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CONTINUOUS VIRAL VACCINE MANUFACTURING AND VIRAL DETECTION
STRATEGIES

By

Dylan Gregory Turpeinen

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Chemical Engineering

MICHIGAN TECHNOLOGICAL UNIVERSITY

2020

This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Chemical Engineering.

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Preface

The work contained in this dissertation was conducted in the department of Chemical Engineering at Michigan Technological University from May 2016 to February 2020. This dissertation was written by Dylan Gregory Turpeinen. All experiments and measurements discussed within this dissertation were performed by Dylan Gregory Turpeinen with the assistance of collaborators who are listed in the following paragraphs.

Chapter 3 discusses the results of analyte detection using printed graphene biosensors. All the sheet resistivity results from printing the graphene ink onto paper and non-paper substrates were obtained by myself in Dr. Julie King's laboratory. I received help in changing ink properties such as ink viscosity and number of printing coats by Clara Peterson (UG). I custom-made different graphene ink during an internship with XG Sciences under Dr. Hiroyuki Fukushima and Thomas Ritch. Design, fabrication, and testing of antibody-functionalized graphene sensors was performed by myself under the direction of Dr. Caryn Heldt. 3-D printing wax barriers onto the graphene paper sheets was performed by Matthew Weiss (UG) and Cameron Miller (UG) under my supervision. During development and testing of the sensors, the work was presented in poster format at the 2017 American Institute of Chemical Engineers (AIChE) national meeting in Minneapolis, MN. The work discussed in Chapter 3 will not be published as no significant, positive results were obtained for analyte detection.

Chapter 4 expands upon the previously published work using gold nanoparticles (AuNPs) for virus detection. From Dr. Xue Mi's AuNP/virus assay, we were able to detect porcine parvovirus (PPV) and bovine viral diarrhea virus (BVDV) using unmodified 15 nm AuNPs aggregated by mannitol. Ellie Lucier (UG) had significant contribution to the work discussed in Chapter 4, from running the experimental assay and collecting dynamic light scattering (DLS) results for my analysis. I learned how to synthesize and characterize various shapes and sizes of AuNPs while at the National University of Singapore working in Dr. James Chen Yong Kah's lab in collaboration with Nural 'Ain Azman (Ph.D. candidate) and Malvin Wei Cherng Kang (Ph.D. candidate). The results obtained during development of the virus detection assay was presented at the 2019 American Chemistry Society (ACS) national meeting in oral presentation format in

Orlando, FL, and at the 2019 AIChE national meeting in poster format in Orlando, FL. The results obtained and discussed in Chapter 4 showed no improvement to limits of detection of PPV and BVDV than the previously tested 15 nm AuNPs, and will be published.

Chapter 5 is about the design and testing of a continuous virus purification technique based on an aqueous two-phase system (ATPS). Using Pratik Joshi's (Ph.D. candidate) batch-mode ATPS work to find an optimized ATPS for purification of PPV, I designed and tested the possibility of the ATPS to be ran continuously without loss in purification performance. Pratik trained me in forming ATPSs and how to determine the composition of the phases using phase diagrams he generated for the PEG/citrate system. With help from Erin Browne (UG), we constructed a continuous ATPS using a static in-line mixer and tubular separator to achieve mixing and separation of the phases. Human immunodeficiency virus type 1 group antigen labelled with green fluorescent protein virus-like particles (HIV VLPs) were donated by Dr. David O'Hagan and Dr. Savita Nikam from Esperovax for testing the continuous purification system. The COMSOL Multiphysics modeling of the helical mixer was performed and analyzed by Dr. Hassan Masoud from the Mechanical Engineering Department at Michigan Technological University. Results obtained during the development of the ATPS process was presented in poster format at the 2019 ACS national meeting in Orlando, FL. The results contained in Chapter 5 are also currently in preparation for submission to the Separation and Purification Technology journal.

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To LiLu, the most caring fiancée I could ever ask for, words are not enough. To my mother for her unconditional love and support. To Danielle, Paige, Rich, and Tyler for bringing fun times and laughter. To Kate and Jack, who I hope will seek scientific and logical answers to questions they have throughout their lives. I love each and every one of you undoubtedly.

Abstract

Viruses are responsible for many human diseases that lead to suffering and death. Acquired immunodeficiency syndrome (AIDS), influenza, and coronavirus disease 2019 (COVID-19) have claimed the lives of millions of people. Two methods have been created to reduce the suffering caused by these diseases. The first is the manufacturing of vaccines that prevent disease and the second is the development of virus detection tests that lead to treatment. The work summarized in this dissertation discusses the development of a continuous virus purification process using an aqueous two-phase system for use in vaccine manufacturing. Additionally, two novel virus detection methods were developed using graphene biosensors and a gold nanoparticle aggregation assay.

This dissertation contains results obtained from a novel continuous viral particle purification technique. The biomanufacturing industry would greatly benefit by switching from traditional batch processes to continuous processing. The reduction in costs and increase in productivity of continuous unit operations are driving the desire for continuous biomanufacturing processes. Switching to continuous processing would eliminate the current downstream processing challenges of viral-based biotherapeutics including limited yield and throughput. The method developed here was a chromatography-free separation technique aimed to increase throughput, purity, and yield of viral particles. The technique utilized an aqueous two-phase system (ATPS) to partition viral particles to a different phase than contaminant proteins. The ATPS consisted of poly(ethylene glycol) (PEG) and sodium citrate to separate viral particles from contaminant proteins from cell culture media. The ATPS was scaled-up from 1 g batch systems and run continuously for porcine parvovirus (PPV) and human immunodeficiency virus-like particles (HIV VLPs). Results from the purification of both viruses showed >70% recovery with a majority of the contaminating proteins and DNA being removed. This system has the potential to be further scaled-up for full-scale continuous biomanufacturing of vaccines.

This dissertation also contains results from the development of two viral detection methods. The first detection method used a graphene ink-based paper biosensor to

electrochemically detect the presence of biomolecules. These graphene biosensors were created with the goal of being lab-on-a-chip devices for rapid detection in low-resource areas without the need of expensive laboratory equipment. The biosensors initially showed promise for portable, inexpensive, and rapid detection of proteins via electrical resistance changes at the sensor surface, but ultimately could not be applied to virus particles. The second detection test utilized osmolyte-induced gold nanoparticle (AuNP)/virus complex aggregation to detect whole viral particles. The AuNP aggregation assay was previously developed to detect two model viruses, PPV and bovine viral diarrhea virus (BVDV), using one size of AuNPs. The AuNP assay was capable of nanomolar level detection of both viruses. Optimization of this previously established virus detection method was performed by utilizing various sizes of AuNPs to lower the limit of detection (LOD). Results from using other sizes of AuNPs showed no improvement to the LOD.

1 Introduction and chapter summaries

1.1 Introduction

Viruses are difficult-to-detect microorganisms capable of infecting living cells and causing numerous communicable diseases. Communicable diseases caused by a wide variety of viruses claim the lives of millions of people each year around the world. Every year, an estimated one billion people get sick and 290,000-650,000 people die due to influenza [1]. Acquired immunodeficiency syndrome (AIDS) resulted in 770,000 deaths and 1.7 million new cases in 2018 [2]. The novel coronavirus causing coronavirus disease 2019 (COVID-19) has already claimed the lives of 500,000 people, with 10 million cases worldwide [3]. These communicable viral diseases and nearly 20 others are responsible for extreme human suffering and death. However, with either prophylactic or early treatment, these harmful effects can be decreased. To have as early treatment as possible, rapid virus detection techniques are necessary. A non-specific virus detection method would be helpful in confirming the presence of any disease-causing virus without the need to test for individual viruses with time-consuming multiple tests. In addition to detecting a disease as early as possible, vaccination remains as one of the best methods of preventing deaths caused by infectious diseases [4]. However, the challenge of developing early detection tests and manufacturing large quantities of vaccines still exists. To help in reducing human suffering and death caused by diseases, my research shown in the dissertation focused on developing two novel virus detection methods and one vaccine manufacturing method.

The work contained in this dissertation explored two different methods for virus detection. The first method utilized graphene/cellulose biosensors to electrochemically detect the presence of biomolecules on the sensor surface. The graphene biosensor initially showed promise for portable, inexpensive, and rapid detection of proteins via electrical resistance changes at the sensor surface, but ultimately could not be applied to virus particles. The second method utilized osmolyte-induced gold nanoparticle (AuNP)/virus complex aggregation to detect viral particles. The AuNP aggregation assay was previously developed to detect two model viruses, porcine parvovirus (PPV) and

bovine viral diarrhea virus (BVDV), using one size of AuNPs. The AuNP assay was capable of nanomolar level detection of both viruses, which is significantly more sensitive than standard commercially available ELISA kits. Optimization of this previously established virus detection assay was attempted by utilizing various sizes of AuNPs to lower the limit of detection of the assay.

This dissertation also contains results from developing a novel virus purification technique that has the potential to be used in large scale manufacturing of vaccines. To create a safe and effective vaccine, the viral product during downstream processing must be purified from contaminants while maintaining high titer. To aid in supplying the world with critical vaccines, the recovery of viral products from downstream processes must also be increase beyond the currently accepted 30% [5]. The developed virus purification method utilizes an aqueous two-phase system (ATPS) of poly(ethylene glycol) and sodium citrate to partition viral particles to a different phase than contaminant proteins with high recovery (>70%) and purity. The ATPS was scaled-up and run continuously for PPV and human immunodeficiency virus-like particles (HIV VLPs). This system has the potential to be further scaled-up for full-scale continuous biomanufacturing of vaccines.

1.2 Chapter summaries

Chapter 2 of this dissertation begins with a literature review of current analyte detection strategies and vaccine manufacturing processes. Each section discusses conventional methods that have their limitations, and current research aimed at breaking through those limitations. The graphene biosensor literature review focuses on the different electrochemical detection techniques, and the advantages and disadvantages of each technique used to detect proteins and viruses. The gold nanoparticle literature review focuses on how functionalized particles have been used for virus detection, and how attempts have been made to use more simple, non-functionalized particle systems. The virus purification literature review focuses on the traditional batch processes used to purify viruses for use in vaccines, and the development of semi-continuous processes.

Chapter 3 discusses the graphene biosensors project aimed at designing and testing an inexpensive paper-based sensor for the electrochemical detection of analytes at

the sensor surface. The ultimate goal of the graphene biosensors was to create a point-of-care device for the detection of a malaria biomarker, plasmodium falciparum topoisomerase I (pfTopI). The biosensors were created from a sheet of blended cellulose/graphene nanoplatelets from XG Sciences. From previous experiments performed and published in 2013, it was shown that an increase in the protein concentration present on the sensor surface caused an increase in electrical resistivity due to an altered electron flow path. This increase in resistivity was also found to be dependent on the protein's molecular weight. In 2012, XG Sciences' manufacturing process of the graphene/cellulose sheets was changed, which caused a project shift to flexographic printing of graphene ink onto various paper and non-paper substrates. Printing the graphene ink onto various substrates allowed us to tune printing parameters such as ink viscosity, number of coats, and ink composition to achieve conditions for analyte detection during testing. Due to the new biosensor creation method, protein detection on the surface of the graphene-ink printed biosensors needed to be confirmed before application to virus particle detection. XG Sciences' standard graphene nanoplatelet ink was printed on common copier paper and on qualitative chromatography filter paper to attempt to mimic the cellulose/graphene biosensors previously created. The graphene ink was also printed onto highly hydrophobic poly(ethylene terephthalate) (PET) and various grades of Tyvek. An additional optimization strategy was altering the composition of the ink to various grades of graphene nanoplatelets to induce different resistivity changes as proteins were applied. The graphene-ink printed biosensors were tested for their ability to detect the presence of proteins in a buffer solution compared to the buffer solution alone as a control. Although numerous combinations of graphene ink types and printing substrates were tested, no combination led to a statistically significant difference in resistivity change when the target protein was applied to the sensor surface, compared to when protein-free solutions were applied. To enhance the electrochemical signal due to analyte presence, the graphene surface was functionalized with antibodies to specifically bind antigens to the sensor surface. Testing the functionalized graphene sensors resulted in the same conclusion that antigen presence could not be distinguished from buffer-only application. Although the number of tests performed covered a large

experimental area, there could exist a combination of parameters not tested which would lead to accurate detection of analytes. As another possible system for analyte detection, a gold nanoparticle virus detection assay was developed and tested in the Heldt lab.

Chapter 4 discusses the gold nanoparticle (AuNP)-virus assay to detect two different viruses at the lowest concentration possible. The goal of the AuNP-virus assay was to find an optimal size ratio between AuNPs and virus particles to enhance electrostatic interactions for binding and signal enhancement using dynamic light scattering (DLS). With an optimal size ratio determined, the assay could be applied to practical applications such as rapid surface cleanliness tests for hospital rooms or equipment. This assay is possible due to the unique ability of osmolytes (mannitol and glycine) to preferentially aggregate virus particles, by decreasing the hydration layer around the virus while leaving proteins un-aggregated in solution. Unmodified, citrate-capped AuNPs are electrostatically bound to the positive patches present on virus surfaces and aggregated in the presence of mannitol and glycine. By performing serial dilutions of the non-enveloped porcine parvovirus (PPV), the limit of detection was 10^6 MTT₅₀/mL [6]. To test the non-specific nature of the assay, a larger enveloped bovine viral diarrhea virus (BVDV) was detected to a limit of 10^4 MTT₅₀/mL [6]. Both of these virus detection tests were performed alongside a control test of various concentrations of a model bovine serum albumin (BSA) protein, which showed no ability to be detected. This AuNP-virus assay demonstrates the ability to detect virus particles at lower concentrations than conventional immunodetection methods with unmodified, nonfunctionalized AuNPs. In an attempt to improve the limit of detection of the AuNP/virus assay, AuNPs of different diameters were synthesized and applied in the assay to find an optimal ratio of diameters between the virus particles and AuNPs.

Chapter 5 discusses the continuous aqueous two-phase system (ATPS) used to purify and recover virus particles directly from crude cell lysate supernatant. An ATPS is a type of liquid-liquid extraction technique; which is formed by mixing two components above critical concentrations, where they separate into two distinct phases over time. The ATPS used here was a mixture of poly(ethylene glycol) (PEG) and trisodium citrate. This project was the developmental beginning of a continuous ATPS. The future goal of the

continuous ATPS will be to scale up and run the process for up to a week to purify various novel vaccine candidates. Batch-mode ATPS experiments performed in the Heldt lab showed $\geq 80\%$ of virus particles can be recovered in a different phase than contaminant proteins [7]. Using the batch-mode results, a bench-scale continuous process was designed and tested for the ability to recover and purify PPV in the PEG-rich phase. The continuous process was optimized by designing and evaluating a mixer capable of mixing the viscous PEG, citrate, and virus streams to allow for proper partitioning of virus particles to the PEG-rich phase and proteins to the citrate-rich phase. Proper mixing and phase separation were confirmed for the various mixers tested by comparing batch-mode and continuous-mode physical property measurements of the PEG-rich and citrate-rich phases after separation to confirm proper ATPS formation. PPV and a human immunodeficiency gag-GFP virus-like particle (HIV gag-GFP VLP) were tested in the continuous ATPS for the ability to recover and purify the viral product in the PEG-rich phase with contaminating proteins in the citrate-rich phase. More than 70% of the viral product was recovered in the PEG-rich phase with high protein purity visualized with SDS-PAGE gels and more than 70% DNA removal. These results indicate that continuous ATPS could be used as a continuous downstream purification method.

Chapter 6 concludes the dissertation with key findings from each project and provides future work theories for further development.

2 Literature review

2.1 Analyte detection systems

2.1.1 Current virus detection methods

Currently, there are three main ways viruses are detected. The first, and oldest, way is to grow host cells via conventional cell culture methods and infect the cells with a virus-containing sample to confirm cell death and presence of a virus [8-11]. The second, and most common today, is the method of polymerase chain reaction (PCR) where specific nucleic acid sequences from the virus are amplified and detected [12]. The third, also common, method is using enzyme-linked immunosorbent assays (ELISAs) where a system of antibodies is used to capture and detect a specific virus [13]. Virus detection methods using biosensors are being developed and have promise of becoming more clinically relevant as they are less expensive, faster, and can be used at the point-of-care [14].

2.1.1.1 Cell culture

Cell culture for virus detection has been regarded as the gold standard method since the beginning of clinical diagnosis of viral diseases [15]. The earliest use of laboratory cell culture was used for the detection of influenza virus using various cell lines including Vero cells, primary epithelial cells of human adenoid, primary rhesus monkey kidney (PMK) cells, and Madin-Darby canine kidney (MDCK) cells [9, 10]. The most conventional method of virus detection is growing host cells, infecting them with virus-containing samples, waiting for virus propagation, and checking for any cytopathic effect (CPE) within the cells. This method was used to detect influenza virus from various specimen locations including the lungs and throats of patients [10]. This traditional technique was also used to detect the presence of human adenovirus in a 5-7 day cell culture in HEp-2 cells [16]. Although the cell culture methods are well understood and has been used for a long time, the major drawback is the amount of time it takes to perform. This length of time between detection and treatment could mean the

difference between life and death for the patient. Another drawback to this technique is the variability in cell lines from lab to lab, which makes it difficult for standardization around the world [17].

2.1.1.2 Polymerase chain reaction

PCR is a type of nucleic acid test (NAT) that is used to make millions of copies of a specific sequence of nucleic acid in order to increase the copy number for detection [12]. Once the nucleic acid of interest is replicated to a certain level, the detection is achieved by co-amplification of fluorescence or luminescent dyes [18]. It is widely used today to detect numerous infectious agents, including up to six different infectious diseases for blood donations in the United States [19, 20]. The PCR technique has been used for the rapid detection of yellow fever virus in under three hours including sample preparation, which is a significant improvement on the five or more days necessary for detection using traditional cell culture methods [21]. PCR has also been used to bring the detection time of influenza A virus strain H3N2 down to approximately three hours, which is much faster than the minimum three days necessary for traditional cell culture techniques [22]. Most recently, PCR has been critical for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); however, several hours are still needed to prepare and test the samples [23]. While PCR has an advantage of decreased detection time compared to traditional cell culture methods, PCR requires skilled laboratory technicians and is considerably expensive for the necessary reagents [18].

2.1.1.3 Enzyme-linked immunosorbent assays

The use of ELISAs has also helped replace the time-consuming traditional virus detection techniques. ELISAs are plate-based assays in which immobilized antibodies are used to capture antigens (viruses) of interest, and secondary, enzyme- or fluorescent-linked antibodies are used for detection. Detection is achieved when a substrate is added to the enzyme-linked antibody to form a measurable product, or fluorescence is measured from fluorescent-linked antibodies [24]. In addition to NATs, ELISAs are used for blood donation screening for up to seven different infectious diseases in the United States,

including Hepatitis B and C, human immunodeficiency virus (HIV), and Zika virus [19, 20]. In the lab, an ELISA was used to detect H5 hemagglutinin from avian influenza virus in as little as 31 minutes, which is much faster than the days needed for traditional cell culture methods and faster than a commercially-available ELISA kit of 260 minutes – without loss of detection at just 0.23 ng/mL [25]. Although more rapid than traditional cell culture techniques, ELISAs require complex sample preparations, skilled laboratory personnel, and monoclonal antibodies used in the kits are expensive [18]. An ideal virus detection technique would be as rapid as possible, sensitive, inexpensive, and easy to perform.

2.1.2 Electrochemical biosensors

Biosensors is a broad term used to describe devices that combine a biological component with physicochemical detectors to detect an analyte of interest [26]. Biosensors are attractive alternatives to the current virus detection methods because they have the potential to simultaneously possess all the characteristics of an ideal detection system. Biosensors have three main components, a biological receptor, a transducer, and a detector, shown in **Figure 2.1** [27, 28]. The biological receptor in the case of viral biosensors can be enzymes, antibodies, or nucleic acid that sense the presence of an analyte, and the transducer transforms the molecular interactions between the receptor and analyte to a quantifiable signal measured by the detector [17, 27]. Three main categories of biosensors exist: electrochemical, piezoelectric, and optical [17, 27]. This review will focus on electrochemical biosensors for virus detection applications.

Figure 2.1: Main components of an electrochemical biosensor [28]

2.1.2.1 Types of electrochemical biosensors

Electrochemical biosensors are the most common and longest-used type of biosensors to date [29, 30]. Three common groups of electrochemical biosensors exist, differing by the electrochemical property being measured: amperometric/voltammetric [31-34], potentiometric [35, 36], and impedimetric/conductometric [37-41]. Each of these have been explored for their virus detection ability using various biological receptors including oligonucleotides, cells, enzymes, and antibodies – discussed later in section 2.1.2.2.

2.1.2.1.1 Amperometric/voltammetric

Within the amperometric category exists two subtypes of measurement strategies: amperometry and voltammetry. Amperometry is when the current changes are measured at a constant voltage, and voltammetry is when the current changes are measured with controlled changes in voltage [28]. The second measurement strategy is more commonly known as cyclic voltammetry. Amperometric biosensors work by measuring the current change from the oxidation or reduction (redox) of an electroactive species and sensing element which is linearly dependent on the analyte concentration applied [42]. Regardless of the measurement strategy (amperometry or voltammetry), the resulting signal from the

redox reaction of electroactive species in a biochemical reaction being monitored is correlated to the analyte concentration [29].

For a redox reaction occur, mediated or non-mediated pathways exist for electron transfer between the electroactive species and the sensing element. A mediator is an artificial electron transferring agent which facilitates the electron transfer between the analyte and electrode [30]. Commonly used mediators include ferrocene and its derivatives [43-45], methylene blue [46, 47], and N-methyl phenazine [48-50]. One graphitized mesoporous carbon-methylene blue mediated nanocomposite biosensor was fabricated for detecting the hepatitis C virus (HCV) core antigen using cyclic voltammetry measurements [47]. The methylene blue was able to effectively transfer electrons from the redox reaction occurring from the reduction of a horse radish peroxidase (HRP) antibodies immobilized on the sensor surface, shown in **Figure 2.2**. While this sensor exhibited a wide linear range of detection from 0.25 pg/mL to 300 pg/mL with a limit of detection (LOD) of 0.01 pg/mL, multiple complex steps were necessary for device creation which could limit the widespread use of the sensor [47].

Figure 2.2: Methylene blue (MB)-mediated electron transfer during the redox reaction between the gold electrode surface and HRP [47]

One mediator-free sensor was created to detect the human immunodeficiency virus type 1 (HIV-1) p24 antigen down to 0.0064 ng/mL in the range of 0.01 to 60.00 ng/mL [33]. As a mediator was not used in the HIV-1 biosensor, the sensitivity was in the ng/mL range which is higher than the HCV biosensor which had a detectable range in the pg/mL. While these example sensors show low limits of detection and good linear ranges, they are limited by their complex fabrication and testing methods requiring equipment capable of generating cyclic voltammograms [51]. These sensors also utilized sensor ligands for the biological receptor to specifically capture the analyte of interest, which will be further discussed in section 2.1.2.3.

2.1.2.1.2 Potentiometric

Potentiometric biosensors operate by measuring the potential at a working electrode with respect to a reference electrode during an electrochemical reaction [30]. Contrary to amperometric biosensors, potentiometric biosensors operate at zero current and monitor the accumulation of charge at an electrode surface. The reaction produces a readable signal caused by a difference in ionic concentration of a target analyte that is reduced during an electrochemical reaction [29]. The Nernst Equation governs the relationship between the concentration of an analyte/its reduced species, and the potential, allowing the concentration to be determined by the measurement of the potential [28]. **Equation 2.1** shows the Nernst Equation:

$$E = E^0 - \frac{RT}{nF} \ln \left(\frac{C_{X^-}}{C_X} \right) \quad (2.1)$$

where E (volts) is the electrode potential at zero current, E^0 (volts) is the standard reduction potential of the cell, R is the universal gas constant (8.314 J/(mol·K)), T (K) is the temperature, n is the number of electrons transferred, F is Faraday's constant (96,485 C/mol), C_{X^-} (mol/L) is the concentration of the reduced species at the electrode, and C_X (mol/L) is the concentration of the non-reduced species at the electrode [28, 52].

One example of the potentiometric technique was used to detect poliovirus in the presence of a contaminating adenovirus mixture to the un-optimized concentration of 0.644 nM (3.10×10^{11} particles/mL) [36]. The group observed significant changes in potential versus the reference electrode when poliovirus alone was present on the sensor surface compared to when a mixture of poliovirus and adenovirus was applied [36]. This is a significant result in the detection of poliovirus as the sensor did not require the use of a biological receptor to capture the virus. The group accomplished this by creating a poliovirus-templated alkanethiol self-assembled monolayer (SAM) onto a gold surface, where only poliovirus could attach during sample testing due to the specific size, shape, and hydrophobicity of the cavity, shown in **Figure 2.3** [47].

Figure 2.3: Fabrication of poliovirus-templated alkanethiol SAM on a gold surface [36]

Another potentiometric biosensor was used to detect the non-structural protein 1 (NS1) from the Dengue serotype 2 virus using egg yolk immunoglobulin (IgY) as the biological receptor [35]. This sensor was able to quickly detect NS1 with an LOD of 0.09 $\mu\text{g/mL}$ with a linear range of 0.10-10 $\mu\text{g/mL}$ in samples containing real serum [35]. Again, although these sensors show promise for viral detection, sensor fabrication contains complex steps and the necessary equipment (a potentiostat) is complex and requires trained personnel to operate and interpret the output.

2.1.2.1.3 Impedimetric/conductometric

Impedimetric biosensors detect analytes by measuring the change in impedance caused by a biochemical reaction or analyte binding events occurring at/on an electrode surface [51]. Impedance describes the opposition to the flow of electrical current, similar to electrical resistance [53]. However, impedance is used when alternating current (AC) is used to drive the flow of electrons instead of direct current (DC). Impedimetric biosensors utilize electrochemical impedance spectroscopy (EIS) where small amplitude sine wave perturbations are used over a wide frequency range to produce changes in impedance as a function of the change in frequency [51]. The resulting impedance versus frequency output is known as the electrochemical impedance spectrum. It is also important to note that conductance is the inverse of resistance, meaning conductance describes the ability of a material to allow the flow of electrical current.

Conductometric biosensors are considered sub-sets of impedimetric sensors as they assume an ideal resistor system is applicable [28, 53]. Conductometric biosensors measure the electrical conductance across electrodes due to the presence of an analyte or solution [28, 54]. Typically, conductivity changes across electrodes are attributed to a change in ionic strength of the medium/analyte during an enzymatic reaction, but conductivity changes can also occur due to binding of analytes to the biosensor or electrode surface [40, 41]. A few advantages over amperometric and potentiometric sensors exist: conductometric sensors do not require a reference electrode, complex electrical equipment, and they can be readily downsized to reduce costs [28, 55]. These advantages make conductometric devices more portable and less complex to use.

Although conductometric sensors have many advantages, they have rarely been explored for their ability to detect viruses and are mostly used to detect small molecules such as arginine and phenol [55-57] and heavy-metal ions in water [58, 59].

One of the only examples of a conductometric biosensor for virus detection was created for the detection of bovine viral diarrhea virus (BVDV). The biosensor used anti-BVDV antibodies as the biological receptor for specific detection of BVDV, and polyaniline as the sensor transducer which provided signal amplification during detection [41]. Signal measurement was accomplished by using a multimeter to monitor the change in resistance across a copper wafer surface when different concentrations of BVDV were applied to the sensor surface. In under ten minutes, 10^2 cell culture infective dose (CCID)/mL BVDV was detected in antigen-spiked blood serum, which is favorable when compared to the three or more hours of assay time using a commercially available ELISA kit with comparable titer detection ability [41].

When considering all the electrochemical techniques discussed, conductometric biosensors have the highest potential to be portable and rapid as they only require the use of a multimeter to record the resistance across electrodes or sensor surfaces. Multimeters can be found in handheld configurations, meaning a new sensor and multimeter can be brought to the point-of-care and used to detect the presence of virus when needed.

2.1.2.2 Biological receptors

A biological receptor (bioreceptor) is the part of a biosensor that captures a specific analyte of interest. The bioreceptors can be whole cells, oligonucleotides, enzymes, or antibodies immobilized onto the sensor surface. The two most popular choices for electrochemical biosensors are immobilized antibodies and complementary oligonucleotides, due to their superior specificity for the target analyte.

2.1.2.2.1 Aptamers

Aptamers are short (20-100 nucleotides) single-stranded DNA or RNA (ssDNA or ssRNA) oligonucleotides that are specific to the analyte of interest to be detected by a biosensor [28, 60]. Immobilization of aptamers onto biosensor surfaces or electrodes is

achieved via covalent attachment, physical absorption, electrostatic/hydrophobic interactions, or π - π stacking [60-63]. Although the immobilization of oligonucleotides does not specifically require complex chemistry pathways for immobilization onto the surface of electrodes, as is required with antibodies, groups still choose to covalently bind the aptamers to their sensor or electrode surfaces. The non-covalent bond pathway of immobilization is possible because the oligonucleotide strands have the ability to π - π stack with commonly-used carbon-based substrates for electrode and sensor creation [64, 65]. This immobilization strategy can reduce sensor preparation steps, leading to faster sensor creation and less chance of fabrication errors. However, it is common for the 5'- or 3'-end of aptamers to be modified with biotin for later attachment to a streptavidin-functionalized surface [60]. Thiolated aptamers are also commonly immobilized on gold nanoparticle-coated substrates and electrodes which allow them to self-assemble onto a gold surface [31, 66]. Regardless of how the aptamer is immobilized, when an aptamer is immobilized onto a sensor or electrode surface, the result is an aptasensor.

An aptasensor can detect the interaction between the immobilized aptamer and a target oligonucleotide strand via any of the electrochemical strategies discussed previously. For example, one amperometric aptasensor was created by immobilizing thiolated tetrahedral-structured ssDNA probes onto a gold electrode via Au-S bonds for the detection of avian influenza A (H7N9) [31]. This aptasensor was able to specifically detect H7N9 to a LOD of 100 fM, even in the presence of other influenza viruses H1N1 and H3N2. Aptasensors offer an attractive virus detection technique due to the highly specific binding events between aptamer probes and target, complementary oligonucleotides. However, the stability of oligonucleotides is of concern, as probe functionality can be reduced with changes in pH, temperature, and ionic strength of the sample and sensor environment [67].

2.1.2.2.2 Antibodies

Antibodies are the most common bioreceptors due to their highly specific nature and sensitivity to target antigens [29]. When an antibody is immobilized onto a sensor or electrode surface, an immunosensor is created. Immobilization of antibodies is

accomplished by electrostatic interactions between the Fc region and sensor surface [35, 68, 69], streptavidin-biotin binding [33], and through carbodiimide-assisted amidation/amine-reactive crosslinking chemistry [70-77]. Carbodiimide-assisted amidation and amine-reactive crosslinking chemistry are the most widely used immobilization technique to form a covalent bond between the biosensor surface and primary amine on the antibody. Carbodiimide-assisted amidation reactions are used to create reactive N-hydroxysuccinimide ester (NHS) groups by reacting carboxylic acid groups located on a biosensor surface with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (EDC) linkers. NHS-amine reactive crosslinking chemistry is performed between NHS esters and primary amines as shown in **Figure 2.4**. In this well understood chemical pathway, NHS esters react with primary amines in near-physiologic pH (7.2 - 9) to create stable amide bonds [78]. The immobilization chemical pathway from a reactive carboxylic acid group to a stable amide bond to a primary amine located on the antibody is used to immobilize antibodies to biosensor surfaces to create highly specific and sensitive devices for target antigens.

Figure 2.4: NHS-amine reactive crosslinking chemical pathway to produce a stable amide bond between a primary amine and NHS ester [78]

One amperometric immunosensor was developed using 1-pyrenebutyric acid N-hydroxysuccinimide ester (PSE) as a linker between graphene oxide sheets and rotavirus-specific antibodies. The PSE linker was used to non-covalently functionalize the graphene oxide sheets through π - π stacking of the pyrene group on PSE and the sp^2 hybridized carbon in the graphene sheets, shown in **Figure 2.5** [72]. Rotavirus-specific antibodies were then crosslinked to the PSE by forming a stable amide bond between primary amines on the antibody and the NHS group on the PSE. A cyclic voltammogram (CV) was collected from the antibody-functionalized graphene electrode and was compared to a CV collected from the application of rotavirus to the antibody-

functionalized graphene electrode, showing a change in current due to increased resistance from rotavirus-bound antibodies. The immunosensor detected rotavirus with an LOD of 10^2 plaque-forming units (pfu)/mL [72].

Figure 2.5: Rotavirus immunosensor fabrication pathway [72]

Another biosensor was created for the detection of the M1 protein from influenza virus by immobilizing polyclonal anti-M1 antibodies onto a boron-doped diamond electrode via the same EDC-NHS chemistry pathway [77]. Electrical impedance spectroscopy was used to detect the presence of decreasing concentrations of M1 protein to an LOD of 50 fg/mL (40 virions) in only six minutes. The immunosensor was compared to other detection methods for influenza including PCR, ELISA, and other biosensors, showing superior testing time and comparable virion detection limits [77].

With low limits of detection being reported, immunosensors developed by immobilizing antibodies to detect specific antigens show promise for being rapid, sensitive, and specific diagnostic tools. However, antibodies are extremely sensitive to their environment, as any change in pH or temperature can change their function due to denaturing. Additionally, immobilizing an antibody and restricting their structure to one conformation could lead to a loss of biological function and a loss of detection ability [28, 79].

2.1.2.3 Graphene-based biosensors

This section of the biosensors review focuses on the material used to create the biosensor. Since electrochemical biosensors are being reviewed, the sensor surface must

be conductive to allow the transport of electrons. Common materials for device and electrode creation include gold [31, 33, 35-37, 66, 68], graphene [39, 40, 72, 75], and other carbon-based materials [32, 38, 45]. Graphene is a popular material to construct biosensors because of its excellent electron transfer ability, high mechanical strength, and high surface area available for functionalization [80]. Graphene surfaces have the potential to replace the solid support surfaces used in conventional ELISA. Using a conductive surface for an ELISA can provide a faster detection method via electrical detection when compared to traditional methods, which were discussed in section 2.1.1.3. In a conventional ELISA, antibodies are immobilized onto a solid plastic support such as the polystyrene walls of a 96-well plate. In this type of system, the antibody that is immobilized on the surface has no way of producing a measurable electrical signal. Therefore, additional antibodies and labels are needed to produce a detectable signal, typically a color change from enzymatic reduction reactions. The most common way to develop a signal is with a sandwich ELISA, shown in **Figure 2.6**. To create this system, a primary antibody is immobilized onto a solid surface (**Figure 2.6A**). An antigen specific to the primary antibody is applied, which binds to the primary antibody (**Figure 2.6B**). After washing, a secondary antibody is attached to the other end of the antigen, which is bound to the primary antibody (**Figure 2.6C**). The last antibody attached is the detecting antibody labeled with a luminescent enzyme or a fluorescent tag to induce a color change after addition of the enzyme's substrate (**Figure 2.6D**) [81, 82]. Instead of relying on a colorimetric readout of antigen-antibody interactions inside of a plastic well, electrochemical biosensors can be used to quantify the antigen presence via electrical signal on a conductive surface, such as graphene.

Figure 2.6: Schematic of a sandwich ELISA. A) Immobilization of the primary antibody to the substrate, B) antigen-primary antibody binding, C) antigen-secondary antibody binding, and D) detecting antibody/label complex binding to the secondary antibody [82]

A sandwich ELISA was constructed on a graphene-sheet coated carbon electrode for the amperometric detection of the H5 gene from avian influenza H5N1 [83]. The graphene sheets were modified with PSE, functionalized with a streptavidin linker to bind a biotin-and fluorescein-labelled PCR H5 gene DNA strand, and finally an anti-fluorescein HRP detecting antibody was applied, as seen in **Figure 2.7**. As a proof-of-concept device, the amperometric response for the H5 gene was above the negative control response down to 0.2659 ng/ μ L, showing promise for a portable, on-site screening point-of-care detection device after further device optimizations [83]. Although this device shows promise for viral gene detection, it does not detect the influenza virus directly, as a PCR-amplified gene was used and diluted as the target analyte, which slows down detection time.

Figure 2.7: Fabrication schematic of the graphene-enhanced electrochemical biosensor for the detection of the H5 viral gene [83]

Another sandwich ELISA system was created for the detection of carcinoembryonic antigen (CEA). This amperometric immunosensor was fabricated in the same way as previously discussed on a graphene/gold nanoparticle nanocomposite surface [84]. The immunosensor showed two linear ranges of CEA detection between 0.5-50 ng/mL and 250-2000 ng/mL with LODs of 0.28 ng/mL and 181.5 ng/mL, respectively [84]. Although this device does not detect a virus particle, it shows the potential application of virus detection with substitutions of the antibodies, while leaving the electrochemical detection technique the same.

In my work, a sandwich ELISA system was created to detect the presence of mouse immunoglobulin G (IgG) antigen and tested using the conductometric sensing strategy. Despite the drawbacks of using a multiple antibody-based system, the sandwich ELISA immunosensor system was chosen to produce the most specific sensors possible. To detect the analytes of choice, the conductometric sensing strategy was chosen as it does not require sophisticated equipment or training to obtain or analyze results, making the sensors easier to transition out of a laboratory setting. The sandwich ELISA system was developed on a graphene biosensor to allow for an electrical signal readout across two electrodes when the target analyte is applied. The goal of the conductometric sandwich ELISA biosensor was to be used for the direct detection of viral particles, which is currently missing from the literature.

2.1.3 Gold nanoparticles used for analyte detection

Although electrochemical biosensors offer an inexpensive, specific, and rapid method to detect analytes, other methods of detection exist that do not require additional fluorescent enzyme labels, electrochemical reactions, or solid sensor surfaces. For example, colorimetric assays based on the aggregation of metal nanoparticles have been developed to detect and quantify analytes [85-87]. The developed colorimetric assays provide rapid and sensitive detection of a variety of viruses and proteins, based on the aggregation or de-aggregation of nanoparticles.

2.1.3.1 AuNP properties and aggregation

Gold nanoparticles (AuNPs) are used for biosensing due to their unique physicochemical and optical properties [88, 89]. Among the most important properties of AuNPs are their biocompatibility, controllable size, surface modification capability, and their intrinsic surface plasmon resonance (SPR) [89]. SPR is a result of the cloud of free electrons present near the surface of AuNPs. The natural oscillations of the free electrons on the surface of the AuNPs are called surface plasmons, which exist at the gold metal-dielectric interface [90]. The surface plasmons can be excited by light, and when coupling occurs between the incident light and the surface plasmons, electromagnetic

waves in the visible range are given off [88, 90]. This resonant oscillation between the incident light and surface plasmons is called SPR [91]. SPR is the property responsible for the AuNPs color in liquid solution, which is bright red, corresponding to a wavelength of 519 nm for 16 nm particles. A red-shift in the absorbance wavelength occurs as the AuNPs size increases and the liquid solution begins to show as a blue color, seen in **Figure 2.8**.

Figure 2.8: 2-150 nm AuNPs color in liquid solution [92]

The wavelength spectrum of the particles is measured using ultraviolet-visible (UV-Vis) spectrophotometry. When AuNPs of the same size aggregate, there is also a red-shift due to reduced inter-particle separation [93, 94]. This can be seen in **Figure 2.9** as AuNPs of diameter 16.3 nm (**Figure 2.9A-C**) are aggregated in the presence of 100 mM NaCl (**Figure 2.9D-F**) [93]. The aggregation of the AuNPs is quantified by the aggregation index (AI) shown in **Equation 2.2** [95]

$$AI = \frac{\lambda_{max+60nm}}{\lambda_{max}} \quad (2.2)$$

where λ_{max} is the absorbance at the maximum wavelength and $\lambda_{max+60nm}$ is the absorbance at a wavelength of 60 nm added to the maximum wavelength. This equation is used to determine the aggregation extent of AuNPs in two ways. First, the UV-Vis peak absorbance decreases as AuNPs aggregate, and second, the UV-Vis peak broadens as AuNPs aggregate. These two phenomena can be seen by comparing the UV-Vis spectrum curves in **Figure 2.9A** and **D**, where the peak absorbance is higher and sharper in **Figure 2.9A**. A larger AI corresponds to a high extent of aggregation [93]. The aggregation of AuNPs can also be measured using dynamic light scattering (DLS), which gives the

hydrodynamic diameter (D_H) of nanoparticles in a liquid medium. The change in D_H (ΔD_H) before and after aggregation can be calculated using **Equation 2.3**

$$\Delta D_H = D_{H,after} - D_{H,before} \quad (2.3)$$

where $D_{H, after}$ is the hydrodynamic diameter (nm) after inducing aggregation, and $D_{H, before}$ is the hydrodynamic diameter (nm) before inducing aggregation.

The red-shift and diameter increase resulting from AuNPs aggregation can be used to develop a colorimetric assay by creating conditions that bring analyte-covered AuNPs together and then quantifies them by AI or diameter increase. For example, the ΔD_H from **Figure 2.9** is $922.2 \text{ nm} - 16.3 \text{ nm} = 905.9 \text{ nm}$.

Figure 2.9: Example of AuNP aggregation resulting in a red-shift. A) UV-Vis spectrum of 16.3 nm AuNP, B) hydrodynamic diameter measurement of AuNP, C) TEM image of 16.3 nm AuNP, D) UV-Vis spectrum of aggregated 16.3 nm AuNP, E) hydrodynamic diameter measurement of aggregated AuNP, and F) TEM image of aggregated AuNP [93]

One AuNP assay was developed to detect clinical samples of hepatitis C virus (HCV) RNA based on the aggregating effects of cationic and anionic AuNP binding [94]. Citrate-capped AuNPs were functionalized with a thiolated-ssDNA probe specific to HCV RNA. When denatured, complementary HCV RNA strands were added, base-pairing between the probe and HCV RNA strand occurred, leaving negatively charged RNA phosphate backbone strands un-bound. Finally, when cationic AuNPs were added, the particles bound to the folded HCV RNA complexes did not allow the cationic and anionic AuNPs to come into contact and aggregate. In contrast, when cationic AuNPs

were added to samples containing non-complementary HCV RNA strands, the anionic AuNPs were able to bind to the phosphate backbone of the RNA probe. This binding reduced the inter-particle distance between cationic and anionic AuNPs, leading to aggregation and a visible color change. The aggregation and anti-aggregation were characterized by UV-Vis absorption peak changes. These results were directly compared to PCR and ELISA techniques, which agreed favorably with both conventional tests. This assay allowed for the detection of unamplified HCV RNA from clinical samples in ~30 minutes with a 94.1% specificity with a limit of detection of 4.57 IU/ μ L [94]. However, one drawback to this type of assay is the need to functionalize the AuNPs with nucleic acid probes, which tend to be expensive.

2.1.3.2 Modified, functionalized AuNPs

Modified AuNPs, commonly through antibody and nucleic acid functionalization, have been used extensively for the detection of analytes. These types of AuNP assays are specific and sensitive due to the use of specific targeting receptors for the analyte of interest. As discussed for bioreceptor immobilization, the modification of AuNPs is very similar. Thiolated probes can be readily bound to the AuNP surface, and antibody immobilization onto AuNPs is achieved through EDC-NHS chemistry. Additionally, antibodies can be passively adsorbed to the surface of citrate-capped AuNPs through ionic and hydrophobic interactions [96]. This was done to conjugate 13 nm AuNPs with anti-influenza A H3N2 HA mouse monoclonal antibodies (mAbs) for the un-amplified detection of influenza A virus H3N2 [96]. When the virus was in the presence of the AuNP-mAb complexes, aggregation lead to a red-shift in the absorbance spectrum due to decreased intra-particle distance. This aggregation was confirmed using DLS, where a ΔD_H of 116 nm (21 to 137 nm) was measured. This assay had a limit of detection of 7.8 hemagglutination units (HAU) with a detection time of only 30 minutes [96]. Another AuNP assay based on DLS measurements was developed to detect influenza A virus H1N1. The AuNPs were conjugated with mAbs specific to the influenza A virus using a 3,3'-dithiobis(sulfosuccinimidyl propionate) bifunctional linker to covalently bind the mAbs to the AuNPs. AuNPs with diameters of 30, 60, and 80 nm were used to evaluate

the size dependence of the influenza assay, revealing that a different AuNP size did not affect the assay performance [97]. By optimizing the concentration of AuNPs, a limit of detection of <100 TCID₅₀/mL was achieved, which is 1-2 orders of magnitude better than conventional ELISA kits and only requires ~30 minutes of detection time [97]. Another virus assay was developed using peptide-functionalized AuNPs for the detection of Newcastle disease virus (NDV) [98]. The AuNPs were functionalized with NDV-specific peptides via strong sulfur-gold bonds. Using optical density measurements with different virus concentrations for varying degrees of aggregation, a limit of detection of 0.125 HAU was found in 20 minutes [98]. These virus detection assays show the aggregation of AuNPs as the detection pathway, but anti-aggregation of AuNPs can also be used.

The anti-aggregation method was highlighted in a gold nanostar (AuNS) assay for the detection of enterovirus 71 (EV71) that causes hand, foot, and mouth disease (HFMD) [99]. In this assay, 105 nm AuNS were conjugated with a receptor protein with a known affinity to EV71 through passive adsorption. Aggregation was prevented in samples that contained EV71 because the receptor protein binding sites were filled, which prevented nonspecific interactions with contaminant proteins present in the sample media [99]. Raman spectroscopy peak intensities after the addition of decreasing concentrations of EV71 were measured, leading to a limit of detection of 10^7 pfu/mL in only 15 minutes [99].

Functionalized AuNPs lead to the rapid detection of various viruses with low limits of detection. However, preparation steps to functionalize and wash the AuNPs increase overall testing time. Increased testing time can cause stability issues because ligand-functionalized AuNPs are temperature and the external-environment dependent.

2.1.3.3 Unmodified AuNPs

To overcome the potential stability issues of functionalized AuNPs, unmodified AuNPs can be used for analyte detection assays. Using unmodified AuNPs reduces the complexity of analyte detection assays, as there are no AuNP pre-treatment functionalization steps. The detection strategy of analytes with unmodified AuNPs is the same as functionalized AuNPs, which is achieved through observing absorbance peak

shifts after aggregation or quantifying the aggregation with DLS. There are also opportunities to develop assays for naked-eye detection of aggregation through a color change of the testing liquid. Most of the developed unmodified AuNP assays are all centered around keeping the AuNPs dispersed in solution to keep the solution color red. This is achieved by adding complementary ssRNA/ssDNA primers to the sample tube, which contains the target virus' RNA/DNA, leading to stable dsRNA/DNA formation that keeps the testing solution red.

This technique was used to detect unamplified BVDV-RNA by naked-eye [100]. In this assay, BVDV probes were added to testing tubes that contained unmodified AuNPs and either non-complementary BVDV-RNA or complementary BVDV-RNA. When NaCl was added to samples containing non-complementary BVDV-RNA, no aggregation was observed because the BVDV probes stabilized the unmodified AuNPs through electrostatic interactions between the negatively charged backbone of the probe and citrate shell around the AuNPs [100]. When NaCl was added to samples containing complementary BVDV-RNA, salt-induced AuNP aggregation occurred because the probes hybridized to the BVDV-RNA, and NaCl was free to aggregate the un-stabilized AuNPs. By using a fixed concentration of AuNPs and probes, BVDV concentration was varied to show a LOD of 80 TCID₅₀/mL by UV-Vis, and 200 TCID₅₀/mL by naked-eye [100]. Despite these promising results, a purified RNA sample was used for detection because unmodified AuNPs can also passively bind to other contaminants in clinical samples, including other DNA fragments and proteins.

Another assay using unmodified AuNPs was developed for the detection of Dengue virus using a similar detection pathway [101]. Instead of inducing aggregation of unmodified AuNPs with NaCl, this assay found that the addition of a neutral-charge peptide nucleic acid (PNA) caused aggregation because PNA removed the charge-repulsion between AuNPs thus reducing the interparticle distance [101]. The PNA was the probe for the target Dengue DNA. In the presence of the Dengue DNA, hybridization occurred, which left the AuNPs un-aggregated in solution. This assay showed a LOD of Dengue DNA at 0.12 μ M [101]. However, this study used a purified ideal Dengue DNA sample that did not originate from the full Dengue virus. This limits the actual application

of these types of assays because the target nucleic acids are located inside the capsid of the virus, which must be extracted prior to detection. While the extraction of the target nucleic acids is possible prior to an AuNP assay, this could result in assay complications because AuNPs non-specifically bind to other biomolecule fragments. One possible solution to avoid this pre-treatment extraction step is to develop an AuNP assay to detect whole viral particles.

Only a limited number of unmodified AuNP assays exist for the detection of whole viral particles. One assay utilized sialic acid-stabilized AuNPs (SA-AuNPs) for the detection of influenza B Victoria and Yamagata [102]. Influenza viral proteins (hemagglutinin) are known to bind to sialic acid, which is present on the surface of lung epithelial cells [103]. By using SA-AuNPs, influenza virus was detected as hemagglutinin-sialic acid binding occurred, aggregating the nanoparticles. The aggregation caused peak broadening of the absorption spectrum at 610 nm, and a change in absorbance at this wavelength was calculated with a decreasing volume of the influenza virus added. A LOD of only 0.09 volume % of influenza virus was achieved [102]. Another viral particle detection assay was developed for human papillomavirus type 16 virus-like particles (HPV16 VLPs) using unmodified citrate-capped AuNPs to explore the interaction between VLPs and AuNPs [104]. In this study, AuNP-VLP complexes formed mainly through hydrophobic interactions, which hindered the salt-induced aggregation of un-complexed AuNPs. To test that hydrophobic interactions were the dominant driving force for complex formation, a higher and higher concentration of ethanol was added to the VLP-AuNP mixture showing that aggregation was increased with higher ethanol concentration. VLP solubility increased at higher ethanol concentrations, resulting in less VLPs complexing with AuNPs, leaving more AuNPs to be aggregated by 70 mM NaCl [104].

As shown in these whole viral particle detection assays with AuNPs, there is some mechanism for the viral particles to aggregate and decrease the interparticle distance between virus-AuNPs complexes. Another option for aggregating viral particles is using osmolytes.

2.1.4 Osmolytes

Osmolytes are water-soluble, naturally-occurring molecules that are produced by cells to enhance protein stability by combating osmotic stress and other environmental stresses [105, 106]. Osmolytes are classified into three groups, carbohydrates/polyols (sucrose, mannitol), amino acids (glycine), and methylamines (TMAO). Additionally, osmolytes can be classified as protecting or denaturing osmolytes. Protecting osmolytes do not affect the native protein structures and promote protein folding and denaturing osmolytes denature proteins into unfolded states [105, 107]. Although the exact mechanism of osmolyte action on protein folding/unfolding is not fully understood, it has been shown that protecting osmolytes are preferentially excluded from the protein surface and do not directly interact with the protein's amide backbone, whereas denaturing osmolytes interact with the protein backbone directly, causing protein unfolding [107, 108]. The theory of protecting osmolytes causing protein stabilization is called the osmophobic effect, which states that there is an unfavorable interaction between osmolytes and the protein peptide backbone [109]. In the unfolded state, the peptide backbone is more readily exposed to osmolyte resulting in a high free energy that is reduced by protein folding back to its native state [109]. Another important action of osmolytes is their ability to alter the water structure around biomolecules. Protecting osmolytes are excluded from the surface of proteins, and they interact with water molecules near the surface, increasing protein stability due to increased intra-protein side chain interactions [105, 110]. This is the solvation effect of osmolytes. Since the osmolytes pull water molecules away from the surface of proteins, the protein surface becomes slightly more hydrophobic, which is stabilized by intra-protein side chain interactions [105, 110].

2.1.4.1 *Preferential virus aggregation*

Protecting osmolytes have been used for the preferential aggregation of both enveloped and non-enveloped viral particles [111, 112]. To test the aggregation ability of protecting osmolytes, 18-26 nm non-enveloped porcine parvovirus (PPV) was filtered through a 0.2 μm filter that would not normally retain the PPV in water or PBS. Over

80% of PPV was removed by the filter using 1.0 M glycine, mannitol, alanine, and trehalose, which aggregated the PPV and allowed for removal by the filter [111]. Even in the presence of model contaminating proteins, BSA and lysozyme, 1.0 M mannitol and alanine were able to remove 85.1% and 80.2% of the PPV, respectively, with less than 5% protein removal [111]. The theory for this preferential virus aggregation versus protein aggregation with osmolytes is based on the relative hydrophobicity between viruses and proteins and the physical structure of the two macromolecules [6, 111]. When osmolytes are introduced into the solutions containing PPV or proteins, there is a partial dehydration layer around the biomolecules resulting in free energy that must be minimized. For proteins, their relatively more hydrophilic and flexible nature allows the excess free energy to be minimized by increased protein side chain interactions resulting in protein structure compaction and stabilization [6, 111, 113]. Since viruses are relatively more hydrophobic and rigid than proteins, the dehydrated and hydrophobic exposed patches are minimized by virus-virus aggregation [111]. This same preferential aggregation phenomenon was confirmed using an enveloped, 60-80 nm Sindbis virus heat resistant strain (SVHR) [112]. In this work, 0.3 M proline and mannitol were able to remove over 80% of SVHR using a 0.2 μ m filter while leaving BSA and lysozyme proteins in solution with less than 23% removed [112]. These studies highlight the ability of protecting osmolytes to preferentially aggregate viral particles and not proteins.

2.1.4.2 Osmolyte-induced AuNP aggregation virus assay

The ideas of preferential virus aggregation, AuNP complexation with viral particles, and decreased intra-AuNP distance leading to a color change and measurable change in complex diameter have been combined to develop a general viral particle detection assay, shown in **Figure 2.10** [6]. When an osmolyte solution was added to virus/AuNP complexes, aggregation occurred if a high enough concentration of virus was present (**Figure 2.10, top left side pathway**). When osmolyte was added to protein/AuNP complexes, aggregation did not occur because the osmolyte preferentially aggregated viruses, not proteins (**Figure 2.10, bottom left side pathway**). To test salt's ability to aggregate virus/AuNP complexes, NaCl was added to AuNP/virus complexes

where aggregation occurred at low enough virus concentration because the virus could not protect the AuNPs from salt-induced aggregation (**Figure 2.10, top right pathway**).

Figure 2.10: Scheme of virus detection using osmolyte-induced aggregation of virus-AuNP complexes [6]

In this assay, AuNPs and viral particles were complexed, aggregated using protecting osmolytes, and the aggregation was quantified by DLS for decreasing titers of PPV and BVDV before and after osmolyte addition. As a control, model proteins BSA and thyroglobulin were complexed with AuNPs, and their aggregation extent due to osmolyte addition was compared to the aggregation of virus-AuNP complexes. As expected, the virus-AuNP complexes aggregated with the addition of 1 M mannitol, and protein-AuNP complexes did not aggregate. PPV was detected in the range of 10^5 to 10^8 MTT₅₀/mL with a LOD of 10^6 MTT₅₀/mL [6]. BVDV was detected in the range of 10^3 to 10^7 MTT₅₀/mL with a LOD of 10^4 MTT₅₀/mL [6].

To expand upon this AuNP virus detection assay, I synthesized different sizes of AuNPs and used them in the same AuNP/virus assay to improve the LOD, discussed in Chapter 4.

2.2 Purification of viral particles

2.2.1 Vaccine manufacturing process

Vaccination is one of the most effective methods of preventing and eradicating deadly viral diseases. Millions of lives are saved every year due to vaccination, but producing vaccines is a difficult undertaking. Vaccine manufacturing is challenging because a high concentration (titer) of viral particles must be produced and purified while ensuring the safety and efficacy throughout the manufacturing process to meet regulatory guidelines. Viruses are complex biomolecules that are susceptible to their environment and the processes they are put through in the vaccine manufacturing process [114]. These manufacturing processes are separated into two broad sections: upstream production where the viral particles are generated in flasks and bioreactors (**Figure 2.11, Upstream**), and downstream purification where the viral particles are separated from contaminants using various unit operations (**Figure 2.11, Downstream**). Common unit operations for upstream production of viral products are batch-mode and perfusion (continuous) bioreactors. Common unit operations for the downstream purification of products are centrifuges, filtration units, and chromatography columns.

Figure 2.11: Vaccine biomufacturing train from upstream production through downstream purification [115, 116]

2.2.1.1 Conventional upstream vaccine production

Perhaps the most well-known and recognizable vaccine is the seasonal influenza vaccine. This vaccine has been historically produced in embryonated hens' eggs, which act as mini bioreactors after being inoculated with the seasonal influenza strain. Many issues exist with this egg-based influenza vaccine platform, such as the need for over 400 million eggs every year for enough doses, the fact that some influenza strains are highly pathogenic and could kill the embryos, and if a downstream purification run fails a large batch of doses must be discarded [117]. To address these issues, the shift to animal cell-culture techniques has been successfully implemented. However, a large majority of influenza vaccine doses are still created in eggs today [117, 118]. The slow transition from producing influenza vaccines in eggs to cell-culture bioreactors is due to the large infrastructure already in place to support the egg-based system, including collaborations between academic and industrial laboratories to ensure the next batch of vaccine will contain appropriate antigenic variants [119]. The current role of large-scale animal cell culture bioreactors is to augment and support the growing demand of the influenza vaccine, not to fully replace the well-established egg-based vaccine production network. However, with further developments and implementation, the reliance on egg-based platforms for the flu vaccine could be fully realized. Numerous cell lines have been considered and researched to produce influenza vaccines. These cell lines include human embryonic kidney (HEK) cells [120], Madin-Darby canine kidney (MDCK) cells [118, 120], Vero cells [120], and *Spodoptera frugiperda* (Sf9) insect cells [120], summarized in **Table 2.1** [121]. Most of the approved vaccines are produced using MDCK and Vero cell lines because they have been extensively characterized and their growth kinetics are well-understood from numerous studies [117].

One study directly compared MDCK and Vero cells for the production of the influenza virus for vaccines in a lab-scale bioreactor [122]. The adherent MDCK and Vero cells were grown on microcarriers inside of a 5 L stirred tank bioreactor (STR) and infected with influenza A Wisconsin strain at $\sim 1.8 \times 10^6$ cells/mL with a MOI of 0.001. The cells were also grown on microcarriers in a 2 L wave bioreactor and infected at $\sim 1.1 \times 10^6$ cells/mL with a MOI of 0.018. To compare the ability of MDCK and Vero cells

to produce influenza virus, a virus productivity value was calculated from quantifying the HA produced during infection per cell. In the STR, MDCK and Vero cells produced 10,000 virions/cell and 4,976 virions/cell, respectively. In the wave reactor, MDCK and Vero cells produced 33,255 virions/cell and 3,990 virions/cell, respectively [122]. This study demonstrated that MDCK cells can produce higher titer of influenza virus compared to Vero cells, which shows why current cell-based influenza vaccines are produced from MDCK cell lines. However, there is still a need to increase the productivity of cell lines to produce even more viral particles to meet the demand and replace egg-based methods.

Table 2.1: Selected list of approved influenza vaccines from cell-culture derived systems

Vaccine Name	Approved Region	Cell Line	Vaccine Type	Approval Year
Optaflu/ Flucelvax	EU/USA	MDCK 33016	Trivalent subunit (H1N1, H3N2, B)	2007/2012
Celtura	Germany	MDCK	Monovalent (H1N1)	2009
Celvapan	EU	Vero	Monovalent (H1N1 or H5N1)	2009
Preflucel	EU	Vero	Trivalent inactivated (H1N1, H3N2, B)	2010
Flublok	USA	Sf9	Quadrivalent subunit (updated annually)	2013
Flucelvax Quadrivalent	EU/USA	MDCK	Tetavalent subunit (H1N1, H3N2, Victoria, Yamagata)	2016

As mentioned, upstream production of viral particles is achieved in a bioreactor. A bioreactor is used to grow host cells which are inoculated with a virus to allow for virus propagation as the cells grow. The classical and commonly used mode for bioreactor operation is batch cultivation where cells and media are introduced into the bioreactor and left to grow and collected after many days of virus replication, shown in **Figure 2.12A** [123]. Bioreactors are equipped with various sensors and probes to monitor the temperature, dissolved oxygen level, pH, and other properties to maintain cell health over time. The six phases of cell concentration occur over time within a batch-mode operated bioreactor, as shown on the growth curve in **Figure 2.12B** [124]. The lag phase

is the short amount of time where cells adapt to their new environment and do not grow. The acceleration phase is when cell growth begins as cells uptake nutrients from the surrounding media and continues at a constant rate through the growth phase. The decline phase starts when the cells utilize all available nutrients, and growth stops through the stationary phase. The death phase begins when cells lose viability and lyse, or break apart [124]. The most critical phase to consider is the growth phase where cells are growing, producing virus particles, and all other biomolecules during normal cell growth.

Figure 2.12: A) Schematic of a bioreactor [123] and B) growth curve of cells within a batch-mode bioreactor [124]

While upstream studies focus on increasing the productivity of cell lines for the desired viral product, additional biomolecules are generated during cell growth. These biomolecules are considered impurities that must be separated from the viral product during downstream purification, so knowing the composition of what exits the bioreactor after growth is important for designing downstream purification methods. These biomolecules include both process- and product-related impurities. Process-related impurities include proteins from serum-based media and compounds added for cell lysis. Product-related impurities include host cell proteins (HCP), host cell DNA (hcDNA), and cell debris after cell lysis.

The amount and type of impurities vary based on the type of expression system used to produce the viral product. For example, in the case of the egg-based vaccines, the allantoic fluid from the eggs is collected, which contains high concentrations of

ovalbumin protein, tissue compounds, and other solids from chicken growth [125]. In contrast, the flu vaccine produced using serum-free media with MDCK or Vero cells have considerably less solids and protein content [125]. Regardless of the expression system or compounds added upstream, the downstream purification train is responsible for removing all impurities to below regulatory-approved concentrations prior to the final formulation of the drug product.

2.2.1.2 Conventional downstream purification processes

The downstream purification train starts directly after the bioreactor harvest step and is called clarification. Clarification aims to remove cells, cell debris, and aggregates. The clarification step varies for each viral product, and different unit operations are used to achieve this removal [125]. The clarification step is a very important step in the purification train as a low solid content stream to other downstream unit operations allows them to operate more efficiently. Numerous unit operations are available for the clarification step, such as low-speed and high-speed centrifuges and various filtration units. The unit operation used for clarification depends on the viral product's physicochemical properties, including size, shape, isoelectric point, and stability. A very common clarification step is using a low-speed centrifuge to pellet cells and cell debris from the reactor harvest and collecting the supernatant which contains the virus and other soluble impurities. This process was used to clarify a rotavirus vaccine produced in Vero cell culture by centrifuging the harvest at $2,831 \times g$ for 30 minutes and $4,424 \times g$ for 10 minutes at 4°C [126]. Another centrifuge-based clarification step ($1,000 \times g$ for 10 min at 4°C) was applied to rotavirus VLPs after a freeze-thaw cycle to lyse the cells [127]. Although neither study directly reported the virus recovery after the primary clarification step, centrifugation was adequate to remove cell debris, which allowed for additional purification steps with filtration and chromatography units. The major drawbacks to a centrifuge-based clarification step are the difficulty of scale-up and continuous operation [125, 128]. At the production level scale, the only way to process more harvest is to use additional centrifuges, which leads to increased capital costs and labor costs [125]. These challenges lead to the development of different clarification steps using filtration units.

Tangential flow filtration (TFF) and dead-end filtration (DEF) are attractive options for the clarification of viral products. Although DEF units are easy to implement at any scale, disadvantages include virus adsorption on the filter surfaces, potential leachables in the product stream, and rapid fouling from cake formation [125]. TFF units have numerous advantages, including simple design and operation, variable flow rate options, and can be operated continuously. As opposed to the conventional perpendicular feed flow of DEF, TFF utilizes a feed flow parallel to the membrane surface, which allows constant cake formation on the filter surfaces, leading to steady-state filtrate flux, shown in **Figure 2.13** [129].

Figure 2.13: A) Feed and product streams in TFF and B) filtrate flux rate profile through filtration time using TFF [129]

The TFF technique has been used extensively at the pilot and production scales [126, 130-132]. This size-based separation technique has received attention because viral particles are in the size range of nanometers, whereas cells and aggregates are in the micrometer range [131]. In one study, a 0.65 μm TFF membrane was used for the primary clarification of 5 L of influenza VLPs produced in a stirred-tank bioreactor with High Five (Hi5) insect cells [133]. The primary harvest clarification was successful based on maintaining 100% HA content before and after clarification, as well as high turbidity reduction [133]. Although the study did not pursue the 0.65 μm filter further for scale-up, the potential exists to use the membrane for similar viruses on a large scale. Another study demonstrated the large-scale recovery of tetanus toxin and toxoid for the diphtheria-tetanus-pertussis (DTP) vaccine using the TFF technique in a cGMP setting

[132]. The conventional primary clarification technique utilizes centrifugation, which shows losses greater than 25%. At a pilot-scale working volume of 400 L, the tetanus toxin broth was clarified by a microfiltration TFF membrane with a pore size of 0.22 μm at >96% recovery. The study also showed that 60,000 L of broth could be processed by the same filter before irreversible fouling occurred [132]. These studies highlight the potential and direct application of TFF for use in the primary clarification of viral products for use in vaccines, which is important in the downstream purification train.

The step after clarification in the downstream purification train is the main purification section, which aims to separate the target viral product from other soluble impurities. In the main purification section, the physicochemical properties of the virus are of great importance in designing purification techniques. These physicochemical properties include the isoelectric point (pI), size, envelope presence, and hydrophobicity which are extensively exploited for use in chromatographic separations [5]. The purification strategies used include ultracentrifugation, ultrafiltration, biphasic extraction systems, and chromatography [5].

Historically, and especially in monoclonal antibody (mAb) production, chromatography has been used as the main unit operation in downstream purification. Chromatography has many different techniques, including size-exclusion, ion exchange, hydrophobic interaction, and affinity. For example, Protein A affinity chromatography has been used for the primary capture step in many production-scale mAb processes [134]. While these chromatography techniques have been used for efficient purification of mAbs, the relatively large size of viral particles limits their adsorption to the column resins leading to decreased purification efficiency and flow rates [135]. To address these issues in viral particle purification, new chromatography resins are being developed and tested including monoliths and larger-pore resins. The main advantage of using a monolith-based resin is the ability to flow at 10-20 times higher than conventional resin columns because mass transfer within porous resin beads is dominated by convection, rather than diffusion [136].

One monolithic column chromatography technique was used for the purification of HIV-1 gag virus-like particles from host cell impurities, including extracellular

vesicles [137]. With a 1 mL monolith column, $41.9 \pm 9.6\%$ of the VLPs were recovered with a 90.6% total protein and 98.2% total dsDNA depletion, comparable to the 48.1% VLP recovery using a standard method of density gradient ultracentrifugation [137]. This process only took 47 minutes and resulted in 2000 doses of the purified VLP. Although not tested during the study, 8 L and 40 L monolith columns are commercially available, leading to possible scale-up opportunities for full-scale production [137]. Another study evaluated the ability of an 8 mL convective interaction media (CIM) quaternary amine (QA) monolith column to purify Vero cell-derived influenza A and B viruses. At physiological pH, the influenza viruses are negatively charged and bind to the QA monolith, which were eluted with 0.5 M NaCl. The four different influenza strains had recoveries between $70.8 \pm 32.3\%$ and $87 \pm 30.8\%$ [138]. Protein removal was between $93.3 \pm 0.4\%$ and $98.6 \pm 0.2\%$ and hcDNA removal was between 76.4% and 99.9% [138].

2.2.1.3 Continuous-mode downstream purification

While there have been advances in the downstream purification of viral particles using conventional techniques like filtration and chromatography, another optimization is necessary to boost the productivity of these unit operations. Switching to the continuous operation for these unit operations would increase throughput and productivity while simultaneously reducing the size of equipment [139, 140]. A reduction in the size of equipment would reduce the facility size of a manufacturing suite, leading to increased capital cost savings [139]. The first attempts of making the downstream purification train continuous was using chromatography for mAbs and a limited number of viruses. In one process for the continuous purification of influenza virus, simulated moving bed (SMB) chromatography was used and compared directly to conventional batch chromatography [141]. SMB chromatography utilizes countercurrent flow between target analytes (solids) and the mobile phases through an arrangement of many columns and valves, shown in **Figure 2.14** [141, 142]. The size-exclusion SMB process continuously moves the inlet and outlet ports along a bed, which allows for increased resolution between solids with similar retention times [143]. Using this size-exclusion SMB technique, 70% of influenza H1N1 produced in adherent MDCK cells was recovered, compared to 80% recovery

using a conventional batch process [141]. Total protein reduction was 60% for the continuous SMB process compared to an 80% reduction in the batch process. The productivity was calculated to be 3.8 times higher for the continuous SMB process, which ultimately leads to the ability to use smaller columns compared to a batch process [141].

Figure 2.14: Four-zone (column) open loop SMB chromatography process [141]

Another SMB process, an open-loop, two-column SMB process, was used for the quasi-continuous purification of adenovirus serotype 5 (Ad5) [144]. This open-loop, two-column SMB process recovered 86% of Ad5 compared to 57% from a single column batch process. Host cell protein removal was 89%, and host cell DNA removal was 90% for the continuous process, which was comparable to the 94% removal of HCP and 94% removal of host cell DNA in the batch process [144]. This study calculated a productivity increase of 6-fold and a 51% increase in Ad5 recovery, which gives strong evidence that quasi-continuous chromatography has the potential to be in a novel downstream processing train.

In addition to SMB, periodic countercurrent chromatography (PCC) has been explored as a continuous purification process. Although no study could be found for the purification of viral particles, PCC has been applied to the purification of therapeutic proteins [140]. The PCC technique utilizes several columns equal to the number of

chromatographic steps necessary for the separation: loading, washing, eluting, and regeneration. A common number of columns is three to allow for loading, eluting, and regeneration, shown in **Figure 2.15** [140]. There are six steps in a full cycle:

1. The first column is loaded until breakthrough
2. The first, loaded column is connected to the second column to begin loading
3. The first column is eluted, and the second column is loaded until breakthrough
4. The first column is regenerated, and the second column is connected to the third column to begin loading
5. The second column is eluted, and the third column is loaded until breakthrough
6. The second column is regenerated, and the third column is connected to the first column

Figure 2.15: Schematic of three-column PCC cycle [140]

This three-column PCC process was used to purify a mAb from a proprietary CHO cell line [140]. The PCC process ran uninterrupted for 30 days, with the continuous recovery of mAb between 70-110%. Compared to a batch chromatography process, the

three-column PCC had a resin capacity of 120%, buffer usage of 75%, and column volume of 3.9% [140]. These comparisons to the batch process highlight the ability of a PCC process to greatly reduce facility size with smaller columns and buffer tanks.

Although these continuous chromatography processes show promise for use in a continuous downstream processing train, several drawbacks exist. First, these processes are not truly continuous, as they require either discontinuous loading onto different columns, or interrupted outlets from valve switches. These issues could lead to the necessity of holding tanks, which increases facility size. Additionally, many chromatographic processes were not designed or optimized for use in viral particle purification, as shown with the limited studies. Lastly, chromatography steps account for up to 60% of downstream processing costs in mAb manufacturing, as Protein A resin can cost over \$1,000,000 for a 1-meter diameter column [145]. For these reasons, chromatography-free downstream processing trains may be an attractive option.

2.2.2 Aqueous two-phase systems

The use of aqueous two-phase systems (ATPSs) could be one solution to replacing chromatography-intensive downstream processing trains. An ATPS is a liquid-liquid extraction technique that can partition biomolecules of different properties to different liquid phases for separation. ATPSs are scalable, do not require labels, are biocompatible, and can be easily operated continuously [146, 147].

2.2.2.1 Formation and characterization

ATPSs are formed by mixing two phase-forming components that are rich in water above certain critical concentrations. The phase-forming components can be water-soluble polymers, salts, sugars, or alcohols [147]. When two of these components are mixed above certain critical concentrations, they separate into two phases – like how oil and water separate when mixed. Phase separation is dominated by three main forces: gravitational, buoyant, and frictional, which act on the phase droplets formed as they coalesce [148]. The density of each phase-forming component affects the gravitational force, and rheological properties such as dynamic viscosity affect the buoyant and

frictional forces [148]. The interfacial tension also plays an important role in the separation of the phases. In the case of a polymer-salt system, the large difference in viscosity plays a major role in phase separation as the salt phase coalesces in the polymer-rich phase and must travel downward through the viscous polymer. For this reason, polymer-salt systems tend to have higher separation times than salt-salt and other less viscous systems. This phenomenon is important when an ATPS is designed for biomolecule separation where a rapid separation is desired; different systems can have vastly different biomolecule separation efficiency.

A phase diagram is used to show the different regions of an ATPS. A phase diagram is specific for each biphasic system of two phase-forming components and can change with temperature, pH, ionic strength, and molecular weight of components. A phase diagram for a polymer-salt system is shown in **Figure 2.16** [7]. The binodal curve on the phase diagram separates the one-phase and two-phase region and is found by titrating the two phases until a turbid mixture, indicative of a two-phase mixture, is formed [7]. Any system formed on a unique tie line within the two-phase region will separate into phases with the same concentration of polymer and salt. For example, the ATPS formed on system point (S) will separate with a polymer-rich top phase at point (T), and a salt-rich bottom phase at point (B). This is true for any system formed on the same tie line connecting points T and B. Tie lines are described by their length (TLL), which is calculated by the length between points T and B [147].

A particular system point can be uniquely identified by the tie line ratio (TLR) calculated as the ratio of lengths between the system point (S) and the top point (T) to the system point (S) and the bottom point (B). Although each system point along a tie line eventually separates into the same top and bottom phase composition, there are differences in mixture properties, especially dynamic viscosity in a polymer-salt system. At low TLR, a higher volume of polymer is present, which makes the total mixture more viscous than a high TLR system on the same tie line. This consideration is important when designing an ATPS where easy fluid processability is necessary, such as in a continuous system. Another important consideration for different systems along tie lines is the differences in biomolecule partitioning.

Figure 2.16: Phase diagram of a polymer-salt ATPS [7]

2.2.2.2 Theories of biomolecule partitioning

When biomolecules are introduced into an ATPS, their partitioning is governed by numerous factors. Phase forming component concentrations, ionic strength, system temperature and pH, and biomolecule properties all affect partitioning to different phases. These factors are all combined into an overall partitioning coefficient, K , which is the concentration ratio between biomolecules in the top phase over the bottom phase at equilibrium ($K = C_T/C_B$), shown in **Equation 2.4**:

$$\ln K = \ln K_e + \ln K_h + \ln K_a + \ln K_s + \ln K_c + \ln K_o \quad (2.4)$$

where K_e is the contribution of partitioning due to electrochemical factors, K_h is the hydrophobic factor, K_a is the affinity factor, K_s is the biomolecule size factor, K_c is the biomolecule conformation factor, and K_o is all other factors such as external environmental factors [149]. Although it is most accurate to consider all of these factors for partitioning behavior [150], the K_e and K_h factors are the ruling factors for partitioning in polymer-salt systems [148, 149].

Two leading and debated theories for biomolecule partitioning exist. The first is based on the excluded volume theory of polymers, which states that the presence of a first molecule (polymer) disallows the presence of a second molecule (biomolecule) [151]. This mechanism suggests the presence of large-chain polymer molecules geometrically

block biomolecules' partition to the polymer-rich phase, making them partition to the opposite, less sterically-hindered phase [152, 153]. This effect was demonstrated by comparing the PEG-rich recovery trends as PEG MW increased using protein from *E. coli* cell lysate during the production of plasmid DNA [153]. As PEG MW was increased from 600 to 4000 Da, protein recovery in the PEG-rich phase always decreased, independent of system pH and lysate load, suggesting the cause of the partitioning was due to an increased steric hindrance of higher MW PEG [153]. However, this effect was directly the opposite in a partitioning study using porcine parvovirus (PPV) with PEG MW from 3000 to 12000 [154]. In this study with PPV particles, an increase in the PEG MW lead to a higher recovery of PPV in the PEG-rich phase [154]. The second biomolecule partitioning theory is based on electrostatic and hydrophobic interactions between the biomolecules and phase-forming components. This theory suggests that biomolecules are salted-out from the salt-rich phase to the interface and then hydrophobic interactions pull the biomolecules from the interface to the PEG-rich phase [7, 154, 155].

2.2.2.3 Purification of proteins, enzymes, nucleic acids

ATPSs have been studied extensively for the purification of proteins [156-164], enzymes [165, 166], and nucleic acids [153, 167-169]. These systems have been run in both batch-mode and continuous-mode with varying degrees of success, summarized in **Table 2.2**.

Table 2.2: Summary of selected batch-mode (B) and continuous-mode (C) ATPSs for the purification of proteins, enzymes, and nucleic acids

ATPS	Type	Biomolecule	Recovery (%)	Purity (%)	Ref
PEG 3350/citrate	B	Human IgG from CHO cells	99	76	[156]
PEG 3350/citrate	B	Human IgG from CHO cells	97	72	[157]
PEG 600/citrate/sulfate	B	Plasmid DNA from <i>E. coli</i>	91	17	[167]
PEG 1000/citrate	C	A-lactalbumin from whey	86	86	[158]
PEG 1450/phosphate then PEG 300/phosphate	B	Plasmid DNA from <i>E. coli</i>	89	Pure by SDS	[168]
PEG 600/sulfate	B	Plasmid DNA from <i>E. coli</i>	67	Pure by SDS	[153]
PEG 1500/phosphate	B	mAb 2F5	95	Pure by SDS	[159]
PEG 550/phosphate	C	Ascorbic oxidoreductase from <i>curcubita maxima</i>	236 ^a	34 ^b	[165]
PEG 3350/phosphate	C	Human IgG from CHO cells and PER.C6 cells	80% (CHO) 100% (PER.C6)	99	[160]
PEG 1000/phosphate	C	C-phycocyanin from <i>spirulina maxima</i>	50	5.8 ^c	[161]
PEG 6000/dextran 500000	B	mAb anti-CD34 from hybridoma cells	84	99.9	[164]
PEG 1000/phosphate	C	Whey protein isolate	90	Pure by SDS	[163]
PEG 1000/phosphate	C	Invertase from spent yeast	31	-	[166]

^arecovery of enzyme activity

^bpurification factor (ratio of enzyme activity after purification to activity in crude extract)

^cpurity index (A_{620}/A_{280})

A commonly studied application of batch-mode ATPSs is for the harvest and primary recovery steps of monoclonal antibody (mAb) manufacturing. This was demonstrated in a study where an ATPS of PEG 3350/6000 Da and dextran 500,000 was used to purify anti-CD34 mAbs produced in hybridoma cells [164]. In this study, a PEG-rich mAb recovery of $84 \pm 7\%$ with $0.1 \pm 0.2\%$ of total cells was achieved in an ATPS of PEG 6000/dextran 500,000 with 150 mM NaCl at pH 3 [164]. This study showed that the conventional clarification step of centrifugation or microfiltration can be skipped by using a one-step ATPS for the primary recovery of mAbs directly from the bioreactor harvest. However, one consideration not addressed in the study is the subsequent downstream purification steps since the purified mAbs are left in a viscous PEG-rich

phase that could pose processing issues. This issue was addressed by introducing a back-extraction step after the first stage of ATPS to partition an IgG product from a PEG-rich phase to a citrate-rich phase for easier processing [156]. The first stage was a system of PEG 3350-citrate with 15% NaCl to recover 99% of the human IgG in the PEG-rich. The second stage introduced fresh citrate to decrease the NaCl concentration to 5%, which back-extracted the IgG product to the citrate-rich phase with 99% recovery and an overall HPLC purity of 76% [156].

Another option to avoid the desired product from being left in a viscous PEG-rich phase was demonstrated by combining an ATPS with hydrophobic interaction chromatography (HIC). A bottom phase from ATPS that was compatible with the loading step of hydrophobic interaction chromatography (HIC) was designed to eliminate any buffer-exchange steps between ATPS and HIC [157]. The bottom phase was 10 w/w% PEG 3350 and 12 w/w% citrate at pH 6, which recovered 97% IgG and 28% overall protein [157]. The citrate-rich bottom phase was directly loaded onto the HIC column and resulted in 99% IgG recovery and 91% purity. While this study shows promise for developing an integrated downstream purification train, these unit operations were all performed in batch-mode. To boost the productivity of purification processes, they could be run in continuous-mode.

The design of continuous ATPSs for biomolecule purification has resulted in the implementation of various devices, such as column contactors and mixer-settler units, to achieve phase mixing and separation [170]. Coming from the chemical industry and traditional liquid-liquid extraction processes, column contactors, such as spray columns, perforated rotating disk contactors (PRDCs), and pulsed cap columns, have been implemented in various continuous ATPSs. A PRDC is constructed by enclosing perforated disks attached to a central rotating shaft, shown in **Figure 2.17**. PRDCs have advantages of high throughput, enhanced mass transfer, and ease of continuous operation. The disadvantages of PRDCs are column flooding and poor separation efficiency at high flow rates [170].

Figure 2.17: Perforated rotating disk contactor for PEG-citrate continuous ATPS

One PRDC was used for an attempted purification of α -toxin (a zinc-metalloenzyme, which can cause hemolysis and necrosis) from *Clostridium perfringens* broth [171]. In this system, PEG 8000 and phosphate were used as the polymer-salt phase forming components inside a 170 mm column with a 32 mm diameter and six perforated disks. A phosphate salt solution containing the α -toxin flowed into the top of the PRDC, and the PEG solution flowed in through the bottom of the column. Optimum conditions for continuous extraction of α -toxin were not found, due to column flooding, highlighting the possible issue of using PRDCs in continuous ATPS processes [171].

Another option for continuous ATPS operation is using mixer-settler units. These types of units achieve proper ATPS formation and separation with a mixing stage (either static or dynamic) and a settling stage (settling tank). This type of continuous ATPS setup has the advantage of being easily constructed and tested compared to column contactors. However, the optimization of mixing and settling in a continuous fashion may be difficult to achieve [170]. One mixer-settler based ATPS process was designed and tested for the partitioning of whey protein isolate (WPI). This simple design utilized a static glass-bead filled mixer followed by separation tubing and, lastly, by a separation tank for phase separation, shown in **Figure 2.18**. In this continuous ATPS prototype, up to 90% recovery of WPI was achieved, which showed the great potential of ATPS to be run in continuous-mode with a mixer-settler unit for recovery of biomolecules [163]. Another

mixer-settler setup for continuous ATPS was used to partition *Cerrena unicolor* laccase in a PEG 6000/phosphate system. Over 5 hours of continuous operation, with 1 hour to reach steady-state, 60% of laccase was recovered in the bottom phosphate-rich phase [172]. These studies show the potential use of mixer settler units to recover proteins and enzymes, but few studies have been developed for continuous virus recovery.

Figure 2.18: Continuous ATPS utilizing static mixer and continuous separator for whey protein isolate partitioning [163]

2.2.2.4 Purification of virus and virus-like particles

Viruses and virus-like particles (VLPs) have seldomly been used in ATPSs, due to complex partitioning behavior. Additionally, viruses and VLPs used in continuous ATPSs have not been studied like proteins and enzymes. However, ATPSs have the potential to be used as a viral particle purification technique, as evidenced by the studies of ATPSs with other biomolecules. The vaccine and gene therapy manufacturing industries would benefit greatly by implementing an optimized ATPS for high recovery and purification of viral particles produced upstream. However, the partitioning behavior of viral particles in various ATPSs must be explored and understood prior to industrial implementation. Batch-mode ATPSs used for the purification of viral particles are summarized in **Table 2.3**.

Table 2.3: Summary of batch mode ATPSs for the purification of viral particles

ATPS	Biomolecule	Recovery (%)	Purity (%)	Ref
PEG 400/phosphate	Rotavirus VLPs from High Five insect cells	85	89-94	[173]
PEG 6000/citrate	FMDV from BHK-21 cells	73	90	[174]
PEG 12000/citrate	PPV from PK-13 cells	79	Pure by SDS	[7]
PEG 12000/citrate	HRV from HeLa cells	36	Pure by SDS	[7]
PEG 8000/phosphate	Bacteriophage T4 from <i>E. coli</i>	38	-	[175]
PEG 12000/citrate	PPV from PK-13 cells	64	Pure by SDS	[154]
PEG 400/phosphate	Human B19 parvo VLPs from Sf9 cells	64	91	[176]
PEG 300/phosphate	Adenoviral vectors from HEK-293 cells	90	-	[177]
PEG 8000/sulfate	Adeno-associated virus from HEK-293 cells	95	Pure by SDS	[178]
PEG 400/phosphate	Bacteriophage M13 from <i>E. coli</i>	80	-	[179]

To address the challenges of complex partitioning behavior of viral particles in ATPS, one study aimed to assess the partitioning behavior of PPV and human rhinovirus (HRV) at various tie lines in a PEG 12000/citrate ATPS [7]. By using two non-enveloped viruses with different pI but similar size within the same system, partitioning behavior was compared at a constant system pH of 7. When PPV was used in the ATPS, 79% recovery was obtained at high purity (by SDS), but only 36% of HRV was recovered [7]. The explanation for this was that, at a system pH of 7, PPV is negatively charged (pI 5), which is readily salted-out of the citrate-rich phase to the interface where it is then pulled to the PEG-rich phase via hydrophobic interactions [7]. However, HRV with a pI of 6.9 has a net zero charge at the system pH, which is not readily salted-out by the citrate-rich phase, lowering the PEG-rich phase recovery. The main outcome from this study was that virus physicochemical properties, such as surface charge and hydrophobicity, play a key role in partitioning behavior in ATPSs and must be considered when designing a new process for a different virus.

One potential issue with using ATPSs for the purification of complex viral particles is inactivation by high concentrations of phase forming components. This was demonstrated in one study for the attempted purification of bacteriophage T4 in a PEG

8000/phosphate system. In this system, high concentrations of PEG and phosphate negatively impacted the infectivity of bacteriophage T4, making quantification via an infectivity assay (plaque assay) unreliable [175]. To solve this challenge, different ATPS components would need to be screened with bacteriophage T4 alone prior to using them in a full ATPS.

One of the best examples of an ATPS with viral particles was the purification of foot-and-mouth disease virus (FMDV) from clarified HEK-293 cell lysate [174]. This 1000-g ATPS utilized a three-stage extraction process to use the first two stages for contaminant protein removal, and the third stage for back-extraction to a citrate-rich phase. Overall, the PEG was removed, 72% of FMDV was recovered, and 90% of the total protein content was removed [174]. This recovery greatly exceeds the currently acceptable industrial viral particle recovery of <30% [180]. To further improve this process, it could be transformed into a continuous three-stage ATPS, possibly using a mixer-settler unit for each stage.

Another ATPS process was used for the purification of rotavirus VLPs as a potential vaccine candidate [173]. Rotavirus VLPs were produced in High Five insect cells using the baculovirus expression system, extracted from cells via disruption, and purified using a PEG 400/phosphate ATPS. The ATPS purification strategy was directly compared to a standard CsCl gradient strategy, showing that 85% of VLPs could be recovered using ATPS, which compared favorably to the CsCl gradient technique with only 2% VLP recovery [173]. The purity of recovered VLPs was 89-94%, which was similar to a purity of 91% by the CsCl gradient. Additionally, the ATPS technique only required two hours of processing time, whereas CsCl gradient purification required more than 24 hours [173]. However, this two-hour processing time could be lowered by removing the centrifuge step in the phase separation process by possibly converting it to a continuous ATPS.

Since the literature on viral particles used in ATPSs is limited, my work focused on developing and testing a continuous ATPS for the purification of viral particles, discussed in Chapter 5. The numerous benefits that continuous unit operations bring to industry are driving the biomanufacturing industry to implement continuous operations.

Since ATPSs can be easily scaled up and run in continuous-mode, the future of downstream processing trains may be ATPS-based.

3 Flexographic-printed graphene biosensors for electrochemical analyte detection

3.1 Introduction

In this chapter, results from testing graphene ink printed biosensors are summarized. The graphene biosensor project first started by fabricating sensors using pre-made graphene/cellulose paper sheets from XG Sciences. The XG Science paper-based sensors lead to the rapid and specific detection of four different proteins applied to the sensor surface via the conductometric detection technique [40]. With this first publication, the graphene biosensors were on track for being applied to detect other analytes, specifically a malaria biomarker. After 2012, XG Sciences changed their production method for the graphene/cellulose blended paper. This change in production method lead to the failure of biosensors constructed from it. Due to this, we began printing our own graphene biosensors onto various paper and non-paper substrates using XG Sciences' graphene-based ink. By starting with a graphene ink that could be applied to any surface desired, there was an opportunity to fully customize the graphene biosensors for analyte detection. These optimizations included different printing substrates, anilox ink roll size, number of printing coats, and different ink properties. The goal of printing custom graphene ink devices was to restore the electrochemical protein detection ability lost from XG Sciences' graphene/cellulose paper, and to use the sensors as point-of-care (POC) devices for other analytes of interest.

Paper-based POC biosensors have been developed to provide inexpensive, disposable, and rapid detection of various diseases. Not only do these devices benefit developing countries, they can also benefit rural parts of developed countries, as they do not require extensive lab equipment or long periods of time. The major advantages of such devices is the simple testing and results read-out, and the ability of wicking aqueous samples through the paper to transport necessary reactants/sample buffers to different areas of the device without the use of pumps [181, 182]. For example, one immunochromatographic strip on nitrocellulose was used to detect foot-and-mouth

disease virus (FMDV) by creating a visible test line using gold nanoparticles in five minutes, similar to an at-home pregnancy test [183]. With rapid POC devices, results can be obtained for diagnosis without a patient ever leaving the test site, reducing follow-up exams [28, 184].

These POC devices need a way to quantify the biomolecule they are designed to detect. The most common technique for quantification of the analyte is through electrochemical detection, discussed in section 2.1.2. Using electrochemical detection, it is possible for the biological information to be processed to an electric signal [28]. However, for these electrochemical techniques to be implemented, a conductive species must be used in the construction of the sensors to carry the electric signal. Our graphene-based sensors avoid this issue by being intrinsically conductive. When an analyte is applied to the graphene sensor surface, a change in the electrical properties of the device can be directly measured.

In this chapter, each sub-section of results will be in order of logical optimizations tested to restore the sensors' ability to detect the presence of protein on the surface.

3.2 Biosensor testing and theory

A circuit board with permanent copper leads was used for rapid testing of up to five graphene biosensor sheets, with the full sensor testing arrangement shown in **Figure 3.1**. To test a graphene biosensor sheet, the amperage was recorded at constant volts (typically between 5-10 mV) for the dry sheet and buffer-only application as starting baselines. Using a micropipet, 10 μ L of the lowest concentration of protein (0.01 mg/mL) was applied to the surface. The amperage was recorded after 30 seconds, 40 seconds, and 50 seconds and averaged for the amperage change resulting from that protein concentration. The solution was wicked from the surface and increasing concentration of protein (0.1 – 5 mg/mL) was applied to measure the resulting change in amperage.

Figure 3.1: Graphene biosensor testing lab set-up

To quantify the presence of varying protein concentration on the surface of the graphene biosensor, sheet resistivity (SR) was calculated from measured amperage changes at constant voltage across a 1-cm wide graphene sheet, shown in **Figure 3.2**. The SR (Ω/sq) was calculated using **Equation 3.1**

$$SR = \frac{V \cdot W}{I \cdot L} \quad (3.1)$$

where V (volts) is the constant potential difference applied across the copper leads, W (cm) is the width of the graphene sheet, I (amps) is the resulting current through the graphene sheet, and L (cm) is the distance between electrodes where the potential is applied (constant, 1 cm).

Figure 3.2: Five sheets of two-terminal graphene/cellulose biosensors applied to reusable circuit board

The Langmuir Isotherm was used to explain and model the change in SR with an increase in the concentration of protein adsorbed to the sensor surface. The Langmuir Isotherm has a few basic assumptions: 1) the surface for adsorbing is perfectly flat and homogeneous, 2) the adsorbing material (protein) adsorbs into an immobile state, 3) all binding sites on the surface are energetically equivalent and require the same binding

energy, 4) each binding site binds exactly one species of adsorbing material, and 5) there are no intra-site interactions between adjacent sites [185]. A simple graphical representation of the Langmuir Isotherm where a solid surface (grey box) with binding sites (black curves) is adsorbing an analyte of interest (red circles) can be seen in **Figure 3.3**. As seen in **Figure 3.3**, the adsorption rate slows as the concentration of adsorbate increasing due to the limited number of binding sites become filled. In the case of the biosensors, the red circles are proteins and the binding sites are individual graphene nanoplatelet sheets. By measuring the SR change as protein concentration increases, we can obtain a Langmuir Isotherm curve like **Figure 3.3**.

Figure 3.3: Langmuir Isotherm representation with solid surface binding sites adsorbing an analyte of interest

The Langmuir Isotherm used to relate the SR change to protein concentration can be seen in **Equation 3.2**

$$\frac{C}{\Delta SR} = \frac{C}{\Delta SR_{max}} + \frac{K_d}{\Delta SR_{max}} \quad (3.2)$$

where ΔSR (Ω/sq) is the change in SR at the protein concentration point compared to the buffer-only application, ΔSR_{max} (Ω/sq) is the maximum change in SR between a protein application step (typically the highest concentration) and buffer-only application step during sample testing, C (mg/mL) is the protein concentration applied, and K_d (mg/mL)

is the equilibrium dissociation constant. The data collected from a tested graphene sheet can be normalized by $\Delta SR_{\max}$ to compare with other sheets by **Equation 3.3**

$$\frac{C}{\Theta} = C + K_d \quad (3.3)$$

where $\Theta = \Delta SR / \Delta SR_{\max}$. Using **Equation 3.3**, K_d is determined for an analyte applied to the surface during testing. The dissociation constant is a measure of the sensitivity of the sensor, which is dependent on the analyte size. A lower K_d value corresponds to a higher amount of protein bound to the sensor surface, increasing the sensitivity of the sensor, shown in **Figure 3.4** [186]. The K_d represents the equilibrium adsorption between the ligand graphene surface (L) and protein (P). When a protein adsorbs to the graphene surface, a complex between the ligand and protein ($P \cdot L$) is formed.

Figure 3.4: Dissociation constant (K_d) definition and chemical equilibrium between graphene surface ligand (L) and protein (P) absorbate [186]

3.3 Results and discussion

3.3.1 Results from 2013 graphene biosensor publication

Graphene biosensors constructed and tested prior to 2012 lead to the stacked graphene nanoplatelet paper sensor publication in 2013 by Heldt *et al* [40]. These biosensors were two-terminal sensors constructed on standard glass slides, seen in **Figure 3.5**. The graphene/cellulose biosensors were able to detect and distinguish between various proteins present on the sensor surface including bovine serum albumin (BSA), chicken egg white lysozyme (LYS), bovine hemoglobin (HEM), and bovine fibrinogen (FIB). These proteins had a range of isoelectric points, hydrophobicity, and molecular weights. Increasing concentrations of each protein was applied to the graphene sensor surface and the electrical surface resistivity (SR) was measured.

Figure 3.5: Two-terminal graphene/cellulose biosensor constructed on glass slide [40]

By fitting the SR versus protein concentration data to the Langmuir Isotherm, the equilibrium dissociation constant (K_d) was determined for each protein. The main finding was that the K_d was a function of the protein molecular weight due to protein adsorption to the graphene surface, seen in **Figure 3.6**. One of the major issues with the graphene/cellulose sensors is the swelling effect when liquid is applied to the sensor surface. Due to the swelling, SR increased by more than three times, likely due to the stacked graphene nanoplatelets separation during liquid application, which reduced the graphene-to-graphene contacts.

Figure 3.6: Equilibrium dissociation constant as a function of molecular weight for LYS, HEM, BSA, and FIB [40]

3.3.2 XG Sciences' graphene paper manufacturing change

In 2013, XG Sciences changed their graphene/cellulose paper manufacturing process. Due to this change, the lab began to see inconsistencies in protein detection compared to the results obtained from the stacked graphene sensor publication. Biosensors created with paper after 2013 resulted in limited increase in SR with increasing protein concentration. As a control, the buffer used to dissolve the BSA, phosphate buffered saline (PBS), was applied to the sensor surface to compare increases in SR between the protein and buffer, seen in **Figure 3.7**. Without the ability to

distinguish between PBS and BSA applications on the sensor surface, the sensors do not work.

Figure 3.7: Graphene (November 2014 batch) sensor ability to distinguish between PBS and BSA applications

Between 2014 and 2016, work was done by previous graduate student, Soontorn Tuntithavornwat, to chemically alter the graphene paper with treatment of 2 w/w% glutaraldehyde (GA), 1 w/w% zinc nitrate (ZN), and 1 w/w% poly(vinyl alcohol) (PVA) to improve the wet and dry strength of the paper. This strength improvement was hypothesized to decrease the swelling effect of buffer application to the surface, which should improve sensitivity of SR measurements with protein application. Treatment with the GA, ZN, and PVA mixture improved the wet strength thirteen times (from 0.5 mPa to 7.2 mPa) while maintaining protein sensing ability, seen in **Figure 3.8**. However, tests comparing protein application versus buffer-only application were not performed to confirm the sensing ability of the treated sensors was specific to protein.

Figure 3.8: BSA sensing ability of GA/ZN/PVA-treated graphene sensors

3.3.3 Graphene ink printing for sensor creation and protein detection

When I joined the Heldt lab in May 2016, we looked for an alternative method to creating the graphene sensors, as the paper received from XG Sciences post-2013 was not performing the same way as paper pre-2013. This alternative method was printing XG Sciences' graphene ink onto various paper and non-paper substrates. The flexographic printing method was chosen because it can use water-based and non-water-based inks and is compatible with a variety of printing substrates. The flexographic proofer (**Figure 3.9A**) used to print the graphene ink lines (**Figure 3.9B**) was purchased from Harper Scientific. Many variables affect the print quality when using the flexographic printing method, including anilox size, ink properties, substrate type, and number of coats. A vital ink property that affects flexographic printing is the ink viscosity. A suitable ink viscosity was determined to be 518 cS, measured with a Zahn cup number 5. The viscosity of the ink was measured prior to every experiment to keep the viscosity constant across substrates. The other printing parameters were changed to determine proper feature formation and sensor detecting ability.

Figure 3.9: A) Flexographic proofer used to print B) graphene ink lines

3.3.3.1 Determination of necessary printing coats

First, the number of coats needed on a paper substrate was determined with SR measurements. Low SR was desired to confirm when enough ink was applied to the substrate surface to allow for proper electron transport across the printed graphene ink. One, two, and three coats of ink were printed on copy paper, and SR was recorded with applying PBS on the surface multiple times, seen in **Figure 3.10**. Three coats (or passes)

was necessary on copy paper to achieve a minimum SR variation across PBS applications, so all future sensors were made from three passes of ink.

Figure 3.10: SR change with one, two, and three coats of graphene ink on a paper substrate

3.3.3.2 Anilox sizing for paper and non-paper substrates

The next optimization step was to determine the size of anilox needed to transfer ink to the paper substrate to print the necessary features. We received two anilox sizes: 75/26 and 70/36. The numbers that define an anilox size have specific meanings. The first number is used to define the transfer volume of ink to a substrate surface. The transfer volume is given in units of billion cubic microns (BCM) of ink per square inch of substrate (BCM/in^2). The second number is used to define the number of cells per inch (LPI) across a straight line on the anilox, also called cell finesse. A larger LPI means more cells per inch, resulting in a smaller cell volume. To determine which anilox performed the best with unmodified graphene ink, three coats of ink were printed onto standard copy paper and SR was measured with increasing BSA concentration on three individual 1 cm lengths of paper. Both anilox produced similar SR measurements, but anilox 70/36 had less variation across sheets tested, and produced a higher ΔSR ($\text{SR}_5 \text{ mg/mL BSA} - \text{SR}_{\text{buffer}}$), seen in **Figure 3.11**. From this result, anilox 70/36 was chosen for all future printing onto paper substrates.

Figure 3.11: SR measurements using anilox 70/36 and 75/26 comparison with increasing BSA concentration

A different size anilox was needed for printing onto non-paper substrates due to the surface roughness and porosity differences between substrates. We received anilox size 120/20 and 200/14 to print onto poly(ethylene terephthalate) (PET) and Tyvek type 1079. These substrates were chosen for controls as they are non-porous, non-swelling, and hydrophobic. Printing with these two anilox showed that only one coat of graphene ink was needed on the surface of each substrate, likely due to the low surface roughness compared to the paper substrates. To determine which anilox roll produced the best graphene sensor sheets, an increasing concentration of BSA was applied to sensors constructed from each roll and substrate type. The criteria for “best sensors” were achieving low SR, low SR variance, plus low and consistent K_d values. Ten sensors were constructed from each anilox roll on PET and tested with increasing BSA concentration applications from 0 to 5 mg/mL, seen in **Figure 3.12**. K_d values were calculated for each sensor, and the average K_d /standard deviation were found for each anilox. Sensors from anilox 200/14 gave a K_d of 1.64 ± 0.46 μ M, and sensors from anilox 120/20 gave a K_d of 1.64 ± 0.35 μ M. Both anilox rolls produced sensors that performed similarly for BSA detection, however, anilox 120/20 produced sensors that started at a lower SR. Tests on the PET substrate alone was not enough to determine which anilox was best. The same anilox were tested on another non-paper substrate, Tyvek.

Figure 3.12: SR values with increasing BSA concentration from sensors constructed using PET with anilox A) 200/14 and B) 120/20

Five sensors constructed from printing graphene ink onto Tyvek type 1079 were tested for their ability to detect BSA, shown in **Figure 3.13**. The K_d from sensors made with anilox 200/14 was $1.76 \pm 0.72 \mu\text{M}$, and $1.89 \pm 0.63 \mu\text{M}$ for anilox 120/20. While comparing K_d values between anilox rolls was inconclusive, considering dry device SR values between anilox for both PET and Tyvek substrates, anilox 120/20 performs the best. For these reasons, anilox 120/20 was chosen for printing on non-paper substrates.

Figure 3.13: SR values for increasing BSA concentration from sensors constructed using Tyvek with anilox A) 200/14 and B) 120/20

With the number of coats and anilox roll necessary for printing on each substrate, sensors were created and tested for their ability to detect BSA versus PBS-only applications. Two paper substrates were tested first: conventional copy paper and chromatography-grade paper. Copy paper was chosen because it is inexpensive and readily available. Chromatography paper was chosen for its controlled purity by the manufacturer, ensuring we could receive the same quality of paper in the future.

3.3.3.3 Protein detection with paper and non-paper sensors

The first graphene ink biosensors tested with BSA and compared to PBS applications were printed on 11"x17" copy paper. The average Δ SR for the five devices tested with BSA was 18 Ω /sq, and the average Δ SR for the five devices tested with just PBS was 2.3 Ω /sq, seen in **Figure 3.14**. Due to the large error, no statistically significant difference was found, meaning there was no reliable way to determine if BSA or PBS was present on the surface of the sensor. The reason for this is hypothesized to be the swelling effect of the graphene/cellulose devices. As any liquid is placed on the sensor surface, the liquid swells the paper and causes sheets of graphene to become separated, regardless of protein presence.

Figure 3.14: Average SR increase with increasing BSA concentration applications and PBS applications on three-coat, copy paper devices printed with anilox 70/36

Next, graphene ink biosensors were printed onto chromatography filter paper. The same BSA versus PBS application test was used for sensors printed on chromatography paper. The average Δ SR for the five devices tested with BSA was 15 Ω /sq, and the average Δ SR for the five devices tested with PBS was 6 Ω /sq, seen in **Figure 3.15**. The average SR difference between BSA-tested and PBS-tested devices was not significantly different. This was additional evidence that any liquid on the sensor surface would produce an SR change, regardless of protein presence. To limit the swelling effect of the paper-based printing substrate, two non-paper substrates were tested: Tyvek and poly(ethylene terephthalate).

Figure 3.15: SR curves for A) BSA and B) PBS tested three-coat, graphene ink devices on chromatography filter paper

Sensors were printed onto PET film and tested for BSA detection versus PBS-only application. The PET film is extremely hydrophobic, which should reduce the swelling effect of the paper substrates and allow for the detection of protein presence based on an increase in the sheet resistivity. The devices with BSA application had an average ΔSR of 22 Ω/sq , and the devices with PBS-only application had an average ΔSR of 28 Ω/sq , seen in **Figure 3.16**. Again, with similar average change in SR between BSA and PBS-only applications, the devices were not able to detect protein on the surface of the sensor. This result indicates that the swelling of the porous substrates was not the only effect causing similar SR changes in the tests. This result shows that any liquid present on the sensor surface causes the SR to change.

Figure 3.16: SR curves for A) BSA and B) PBS tested one-coat, graphene ink devices on PET film

As another control for a non-paper substrate, Tyvek grade 1079 was used for sensor printing and tested for its detection ability. Devices tested with BSA had an average Δ SR of 27 Ω/sq , and the devices tested with PBS-only had an average Δ SR of 23 Ω/sq , seen in **Figure 3.17**. Again, the same result as PET film was obtained with Tyvek grade 1079. These results combined show that liquid present on the sensor surface is causing the change in SR, not purely the presence of protein on the surface. One possible reason for this could be the fact that liquid has the ability to contact the electrical leads during resistivity measurements, which could alter the electrical performance of the sensors. To test this theory, hydrophobic wax was printed onto the paper substrates to create a barrier between the testing region and the electrical leads on the sensor surface, discussed in Section 3.3.4.

Figure 3.17: SR curves for A) BSA and B) PBS tested one-coat, graphene ink devices on Tyvek grade 1079

3.3.3.4 Custom graphene ink testing

Custom graphene inks were formulated during a month-long internship at XG Sciences to increase the compatibility of ink with protein application. Specifically, poly(acrylic acid) (PAA) was added to the ink formulation solvent to make the resulting ink more hydrophilic compared to the normal ethyl cellulose used in the ink. Additionally, a different size of graphene nanoplatelets was used to formulate the ink to increase the available binding surface area from $\sim 50 \text{ m}^2/\text{g}$ to over $500 \text{ m}^2/\text{g}$. Three devices created using the PAA-custom ink were tested with BSA applications (**Figure 3.18, solid lines**), and two devices created using the same ink were tested with PB only applications (**Figure 3.18, dashed lines**). The Δ SR for BSA-tested devices was 1080

Ω/sq , and PB-tested devices had a ΔSR of $1800 \Omega/\text{sq}$. Although this ink was slightly more hydrophilic than the standard ink tested, it did not result in acceptable protein detection.

Figure 3.18: SR curves for PAA-custom graphene ink devices on GP copy paper

Three devices created using the size-custom ink were tested with BSA applications (**Figure 3.19, solid lines**), and two devices created using the same ink were tested with PB only applications (**Figure 3.19, dashed lines**). The ΔSR for BSA-tested devices was $\sim 7090 \Omega/\text{sq}$, and PB-tested devices had a ΔSR of $13000 \Omega/\text{sq}$. This custom ink had large variance from device to device, immediately making this ink unsuitable for repeatable device sensing ability.

Figure 3.19: SR curves for custom-size graphene ink devices on GP copy paper

3.3.4 Wax printing hydrophobic barriers

Printing barriers onto the paper substrates requires the optimization of 3D printing wax onto the surface of the sensor, and the subsequent heating of the wax to penetrate through the paper to form a complete barrier. 3D printing was achieved using a replication rapid prototyper (RepRap) with a heated syringe extruder for printable wax. The RepRap and the printed wax features to create a liquid flow channel can be seen in

Figure 3.20. The RepRap is only capable of printing the heated wax onto the top surface of the substrate, so additional heating of the wax was necessary to penetrate the wax through the thickness of the substrate to form the complete hydrophobic barrier.

Figure 3.20: RepRap 3D printer with printed wax lines

After graphene ink lines were printed onto copy paper and chromatography paper by the flexographic proofer, wax was 3D printed across the graphene ink lines to create the hydrophobic barriers. To fully penetrate the wax through the graphene ink lines and paper substrates, the wax was melted at 90°C for five minutes. The fully melted wax barriers on the graphene lines can be seen in **Figure 3.21**. With the hydrophobic barriers in place, the liquid application region is the ~2 mm gap between wax lines.

Figure 3.21: Hydrophobic wax barriers printed onto graphene ink lines

3.3.4.1 Wicking tests

A wicking test was designed to confirm proper wax barrier formation through the paper substrate and to show the hydrophobicity of both the wax and graphene ink line. A drop of red-dyed water was applied to one side of the paper substrate, and the time to cross to the other side of the paper was recorded (**Figure 3.22**). The wicking test was

performed on copy paper with and without the graphene line, chromatography paper with and without the graphene line, and on the cellulose/graphene paper blend from XG Sciences. As expected, the copy paper without the graphene line had the shortest time to cross at 10 seconds, and chromatography paper with the graphene line had the longest time to cross at 30 seconds. The long time to cross is because chromatography paper is thicker than the copy paper and cellulose/graphene paper blend. Interestingly, the application of the graphene line caused the time to cross to increase by 10 seconds in the case of copy paper and chromatography paper, shown in **Figure 3.23**. During the tests, no wicking to the outside of the wax lines was observed, confirming that the hydrophobic barriers were fully formed and retained the liquid between the wax lines. From this test, the graphene lines with wax barriers were used to electrochemically detect the presence of protein on the surface without liquid interference on the conductive leads.

Figure 3.22: Wicking test schematic for the wax barriers on various paper substrates

Figure 3.23: Wicking test results on paper substrates with and without graphene lines

3.3.4.2 Paper substrate device testing with wax barriers

Biosensors were fabricated by three-pass printing graphene ink onto copy paper and wax barriers were 3D printed onto the surface. Four sensors were tested for their ability to detect BSA from 0.01 to 5 mg/mL and compared to a control PB-only application sensor, shown in **Figure 3.24**. Sensors tested with BSA had an average ΔSR of 3.3 Ω/sq , and the sensor tested with PB-only had an average ΔSR of 4.3 Ω/sq . As seen in **Figure 3.24**, the PB-only sensor fell directly in the same range of SR changes as the BSA-tested sensors meaning there is no different protein application compared to buffer-only application.

Figure 3.24: SR curves for BSA-tested sensors (1-4) and PB-tested sensor (%) with 3D-printed wax barriers on copy paper

Additional biosensors were fabricated by three-pass printing graphene ink onto chromatography paper and wax barriers were 3D printed onto the surface. Four sensors were tested for their ability to detect BSA from 0.01 to 5 mg/mL and compared to a control PB-only application sensor, shown in **Figure 3.25**. Sensors tested with BSA had an average Δ SR of 33000 Ω /sq, and the sensor tested with PB-only had an average Δ SR of 760 Ω /sq. Although the comparison of Δ SR between BSA and PB sensors seems to suggest there is a significantly large increase in SR for BSA and not PB, the large variability between sensors 1-4 is unacceptable for reliable analyte detection.

Figure 3.25: SR curves for BSA-tested sensors (1-4) and PB-tested sensor (%) with 3D-printed wax barriers on chromatography paper

These results from paper-based sensors with wax barriers indicate that the liquid contact to the electrical leads is not the dominating effect of SR increase. Regardless of protein presence on the sensor surface with wax barriers, SR increases. This means the sensors are not specific enough to what was applied on the surface and the specificity must be increased for proper analyte detection. One attempt to increase the specificity of the sensors was by functionalization with primary antibodies.

3.3.5 Functionalization of graphene sensors

Before a complete sandwich ELISA system was constructed on the graphene biosensor surface, proper immobilization of antibodies on the graphene surface was tested. As discussed in section 2.1.2.2.2, the immobilization of antibodies onto graphene is accomplished by π - π stacking an amine-reactive probe onto the graphene surface and applying antibodies onto the probe for immobilization via EDC-NHS chemistry between

the NHS probe and primary amine on the F_C region of antibodies. The amine-reactive linker applied to the graphene surface was 1-pyrenebutanoic acid succinimidyl ester (PBA-NHS) (**Figure 3.26A**). After linker attachment, donkey anti-goat IgG antibodies labelled with HRP were applied and immobilized on the surface by EDC-NHS chemistry (**Figure 3.26B**). Ethanolamine was used to cap unreacted PBA-NHS linkers (**Figure 3.26C**). This functionalization procedure was performed in non-stick test tubes by adding the appropriate liquid component and rocking. After numerous washing steps, a 3,3',5,5'-tetramethylbenzidine (TMB) substrate and hydrogen peroxide was added. TMB is a colorless chromogenic substrate for HRP which acts as a hydrogen donor during the redox reaction of H₂O₂ to water by the enzyme HRP. When HRP is in the presence of TMB and H₂O₂, the enzymatic reaction produces a blue colored 3,3',5,5'-tetramethylbenzidine diimine. The reaction is stopped using sulfuric acid which turns the solution color to yellow and can be read at 450 nm for quantification.

Figure 3.26: Primary antibody immobilization onto graphene sheet

To test the immobilization procedure, sheets of graphene were fully functionalized and the absorbance at 450 nm was compared to non-functionalized graphene sheets, sheets with PBA manually pipetted onto the surface, and sheets without the PBA-NHS linker added. Additionally, plain copy paper was used as a negative control for functionalization. Functionalized graphene sheets had an average absorbance of 1.4 ± 0.4 and manually applied PBA functionalized graphene sheets had an average absorbance of 1.7 ± 0.5 , suggesting that the PBA application process does not affect the immobilization of antibodies, seen in **Figure 3.27**. Non-functionalized graphene sheets had an average absorbance of 0.04 ± 0.001 , confirming that if no antibodies are added to

the sheets, no color change is observed. Functionalized plain paper, no PBA on graphene sheets, and no PBA on plain paper had average absorbances of 1.2 ± 0.6 , 0.8 ± 0.3 , and 1.4 ± 0.2 respectively. Although these values do lie within the error of fully functionalized graphene sheets, plain paper would lack the conductivity provided by the graphene to be viable in electrochemically detecting the antigen. This high absorbance from functionalized plain paper is likely due to antibodies passively absorbed into the porous paper. From this functionalization test, it was confirmed that our procedure immobilizes antibodies onto the graphene sheets.

Figure 3.27: Absorbance measurements of HRP-labelled antibody functionalized graphene sheets and plain copy paper sheets

3.3.5.1 Sandwich ELISA system design and testing

Once proper immobilization of antibodies onto the graphene sheet was confirmed, a full sandwich ELISA system was designed and tested for the specific detection of our analyte of interest, mouse IgG. To functionalize the graphene biosensors, goat anti-mouse IgG antibodies were immobilized on the surface of the graphene shown in **Figure 3.28**. The PBA-NHS linker was applied to the surface of the graphene sensor (**Figure 3.28A**). Next, anti-mouse IgG antibodies were applied to the surface to be immobilized by the NHS on the PBA and primary amines on the F_C regions of the antibodies (**Figure 3.28B**). BSA was added to the sensor surface to block non-specific binding of the antigen to the sensor surface itself, and ethanolamine was used to cap unreacted PBA-NHS linkers (**Figure 3.28C**). The antigen of interest, mouse IgG, was applied to the surface and captured by the immobilized antibodies by specific antibody-antigen interactions (**Figure**

3.28D). Finally, detecting goat anti-mouse antibodies labelled with a AuNP were applied to the antigen (**Figure 3.28E**). The detecting antibodies act as signal-enhancing molecules as the immobilization of a AuNP-labelled antibody should drastically change the electrical properties of the sensor surface.

Figure 3.28: Sandwich ELISA system on graphene sheet

To test the functionalized graphene sheets using a sandwich ELISA system, a constant concentration for the detecting antibody was used at 0.1 mg/mL and an increasing concentration of mouse IgG was applied to the primary antibody-functionalized graphene sheets from 10^{-5} to 10^{-1} mg/mL. With an increased amount of mouse IgG bound to the sensor, which would result in an increase amount of AuNP-labelled detecting antibody, a decrease in the resistivity was expected. The AuNP should act as an electron-mediator to increase the conductivity of the bound complex when electrochemically tested. Five sensors were tested, and the SR curves before (black curve) and after (red curve) detecting antibody application for individual sensors is shown in **Figure 3.29A-E**. This data is shown as individual functionalized devices to show the inconsistent results obtained for changes in the SR as mouse IgG and dAbs were applied to the surface. Sensors A, C, and D have the expected drop in SR with increasing mouse IgG concentration, and sensors B and E have an increase in the SR with increasing mouse IgG concentration. Another issue is that there should be an observable difference in SR curves before and after dAb application, and there is no such result. This suggests that the application of a AuNP-labelled species onto the graphene sheets did not

significantly change the electrical properties. Without a change in the SR during testing, there is no reliable method to detect the presence of the target analyte.

Figure 3.29: SR curves for 5 functionalized graphene sheets tested using a sandwich ELISA system for mouse IgG detection

3.4 Conclusions

The results discussed here for the graphene biosensors project show limited success in the detection of target analytes. Throughout biosensor creation, various conditions were explored to obtain positive results. Paper and non-paper substrates were used for biosensor creation with no change in SR when comparing BSA-applications to buffer-only applications. The number of ink coats, ink conductivity, and graphene sheet hydrophobicity/size were varied in attempts to change the electrical and chemical properties of the biosensors without success. Wax barriers were used to reduce swelling effects of the biosensors also without successful protein detection. Additionally, functionalization of the graphene sheets with a sandwich ELISA system was performed which also lead to insufficient data to support the detection of target analytes. Overall,

with a wide variety of parameters tested for the biosensors project, no positive and publishable results were obtained. The results discussed in this dissertation should act as a review of optimization attempts for creating graphene-based biosensors for rapid analyte detection.

4 Virus particle detection using various sizes of gold nanoparticles aggregated by osmolytes

4.1 Introduction

Up to nine different infectious diseases are tested for in blood donations in the United States [19]. These infectious diseases are detected by confirming the presence of antibodies to the disease with immunoassays or amplifying the nucleic acid from the virus itself using a nucleic acid test (commonly polymerase chain reaction). Although effective, these tests are specific to only one virus at a time, are time consuming, and expensive. A faster, non-specific test would increase throughput of samples and reduce costs by being able to confirm the presence of any viral particle in each sample. One potential way to accomplish this is utilizing gold nanoparticles (AuNPs) in a virus detection assay.

AuNPs have unique optical properties that can aid in detection and is called surface plasmon resonance (SPR). SPR dictates the AuNP's color in solution and changes with different sizes of AuNPs and their inter-particle distance [187]. SPR is a result of exciting the cloud of free electrons present near the surface of metals, including AuNPs. The natural oscillations of the free electrons on the surface of the AuNPs are called surface plasmons, which exist at the gold metal-dielectric interface [90]. When excited by light, the surface plasmon oscillations can couple with the incident light, which emits electromagnetic waves in the visible range [88, 90]. This resonant oscillation between the incident light and surface plasmons is called SPR [91]. AuNPs with a certain size have a characteristic ultraviolet-visible (UV-Vis) absorption spectrum and absorbance peak. For example, 15 nm diameter AuNPs have an absorbance peak at a wavelength of 519 nm, corresponding to a deep red solution [6].

AuNPs and other metallic nanoparticles are capable of Rayleigh scattering, which is the phenomenon of elastic scattering of light by particles smaller than the wavelength of the incident light [188]. Within the Rayleigh scattering range, which is appropriate for sub-100 nm nanoparticles, the scattering light intensity is proportional to the sixth power

of the particle diameter [189]. AuNPs light scattering ability can be used to determine their size using dynamic light scattering (DLS). It has been shown that assays developed using a light scattering detection system have a lower limit of detection up to four orders of magnitude compared to absorbance based systems [187]. For this reason, light scattering-based assays are more appropriate for detecting low concentrations of virus present in samples, which has been used to detect whole virus particles [97], viral nucleic acids [94], and viral antibody markers [190].

The detection pathway of these virus detection tests typically occurs in two steps. First, AuNPs are functionalized with virus-specific nucleic acid probes or capture antibodies (**Figure 4.1A**). Then, the functionalized AuNPs aggregate in the presence of the target viral oligonucleotides or virus particles (**Figure 4.1B**). This aggregation occurs when target virus is added because viral particles are symmetrical. The symmetrical nature of viruses allows multiple antibodies to bind to the same virus, causing a cross-linking like pattern to occur, shown in **Figure 4.1B**. The same would not occur in the presence of proteins because proteins cannot bind to multiple antibodies. The aggregation results in a measurable change in size directly measured using DLS.

Figure 4.1: A) AuNP functionalization with virus-specific antibodies and B) induced aggregation of AuNPs after the addition of target viral particles

One example of a functionalized AuNPs aggregation assay was detection of influenza virus with influenza-specific antibody functionalized AuNPs [97]. When influenza virus was added to a solution of antibody-functionalized AuNPs, aggregation occurred and was measured by DLS with a limit of detection (LOD) of <100 TCID₅₀/mL, which was 1-2 orders of magnitude lower than conventional diagnostic kits [97]. These

tests have shown improved detection ability compared to ELISA-based and PCR-based tests. However, like these conventional tests, they also depend on the use of nucleic acids and antibodies that must be immobilized onto the AuNPs, which increases preparation complexity and cost. Stability issues are also a concern when nucleic acids and antibodies are used. Thus, an unmodified AuNP, whole viral particle test is desired to reduce the cost and increase simplicity.

The goal is to create a viral particle assay using unmodified AuNPs. Without functionalizing the AuNPs, a different aggregation technique must be used. It has been well studied that NaCl has the ability to aggregate citrate-capped AuNP by neutralizing the negative charge distribution with Na^+ cations in as little as 100 mM NaCl [93, 94]. One assay was developed that utilizes NaCl to aggregate unmodified, non-stabilized AuNP. In this assay, hepatitis C virus (HCV) RNA was detected based on NaCl-induced AuNP shown in **Figure 4.2** [191]. First, extracted HCV RNA from clinical samples was added to a tube containing a hybridization buffer with the targeting RNA primer. The mixture was then denatured and annealed to allow the primer to bind to the HCV RNA. NaCl and AuNPs were then added to the tube and the color was observed after 1 minute of incubation (**Figure 4.2, Complementary Target**). In this case, the primer hybridized with the target, which left the AuNPs unprotected and thus aggregated by NaCl. In the case where a non-complementary target RNA was added in the sample tube, the RNA primers were adsorbed to the surface of the AuNPs through electrostatic interactions between the nitrogenous backbone of RNA and the AuNPs, which protected the AuNPs from NaCl-induced aggregation (**Figure 4.2, Non-complementary Target**). This assay had a LOD of 50 copies/reaction, which was 1,200 times lower than a conventional ELISA for the HCV core antigen [191]. Although this assay significantly improved upon the conventional method, it still utilized oligonucleotide probes that have stability issues. Additionally, the sample preparation steps to extract HCV RNA are complex.

Figure 4.2: Colorimetric detection pathway for HCV RNA using unmodified AuNPs via NaCl-induced aggregation [191]

One different method of causing AuNP aggregation is using osmolytes, which are known to aggregate viral particles [111, 112]. Osmolytes are water-soluble, naturally-occurring molecules that are produced by cells to enhance protein stability. Osmolytes work by combating osmotic stress and other environmental stresses to help cells maintain their structure by regulating water content within the cell [105, 106]. Osmolytes have been used for the preferential aggregation of both enveloped (Sindbis virus) and non-enveloped (PPV) viral particles [111, 112]. The theory for this preferential virus aggregation versus protein aggregation with osmolytes is based on the relative hydrophobicity between viruses and proteins and the physical structure of the two macromolecules [6, 111]. When osmolytes are introduced into solutions containing PPV or proteins, there is a partial dehydration around the biomolecules. This is because osmolytes have a higher affinity for water molecules than does the protein backbone [108]. Excess free energy is generated that must be minimized when water molecules are pulled away from the biomolecule surface. For proteins, their relatively more hydrophilic and flexible nature allows the excess free energy to be minimized by increased protein side chain interactions, resulting in protein structure compaction and stabilization [6, 111, 113]. The mechanism of free energy reduction due to osmolyte-induced dehydration in

the case of viruses is different. It has been shown that viruses are relatively more hydrophobic and rigid than proteins [113]. Due to the relatively high hydrophobicity of viruses, they are dehydrated to a greater extent than proteins when exposed to osmolytes. Since viruses cannot reduce the excess free energy by structure compaction like proteins, the hydrophobic exposed residues are minimized by virus-virus aggregation [111]. This difference in free energy reduction mechanism between proteins and viruses gives the virus detection assay its specificity because AuNP/protein complexes should not aggregate to the same extent as AuNP/virus complexes when exposed to osmolytes.

This osmolyte-induced, preferential aggregation of viral particles action was used to develop a PPV and BVDV detection assay [6]. The viral particles were capable of non-specifically binding to AuNPs through a combination of electrostatic [192] and hydrophobic interactions [104]. The hypothesized mechanism of this interaction is that viral particles have surface-exposed, positively charged patches on the capsid which can electrostatically bind with the citrate-capped AuNPs. After complexing viral particles with 15 nm AuNPs via these electrostatic interactions, mannitol was used to aggregate the AuNP/virus complexes. The addition of 1M mannitol dehydrated the viral particles and caused the complexes to aggregate. This aggregation was detected using DLS. The AuNP/virus complex size before and after aggregation was measured by DLS and compared to AuNP/protein complex size before and after aggregation. This resulted in a LOD of 6 log MTT₅₀/mL for PPV and 4 log MTT₅₀/mL for BVDV [6].

The previous virus detection study using 15 nm AuNPs served as the baseline to be improved using different sizes of AuNPs in the same aggregation assay. To improve the virus detection assay, different sizes of AuNPs were synthesized and used to potentially lower the LOD for the same viruses. To explore how different sizes of AuNPs interact with viruses and effect the assay, AuNPs with a diameter of 50 nm were used with BVDV particles (**Figure 4.3E**), and AuNPs with diameters of 5, 15, and 40 nm were used with PPV particles (**Figure 4.3A-C**). These sizes were chosen to have the size ratio between the AuNP and virus diameters to be <1, ~1, and >1, shown in **Figure 4.3**. The diameter ratios between viral particles and AuNPs were expected to change the detection ability two different ways. First, a diameter ratio >1 should lead to both a larger surface

for viruses to bind to (**Figure 4.3C and F**), and an enhanced DLS signal with larger AuNPs should be observed. This enhanced signal is expected because the intensity of the scattered signal in DLS to changes in the radius of the AuNPs has the relation of (DLS intensity $\propto$ AuNP radius⁶) [193]. Second, a diameter ratio <1 should cause smaller AuNPs to electrostatically bind to the viral particles more readily since the positively charged patches on the virus surface may be hard for larger AuNPs to access. This would result in the virus surface being more covered by the smaller AuNPs, shown in **Figure 4.3A and D**. At a diameter ratio of ~ 1 , there are less AuNPs covering the virus surface as the AuNPs would be electrostatically hindered from completely covering the surface, shown in **Figure 4.3B and E**.

Figure 4.3: AuNPs binding to A-C) PPV and D-F) BVDV particles in size ratios of <1 , 1, and 2

However, from this work, the use of the osmolytes mannitol and glycine as aggregating agents for the various sizes of AuNP/virus complexes did not result in an improved virus detection assay, as the LODs for both viruses were unchanged compared to the original assay using 15 nm AuNPs.

4.2 Materials and methods

4.2.1 AuNP and cell culture materials

Gold (III) chloride trihydrate ($\geq 99.9\%$), trisodium citrate (ACS grade, $\geq 99.0\%$), bovine serum albumin ($\geq 98\%$), D-mannitol ($\geq 98\%$), and sodium phosphate dibasic heptahydrate (ACS grade, 98.0-102.0%) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate monobasic monohydrate (ACS grade, 98.0-102.0%) and sodium chloride (ACS grade, $\geq 99.0\%$) were a gift from MilliporeSigma (Burlington, MA). Barnstead™ nanopure water (Thermo Scientific, Waltham, MA) with an electrical resistance of $\geq 18 \text{ M}\Omega$ was used to create the aqueous solutions. AuNPs with a diameter of 5 nm (typical diameter 5.3-5.8 nm) were purchased from Ted Pella (Redding, CA) and were used without any modification. The 5 nm AuNPs had a particle concentration of $8.3 \times 10^{-8} \text{ M}$.

Minimum essential medium, Dulbecco's modified eagle's medium, fetal bovine serum (Mexico origin), horse serum (New Zealand origin), 10,000 U/mL penicillin streptomycin, 0.25% trypsin-EDTA (1X), phosphate buffered saline pH 7.2 (1X), and sodium bicarbonate (7.5%) for cell culture and virus propagation were from Gibco™ (Grand Island, NY). All solutions were filtered through a 0.2 μm Nalgene™ Rapid-Flow™ sterile disposable bottle-top filter with PES membrane from Thermo Scientific into an autoclaved vessel for storage before use.

4.2.2 Virus production and purification

Porcine kidney cells (PK-13) were purchased from the American Type Culture Collection (ATCC®) (cat# CRL-6489™) and cultured in minimum essential medium (MEM) supplemented with 10 vol% fetal bovine serum (FBS) and 1 vol%

penicillin/streptomycin (pen/strep). The cells were incubated at 37°C, 5% CO₂, and 100% humidity. PPV strain NADL-2 was a gracious gift from Dr. Ruben Carbonell at North Carolina State University (Raleigh, NC). PPV strain NADL-2 was propagated in PK-13 cells as described previously [194]. After PPV propagation in PK-13 cells, the cell lysate was clarified by centrifugation at 5,000 rpm at 4°C for 15 minutes in a ST16R centrifuge with a TX-400 swing-bucket rotor (Thermo Scientific). The collected supernatant was dialyzed using Biotech cellulose ester 1000 kDa dialysis tubing at 4°C with two 4 mM phosphate buffer (pH 7.2) exchanges over two days. The dialyzed PPV was further purified using an Econo-Pac 10DG size-exclusion desalting column and used within one week of purification.

Bovine turbinate cells (BT) were purchased from ATCC (cat# CRL-1390™) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10 vol% horse serum and 1 vol% pen/strep [195]. The cells were incubated at 37°C, 5% CO₂, and 100% humidity. BVDV strain NADL was purchased from USDA APHIS. BVDV was propagated in BT cells [6, 196]. After BVDV propagation, the cell lysate was clarified and stored the same as PPV.

4.2.3 Synthesis and characterization of AuNPs with various sizes

Various sizes of AuNPs were synthesized and characterized. AuNPs ranging from ~27 to 106 nm in diameter were synthesized by the seed-mediated, room temperature growth method with trisodium citrate (Na₃Ctr) and chloroauric acid (HAuCl₄) [193]. The synthesis of larger AuNPs started with monodisperse 15 nm diameter AuNP seeds that were synthesized by the conventional Turkevich method of adding Na₃Ctr to reduce boiling HAuCl₄ [6]. By changing the volume of water and AuNP seed additions to constant volumes of HAuCl₄ and Na₃Ctr, different diameters of AuNPs were synthesized over time at room temperature, shown in **Table 4.1** [193]. The concentration of HAuCl₄ was 44.7 mM, the concentration of the seed AuNPs was 6.54×10^{12} particles/mL, and the concentration of Na₃Ctr was 38.8 mM [193].

Table 4.1: Reactants volumes for synthesis of AuNPs with various diameter [193]

AuNP size (nm)	V_{water} (mL)	V_{HAuCl4} (mL)	V_{seed} (mL)	V_{Na3Ctr} (mL)
22.7	36.220	0.227	3.377	0.176
34.2	38.786	0.227	0.811	0.176
45.7	39.270	0.227	0.327	0.176
57.1	39.432	0.227	0.165	0.176
68.6	39.502	0.227	0.095	0.176
80.0	39.537	0.227	0.060	0.176
91.4	39.557	0.227	0.040	0.176
102.9	39.569	0.227	0.028	0.176
114.3	39.577	0.227	0.020	0.176

4.2.4 Concentration determination of different AuNP sizes

The concentration of AuNPs was determined by the molar extinction coefficient method after collecting the UV-Vis spectra and size via DLS, which has been shown to be accurate for AuNPs between 5 and 100 nm [197]. The concentration of AuNPs calculated from the UV-Vis spectra method was adjusted so that all reactions would have the same binding surface area across all sizes. The concentration of 3.5 nM was used for 15 nm AuNPs for the detection of PPV and BVDV [6]. Using **Equation 4.1**, the surface area was held constant by varying the concentration of AuNPs with different diameter,

$$\frac{c_2}{c_1} = \frac{\left(\frac{D_1}{2}\right)^2}{\left(\frac{D_2}{2}\right)^2} \quad (4.1)$$

where C_1 is the concentration of the 15 nm AuNPs (3.5 nM), C_2 is the desired concentration of any other sized AuNPs, D_1 is the diameter of 15 nm AuNPs (15 nm), and D_2 is the diameter of any other sized AuNPs. This equation shows a higher concentration of larger diameter AuNPs are necessary for the virus detection assay.

4.2.5 AuNP/virus and protein complex formation

Different concentrations of PPV, BVDV, and BSA were added to the AuNPs of different sizes to form complexes to be aggregated by osmolyte or salt. PPV from 8 log MTT₅₀/mL to 4 log MTT₅₀/mL was added in a 1:1 volume ratio (total volume of 1 mL) with AuNPs and mixed overnight on a Roto-shake Genie rocker (Bohemia, NY) with end

over end rotation. The same overnight mixing was performed for BVDV from 6 log MTT₅₀/mL to 2 log MTT₅₀/mL, and BSA at 0, 1 nM, and 1 µM to form AuNP complexes. For AuNPs with diameter 40 and 50 nm, the complexes were centrifuged at 6000×g for 20 minutes in a ST16R centrifuge with a microliter 30 x 2 mL fixed angle rotor (Thermo Scientific) to remove un-complexed material in the supernatant, and resuspended in 1 M mannitol, glycine, or NaCl.

For AuNPs with a 5 nm diameter, the manufacturer did not recommend centrifugation of the complexes as they are readily aggregated by centrifugation. Instead, 250 µL of 7.0 nM AuNPs and 250 µL of PPV were combined and mixed overnight the same way as above. The concentration of aggregating agent was doubled to 2 M to allow for a 500 µL addition to achieve the same final concentration of components with a final volume of 1 mL after aggregation.

4.2.6 Quantification of aggregated AuNP/virus complexes by DLS

A Malvern Nano ZS Zetasizer (Malvern, UK) was used to collect DLS curves of intensity versus hydrodynamic diameter (D_H) for samples before and after aggregation. The software-generated Z-average for the size distribution was used as the D_H for each sample. The AuNP material properties were set at a refractive index of 0.20 and an absorption of 3.32 [198]. The water dispersant properties were set to a temperature of 20°C, a viscosity of 1 cP, and a refractive index of 1.333. Samples were placed in disposable acrylate cuvettes at a volume of 1 mL.

The quantification of AuNP/virus complexes was achieved using DLS to determine the D_H before and after aggregation by osmolyte or salt. The change in hydrodynamic diameter (ΔD_H) was used to quantify the extent of aggregation, which was inferred by measuring the change in size of the aggregates using **Equation 4.2**,

$$\Delta D_H = D_{H, \text{complexes after treatment}} - D_{H, \text{complexes before treatment}} \quad (4.2)$$

where $D_{H, \text{complexes after treatment}}$ is the hydrodynamic diameter of AuNP/virus complexes after aggregation by osmolyte or salt (nm), and $D_{H, \text{complexes before treatment}}$ is the hydrodynamic diameter of AuNP/virus complexes before aggregation by osmolyte or salt (nm).

4.3 Results and discussion

4.3.1 Virus interaction with various AuNP sizes

The interaction between different sizes of AuNPs and virus particles was explored. The two model viruses to be detected in the AuNP assay, PPV and BVDV, have diameters of ~20 nm [199] and ~50 nm [200]. To explore how different sizes of AuNPs interact with the two viruses, AuNPs with diameters of 5, 15, and 40 nm were chosen for PPV (**Figure 4.3A-C**), and AuNPs with a 50 nm diameter were chosen for BVDV (**Figure 4.3D-E**). These sizes were chosen to have the size ratio of AuNP to virus diameters be <1, ~1, and ~2. For AuNPs that are smaller in diameter than the virus particles, it is believed that the AuNPs surround the virus particles (**Figure 4.3A and D**). The size ratio of ~1 for PPV has already been performed and serves as the base line control to compare the other size ratios (**Figure 4.3C**) [6]. When sufficiently large AuNPs are used, the opposite should occur, where many virus particles surround a single AuNPs (**Figure 4.3C and F**).

It was hypothesized that the configuration where virus particles surround the larger AuNPs (ratio ~2) would result in a higher DLS signal, and hence a lower LOD if enough virus is present to be aggregated by the osmolytes. This is because the inter-particle distance decrease of larger AuNPs results in more scattered light than decreasing the inter-particle distance between smaller AuNPs. Additionally, the larger AuNPs have a higher accessibility of the virus to bind due to the increased surface area. This should help ensure the virus particles are complexed with AuNPs prior to aggregation so the virus can be detected. This hypothesis was tested by measuring the size of AuNP/virus complexes before and after osmolyte-induced aggregation with DLS. The results indicated that neither larger AuNPs nor smaller AuNPs resulted in an improved virus detection assay because the LOD was not reduced.

4.3.2 Synthesis and characterization

After synthesis, the particles were characterized by UV-Vis, DLS, and TEM. The UV-Vis absorption spectrums for 27, 68, 96, 100, and 106 nm diameter AuNPs are

shown in **Figure 4.4A**. A red-shift in absorbance peak is clearly visible due to the increased size of AuNPs, and the absorbance peaks are very close to the expected literature values [193]. To determine the size of the different diameter AuNPs, DLS curves collected shown in **Figure 4.4B**. The Z-average hydrodynamic diameter collected during DLS measurements were used as the reported sizes of AuNPs, which is the diameter at peak intensity. The synthesized AuNPs of different diameter are monodisperse based on the presence of only one peak on the DLS curves, except for 68 nm particles, possibly due to a small amount of contamination. To confirm the correct size and shape of AuNPs, TEM images were collected and can be seen in **Figure 4.4C** for 68 nm and 100 nm AuNPs. The TEM image confirmed the size of the 100 nm AuNPs. The peak-broadening of the larger AuNPs (>100 nm) seen in UV-Vis (**Figure 4.4A**) is caused by dephasing of the surface plasmons from an enhanced mean free path of electrons, which causes new plasmon modes to appear at higher wavelengths [201-203]. The measured sizes of the AuNPs from the TEM images were similar to the sizes determined by DLS, with a slight over-estimation of the size by DLS. This result has been observed by others characterizing AuNPs and is due to DLS measuring the hydrodynamic diameter of the particles, which includes the solvation layer, whereas TEM measures the absolute size [188].

Figure 4.4: Characterization of various sizes of AuNPs showing the A) UV-Vis absorption spectrums, B) DLS curves, and C) TEM images of 68 nm and 100 nm AuNPs

4.3.3 Aggregation of 40 nm AuNP/PPV complexes

AuNPs with a diameter of 40 nm (AuNP₄₀) were complexed with PPV in the range of 8 log MTT₅₀/mL to 4 log MTT₅₀/mL and aggregated using 1 M mannitol, 1 M glycine, and 1 M NaCl. 1 nM and 1 μ M BSA were used as controls to show the specific nature of osmolytes to aggregate AuNP₄₀/virus complexes, but not AuNP₄₀/BSA complexes. Aggregation of the AuNP₄₀/virus and BSA complexes by mannitol, glycine, and NaCl are shown in **Figure 4.5**. At 7 log MTT₅₀/mL, there began to be a difference between AuNP₄₀/PPV complexes before and after treatment with mannitol (**Figure 4.5A**) and glycine (**Figure 4.5B**). Below that concentration, not enough virus was present to be aggregated and measured. A concentration of 8 log MTT₅₀/mL was necessary to halt salt-induced aggregation of the AuNPs (**Figure 4.5C**). From the previous assay with AuNP₁₅,

6 log MTT₅₀/mL was needed to halt salt-induced aggregation [6]. This difference is likely because of the larger AuNP surface exposed to the salt environment when compared to using smaller AuNPs. Diameters of ~75 nm (**Figure 4.5D**) and ~55 nm (**Figure 4.5E**) were achieved by aggregating AuNP₄₀/BSA control complexes with mannitol and glycine, respectively. Using **Equation 4.2**, ΔD_H values of ~25 nm and ~7 nm were calculated for mannitol-treated AuNP₄₀/BSA complexes and glycine-treated AuNP₄₀/BSA complexes, respectively. The ΔD_H values for BSA complexes represent the limit of the DLS to distinguish between AuNP/virus and AuNP/BSA complexes aggregated by mannitol and glycine. ΔD_H was calculated for mannitol and glycine aggregated AuNP₄₀/PPV complexes and compared to ΔD_H calculated for aggregated AuNP₄₀/BSA complexes, showing that the LOD for mannitol-treated complexes was 7 log MTT₅₀/mL (**Figure 4.5G, red line**). The LOD for glycine-treated complexes was also 7 log MTT₅₀/mL (**Figure 4.5H, red line**).

The baseline LOD results from the AuNP₁₅/PPV assay was 6 log MTT₅₀/mL for PPV [6]. Neither mannitol-treated nor glycine-treated AuNP₄₀/PPV complexes led to an improved LOD. This result did not support the hypothesis that larger diameter AuNPs would increase the DLS intensity signal at lower concentration of virus.

Figure 4.5: AuNP₄₀/PPV complexes aggregated by A) 1 M mannitol, B) 1 M glycine, and C) 1 M NaCl. AuNP₄₀/BSA complexes aggregated by D) 1 M mannitol, E) 1 M glycine, and F) 1 M NaCl. G) ΔD_H as a function of AuNP₄₀/PPV complexes aggregated by mannitol with the red line representing the LOD. H) ΔD_H as a function of AuNP₄₀/PPV complexes aggregated by glycine with the red line representing the LOD.

4.3.4 Aggregation of 50 nm AuNP/BVDV complexes

AuNPs with a diameter of 50 nm (AuNP₅₀) were complexed with BVDV in the range of 6 log MTT₅₀/mL to 2 log MTT₅₀/mL and aggregated using 1 M mannitol, 1 M glycine, and 1 M NaCl. BSA was used as a control. Aggregation of the AuNP₅₀/virus or

protein complexes by mannitol, glycine, and NaCl are shown in **Figure 4.6**. At 5 log MTT₅₀/mL, there began to be a difference between AuNP₅₀/BVDV complexes before and after treatment with mannitol (**Figure 4.6A**). At 6 log MTT₅₀/mL, there began a difference between AuNP₅₀/mL complexes before and after treatment with glycine (**Figure 4.6B**). Below these concentrations, not enough virus was present on the AuNP surface to be aggregated and measured. A concentration of 5 log MTT₅₀/mL was necessary to halt salt-induced aggregation of the AuNPs (**Figure 4.6C**), which is lower than AuNP₄₀ used with PPV likely because of the larger size of BVDV compared to PPV capable of shielding the NaCl from aggregating the AuNPs. Diameters of ~80 nm (**Figure 4.6D**) and ~60 nm (**Figure 4.6E**) were achieved by aggregating AuNP₅₀/BSA control complexes with mannitol and glycine, respectively, leading to ΔD_H values of ~30 nm and ~6 nm. ΔD_H values of ~30 nm and ~6 nm were calculated for mannitol-treated AuNP₅₀/BSA complexes and glycine-treated AuNP₅₀/BSA complexes, respectively. The ΔD_H values for BSA complexes represent the limit of the DLS to distinguish between AuNP/virus and AuNP/BSA complexes aggregated by mannitol and glycine. ΔD_H was calculated for mannitol and glycine aggregated AuNP₅₀/BVDV complexes and compared to ΔD_H calculated for aggregated AuNP₅₀/BSA complexes, showing that the LOD for mannitol-treated complexes was 4 log MTT₅₀/mL (**Figure 4.6G, red line**). The LOD for glycine-treated complexes was 6 log MTT₅₀/mL (**Figure 4.6H, red line**).

The baseline LOD results from the AuNP₁₅/BVDV assay was 4 log MTT₅₀/mL for BVDV [6]. These results did not improve the LOD compared to when 15 nm AuNPs were used, which did not support the hypothesis that larger diameter AuNPs would increase the DLS intensity signal at lower concentration of virus.

Figure 4.6: AuNP₅₀/BVDV complexes aggregated by A) 1 M mannitol, B) 1 M glycine, and C) 1 M NaCl. AuNP₅₀/BSA complexes aggregated by D) 1 M mannitol, E) 1 M glycine, and F) 1 M NaCl. G) ΔD_H as a function of AuNP₅₀/BVDV complexes aggregated by mannitol with the red line representing the LOD. H) ΔD_H as a function of AuNP₅₀/BVDV complexes aggregated by glycine with the red line representing the LOD.

4.3.5 Aggregation of 5 nm AuNP/PPV complexes

Since larger AuNPs complexed with the virus did not result in a lower LOD, it was decided to try AuNPs that were smaller than the virus. AuNP₅ (5 nm particles) were

complexed with PPV in the range of 4 to 8 log MTT₅₀/mL with the same osmolytes and salt. BSA was used as the control. Aggregation of the AuNP₅/PPV or BSA complexes are shown in **Figure 4.7**. Regardless of the concentration of PPV used to complex with the AuNP₅, the same level of aggregation was observed when mannitol and glycine were added, shown in **Figure 4.7A and B**. However, as expected, salt-induced aggregation was stopped at higher concentration of PPV (6 log MTT₅₀/mL), meaning PPV shields the AuNPs from being aggregated, as shown in **Figure 4.7C**. Regardless of the BSA concentration used, the same degree of aggregation was observed when compared to PPV samples (**Figure 4.7D and E**). The ΔD_H values calculated for AuNP₅/BSA complexes were ~130 nm for mannitol treated complexes and ~30 nm for glycine treated complexes. Using these ΔD_H values for AuNP₅/BSA complexes as the LOD for AuNP₅/PPV complexes, there is no detection ability when aggregated by mannitol or glycine (**Figure 4.7G and H, respectively**). These results indicated that the AuNP₅ were aggregating due to osmolyte addition regardless of the biomolecule or biomolecule concentration.

Figure 4.7: AuNP₅/PPV complexes aggregated by A) 1 M mannitol, B) 1 M glycine, and C) 1 M NaCl. AuNP₅/BSA complexes aggregated by D) 1 M mannitol, E) 1 M glycine, and F) 1 M NaCl. G) ΔD_H as a function of AuNP₅/PPV complexes aggregated by mannitol with the red line representing the LOD. H) ΔD_H as a function of AuNP₅/PPV complexes aggregated by glycine with the red line representing the LOD.

To test this theory, AuNP₅ were mixed with an equal volume of nanopure water, 1 M mannitol, 1 M glycine, and 1 M NaCl, and DLS curves were collected for each condition to test if the size increased just with osmolyte treatment without biomolecule presence. The results are shown in **Figure 4.8**. The AuNP₅ straight from the manufacturer

and in water showed a D_H of ~ 11 nm, see **Figures 4.8A and B**. Although the size is larger than the expected 5 nm, it is common for DLS to over-estimate the true size of the particles as it is measuring the hydrodynamic diameter, and not true diameter of the particles [188]. The size of the particles was confirmed with TEM imaging, showing a size between 6-7 nm, shown in **Figure 4.9**. AuNP₅ mixed with glycine showed only a slight increase in hydrodynamic diameter to 16 nm, (**Figure 4.8C**). AuNP₅ mixed with mannitol and NaCl showed an increase in diameter to 113 nm and 950 nm, respectively. Although the salt-induced aggregation is expected for bare AuNPs, the high degree of aggregation due to mannitol treatment was unexpected and undesired. One possible reason for this aggregation by mannitol could be that the high number of hydroxyl groups present on mannitol can substitute citrate ions on the surface leading to a destabilization of the AuNPs. This increased hydroxyl group affinity to smaller AuNPs was noticed when a reducing agent with a high number of hydroxyl groups was more efficient at synthesizing smaller diameter AuNPs, compared to reducing agents with a lower amount of hydroxyl groups [204]. Due to the challenge of osmolytes aggregating the plain AuNP₅, they were not investigated for further use in virus detection.

Figure 4.8: DLS curves showing the size of A) AuNP₅ straight from the manufacturer's bottle and B) AuNP₅ in nanopure water. DLS curves of AuNP₅ aggregated by C) 1 M glycine, D) 1 M mannitol, and E) 1 M NaCl.

Figure 4.9: TEM images of 5 nm AuNPs at two different magnification levels

Overall, by utilizing AuNP₄₀ with PPV, AuNP₅₀ with BVDV, and AuNP₅ with PPV, there was no improvement to the virus detection assay previously published using AuNP₁₅ for PPV and BVDV. The LODs remained at 6 log MTT₅₀/mL for PPV and 4 log MTT₅₀/mL for BVDV.

4.4 Conclusions and future work

Synthesis of various sizes of AuNPs was performed and characterized for use in a PPV and BVDV detection assay. AuNP₄₀ were complexed with PPV, resulting in a LOD of 7 log MTT₅₀/mL when aggregated by 1 M mannitol. AuNP₅₀ were complexed with BVDV, resulting in a LOD of 5 log MTT₅₀/mL when aggregated by 1 M mannitol. AuNP₅ were used in an attempt to detect PPV, but the high concentrations of osmolytes used in the aggregation step led to AuNP aggregation regardless of the presence of virus or control protein. Neither an increase nor decrease in the size of AuNPs lead to an improved LOD compared to the published work of using 15 nm AuNPs in the same detection assay. These results indicate that the size of AuNPs used in the virus detection assay do not play a critical role in detection.

Future work for the AuNP project includes using 100 nm AuNPs (AuNP₁₀₀) to test if a size ratio between AuNP and BVDV of 2 would improve the detection ability. The size ratio of 2 between PPV and AuNP₅₀ did not lead to an improved LOD, but BVDV is a larger virus that could complex with a few number of AuNP₁₀₀. By complexing with fewer, larger AuNPs BVDV could be detected due to the increase in scattered light intensity with larger AuNPs.

The system pH could be varied to improve the virus detection assay. Since the virus pI dictates the surface charge on a virus particle, the system pH could be lowered to cause more electropositive patches to be present. With more electropositive patches present, the electrostatic interaction may become stronger between citrate-capped AuNPs and virus particles. The stronger electrostatic interaction may lead to the ability to use a lower concentration of virus particles to bind to AuNPs, and thus a lower amount of virus would be needed for osmolyte-induced aggregation.

Additionally, other osmolytes such as alanine and trehalose have been shown to aggregate enveloped and non-enveloped viruses, which could be used to aggregate the AuNP/virus complexes [111, 112]. Alanine is an amino acid with a hydrophobic side chain that may be able to aggregate viral particles more effectively. Alanine residues in protein molecules are normally buried within the protein structure to protect it from solvent exposure [205]. This effect could be exploited for the aggregation assay because the hydrophobic residues from alanine could aid in stabilization and enhanced aggregation of the dehydrated hydrophobic patches of virus particles. Trehalose is a sugar with two molecules of glucose linked by an α , α -1,1-glucosidic bond [206]. Trehalose has a high affinity for water due to multiple hydrogen bonding events between its two glucose rings and water molecules [207]. The high affinity for water would rapidly dehydrate the virus surface, potentially causing more virus aggregates to form as more and more water is pulled away from the virus surface. This could improve the AuNP assay by allowing a lower concentration of osmolyte to be used in the aggregation step, or to dehydrate a smaller concentration of virus particles due to the strong dehydration ability of trehalose.

5 Continuous downstream purification of viral products using an aqueous two-phase system

5.1 Introduction

Vaccination remains the best method to prevent the transmission of communicable human diseases. According to the World Health Organization (WHO), in 2018, 86% of infants worldwide (116.3 million) received three doses of the diphtheria-tetanus-pertussis vaccine. But that left 14% of children under the age of 1 (19.4 million) unvaccinated [208]. One reason for this is due to vaccine supply shortages. Over the past 15 years, significant manufacturing problems have led to numerous vaccine shortages. These shortages have led to the inability for even the United States to meet past demands of vaccines for hepatitis A/B, rabies, and influenza [209, 210]. The ever-expanding world population will continue to increase the demand for vaccines. The biomanufacturing industry must develop and implement novel production methods to supply the market quickly and reliably with vital virus-containing biotherapeutics. One such solution is to switch to continuous operation.

The switch to continuous operation would increase profits by increasing productivity and flexibility, while decreasing capital and operating costs [211-213]. This switch requires a fully integrated, end-to-end, continuous biomanufacturing process. Upstream unit operations that operate continuously are currently in operation [214, 215], but truly continuous downstream processes are still being developed. Truly continuous downstream processes must also handle the increasing titers produced in the bioreactor to purify viral products safely and quickly.

Current semi-continuous downstream processes are focused on simulated moving bed (SMB) chromatography. There are only a limited number of SMB virus purification processes due to the complex nature of designing an SMB process [139, 141, 216]. One SMB chromatography process purified human influenza virus from a serum-free MDCK cell culture supernatant. The process resulted in 89% virus recovery, 52% protein removal, and 99.7% DNA removal using a quaternary amine anion exchange monolith

[139]. However, to achieve the FDA-required DNA maximum amount of 10 ng/dose, a Benzonase®-treatment was necessary and adds an additional step of Benzonase® removal later in the process. In addition, a 10% column load was required to keep contaminant protein and DNA levels low [139]. This low of a column load leads to higher processing time and cost due to under-utilized column resin. Another SMB chromatography process used size-exclusion chromatography (SEC) to purify adenovirus from a filtered harvest of Triton X-100 and Benzonase®-treated HEK-293 cell media. Use of the two-column SEC technique resulted in 86.3% virus yield, 90% DNA removal, and 89% host cell protein (HCP) removal [144]. These two SMB chromatography processes show promise for operating a semi-continuous downstream purification, since most SMB processes are continuous loading and semi-continuous elution. Since SMB processes are not fully continuous and resin costs remain high, other unit operations need to be explored.

Aqueous two-phase systems (ATPSs) can be run as fully continuous processes. An ATPS is a liquid-liquid extraction technique that is used to partition molecules between two semi-miscible liquid phases and is typically comprised of a polymer and salt for biomolecule separations [147, 149]. Water is the major component in ATPS, making the materials inexpensive and environmentally-friendly compared to common chromatography resins [217]. ATPSs have been tested extensively in batch-mode to separate and purify proteins [161, 163], phages [175], and viruses [7, 154, 173]. ATPS was used for the separation and purification of porcine parvovirus (PPV) with an infectious titer recovery of 79%, DNA removal of 78%, and high protein removal using a mixture of poly(ethylene glycol) (PEG) and citrate [7].

There have been several continuous ATPS designs used for the recovery of proteins [158, 161, 163] and enzymes [165, 166, 218]. Two vital considerations for continuous ATPS operation are effective mixing and phase separation. The most common designs for continuous ATPSs include the use of column contactors, such as rotating disk contactors (RDC) and mixer-settlers [170]. The key difference between these types of designs are how mixing is achieved. RDC uses mechanically spun plates to form mixing vortices between plates and in-line turbulence generators mix the ATPS in mixer-settles. One continuous ATPS used an RDC to extract α -lactalbumin from a mixture of α - and β -

lactalbumin [158]. This was performed by spiking α - and β -lactalbumin into the citrate-rich phase, then a PEG-rich phase was flowed through the bottom of the citrate-rich phase inside the RDC. Adequate contact between phases was provided and 86% of the α -lactalbumin was recovered in the PEG-rich phase with an 86% purity. Although high recovery and purity of the product was achieved using the RDC, two hours was required to achieve steady-state operation while flowing at 3 mL/min [158]. Another draw-back to column contactors is the issue of column flooding, which decreases separation performance [170].

Mixer-settlers avoid issues of flooding and mechanical failures by employing various in-line static mixer designs, such as a tube filled with glass-bead or baffles. These types of mixers use in-line obstacles to disrupt the streamlines of the flowing phases, creating droplets of each phase. Continuous ATPSs with a polymer as one of the phase forming components will typically operate in the laminar flow regime due to the high viscosity of the polymer. For this reason, in-line mixers are appropriate because they can blend the two phases by disrupting the flow pattern and creating convective radial mixing. Separation of mixed phases is achieved using long lengths of tubing and decanters. Continuous ATPS using a mixer-settler was used to study the partitioning of bovine whey protein isolate (WPI) proteins (α - and β -lactalbumin) between PEG-rich and phosphate-rich phases [163]. After 12 minutes of continuous operation, 72% of the initial total protein was partitioned to the phosphate-rich phase, while 30% of the total protein was collected in the PEG-rich phase [163]. Although the purity of the collected phases was not quantified, SDS-PAGE gels showed the phosphate-rich phase contained only bands corresponding to β -lactalbumin, demonstrating that a high purity was achieved [163]. This study demonstrated the ease of scale-up from batch mode experiments to a fully continuous separation process.

In this work, we developed a fully continuous ATPS for the purification of PPV and human immunodeficiency virus type-1 group antigen virus-like particles (HIV VLPs) from crude cell supernatant without pre-treatment. Optimizations of mixer types was performed to achieve proper mixing of phases and partitioning of viral particles to the PEG-rich phase while partitioning most contaminant proteins and DNA to the citrate-rich

phase. Recovery and purity of each viral product was determined over approximately 40 minutes.

5.2 Materials and methods

5.2.1 Materials

Poly(ethylene glycol) 12,000 (MW_{avg} 11,000-15,000), citric acid monohydrate (ACS grade $\geq 98\%$), sodium dodecyl sulfate (BioReagent, $\geq 98.5\%$), sodium citrate tribasic dihydrate (ACS reagent, $\geq 99\%$), and 2-mercaptoethanol were from Sigma-Aldrich (St. Louis, MO). Minimum essential medium, fetal bovine serum (Mexico origin), 10,000 U/mL penicillin streptomycin (pen-strep), 0.25% trypsin-EDTA (1X), phosphate buffered saline pH 7.2 (1X), and sodium bicarbonate (7.5%) for cell culture and virus propagation were from Gibco™ (Grand Island, NY). Thiazolyl blue tetrazolium bromide (98%) was from Alfa Aesar (Ward Hill, MA). SeeBlue™ Plus2 prestained standard, NuPAGE™ 3-(N-morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer (20X), SimplyBlue™ SafeStain, and NuPAGE™ 4-12% Bis-Tris gels were from Invitrogen (Carlsbad, CA). Pierce™ lithium dodecyl sulfate (LDS) sample buffer non-reducing (4X) was from Thermo Scientific (Waltham, MA). Barnstead™ nanopure water (Thermo Scientific) with an electrical resistance of $\geq 18\text{ M}\Omega$ was used to create all solutions. All solutions were filtered through a 0.2 μm Nalgene™ Rapid-Flow™ sterile disposable bottle-top filter with PES membrane from into an autoclaved vessel for storage before use.

5.2.2 Methods

5.2.2.1 Cell maintenance

Porcine kidney cells (PK-13) were purchased from the American Type Culture Collection (ATCC®) (cat# CRL-6489™) and cultured in minimum essential medium (MEM) supplemented with 10 vol% fetal bovine serum (FBS) and 1 vol% pen/strep. The cells were incubated at 37°C, 5% CO₂, and 100% humidity. PPV strain NADL-2 was a

gracious gift from Dr. Ruben Carbonell at North Carolina State University (Raleigh, NC). PPV strain NADL-2 was propagated in PK-13 cells as described previously [194]. After three freeze-thaw cycles, the cell lysate was clarified by centrifugation at 5,000 rpm at 4°C for 15 minutes in a ST16R centrifuge with a TX-400 swing-bucket rotor (Thermo Scientific). The PPV-containing supernatant was stored at -80°C until use.

5.2.2.2 MTT assay for PPV titer

The titer of PPV was found by the MTT colorimetric cell viability assay [219]. PK-13 cells at a seeding density of 8×10^4 cells/mL were placed in 96-well plates and incubated overnight. The next day, the cells were infected with PPV by serially diluting across the plate at a ratio of 1:5 and placed back in the incubator for 6 days. 6 days after infection, 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt in 1X pH 7.2 PBS was added to each well to allow living cell's mitochondrial reductase to reduce the MTT to (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). The solubilizing solution, consisting of 10 w/v% sodium dodecyl sulfate (SDS) with 0.01 M hydrochloric acid (HCl), was added to dissolve the formazan. The absorbance at 550 nm was measured with a Synergy™ Mx microplate reader from BioTek (Winooski, VT), and the 50% viral infectious dose was determined in units of MTT₅₀/mL.

5.2.2.3 Fluorescence measurements for HIV VLP detection

The HIV VLPs were expressed in proprietary *Hansenula polymorpha* cells donated by Esperovax (Plymouth, MI). The fluorescence from the GFP was recorded with a Synergy™ Mx microplate reader in a black, flat-bottom 96-well plate at an excitation wavelength of 485 nm and emission of 509 nm.

5.2.2.4 ATPS formation

Unpublished work in our lab from an ATPS tie line study using a 12 kDa PEG/citrate ATPS determined the highest recovery of PPV in the PEG-rich phase at a tie line length (TLL) of 32 w/w% and tie line ratio (TLR) of 0.5. For this reason, this system of PEG and citrate was chosen as the ATPS to be run continuously. To form the TLL

32%/TLR 0.5 system, stock solutions of 45 w/w% PEG ($\rho = 1.065 \text{ g/mL}$), 30 w/w% citrate ($\rho = 1.158 \text{ g/mL}$), and nanopure water were mixed to achieve starting system concentrations of 18 w/w% PEG, 12 w/w% citrate, and 70 w/w% water. To achieve these starting system concentrations, 41% of the total volume was 45 w/w% PEG, 38% of the total volume was 30 w/w% citrate, 11% of the total volume was viral product, and 11% of the total volume was water.

5.2.2.5 Mixer-settler construction

An air-powered mixer operating at 1,500 rpm with a 5 cm diameter Cowles blade was used to mix ATPS components. This mixer was used as the positive control of mixing for the in-line mixers. Two in-line mixer designs were tested for their ability to sufficiently mix the PEG, citrate, and water prior to testing the separation ability and ATPS formation of the full system. The first mixer was constructed by filling a 15 cm length of Tygon S3 E-LFL (Saint Gobain, Malvern, PA) polyvinyl chloride (PVC) tubing (6.35 mm inner diameter) with approximately 150, 3 mm outer diameter glass beads from Fisher Scientific (Waltham, MA) until fully packed. This was designated the “glass bead mixer”. The second mixer was assembled by inserting 2, 12-element helical blades (4.76 mm outer diameter) from Cole-Parmer (Vernon Hills, IL) into a 15 cm length of 4.76 mm ID Tygon S3 E-LFL PVC tubing, which was designated the “helical mixer”.

After each in-line mixer, 45.7 cm of 2 mm ID Tygon tubing was shaped into a spiral by winding around a 100 mL graduated cylinder (9.5 cm outer diameter) to begin phase separation before the settler. The settler was a 15 mL centrifuge tube with 2 mm OD holes drilled into the top of the cap, direct bottom of the tube, and half-way up the vertical wall of the tube for PEG-rich phase collection, citrate-rich phase collection, and feed inlet lines, respectively. Each hole was sealed with DAP® RapidFuse® Fast Curing All Purpose Adhesive with Plastic Primer glue to prevent leaking. The full system process flow diagram and in-lab set-up are shown in **Figure 5.1A** and **B**, respectively. Close-up images of the helical mixer and the separator section of the continuous ATPS set-up are shown in **Figure 5.1C** and **D**, respectively.

Figure 5.1: A) Process flow diagram of continuous ATPS and B) in-lab bench scale set-up. C) Close-up picture of the in-line helical mixer and D) separator section.

5.2.2.6 COMSOL Multiphysics simulation of helical mixer

COMSOL Multiphysics was used to further evaluate the helical mixer performance. The program simulated the two-phase laminar flow through the helical mixer (**Figure 5.1C**). A schematic of the numerical model and the associated computational grid are shown in **Figures 5.2A and B**, respectively. A finite-element approach was employed as implemented in COMSOL Multiphysics to solve the incompressible Navier-Stokes equations for the two-phase flow of the PEG-rich (stream 1) and the citrate-rich (stream 2) mixture [220, 221]. A phase-field approach was adopted to model the interaction between the two immiscible components [222]. The governing equations were solved iteratively, where within each iteration first velocity and pressure fields were calculated together directly, and then the phase-field variable was computed in the same manner. The fluid flow and phase-field equations were discretized via P2+P1 scheme and quadratic elements, respectively. Tetrahedral elements were used to mesh the computational domains (see **Figure 5.2B**), and the grid was chosen such that the changes in the results due to the mesh refinement were marginal.

Figure 5.2 : COMSOL Multiphysics modelling of in-line helical mixer showing the A) mixer geometry and B) mesh pattern used in the simulation

The flow within the helical mixer was driven in the simulation by applying inlet and outlet boundary conditions upstream and downstream of the mixer. Consistent with stream 1 and stream 2 in the experiments, the fluid velocity (measured in m/s) and the phase-field variable are specified as

$$\mathbf{u}_{in} = \begin{cases} 4.0404 \times 10^{-3} \mathbf{e}_z & \text{for } x > R \\ 2.869 \times 10^{-3} \mathbf{e}_z & \text{for } x \leq R \end{cases} \text{ and } \Phi = \begin{cases} 1 & \text{for } x > R \\ -1 & \text{for } x \leq R \end{cases}$$

at the inlet (see **Figures 5.4A and B**) and the outlet pressure is fixed to zero.

Additionally, no-slip and 90° contact angle conditions were imposed on wetted walls.

Furthermore, the properties of PEG-rich and citrate-rich phases, and the surface tension between them were set to $\mu_{\text{PEG}} = 0.0912 \text{ Pa}\cdot\text{s}$, $\rho_{\text{PEG}} = 1015 \text{ kg/m}^3$, $\mu_{\text{citrate}} = 0.001 \text{ Pa}\cdot\text{s}$, $\rho_{\text{citrate}} = 1068 \text{ kg/m}^3$, and $\gamma = 0.000463 \text{ N/m}$, respectively.

5.2.2.7 Continuous ATPS operational procedure

A mass balance was performed around the mixer to determine proper flow rates of the stock PEG, citrate, and water into the mixer to achieve the desired TLL 32% TLR 0.5 ATPS (**Appendix Figure A.1**). To confirm the proper ATPS was formed by the mixer, physical property measurements of the PEG-rich and citrate-rich phase after separation were performed on the density, dynamic viscosity, and refractive index, as described previously [7]. Density measurements were performed by weighing a 1 mL sample on a Mettler-Toledo analytical balance (Columbus, OH). Dynamic viscosity measurements were performed with an SV-10 sine-wave vibro viscometer from A&D

(Tokyo, Japan). Refractive index measurements were performed using a Digital Brix/RI-Chek refractometer from Reichert Technologies (Depew, NY). These measurements were used to compare batch-mode and continuous-mode phase samples to confirm proper ATPS formation.

To equilibrate the continuous ATPS, 45% PEG, 30% citrate, and water were pumped through a mixer at 1.45 g/min ($\rho=1.065$ g/mL), 1.45 g/min ($\rho=1.158$ g/mL), and 0.73 g/min ($\rho=1.0$ g/mL), respectively, for a total mixture flow rate of 3.63 g/min ($\rho=1.209$ g/mL). The PEG, citrate, and water flowed through the entire system for 15 minutes to allow for steady-state operation prior to introducing any viral product. After system equilibration, the water line was switched to a crude viral product tube for purification for continuous operation. PEG-rich and citrate-rich fractions were collected by two individual peristaltic pumps set at different flow rates (typically between 1.0-1.5 mL/min) to keep the interface of the phases within the separator at the same level. Fractions were collected every 2 minutes for 10 seconds. After fraction collection, PPV was quantified by MTT assay to assess recovery in both phases. Recovery of PPV in the PEG-rich and citrate-rich phases was calculated using **Equation 5.1**:

$$PPV \text{ Recovery (\%)} = \left(10^{T_{P,C}} \cdot V_{P,C} / 10^{T_M} \cdot V_M \right) \cdot 100 \quad (5.1)$$

where T is the titer of PPV ($\log_{10}$ MTT₅₀/mL) in the PEG-rich phase (P), citrate-rich phase (C), or PPV starting material (M), $V_{P,C}$ is the volume of the fraction collected for 10 seconds (mL), and V_M is the volume of starting material flowed into the system for the total testing time (mL).

Recovery of the HIV VLPs in the PEG-rich and citrate-rich phases was calculated from fluorescence measurements using **Equation 5.2**:

$$HIV \text{ VLP Recovery (\%)} = \frac{RFU_{P,C} \cdot V_{P,C}}{RFU_M \cdot V_M} \cdot 100 \quad (5.2)$$

where RFU is the constant-volume relative fluorescence units per 100 μ L of the PEG-rich phase (P), citrate-rich phase (C), or starting HIV VLP material (M), and V is the phase volume measured (μ L).

5.2.2.8 SDS-PAGE, Bradford assay, PicoGreen DNA assay

Contaminant protein partitioning was visualized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run using 4-12% Bis-Tris 1.0 mm 12-well NuPAGE™ gels with NuPAGE™ MOPS running buffer in an XCell SureLock Mini-Cell from Life Technologies (Carlsbad, CA). Samples were prepared and denatured by mixing 25 µL of sample in 25 µL 4X sample buffer with addition of 4 µL of 2-mercaptoethanol. Samples were placed in a dry block heater at 95°C for 5 minutes prior to loading. The SeeBlue Plus2 pre-stained protein ladder (191-14 kDa) was used as the protein band standard. 10 µL of protein standard and sample was loaded into the gel and ran at a constant 200 V for 50 minutes. Gels were stained with SimplyBlue™ SafeStain as per the manufacturer's microwave protocol for a 5-ng detection limit.

Contaminant proteins present in each ATPS phase were quantified using the Pierce™ Bradford Coomassie assay from Thermo Scientific according to the manufacturer's microplate protocol. The concentration of virus present in samples is much lower than the limit of detection for the Bradford assay and therefore did not interfere with contaminant protein quantification. To eliminate interference from high concentrations of PEG and citrate present in samples, PEG-containing samples were diluted 1:33 in nanopure water and citrate-containing samples were diluted 1:22 in nanopure water prior to addition of assay reagents. Contaminant protein recovery was calculated using **Equation 5.3**:

$$\text{Protein recovery (\%)} = \frac{C_{P,C} \cdot V_{P,C} \cdot DF}{C_M \cdot V_M \cdot DF} \cdot 100 \quad (5.3)$$

where C is the protein concentration (µg/mL) in the PEG-rich phase (P), citrate-rich phase (C), or PPV starting material (M). DF is the dilution factor for each sample.

DNA content in the PEG-rich and citrate-rich samples was quantified using the Quant-iT PicoGreen dsDNA kit from Thermo Scientific. Standard curves were created by diluting the stock λ DNA standard in a range of 1-10 ng/mL in 2 w/w% PEG and 0.24 w/w% citrate diluted in 1X TE buffer for quantitating dsDNA in PEG-rich and citrate-rich samples. A standard curve from 10-200 ng/mL in 1X TE buffer was created to quantitate starting viral product dsDNA content. The PEG-rich samples were diluted in

1X TE buffer to 2 w/w% PEG according to the manufacturer's recommendation, and citrate-rich samples were diluted in 1X TE buffer to 0.24 w/w% citrate to reduce assay interference. Samples were compared to the total amount of dsDNA present in the crude starting PPV to quantify DNA removal. DNA recovery was calculated using **Equation 5.4**:

$$DNA\ recovery\ (\%) = \frac{C_{P,C} \cdot V_{P,C} \cdot DF}{C_M \cdot V_M \cdot DF} \cdot 100 \quad (5.4)$$

where C is the DNA concentration (ng/mL) in the PEG-rich phase (P), citrate-rich phase (C), or PPV starting material (M).

5.3 Results and discussion

5.3.1 Model virus and virus-like particles

PPV and HIV VLPs were used as viral products to test the ability of continuous ATPS to partition viral products to the PEG-rich phase while leaving contaminant proteins and host-cell DNA in the citrate-rich phase. PPV was chosen as a model non-enveloped virus for purification due to its small size and physicochemical properties making it difficult to recover from traditional filtration or chromatographic techniques. The physicochemical properties of PPV are summarized in **Table 5.1**.

HIV VLPs were expressed by genetically modified *Hansenula polymorpha* cells donated by Esperovax. These VLPs are created for their potential use as an oral vaccine candidate. The VLPs self-assemble after the core HIV-1 structural polyprotein gag is made by the cells and bud through the cell membrane (**Appendix Figure A.2**). The physicochemical properties of the HIV VLPs are summarized in **Table 5.1**. The pI of the VLPs is unknown but is likely less than 7 for two reasons: first, the pI of the proteins present in the gag polyprotein are less than 7 [223, 224], and second, glycoproteins comprising the *Hansenula polymorpha* cell membrane have high-mannose type N-glycans. The envelope proteins terminate with mannose residues that have excess hydroxyl groups, likely resulting in a pI well below 7 [225]. The enveloped HIV VLP was chosen to demonstrate the flexibility of the continuous ATPS to purify a potential vaccine candidate.

Table 5.1: Virus and VLP physicochemical properties

Virus	Model For	Genome	Shape	Envelope	Size (nm)	pI
PPV	Human B19 parvovirus [226]	ssDNA [227]	Icosahedral [228]	No [228]	18 – 26 [227]	4.8 – 5.1 [229]
HIV VLP	HIV oral vaccine	None	Pleomorphic spherical [230]	Yes [230]	80 – 120 [137]	<7 [223, 224]

5.3.2 Selection of in-line mixer

The first unit operation to be designed was the in-line mixer. The chosen mixer needed to produce a homogeneous mixture of the PEG, citrate, water, and viral particles to allow for proper mass transport of viral particles to each phase. The in-line mixer performance was evaluated by comparing each one to the mixing ability of an air-powered Cowles blade mixer. The air-powered mixer (**Figure 5.3A**) was used as the gold standard for mixing the components, as it produces turbulent mixing with a spinning blade for high shear rates. A straight length of tubing the same length as the in-line mixers was used as the negative control to demonstrate no mixing, as the system was run in laminar flow (**Figure 5.3B**). Both in-line mixers (**Figure 5.3C and D**) were compared to the air-powered mixer's ability to create a homogeneous mixture. Due to the high viscosity of PEG and low flow rate of the system, the helical mixer and glass bead mixer were tested, which are both capable of dispersing high viscosity fluids in the laminar flow regime [231, 232]. A helical static mixer redistributes fluid in the radial and tangential directions using only the process stream's energy, which achieves mixing by disrupting the streamlines in laminar flow [233].

Figure 5.3: Collage of different mixing modes for continuous ATPS. Mixing ATPS with an A) air-powered mixer, B) straight length of tubing, C) glass bead mixer, D) and helical mixer.

The two in-line mixers, the glass bead mixer and the helical mixer, were tested for their ability to produce a homogeneous mixture at their outlet, and for proper phase separation after mixing. Mixing and settling was visualized with the addition of neutral red. Neutral red was added to the citrate phase and then partitioned to the PEG-rich phase. Mixing the ATPS components with the air-powered mixer for 5 minutes resulted in a completely homogeneous mixture shown in **Figure 5.3A** at 0 minutes of settling time. After 20 minutes of settling time, the neutral-red dye partitioned to the PEG-rich top phase, leaving the citrate-rich bottom phase clear seen in **Figure 5.3A**. The mixing and partitioning shown in **Figure 5.3A** was used as the benchmark for the in-line mixers to determine if proper mixing was achieved.

A flow rate of 3 mL/min (3.63 g/mL) was chosen as a starting basis to flow through the glass bead mixer and the helical mixer. The glass bead mixer was expected to produce a highly homogeneous mixture due to flow distribution as the liquid components encountered the beads [163]. Mixing is achieved in a glass bead mixer because as the laminar flow streamlines encounter a bead, they are deflected around the bead causing crowding between phases next to the sphere. This crowding results in a change in the velocity of the streamlines close to and far away from the bead. The difference in

streamline velocity causes convective mixing [234]. However, the glass bead mixer did not produce a fully homogeneous mixture as there is a clear interface between the phases, indicating that proper mixing was not achieved, seen in **Figure 5.3C (red arrow)** at 0 minutes of settling time. Additionally, a high pressure drop was observed across the glass bead mixer during testing due to the high viscosity of PEG. This high pressure drop, even at a relatively low flow rate of 3 mL/min, excluded it for further scaled up experiments. The helical mixer was able to produce a more homogeneous mixture (**Figure 5.3D**, 0 min) which was similar in appearance to the air-powered mixer. This successful mixing was achieved because the helical elements in the mixer disrupt and disperse the laminar flow streams radially towards the tube wall to induce convective mixing [233]. By redistributing laminar flow streams radially, striations, or unmixed regions, are minimized [232]. The mixing action of the helical mixer was greater than the glass bead mixer possibly because in the glass bead mixer, especially in the creeping flow regime, the streamlines may not have been sufficiently dispersed radially. Additionally, the helical mixer does not have the issue of generating a high pressure drop since most of the mixer is an open channel compared to the glass bead mixer.

The helical mixer was able to closely replicate the mixing ability of the air-powered mixer, and for this reason, was chosen as the in-line mixer type for the continuous ATPS. A still image from a video of the helical mixer action is shown in **Appendix Figure A.3**. The video shows a neutral red dye being dispersed evenly throughout the mixture as it travels down the length of the mixer.

5.3.3 Helical mixer performance simulated in COMSOL Multiphysics

A simulation of the helical mixer was generated in COMSOL Multiphysics to further confirm mixing action of the PEG and citrate phases. The steady-state flow structure inside the mixer was visualized by plotting the magnitude of the fluid velocity contours at several cross-sections along the tube and by drawing streamlines originating from the inlet, shown in **Figure 5.4A**. The tortuous paths of the flow streamlines highlighted the critical role of advection in facilitating mixing. Additionally, the degree of mixing along the tube was visualized in the form of cross-sectional contours of the

phase-field variable, shown in **Figure 5.4B**. The simulation revealed that the red colored PEG phase and blue colored citrate phase are efficiently blended by the helical blades. By 10th element of the helical mixer, the red and blue color are nearly completely mixed, represented by the green color shown in **Figure 5.4B**. These results illustrated how well the PEG and citrate phases were blended efficiently by flowing it through a properly designed static mixer consisting of a tube embedded with a series of twisted blades. Overall, numerical calculations corroborated nicely with the experimental observations, and provide further confirmation of the excellent performance of the helical mixer.

This simulation will be helpful in future scale-up designs of the continuous ATPS to simulate increased flow rates, different phase forming concentrations, and tubing sizes without extensive experimental trials.

Figure 5.4: COMSOL Multiphysics modelling of in-line helical mixer showing the A) mixer geometry and B) mesh pattern used in the simulation. C) Streamlines and fluid velocity magnitude contours and D) phase field contours

5.3.4 Settler design

A settler must be used to allow the PEG-rich and citrate-rich phases to separate after mixing. From the experimental study of the different in-line mixers, 20 minutes of gravity-driven settling time was necessary to achieve phase separation in batch systems. This settling time was achieved in the settler section of the bench scale set-up, seen in **Figure 5.1D**. The first part of the settler section used a straight length of tubing to begin phase separation. The second part of the settler section was a separator column to allow for phase hold-up before collection. Half of the separation time was achieved with a 15 mL tube separator, and the other half of the separation time was achieved using tubing between the mixer and settler. To achieve the separation time, 3 m of 2.0 mm ID tubing was used.

To determine if the continuous settler section was allowing the phases to separate properly, refractive index (RI), dynamic viscosity, and density of the PEG-rich was collected and compared to the batch ATPS. For the citrate-rich phase comparison between batch and continuous modes, only density was measured, as viscosity and RI measurements are unaffected by varying citrate concentrations. The comparison between batch mode and continuous mode measurements, along with the percent difference between the measurements can be seen in **Table 5.2**. The physical property measurements confirmed that the proper PEG-rich and citrate-rich phases were obtained after separation evidenced by low percent difference values.

Table 5.2: Physical property measurement comparison between batch mode and continuous mode ATPS TLL 32% TLR 0.5

System	PEG-rich Density (g/mL)	Citrate-rich Density (g/mL)	PEG-rich Viscosity (mPa·s)	PEG-rich Refractive Index
Batch	1.015 ± 0.009	1.068 ± 0.008	320 ± 130	1.3852 ± 0.0001
Continuous	1.018 ± 0.005	1.070 ± 0.016	310 ± 31	1.3847 ± 0.0002
% Difference	0.20	0.10	4.5	0.03

5.3.5 HIV VLP recovery and purification

The HIV VLPs were first introduced as the viral product to be purified by the continuous ATPS due to the simple detection of the GFP label. Purification of HIV VLPs was achieved using the continuous ATPS set-up shown in **Figure 5.1B**. After 15 minutes

of system equilibration without HIV VLP flow, HIV VLP was continuously injected into the system for 40 minutes to test HIV VLP partitioning to the PEG-rich and citrate-rich phase. 10 minutes after HIV VLP injection, HIV VLPs began to partition to the PEG-rich phase more favorably compared to the citrate-rich phase, shown in **Figure 5.5A**.

Although the expected residence time through the separator section was 20 minutes, HIV VLPs were collected in the outlet sooner possibly because the first HIV VLPs to enter the filled separator column could travel quicker through the PEG-rich phase due to unhindered transport. After 20 minutes of operation, the partitioning between the two phases reached a plateau, indicating 20 minutes was the proper separation time to reach steady-state, which matched the batch system separation time. HIV VLP flow was stopped at 40 minutes to demonstrate the ability of the system to achieve equilibrium again after HIV VLP flow stopped (**Figure 5.5A**, vertical dashed line). No HIV VLP was collected in either phase 20 minutes after HIV VLP flow was stopped, indicating a 20-minute residence time. For the 40 minutes of continuous operation with HIV VLP flow, $69 \pm 1\%$ of the HIV VLP was recovered in the PEG-rich phase and $41 \pm 7\%$ of the HIV VLP was recovered in the citrate-rich phase. The recovery and purification of HIV VLPs are summarized in **Table 5.3**.

This was an unoptimized system using partitioning parameters that were established for PPV in batch-mode. With ATPS parameters specifically determined for the HIV VLPs, recoveries of the VLPs would likely increase.

Figure 5.5: A) HIV VLP recovery in the PEG-rich and citrate-rich fractions collected over 60 minutes of continuous separation, B) PPV recovery in the PEG-rich and citrate-rich fractions collected over 30 minutes of continuous separation, C) DNA recovery in the PEG-rich and citrate-rich fractions collected from HIV VLP purification, D) DNA recovery in the PEG-rich and citrate-rich fractions collected from PPV purification, E) SDS-PAGE gel showing contaminant protein content in the PEG-rich and citrate-rich phases from HIV VLP purification, and F) SDS-PAGE gel showing contaminant protein content in the PEG-rich and citrate-rich phases from PPV purification.

Table 5.3: Recovery and purification of PPV and HIV VLPs summary

Virus	PEG-rich Recovery (%)	DNA Removal (%)	Protein Purity
HIV VLP	69 ± 1	73 ± 1	Pure by SDS
PPV	107 ± 56	96 ± 3	Pure by SDS

The partitioning of host cell dsDNA was observed by quantifying the DNA in each PEG-rich and citrate-rich fraction using the PicoGreen assay. With the high concentration of PEG and citrate present in the samples, PEG-rich samples were diluted

to a final concentration of 2 w/w% PEG, and citrate-rich samples were diluted to a final concentration of 0.24 w/w% citrate. The DNA partitioning over 40 minutes of continuous operation of the ATPS can be seen in **Figure 5.5C**. The DNA recovery in the PEG-rich phase was $27 \pm 1\%$, and DNA recovery in the citrate-rich phase was $78 \pm 5\%$. This demonstrates cATPS can remove a majority of host cell DNA in single step.

The purity of the collected HIV VLP product in the PEG-rich phase was visualized with SDS-PAGE. Protein bands from PEG-rich and citrate-rich fractions collected during continuous separation of VLPs can be seen in **Figure 5.5E**, with all detectable proteins partitioning to the citrate-rich phase. The figures show that the PEG-rich phase containing the HIV VLP product is highly purified from contaminant proteins.

5.3.6 PPV recovery and purification

With the successful recovery and purification of HIV VLPs, PPV was introduced into the continuous ATPS as the viral product. However, initial trials showed low recovery of PPV in the PEG-rich phase using the same continuous ATPS used for HIV VLPs. Typical PPV recovery was 10-15% in the PEG-rich phase, and 60-80% in the citrate-rich phase, which was not expected. Batch experiments using the same ATPS concentrations had over 70% recovery in the PEG-rich phase, with rarely any PPV in the citrate-rich phase, as it was readily salted-out of the citrate when mixed with a vortex mixer [7]. It was hypothesized that PPV takes longer to partition to the PEG-rich phase compared to HIV VLPs due to the difference in pI (5 for PPV and likely <5 for HIV VLPs), and differences in the viral surface affinity to the relatively more hydrophobic PEG-rich phase. The pI of the viral product in ATPS directly effects the salting-out ability of the citrate. The net negative surface charge on PPV at a system pH of 7 is of less magnitude when compared to the net negative surface charge on the HIV VLPs because of the lower pI. This makes the HIV VLPs electrostatically repulsed out of the citrate-rich phase with more force than PPV. Additionally, HIV VLPs have a lipid bilayer which is hydrophobic due to the presence of many mannose N-glycans on the lipid surface [235]. This allows the HIV VLPs to have a higher affinity to the relatively more hydrophobic PEG-rich phase compared to PPV, which has been shown to be slightly

more hydrophobic than common proteins [113]. It was hypothesized that HIV VLPs were quickly salted-out from the citrate-rich phase to the interface and pulled into the PEG-rich phase by hydrophobic interactions between the HIV VLP lipid bilayer and high MW PEG.

A different mixing strategy was implemented for the PPV-containing ATPS to ensure PPV had time to partition to the PEG-rich phase after being salted-out from the citrate-rich phase. This was achieved by mixing a 250 g system with 10 vol% PPV in a glass bottle with a stir bar and flowing directly into the continuous settler. This design change for PPV purification can be seen in **Figure 5.6** (note the change from the helical mixer in **Figure 5.1B**).

Figure 5.6: Mixer set-up for ATPS TLL 32 TLR 0.5 with 10 vol% PPV for continuous purification

After the 250 g system was mixed in the glass bottle with a stir bar for 10 minutes, the PPV-containing ATPS was continuously flowed through the separator tubing and settler sections. PPV favorably partitioned to the PEG-rich phase as expected during the 30 minutes of continuous operation, shown in **Figure 5.5B**. For the 30 minutes of continuous operation, $107 \pm 56\%$ of the PPV was recovered in the PEG-rich phase and $0.9 \pm 0.3\%$ of the PPV was recovered in the citrate-rich phase. The recovery and purification of PPV are summarized in **Table 5.3**.

The DNA recovery in the PEG-rich phase was $1.1 \pm 2.7\%$, and the DNA recovery in the citrate-rich phase was $85 \pm 10\%$, shown in **Figure 5.5D**. The high recovery of

DNA in the citrate-rich phase is consistent with another study that recovered plasmid DNA in the salt-rich phase of ATPS [167]. Thus, this continuous ATPS is a good unit operation for the removal of DNA from viral products and increased mixing may assist in DNA removal from the PEG-rich phase.

SDS-PAGE was performed on every other collected fraction to visualize the purity of the PEG-rich and citrate-rich phase, shown in **Figure 5.5F**. The main contaminant in the crude PPV injected into the system was BSA. The PPV was made with 10 vol% FBS added to the cell culture media, which contains 3-5 g/dL of total protein [236]. The citrate-rich fractions contained a majority of the contaminant proteins, with only minimal amounts of protein seen in the PEG-rich fractions. The protein band present in the PEG-rich fractions correspond to BSA present in the sample with high concentration of PEG. Proteins present in high concentrations of PEG are known to cause smearing and protein band migration [237], which was also tested by spiking known concentrations of BSA into water and PEG to compare the protein band locations (**Appendix Figure A.4**).

The Bradford assay was used to attempt to quantify the protein content in the PEG-rich and citrate-rich phases. Due to high concentrations of PEG and citrate in the samples, 27 w/w% and 24 w/w% respectively, samples were diluted to 1 w/w% to minimize the interference. However, batch-mode experiments showed diluting the samples to 1 w/w% PEG caused the protein concentration to be below the lowest limit of detection possible by the assay at 2.5 µg/mL. This resulted in <56% protein recovery in the PEG-rich phase, however, the true protein content is expected to be much lower in the PEG-rich phase. Protein recovery in the citrate-rich phase was $15 \pm 7\%$, with the remaining protein likely trapped in the interface between the PEG- and citrate-rich phases. While it was difficult to quantify, high levels of proteins were removed from the stock PPV.

5.4 Conclusions

This is the first demonstration of continuous ATPS for viral particle purification. This system was able to purify both an enveloped (PPV) and non-enveloped (HIV VLP)

virus from their contaminants. The continuous ATPS was able to recover $107 \pm 56\%$ of PPV in the PEG-rich phase with high protein and DNA removal. The system was also able to recover $69 \pm 1\%$ of HIV VLPs in the PEG-rich phase with similar contaminant removal. The scale up and continuous operation of this ATPS was achieved by ensuring continuous mixing and settling of the ATPS components was achieved. Mixing was the critical step to partition the viral product to the PEG-rich phase while leaving the contaminants in the citrate-rich phase. HIV VLPs were readily partitioned to the PEG-rich phase when mixed by the helical mixer, but PPV required the use of a mechanical mixer to ensure adequate phase contact between ATPS components. This difference in partitioning ability between the two viral products was hypothesized to be due to the difference in physicochemical properties of the virus. The pI of the viral products dictated the salting-out ability of the citrate-rich phase and the presence of a lipid bilayer envelope of HIV VLPs allowed them to easily partition to the PEG-rich phase. With further scale-up and optimization of the continuous ATPS for a target viral product, this purification process has the potential to be used in large-scale, fully continuous downstream processing trains in vaccine manufacturing.

6 Conclusions and future work

6.1 Conclusions

This dissertation discussed the development of two novel virus detection methods and one virus purification method. The first detection method used graphene biosensors and was discussed in Chapter 3. The second virus detection method utilized gold nanoparticle (AuNP) aggregation and was discussed in Chapter 4. The virus purification technique was a scaled up continuous aqueous two-phase system (ATPS) and was discussed in Chapter 5.

Graphene ink printed biosensors were created for potential use as inexpensive point-of-care devices. The goal was to flexographically print electrochemical biosensors that were capable of distinguishing between analyte-containing samples compared to buffer-only samples. The sheet resistivity (SR) on the sensor surface was monitored as different concentrations of analyte was applied and compared to the SR changes due to buffer-only applications. Biosensors created with paper and non-paper substrates showed no ability to distinguish between either sample applied to the surface. The number of ink coats, ink conductivity, and graphene sheet hydrophobicity/size were varied in attempts to change the electrical and chemical properties of the biosensors without success. Each set of tested biosensors resulted in the same conclusion: SR changes were due to applying any liquid to the surface, independent of what biomolecule was present in the sample. This conclusion led to the strategy of applying hydrophobic wax barriers on the graphene surface to contain the liquid sample in a testing region without contacting the electrical leads. However, wax barriers were reduced the swelling effects of the biosensors but were also unsuccessful at protein detection. Additionally, functionalization of the graphene sheets with a sandwich enzyme linked immunosorbent assay (ELISA) was performed which also lead to insufficient SR differences to detect target analytes. Overall, with a wide variety of parameters tested for the biosensors, no positive and publishable results were obtained.

The virus detection ability of the AuNP aggregation assay was studied using different sizes of AuNPs. The original assay utilized 15 nm AuNPs for the detection of

porcine parvovirus (PPV) to 6 log MTT₅₀/mL and bovine viral diarrhea virus (BVDV) to 4 log MTT₅₀/mL when aggregated by 1 M mannitol. The goal was to synthesize other sizes of AuNPs and use them in the assay to lower the limit of detection (LOD). Seed-mediated, room temperature synthesis of 27, 68, 96, 100, and 106 nm diameter AuNPs was successfully performed. The AuNPs were characterized by ultraviolet-visible spectroscopy (UV-Vis), dynamic light scattering (DLS), and transmission electron microscopy (TEM). The different sizes of AuNPs were used to explore how the LOD of the assay would change based on the differences in interactions between AuNPs and viral particles. AuNPs with a 40 nm diameter were complexed with PPV, resulting in a LOD of 7 log MTT₅₀/mL when aggregated by 1 M mannitol. AuNPs with a 50 nm diameter were complexed with BVDV, resulting in a LOD of 5 log MTT₅₀/mL when aggregated by 1 M mannitol. AuNPs with a diameter of 5 nm were used in an attempt to detect PPV, but the high concentrations of osmolytes used in the aggregation step led to AuNP aggregation regardless of the presence of virus or control protein. Neither an increase nor decrease in the size of AuNPs lead to an improved LOD compared to the published work of using 15 nm AuNPs in the same detection assay. These results indicated that the size of AuNPs used in the virus detection assay do not play a critical role in detection.

A novel continuous virus purification technique using an aqueous two-phase systems (ATPS) was developed. The goal was to scale up a batch ATPS and confirm the recovery and purification of PPV and human immunodeficiency virus-like particles (HIV VLPs) was not lost. The continuous mixing section of the ATPS was identified as the unit operation that influenced proper viral particle partitioning to the poly(ethylene glycol) (PEG) rich phase. Different in-line mixer types were tested for their ability to properly mix the citrate salt and viscous PEG components. The components flowing through the system were in the laminar flow regime due to the low flow rate tested for the bench-scale operation and high viscosity PEG. Testing revealed that an in-line helical mixer was able to disperse the phases in the laminar flow regime. HIV VLPs were readily partitioned to the PEG-rich phase when mixed by the helical mixer, but PPV required the use of a mechanical mixer to ensure adequate phase contact between ATPS components. This difference in partitioning ability between the two viral products was hypothesized to

be due to the difference in physicochemical properties of the virus. The pI of the viral products dictated the salting-out ability of the citrate-rich phase and the presence of a lipid bilayer envelope of HIV VLPs allowed them to easily partition to the PEG-rich phase. The continuous ATPS was able to recover $107 \pm 56\%$ of PPV in the PEG-rich phase with high protein and DNA removal. The system was also able to recover $69 \pm 1\%$ of HIV VLPs in the PEG-rich phase with similar contaminant removal. With further scale-up and optimization of the continuous ATPS for a target viral product, this purification process has the potential to be used in large-scale, fully continuous downstream processing trains in vaccine manufacturing. This work was the first demonstration of continuous ATPS for viral particle purification.

6.2 Future work

This dissertation contains numerous tests to develop a graphene biosensor for rapid analyte detection, an optimization strategy for the AuNP virus detection assay, and a scale up design of a continuous ATPS. However, there are other experiments that can be performed to further improve each method.

To improve the graphene biosensors for analyte detection, the graphene ink properties could be altered. Results were discussed from biosensors created with a different ink binder and graphene nanoplatelet grade, but other binds and grades of graphene could be used. For example, a more hydrophilic ink binder could be used during the formulation of the ink which could provide biomolecules a more suitable graphene ink surface during sample application and testing.

The LOD of the AuNP aggregation assay was not improved using different sizes of AuNPs. This indicated that AuNP size was not the main influencer in virus detection. Many other system parameters exist that could influence the detection ability. The system pH could be lowered closer to the virus pI, which would create more positively charged patches on the virus surface. With more positively charged patches, the negatively charged citrate-capped AuNPs could electrostatically bind to the AuNPs more readily. With more AuNPs complexed with the virus, the light scattering ability of the aggregated complexes with less viral particles could be improved, leading to a lower LOD. One

possible issue with this change would be the stability of the AuNPs at a lower pH, as the change in pH could cause the citrate-capped AuNPs to aggregate. Different shapes of AuNPs also exist, such as gold nanorods (AuNRs), gold nanostars (AuNSts), and gold nanocubes (AuNCs). The different shapes of AuNPs have different characteristic UV-Vis spectra and light scattering ability. For example, AuNRs have a characteristic two-peak UV-Vis spectra due to the longitudinal and transverse surface plasmon resonance (SPR) properties due to the large aspect ratio of rods compared to spheres [238]. This could be exploited because as AuNRs aggregate, one of their characteristics peaks could disappear due to losing that characteristic aspect ratio of monodisperse AuNRs. Additionally, different osmolytes could be used to induce aggregation in the assay. Other osmolytes such as alanine and trehalose have been shown to aggregate enveloped and non-enveloped viruses, which could be used to aggregate the AuNP/virus complexes [111, 112]. Alanine is an amino acid with a hydrophobic side chain that may be able to aggregate viral particles more effectively. Alanine residues in protein molecules are normally buried within the protein structure to protect it from solvent exposure [205]. This effect could be exploited for the aggregation assay because the hydrophobic residues from alanine could aid in stabilization and enhanced aggregation of the dehydrated hydrophobic patches of virus particles. Trehalose is a sugar with two molecules of glucose linked by an α , α -1,1-glucosidic bond [206]. Trehalose has a high affinity for water due to multiple hydrogen bonding events between its two glucose rings and water molecules [207]. The high affinity for water would rapidly dehydrate the virus surface, potentially causing more virus aggregates to form as more and more water is pulled away from the virus surface. This could improve the AuNP assay by allowing a lower concentration of osmolyte to be used in the aggregation step, or to dehydrate a smaller concentration of virus particles due to the strong dehydration ability of trehalose.

The ATPS was the first of its kind technique for continuous viral particle purification, which leaves it open for numerous optimization opportunities and future work to make it a complete and robust process. The current system could be improved by implementing a true continuous mixer for PPV purification. One possible way to accomplish this would be to use the same glass bottle/stir bar configuration but purchase

another 3-channel pump to flow the stock components into the mixer. The current system could also benefit from additional scale-up beyond 3 mL/min. At an increased flow rate, the helical mixer should have increased performance as turbulence is reached in the mixer. If sufficient mixing is not achieved with the single 12-element helical mixer, additional mixers could be put in series, or a larger-diameter helical mixer could be constructed from larger inserts. Proper quantification of contaminant proteins in the PEG-rich and citrate-rich phases also needs to be performed. Attempts to quantify the protein in both phases using the Bradford assay, microBCA, and UV adsorption were mostly unsuccessful due to the high concentrations of PEG and citrate interfering with the assay reagents. One quantification technique that may possibly work is densitometry from SDS-PAGE gels. This technique has been used before in a PEG 400 Da-potassium phosphate ATPS, and could be implemented for our PEG 12,000 Da-citrate ATPS [179]. Ideally, in-line biomolecule quantification techniques should be implemented because this is a fully continuous ATPS. With robust in-line quantification techniques, industrial implementation of a continuous ATPS could be realized. This is because industrial bioprocesses are under strict regulatory guidelines that require quantification of the bioproducts at each stage of the manufacturing process. One possible in-line quantification technique could utilize nanoparticle tracking analysis (NTA), but issues of resolution to detect viral particles is a concern. Additional changes to the ATPS itself could result in improved recovery and processability. Unpublished work in the Heldt lab has shown the addition of osmolytes to ATPS improves the recovery of the viral product on a lower TLL. A lower TLL means a lower w/w% PEG-rich phase is collected, which has a much lower viscosity without a loss of recovery. This change would increase the processability of the PEG-rich product containing phase for further downstream processes. One of these downstream processes is the recovery of the viral product out of the PEG-rich phase. Two methods for recovering the viral product out of the PEG-rich phase are back extraction using a second stage of ATPS or filtering the viral product out of the PEG-rich phase. Back extraction is the technique to partition the product from the PEG-rich phase into the citrate-rich phase using a different ATPS that more favorably partitions the virus to the citrate-rich phase. This technique has been used before to purify

and back extract foot-and-mouth disease virus in a batch system [174]. Lastly, to convince industry to implement a continuous ATPS for virus purification, an economic analysis should be performed.

7 References

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A Appendix for Chapter 5

System Schematic:

Basis:	
$Q_{mixture} =$	3 mL/min
Mixture Stream (36% TLL, 0.1 TLR)	
ρ_{mix}	1.2085 g/mL
$\dot{m}_{mix}$	3.6256 g/min
$wt\%_{PEG}$	18 %
$wt\%_{Cit}$	12 %
$wt\%_{Water}$	70 %
$\dot{m}_{PEG}$	0.6526 g/min
$\dot{m}_{Cit}$	0.4351 g/min
$\dot{m}_{Water}$	2.5379 g/min
Stream 1 (45 w/w% PEG)	
$wt\%_{PEG}$	45 %
$\rho_{45\%\ PEG}$	1.0654 g/mL
$\dot{m}_{total,1}$	1.4502 g/min
$Q_{total,1} =$	1.3613 mL/min
$\dot{m}_{water,1}$	0.7976 g/min
Stream 2 (30 w/w% Cit)	
$wt\%_{Cit}$	30 %
$\rho_{30\%\ Cit}$	1.1579 g/mL
$\dot{m}_{total,2}$	1.4502 g/min
$Q_{total,2} =$	1.2525 mL/min
$\dot{m}_{water,2}$	1.0152 g/min
Stream 3 (Dilution water)	
$\dot{m}_{water,3}$	0.7251 g/min

Figure A.1: Mass balance and schematic of 32% TLL 0.5 TLR ATPS. The green boxes are boxes that should be changed for each run to ensure the density of PEG and citrate result in the correct flow rate.

Figure A.2: TEM images of HIV gag-GFP VLPs purified by ultra-centrifugation

Figure A.3: Helical mixer video still showing neutral red dye dispersing through the phases

Figure A.4: SDS-PAGE of 0.01-4 mg/mL BSA in water and 33 w/w% PEG showing band migration

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