Alexa tagged PPV. A 500 µl of PPV (8 $\log_{10}$ MTT_{50}/ml), 0.1% $NaHCO_3$ was tagged with 5 µl Alexa fluor dye and eluted from a desalting column. By passing PBS elution buffer 7 samples were collected of 1 ml each and subjected to MTT assay for infectivity determination. The data points shown are results from a single test only.

The PPV titer was high for the initial samples and then reduced for the later samples. The result was expected since the desalting column had a molecular weight cut off of 7000 Daltons, which is approximately a 0.7 nm nominal pore size allowing the large 18-26 nm virus to elute early in the void fraction. The figure in comparison to log 8 stock control did not lose infectivity and hence confirmed that the dye does not prevent infection on the cells.

After confirming the titer had not altered with the tagging protocol, it was necessary to identify the impurities that had been tagged in the sample along with the virus and remove them. The virus production in the lab comes with media and cellular proteins including BSA. The presence of any tagged impurity in the sample would stray us from our observation in virus infection imaging process. To circumvent this issue, we injected 10 µl of tagged virus into a sephacryl size exclusion column and measured the fluorescence. The size exclusion column was designed to identify and discriminate the virus from smaller impurities. Experiments were again performed for 1 µl, 3 µl, 5 µl and 9 µl dye on the virus. An example of the 5 µl Alexa fluor tagged PPV sample is shown in **Figure 4.4**. Samples from the desalting column were combined into 2 ml fractions, so sample 1 corresponds to fractions 1 and 2 in **Figure 4.3**. For the desalted sample 1, we were interested in the peak at 2 minutes. We proposed that this peak belonged to the virus as the sample provided the highest titer on the MTT assay, and the virus should elute in the void fraction of an SEC column. We hypothesized the peak at 4 minutes belonged to the tagged BSA and extraneous proteins based on the results of Chapter 3 (**section 3.3.2**). In this section, the PPV stock, which included BSA-containing media proteins, obtained a broad peak ranging from 2 min to 6 min due to the PPV and the protein contaminants from the cell media. By desalting the sample in the current investigation we are able to remove majority of the proteins in the sample 1 that has created distinct peaks at 2 and 4 min. We also used fluorescence detection, which is more sensitive than the UV detection used earlier. The hypothesis that the initial 2 min peak belongs to PPV and 4 min to contaminants bodes well with the results from remaining desalt samples as well. For remaining samples the 2 min peak is

negligible and the 4 min peak has a high fluorescence reading. The high fluorescence is owing to smaller and more abundantly present proteins which elute out behind the PPV due to size exclusion. An alternate theory may also be possible that the unincorporated dye is the eluting at the 4 min peak range. Even if this is the case the unincorporated dye is an unwanted impurity as it may hinder the epitope selection in the infection process. With a goal of having purified virus for imaging trafficking study, the sample 1 seemed to be the best sample. However even sample 1 had a significant impurity peak with larger fluorescence units than virus peak. We were interested to decrease the impurity significantly and hence tagging the PPV after its purification using the ion exchange chromatography (IEC) was planned. As discussed in Chapter 3, the IEC had removed BSA and media proteins from the PPV stock and additionally if we happen to note a reduction in the 4 minute peak we can be sure the peak belonged to protein impurities and not unincorporated dye.

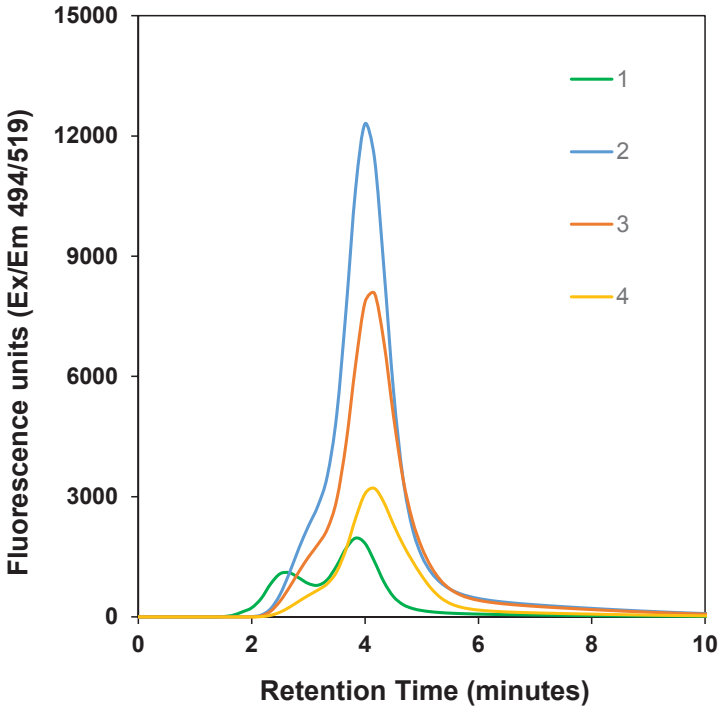

Figure 4.4 Fluorescence measurement of desalted and Alexa fluor tagged PPV using SEC. A 500 µl PPV (2mg/ml), 0.1% $NaHCO_3$ was tagged with 5 µl Alexa fluor and desalted through a desalting column. Four 1 ml samples were collected and subjected to SEC and fluorescence measurement in a 2475 Water fluorescence detector.

Several samples of 500 µl of purified PPV (from IEC, as described in **section 3.3.1**) were tagged with Alexa fluor dye. First it was necessary to show the tagged virus was not preventing infection steps. This was proven by MTT assay which showed the virus titer of the samples with different dye concentrations was comparable to the control (see **Figure 4.5**).

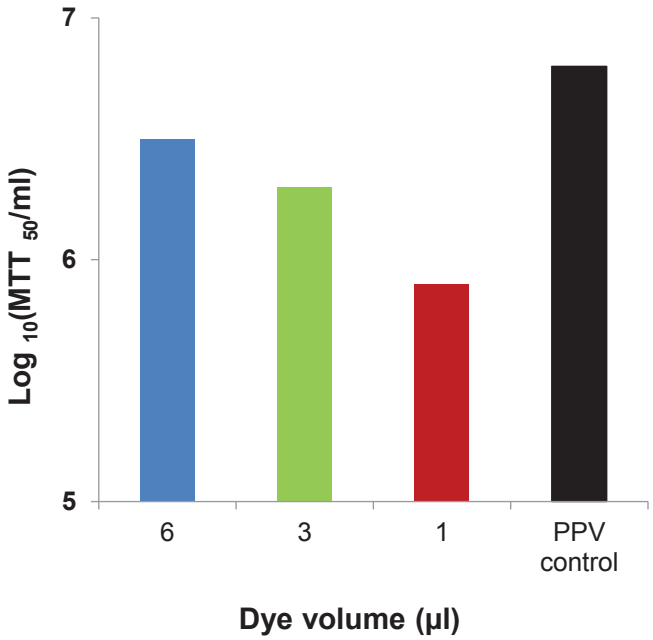

Figure 4.5 MTT assay of IEC purified PPV with different dye volume. A 500 µl of PPV (purification of PPV stock using IEC), 0.1% $NaHCO_3$ was tagged with different volumes of Alexa fluor for immunogenic testing or transfection efficiency. The data points shown are results from a single test only.

The labeled PPV samples were injected on to the SEC column for detecting virus and impurity peaks (see **Figure 4.6 A**). The injection run for the initial sample 1 shows the PPV and protein impurity dye peaks at 2 and 4 minutes, similarly as seen in the **Figure 4.4**. A PPV peak with higher fluorescence at 2 minutes compared to the 4 minute impurity peak unlike the **Figure 4.4** was noted. The result was encouraging confirming the 4 min peak belonged to impurity that was reduced sufficiently. To satisfy the unincorporated dye theory we analyzed dye concentration using a fluorescent plate reader (see **Figure 4.6 B**) for the desalted samples 1-4. If unincorporated dye impurity is available in the samples we wanted it to be at a minimum to prevent the dye from blocking active sites for virus transfection. From the IEC purified tagged virus investigation, we inferred the 3 µl of dye provided the

best virus: impurity peak height ratio (**Figure 4.5 A**) and a minimum unincorporated dye if any (F**igure 4.5 B**).

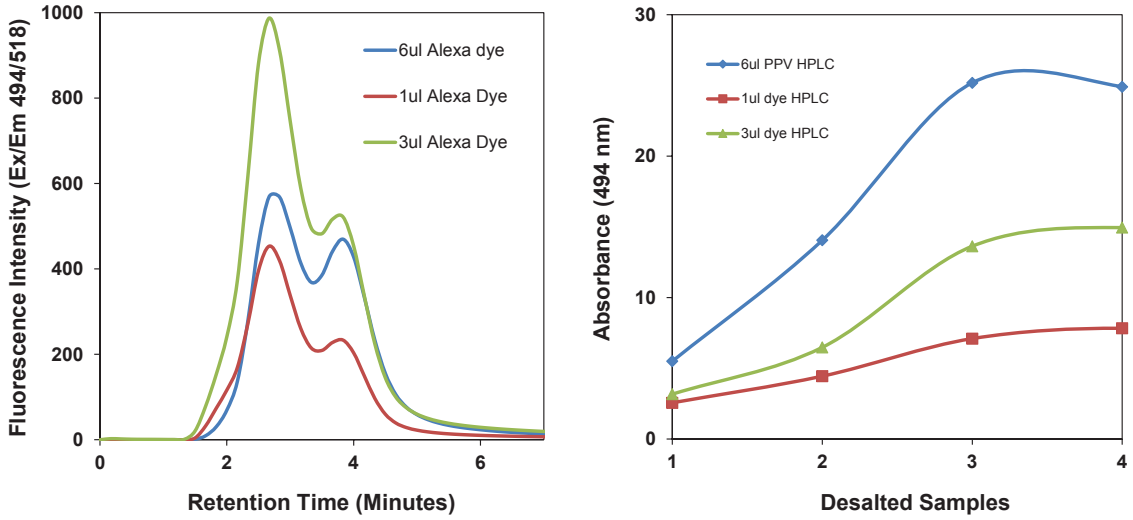

Figure 4.6 Fluorescence emission for samples (A) SEC (B) Fluorescence plate reader. A 500 µl of IEC purified PPV, 0.1% $NaHCO_3$ was tagged with different volumes of Alexa fluor and measured for fluorescence to determine the optimum dye: virus ratio.

4.3.1.2 Cellular trafficking of PPV in live cells

We then proceeded to observe the cellular trafficking of PPV through cells with the tagged PPV samples. Images were procured at 1, 8 and 20 hour post infection of cells with Alexa fluor tagged PPV, as shown in the **Figure 4.7** (A), (B) and (C) respectively. The virus in (A) was seen in the cell vesicle, in (B) at the perinuclear region and in (C) it was mainly observed in the cell organelle with a few particles in the nucleus as well. The virus, as per the figures, appears to be trafficking in a non-linear, multiple pathway system, as also noted by other research groups [8]. Studies involving parvoviridae family have shown multiple cell entry mechanism or receptor mediated endocytosis as sialic acid [27], macropinocytosis [8], clathrin [28] which

occurs within the initial 0 – 2 hours [8]. It is the process of endosomal trafficking and the compartments which virus chooses before entering the cytoplasm and nucleus that gets more complicated and unclear [29]. Particles can reside in different organelle at different time periods [7] and this seems likely from the **Figure 4.7** where the virus is present within an organelle at 1 hour and also again at 20 hours. It is not well documented but the stock virus particle: infectivity ratio can be as large as 1000: 1 that can reduce the ability to distinguish between an infectious and non-infectious particle. IEC cannot differentiate between infectious and non-infectious particle. Another phenomenon that is difficult to account for is the presence of individual and aggregated virus particles. Infection pathways can be different for aggregated and individual particle. Isolated and purified PPV has been found to follow clathrin mediated endocytosis whereas aggregates follow the macropinocytosis pathway [8]. Due to these concerns the multiple pathway system required a more dynamic and informative live cell imaging for tracking a single virus particle in real time, which we did not have access to obtain. The time analysis to study virus trafficking with tagged PPV could not be successfully implemented. However this investigation helped us to establish and implement the virus time study using IHC, which was successfully implemented to understand the effects of antiviral compounds on the PPV infection cycle.

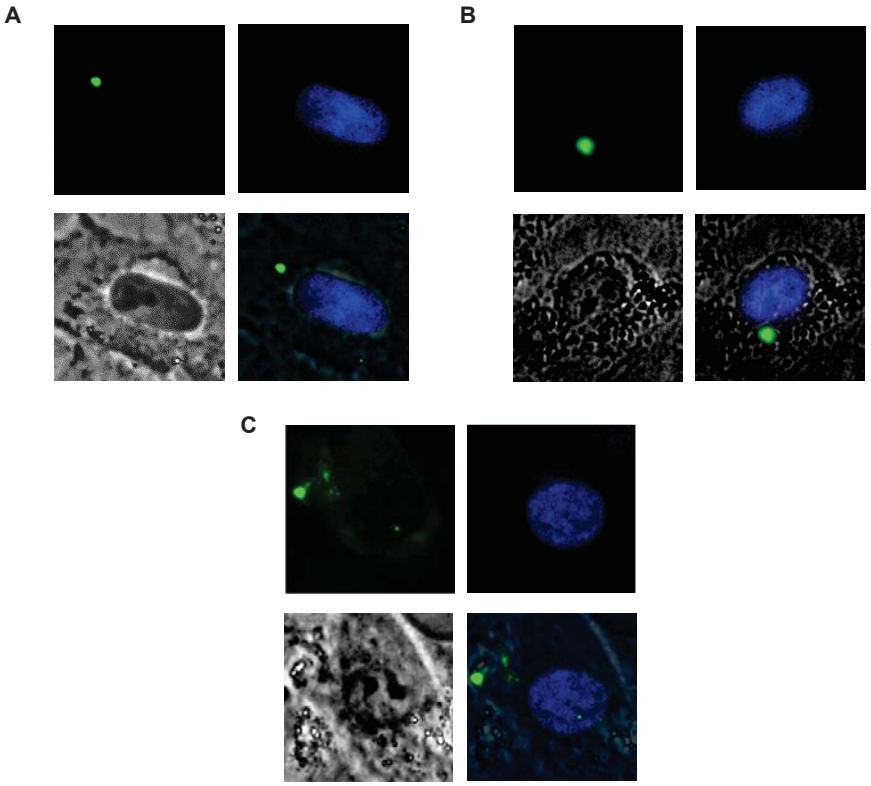

Figure 4.7 Alexa tagged PPV trafficking in PK-13 cells. Fixed cell images of Alexa fluor (3 µl) in IEC purified PPV (500 µl) on infected cells at (A) 1 hour, (B) 8 hour, (C) 20 hours.

4.3.2 Determining the action of virus on cells with and without the presence of osmolytes

Osmolytes are natural organic compounds found in water-stressed organisms to maintain cell volume by stabilizing intracellular proteins. Protecting osmolytes fold proteins by structuring water around themselves and changing the interaction between water and the protein backbone [30]. In work mainly done by my fellow graduate student, Maria Tafur, several protecting osmolytes were found to have antiviral activity against PPV. For antiviral activity, TMAO and glycine at a concentration of 0.20 M were observed to reduce the infectivity of PPV by a

promising 4 LRV, as defined in **Eq. 4.1**. It was noted that the treatment of virus with osmolytes prior to the PPV infection showed no reduction in PPV infectivity. It shows that the osmolytes played no role in hindering the virus-host cell attachment process. However, the compounds worked even when added to the cells up to 20 hours post-infection. Antiviral activity past 20 hours of infection is quite a long time post-infection process. Upon examination of the PPV infection cycle (see **Figure 4.8**), we decided to explore the mechanism of the antiviral activity by starting at the end of the infection cycle and proceeding backwards. To determine the mechanism of action of the osmolytes, we examined mechanisms from the end of the virus infection cycle because we believed that the mechanism of action was late in the infection cycle. To study if virus capsid proteins were created, we used IHC. [31].

$$\text{Log Reduction} = -\log\frac{(virus\ titer\ with\ osmolyte)}{(virus\ titer\ of\ control)} \quad \ldots\ldots\ldots\ldots\ldots \quad (4.1)$$

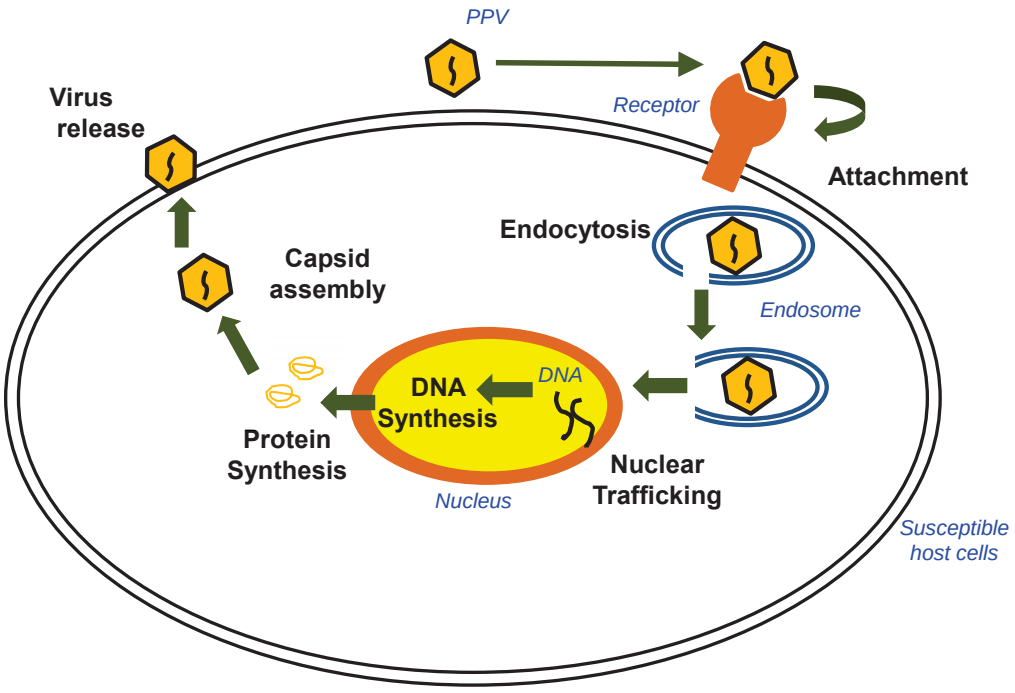

Figure 4.8 The PPV infection cycle

To examine the function of osmolytes on the PPV infection cycle, we employed IHC for a detailed time study from 0 -20 hours post-PPV infection to determine the amount of PPV capsid found in infected cells. The samples under investigation were PPV+0.2 M TMAO, PPV + 0.2 M glycine, media containing TMAO and glycine only (no PPV) and the positive control of only PPV and the negative control of PBS. **Figure 4.9** is a representation of virus at 16 hours post infection. PPV capsid proteins were found in all samples infected with PPV, including those containing 0.20 M TMAO and 0.20 M glycine. PBS without virus was used as a negative control, and did not show any virus capsid protein formation. TMAO and glycine were also tested without virus present and no virus capsid protein formation was detected (not shown).

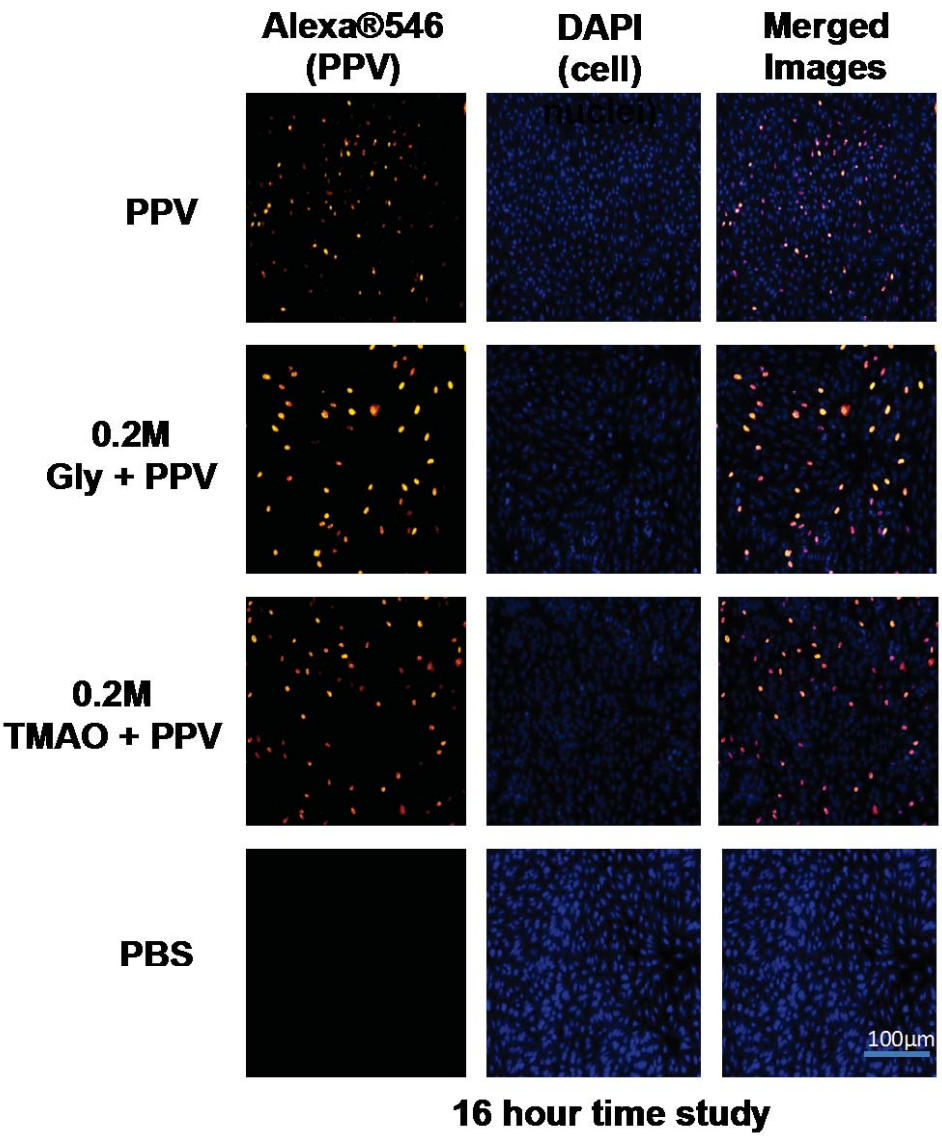

Figure 4.9 Impact of osmolytes on virus capsid protein formation (A) Images of PPV, 0.20 M glycine + PPV, 0.20 M TMAO + PPV and PBS mock infected cells at 16 hours. TMAO and glycine were tested in order to determine osmolytes cross-reactivity with antibodies and no virus capsids were found (data not shown). Reprint permission granted from [34].

To quantify PPV capsid proteins per cell, we converted multi-color images into 16-bit binary images using ImageJ and selected thresholds such that noise disturbances were removed, leaving only virus or nuclei in each image (see **Figure 4.10**). The PPV per cell could be analyzed by using either PPV capsid (by count) or

PPV capsid (area) for each DAPI nuclei (count). For PPV capsid (count) to DAPI (count) we do a summation of all the assembled virus capsid particles shown as orange-fluorescent dye particles in **Figure 4.9** and divide against the number of nuclei represented in DAPI blue stain. For PPV area to DAPI count we do a summation of cross-sectional area covered by PPV fluorescence and divide against the available DAPI nuclei count. For PPV, our initial proposition was to analyze on a count basis but this changed after beginning our hands-on microscope operation. While analyzing PPV by count we noticed that minor disturbances that were difficult to control created many noise specs. The noise specs could not be removed completely by the pixel threshold and they accounted for large unreasonable value and errors during particle count. On the other hand, analyzing the PPV by area accounted the noise specs as tiny surfaces in terms of area, giving us smaller errors. Also, it allowed for the possibility that one large PPV fluorescent spot could be multiple capsids. Hence we chose PPV capsid analysis by area and for nuclei we chose the DAPI count since it did not have noise reduction issues as PPV.

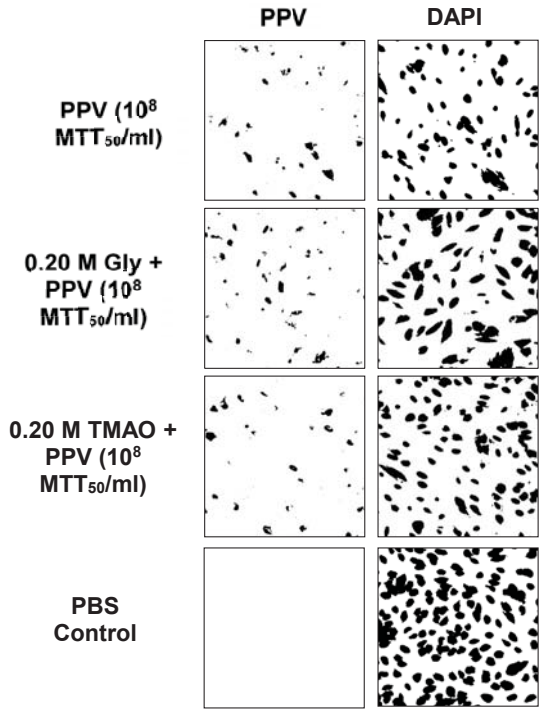

Figure 4.10 Binary images for virus per cell quantification. ImageJ software was used to create binary Images of PPV, 0.20 M glycine + PPV, 0.20 M TMAO + PPV and PBS mock infected cells at 16 hours to quantify virus/cell. Reprint permission granted from [34].

The graph of PPV area/DAPI count (**Figure 4.11**) shows for the sample involving PPV with osmolytes, the fluorescence per cell started to increase after 8 h. Similar trends were observed with the positive control PPV. These results show that viral capsid proteins are produced under these conditions. Since the osmolytes were shown to reduce infectivity by 4 LRV, we infer that although capsid proteins are produced they are not viable particles.

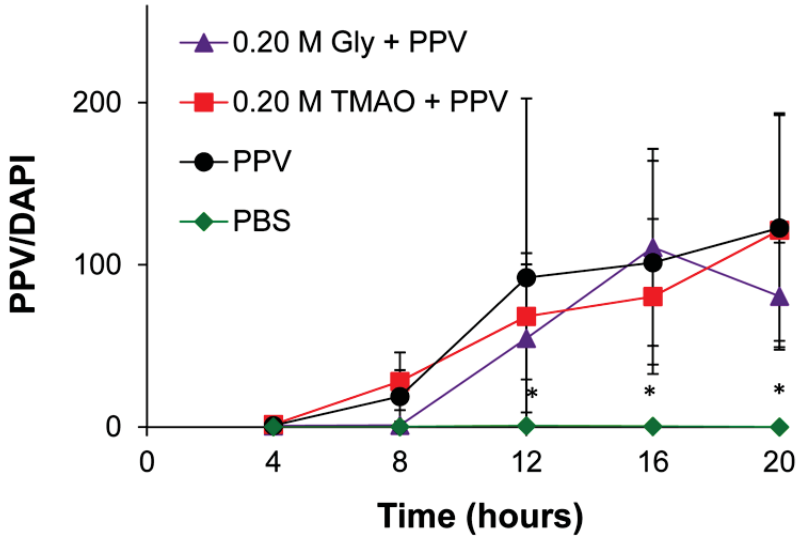

Figure 4.11 Impact of osmolytes on virus capsid protein formation by determining the ratio of the fluorescence of PPV capsid proteins to the count of DAPI. All data points are the average of three independent slides with 10 images per slide and the error bars represent the standard deviation. TMAO and glycine were tested in order to determine osmolytes cross-reactivity with antibodies and no virus capsids were found (data not shown). Student's t-test was used to evaluate the statistical significance between cells infected with PPV (with or without osmolyte) and the PBS negative control *p value of <0.05. Reprint permission granted from [34].

The fact that capsids are produced but not viable proves that the osmolytes are working post-virus infection process as shown in the **Figure 4.12**. We hypothesize that protecting osmolytes are disrupting capsid assembly by stabilizing viral capsid proteins and preventing the assembly process. Osmolytes are known to stabilize proteins by preferential hydration [32]. This can cause the proteins to adjust to a more compact configuration [30]. It is likely the compact structure of VP2 proteins, which constitute 90% of the PPV is not able to assemble into viable infectious capsid. The other alternate hypothesis is the presence of osmolytes is preventing the assembly mechanism from guiding the DNA into the capsid. However since enzymes have to been known to be stable under the presence of osmolytes conditions [33] we would suppose the osmolytes are disrupting the self-assembly of

virus capsid proteins. A third option that we never tested was the DNA was not being created due to the presence of the osmolytes.

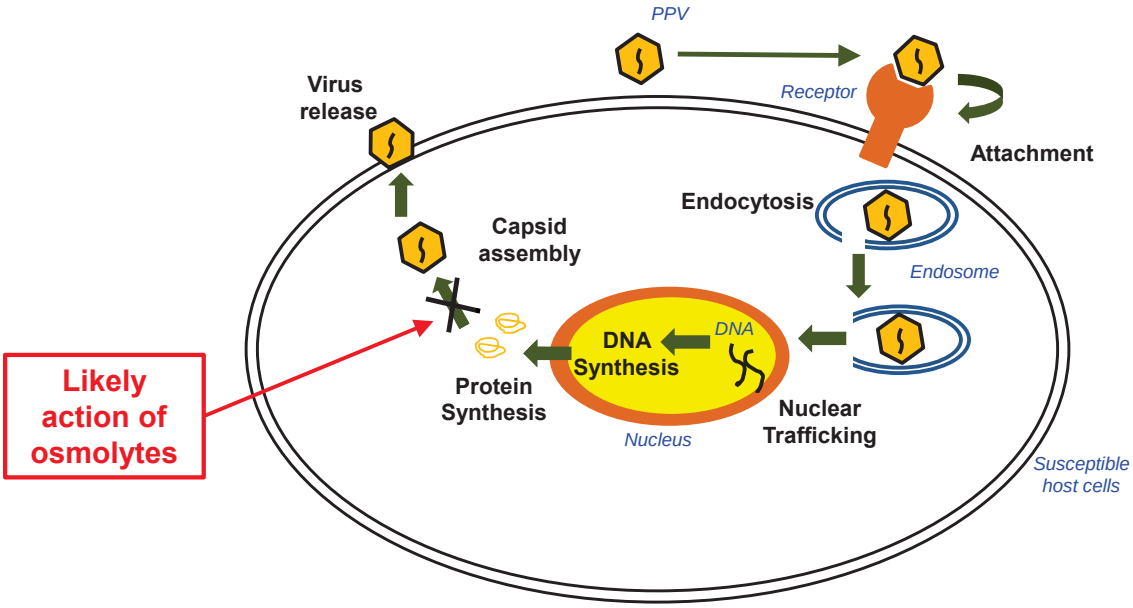

Figure 4.12 PPV infection cycle and the osmolytes action on virus infectivity

4.4 Conclusions

Virus infection and trafficking through cells is a very challenging and complicated process. Understanding the virus pathway through cells can greatly benefit gene vector therapy and antiviral drug design. We were interested in understanding the PPV trafficking through PK-13 cells. Research groups studying PPV pathway had found that PPV entered through receptor mediated endocytosis, internalized through endosomes, entered the cytoplasm and then nucleus. The DNA replication step is poorly understood and following the genome synthesis cells are lysed releasing virus for further replication. Our objective was to tag the virus with a fluorescent dye and observe its trafficking under fluorescence microscope.

The virus was tagged with Alexa fluor 488 and desalted using a column. We analyzed the desalted samples using MTT assay to make sure the virus had not lost its immunogenic properties upon tagging. The protein impurities present along with the virus from virus production protocol were identified using fluorescent signal from the SEC. Any impurities, especially if tagged, could change our virus trafficking observation under the microscope. Hence it was important to remove as many impurities as possible. In order to remove the impurities we made use of IEC with Q-sepharose beads in an omega 4.6 mm x 25 cm column. After the purification, we tagged the virus and used for trafficking purpose. Tagged virus was observed at vesicle in the first hour, at the perinuclear region after 8 hours and at 20 hours it was observed in nucleus, vesicle and perinuclear region. In our observation the virus seemed to be trafficking in multiple ways through the cellular machinery. The low infective particle ratio 1:1000 along with PPV multiple pathways made it difficult to make a valid conclusion for single virus trafficking project. Our learning and experimental working here was used to study the infectivity mechanism of PPV in the presence of antiviral drugs.

Osmolytes are natural compounds that are found in stressed animal to maintain cell volume by stabilizing intracellular proteins. Protecting osmolytes are a special type of osmolytes which structure water around themselves causing protein folding. Several osmolytes were screened for observing antiviral activity. Among them TMAO and glycine at 0.20 M concentration were found to inhibit PPV infection by a promising 4 LRV measured using the MTT assay. In order to further understand the mechanism of osmolytes on the infection process, we made used of IHC. We performed a 0-20 hour timed study post PPV virus infection in the presence of

osmolytes. The fact that the PPV capsids are seen using IHC but inhibited by 4 LRV using cell viability assay shows that osmolytes are preventing the formation of viable virus particles. We hypothesize the protecting osmolytes TMAO and glycine are disrupting virus capsid assembly process by stabilizing and preferentially hydrating the VP2 capsid proteins.

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Chapter 5

Purification of porcine parvovirus aqueous two-phase system

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K. Saagar Vijayaragavan, Amna Zahid, Jonathan W. Young and Caryn L. Heldt

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5.1 Introduction

The goal of public sector immunization programs is to ensure high quality vaccines are produced in adequate quantity and at affordable prices. With industrialized countries producing original, higher profitable vaccines in lieu of traditional vaccines, vaccine manufacturing of older vaccines is *steadily* being outsourced to developing and emerging economy countries. This poses an overwhelming challenge of meeting the demand of vaccines for public sector immunization programs while re-engineering the manufacturing steps to reduce the financial burdens [1]. With 70% of the overall vaccine production costs being spent on downstream processing, improving vaccine purification is an appropriate target to reduce manufacturing costs [2].

The purification strategies for the downstream processing of viral vaccines have included a variety of methods. Some of the typical unit operations are precipitation, centrifugation, ultrafiltration and chromatography. Precipitation using ammonium sulfate and polyethylene glycol (PEG) has been used for virus precipitation based on particle-particle interaction and hydrophobicity [3]. However, the lack of process robustness and co-precipitation of impurities has limited the application of this method. Centrifugation has been predominantly used in lab-scale production of large biomolecules based on density gradients of cesium chloride or sucrose. Particle degradation from pressure and osmotic shock and lengthy processing times plague density gradient centrifugation, along with scale-up difficulties [4]. Tangential flow filtration (TFF) has been used to purify viruses. However, the high transmembrane pressures in TFF can reduce virus infectivity and membrane fouling can reduce permeate flux [5, 6].

Column chromatography using porous beads is the most routine method used for virus purification. Chromatography is designed for biomolecules and nanoparticulates < 5 nm in diameter and virus particle diameters typically range between 20-200 nm. This creates issues pertaining to pore diffusion and pore plugging, which severely restricts virus adsorption within the inner surface area of the solid matrix [7, 8]. Due to the reduced adsorption surface accessibility, monolith and membrane chromatography have gained considerable attention as adsorption matrices. However, membrane absorbers face a similar drawback as TFF, which is the degradation of liable virus particles due to shearing effects [8, 9]. For each physically different stationary phase configuration, chromatography still requires a change in mobile phase. The change in salt concentration, pH, or addition of a solvent equally run the risk of inactivating virus particles [10]. In vaccine manufacturing, chromatography also has a tendency to fail to discriminate between virus and protein contaminants [11], likely due to the different amount of surface area available to the proteins and virus particles that can alter the balance of the equilibrium.

Currently, the downstream processing of viral products combines several of the previously mentioned unit operations. The purification of viruses using clarification or filtration along with a chromatographic step have demonstrated recoveries of 32% for adenovirus [12], 25% for baculovirus [13], and 30-50% for adeno-associated virus [14]. Overall, virus recoveries of 30% are considered an acceptable standard for the entire vaccine purification train [15]. Our goal is to design an alternative unit operation that is capable of creating a high infectious yield. In a quest to fulfill this goal, we have been exploring aqueous two-phase systems (ATPSs) as an optional

mechanism to purify virus. ATPS has been unable to gain widespread implementation mainly due to loss of virus infectivity, co-purification of proteins, and difficulty of polymer recycling [16]. Enveloped viruses have a sensitive lipid bilayer that makes them susceptible to inactivation in high ionic strength environments. The majority of non-enveloped virus families, reovirus, picornavirus, polyomavirus [17] and parvovirus [18], are known to withstand contact with high concentrations of polyethylene glycol (PEG).

ATPS is formed by mixing water soluble polymers, or a water soluble polymer and a salt, above a critical concentration that results in two immiscible aqueous phases [19, 20]. It is a versatile method used for the separation and purification of biological molecules such as proteins [21], enzymes [22], nucleic acids [23] , virus [24], antibodies [25] and cell organelles [20]. The partitioning is governed by many physicochemical properties, such as surface hydrophobicity, electrostatic interactions, van der Waal's forces and hydrogen bonding [26]. ATPS combines clarification, concentration and purification into a single, integrated step to obtain a high yield with a low financial burden. A monoclonal antibody was purified with ATPS and obtained a similar yield as protein A chromatography at 39% reduced operating costs [27]. In the recovery of penicillin acylase from recombinant *E.Coli*, ATPS was able to reduce the number of unit operations from 7 to 4 and achieve a 97% yield with a gross cost reduction of 37% compared to ion exchange chromatography [28]. ATPS also boasts other advantages: easy scale-up, environmentally-friendly, low cost and high mass transfer [20]. Some of the disadvantages of ATPS include the removal of the PEG from the final biological product, dilution of the product, and

large buffer volumes that would need to be recycled. As we pursue the purification of virus with this method, we will tackle many of these disadvantages.

Chemical cost is always a dominant factor in any biochemical process. Due to this, the inexpensive PEG-salt ATPS has been widely favored for commercial use instead of the PEG-dextran system. The drive to eliminate PEG is not so great as it is inexpensive when purchased in a large MW range and forms two phases with most natural polymers and salts [29]. In order to achieve an extraction with high recovery of virus and purity from cell contaminants, the composition of ATPS needs to be carefully chosen. ATPS has been frequently used in the past for recovery of virus-like particles (VLPs). VLPs are multiprotein structures that contain the same or similar immunogenic features of infectious viruses, but lack the viral genome the is required for virus replication [30]. A VLP vaccine against the human papillomavirus was obtained with a 54% recovery in a PEG 1000-phosphate system [31]. A DNA plasmid vaccine was recovered from a PEG 400-phosphate system with a 37% yield [32]. However, appropriate technologies to obtain high yields for infectious virus particles are still being sought. Infectious bacteriophage T4 in a PEG 8000-phosphate system obtained a recovery of 30-38% [33]. The yields of < 55% demonstrate a need to find an alternative purification method for large biomolecular vaccines.

The aim of this study is to recover infectious porcine parvovirus (PPV) using a PEG-salt system. PPV was chosen due to its small size, simplicity and its structural similarity to adeno-associated virus (AAV), a commonly studied gene therapy vector. It is also used as a surrogate for Hepatitis A and poliovirus, both of which have vaccines. PPV is also a model non-enveloped virus for the human B19 parvovirus, a

known blood-borne pathogen in humans. We successfully achieved 64% virus recovery in a PEG-citrate ATPS and eliminated the major contaminant protein, bovine serum albumin (BSA), which comes from the addition of serum to the media. Although many vaccines are now produced in serum-free media, we used this as a proof-of-concept study that a major protein contaminate could be separated from the virus with ATPS. The partition behavior of virus has been explained primarily on the basis of electrostatic interactions, surface hydrophobicity and ATPS surface tension.

5.2 Materials and Methods

5.2.1 Materials

PEG samples with molecular weights (MW) of 3K, 8K and 12 KDa were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate and sodium sulfate were purchased from VWR (Radnor, PA). For the phosphate buffer solution, sodium hypophosphite ($NaH_2PO_4.H_2O$) was purchased from VWR (Radnor, PA) and sodium triphosphate (Na_3PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide was purchased from Acros Organics (New Jersey, NY). Sodium chloride was purchased from Macron Chemicals (Center Valley, PA). Sodium citrate dihydrate and hydrochloric acid were purchased from EMD Chemicals (Billerica, MA).

Phosphate-buffered saline (PBS) pH 7.2, 0.25% trypsin/EDTA, penicillin/streptomycin (pen/strep) and minimum essential medium (MEM) for cell propagation were purchased from Life Technologies (Grand Island, NY). For virus titration, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO).

High pressure liquid chromatography (HPLC) grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA), respectively. BSA was purchased from Sigma-Aldrich (St. Louis, MO). All solutions were made with water from a NanoPure water system (Thermo Scientific, Waltham, MA) to a resistance of >18 MΩ.

5.2.2. Cells, virus and titer assay

Porcine kidney cells (PK-13) were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% pen/strep at 37°C, 5% CO_2 and 100% humidity. The cells were propagated every 3-4 days at a split ratio of 1:5. PPV strain NADL-2, also a gift from Dr.Ruben Carbonell at North Carolina State University, was propagated in PK-13 cells, as described previously [34]. PPV was clarified with centrifugation prior to use.

PPV was titrated with a cell viability assay, the colorimetric MTT assay, as described previously [35]. Briefly, PK-13 cells were seeded in 96-well plates. Plates were infected with 25µl of PPV and serially diluted across the 96-well plate. After five days of incubation, the MTT salt solution was added. Four hours later, solubilizing agent was added. Plates were read on a Synergy Mx microplate reader (BioTek, Winoski, VT) at 550 nm between 18-24 hours after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that corresponded to an absorbance of 50% of the uninfected cell absorbance. The value was converted to a per millimeter basis and stated as the MTT_{50}/ml titer [34]. The

MTT titer for PPV has been shown to be linearly correlated to the more common $TCID_{50}$ [34].

5.2.3 Binodal Curve

To study the experimental space of a PEG-salt system, we determined the binodal curves by the turbidmetric titration method [21]. Different bulk salt: bulk PEG ratios were made to obtain multiple phase transition lines transcending towards the origin. The unique aqueous combinations were initially stirred for 1 minute until the solution was turbid. Small volumes of water were then added using a burette until a clear solution was obtained. The composition of the mixture when the clear solution was obtained was determined to be the binodal point.

5.2.4 ATPS

All the experiments presented are mini ATPS performed using 1.7 ml microcentrifuge tubes for a total of a 0.9 g system. The scale-up and scale-down of ATPS have not been considered problematic [36]. PEG solutions with bulk concentration of 33 w/w% were prepared and mixed with salt solutions of varying concentrations for a total of 0.8 g [37]. The pH of the system was kept at 7 using HCl or NaOH, unless otherwise stated. To this system 0.1 g of 10^8 MTT_{50}/ml of PPV was added and the mixture was vortexed for 30 seconds and centrifuged using a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) for 3 min at 19-22°C. Visual estimates were made on the graduated microcentrifuge tubes for determining volume ratios. The PEG-rich phase was extracted using a 10-100 µl pipette tip and the salt-rich phase using a rounded gel tip (USA Scientific, Oscala, FL) to minimize disturbances and sample withdrawal from the interface. The samples were tested for

infectious PPV with the MTT assay, as described in **Section 5.2.2**. The final virus concentration in each phase is reported in percent recovery (see Eq. 5.1), taking into consideration the volume of each phase for closing the mass balance of the system. We tested the infectivity of the unseparated PEG-salt systems and used this as the starting value for the mass balance. The mixed PEG-salt systems did not lose infectivity, as calculated from the known stock infectivity. The % recovery is defined as,

$$\% \, Recovery = \frac{\frac{MTT_{50}}{ml}_{p,s} \times V_{p,s}}{\frac{MTT_{50}}{ml}_{i} \times V_i} x \, 100\% \quad \text{.....(5.1)}$$

where *p, s, and i* represent the virus concentration in the PEG-rich phase, salt-rich phase, or initial concentration, respectively.

5.2.5 RP-HPLC and SDS-PAGE

In order to validate the removal of the main contaminating protein BSA, we monitored the BSA separation in ATPS. Reverse-phase high pressure liquid chromatography (RP-HPLC) was performed with a Waters XBridge BEH 130 C_{18} column on a Waters Alliance HPLC equipped with a photo diode UV-Vis detector operating at 254 nm wavelength. Samples of 200 µl from the PEG-rich and salt-rich phases were withdrawn from multiple ATPS experiments until a final volume of 800 µl was obtained. The samples were dialyzed using a 20 kDa MWCO cellulose membrane purchased from Spectrum Laboratories (Dominguez, CA) to remove the high concentration of salt or polymer in the system that would interfere with the RP-HPLC analysis. The dialysis was conducted against 50 mM phosphate, 150mM NaCl at pH 7.2 for 48 hours at 20°C with two buffer exchanges. Dialyzed samples were

collected and filtered through a 0.2 μm syringe filter and 25 μl was injected onto the RP-HPLC C_{18} column at a flow rate of 1.0 ml/min. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. A linear gradient of increasing mobile phase B was performed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run using 4-12% Bis-Tris NuPage gels in NuPage MOPS running buffer purchased from Life Technologies (Grand Island, NY). Samples were reduced in DTT and heated to 70°C for 10 minutes prior to loading onto the gel. SDS-PAGE was stained with the SilverXpress kit (Life Technologies, Grand Island, NY).

5.3 Results

5.3.1 Model Virus

The virus used in this study was porcine parvovirus (PPV). PPV is a pathogen causing reproductive failure in swine [38]. PPV is a non-enveloped, single-stranded DNA virus with a diameter of 18-26 nm [39] and a model for the human B19 parvovirus [40]. B19 causes fifth disease, a mild rash illness that generally affects children. PPV and other parvoviruses are commonly used to test virus removal in biotherapeutic manufacturing [41, 42]. The PPV was produced in serum-containing media; therefore, bovine serum albumin (BSA) removal was studied as a model contaminating protein. Many vaccines are currently produced in serum-free media, and future work will be to study the removal of host cell proteins (HCPs) from the virus system.

5.3.2 Binodal Curve

Binodal curves are needed to predict the two-phase region that can be utilized for ATPS extraction and partitioning of biomolecules. The binodal curve divides the region of component concentration into two phases; above the curve is the two-phase region and below the curve is the miscible, one-phase region. From **Figure 5.1**, it can be seen that there is an expanded two-phase zone with increasing polymer molecular weight for the PEG-salt system. There was a similar trend to binodal curves found by others [21, 43]. Due to the high PEG MW that was used in this work, binodal curves from other studies could not be utilized.

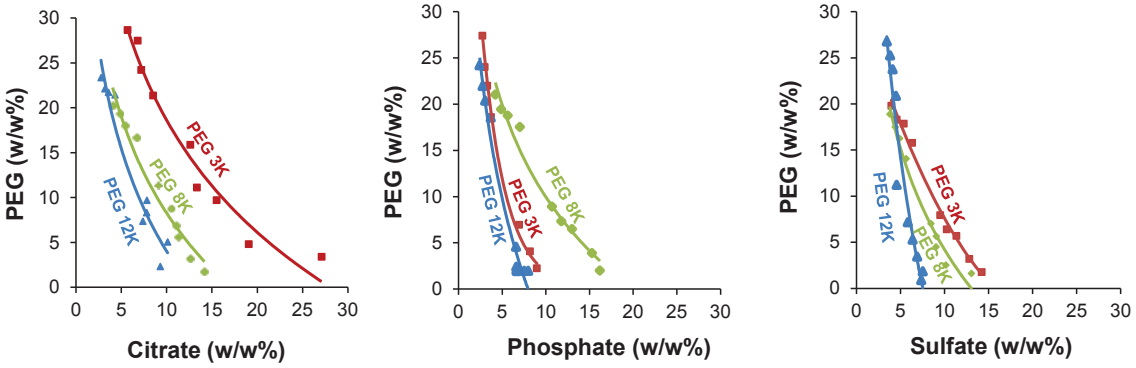

Figure 5.1 Binodal curves for (A) PEG-citrate, (B) PEG-phosphate and, (C) PEG-sulfate

5.3.3 Effect of anion

Before choosing the appropriate PEG-salt system, it was important to identify the anionic salt which was well-suited for PPV separation. Three different salts, citrate, phosphate and sulfate, were individually tested for ATPS separation containing PEG 12K. After extraction and cytotoxicity analysis, the results are shown in **Figure 5.2**.

Citrate demonstrated the greatest recovery of PPV in the PEG-rich phase. All three salts had minimal PPV found in the salt-rich phase. We assumed that the remaining virus resided at the liquid-liquid interface between the PEG-rich and salt-rich phases, shown in **Figure 5.2C**. We also assumed no loss of infectivity. We tested the infectivity of many unseparated PEG-salt systems and used this as the starting value for the mass balance. The mixed PEG-salt systems did not lose infectivity, as calculated from the known stock infectivity. All further experiments were carried out in the PEG-citrate system.

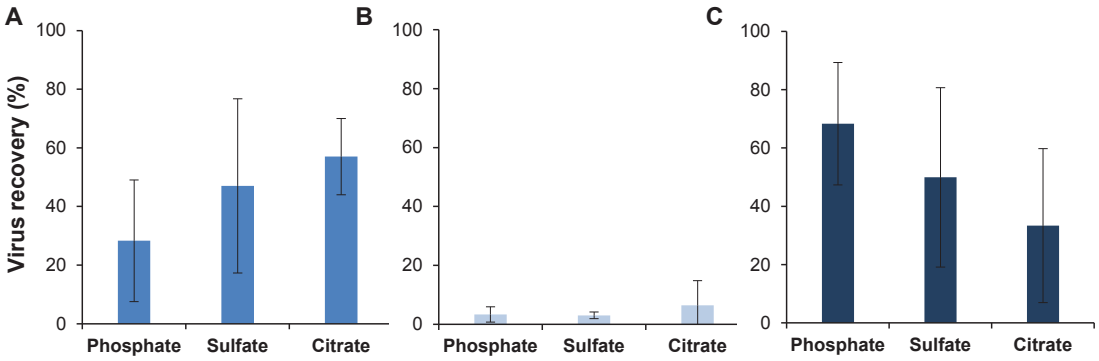

Figure 5.2 Effect of salt type on PPV separation. Partition behavior of PPV in 15 w/w% PEG 12K and salts at 14 w/w%. Virus concentration in (A) PEG-rich phase, (B) salt-rich phase, and (C) interface. The interface is calculated from the mass balance of the recovery from the PEG-rich and salt-rich phases. All data points are the average of three separate tests and error bars represent the standard deviation.

5.3.4 Effect of polymer size and salt concentration

PPV partitioning was conducted on PEG 3K, 8K and 12K with a constant polymer concentration and various salt concentrations, as shown in **Figure 5.3**. The concentration of salt and the PEG MW affect the partitioning of infectious virus in ATPS. As the MW of PEG increased, the recovery of PPV in the PEG-rich phase increased (**Figure 5.3A**), except for the highest salt concentration tested. For PEG

12K, a maximum recovery was reached at 14 w/w% citrate, and additional citrate decreased the recovery. At higher citrate concentration than 14 w/w%, the virus was pushed into the interface (**Figure 5.3C**). At lower citrate concentrations than 14 w/w%, the virus was found both at the interface, but also in the salt-rich phase (**Figure 5.3B**). As the salt concentration encroached the binodal curve (**Figure 5.1**), more virus was found in the salt-rich phase. For the PEG 8K, there also appears to be an increase in recovery in the PEG-rich phase as the citrate concentration increases, but it occurred at the higher citrate concentrations tested (16-21 w/w%). The PEG 8K also had much more virus at the interface than the PEG 12K at the optimal citrate concentration for that particular PEG MW. There was an increase in recovery of the PPV in the PEG-rich phase for PEG 3K with increasing salt concentration, but it was just beginning at the highest citrate concentrations tested. The PEG 3K also had most of the virus residing at the interface. It is undesirable to have the virus reside at the interface because it is difficult to recover and is likely aggregated and not infectious.

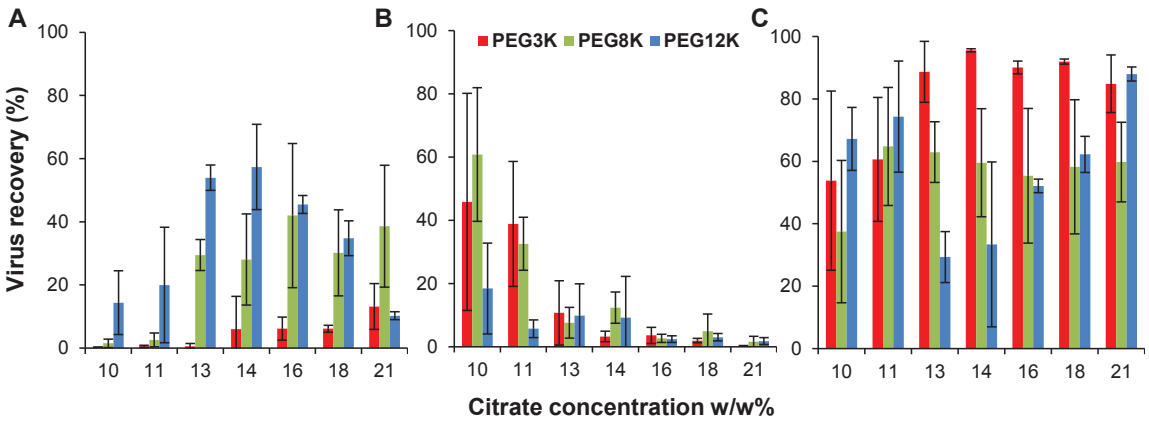

Figure 5.3 Effect of salt concentration on PPV separation. Partition behavior of PPV in 15 w/w% PEG and different citrate concentrations. Virus concentration in (A) PEG-rich phase, (B) salt-rich phase, and (C) interface. The interface is calculated from the mass balance of the recovery from the PEG-rich and salt-rich phases. All data points are the average of three separate tests and error bars represent the standard deviation.

5.3.5 Effect of ionic strength and pH

Addition of sodium chloride has been known to improve protein recovery in the PEG-rich phase [44]. It is hypothesized that the increased ionic strength in the salt-rich phase increases the hydrophobic interactions between PEG and protein [45]. To determine the effect of sodium chloride on the partitioning behavior of PPV, the addition of NaCl to 15 w/w% PEG 12K and 14 w/w% citrate was studied. In **Figure 5.4A**, we see that at all concentrations of NaCl tested, the virus partitioned less to the PEG-rich phase and more to the interface. The addition of NaCl may tell us more about the mechanism of virus separation, but it did not improve the virus recovery.

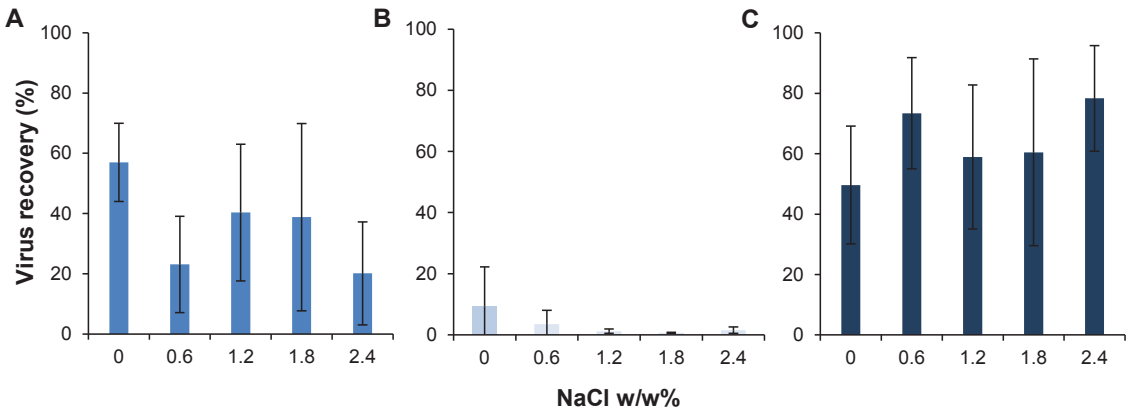

Figure 5.4 Effect of addition of NaCl on PPV separation. Partition behavior of PPV in 15 w/w% PEG 12K and 14 w/w% citrate with addition of NaCl. Virus concentration in (A) PEG-rich phase, (B) salt-rich phase, and (C) interface. The interface is calculated from the mass balance of the recovery from the PEG-rich and salt-rich phases. All data points are the average of three separate tests and error bars represent the standard deviation.

The pH of the ATPS solution was varied by controlling the pH of the bulk PEG and salt solutions prior to ATPS separation. The PPV partitioning is shown for 15 w/w% PEG 12K and 14 w/w% citrate ATPS in **Figure 5.5**. PPV is stable in the pH range of 3-9 [46], but lower pH in the 3-4 range resulted in miscible solutions in the chosen ATPS. From **Figure 5.5A** we observe that between pH 5 to 8, the separation to the PEG-rich phase increases from 7% to 64% and drops to 58% at pH 9. Virus recovery shifts from the salt-rich phase near the virus pI (~5 [47]), as shown in **Figure 5.5B**, to the interface at pH 6 and then to the PEG-rich phase from pH 7-9. This demonstrates that a neutral virus prefers the salt-rich phase, whereas a negatively charged virus prefers the PEG-rich phase. A change in pH does not change the amount of virus at the interface except at pH 6 (**Figure 5.5C**).

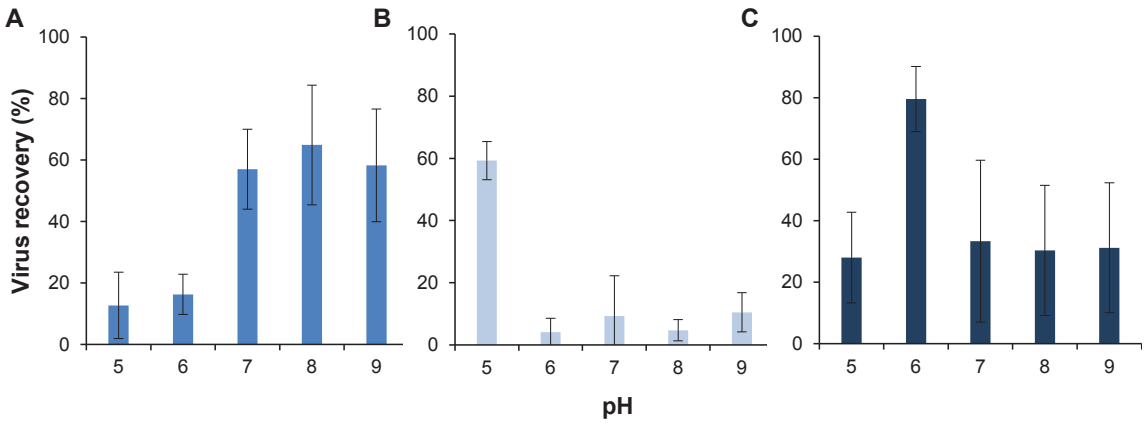

Figure 5.5 Effect of pH on PPV separation. Partition behavior of PPV in 15 w/w% PEG 12K and 14 w/w% citrate with pH controlled for the PEG-rich and salt-rich phases before ATPS was created. Virus concentration in (A) PEG-rich phase, (B) salt-rich phase, and (C) interface. The interface is calculated from the mass balance of the recovery from the PEG-rich and salt-rich phases. All data points are the average of three separate tests and error bars represent the standard deviation.

5.3.6 Effect of polymer dehydration

We also explored the possibility that dehydration of the polymer phase would affect the partitioning of the PPV. As shown in **Figure 5.6A**, the volume ratio of the PEG-rich and salt-rich phases change as the citrate concentration changes in the PEG 12K system. The highest recovery of the virus in the PEG-rich phase is at a citrate concentration of 13-14 w/w%, which corresponds to even volumes of each phase. At lower salt concentrations, the virus tends to partition more to the salt phase and the salt-phase volume decreases. At higher salt concentrations, the virus tends to partition to the interface and the PEG-phase volume decreases. To test the hypothesis that the virus favors a system where the salt-rich and PEG-rich phases are close to equal, we explored the volume ratios of the different salts tested and the addition of NaCl, shown in **Figure 5.6B and 5.6C**. However, these systems did not agree with the hypothesis that equal volumes of the phases favors PPV partitioning

to the PEG-rich phase. All of the ATPSs with different NaCl concentration and different salt types had equal volume ratios; however the partitioning did not favor the PEG-rich phase (see **Figure 5.4** and **Figure 5.2**, respectively).

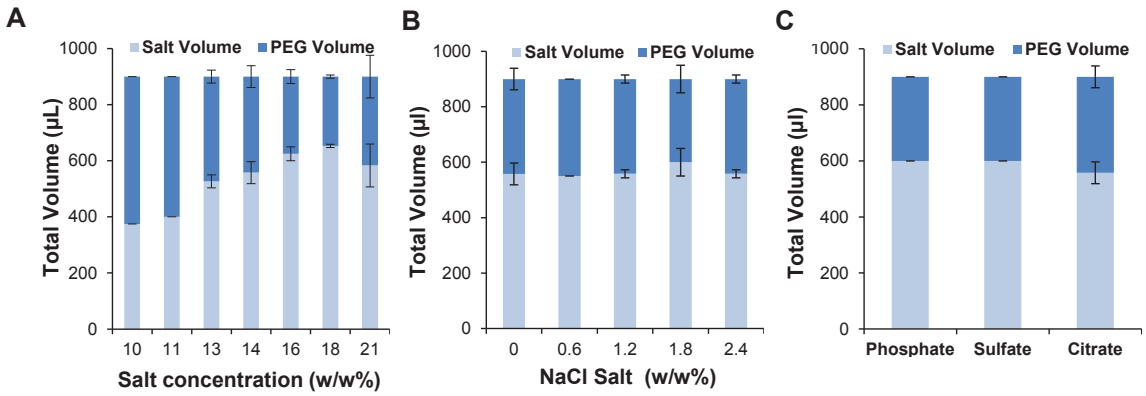

Figure 5.6 Effect of polymer dehydration on PPV separation. (A) Volume ratios of PEG-rich phase and salt-rich phase of ATPS for 15 w/w% PEG 12K and various citrate concentrations. (B) 15w/w% PEG 12K and 14 w/w% citrate with addition of NaCl. (C) 15w/w% PEG 12K and 14 w/w% salts. All data points are the average of three separate test and error bars represent the standard deviation

5.3.7 Separation of PPV from contaminant proteins

After partitioning PPV to the PEG-rich phase, we validated the purification and separation of virus particles from the most abundant contaminant, bovine serum albumin (BSA). BSA comes from the fetal bovine serum (FBS) used in virus production. Previously, it has been shown by Lu et al. that BSA partitions to the PEG-rich phase in a PEG 1K-citrate system, but partitions to the salt-rich phase when the polymer MW is increased [21]. We therefore hypothesized that the BSA would partition to the salt-rich phase in our PEG-citrate system since we were using a high MW PEG.

SDS-PAGE is generally considered the first choice as an assay for protein purity, however, PPV and BSA run closely together in SDS-PAGE. The main protein in the PPV capsid is VP2, and it has a MW of 64 kDa [48] and the MW of BSA is 66 kDa. Over 80% of PPV capsid is VP2, making the capsid fairly homogeneous. Not being able to determine the separation of PPV and BSA with SDS-PAGE, we sought a different method to distinguish the two biomolecules.

RP-HPLC was used to identify the presence of BSA in ATPS. We first used pure BSA to test our RP-HPLC method with high concentrations of BSA and without PPV present (**Figure 5.7A).** The BSA eluted at 21.5 minutes. **Figure 5.7B** shows chromatograms of BSA partitioning in the polymer and salt phases of ATPS, with an insert that focuses on the region of BSA elution. The concentration of BSA in **Figure 5.7B** is about 10 times lower than in **Figure 5.7A**, therefore many of the baseline peaks from the water used to make the solutions can be seen. We show that the BSA partitions to the salt-rich phase and none is found in the PEG-rich phase. The reduction in the PEG-rich phase is not due to dilution because the control system is the unseparated ATPS system, which contains the PEG, salt and BSA. For this reason, the salt-rich phase is able to have a higher peak, and therefore concentrates the BSA in a smaller volume than the total system control. The peak at 21.9 minutes is believed to be contamination from the water used, as it is also found in the PBS control. Using higher concentrations of BSA than found in the PPV extract, we confirmed the RP-HPLC method with SDS-PAGE. **Figure 5.7C** demonstrates that there is no BSA in the PEG-rich phase, whereas it can easily be found in the salt-rich phase, confirming the results of RP-HPLC in **Figure 5.7B**.

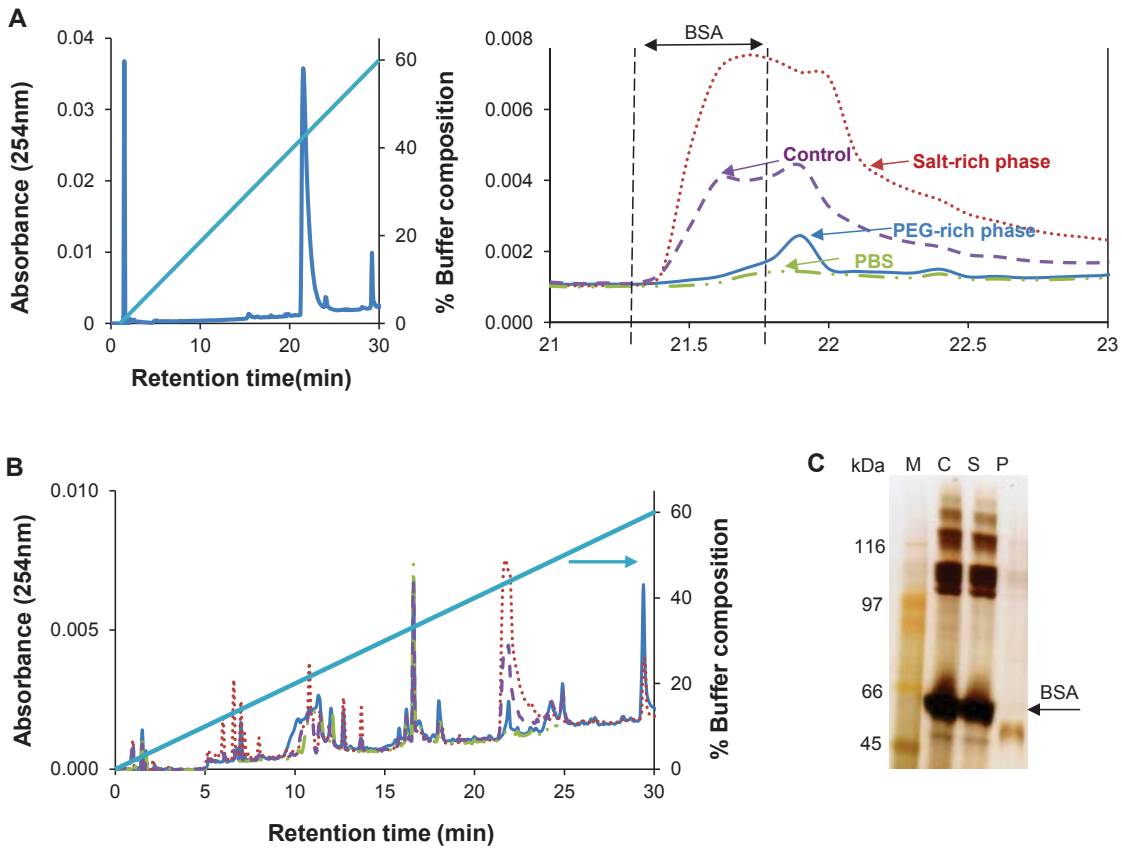

Figure 5.7 RP-HPLC of BSA in ATPS. (A) Chromatogram of 0.05 injected milligrams of pure BSA eluted with a linear acetonitrile gradient. (B) RP-HPLC of 0.005 injected milligrams of pure BSA within the control ATPS. The insert highlights the BSA peak at 21.5 minutes. (C) SDS-PAGE of samples from B: M=marker, C=control, S=salt-rich phase, and P=PEG-rich phase. The control was the unseparated ATPS containing PEG, salt and BSA.

Once we determined that the BSA partitions to the salt-rich phase, we used RP-HPLC to investigate the actual ATPS separation with PPV containing FBS from the cell culture media used to produce the virus (**Figure 5.8**). Again, the results show that all of the BSA partitions to the salt-rich phase and none is found in the PEG-rich phase. This demonstrates that the 15 w/w% PEG 12K and 14 w/w% citrate system was able to remove all detectable BSA.

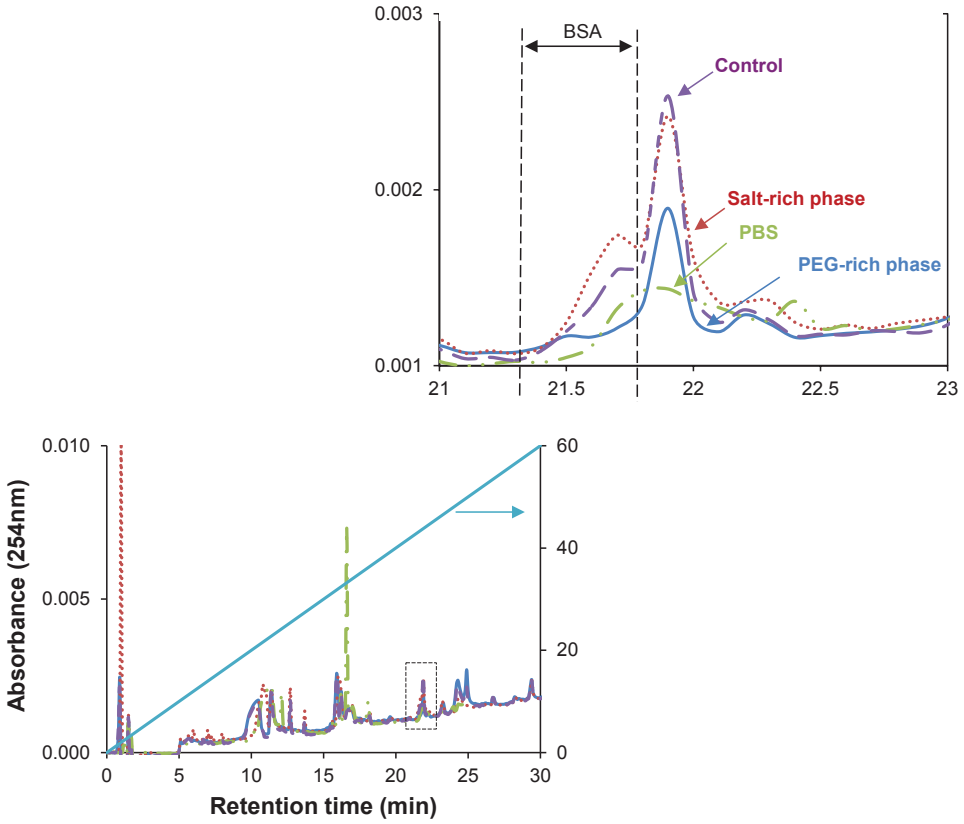

Figure 5.8 RP-HPLC of BSA in ATPS containing PPV. RP-HPLC of BSA after ATPS containing 0.1 g of PPV (which contains FBS from the PPV preparation), 0.4 g of 15 w/w% PEG 12K and 0.4 g of 14 w/w% citrate. The insert highlights the BSA peak at 21.5 minutes.

5.4 Discussion

From this study of PPV separation on a PEG-citrate system, we hypothesize that the dominant forces that are dictating the separation are biomolecule charge, surface hydrophobicity and the ATPS surface tension. Although others have suggested that the excluded volume of high MW PEG also plays a dominant role in ATPS [49, 50], we did not observe this effect.

High salt concentrations can drive biomolecules out of the salt-rich phase. When this occurs in ATPS, the biomolecule can either partition to the PEG-rich phase or occupy the interface. Virus separation has been plagued with partitioning of the virus to the interface. The majority of bacteriophage T4 particles in a PEG 0.6K-sulfate system [33], adenoviral vectors in a PEG 8K-phosphate system [51] and B19 VLPs in a PEG 1K-sulfate system [36] partitioned to the interface. However, we have found that the strongly hydrating citrate ion, as compared to the sulfate or phosphate ion, is able to drive PPV to the PEG-rich phase. This is advantageous because it allows easier recovery of infectious virus as compared to virus that occupies the interface. Citrate has only recently been used in ATPS and is gaining popularity due to its environmentally friendly nature as compared to phosphate [21].

5.4.1 Biomolecule charge

The molecular weight, surface net charge, size, shape, and specific binding sites of biomolecules play a crucial role in the molecular partitioning mechanism [20]. The electrical potential created due to distribution of salt ions between the aqueous phases causes negatively charged protein molecules to partition to the PEG-rich phase and positively-charged proteins to partition to the salt-rich phase [22]. This is observed as the pH is changed in the separation of PPV in **Figure 5.5**. As the pH is increased beyond the pI of PPV~ 5 [47], the virus surface becomes negatively charged and the recovery of the virus in the PEG-rich phase increases.

Water plays a significant role in the partitioning of proteins between phases. ATPSs typically consist of 70-90 w/w% of water. In PEG–salt systems, the distribution of anions and cations between the phases is highly dependent on their

ability to structure water [52]. The efficiency in virus separation can also be compared to the lyotropic Hofmeister series (see **Figure 5.2**). The order of anions as per Hofmeister are citrate^{3-} > SO_4^{2-} > PO_4^{2-}> acetate^{-}> Cl^{-}>NO_3^{-}. The sequence is in line with higher surface tension, stronger hydration, better protein stability and greater destruction of bulk water structure [53]. Citrate, a strongly hydrating kosmotrope, creates a charge difference between the PEG-rich and salt-rich phases, drastically changing the order of water molecules in the system. Hence, as citrate concentration is increased to 14 w/w% for the PEG 12K system, we can see a clear trend with increasing partitioning of PPV to the PEG-rich phase.

5.4.2 Excluded volume and hydrophobicity

The molecular mass of PEG used in these experiments is much higher than most other ATPS investigations. Several researchers in ATPS studies have acknowledged a necessity to identify optimal PEG MW. Increased polymer MW has a tendency to cause polymer compaction due to the increased intermolecular hydrophobic bonds formed by the high MW PEG, therefore decreasing free volume [49]. This volume exclusion principle decreases the partition coefficient and increases the separation of the desired protein toward the salt-rich phase [21, 50, 54]. The data shown in **Figure 5.3A** does not support the volume exclusion theory. The higher PEG MW enhances PPV partitioning to the PEG-rich phase. This is likely due to the interaction between the hydrophobic residues on lengthy high MW PEG and hydrophobic sites on the viral surface. It has been shown that hydrophobic proteins predominantly partition to the PEG-rich phase [55].

There is a growing body of evidence that virus surfaces are hydrophobic. Phages (viruses that infect bacteria) were shown to have a varying degree of hydrophobicity, as demonstrated by carbon adsorption [56]. B19 human parvovirus [57] and PPV [58] were precipitated with glycine and it was concluded that this was due to the highly hydrophobic surface of the virus. Our past work on virus binding peptides concluded that both hydrophobicity and charge play a major role in the binding of peptides to PPV [59-61]. The work presented here adds to the body of evidence that viruses have a strong surface hydrophobicity.

In support of the theory that hydrophobicity plays a major role in the partitioning of PPV to the PEG-rich phase, it has been shown that BSA partitions to the salt-rich phase of a PEG-citrate system when the PEG MW is greater than 1K [21]. Two theories were presented to explain this result, the increased PEG MW caused an excluded volume effect in the PEG-rich phase and the increased PEG MW increased the hydrophobicity of the PEG-rich phase and therefore the BSA preferred the salt-rich phase. Our results with PPV do not support the theory of excluded volume, but it does support the exclusion of the hydrophilic BSA from the hydrophobic PEG-phase.

5.4.3 Surface tension

When it comes to the partitioning of biomolecules in ATPS, the surface tension of the solution is an important parameter. Upon salt addition, the surface tension of water increases due to additional ionic charge that causes highly structured networks of hydrogen-bonded water. This increasing surface tension can cause an increase in protein surface free energy [62] which brings about protein-protein

interactions mainly due to hydrophobic bonding and weak van der Waals forces. However, as PEG MW decreases, the surface tension increases [63]. Therefore, it appears that if surface tension is an important parameter in the separation of PPV, then the balance of the higher surface tension citrate salt (**Figure 5.2**) and the lower surface tension 12K PEG (**Figure 5.3**) have the correct combination to effectively recovery the PPV in the PEG-rich phase.

To further explore the effect of surface tension on the separation and recovery of PPV, we added NaCl to the PEG-citrate system (see **Figure 5.4**). The addition of NaCl increases the partitioning to the interface (**Figure 5.4C).** We postulate that the PPV is residing at the interface upon high NaCl concentrations due to the increased surface tension of the system. The Cl^- ion is weakly hydrating in comparison to the citrate anion, which is likely why higher salt concentrations are needed to see an effect on PPV separation. The increase in surface tension would also explain why the PPV begins to partition to the interface at high citrate concentrations (**Figure 5.3C**).

5.5 Conclusions

A model system of infectious PPV in a serum-containing media was used to determine a virus purification system to model a non-enveloped vaccine production. A system containing 15 w/w% PEG 12K and 14 w/w% citrate at a pH of 8 was found to have a recovery of infectious PPV of 64% in the PEG-rich phase. This is a high recovery for an infectious virus that could possibly improve the 30% industry standard for a series of vaccine purification steps [15]. High PEG MW was needed for a high infectious recovery in the PEG-rich phase; however, it was not likely due to

volume exclusion. A more likely theory is that the high hydrophobicity of the PPV allowed for the virus to separate into the highly hydrophobic, high MW PEG-phase. The electrostatic potential between the PEG and citrate phases also likely assisted in the PPV partitioning at a high PEG MW. ATPS has to be balanced so that the hydrophobic driving force is greater than the surface tension, so that PPV can enter the hydrophobic PEG-rich phase and not be deterred at the interface. High citrate concentration or high NaCl concentration created a high enough surface tension to entrap the virus at the interface. This is undesirable since it is difficult to recover infectious virus from the interface. Now that a system that partitions the virus to the PEG-rich phase has been found, other disadvantages of ATPS for large-scale manufacturing, including virus dilution and removal of PEG, can be tackled in the future. This system needs to be tested with other hydrophobic, non-enveloped viruses to determine if ATPS has the potential to become a platform purification approach for vaccine production.

5.6 References

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Chapter 6

Virus removal using peptide functionalized chitosan nanofibers

The material contained in this chapter is planned for submission in a research journal.

6.1 Introduction

Water is the key to existence. Yet, in spite of the importance of water, 884 million people lack access to safe clean water [1]. Water appropriated for human consumption originates either from surface water bodies (lakes, rivers, streams etc.) or is pumped from ground water sources. Infection is a very common problem when there is a shortage of adequate sanitation, hygiene and clean water for drinking and household activities. A few of the common waterborne diseases are diarrhea, cholera and schistosomiasis. The major pathogenic organisms responsible for water borne diseases are bacteria (*E. Coli* and *V. cholera*), viruses (hepatitis A, and poliovirus) and parasites (*E. histolytica*, Giardia, Hookworm, *Schistosoma*). Treatment is therefore needed to produce clean drinking water. Municipal water treatment plants typically treat water with coagulation, sedimentation, filtration, disinfection and storage [2]. The physical filtration and disinfection steps play a key role in the removal of pathogenic organisms. Most countries use coliphages, phages that infect *E. coli*, to assess microbiological quantities in drinking water [3]. However, bacterial indicators do not reflect the risks of viral pathogens [4]. Pathogenic viruses are smaller than bacteria and therefore capable of escaping the filtration mechanisms. Some viruses are highly resistant to conventional treatment systems such as chlorination [5]. Chlorination and ultraviolet (UV) radiation are the conventional techniques in disinfection to achieve 4 LRV as required by the EPA for drinking water standards. Chlorination and UV inactivation studies have often demonstrated the susceptibilities of enteric viruses that have the potential to create waterborne outbreaks, such as poliovirus [6] and adenovirus [7]. In third world countries where municipal water treatment is unavailable, water supplies are

generally provided from unprotected dug wells, unprotected springs, small carts with water drums, tanker trucks, surface water and bottled water [8]. Even a small dose of virus is capable of causing severe damage to human health. This necessitates the development of sustainable pathogen removal systems that can be applied worldwide.

Methods used for physical filtration include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). MF has a pore size (0.1 – 10 μm) which will restrict bacteria but allow viruses to seep through the filter pores due to their small size [9]. UF has a pore size of 0.01 – 0.1 μm that prevents some but not all viruses [10-12]. Better results using UF is reported in literature due to the charge of the virus, secondary interactions of virus with membrane surface, pore block from organic matter, pore constriction, and sieving [13, 14]. The virus removal due to the featured characteristic of UF i.e. size exclusion is not a foolproof system. The evidence for this has been witnessed during an inconsistent 1 to 6 LRV for MS2 and GA bacteriophage [15] and 3 to 4 LRV of influenza virus [16] when filtered with a UF membrane. Despite a lack of reliable system for virus removal from UF, up to 1.8 billion gallons per day of drinking water worldwide is processed through UF or MF mechanism only [17]. The alternate techniques of nanofiltration and reverse-osmosis have been proposed. Nanofiltration is accompanied with membrane fouling and low permeability effects due to its small pores [18]. NF and RO experience membrane design imperfections which can cause large virus escape [19, 20] and both methods are expensive to produce and maintain on a consistent basis [21]. A more detailed description of the ability of filters for virus removal from water has been explained in the literature review **section 2.3.**

To overcome the processing problems that occur with standard size-based filtration, we propose to use nanofibers adsorption, which is a novel method to remove viruses. Nanofiber filtration has low pressure drops, high water fluxes and decreased fouling. Our proposal is to make nanofibers from the biocompatible, inexpensive and environmentally-friendly natural polymer, chitosan. Nanofibers have an increased surface area to volume ratio, which increases their adsorption capacity closer to that of chromatography, as compared to standard membrane absorbers. Nanofibers are also on the same size scale as viruses, creating a curved surface to increase virus adsorption. Ultrafine cellulose nanofibers (UCCN) modified with polyethylenimine infused into electrospun polyacrylonitrile nanofibrous scaffolds have removed *E.Coli* and MS2 bacteriophage by a LRV of 6 and 4, respectively, for water purification [22]. Polyurethane-based N,N-dodecyl,methyl-polyurethane (Quat-12-PU) electrospun nanofibers exhibited antimicrobial properties against gram-positive *Staphylococcus aureus* and gram-negative *E.coli* bacteria close to 7.5 LRV [23]. Chitosan polycaprolactone (PCL) nanofibers were able to remove almost all of *Staphylococcus aureus* [24]. [(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) and polyvinyl alcohol (PVA) in ratio 4:6 were electrospun into nanofibers crosslinked with 30% glutaraldehyde vapor for 4 hours. This system was filtered for viral clearance studies with water which was able to remove up to 3.6 LRV for porcine parvovirus (PPV) and 4.1 LRV for Sindbis virus [25]. Here, we recognize the potential of virus removal using nanofibers and propose to remove a model non-enveloped virus with an electrospun chitosan membrane.

6.1.1 Electrospinning

Electrospinning is a technique that allows the fabrication of continuous nanofibers with diameters of 100 nm or less under the application of external electric field [26]. Electrospun nanofibers has gained considerable importance and studies in the areas of tissue engineering [27], drug delivery [28], water filtration [25] and wound dressing [29]. Nanofibers are desired due to their high surface-to-volume ratio, large pore size and the ease of functionalization of fiber membranes [30, 31]. Electrospun nanofibers possess high porosity, consistently interconnected pores which improve flux performance [32]. The pore size of the range of MF 0.1 μm also causes low membrane fouling and low backpressures [9]. MF can remove bacteria by size-exclusion, but not viruses. However, the electrospun fibers can be functionalized which can result in adsorption of viral pathogens. Microcrystalline cellulose nanofibers infused with polyethylenimine gave promising results up to 4 log of MS2 bacteriophage [22]. Positively charged and functionalized polyacrylonitrile membrane demonstrated not only 2-3 times higher permeation flux and low pressure drop compared to commercial MF but yielded a high 4 log removal of MS2 bacteriophage [33] .

A typical setup for electrospinning consists of three major components: a high-voltage power supply, a syringe pump and an electrically conductive collector [34]. In electrospinning, a polymer solution is held by its surface tension at the end of a needle and subjected to an electric field. Once the electric field reaches a critical value, mutual charges on the liquid surface cause a repulsion stronger than the surface tension. It induces an ejection of liquid from the tip of the needle, which then elongates from a hemispherical droplet into a cone-like structure known as a Taylor

cone [35]. As the viscous polymer solution is pulled toward the rotating drum collector, the solvent evaporates leaving a charged polymer fiber on the collector, forming a random, non-woven nanofiber mat. A schematic diagram is as shown in **Figure 6.1** [36].

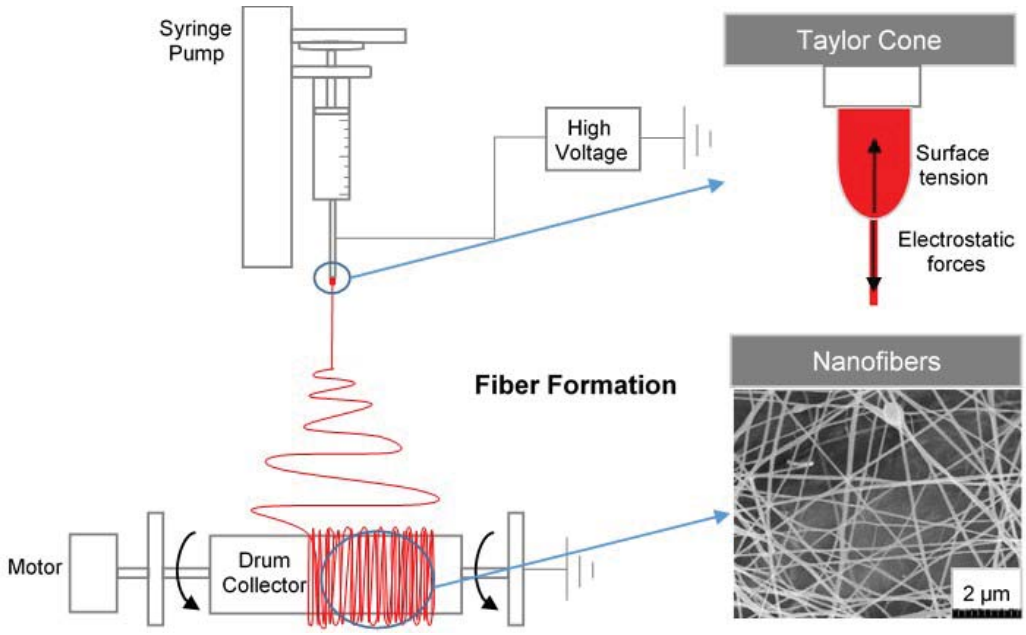

Figure 6.1 Schematic of electrospinning apparatus [36]. The polymer solution held on syringe is collected as dry polymer fibers on an electrically conductive collector after subjecting the syringe to an optimum electric field (copyright permission granted).

6.1.2 Chitosan

Chitin (poly *β*-(1→4)-*N*-acetyl-D-glucosamine) is the second most abundant natural polysaccharide, after cellulose, and it provides structural integrity to insect and crustacean shells. These polymers are biocompatible, biodegradable, non-toxic, antimicrobial and have low immunogenicity, attracting immense future potential [37]. Chitosan is synthesized by deacetylation of chitin, which then exposes amine groups on the carbohydrate surface. Chitosan has been preferred over chitin for research

and commercial applications as it is easily soluble in acidic, aqueous solutions. Chitosan is popularly known for its antimicrobial properties [38-40]. Although the complete mechanism is yet to be understood, several hypotheses attribute it to the presence of the NH_3^+ group on the glucosamine monomer at a pH below the pI of 6.3 [41]. At low pH, chitosan amines are protonated, making them a water soluble cationic polyelectrolyte [37, 42]. At high pH, chitosan is deprotonated, and the neutral polymer is insoluble in water. Chitosan has shown antiviral activity in plant and animal biological system. A few proposed antiviral mechanisms include binding to the proteins in the viral capsid, causing structural damage, blocking viral replication, and inhibiting replication of bacteriophages [43]. However the mechanism is poorly understood and limited literature is available on the effect of chitosan on viruses. We hypothesis that chitosan is a polycationic polymer, which can adsorb negatively charged viruses by electrostatic forces. Implementation of this research technique focusing on water purification is supported by the application of chitosan for extraction and separation of aromatic compounds (phenolic and polycyclic) for water decontamination. It also has the capacity to remove metals, chemicals and bacteria. Chitosan has been identified in reducing infection of bacteriophage MS2 and feline calicivirus FCV-F9 (from initial ~5 log *plaque-forming unit* (*PFU*)/ml viral titers) by 1.70 and 4.21 log PFU/ml [44]. For non-enveloped PPV, functionalized electrospun chitosan nanofibers with a positively charged quaternized amine forming HTCC has 2.0 LRV with 12% w/v HTCC:graphene blend in water [45]. One of the potential ways of improving the virus adsorption to chitosan nanofibers is to attach small peptide ligands to the fibers. For PPV removal it was noted that trimeric peptide WRW and hexamer peptide YKLKYY achieved 4.5-5.5

LRV in human blood plasma [46] on a chromatographic system. We propose to conjugate the CWRW peptide to the chitosan fibers and design a point-of-care filtration system for potable water from viruses.

In this chapter we will show the results found from accomplishing the following objectives:

Objective 1: Explore linker chemistry (carbodiimide and maleimide) required to synthesize peptide affinity ligands on electrospun chitosan fibers

Objective 2: Determine the virus removal capability of chitosan membrane matrix from virus contaminated water

6.2 Materials and Methods

6.2.1 Materials

Chitosan of 310,000 molecular weight and 75-85% deacetylated, polyethylene oxide (PEO) (MW 90,000) and glacial acetic acid required for making electrospinning polymer solution was purchased from Sigma (St.Louis, MO). Whatman quantitative Filter Paper Circles (Clifton, NJ), Grade 1, 55 mm and 10 mm diameter were used as nanofiber supports. Iodoacetic acid (IAA) (≥ 98.0%, MW 185 g/mol), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) (≥ 98.0%, MW 191 g/mol) for carbodiimide reactions was purchased from Sigma (St.Louis, MO). Sulfo-SMCC (MW 436 g/mol) purchased from Pierce (Rockford, IL) was used for maleimide crosslinking reaction. Sulfo-N-hydroxysuccinimide (Sulfo-NHS) and 2-Mercaptoethanol were purchased from Sigma (St.Louis, MO). MES sodium salt (≥ 98.0%, MW 217 g/mol) for buffer and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). For the phosphate buffer saline (PBS) sodium

phosphate and sodium hypophosphite ($NaH_2PO_4.H_2O$) were purchased from VWR (Radnor, PA) and sodium triphosphate (Na_3PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Hydrochloric acid was purchased from EMD Chemicals (Billerica, MA) and sodium hydroxide was purchased from Acros Organics (New Jersey, NY) for pH control. All aqueous solutions were prepared using purified water with a resistivity of ≥ 18 MΩ·cm with a Nanopure filtration system (Fisher Scientific, Pittsburgh, PA). Peptide CWRW (MW 649.7 g/mol) was purchased from Biomatik LLC (Wilmington, DE).

Minimum essential medium (MEM) for cell propagation was purchased from Life Technologies (Grand Island, NY). For virus titration, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). High pressure liquid chromatography (HPLC) grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA) respectively.

6.2.2 Methods

6.2.2.1 Electrospinning of chitosan nanofibers

The electrospinning was done using an in-house facility setup. Contents including 0.4 g of chitosan and 0.04 g of PEO was mixed in a 50 ml tube (VWR, Radnor, PA) with 90% acetic acid solution. After contents were mixed using a vortex it was let to sit on a bench top for 3 hours. Three ml of the contents was drawn into a syringe and then attached to a syringe pump (Braintree Scientific INC, Braintree, MA). The needle inserted into the syringe tip was connected to the high voltage supply (Glassman high voltage, INC, High Bridge, NJ), and the ground was attached

to the rotating drum collector that was run by a pump (ElectroCraft TorquePower™, Ipolis, OH). Whatman circle papers were taped to the drum covered with aluminum foil for collecting fibers. The needle syringe was placed 6 cm away from the drum which was rotated at a speed of 2000 rpm as the fibers spun at flow rate of 6 ml/hr and voltage of 20 kV.

6.2.2.2 Attachment of peptides onto chitosan electrospun fibers

In our investigation, we used reagents capable of linking the amine reactive chitosan polymer to the thiol cysteine on the peptide. We have explored the carbodiimide with iodoacetic acid and maleimide to achieve our objective.

6.2.2.2.1 IAA-EDC crosslinker

Method 1: The amount of chitosan on the 23 cm^2 Whatman circle paper was determined by weighing the paper before and after electrospinning. The adsorbed chitosan on the paper was assumed to be uniform and 1 layer of 0.5024 cm^2 of paper was punched containing approximately 0.6 µmoles of chitosan or its equivalent amines. 50 mM of iodoacetic acid (IAA) and 50 mM of *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was dissolved in PBS in a micro-centrifuge tube. The contents of the tube were pH adjusted to 7, transferred to 20 ml glass containers and allowed to react with punched chitosan for 2 hours. The punched paper was then removed and excess IAA-EDC from the matrix was rinsed off with 10 mM phosphate buffer by rocking in a micro-centrifuge tube for 10 min on a Roto-shake Genie rocker (Scientific Industries Inc., Bohemia, NY). The rinsing procedure was repeated 3 times. Two separate tests were then performed; one to validate the coupling of amines using ninhydrin test and the other

to visually check for dissolving fibers. Ninhydrin reagent in practice is used for determination of primary and secondary amines in a solution or on a substrate. A ninhydrin kit test (2% solution) from Sigma (St.Louis, MO) was used to verify if the amine has been coupled to iodo group. For the ninhydrin test, we immersed the reacted chitosan in a solution of 100 µl ninhydrin reagent and 200 µl 10 mM phosphate buffer. We heated the contents to 100 °C for 10 mins, allowed it to cool and diluted in 500 µl ethanol before reading the absorbance at 570 nm. The punched chitosan was examined for dissolving fibers using electron microscopy, detailed in **section 6.2.2.3.**

Method 2: The procedure for obtaining punched chitosan after electrospinning was followed as per **section 6.2.2.2.1**. 25 mM of IAA and 25 mM EDC were dissolved in MES buffer using a micro-centrifuge tube. Immediately 50 mM NHS was added, pH adjusted to 4-5 and the reaction allowed to proceed for 15 minutes. 2 µl mercaptoethanol was added to quench the EDC reaction and contents in the tube were desalted for terminating the reaction using an Econo-Pac 10DG column purchased from Bio-Rad Laboratories (Hercules, CA). PBS was used to elute the different 1 ml samples from the column, which were collected in separate micro-centrifuge tubes. The desired 1ml sample was identified by reading the absorbance on a Synergy Mx microplate reader (BioTek, Winoski, VT) at 280 nm, where NHS activated IAA can be measured [47]. The sample pH was adjusted to 7-8 and allowed to react in the presence of punched chitosan (0.5024 cm^2) in a 20 ml glass container. The reaction to bind iodo group onto the amine reactive chitosan was allowed to proceed for 2 hours following which the unreacted IAA and EDC were rinsed off with 10 mM phosphate buffer (pH ~ 7) in a Roto-shake Genie. The

ninhydrin kit test on chitosan as a validation step was followed due to the explanation given in **section 6.2.2.2.1**. Continuing from the IAA-EDC rinsing procedure, 1.5 mM CWRW peptide was adsorbed to the chitosan by dissolving CWRW peptide in 1 ml of 10 mM phosphate buffer in a micro-centrifuge tube. The reaction was performed overnight with rocking at room temperature. Peptide adsorbed on chitosan was quantified by measuring the aqueous tube contents using C_{18} reverse phase chromatography (RP-HPLC), detailed in **section 6.2.2.4.** The chitosan was carefully removed and retained for virus removal studies using MTT assay, detailed in **section 6.2.2.5**. Fiber morphology was examined in intermittent steps using electron microscopy, detailed in **section 6.2.2.3.**

6.2.2.2.2 Maleimide crosslinker

Method 3: The procedure for obtaining punched chitosan after electrospinning was followed as per **section 6.2.2.2.1**. 4.6 mM of Sulfo-SMCC in 10 mM phosphate buffer was prepared in a micro-centrifuge tube and the pH was adjusted to 7. Sulfo-SMCC was treated with punched chitosan for 2 hours at room temperature in a 20 ml glass container. The punched chitosan was removed from the container and rinsed using 10 mM phosphate buffer in a micro centrifuge tube for 10 minutes. Ninhydrin test was performed as a validation step to confirm the reaction of amines on the chitosan. The punched chitosan was immersed in 1.5 mM CWRW peptide in 10 mM phosphate buffer from anywhere between 1 hour to 24 hours. All quantification was conducted, as described in **section 6.2.2.1**.

6.2.2.3 Scanning electron microscope

The morphology of the nanofibers was observed by a Hitachi S-4700 field emission scanning electron microscope (FE-SEM) (Tustin, CA) which is a cold field, emission high resolution scanning electron microscope. The electrospun fibers were coated with a 5 mm layer of platinum/ palladium (Pt/Pd) using a Hummer 6.2 Sputter Coater (Union City, CA) before being examined by FE-SEM. The accelerating voltage for the FE-SEM was 5 kV, and the magnification was from 1,000× to 10,000×. All fibers were dried in a Gold series DP-32 vacuum drying oven (Ontario, Canada) at 80°C for 2 hours before Pt/Pd coating and imaging with FE-SEM.

6.2.2.4 Reverse phase chromatography

For quantification of the CWRW peptide adsorbed to the chitosan membrane, RP-HPLC was performed with a Waters XBridge BEH 130 C_{18} column on a Waters Alliance HPLC equipped with a photo diode UV-Vis detector operating at 220 nm wavelength. Aqueous contents of the tube during peptide-chitosan reaction were collected before and after the reaction separately. Samples were filtered through a 0.2 µm syringe filter and 25 µl was injected onto the RP-HPLC C_{18} column at a flow rate of 1.0 ml/min Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. A linear gradient of decreasing Buffer B was performed.

6.2.2.5 Virus removal assessment

Log 6 PPV was prepared by diluting stock virus in PBS or water. Peptide was attached on one or three layers of punched chitosan which were immersed in 500 µl virus in a micro-centrifuge tube. The contacted was performed for 1-3 hours with

rocking. Loose fibers were spun down using the Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) at 5000 RPM for 3 mins. The chitosan was then discarded and a minimum of 100 μl supernatant was extracted using a 10-100 μl pipette tip (USA Scientific, Oscala, FL). The extract was used for determining virus concentration using the MTT assay, described in **section 6.2.2.6**.

6.2.2.6 Cells, virus and titer assay

Porcine kidney cells (PK-13) were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% pen/strep at 37°C, 5% CO_2 and 100% humidity. The cells were propagated every 3-4 days at a split ratio of 1:5. PPV strain NADL-2, also a gift from Dr.Ruben Carbonell at North Carolina State University, was propagated in PK-13 cells, as described previously [48, 49]. PPV was clarified with centrifugation prior to use.

PPV was titrated with a cell viability assay, the colorimetric MTT assay, as described previously [48]. Briefly, PK-13 cells were seeded in 96-well plates. Plates were infected with 25 μl of PPV and serially diluted across the 96-well plate. After five days of incubation, the MTT salt solution was added. Four hours later, solubilizing agent was added. Plates were read on a Synergy Mx microplate reader (BioTek, Winoski, VT) at 550 nm between 18-24 hours after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that corresponded to an absorbance of 50% of the uninfected cell absorbance. The value was converted to a per millimeter basis and stated as the MTT_{50}/ml titer.

6.3 Results and Discussions

6.3.1 Characterization of peptide functionalized chitosan

We selected two different conjugation chemistries in order to attach the terminal cysteine residue on the CWRW peptide to chitosan electrospun fibers. This peptide was selected due to its previously found properties of removing PPV from solutions containing human blood plasma (citation needed). Electrospun fibers have been shown to adsorb viruses (cite Michelle's papers) and we therefore desire to improve the virus removal capabilities by combining these two powerful virus removal techniques.

6.3.1.1 Method 1: IAA-EDC crosslinker

The chitosan was electrospun on Whatman filter paper. The spun fibers were observed for fiber density and morphology using the FE-SEM and images are shown in **Figure 6.2.**

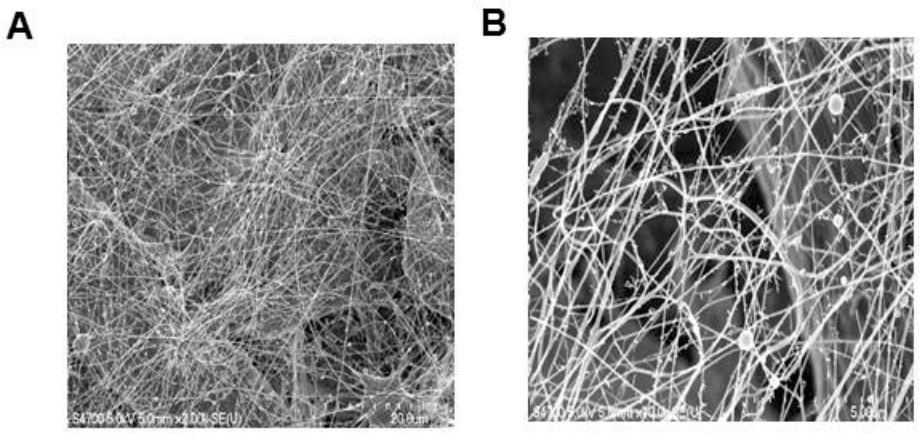

Figure 6.2 FE-SEM micrographs of electrospun chitosan/PEO in 90% acetic acid. The feed concentration was 2.5 wt%, feed rate was 6 ml/h, distance between the needle and collector was 6 cm, collector was rotated at 2000 rpm, volume of electrospun solution was 3ml and applied voltage of 20 KV (A) 2,000× (B) 10,000×.

Linking agents were used to conjugate the peptides to the amine group on the chitosan. We used IAA (iodo and carboxyl group) and EDC conjugate reactants in water or PBS as a coupling agent to react with chitosan. The carbodiimide reaction using the IAA-EDC conjugate will form active O-acylisourea ester and the chemistry is shown in **Figure 6.3.** The most important feature and requisite of this reaction was the coupling of iodo group on the primary amine of the chitosan.

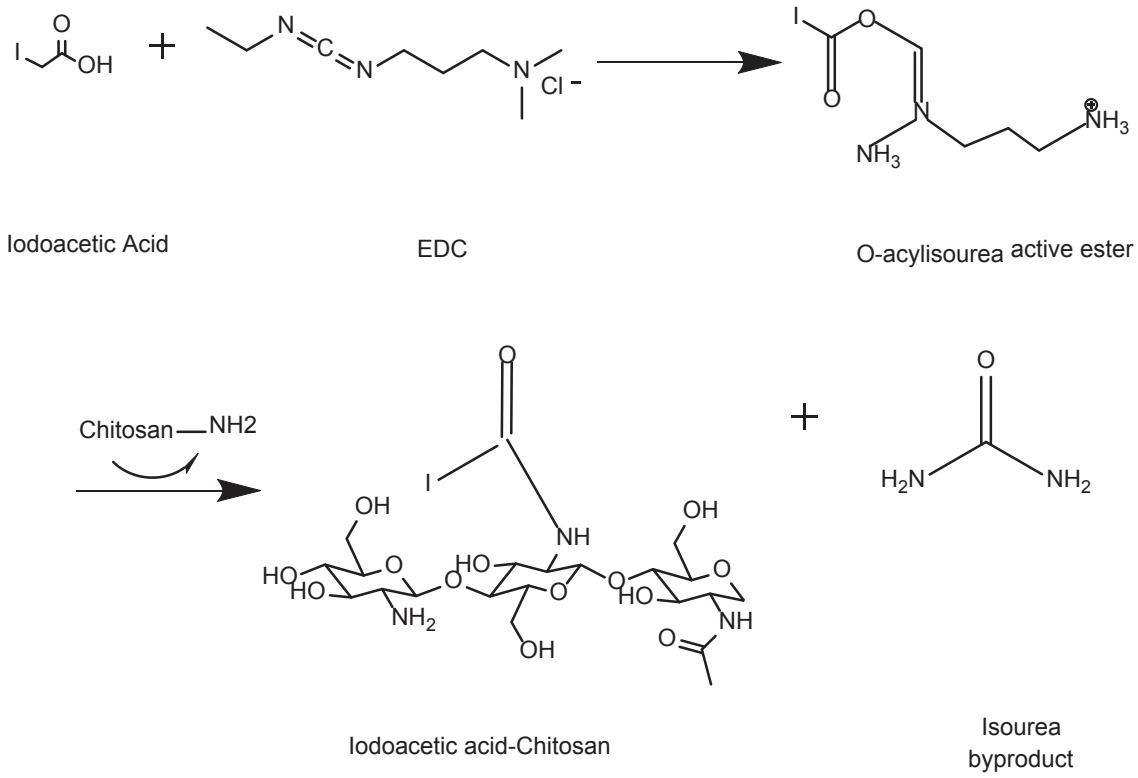

Figure 6.3 O-acylisourea ester and IAA-chitosan intermediate. 50 mM of IAA and 50 mM of EDC in PBS (~ 7) were treated with 0.5024 cm^2 chitosan for 2 hours. It was to create a functional iodo group coupled to the primary amines on the chitosan fibers.

Before proceeding with the peptide synthesis it was necessary to confirm the stability of fibers after the ester reaction. The initial few experiments were performed in water which dissolved the fibers (see **Figure 6.4 (A))**. Chitosan is soluble at pH <

6.3 [37] and it was necessary to maintain the pH > 6.3 during the carbodiimide reaction which was difficult for water due to its poor buffering capacity. The medium was replaced with PBS (~ pH 7) which improved the buffering capacity. The chitosan did not dissolve and fiber morphology was maintained (see **Figure 6.4 (B))**.

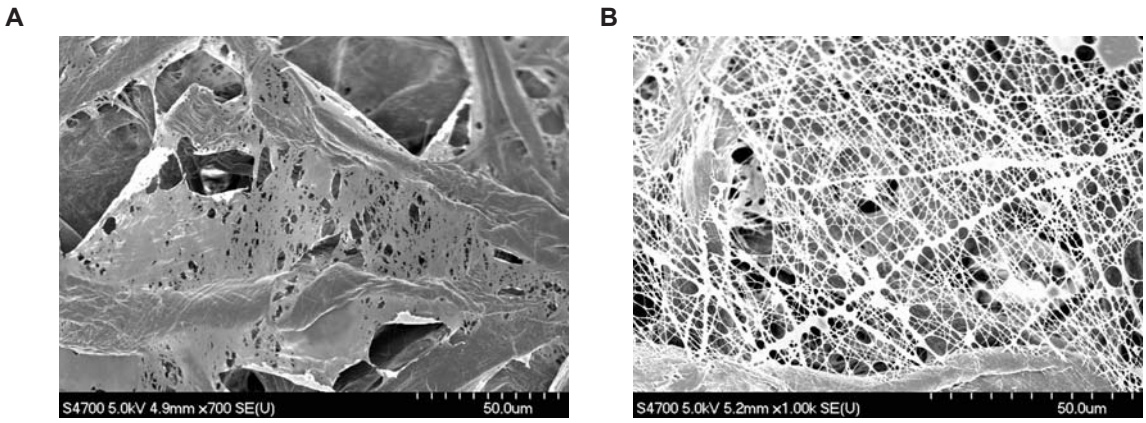

Figure 6.4 FE-SEM micrographs of electrospun chitosan/PEO after coupling reaction between O-acylisourea and chitosan. 50 mM of IAA and 50 mM of EDC in PBS (~ 7) were treated with 0.5024 cm^2 chitosan for 2 hours (A) Reactants in water showing dissolved fibers at 700× (B) the reactants in PBS showing intact fiber at 1,000×.

The ninhydrin test was performed to determine the concentration of amines on the chitosan. A higher absorbance would mean the inability of iodine in the IAA to couple to chitosan or in other words leading to an incomplete reaction. The result of the ninhydrin test on chitosan samples treated with IAA-EDC along with the controls are shown in **Figure 6.5.** The three samples shown on the x-axis were (1) chitosan treated with IAA-EDC, (2) plain paper without chitosan treated with IAA-EDC (negative control) and (3) chitosan treated with PBS only (positive control). Ideally it was expected for chitosan sample to have a lower absorbance since the amines should have been coupled with the iodine. However we observed amine detection in

decreasing order i.e. paper control, the chitosan sample and finally the chitosan control.

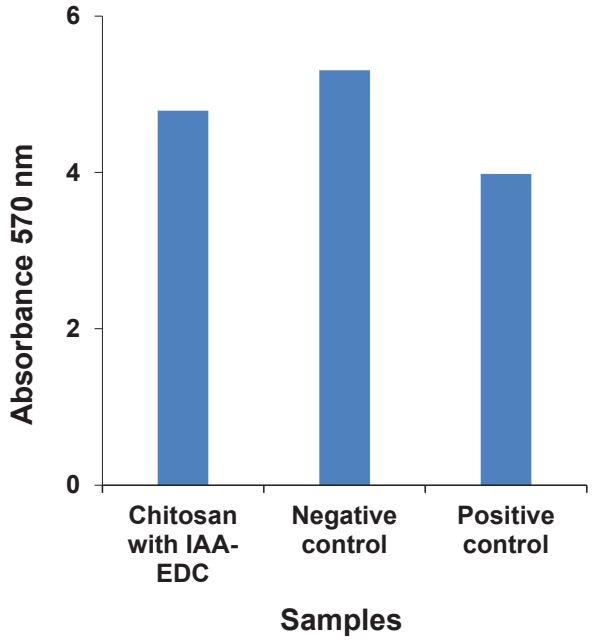

Figure 6.5 Ninhydrin test kit. Determination of amines for samples (1) chitosan with IAA and EDC, (2) plain paper reacted with IAA and EDC (negative control) and, (3) chitosan treated with PBS only (positive control) treated with 50 mM IAA and 50 mM EDC in PBS at pH 7 for 2 hours. The data points shown here are results from a single test only.

The O-acylisourea intermediate is known to be unstable and undergo hydrolysis when it doesn't react with amines [50]. It leads to regeneration of carboxyl group and un-substituted urea (see **Figure 6.6).** The short half-life of the O-acylisourea and excess unreacted amines from urea can complicate the reaction scheme. We suspect the ninhydrin test results to be a resultant of the unstable ester. The issue was overcome by combining new procedural steps which is detailed in the following method 2 of IAA-EDC chemistry.

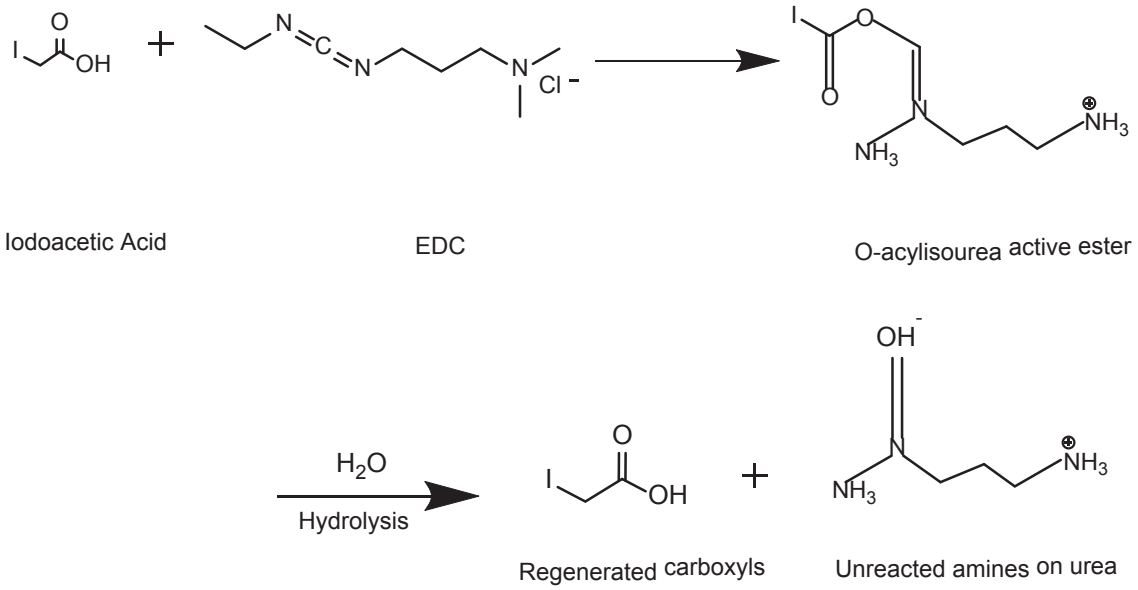

Figure 6.6 Hydrolysis of O-acylisourea. Short half-life of O-acylisourea can create unstable and unsuitable conditions for IAA-chitosan coupling reaction in the presence of EDC conjugate.

6.3.1.2 Method 2: IAA-EDC conjugate in Sulfo-NHS

The IAA-EDC chemistry was performed in the presence of Sulfo-NHS to maintain stable amine reactive groups for the ester intermediates. The chemistry is shown in **Figure 6.7.** The PBS was replaced with MES buffer, which has a buffering capacity in the desired pH range of 4-5. The low pH was a more suitable reactive pH condition for the EDC chemistry. The EDC reactions were quenched by adding mercaptoethanol that would form stable complex with carbodiimide and the excess reagents were removed by desalting the aqueous contents. The desalted sample was reacted with punched chitosan.

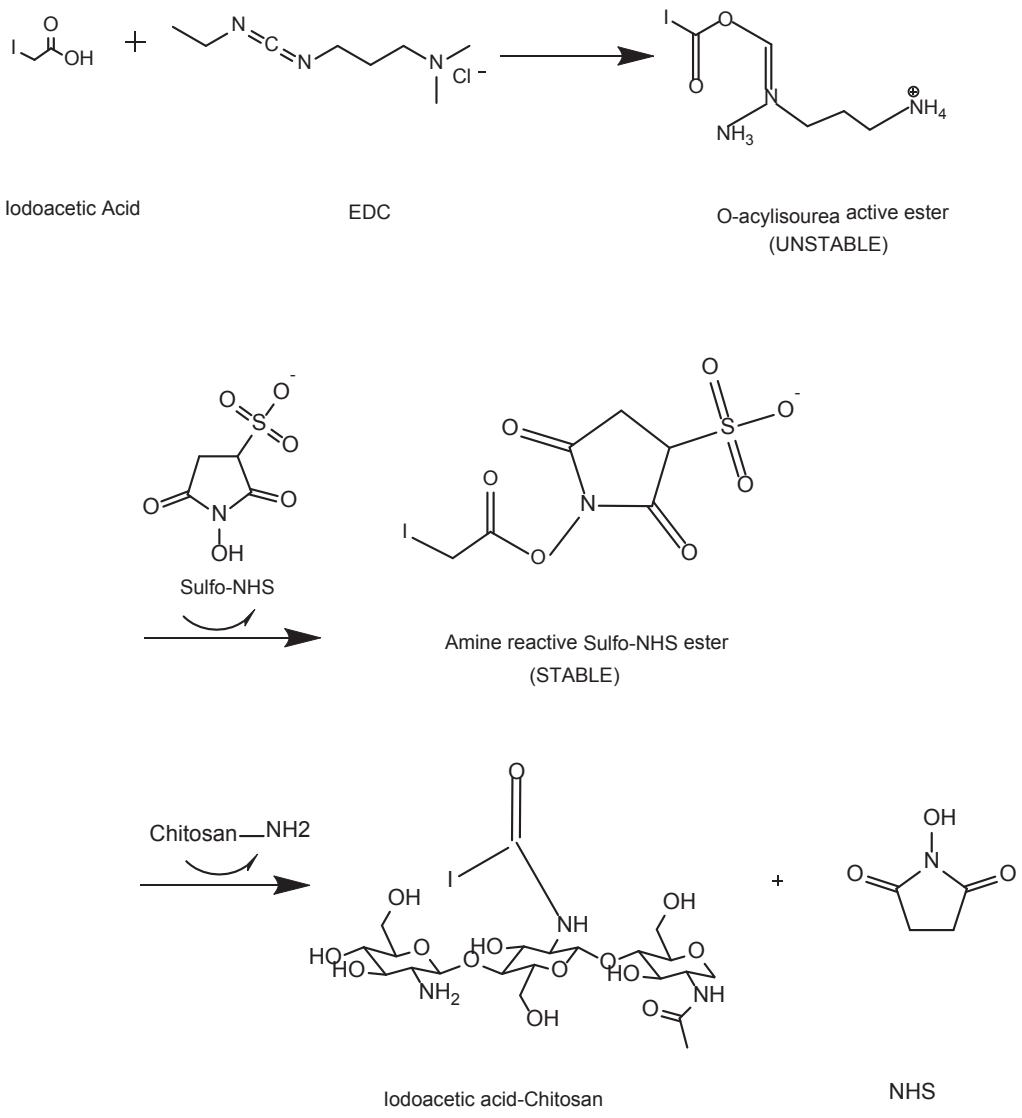

Figure 6.7 Stabilizing the IAA – EDC ester reaction. 25 mM IAA, 25 mM EDC in MES buffer at pH 4-5 was reacted in the presence of 50 mM NHS. After 15 min reaction was quenched with 2- mercaptoethanol and desalted. The samples with maximum absorbance at 280 nm was collected and reacted with 0.5024 cm^2 punched chitosan (pH 7-8) to obtain IAA-chitosan complex.

The ninhydrin test was performed on samples (1) chitosan treated with IAA-EDC, (2) plain paper without chitosan treated with IAA-EDC (negative control) and (3) chitosan treated with PBS only (positive control). The chitosan sample with an absorbance (570 nm) in between the two controls indicated the amines were

coupled to the iodo functional group (see **Figure 6.8**). The chemistry for the reaction is shown in **Figure 6.7**. It must be noted here that inconsistencies were noted in the ninhydrin kit test when the test was repeated for verification after a few days. The possible inconsistency was suspected to be the oxidation of the ninhydrin, which is sensitive to air. Since the test was only a validation step and not an actual procedure in peptide adsorption we proceeded with the peptide conjugation to iodo-substituted chitosan.

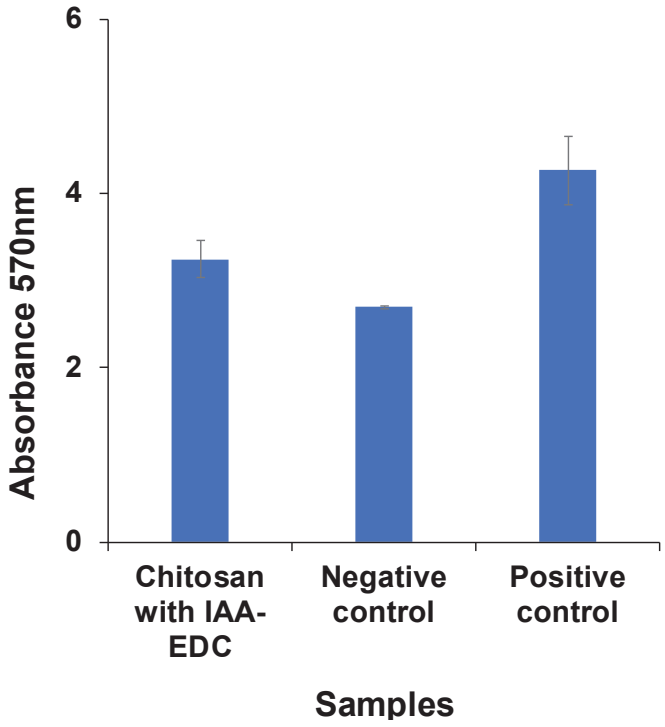

Figure 6.8 Ninhydrin test kit. Determination of amines for samples 1) chitosan treated with IAA and EDC, (2) plain paper treated with IAA and EDC (negative control) and, (3) chitosan treated with PBS only (positive control 2). 25 mM IAA, 25 mM EDC in MES buffer at pH 4-5 was reacted in the presence of 50 mM NHS. After 15 min reaction was quenched with 2- mercaptoethanol and desalted with PBS. The sample with maximum absorbance at 280 nm was collected pH to 7-8 and reacted with punched chitosan (0.5024 cm^2) or punched paper. All data points are the average of three separate tests and error bars represent the standard deviation.

We confirmed that the fibers were still present before continuing with the peptide adsorption using FE-SEM. The result of undamaged and smooth fiber morphology is shown in the F**igure 6.9.**

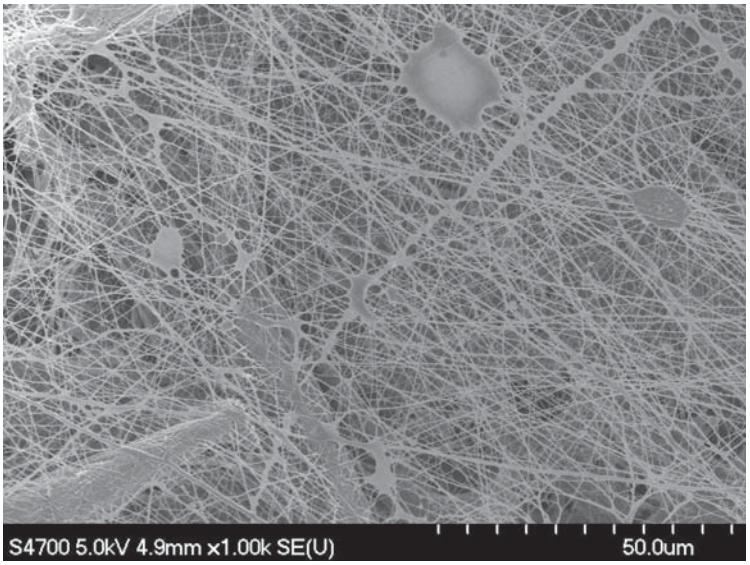

Figure 6.9 FE-SEM micrographs of electrospun chitosan/PEO after the treatment of chitosan with stable amine-reactive Sulfo-NHS ester at 1000×. 25 mM IAA, 25 mM EDC in MES buffer at pH 4-5 was reacted in the presence of 50 mM NHS. After 15 min reaction was quenched with 2- mercaptoethanol and desalted with PBS. The sample with maximum absorbance at 280 nm was collected pH to 7-8 and reacted with punched chitosan (0.5024 cm^2).

The conjugation of peptides on IAA-chitosan was performed by mixing with cysteine-terminal CWRW peptide**.** The reaction proceeds through substitution of carboxymethyl group (-CH_2-COOH) of IAA for the hydrogen of the thiol group on cysteine with a byproduct formation of HI. The reaction is shown in **Figure 6.10.**

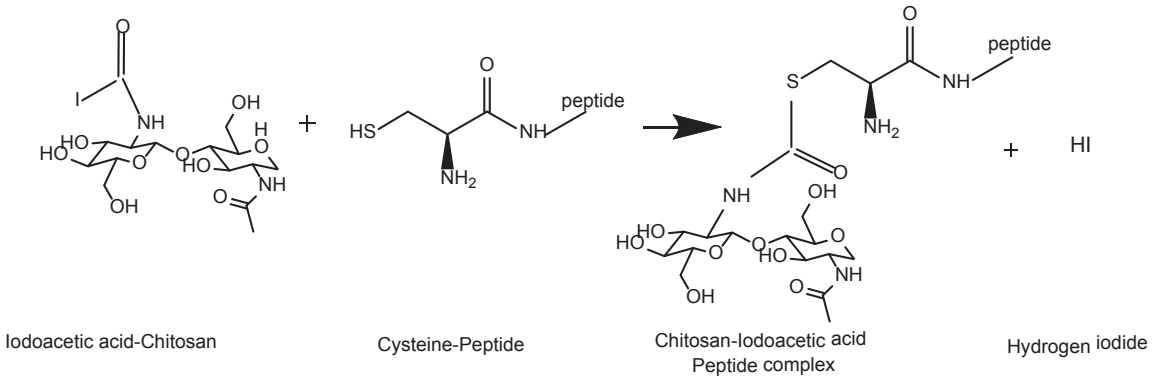

Figure 6.10 Conjugation of CWRW peptide to chitosan using IAA-EDC crosslinker. Weighed peptide was dissolved in 10 mM phosphate buffer (pH ~ 7).

Peptide was prepared in 10 mM phosphate buffer (pH ~ 7). The measurement of only 1 mg had the possibility of encountering measurement errors. This lead us to create a one-time calibration curve before proceeding with the peptide coupling to chitosan. The calibration graph will importantly be used for estimating the peptide adsorbed on the chitosan membranes by injecting aqueous sample in RP-HPLC. The CWRW peptide stock injection at 220 nm is shown in **Figure 6.11 (A)** and the calibration graph in **Figure 6.11 (C)**. The calibration was linear and so we continued to use RP-HPLC as a method to quantify the peptide.

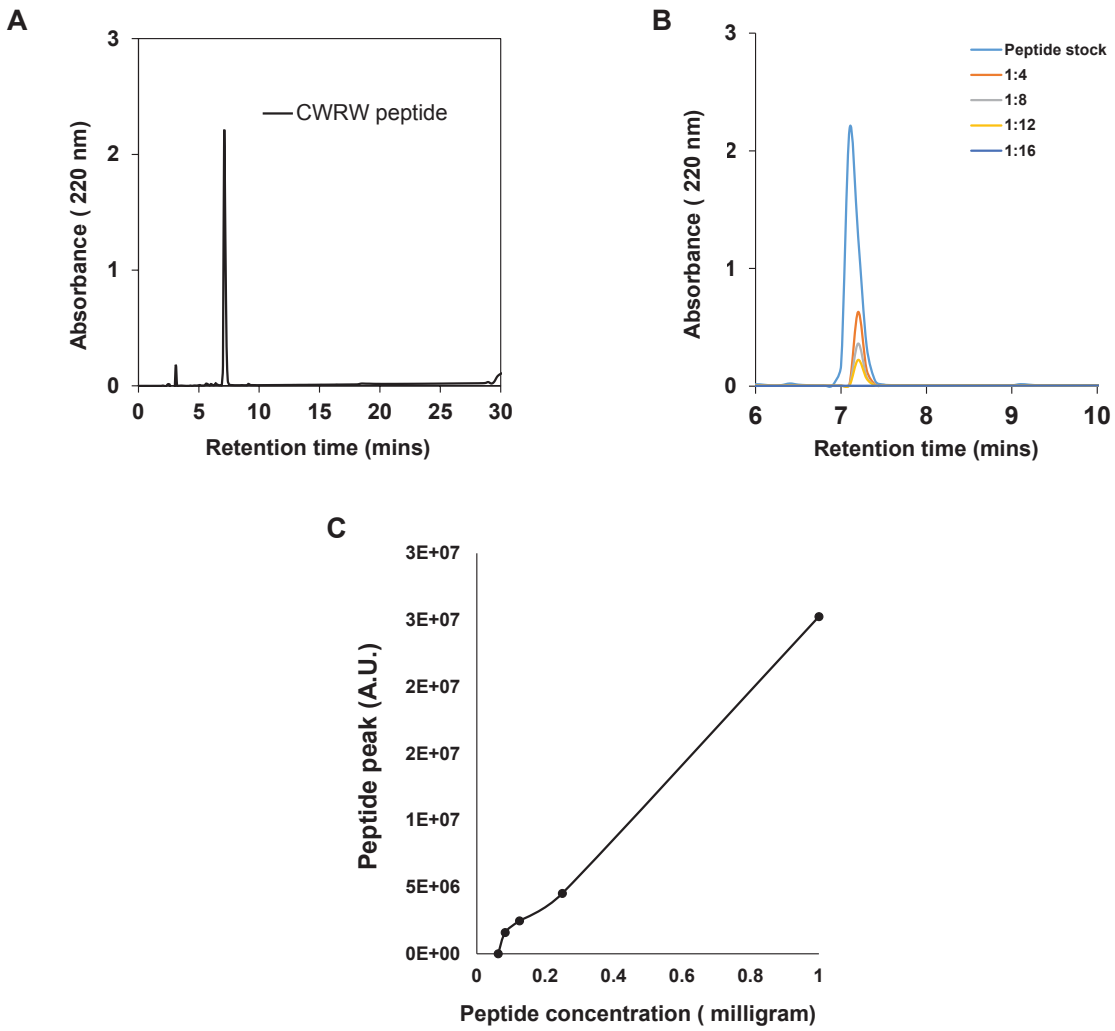

Figure 6.11 CWRW peptide peak and peptide calibration data. 1 mg of peptide in 1 ml of 10 mM phosphate buffer was prepared. (A) peptide injection at 220 nm (B) Samples expressed as peptide: buffer (a) 1, (b) 1:4, (c) 1:8, (d) 1:12, (e) 1:16 injected through C_{18} RP-HPLC and measured for peptide peaks at 220 nm and (C) plot for peptide peak area vs. peptide concentration to estimate the peptide coupled during chitosan reaction. The data points shown here are results from a single test only.

Approximately 1 mg of peptide was weighed, dissolved in buffer and treated with chitosan. After overnight contact with the chitosan, the peptide (aqueous) was injected and tested for peptide adsorption. The solution showed no reduction in peptide concentration after contact with chitosan (see **Figure 6.12**). The results from RP-HPLC along with a few inconsistent results during the ninhydrin test had not

provided satisfactory results. The pH for IAA-EDC carbodiimide reaction in the presence of Sulfo-NHS required a low pH 4-5 and the thiol substitution required a high pH 7-8. We hypothesized the change in pH between reactions were hindering the conjugation of EDC and the stability of iodo-coupled chitosan. However a clear understanding on the failure of this mechanism is yet to be fully understood.

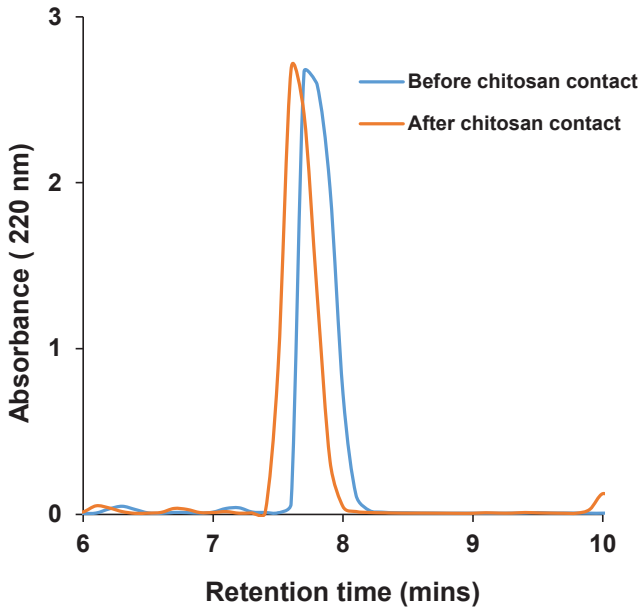

Figure 6.12 CWRW peptide after IAA-EDC reaction for peptide-chitosan complex. 25 µl injection of samples (a) peptide starting stock and (b) after chitosan contact using C_{18} RP-HPLC. The reaction was performed after reacting iodo coupled chitosan fibers with 1 mg peptide in 10 mM phosphate buffer (overnight rocking).

6.3.1.3 Method 3: Maleimide crosslinker

After no peptide was adsorbed on the chitosan through IAA-EDC chemistry, the maleimide chemistry from Sulfo-SMCC was investigated. The Sulfo-SMCC has a NHS ester at one end to conjugate with primary amine in pH 7-9 and maleimide group on other to conjugate with sulfhydryl group of cysteine available on the WRW

peptide around pH 6.5-7.5. It allowed us to operate the amine and thiol substitution at a pH 7 which was not available earlier technique. The chemistry is shown in **Figure 6.13.**

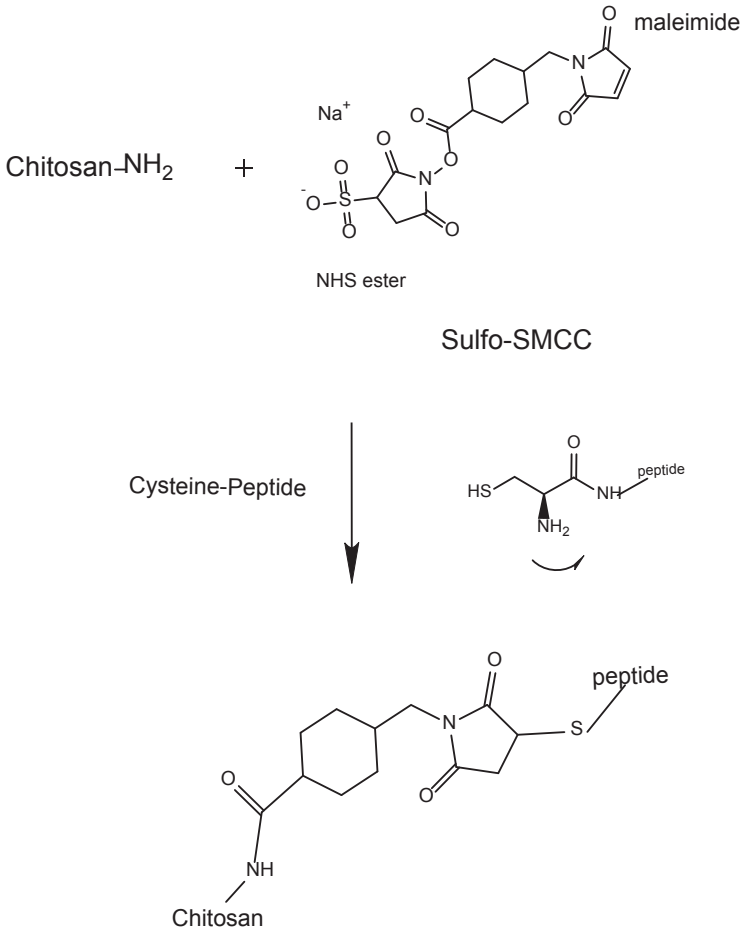

Figure 6.13 Peptide conjugation to the chitosan using Sulfo-SMCC (maleimide crosslinker chemistry). 4.6 mM of Sulfo-SMCC in 10 mM phosphate buffer was prepared (pH ~ 7) and treated with chitosan for 2 hours. The chitosan was treated with peptide in same buffer conditions for 3 hours.

The ninhydrin test was performed with a new bottle of reagent and always stored under inert nitrogen to prevent oxidation. The test was performed on two samples (1) chitosan with Sulfo-SMCC and, (2) chitosan with PBS (positive control). The result was promising showing less amines on Sulfo-SMCC treated chitosan (see **Figure**

6.14) compared to control. The reduced amines on chitosan treated sample was due to substitution of amine on chitosan with the NHS ester group on the linker. The FE-SEM showed undissolved fibers (**Figure 6.15**) and it was expected since the pH was maintained above 6.3 where the chitosan is soluble.

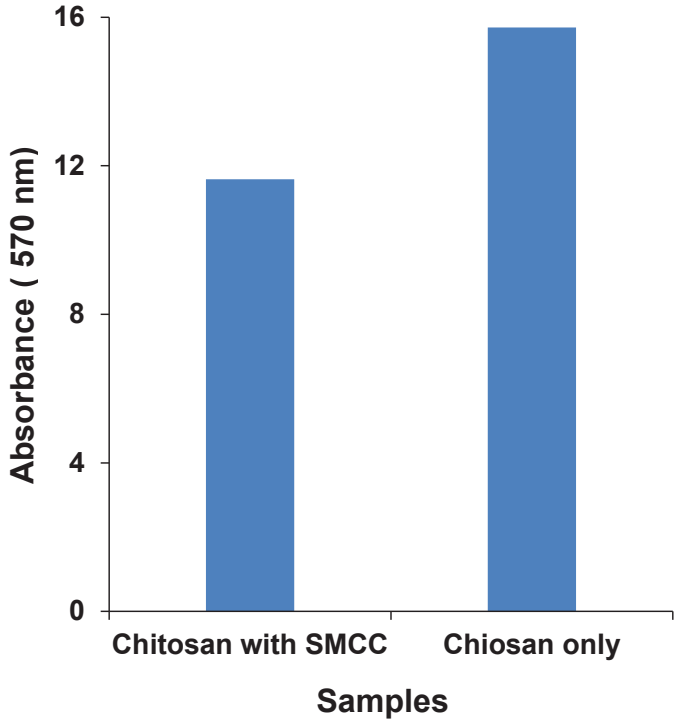

Figure 6.14 Ninhydrin test kit for maleimide. Determination of amines for samples (1) chitosan treated with Sulfo-SMCC, (2) chitosan treated with PBS only (positive control). 4.6 mM of Sulfo-SMCC in 10 mM phosphate buffer was prepared (pH ~ 7) and treated with chitosan for 2 hours. The data points shown are results from a single test only.

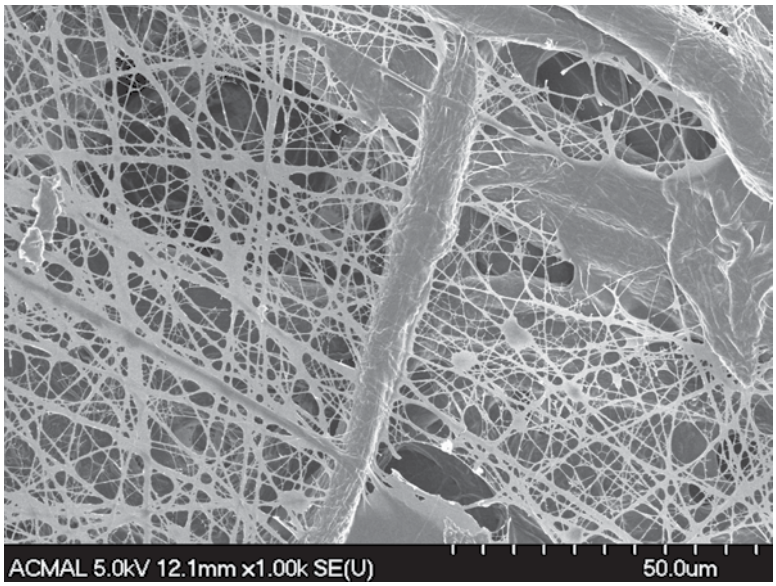

Figure 6.15 FE-SEM micrographs of electrospun chitosan/PEO after the treatment of chitosan with stable amine-reactive NHS ester. 4.6 mM of Sulfo-SMCC in 10 mM phosphate buffer was prepared (pH ~ 7) and treated with chitosan for 2 hours. The chitosan was treated with peptide in same buffer conditions for 3 hours.

About 0.4 mg of peptide was weighed and contacted with NHS-substituted chitosan for the still active available maleimide group to bind to the thiol on the cysteine of WRW peptide. After 2 hours the peptide solution was injected into RP-HPLC to confirm adsorption to chitosan. The data in **Figure 6.16** showed a 38% peak reduction which qualifies to 0.15 mg of peptide or 0.2 μmole. Our initial approximation of the amount of amines on the punched chitosan paper was 0.6 μmole / paper layer. The 0.2 μmole of peptide attaching to three layers here was definitely an encouraging result. The important challenge overcome during the peptide-chitosan conjugation was the narrow pH working range and the complexities of the multiple reaction stages without disturbing the stability of the fibers.

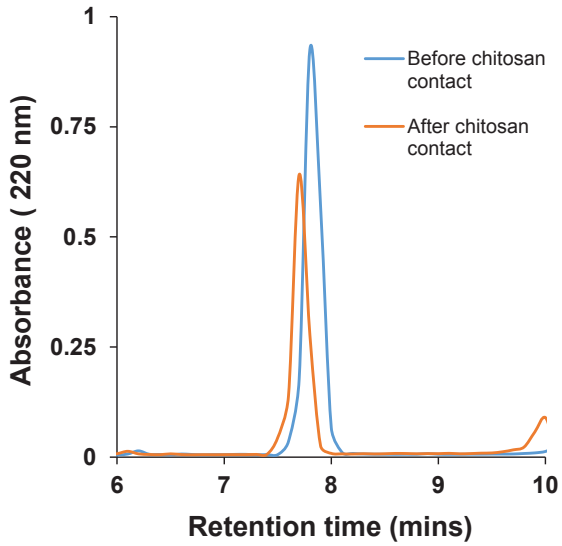

Figure 6.16 CWRW peptide after maleimide reaction for peptide-chitosan complex. 25 µl injection of samples (A) peptide starting stock and (B) after chitosan contact using C_{18} RP-HPLC. 0.4 mg of peptide was weighed and treated with maleimide active chitosan fibers in 10 mM phosphate buffer for 3 hours.

For virus removal we experimented with three layers of punched chitosan (0.5024 cm^2) which were contacted with either virus in 10 mM phosphate buffer or PBS (10 mM phosphate and 150 mM NaCl). The MTT assay results are shown in **Figure 6.17** (a) for 10 mM phosphate buffer and (b) PBS. The punched samples subjected to virus were (1) peptide-chitosan treated with Sulfo-SMCC, (2) peptide-chitosan treated in 10 mM phosphate buffer or PBS (control A), (3) chitosan treated with 10 mM phosphate buffer or PBS only (control B), and (4) virus MTT control. **Figure 6.17 (A)** shows 1-2 LRV for sample (1) for maleimide conjugated peptide in 10 mM phosphate buffer. The controls A and B for this experiment showed < 1 LRV. It can be safely accepted that the PPV removed from 10 mM phosphate buffer was primarily due to trimer WRW achieved from the conjugation chemistry. **Figure 6.17 (B)** were the experiments performed on PBS which showed no significant difference between all of the samples.

Virus particle adhesion on membranes can be caused due to electrostatic charge, hydrophobic bonds, ionic strength, aggregation and ion composition. PPV is negatively charged at neutral pH conditions due to its pI ~ 5 [51]. The membrane is positively charged due to the polar basic amino acid arginine (R) on WRW (tryptophan-arginine-tryptophan) and the unreacted primary amines ($-NH_3^+$) on chitosan. The amino acid structures of WRW is shown in **Figure 6.18.** When the virus and membranes are oppositely charged the electrostatic attraction exists between the two surfaces due to the electrical double layer effect bringing them close together. The peptide ligand WRW also has 2 non-polar aromatic amino acids in the form of W (tryptophan) that is capable of forming hydrophobic bonds with the hydrophobic PPV. Viruses and bacteriophages are generally considered hydrophobic in nature [46, 52-54]. We hypothesize that the virus is drawn close to the membrane matrix due to their differential charge and then the virus adsorbs on the peptide majorly due to hydrophobicity of W and some due to positive charge on R amino acid. Hence an electrostatic enriched hydrophobic effect [55] could cause virus attachment to the peptide in 10 mM phosphate buffer. The dynamic binding effect and virus removal seems to be absent when the buffer is changed to PBS. The high salt conditions of PBS can cause Na^+ ions to create an electrical double layer around the virus particles [55] leaving the virus boundary and the membrane surface with positive charges. The charge shielding effect will create an electrostatic repulsion of virus from the membrane surface causing reduced LRV as in the case of PPV removal in PBS < 1 LRV. An alternate hypothesis for low LRV in PBS could be aggregation behavior of PPV at higher salt conditions. SP and GA bacteriophage which are highly hydrophobic have aggregated at a wide pH range (1.5-7.5) and

ionic strength (1-100 mM $NaNO_3$) [54]. The aggregation from protein-protein interactions could cause conformational changes in virus structure or even reduce hydrophobic bonding strength with the aromatic ligand W leading to reduced sorption of PPV on membrane surface. The hypothesis of aggregation can be further tested by using a filter design with solution subjected through the filter membrane pores. In our study we have rocked the tube contents to observe binding interactions of virus with peptides. If the viruses are aggregating then the virus removal in filter design would be achieved by size exclusion which is not in our interest.

Virus removal of 4.5 – 5.5 LRV was achieved with trimer peptide WRW in PBS and blood plasma in the earlier investigation [46, 52] unlike 10 mM phosphate buffer in our project. The earlier study was performed on amino resin chromatographic column and not a membrane fiber matrix system as in our case. The peptide screening library for membrane fibers can be very different than column beads due to lack of diffusional limitation. Also the WRW peptide density in amino resin column was in the millimoles range [52] compared to μmoles in our approach

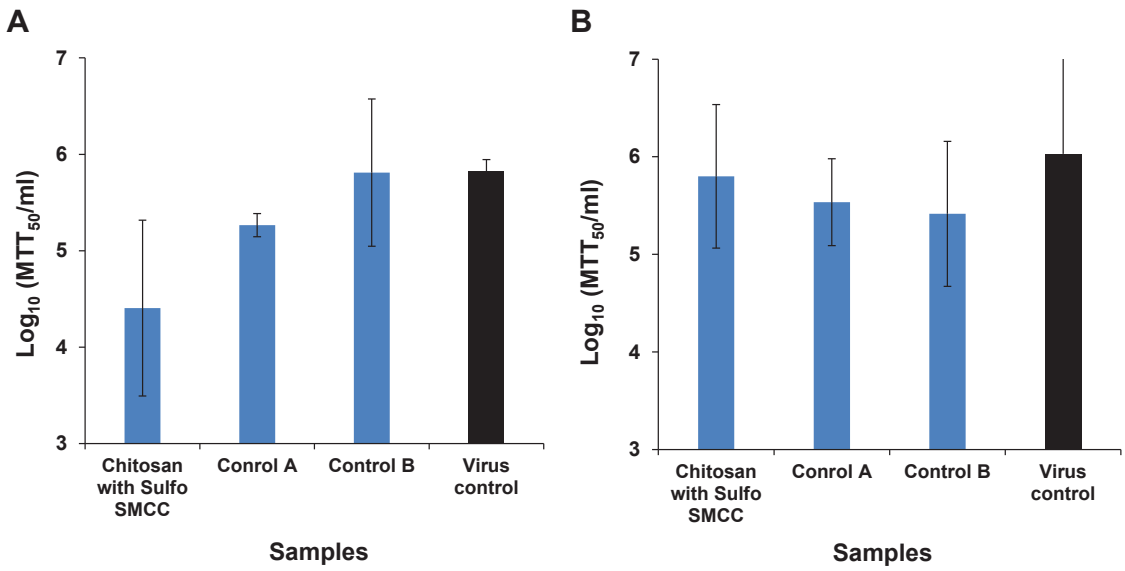

Figure 6.17 Virus removal assessment from peptide attached to chitosan using Sulfo- SMCC crosslinker. 6 $\log_{10}$ MTT_{50}/ml 500 μl virus solution in (A) 10 mM phosphate buffer (B) PBS was contacted with 3 layers of peptide conjugated chitosan (0.5024 cm^2) for 3 hours. The samples subjected to virus are shown in the x-axis (1) peptide-chitosan treated with Sulfo-SMCC, (2) peptide-chitosan treated in 10 mM phosphate buffer or PBS (control A), (3) chitosan treated with 10 mM phosphate buffer or PBS (control B), and (4) virus MTT control. The solution was spun in centrifuge at 5000 rpm for 3 min and supernatant was extracted for performing MTT assay. All data points are the average of two separate tests and error bars represent the standard deviation.

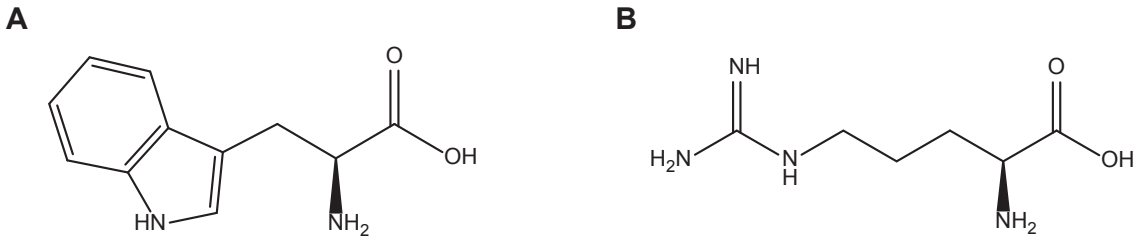

Figure 6.18 Tryptophan (W) and arginine (R) amino acids.

6.4 Conclusions

Membrane surface matrix was designed for virus removal using discovered trimer WRW. The trimer was able to remove up to nine chromatographic column volumes of PPV from PBS and human blood plasma [46, 52]. On a membrane

surface, the trimer was expected to bind and remove PPV more effectively since the column porous beads do not support large virus capture.

The crosslinking chemistry to adsorb peptides on chitosan nanofibers was investigated using the carbodiimide and maleimide chemistry. The peptide adsorption using carbodiimide chemistry was unsuccessful due to either lack of amine's ability from chitosan to conjugate to the iodine required for thiol substitution or the lack of stability of iodo-substituted chitosan in the presence of mercaptoethanol during the peptide reaction. Mercaptoethanol was added for stabilizing the carbodiimide reaction but we suspect in the case of incomplete reaction the mercaptoethanol could have deactivated the iodo group. The maleimide chemistry was able to attach peptides and remove virus in 10 mM phosphate buffer. The peptides showed 1-2 LRV which is equivalent to 90-99% of virus. Higher salt concentration of 10 mM phosphate and 150 mM NaCl lead to almost no LRV. For 10 mM phosphate buffer we hypothesize the electrostatic attraction between virus negative surface and membrane positive surface brings them close together. This is followed by hydrophobic bond between the aromatic amino acids of CWRW ligand and hydrophobic patches on the PPV. This electrostatic enriched hydrophobic binding pattern between the virus and membrane helped achieve a 1-2 LRV (90-99%).

Nevertheless 1-2 LRV (or 90-99%) virus removal using membrane system is a promising start. These values can be improved to EPA standards of 4 LRV (99.99%) by increasing ligand density to increase hydrophobic strength and including spacer arm for peptides to inhibit steric hindrance effect from membrane surface matrix. In addition a study on virus removal studies for different salt

strengths may provide further understanding to improve the binding effect between the virus and membrane.

6.5 References

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Chapter 7

Conclusions and recommendations

7.1 Conclusions

This work began in Chapter 3 on the purification of PPV with chromatography. IEC with Q-sepharose resin was able to purify PPV from media proteins on a small scale and large scale column. We were able to use the IEC purified virus to aid in the IHC work on the action of antiviral compounds. The purity of the PPV from media proteins could not be confirmed using the gold standard of SDS-PAGE due to similar MW of VP2 capsid protein and BSA. Virus purification was also investigated on SEC using sephacryl resin. The SEC was unable separate the PPV from BSA since both were eluting almost together with a very short time window.

Also in Chapter 3, we examined the surface properties of PPV. Understanding the hydrophobicity of a biomolecule can help in developing virus separation strategies. The presence of hydrophobic residues on PPV was found to be more than panel of proteins (insulin, lysozyme, BSA, fibrinogen, hemoglobin) by using a C18 RP-HPLC and evaluating on the basis of acetonitrile elution strength. To further prove the hypothesis that virus surface are more hydrophobic than proteins, ANS fluorescence was employed. PPV gave false reading due to the presence of media and cell proteins that were procured from the virus production process.

In Chapter 4, we examined single virus trafficking and the exploration of virus capsid formation by IHC. Single virus trafficking was studied by tagging the PPV with Alexa fluor 488 succinimidyl ester and observing on the virus in a time-based study. The multiple pathways of virus trafficking through cells made it very difficult to account for steady virus progress. Though trafficking studies were not successful, the protocol designed was used for studying the action of antiviral drugs using immunohistochemistry. TMAO and glycine at 0.20 M showed 4 LRV or 99.99 %

reduction in PPV infectivity. IHC was used to study the mechanism of action of osmolytes using a time-based study. IHC showed that despite the 4 LRV reduction the fluorescent capsid proteins were still produced at time 8, 12, 16 and 20 hours similar to PPV positive control. Hence we hypothesize the osmolytes are stabilizing the VP2 capsid proteins and preventing the virus assembly process.

In Chapter 5, PPV was purified using an aqueous two-phase system (ATPS). A 15 w/w% PEG 12K and 14 w/w% citrate at pH 8 was able to recover 64% infectious virus recovery in the PEG-rich phase of the system [1]. Citrate was chosen since it was proven to be a stronger hydrating salt, thus creating a large hydrophobic difference between the two phases. The reason for high virus recovery was electrostatic potential causing the negatively charged virus to move away from the salt-rich into the PEG-rich phase. The hydrophobic interaction between the virus residues and PEG-rich phase from high PEG MW allowed increased partitioning. The surface tension from increasing salt tends to cause the virus to be drawn into the interface, which was undesirable. Hence it was important to maintain a balance of high hydrophobicity and electrostatic potential to prevent from virus residing at interface. The major media protein, bovine serum albumin (BSA), was separated into the salt-rich phase. C_{18} RP-HPLC was used to validate the concentration and partitioning of BSA into the salt-rich phase.

Finally, in Chapter 6, membrane filters were designed for achieving virus removal from potable water. The membranes were created by conjugating CWRW peptides to electrospun chitosan nanofibers. The conjugation of terminal amines on chitosan to the cysteine residue on the CWRW peptide was obtained using maleimide chemistry. Upon subjecting the filters to virus in 10 mM phosphate buffer, we

obtained 1-2 LRV or 90-99% virus removal. When PBS was used as a buffer we obtained almost no virus removal. We hypothesize that the virus was attracted to the membrane because of positive charge on residual primary amines of chitosan and the R amino acid. This was followed up with hydrophobic interaction between the W amino acid and hydrophobic PPV [2, 3].

7.2 Recommendations

The work has some interesting insights in improving the virus recovery for aqueous two-phase system and synthesizing ligands on electrospun chitosan for virus removal from water. In ATPS, the virus recovery was increased to 64% in the PEG-rich phase by controlling pH, surface tension, hydrophobicity and electrostatic potential. Also we showed that the removal of the major protein contaminant BSA. For virus removal, we were able to show 90-99% virus removal from water by conjugating the peptide with maleimide chemistry and using 3 layers of 0.5024 cm^2 chitosan paper circles. Based on these conclusions we can make future recommendations for improving the yield and purity for ATPS. For membrane filtration we can make suggestions for improving the virus removal from CWRW and experimenting on CYKLKYY peptides for removal.

7.2.1 Improving recovery on ATPS

ATPS experiments were performed for virus produced from fetal bovine animal serum. However the current vaccine manufacturing trend is to use serum-free media and avoid adventitious agents [4]. Now that we have shown removal of BSA from the PEG-rich phase, we could next investigate to remove the host cell DNA to the FDA limit of 10 ng/dose [5, 6]. In our study, we still need to explore back-extraction of the

virus from the PEG-rich phase into the salt-rich phase and make it available for final use. It is possible that the PPV is encapsulated in the PEG molecules and understanding the PEG-virus coat will help in the designing of the recovery into salt-phase. Our initial back-extraction of virus has shown 70-90 % recovery at low pH 5 and there is a great potential for improving the value.

7.2.2 Higher virus removal on affinity membrane substrate

The EPA requires treatment technology which can reliably achieve 99.99% or 4 LRV inactivation or removal of viruses [7, 8]. We are currently achieving 90-99% or 1-2 LRV in 10 mM phosphate buffer. The virus removal can be increased by increasing peptide concentration. The current results were produced with only 0.2 µmole of peptide attached to the membrane. Peptide WRW has demonstrated best virus removal ability in the presence of ethylene oxide spacer arm [9]. The spacer arm can facilitate increased contact between the ligand and PPV. The other peptide that has shown virus removal is the YKLKYY. Molecular docking study had shown the secondary structure of YKLKYY allowing the PPV to bind to the peptide [3]. It would be interesting to investigate the virus removal using CYKLKYY.

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Permission Figure 6.1

Sample Permission Letter for Use of Previously Copyrighted Material

November 11, 2014

Ms. Xue Mi
Haidian Disctrict
Beijing, China

Dear Xue,

I am completing a doctoral dissertation at Michigan Technological University entitled "Virus purification, detection and removal." I would like your permission to reprint in my dissertation excerpts from the following:

Mi, Xue, "Electrospin Quaternized Chitosan Fibers for Virus Removal from Drinking Water", Master's Thesis, Michigan Technological University, 2013.

The excerpts to be reproduced are **Figure 2.3 Schematic of a typical electrospinning laboratory setup.**

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If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope or via email. Thank you very much.

Sincerely,
Khrupa Saagar Vijayaragavan

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Name: Xue Mi

Sign: [redacted]

Date: Nov. 12, 2014

Permission from Novo-Nordisk company to use their Insulin samples for experimentation

---------- Forwarded message ----------
From: **AST (Arne Staby)** <ast@novonordisk.com>
Date: Mon, Nov 3, 2014 at 8:25 PM
Subject: RE: Chapter submission to Novo-Nordisk for permission
To: Caryn Heldt <heldt@mtu.edu>

Hi Caryn,

Yes, it was great meeting you.

With respect to the paper, I have no comments and you are welcome to publish it – please wish your student good luck with his dissertation (a small comment though for page 93 – the weight of monomeric insulin is around 6 kDa, not 12…).

Best regards, Arne

From: Caryn Heldt [mailto:heldt@mtu.edu]
Sent: 3. november 2014 15:02
To: AST (Arne Staby)
Subject: Fwd: Chapter submission to Novo-Nordisk for permission

Dear Arne,

It was great to finally meet you at the Recovery Conference this summer. I have a student who is submitting his final dissertation and has worked with your insulin. I would like your permission to publish this work, please see attached. It is the same work I have sent to you before, just in a different form. He has highlighted where the insulin in discussed. Not shown is that he also thanks NovoNordisk in the acknowledgement section of the dissertation.

Thank you for your time,

Caryn

Caryn Heldt

Michigan Technological