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Complementary Charged Molecular Imprints of West Nile Virus Antibodies

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COMPLEMENTARY CHARGED MOLECULAR IMPRINTS OF WEST NILE VIRUS ANTIBODIES

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Dedication

To my family who has been there with me every step of the way.

COMPLEMENTARY CHARGED MOLECULAR IMPRINTS OF WEST NILE VIRUS ANTIBODIES

by

JULIO RINCON, M.S. INTERDISCIPLINARY ENGINEERING

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Abstract

There is a significant demand for robust and stable receptor molecules that can mimic biological molecules, such as antibodies. Relying only in natural recognition molecules have greatly limited the uses and capabilities of many aspects of health sciences due to product expense and stability. This is especially important in medically underserved areas where the lack of resources and faulty or limited cold-chain makes antibody based diagnostics very difficult to implement.

With molecular imprinting, it is possible to recognize diseases with the added advantage of product stability, long term use, fast preparation and ease of scalability; all while being cost effective. Despite of these great advantages, molecular imprinting also suffers from lower binding capacities and affinities. Most importantly, molecular imprints are limited to molecules less than 1,500 Dalton (Da).

We developed a molecular imprinting method by matching the physiological pH and by complementary matching electrostatic and hydrophobic charges in West Nile virus antibody (WNVA). Charge matching was achieved by downloading the crystallographic data of WNVA. After electrostatic and hydrophobic aminoacid ratios are determined, MIPs active monomers ratios are counter matched to the template. This method allows MIP formulations to be tailored to their required template easily.

The successful imprint of WNVA, a 150 kDa molecule, enables an emerging technology where artificial recognition molecules can complement and expand upon current applications of antibodies. Future applications of molecularly imprinted particle range from molecular biology to healthcare including disease diagnostics and passive immunization.

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Chapter 1. Introduction

Modern medicine has witnessed interdisciplinary technology innovations in healthcare with a continuous growth in life expectancy across the globe. However, there is also a growing global concern about its affordability of rapidly rising costs. Providing minimal quality healthcare to all, including large and sparsely distributed communities in rural areas, is the most vital challenge to developing nations [1], [2]. With quality and affordable diagnostics, it is possible to increase availability to medically underserved areas, where diagnostics can serve a fundamental role in the health value chain by down streaming resources. By reducing misdiagnosis and the prescription of ineffective remedies, the cost of healthcare can be lowered [3].

Although diagnostics are widely used in developed countries, they are not available in low resource settings commonly found in underdeveloped countries. In low resource settings, the absence of diagnostic tests, due to costs or insufficient infrastructure, has led to diseases being treated only by clinical symptoms and the local prevalence of the disease. Whereas this approach captures most patients requiring treatment, it also unnecessarily treats others which do not require treatment. Equally important, this latter group of patients is not being treated for their disease. Finally, syndromic management of disease may also accelerate drug resistance [4]. Currently, lateral flow tests have become the standard bioassay format in low-resource settings because it is rapid, easy to use, affordable, uses reagents stored in dry form, and is equipment-free.

Unfortunately, lateral flow tests suffer from various limitations, such as poor sensitivity of multiple analytes and inability to multiplex [5], [6]. The main component in lateral flow assays is the antibody, a recognition biomolecule which typically suffers from product stability, slow and difficult manufacturing, and some require strict cold chain requirements [7–10]. Usually, it takes 2 to 6 months and costs between $4,000–$30,000 to develop monoclonal and polyclonal antibodies [11]. Because of difficulties associated with the development of new diagnostics, companies are spending ten times the average of all industries for research and development [12]. Hence, it may

be advantageous to develop affordable, robust, and stable receptor molecules that can mimic biomolecules [13–16], such as antibodies and enzymes, in immuno assays.

With molecular imprinting, it is possible to develop specific recognition molecules. This technology can be used to design affordable diagnostics, with the added advantage of rapid manufacturing at any scale [17], [18]. Molecular imprinting is achieved by guiding the assembly of polymers in the presence of a print molecule called template. As monomers surround the template, due to non-covalent attractions, these become fixed by a rapid polymerization of the network. The subsequent removal of the template leaves behind specific binding sites that are capable of selectively recognizing their original template again [19–21]. These sites are used as the recognition molecules in the newly formed molecularly imprinted polymer particles (MIPs).

MIPs are capable of selectively recognizing molecules with similar binding strengths as those seen in antibodies [13], while being stored dry for several years and reused up to a hundred times before losing recognition capabilities as shown by Tan et al. [22] and Kupai et al. [23]. Whereas antibodies can only be used a few times with reducing binding strengths in each use and usually can be stored for up to a year [24], [25]. Despite MIPs advantages, the imprinting process is hindered by multiple factors such as size, molecule complexity and the conformational structure of templates [26–29]. Also, the use of organic solvents during synthesis and testing of MIPs is necessary by the majority of imprinting technologies to date. While solvent increases protein identification, it causes severe protein disruption, where proteins will not behave as in physiological conditions [30].

Most importantly, there are still limitations when imprinting molecules larger than 1,500 Da, whereas antibodies are produced by the immune system for molecules greater than 6,000 Da without the use of adjuvants [31], [32]; hence, the use of MIPs as artificial antibodies is limited. Despite this, significant advances have been achieved. Rachkov and Minoura produced MIPs by the epitope approach [33], where only a portion of the template is used during molecular imprinting. Although a fraction of the template was used during imprinting, MIPs are still capable

of selectively recognizing the whole molecule. Another significant achievement is the use of anti melittin MIPs developed by Hoshino et al. [34–36] for the neutralization of bee venom in rats, where the use of MIPs neutralized melittin in circulating blood with reduced mortalities.

Therefore, MIPs show potential as biological recognition molecules with the possibility of applications in diagnostic devices and therapeutics. Because of this, we focused on developing MIPs against West Nile Virus (WNV). WNV is an emerging disease and a nationally notifiable condition. Despite the dangers of WNV, many cases are not reported because they are not recognized. Symptoms can resemble a cold or mild influenza-like illness, for which medical care is not sought. In low resource countries, the additional cost for testing a severe WNV infection provides little value to a patient as it does not provide with improved medical attention [37]. The high cost of diagnosis and lack of targeted treatment prevents early disease monitoring and delays disease vector control. Thus diagnostic costs must be lowered to improve targeted early vector control.

1.1 West Nile Virus

Viruses are particles that consist of genetic material and a protein coat. They are not cells and are not considered living organisms. Viruses invade cells and direct them to generate new copies along with proteins that cause the host cell to lyse and release new viruses; killing the cell in the process and releasing thousands of new virus copies. Viruses cause common infectious diseases such as the common cold, flu, and warts. They also cause severe illnesses such as HIV/AIDS, smallpox and hemorrhagic fevers [38]. Particularly, WNV is an arthropod-borne virus, with human infection most often resulting from bites of infected mosquitoes. Mosquitoes become infected when they feed on infected birds, which circulate the virus in their blood for a few days. During later blood meals (when mosquitoes bite), the virus is injected into humans and animals, where it can multiply and cause illness.

In 1999, WNV was introduced into North America, and over the last 18 years, it has spread throughout the continental United States, Mexico, Canada, Caribbean, Central and South America.

Because of its increased range, WNV has emerged globally as a significant cause of viral encephalitis, with steadily increasing incidence worldwide. Overall, a small percentage of infections result in severe neurological disease [39]. Infection with WNV is usually asymptomatic (no symptoms) in around 80% of infected people. About 20% of people who become infected with WNV will develop West Nile fever. Symptoms include fever, headache, tiredness, and body aches, nausea, vomiting, occasionally with a skin rash (on the trunk of the body) and swollen lymph glands. The incubation period is usually 3 to 14 days [40].

Afterward, approximately 1 in 150 persons infected with the WNV will develop a more severe form of the disease. Serious illness can occur in people of any age, however people over the age of 50 and some immunocompromised persons (for example, transplant patients) are at the highest risk for getting severely ill when infected with WNV [41], [42]. Symptoms of severe disease include headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, and paralysis. Models estimate that between two million and four million people have been infected with WNV since 1999. Unfortunately, the management of WNV disease is based exclusively on the control of the complications of this infection, including seizures, hyponatremia, and raised intracranial pressure [43].

Any additional testing to confirm WNV provides with no effective treatment of the disease. The sole value of diagnosis lies exclusively in vector control to prevent further infections. Because there is no drug prophylaxis, human vaccine, or treatment available for WNV, integrated pest management and personal mosquito protection remain the only options for reducing human illness and death, and early warning of high-risk areas allows for these measures to be implemented in a timely and effective manner [44]. Reporting can assist local, state, and national authorities to recognize outbreaks and to implement control measures to reduce future infections.

The lack of value in WNV diagnosis, due to considerable cost [45] , prevents proper disease monitoring, in which only severe cases are reported. The under diagnosis of WNV disease reduces the capability of preventing WNV disease outbreaks. To improve disease monitoring and reduce

the dependence on human samples early diagnosis methods were developed. These methods include the monitoring of statistically significant spatiotemporal clustering of dead birds (DYCAST), as deaths of birds have been proven significantly associated with human cases. As an alternative, molecularly imprinted particles (MIPs) can be used to develop affordable diagnostics for use with humans and animals. To improve DYCAST specificity, MIPs could be used for animal testing of WNV infections, at a reasonable price.

1.2 Diagnostics and its Limitations

According to the Institute of Medicine, $750 billion in healthcare spending could have been saved in 2009 by using quality and effective diagnostic tools, in the US [46]. Diagnostics serve a fundamental role in the health value chain by influencing the quality of patient care, health outcomes, and downstream resource requirements. From consumer-friendly at-home pregnancy and glucose monitoring tests to more complex automated laboratory-based systems, these tests are often first-line health decision tools. While diagnostics comprise about 2% of healthcare costs, test results influence as much as 70% of healthcare decision-making. The value of diagnostics accrues not only to clinicians and patients but to health care managers, third-party payers, and quality assurance organizations that use diagnostic to measure and improve health care quality [47].

Due to the high necessity of better diagnostics, companies are spending ten times the average of all industries in research and development. Today most diagnostic devices rely on molecular recognition by using biological tools such as antibodies, enzymes, nucleic acids, and cells. Without the use of natural recognition molecules, diagnosing diseases would be almost impossible. This dependence in bio-recognition molecules has proven vital to the success of our current medical technologies. Unfortunately, relying only on natural components have significantly limited the uses and capabilities of many aspects of medicine in the use of vaccines, treatment, and diagnostics. Current technologies can produce known antibodies in as little as three weeks but depending on the diagnostic or therapy, time and cost can significantly rise. As time

and difficulty to produce an antibody (or any biorecognition molecule) increases, so does its price [48].

In low resource areas, as defined by limited access to healthcare, the storage of biomolecules becomes complicated. The use of costly and delicate molecules in these areas, where electricity and equipment are often unreliable, results in material waste. It is not uncommon for reagents to degrade before they are used, resulting in reduced availability of diagnostics and treatment. The advantage of developing robust and cost effective recognition molecules translates to an increase in availability of diagnostics at local health clinics. For example, a patient can verify if his/her sore throat is caused by an infection treatable with antibiotics, all in a single visit with the medical practitioner. This practice effectively reduces antibiotics misuse [49], [50] and streamlines limited resources. However, the use of rapid diagnostics is not a common practice where treatment is close to the price of the diagnostic. Because of this, the value of information over the cost of diagnostic is not high enough as to justify an additional expense on simple diseases, even if a disease is misdiagnosed and improperly treated.

The need for better diagnostics also extends to developed countries, where rare infections require patient's sample to be collected, stored, and shipped to a facility capable of testing for the particular disease. Centralized testing is necessary for diseases where the number of tests required by the reagents is too low to be carried locally. The sample turnaround may take more than ten days before an answer is available. Because of this, critical diseases might be treated empirically regardless if the patient is infected or not with such disease; meanwhile, the disease is confirmed. It is not uncommon for patients to be given unneeded treatments or the lack of appropriate treatment results in delayed therapy.

Currently, developing nations have the greatest need for robust and affordable diagnostics, especially those with widely scattered rural areas. The need of diagnostics is due to infectious diseases being the leading cause of death in developing countries. The lack of resources and poor infrastructures creates challenges in delivering healthcare where access to electricity, purified

water, and specialized equipment becomes difficult. Furthermore, in scattered rural areas, temporary health stations are required to deliver healthcare to the medically underserved. Unfortunately, they are installed only in seasons of high endemic incidence to monitor and protect citizens with the least amount of resources; diagnostic capabilities are often null or limited. Due to the described challenges, available diagnostics are typically cost prohibitive or if not the proper storage and handling conditions cannot be met during and after transit to an established location [51].

In rural areas, patients must travel from their remote establishments to temporary health stations. Due to the distance traveled or their weakened health conditions, it is likely for a patient to show up once, follow-ups are unlikely to happen. Because of this, it is imperative for the health personnel to have the capability of rapid diagnosing without the need of specialized equipment, aside from the diagnostic device. Finally, they must be able to gather results during the time patients remain on the establishment. Hence, diagnostics in rural areas are mostly limited to point of care (POC) devices [52], [53]. POC devices can benefit diagnostic and monitoring of diseases such as HIV, where patient attrition is high, such as in Mozambique [52].

Particularly, in Cd Juarez, Mexico the standard procedure for testing HIV in children may take from 2 to 4 weeks through "Seguro Popular" or the government's medical insurance for people living in poverty or the uninsured. Although local screening is available at private laboratories, samples are collected and centrally processed due to the unaffordability of local processing of samples. While this approach enables cost effective HIV screening, patient attrition is high, and treatment is often not delivered. In cases where patients might not return, it may be more important to obtain an immediate, yet less sensitive test at the point of care (POC), than a highly reliable test that takes weeks to process. Reasons for high patient attrition are due to the time required to obtain health care services, the stress induced by lengthy turnarounds (not knowing if positive or negative), patient relocation, and many other factors.

It is common for patients living at the edge of the city to require 2 to 3 hours to arrive at the clinic; in rural areas travel time increases up to a week. Also, while waiting for results, infected patients remain untreated. In fact, many patients do not return for the results until they developed serious complications, due to the lack of treatment. Hence, patient outcomes are widely varied. While specificity and selectivity of standard diagnostics cannot be rivaled by POC tests, it is important to realize that limited resources can render a standard diagnostic undeliverable. Hence, affordable POC tests offer unique solutions for limited resource areas; whether the limitations are economical, spatial, or temporal.

While POC tests have unique advantages, there are other ways countries manage to deliver healthcare to rural areas. For example, Argentina and Mexico offer health services to medically underserved areas by train, as seen in figure 1. This approach allows a significant amount of supplies, equipment, and personnel to be delivered, but it is limited to areas accessible by train. Additionally, this requires patients to travel to the health station, where diagnostics need to be delivered in a single visit to achieve proper care during the short visit of the health station. Even with sufficient storage capacity for standard diagnostic equipment, it is beneficial to use POC tests as they reduce the time required for diagnosis and the required space for laboratory testing, a space that can be used to carry additional treatments.

Figure 1. Health train “Dr. Vagon” delivering services in a rural area [54].

1.2.1 POC devices

Point of care devices, as defined by the world health organization (WHO), are diagnostic tests that must be affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and must be deliverable to end users. These guidelines are known as the ASSURED criteria where table 1 further defines them. Due to the compelling need for equipment free diagnostics, in low resource settings, many point of care devices are being developed. Research objectives vary from multiplexed devices to assays with greater sensitivity and specificity. Particularly, microfluidic immunoassays developed as lab-on-a-chip tests have gain popularity and are being investigated. However, few are capable of delivering sample to result reliably, and many are cost prohibitive for developing countries. Regardless of the POC device most of them use antibodies to detect diseases [55].

Currently, the most successful POC diagnostics are lateral flow immunoassays. These POC tests incorporate antibodies to immobilize a target molecule, which detects a disease or condition. Because of their speed, ease of use, and relatively low cost, lateral flow assays have become the standard POC test in low resource settings. A typical lateral flow immunoassay is a pregnancy test, where antibodies are used to immobilize human chorionic gonadotropic, a hormone normally produced during pregnancy, in nitrocellulose paper. When the pregnancy hormone is detected, two red lines are shown, indicating a positive result [56].

Table 1. ASSURED guidelines for POC test for the developing world [57].

Characteristic	Target specification
Affordable	Less than US$ 500 per machine, less than US$ 10 per test
Sensitivity, specificity	Lower limit of detection: 500 HIV RNA copies per mL, 350 CD4+ T-cells per µL
User-friendly	1–2 days of training, easy to use
Rapid and robust	< 30 minutes for diagnosis, < 1.5 hours for HIV load monitoring, minimal consumables (i.e. pipettes), shelf life > 1 year at room temperature, high throughput
Equipment-free	Compact, battery powered, on-site data analysis, easy disposal, easy sample handling, no cold chain
Deliverable	Portable, hand-held

Although lateral flow tests offer great portability and cost effectiveness, it is a single use system, limiting testing to a single patient. Furthermore, they suffer from limitations such as poor sensitivity for many analytes of clinical importance and the inability to multiplex [5], [58]. Research is being carried to increase the sensitivity of these devices, and few to add the capability of multiplexing, but their reliability in the field needs to be proven. Currently, multiple steps are required to increase the sensitivity of lateral flow assays; thus, their manufacturing complexity increases. An amplified lateral flow immunoassay with higher sensitivity is shown in figure 2.

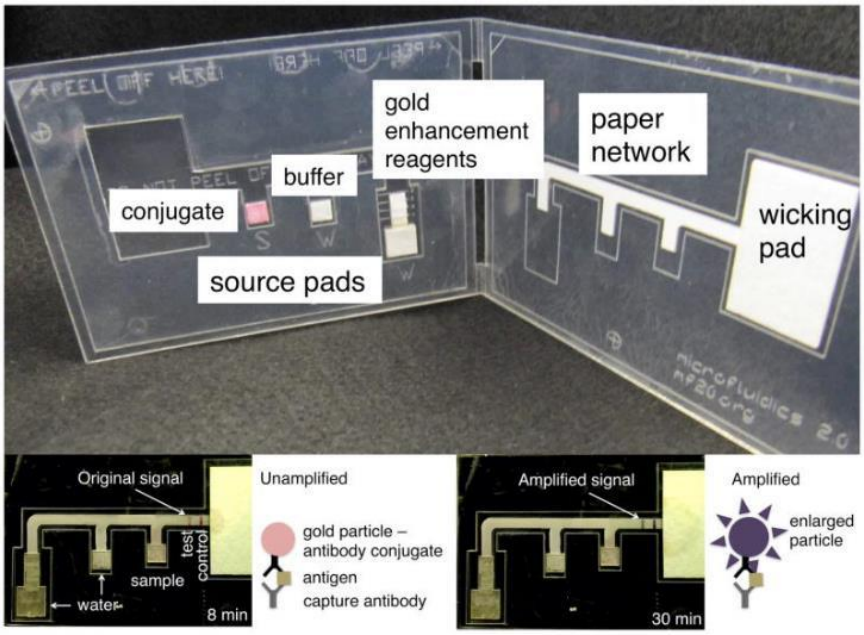

Figure 2. Amplified lateral flow immunoassay [5].

The most significant limitation of POC test is the stability during transportation and storage, which results in reduced sensitivity when improperly handled [59], [60]. Thus, it is of great interest to develop new recognition molecules that offer similar selectivity as antibodies, while offering increased molecule robustness. With molecularly imprinted polymers it is possible to develop synthetic recognition molecules which are capable of antigen recognition even after autoclaving them [61], [62]. The robustness of MIPs will offer unique capabilities over conventional tools such as extended shelf life and simpler transportation and storage.

1.2.2 WNV diagnostics

In 2003 there were an estimated 730,000 undiagnosed WNV infections. A total of 9,862 cases and 264 fatalities were reported. Whereas in 2012, there were 5,674 cases reported and 286 fatalities [63], [64]. Despite that 2012 had more WNV fatalities, almost half of the cases were reported from 2003. The reduction of WNV screening, especially for patients with mild symptoms, is due to the expense of diagnostics which offer little to no value to patients. According to the CDC, the average cost to diagnose WNV was in average $302 for outpatients.

As an overview, available tests for WNV are:

- IgG and IgM antibody seroconversion
- Plaque neutralization assays
- Viral detection by reverse transcription polymerase chain reaction (RT-PCR) assay
- Virus isolation by cell culture

Specifically, IgG and IgM seroconversion tests detect antibodies in serum. These tests are possible after an immune response is developed against an infection, in which antibodies are produced. Seroconversion assays are carried after at least 5 days after signs appear and about 2 weeks later on another sera sample to demonstrate a four-fold increase in titer as a confirmed seroconversion. If seroconversion assays are carried before the immune system can respond or if a patient is immuno compromised, these will yield a false positive. Particularly for a new infection, IgM test can be carried as soon as 5 days after infection, whereas IgG assays require 7-14 days before they can be used. Regardless of the immunoglobulin screened, detecting antibodies is simpler due to their high concentration available in serum, whereas viruses are present at continuously lowering titers; hence, detecting viruses in plasma is increasingly difficult after an infection when using antibodies as the detection molecule.

Other tests available are the plaque neutralization assay, in which patient's serum is incubated in a cell culture plate infected with WNV. A reduction of plaques, formed by the virus, indicates the presence of neutralizing antibodies. Unfortunately, this test requires days of incubation. For viral detection, virus isolation and incubation from patient's serum can be used to amplify any virus which is later screened with other detection methods; such as an ELISA or PCR test. Particularly, RT-PCR is used as a highly sensitive test against viruses, where the tests amplify disease specific DNA or RNA. PCR assays can consistently detect virus during viremia, but it is expensive and requires trained operators. Regardless of the test used, it is important to consider the days passed after the contraction of an infection as the suitability of the assay varies by the time passed. The recommended tests for a WNV infection, based on days, is shown in figure 3. MP-TMA refers to a mini pool test of 6 to 8 samples using transcription mediated amplification

assay, whereas ID-TMA refers to individual testing, which is more sensitive. MP-NAT and ID-NAT refers to nucleic acid tests in mini pool or individual test format. RNA, IgM, and IgG refer to viral and antibody levels in plasma.

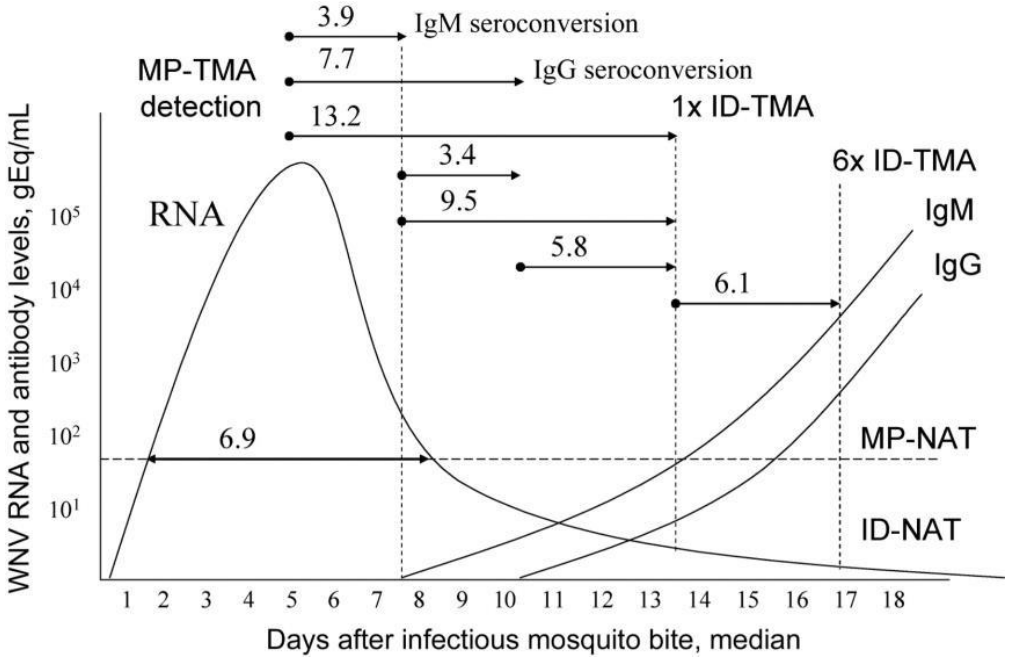

Figure 3. Dynamics of WNV RNA and WNV-specific antibody positivity and negativity from available detection methods [65].

Currently, there are few POC test available for WNV. The most recently POC test is a lateral flow assay, which the FDA approved in 2006 [66]. Furthermore, there are two vector diagnostics available, the VecTest® and RAMP®, but their use is limited to insect testing, and only a few countries can afford their use.

1.3 Disease Treatment and Drug Delivery

Currently, Arthropod-Borne viruses, including WNV, have no specific therapeutics. Because of this, the treatment of WNV infections is based solely on the control of complications. Despite this, there are nonconventional methods for treating WNV. In life threatening cases, passive immunization has been applied with positive effects [67]. This treatment adds foreign antibodies against WNV, which then the immune system can use for the recognition and neutralization of WNV.

Particularly, antibodies can be used as therapeutic agents but there are functional limitations, such as in WNV infections antibody treatment is ineffective. The main limitation in antibody therapeutics is due to the large quantities of antibodies required during treatment; since antibodies are expensive it is usually not cost effective [68]. Second, with non-human antibodies, the patient's immune system identifies them as foreign molecules and eventually eliminates them; thus the immune system renders the therapeutic useless. To prevent the immune system from attacking the therapeutic, monoclonal antibodies have to undergo a series of complicated modifications to reduce their immunogenicity, such as the development of chimeric antibodies [69]. After all the additional processing, antibodies used as therapeutics are more expensive than those used for diagnostics.

The expense of passive immunization has limited the treatment to mostly life threatening situations. While macromolecular imprinting can be used as artificial antibodies in diagnostics, it has not yet been used for passive immunization. Nonetheless, new drug delivery methods [70], [71] are being developed. MIP have been developed for the treatment of toxins [34], high cholesterol [72], and as anti-acids [73]. Interestingly, MIPs imprinted with mellitin were used for the recognition and neutralization of toxic mellitin in mice [35]. MIPs were used to reduce the tissue damage caused by the toxin, effectively reducing the mortality of mice against the control groups. A comparative image of mice treated with and without MIPs is shown in figure 4.

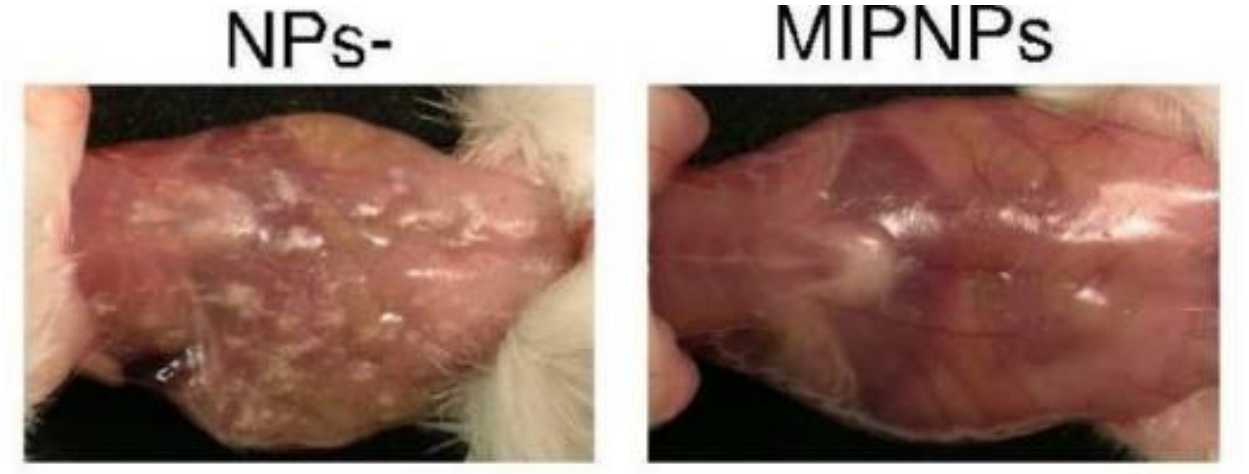

Figure 4. Macroscopic pathology of peritoneal inflammation of mice injected with mellitin without (left) and with (right) MIPs [35].

Chapter 2. Literature Review

To achieve molecular imprinting of large proteins, we reviewed current methodologies and their limitations. We then evaluated their synthesis methods, and after an in depth review, a synthesis technique was chosen. To refine our molecular imprinting formula, we looked to biology for guidance. Particularly, we used antibodies as inspiration by examining how they are capable of recognizing antigens selectively. By understanding the strategies and processes of the immune system to identify pathogens, improving MIP synthesis is considerably easier than designing specific protein interactions by pure chemistry alone.

2.1 Molecular Imprinting

Molecular imprinting is a synthesis technique that uses monomers as building blocks which self-assemble around a molecule called template. As monomers conform to the shape of the template, due to covalent and noncovalent attractions, they are fixed by a rapid polymerization of the network. After polymerization, a semi rigid matrix is formed. Meanwhile, the template remains trapped. Afterwards, the template is forcibly removed from the polymer by denaturalizing it. Once the template is removed, the leftover space becomes an active binding site with specific selectivity to the template. Thus, the polymer network is capable of rebinding the original template. The molecular imprinting process is simplified in figure 5.

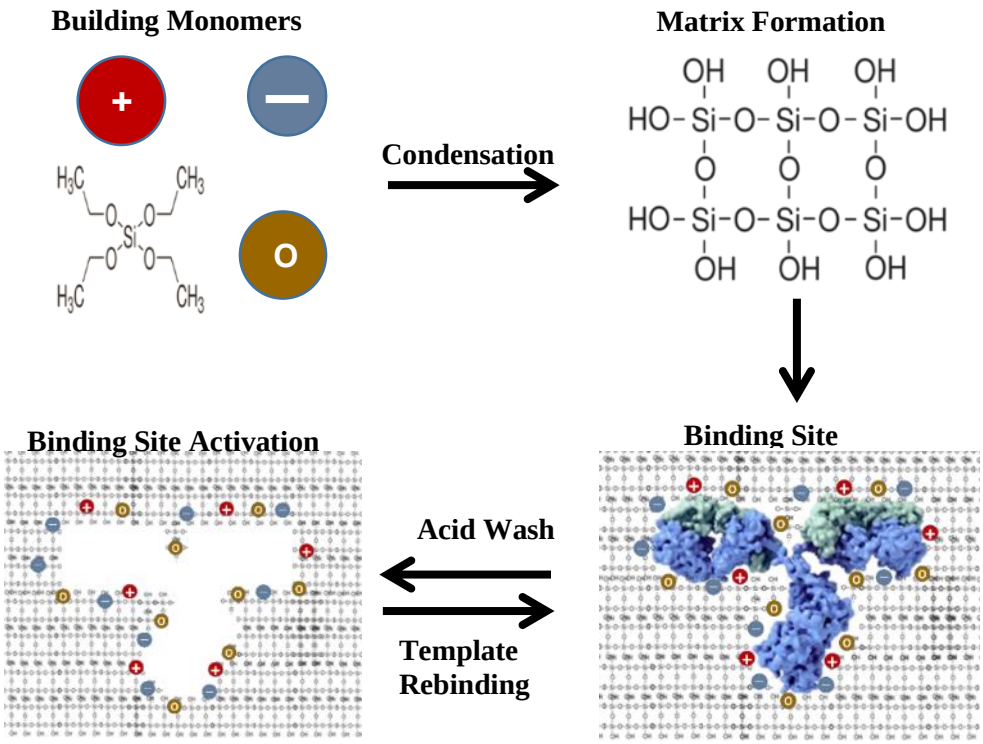

Figure 5. Simplified model of molecular imprinting [74].

For a successful molecular imprinting, template's properties during and after synthesis must be preserved to create recognition sites and potentially reuse template for further imprinting; thus reducing production costs. There are four steps involved in the preparation of molecular imprints [14], [28]:

- Effective selection of monomers that allow for noncovalent interactions, such as hydrophobic, hydrogen bonding, van der Waals forces, and electrostatic interactions, with the template
- Self-assembly of template and monomers
- Polymerization of monomers in a polymeric matrix
- Template and unreacted monomer removal

While the imprinting chemistry varies depending on the polymers used, there are two variations regarding the location of the template. The simplest method is bulk imprinting, were template is added during the polymerization of monomers and becomes randomly dispersed within the synthesis. This method produces multiple recognition sites in the molecular imprinted material. Unfortunately, the process permanently entraps some of the template and it cannot be recovered.

Furthermore, the template requires longer incubation to reach the deep binding sites within the particle; this is due to the limited protein diffusion to the inner matrix of the particles. Finally, bulk molecular imprinting yields the highest binding capacity per milligram of imprinted polymer. A representation of a bulk imprinted particle is shown in figure 6.

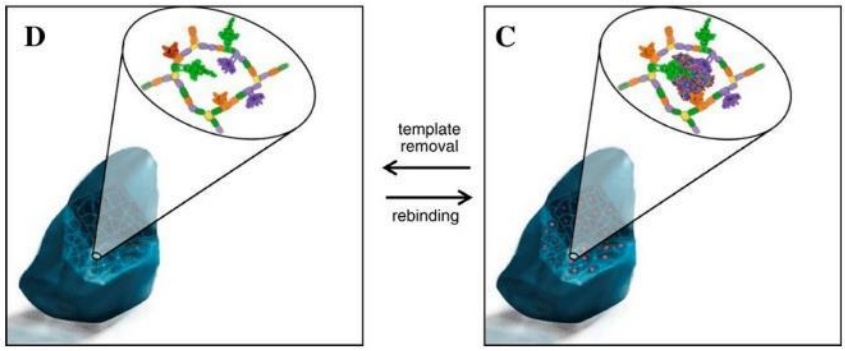

Figure 6. Representation of bulk imprinting after monomer polymerization. (C) Formation of binding sites and (D) available binding sites after template removal inside a porous particle [14].

The second molecular imprinting variation requires using template only at the surface of the imprinted polymer. This method uses less protein to form recognition sites with the tradeoff of smaller binding capacity per mg of polymer. The advantages of surface imprinting are faster template diffusion and easier sensor integration [7], [75]. A schematic representation of surface imprinting is shown in figure 7.

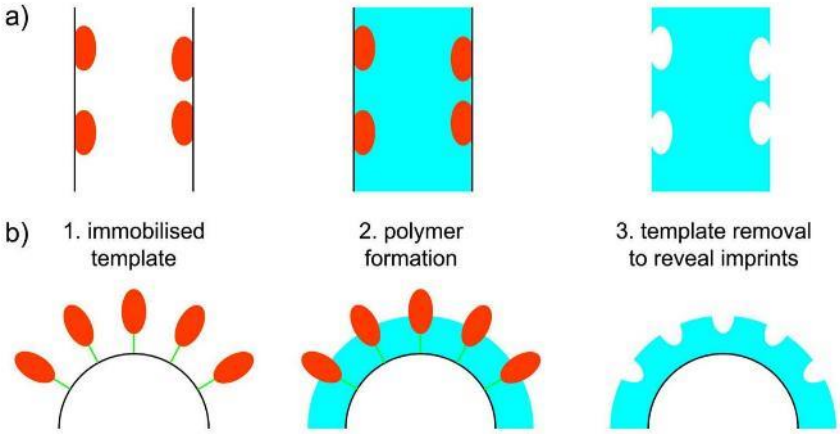

Figure 7. Schematic representation of (a) top-down and (b) bottom-up surface imprinting [27].

Additionally, the template can be added during the synthesis of MIPs to form covalent or non-covalent bonds with the polymer network. With covalent imprinting, the template is immobilized during polymerization; thus covalent imprinting offers superior leakage prevention of the template during synthesis. To remove the template from the polymer matrix, the covalent bonds need to be broken. This process denaturalizes the template, and its reusal is eliminated. Whereas with non-covalent imprinting, the template is weakly encapsulated by electrostatic and hydrophobic interactions. This method produces a more direct and flexible approach, where the template is easier to remove, and template can be reused [76]. Regardless of the method, 1-2 millimoles is commonly used for molecular imprinting of molecules less than 1600 Daltons [77–79], while higher molecular weight templates are mostly used in micro molar concentrations. Figure 8 shows a representation of covalent and non-covalent molecular imprinting.

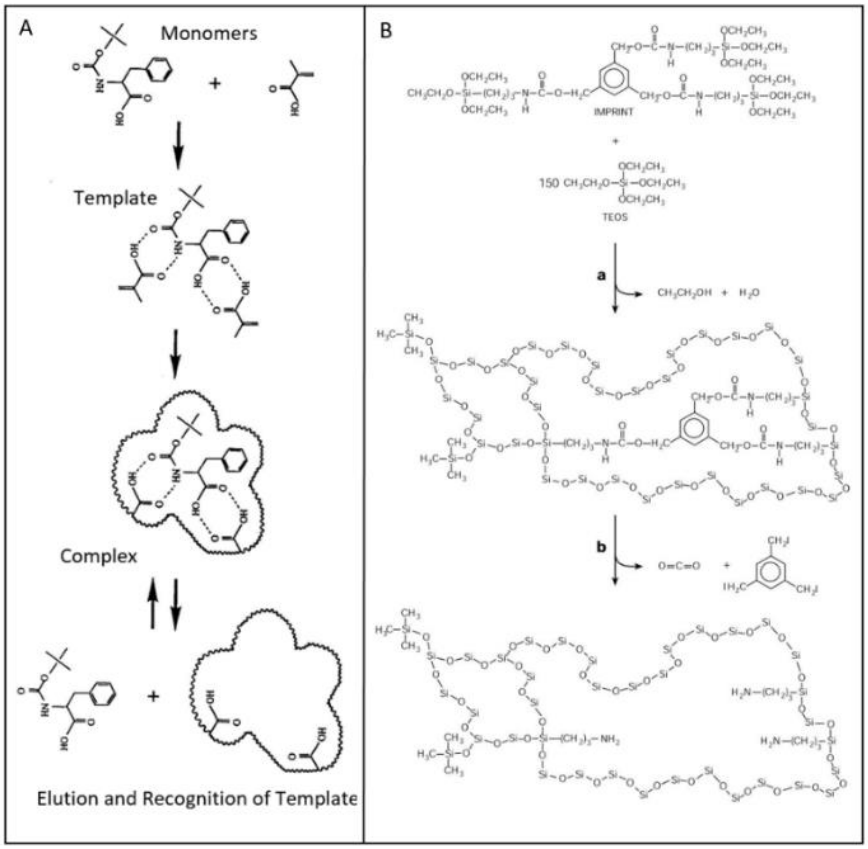

Figure 8. Schematic of non-covalent (A) and covalent molecular imprinting (B). Particularly, the template in A is the same before and after polymerization, whereas the template in B is cleaved [20], [25].

Regardless of the methods used, the main limitation of molecular imprinting is the synthesis environment in which proteins are exposed during MIP synthesis. While the ideal solvent for proteins is water, the majority of imprinting technologies require synthesis and testing of MIPs in organic solvents. Particularly, in aqueous solutions the electrostatic and van der Waals attractions are reduced by the competition between ions and water molecules; hence, binding with the template is inhibited [3]. To improve electrostatic interactions, the use of organinc solvents is necessary [9], [28].

Although it is possible to use organic solvents with proteins, their use is a major challenge. Before a protein can be used, a transfer protocol needs to be developed. This process reduces protein denaturation during the change from an aqueous solutions to an organic solvent. Additionally, many proteins have low solubility in organic solvents. While complexation of proteins with ionic or amphiphilic detergents or polymers is possible, this can substantially mask protein polarity, increasing the overall hydrophobicity thus protein becomes distorted in organic solvents. It is also possible to increase protein solubility with the addition of inorganic salts, such as lithium halides [80], but it strongly reduces protein interactions due to ionic competition. Thus, molecular imprinting with organic solvents has major hurdles and challenges that we wish to avoid.

While many MIP articles have been published within the last decade, more than 90% of published papers are about molecular imprinting of templates less than 1,500 Da. Molecular imprinting with larger molecules has been difficult due to the complexity of large molecules greatly increases with size. The bigger the size, the fewer ways of obtaining a match with a recognition site than a mismatch [81]. Because of this, a fraction of protein template can be used to later recognize the whole molecule. This approach is similar to antibody behavior, where antibodies only recognize a small but unique area of the antigen, called the epitope. Thus, the imprinting process is known as the epitope approach.

While the epitope approach has seen limited success, the process was used successfully to recognize and neutralize the toxin melittin [34–36]. By imprinting a 26 amino acid sequence of

melittin, in a mild aqueous solution, it was possible to recognize the whole melittin molecule. Unfortunately, the epitope approach requires fractionated protein, which must be available commercially or it must be produced in the laboratory. Furthermore, the fractionated protein most retain its conformational structure as much as possible. Figure 9 shows a schematic representation of the epitope approach.

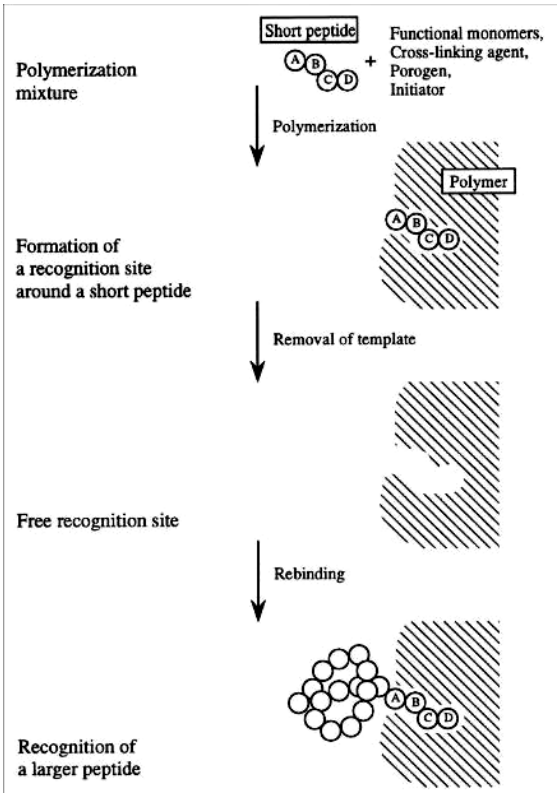

Figure 9. Schematic representation of the epitope approach [33].

In molecular imprinting, studies have found that the adsorption capacity depends significantly on the use of functional monomers. Furthermore, the affinity and selectivity of MIPs correlated with the use of hydrophobic templates. Finally, the template required hydrogen bonding to achieve a strong bind with the recognition sites. These behaviors pose striking similarities with the molecular recognition principles of antibodies. The importance of having a matrix capable of short range interactions was identified. Furthermore, proper ionic and hydrophobic monomer ratios are key components for molecular imprinting of high molecular weight templates [76], [78], [79], [82].

2.2 Applications with Molecular Imprinting

An important advantage of molecular imprinting is their wide application possibilities. Molecular imprints have been used as lateral flow immunoassays [83], particle agglutination assays [84], immunofluorescent assays, immunohistochemistry, bio sensors [85], [86], high-pressure chromatography columns [87], magnetic core imprinted particles for protein isolation or purification [88], to name a few. Particularly, to develop lateral flow immunoassay, a narrow size distribution of molecularly imprinted particles is required. If molecular imprinting yields wide particle size distribution, particles will become trapped irregularly in the assay, causing false positive and negative results.

The development of MIPs for agglutination assays is of great interest as they are simple to use. Unfortunately, agglutination assays are difficult to design. These assays require reagents to remain suspended during testing of samples. Only if the antigen is detected, particles must precipitate and a color change must be visible. When the sample is negative, particles need to remain suspended. Furthermore, agglutination assays require larger amounts of sample than other techniques. Hence, agglutination assays have limited applications, where screening for seroconversion antibodies is the only possible application. Finally, the assay must show clearly the difference between positive and negative samples, which might be difficult to achieve. Figure 10 shows a weak agglutination assay with MIPs, where the positive sample has red particles at the bottom of the vial, while negative sample has no red particles whatsoever.

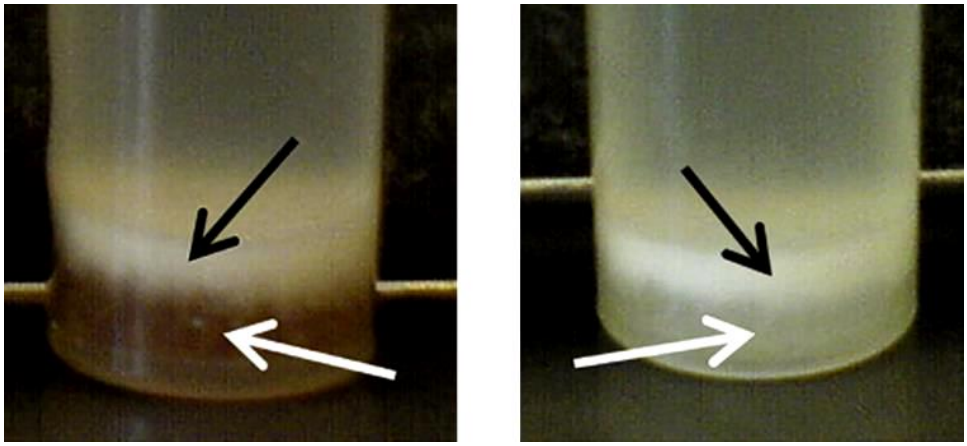

Figure 10. Particle agglomeration assay. Left positive test and right negative control [29].

While MIPs have unique advantages as single use assays, these can be used with other technologies, including bio-sensors. Currently, bio-sensors use natural recognition molecules such as antibodies [89], [90]. While bio-sensors are capable of quantitatively determining antigens with great accuracy and reliability, these tend to be expensive and with little to no added diagnostic value; thus, the added expense is not justified. Due to the robustness of MIPs, these can be used in bio-sensors with unique advantages of picomolar sensitivity as developed by Cai et al [91], while allowing for sensor reutilization [62]. Figure 11 shows a schematic of a bio-sensor developed using molecularly imprinted polymers.

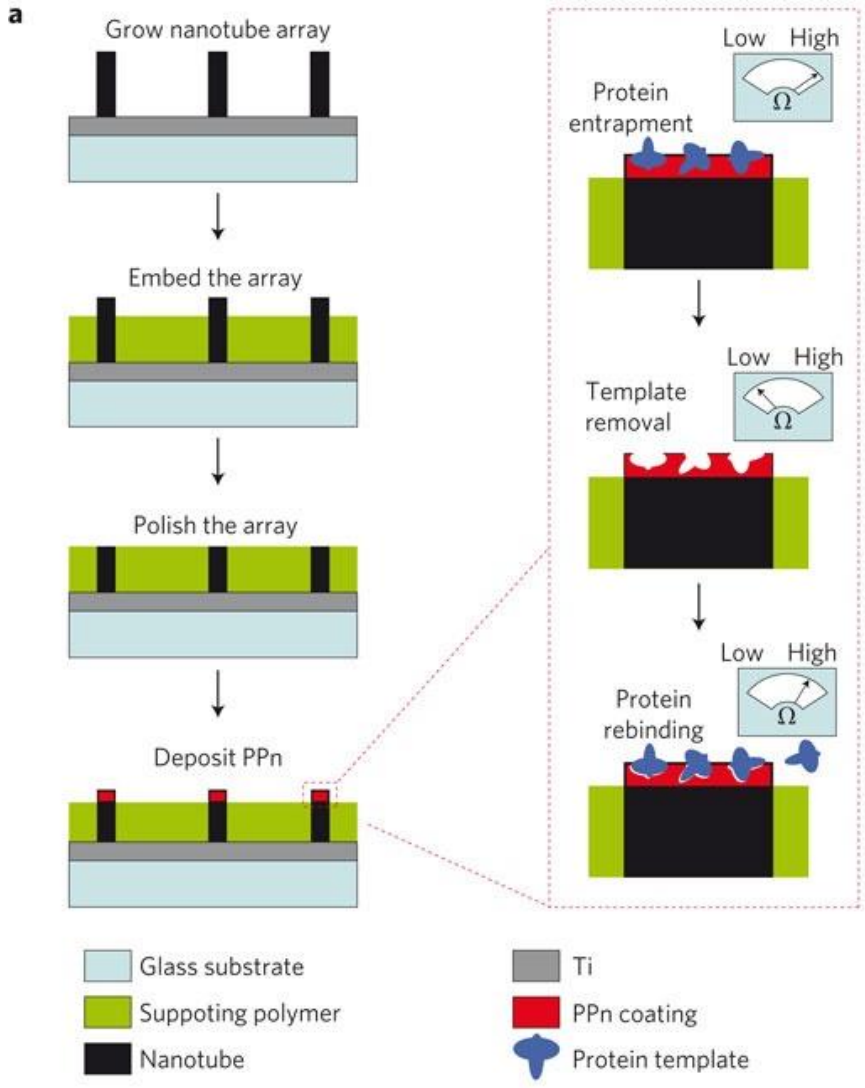

Figure 11. Schematic of nanosensor fabrication and protein detection [91].

2.3 Preparation of Silica Particles

Upon evaluation of polymers for molecular imprinting, we opted to use silicon alkoxides for developing molecularly imprinted silica particles. Silica nanoparticles occupy a prominent position in scientific research because of their ease of preparation and their wide applications. These particles have been proven as chemically and thermally stable, with large surface area, excellent suspendability, and environmental inertness. Furthermore, silica particles are highly soluble in aqueous solution, making them ideal for water based assays. Also, the high silanol concentration, allows for multiple electrostatic interactions; hence, silica particles are ideal as protein carriers. Additionally, silica nanoparticles are of no or very little interest to microbes. Due to the versatility of silica particles, they have been used in catalysis, pigments, pharmacy, thin film devices, and many others [92–94].

For the synthesis of silica particles, two main synthesis methods are available, the Stöber and the reverse micro emulsion process. In both approaches, particles are formed by self-assembly and polymerization of monomers. Particularly, the reverse emulsion process uses large amounts of surfactants to form inverse micelles and particles need to be extensively washed. Proteins will most likely denature under these conditions. Hence, we opted to synthesize particles using the Stöber process. The Stöber method is regarded as the simplest and most efficient way to prepare silica spheres. Particle synthesis is possible by the hydrolysis and polymerization of silicon alkoxides in aqueous solutions. Depending on the chosen parameters, the formation of either silica particles or gels is possible [95], [96]. The chemical reactions can be schematically represented as:

$$Si(OR)_4 + H20 \longrightarrow Si(OH)_4 + ROH \text{ (Hydrolysis)}$$

$$\equiv Si{-}OH + OH{-}Si\equiv \longrightarrow \equiv Si{-}O{-}Si\equiv + H20 \text{ (Polymerization)}$$

While many silicon alkoxides are available, tetraethyl orthosilicate (TEOS) is commoly used. Particularly, TEOS and water are added at minimum 1:2 molar ratio, with ammonia as a catalyzer and ethanol as a destabilizer. The TEOS to water ratio is required as the production of one mole of TEOS consumes two moles of water and produces four moles of ethanol. The addition

of ammonia accelerates TEOS hydrolysis and condensation. Also, ammonia is used to stabilize the silica colloid suspension. The suspension is stabilized by the hydroxyl groups of the ammonia which adhere to the surface of the silica particles; thus, static repulsion is increased between particles. Although, if the concentration of ammonia is too high, particle coagulation will occur. Alternatively, low ammonia concentrations slows particles synthesis [96], [97]. Although ammonia is important for silica particle formation, it may cause protein denaturation if overused, so it must be used cautiously.

The silica particle formation is a multistep process in which hydrolysis of alkoxides occurs, followed by nucleation of monomers, particle growth, and then condensation. In the early stage of silica particle formation, particle nucleation occurs mostly. Eventually, particle growth becomes the dominant process and the number of particles remains constant as the rate of nucleation decays to insignificant levels. Particle nucleation is rate-limited by the hydrolysis of TEOS; thus, the hydrolysis of TEOS directly influences the particles produced. When monomers are produced faster than they react, their concentration remains high; therefore, nucleation is favored. Once the rate of hydrolysis drops and monomers react faster than they are produced, particle growth is favored [98], [99].

A critical factor in silica particles is the full hydrolysis of TEOS, where uniformly compact particles with smooth boundaries are produced when monomers are fully hydrolyzed. Whereas, partly hydrolyzed monomers will produce progressively irregular boundaries. Since ammonia must be used conservatively, we are expecting particles with irregular boundaries. During particle synthesis, temperature was found to have an effect on particle formation. In low temperatures, the formation of larger particles was favored. Finally, since we will use proteins as templates, the use of isotonic solutions might be necessary. Unfortunately, the addition of sodium chloride in the range of 0.002-0.02 M has resulted in particle aggregation. The addition of salts causes a reduction of the electric double layer thickness making repulsive potential decline and leading to a dispersion that is more easily flocculated [100], [101].

A typical synthesis of silica particles is composed of a solution with 0.2 M TEOS, 1.0 M NH_3, and 6.0 M H_2O, carried at a temperature of 55°C and constant stirring. Since molecular imprinting has unique synthesis requirements, we need to develop a modified Stöber process. Figure 12 shows silica particles produced with the standard Stöber process.

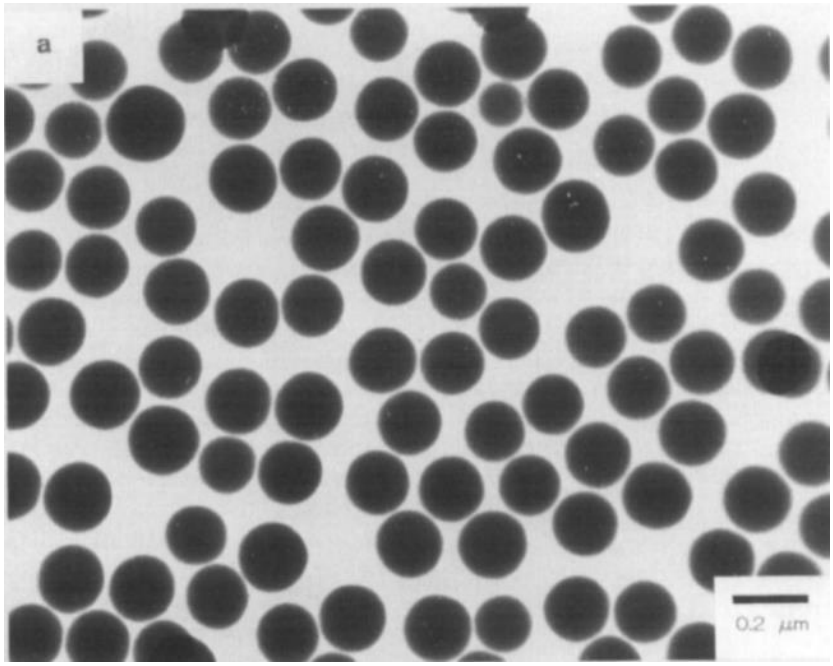

Figure 12. Particles synthesized at 55°C in a solution of 0.2 M TEOS, 1.0 MNH_3, and 6.0 M H_2O [97]

2.4 Colloid stability

Due to our interest in developing molecularly imprinted silica particles, it is important to discuss colloid stability. While the synthesis of silica particles produces nanometer spheres, these can be made to flocculate producing micrometer sized particles. Particularly, all particles visible to the naked eye are formed by the aggregation and agglomeration of nanometer sized primary particles; thus, colloid stability is an important factor in developing molecular imprints. According to DLVO theory [102], a stable suspension is formed when the energy profile is strongly repulsive. When this profile is attractive, the particles will approach each other until they stick together forming aggregates and then agglomerates; this is an unstable suspension. Figure 13, shows a representation of stable and unstable colloidal suspensions.

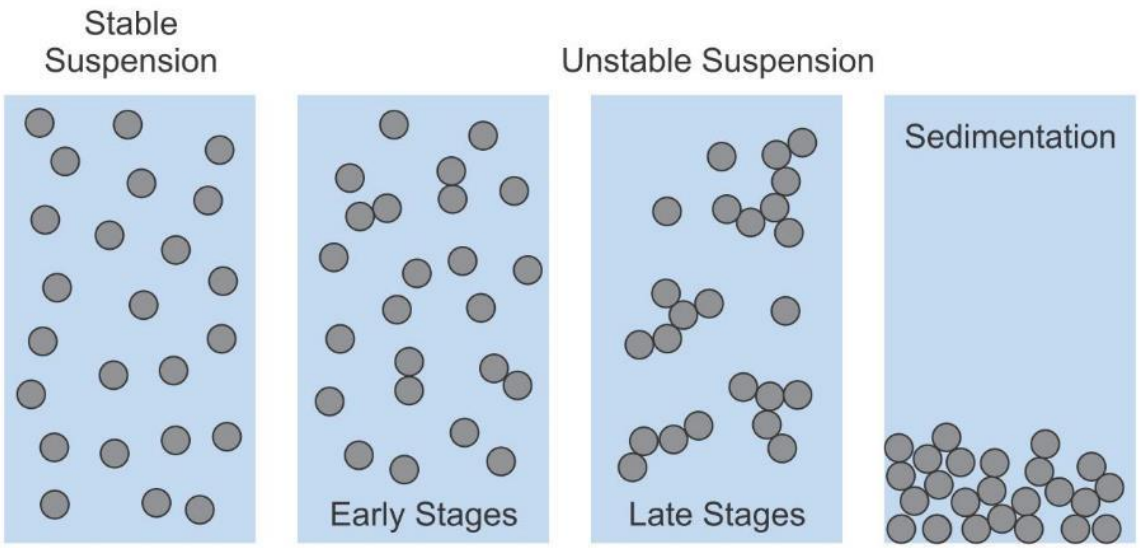

Figure 13. Representation of stable colloidal suspensions and the aggregation stages of unstable suspensions [102], where selective binding sites are formed during the late stages of an unstable suspension.

In stable suspensions, molecular imprinting is inhibited by the difficulty of the template to approach primary particles; thus the formation of selective binding sites are inhibited. To facilitate molecular imprinting, the energy profile of particles needs to be controlled to promote slightly unstable suspensions. By designing a solution that permits aggregation of particles and template, molecular imprinting can occur. By reducing repulsive energies, with the careful addition of salts, one can optimize the synthesis of MIPs. It is important to note that a highly unstable solution will most likely be detrimental to the formation of MIPs. Thus, there is an ionic content ratio to be determined, where Zeta potential can be used to identify this value [103]. An example of molecular imprinting with an unstable solution is shown below in figure 14, where primary particles and template aggregate irreversibly. These aggregates then form larger micrometer sized agglomerates which can be disrupted by probe sonication.

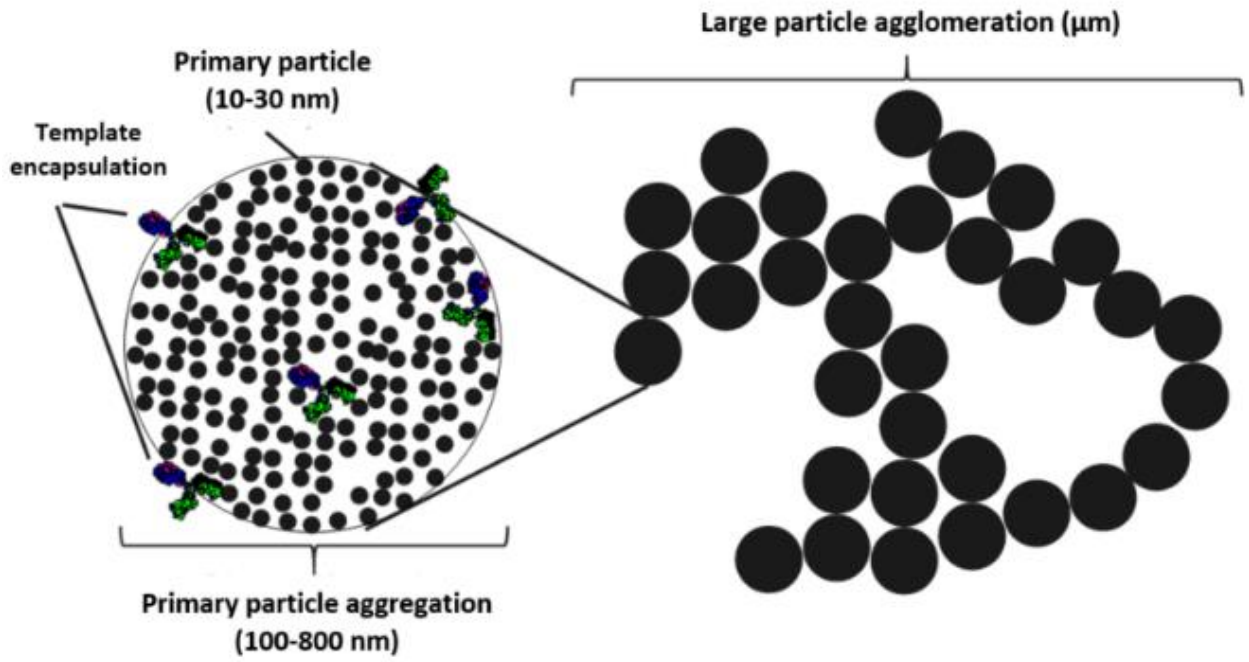

Figure 14. Schematic of molecular imprinting process during the unstable colloidal synthesis of silica particles.

2.5 Biorecognition Molecules

2.5.1 The Antibody Structure

Currently, the majority of imprinting technologies involve the synthesis and testing of MIPs in organic solvents. In aqueous solutions, electrostatic and Van der Waals attractions are reduced due to competition between ions and water molecules, hence preventing a successful bind [30]. Despite short interactions are reduced by ions and water molecules, natural antibodies are capable of displacing hydration layers in proteins with the aid of long range interactions. Once the hydration layer has been removed, an intimate contact with the antigen can be achieved [104]. Since antibodies are successful in aqueous solutions, antibodies and their optimal binding conditions were investigated to generate bio-inspired molecular imprints.

While there are many types of biorecognition molecules, such as enzymes and nucleic acids, the most used biomolecule is the antibody. The antibody is a recognition molecule produced by the immune system with high specificity and affinity; hence, antibodies are used for the identification of many diseases. Particularly, antibodies are proteins secreted from B-cells.

Antibodies are soluble, secreted in large quantities, easily obtainable, and easily studied. Antibodies function by tagging invaders for destruction. Tagging and destruction of antigens is accomplished by the antibody's dual binding regions, where one end can bind to a wide variety of antigens, and the other to a limited number of effector molecules and cells. The most abundant antibody molecule is the IgG, and it is made of four polypeptide chains, comprising two light chains and two heavy chains; IgG antibodies can be thought of as a flexible Y-shaped structure. Each of the four chains has a variable (V) domain, which contributes to the antigen-binding site, and a constant (C) region, which is used to bind specialized immune cells [105].

Particularly, the light chains are bound to the heavy chains by many non-covalent interactions and disulfide bonds. The antibody V regions of the heavy and light chains pair in each arm of the Y to generate two identical antigen-binding sites, or Fab fragments, which lie at the tips of the arms of the Y. The possession of two antigen-binding sites allows antibody molecules to cross-link antigens and to bind them more securely. The trunk of the Y, or the Fc fragment, is composed of the heavy chains, and joining the arms of the Y to the trunk are the flexible hinge regions. The Fc fragment and hinge regions differ in antibodies of different isotypes. Because of this, there are four antibody isotypes, being IgG, IgE, IgA, and IgM [106]. Antibody isotypes determine functions in the immune system, and it is beyond our need of understanding for molecular imprinting purposes. In figure 15 an IgG antibody is rendered, where the blue and green proteins form the antibody's heavy chains, while the pink and brown proteins form the light chains.

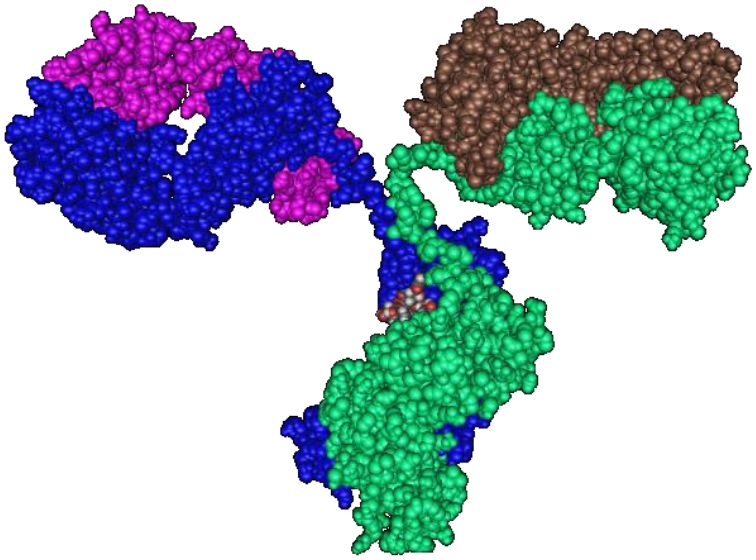

Figure 15. Space fill rendering of IgG. Blue and green represent heavy chains, pink and brown light chains. Top regions with light chains are the Fab fragments, whereas bottom region is the Fc fragment [107].

Interestingly, antibodies only recognize a small fragment of a large molecule such as a polysaccharide or protein subunit. The structure recognized by the antibody is called an antigenic determinant or the epitope. Whereas, the recognizing portion of the antibody is known as the paratope. Commonly, many pathogens create an immune response by their polysaccharide coats, which antibodies then use to neutralize these molecules. However, in many cases antigens can provoke an immune response just by proteins alone. For example, protective antibodies against viruses recognize viral envelope proteins. As a general principle, antibodies will selectively bind to ligands whose surfaces are complementary to that of the antibody [108].

2.5.2 Antigen-Antibody Interactions

The way antibodies interact with antigens is complex, but it can be generalized by types of non-covalent interactions that are involved in protein binding. These forces can be classified by their interaction distance, where long range interactions are composed of electrostatic and hydrophobic forces and short range forces are made from Van der Waals forces and hydrogen bonds. As presently understood, events at a molecular level occur as follows [104]:

- The epitope and paratope casually come to a distance of several nanometers
- They are then attracted by long-range ionic and hydrophobic interactions
- The attractive forces locally overcome hydration layers present in both protein molecules
- Water molecules are expelled and proteins approach each other closely
- Once the hydration layer has been removed, short range Van der Waals forces predominate, but ionic interactions still play a role
- After antigen antibody fitting, the overall strength of the binding is dependent on the goodness of the fit between the two surfaces and their total contact area

Long Range Interactions

Before specific protein recognition can occur, an intimate contact between an antibody and antigen needs to occur. In water, this is difficult as proteins are surrounded by a hydration layer, as shown in figure 16. Fortunately, long range interactions are capable of displacing the surrounding water molecules between both proteins. During long range interactions, proteins are attracted with no specific orientation. Although long rang interactions have no specificity, they improve collisions between proteins and helps reduce repulsion barriers between the molecules. Interestingly, antibodies are capable of adding charged and hydrophobic amino acids to their structure with their hyper variability loops [109], [110]. By doing so, antibodies optimize their long range interactions, as seen with antibody maturation. In fact, by introducing residues with counter ions against antigens, long range interactions improve the affinity of an antibody for the target antibody; thus, increasing their association rates [111].

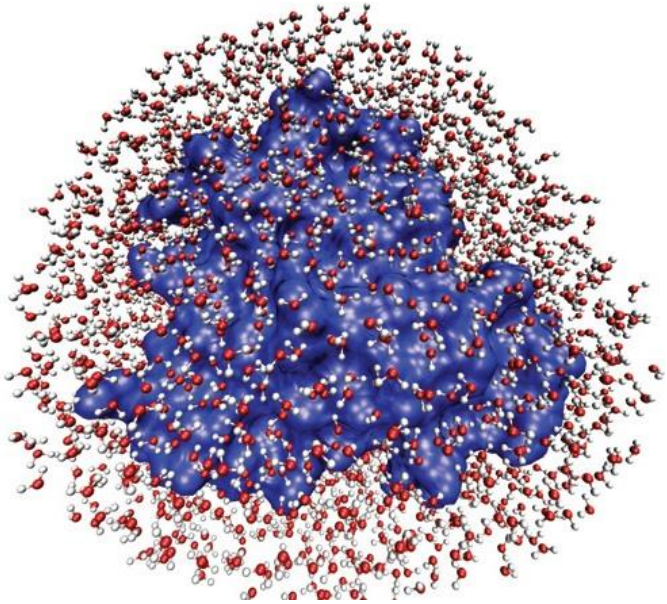

Figure 16. Hydration layer of myoglobin [112].

Short Range Interactions

After the displacement of water molecules, short range interactions, as characterized by weak Van der Waals attractions and hydrogen bonds, can occur. A specific and stable binding is achieved by the collection of several short range bonds occurring contemporaneously; this can only occur when antigens and antibodies have a good steric complementarity. Particularly, antigenic determinants of red cells and antibody's paratopes, require a contact area between 0.4 to 8 nm^2 between molecules for a stable bind [104]. Although long range interactions have no specific orientation, short range interactions are capable of aligning themselves to complement their dipoles; thus aligning the epitope and paratope, until a good fit is achieved. After antigen recognition, a stable bind is characterized by a slow dissociation, antigen separation from the antibody, and a fast association rate, antigen-antibody complex formation [113]. Most importantly, antigen recognition occurs with high specificity and selectivity.

An important advantage of antibodies, is their capability of recognizing a wide array of proteins regardless of their size. Depending on the size of the protein being recognized, antibodies will bind to antigens with unique pockets, grooves, or extended surfaces, as required to identify the antigen [108]. As the proteins become larger, so do the binding pockets in antibodies. The

better the shape complementarity between antigen and antibody, the stronger the affinities become. When the conformational structure of an antibody or antigen is deformed, the binding strength of the antibody is greatly reduced, as short range interactions require a close fit to achieve multiple short range bonds. Thus, it is important to use antibodies at their physiological pH, to prevent changes to their conformational structure.

2.5.3 Factors Affecting Antigen-Antibody Interactions

When antigen-antibody complex formation (AACF) occur in vitro, these can be easily disrupted with high salt concentrations and changes of solution's pH. AACF can be disrupted by inhibiting long range interactions with ionic competition with the addition of salts to the solution. While, short range interactions can be eliminated by forcing a conformational change with pH modification of the solution. Because AAC is a reversible process, mild changes in pH and salt concentrations are used for the removal of antigens from antibodies. In fact, affinity columns with immobilized antibodies are capable of binding antigens in gel and once the pH is reduced, antigens are released [108]; thus a purified antigen sample is recovered.

While adding salts can inhibit AACF, reducing ionic concentration from 0.17 to 0.03 M NaCl results in an association rate increase of a 1000 fold [104]. Unfortunately, this increase is accompanied with reduced specificity as well. Finally, the use of lower ionic concentrations led to no further improvements and in cases protein agglomerated. Other factors affecting the AAI are temperature, concentrations of antigen and antibody, sample purity, and incubation time.

For optimum binding temperatures, results varied based on the chemical nature of both the antigen and the antibody. While some antibodies are found to strongly bind at 4-7°C, others were found to bind with similar strength at room temperatures but with faster association rates. Nonetheless, it is thought that more stable hydrogen complexes can exist at low temperature and short range interactions are improved [104]. At room temperature, the increased entropy improves collisions between antibodies and their antigens favoring long range interactions, but weaker antibody-antigen binding might occur.

Antigen and antibody concentrations also play a major role in AACF. At low protein concentrations, where the distances among molecules are large, only long-range interactions are expected to affect the overall AACF. However, as the concentration is increased and inter-protein distance decreases, long-range interactions become stronger and, short-range interactions become significant. In diluted samples, the dilution effect is much more pronounced for low-affinity antibodies [104], [113]. Furthermore, sample purity also plays a role. When using high-affinity antibodies with low antigen quantity, AAI might be inhibited by the presence of the high concentration of a nonreactive protein in the sample. Hence, sample dilutions increases the selectivity of antibodies for some antibodies, at the cost of signal strength.

Finally, for maximum sensitivity, the antigen-antibody reaction should be allowed to reach equilibrium. Equilibrium may occur within minutes or up to several hours. Depending on the type of study, waiting for equilibrium is not necessary, especially in cases where binding is very strong. With very strong association constants, dissociation rates may take up to days to occur; hence, binding is considered nonreversible [114], [115]. Since disassociation constants (K_d) predict the degree of the effect of the factors considered, it is a significant value to obtain. Typical antibody properties of commercial products have high affinity K_d values of $K_d < 10^{-7}$ M, with antibody concentration from 100 to 1000 µg/ml. Sample purity, purification method, and its storage buffer are commonly listed. Finally, antibody clonality and isotype are shown along with the known cross reactivities.

2.5.4 Antigen Molecular Complexity

Particularly, antibodies can be used to compare molecular imprints behavior, where templates are analogs to antigens and antibodies to MIPs. Because of this, we investigated methods to optimize recognition of antigens. One of the most important factors in antigens is its size and molecule complexity. Molecule complexity pertains to the uniqueness of a given molecule and the way it interacts or behaves with its surroundings (in our case with antibodies). The importance of molecular complexity perhaps might not be evident, but it is immediately related to how a

recognition molecule will be able to detect its complementary molecule. As molecular complexity increases, so does the probability of finding a unique binding event (i.e. where the ligand can match the receptor in just one way). Particularly, simple ligands can find multiple binding modes, while large and complex ligands require a long time before achieving a binding event.

In fact, the probability of binding decreases rapidly as the complexity of the ligand increases. The probability decrease is due to the fewer ways of obtaining a match than a mismatch between the antibody and the antigen. As a consequence, it will take longer for a recognition molecule to immobilize its ligand. The additional time is due to the receptor molecule need to match its ligand in a manner that coincides with the three dimensional void of the receptor, as it is approaching. The larger the receptor's void the more difficult it becomes for the ligand to precisely match this space purely by coincidence. Finally, if the receptor is too small, it will likely have large cross reactivity and interact with multiple substances. Hence, there is an optimal size for a ligand and receptor to interact [81].

Because antibodies limit recognition to small protein units, they are capable of selectively binding to macromolecules, i.e. a virus envelope, by only recognizing a protein unit that is repeated multiple times. For example, antibodies can recognize the red-rendered proteins in the protein envelope of dengue virus, shown in figure 17. Since multiple red proteins are available on the surface of the envelope, multiple antibodies bind to the surface of the viral envelope. With this method, the immune system balances molecular complexity by identifying protein units, which are usually larger than 6,000 Da, digesting them with antigen presenting cells, and then presenting them as small polypeptides between 7 and 30 amino acids. These polypeptides are then used to develop antibodies. By limiting protein recognition to a few polypeptides, antibodies are capable of recognizing a unique area of the protein, rather than binding the whole protein structure.

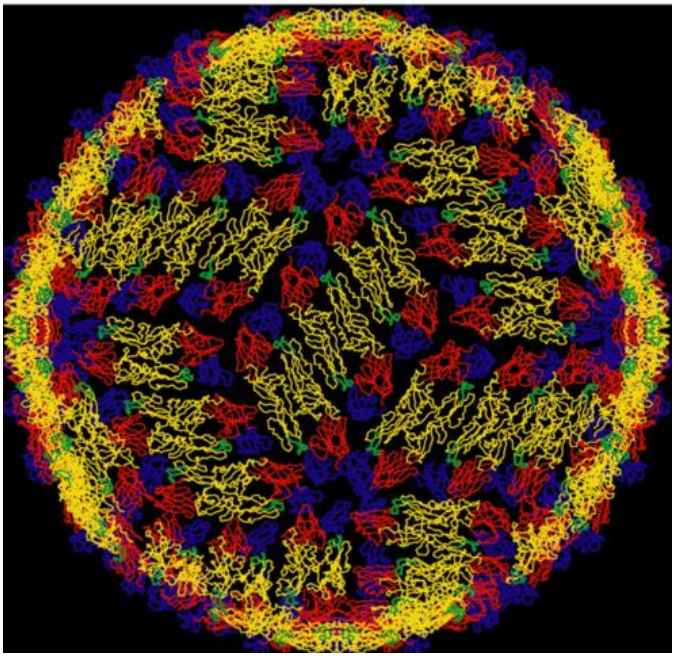

Figure 17. Dengue virus envelope [116].

Finally, an important consideration is the degree of chemical complexity in the tertiary structure, were linear amino acids and simple polysaccharides rarely produces immunogenicity. Large linear molecules, even if greater than 6,000 Da, may not elicit any response, whereas highly complex structures tend to be highly immunogenic, even if they are smaller [32]. Since molecular complexity plays a key role in AACF, we determined that the use of proteins with labile tertiary structures could become problematic with molecular imprinting, as synthesis occurs in unideal conditions for proteins. If a given protein loses its tertiary structure during synthesis conditions, molecular imprinting will likely fail.

2.5.5 Effects of pH on the Conformational Structure of the Antibody

Depending on the solution's pH, amino acids can become positively, neutral, or negatively charged. This charge variability is partly responsible for folding and preserving the protein's conformational structure. The ionic charges of amino acids attract sections of oppositely charged amino acids and repel others with similar charge within the protein; hence ionic charges act like holding braces of the physical structure of the protein. Once the pH changes, the amino acids charges are modified and depending on how pronounced the change is, the conformational

structure can be stretched, weakened, or destroyed. Particularly, antibodies can tolerate pH changes from 4 to 8 with only mild protein stretching. Because of this, antibodies can be forced to unbind antigen with the use of acids.

By reducing the pH in solution, mild conformational changes occur in the antibody and the antigen, eliminating the required steric complementarity; thus, releasing the antigen. Afterward, antibody containing solution is neutralized, and it is now capable of recognizing the same antigen again. Studies have shown that the process can be repeated for a limited amount of times, with reduced binding strengths after each use. It is this particular protein resiliency that makes antibodies attractive to molecular imprinting since they are capable of tolerating pH changes without major protein activity loss and with minor conformational structure distortions. Because of this, molecular imprinting can occur slightly outside of physiological pH.

Chapter 3. Synthesis Development of Molecularly Imprinted Polymers

3.1 Introduction

Although molecular imprinting has existed for longer than 20 years, there are yet challenges when imprinting molecules larger than 1,500 Da. Imprinting is hindered by multiple factors such as size, molecule complexity and conformational structure of the template [14]. Furthermore, the synthesis environment is usually too aggressive for the template, where the solvent might denature the template before an imprint is formed. To achieve macromolecular imprinting, the synthesis of molecularly imprinted polymers (MIPs) must occur in an environment where the template can subsist for as long as the formation of the imprint occurs.

To date, the majority of imprinting technologies involve the synthesis and testing of MIPs in organic solvents [13]. Organic solvents are necessary to increase short range interactions between the template and MIP. In aqueous solutions, electrostatic and Van der Waals attractions are reduced due to competition between ions and water molecules, hence preventing a successful bind [30]. A MIP capable of template recognition at aqueous environment is of great importance as it greatly reduces materials and skills necessary for the recognition and separation of molecules of interest.

To develop effective molecular imprints, MIP recognition interactions must be compared with the antigen antibody complex formation process. Particularly, antibodies are capable of displacing hydration layers of proteins by using long range interactions with ionic and hydrophobic aminoacids. For MIPs, long range interactions can be formed by using hydrophobic and ionically charged monomers. These monomers are not required for the formation of silica particles but are essential for molecular imprinting. In antibodies, once the antigen is near, short range interactions begin to occur. These interactions are formed by Van der Waals and hydrogen bonding, where the steric complementarity of both molecules permits multiple bonds to occur simultaneously.

To generate short range interactions in silica particles, Van der Waals and hydrogen bonding need to occur with the particle and the template. Fortunately, silica particles have multiple

hydroxyl sites at their surface. It is then possible to compare the short range interactions that occur in a silica thin layer chromatography (TLC) paper, where these interactions occur at the multiple hydroxyl sites available in the silica matrix of the paper. Figure 18 illustrates how TLC paper interacts with molecules.

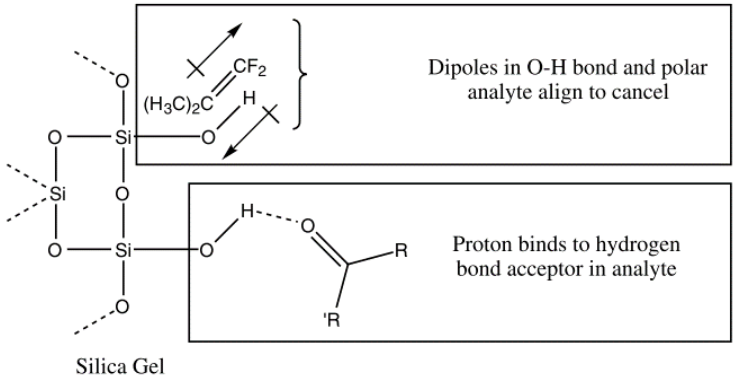

Figure 18. Modes of Interaction of analytes in silica thin layer chromatography paper [117].

3.2 Materials and Methods

3.2.1 Materials

Tetraethyl orthosilicate (TEOS) and 3-aminopropyl triethoxysilane (APS). Phosphate buffered saline as tablets, sodium dodecyl sulfate (SDS), succinic anhydride, sodium hydroxide, and ammonium hydroxide (30%) were obtained from Sigma-Aldrich (St. Louis, MO) reagent grade. Hydrochloric acid (37%), anhydrous acetic acid, methanol, and ethanol were acquired from Fisher Scientific (Pittsburgh, PA), reagent grade. Polymer grafted carbon black was obtained by collecting ink from HP 33 cartridges (HP, Palo Alto, CA). Ultrapure water was obtained from a Milli-Q Millipore unit with a water quality of 18.2 MΩ. PBS buffer was prepared as a 150 mM NaCl solution with 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. Human Chorionic Gonadotropin (HCG) was obtained lyophilized from Sigma and used as the template; a solution of 20 mg/ml was prepared in 1X PBS buffer. West Nile virus antibodies (WNVA) were obtained from hyper immune mouse ascitic fluid (HMAF). Elution solution was made with a 50% v/v mixture of glacial acetic acid and methanol

3.2.2 Preparation of West Nile antibodies

Hyper immune mouse ascitic fluid (HMAF) WNVA were prepared as described in the article [118]. WNVA were then purified by affinity gel goat antibody to mouse IgG column from MP Biomedicals (Santa Ana, CA). Briefly, the affinity column was equilibrated with a 2X PBS buffer solution. Afterward, 1.0 ml of serum containing WNVA was added. The column was then rinsed with 10 ml of PBS. Finally, the antibody was eluted from the column with elution buffer; the sample was collected. Eluted antibodies were then desalted with a Hitrap 5 ml desalting column (General Electric Healthcare Life Sciences, Wauwatosa, WI), previously equilibrated in 1X PBS buffer.

3.2.3 Synthesis of Molecularly Imprinted Silica Particles

To form silica particles we used tetraethyl orthosilicate (TEOS) as the backbone monomer, while black ink [119], [120], was added as a visualization agent. Particularly, for every TEOS molecule consumed, there are 4 molecules of ethanol produced, and for every active monomer consumed, there are 3 ethanol molecules produced. Because of this synthesis of MIPs will produce ethanol, which can denature proteins in high concentrations. Although there are other monomers available that produce salt as a byproduct, these tend to be extremely reactive to water; hence, they were not used. The usage of ink allows simple visualization of an otherwise semitransparent particle. We chose 3-aminopropyl triethoxysilane (APS) as the active monomer. To reduce protein denaturation, the reagent order of addition was arranged to minimize the template exposure to the synthesis. Since negative and hydrophilic monomers do not influence particle formation, these will not be used in this chapter.

Our primary objective was to develop a stable imprinting synthesis. To do so, the effect of particle formation was investigated by varying reagents concentrations. Since the hydrolysis of TEOS is dependent to its concentration in water, the optimal concentration must be determined. When particle synthesis occurs in a concentrated monomer solution, greater protein concentrations can be used; this increases the quantity of binding sites formed during MIP synthesis.

Unfortunately, high concentration of monomers result in slower synthesis of particles and higher ethanol concentrations; thus the template might denature. When monomers are used in low concentrations, TEOS hydrolysis is favored, and particle formation is accelerated. Additionally, ethanol concentration is reduced, but the concentration of the template is reduced; thus, fewer imprints can be formed.

Also, the ionic content of water needs to be determined. When a low ionic content is used, protein agglomeration can occur, and the protein's conformational structure might be distorted as well [104], but the formation of particles is improved. While MIPs produced in high ionic concentrations allow for protein stability, MIP synthesis will suffer from ionic competition. Thus the active monomer self-assembly might not occur as it is required. Hence, ionic content needs to be determined and controlled as either a lack or an excess of ions will prevent a successful molecular imprinting of large proteins.

Another important consideration is the concentration of alcohol in the synthesis of particles. Particularly, alcohol content can be used to synthesize particles or gel, and its absence can prevent the formation of particles entirely. Unfortunately, alcohol disrupts protein interactions. Hence, the minimum concentration of alcohol for MIP synthesis must be identified. If needed, ethanol concentrations can be kept at a minimum by synthesizing MIPs under high vacuums. The ethanol's high vapor pressure causes ethanol to be removed by evaporation as it is produced [92]. This method will not be used unless needed.

While the majority of MIPs use 1-2 mmol of template during synthesis, this is not possible with macromolecules. Using millimolar concentrations of large proteins is difficult and expensive. Thus, MIPs with macromolecules are mostly produced in micro molar concentrations. Particularly, we formulated MIPs with human chorionic gonadotropin (HCG) and West Nile virus IgG antibodies (WNVA). For MIPs developed with WNVA, it is possible to imprint only the Fab section to prevent cross reactivity with other antibodies. This was not done as antibody fractionation reduces template concentration to below usable concentrations. Hence, the whole

WNVA was imprinted. Because of this, WNVA MIPs have cross reactivity to other non-specific antibodies within the same antibody isotype. Particularly, we synthesized bulk MIPs where template was non-covalently imprinted.

To improve template diffusion in MIPs, sodium dodecyl sulfate (SDS) was added to generate mesopores. Nonetheless, SDS denatures proteins, and its benefit needs to be determined. Finally, the minimum concentration of ink to generate black particles needs to be found. To investigate all the above variables, a multivariable and single variable tests where carried.

Multivariable Test

Before any variable can be optimized, we need to understand particle reaction kinetics; thus, a multivariable test was performed. We tested high and low TEOS concentrations in either deionized water (DI) or 0.2 MES buffer. Furthermore, we tested synthesis with or without ethanol and SDS. For the all the synthesis, we used ammonium hydroxide as a catalyzer, and APS was fixed at 170 µl. To minimize template exposure and increase repeatability of the synthesis, two solutions were prepared. A total of 16 parallel reactions were performed for the multivariable test.

Preparation of solution 1a and 1b

Because a significant amount of time is required to add each reagent individually, we separated the synthesis in two solutions. Particularly, solution 1 mixes all the reagents that do not polymerize until added to solution 2. By premixing reagents, a large stock can be prepared beforehand. Thus, solution 1a and 1b is made for the total amount of MIPs that are going to be synthesized for the study. Solution 1a was prepared with 8 ml of ink, 6.08 ml of 1.48 M ammonium hydroxide, and 8 ml of SDS. Solution 1b was prepared with 8 ml of ink and 6.08 ml of 1.48 M ammonium hydroxide. SDS was not used in solution 1b.

Preparation of Solution 2

Solution 2 uses reagents that begin to polymerize immediately as they are added. Because of this, each MIP was prepared individually. Particularly, solution 2 has three variables which are

total reagent concentration, ethanol concentration, and ionic content, where either DI water or 0.2M MES buffer was used. Each MIP was produced in the following order, where the volumes of the reagents are listed in tables 2 and 3. First, 2.4 ml of TEOS was added to a 15 ml centrifuge tube, followed by either DI or MES buffer. Afterwards, ethanol was added, and the solution's pH was lowered to ~3.5 with 6 µl of 1.2M HCl. The solution was then gently agitated by hand. Once solution returned to room temperature, 35 µl of APS and 25 µl of 1.2M HCl was added to maintain the solution's pH at ~3.5. Then, 65 µl of HCG template and 135 µl of APS was added; the solution was strongly agitated with a pipette and pH increased. Finally, either 2.25 ml of solution 1a or 1.45 ml of solution 1b was added.

Table 2. MIPs produced with solution 1a, which adds 820 µl of SDS to each MIP listed.

MIP	Deionized Water	0.2 MES	Ethanol
1	1.5 ml	0 ml	820 µl
2	1.5 ml	0 ml	0 µl
3	0 ml	1.5 ml	820 µl
4	0 ml	1.5 ml	0 µl
5	2.4 ml	0 ml	820 µl
6	2.4 ml	0 ml	0 µl
7	0 ml	2.4 ml	820 µl
8	0 ml	2.4 ml	0 µl

Table 3. MIPs produced with solution 1b, which does not add any SDS to any MIP listed.

MIP	Deionized Water	0.2 MES	Ethanol
9	1.5 ml	0 ml	820 µl
10	1.5 ml	0 ml	0 µl
11	0 ml	1.5 ml	820 µl
12	0 ml	1.5 ml	0 µl
13	2.4 ml	0 ml	820 µl
14	2.4 ml	0 ml	0 µl

15	0 ml	2.4 ml	820 µl
16	0 ml	2.4 ml	0 µl

Once particles were synthesized, they were centrifuged at 2880 g's for 10 minutes and rinsed with 40 ml of DI water in triplicate to remove any unreacted monomers. Afterwards to remove the template, particles were washed with 40 ml of elution buffer at room temperature under sonication for 10 minutes. Particles were then centrifuged at 2880 g's RPM for 10 minutes, 2 ml of supernatant was collected per solution. The absorbance of the elution supernatant was measured by UV-VIS spectrometry at 280 nm. If absorbance was measured in the supernatant, due to the presence of template or unreacted monomers, particles were washed again in the elution buffer. Particles were then rinsed in triplicate with 40 ml of DI water. Particles were centrifuged at 2880 g's for 10 minutes and suspended in a concentrated PBS solution and stored at room temperature until needed.

Individual Variable Tests

To determine the individual effects of reagents, particles were synthesized under similar conditions with variable volumes of the reagent in question. We tested 5 different volumes of water, ethanol, and ink. Particles were not molecularly imprinted. Finally, the volumes of reagents were dramatically reduced from 2.4 ml of TEOS to 146 µl; each reagent was downscaled identically as well.

Solvent volume variable

Imprinted silica particles were prepared by a modified sol-gel method. The synthesis was developed as a four-step procedure, where the volume of water used is listed in table 4. Briefly, 0.03 M NaCl was added to a 2 ml centrifuge tube, followed by 146 µl of TEOS. Afterward, 7.5 µl of APS and 62 µl of black ink were added and mixed by pipetting. Particles were then left overnight in a rotisserie. Particles were then centrifuged at 3200 g for 5 minutes. The centrifuged particles were resuspended in 400 µl of DI; particles were made in triplicate.

Table 4. Water volume used during the synthesis of MIPs.

Reagent	Sample 1 (µl)	Sample 2 (µl)	Sample 3 (µl)	Sample 4 (µl)	Sample 5 (µl)
Water	146	256	365	548	730

Ethanol variable

Synthesis of imprinted particles with ethanol variable was a five-step procedure. First 548 µl of 0.03 M NaCl was added to a 2 ml centrifuge tube, followed by 146 µl of TEOS. Afterward, 7.5 µl of APS and 62 µl of black ink were added and mixed by pipetting. Finally, ethanol was added in the volumes defined by the table 5 below. Particles were made in triplicate and left overnight in a rotisserie. Particles were then centrifuged at 3200 g for 5 minutes or longer if required. The supernatant was removed and placed in a different centrifuge tube, while precipitated particles were resuspended in 400 µl of DI.

Table 5. Ethanol variable volumes used.

Reagent	Sample 1 (µl)	Sample 2 (µl)	Sample 3 (µl)	Sample 4 (µl)	Sample 5 (µl)
Ethanol	0	18.75	36	63	152

Ink variable

Synthesis of imprinted particles with ink as a variable was a four-step procedure. First 548 µl of 0.03 M NaCl was added to a 2 ml centrifuge tube, followed by 146 µl of TEOS. Afterward, 7.5 µl of APS and variable amounts of black ink were added and mixed by pipetting, see table 6 below. Particles were made in triplicate and left overnight in a rotisserie. Particles were then centrifuged at 3200 g's for 5 minutes or longer if required. The supernatant was discarded, and precipitated particles were resuspended in 1X PBS. This test was also repeated with the substitution of black ink with a solution labeled as pseudo ink. The solution was prepared with 15% w/v polyvinyl pyrrolidone and 2.5 % v/v isopropyl alcohol in DI.

Table 6. Ink variable volumes used.

Reagent	Sample 1 (µl)	Sample 2 (µl)	Sample 3 (µl)	Sample 4 (µl)	Sample 5 (µl)
Ink	7.5	15	31	62	93

To further analyze the role of black ink in molecular imprinting, three MIPs were synthesized with black ink, red ink, or with no ink whatsoever. All MIPs were synthesized with template. The synthesis of MIPs was as follows: 484 µl of 0.03 M NaCl was added to a 2 ml centrifuge tube, followed by 146 µl of TEOS. Afterward, 7.5 µl of APS, 100 µl WNVA, and 15 µl of the corresponding ink was added. Particles were made in triplicate and left overnight in a rotisserie. Particle washing was carried in triplicate using 1.5 ml of elution solution consisting of 50% v/v mixture of glacial acetic acid and methanol at room temperature under sonication for 10 minutes to remove the template. Particles were then centrifuged at 3200 g's for 5 minutes, 2 ml of supernatant was collected per solution. The absorbance of the elution supernatant was measured by UV-VIS spectrometry at 280 nm. If absorbance was measured in the supernatant, indicating the presence of template or unreacted monomers, particles were washed again in elution solution. Particles were then rinsed in triplicate with 1.5 ml of DI. Particles were centrifuged at 3200 g's for 5 minutes and suspended in a 1X PBS solution and stored at room temperature until needed.

3.2.4 Particle Characterization

To observe MIPs under high magnification, SEM images were taken. Samples were prepared by loading 10 µl of a diluted particle solution directly to a 12 mm carbon conductive tab. Particles were then lightly coated with 80:20 gold/palladium target in a sputter coater. Images were obtained using a Hitachi S-4800 SEM. To identify material yield, 1 ml of suspended particles were obtained, dried, and weighed. To quantify color intensity, pictures were evaluated using Adobe Photoshop CS5.1, where black saturation percentage was obtained by selecting the center of the solution. Images were taken under same lighting conditions, and black color intensity of the solutions was observed. Particle precipitation was recorded using a Biomate 3 UV-VIS spectrophotometer at a wavelength of 621 nm. Finally, particle behavior during centrifugation and

resuspension was observed. The supernatants of single variable studies were filtered with progressively smaller nitrocellulose filters with pore sizes of 8 µm, 0.8 µm, 0.60 µm, and 0.45 µm until a clear solution was observed.

3.2.5 Agglutination Assays

An agglutination assays for the different ink types was carried. Briefly, 1 mg of black MIPs were suspended in 10 ml 1X PBS buffer, in a 15 ml centrifuge tube; a total of 3 black, 3 red, and 3 white MIPs were suspended. For positive tests, the solutions were spiked with 100 µg of WNVA. For false positives, solutions were spiked with 100 µg bovine serum albumin. Finally, for negative controls solutions were not spiked. Precipitation rates were noted, and particles were compared.

Since multivariable studies required observation of multiple MIPs, a device was built with 5 sensors capable of monitoring the solution's infrared transmittance per time in standard disposable cuvettes. The device is capable of collecting changes from full dispersion up to near total precipitation with a resolution of 1024 bits. The device was programmed to collect values from all sensors simultaneously every 2 seconds. The data was then transferred to an Excel sheet for processing and evaluation. The device is shown in figure 19.

Particle characterization of multivariable MIPs was done by monitoring the rate of precipitation. Each test was carried with 2 mg of the respective MIPs with 400 µg of HCG in 3 ml of 1X PBS solution. The same process was repeated but with the absence of HCG, as the negative control. The rate of precipitation per solution was recorded. Tests were performed in pairs of solutions with their respective template and control per particle type. Data collection was initiated once all MIPs were dispersed. After data collection, the rate of precipitation was determined by the slope generated by each sensor. Successful imprinting was determined by a precipitation difference of at least 500 seconds, meaning that the rate of precipitation for the template containing solution must precipitate noticeably faster or slower than its control. Finally, to evaluate the effect of ionic content in agglutination assays, particles 9 were further tested. MIPs 9 were loaded with 400 µg of HCG in 3 ml of 1X PBS, 2X PBS, 1X MES, 2X MES, and DI respectively.

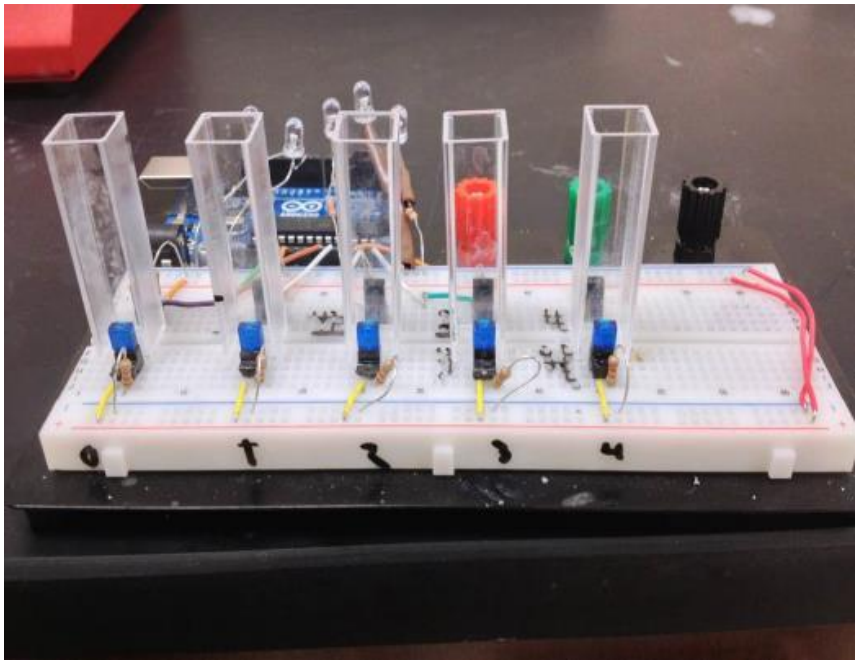

Figure 19. Turbidity sensor array.

3.3 Results and Discussion

3.3.1 Multivariable Results

After synthesis and washing of particles, color density, and particle concentrations were noted in table 7 below. The average concentration of MIPs was 58 mg/ml with a standard deviation of 18 mg/ml in the batch process. All particle groups were measured for their rate of precipitation, slopes were then collected, and the ratio of template against the control slope was obtained for each particle type. Interestingly, some MIPs exhibited a stepped behavior during agglomeration only when the template was in the solution. Figures 20 and 21 show MIPs agglomerating over time with the presence of template. Table 8 lists the measured precipitation ratio.

Table 7. Batch process particle yield and color intensity.

MIP	mg/ml	Black %
1	33	95
2	32	95
3	35	95
4	64	95
5	64	80
6	80	90
7	66	90
8	66	85

MIP	mg/ml	Black %
9	54	95
10	47	95
11	102	95
12	67	95
13	70	80
14	50	80
15	48	85
16	49	85

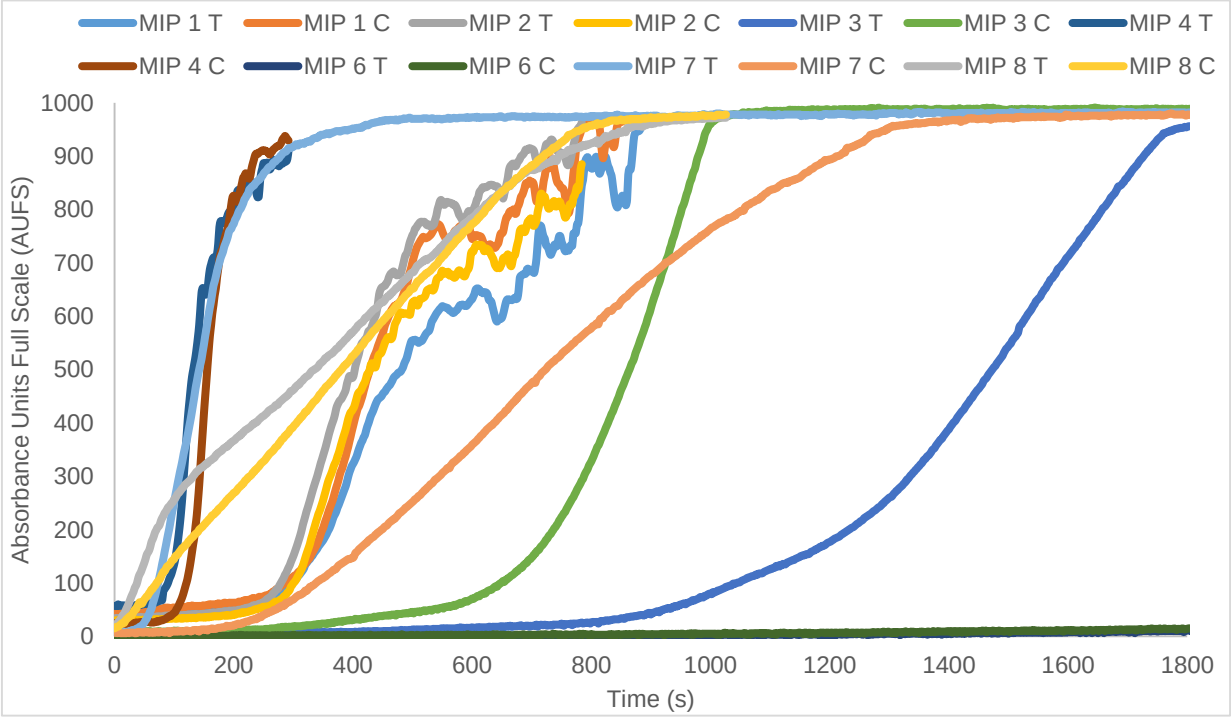

Figure 20. Particle precipitation rates for MIPs 1-4 and 6-8. MIP T refers to particles with positive template and C to MIPs with negative control. MIPs 5 are shown in figure 23.

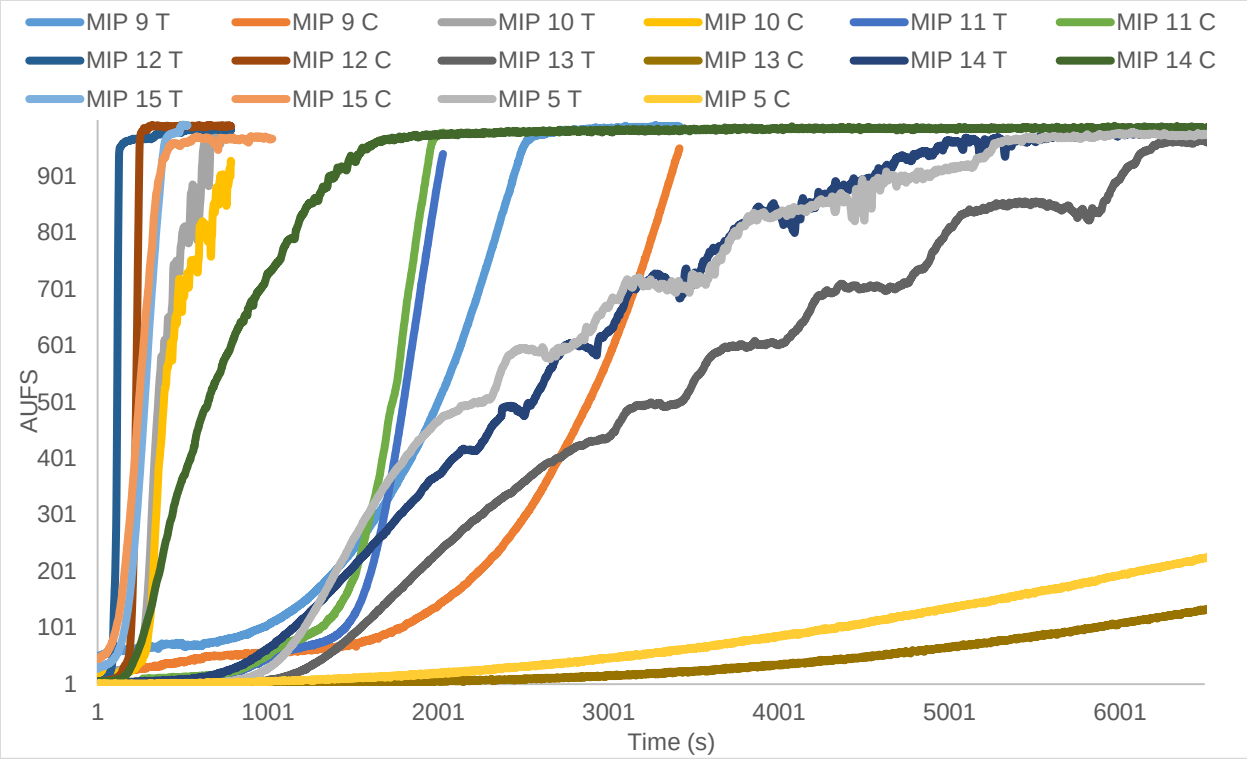

Figure 21. Particle precipitation rates for MIPs 9-15 and MIPs 5. MIP T refers to particles with positive template and C to MIPs with negative control.

Table 8: MIP precipitation ratios. MIPs 1-8 were produced with SDS and MIPs 9-16 without it.

MIP	Ratio
1	1
2	1
3	2
4	1
5	4.15
6	1
7	5.08
8	1

MIP	Ratio
9	1.4
10	1
11	1
12	1
13	4.22
14	2.83
15	1
16	6

The effect of reagent concentration resulted in more successful imprints when used in lower concentrations, producing 5 out of 8 imprints for MIPs with 2.4 ml of water. Whereas only 2 out of 8 successful MIPs were produced with 1.5 ml of water. Since lower reagent concentrations improved molecular imprinting, single variable studied even lower reagent concentrations. Interestingly, the rate of precipitation was also fastest when 2.4 ml of water was used in all successfully imprinted MIPs. This effect was most likely due to dilution of black ink, which was indicated by the solution's lower black density, see table 7. Particularly, black ink has dispersants which help particles stay suspended in solutions. Where the dilution of ink prevents MIPs from staying suspended for longer periods; hence, black ink plays an important role for agglutination assays.

MIPs developed with SDS had a total of 3 successful imprints out of 8. Particles synthesized without SDS produced 4 out 8 successful imprints. The rate of precipitation from each particle was added and compared by their corresponding variable. Particularly, particles with SDS summed 11 points, while none SDS particles totaled 14.5 points. Also, the removal of SDS proved difficult as it required extensive washing. These observations showed that SDS is not beneficial nor necessary for MIPs. Because of this, the use of SDS was discontinued.

For particles produced in the presence of ethanol, 5 out of 8 particles were imprinted, while no ethanol particles produced only 2 out of 8 imprinted particles. Thus, ethanol was found crucial for molecular imprinting. The role of ethanol during synthesis is to decrease the colloid stability of activated monomers, forcing them to polymerize as they precipitate. Furthermore, alcohols are commonly used for the precipitation of proteins in biology during centrifugation; thus, alcohols act as a solution destabilizer. As explained in the literature review, molecular imprinting can only occur in unstable colloid solutions, as the protein needs to come in direct contact with adjacent particles to form active binding sites. In a stable colloid solution, proteins will unlikely stick to adjacent particles, where few to no imprints will occur. Hence MIPs without ethanol were not as successful.

MIPs produced with either MES buffer or DI water were mostly undistinguishable. Particularly, 0.2 M MES had 3 out of 8 successful imprints, where the total rate of precipitation resulted in 13. For DI water, 4 out of 8 imprints where successful, and the rate of precipitation equaled 12.5. It was then determined that ionic content during MIP synthesis has minimal effects. Hence, as long as ionic contents are above 0.03 M and below twice the physiological ionic content, molecular imprinting will most likely not be affected.

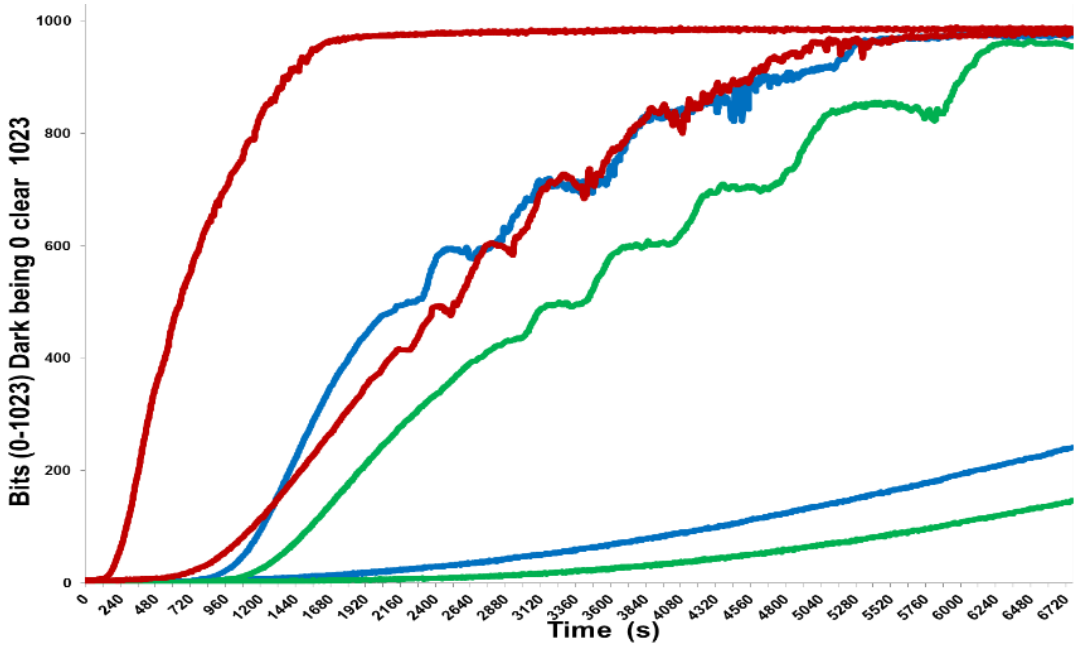

Figure 22. Particle precipitation rates. Green trace represents MIPs 13, blue trace MIPs 5, and red trace inappropriately stored MIPs 13. All positive samples exhibited stepped behavior.

Interestingly, during data collection, some MIPs degraded when stored diluted for longer than a month. This was seen by a change in the rate of precipitation, were negative control samples precipitated faster than positive samples with the same MIPs. Interestingly, positive samples retained the same precipitation rate and their stepped trace, see figure 22 above. This behavior was not observed when particles were stored in high concentrations or lyophilized.

For ionic content studies, particles in DI remained suspended for longer periods, while MES showed an insignificant change rate against DI. The rate of precipitation increased in the following manner: DI, MES 2X, MES, PBS 2X, and finally PBS. Particularly, PBS buffered

solutions had the fastest precipitation rate, while MES precipitated particles slower. Finally, both buffers precipitated MIPs slower with increasing ionic content of the same buffer. Figure 23 shows the rate change based on ionic content and concentration.

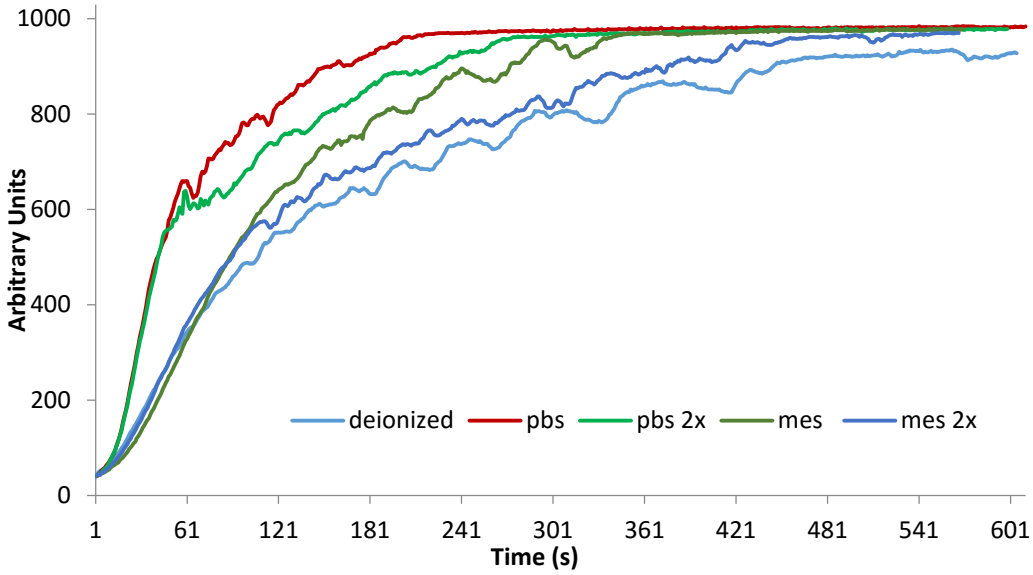

Figure 23. Ionic content effect on rate of precipitation.

3.3.2 Single Variable Effects

MIPs with water volume as a variable were centrifuged, resuspended, and their precipitation rate was determined using UV-VIS spectrophotometry, see figure 24 below. Particularly, MIPs precipitated without the need of a centrifuge with increasingly slower precipitation rates as the water volume increased, except 730 µl, which precipitated faster than the 584 µl sample. During centrifugation, MIPs precipitated faster with increasing water volumes, visually there were no differences between them. Water was determined optimal for MIPs at 4 times the volumetric amount of TEOS based on the slowest precipitation rate of particles.

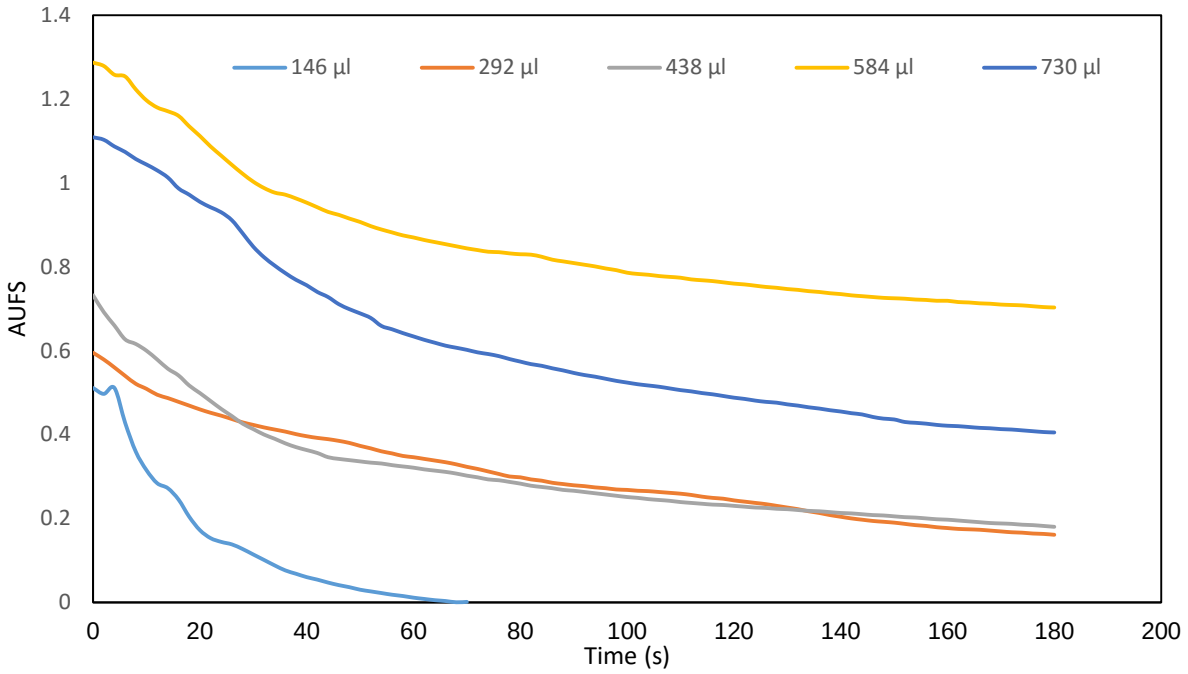

Figure 24. Precipitation rate of particles of different water volumes used during synthesis.

Ethanol studies provided interesting results, where nanoparticle color changed from pink, red, brown, and blue as the ethanol concentration increased; this indicates a change in the size of the nanoparticles produced. An image of the ethanol influence on color nanoparticle is shown below, in figure 25. Furthermore, when ethanol was not used fewer particles precipitated after centrifugation. Consecutively, ethanol improved the precipitation of particles during centrifugation with increasing amounts of ethanol up to 63 µl, where particle precipitation occurred the most. Afterwards as ethanol increased above 63 µl, particles were harder to precipitate, where MIPs with 152 µl ethanol produced the least amount of precipitated particles.

For the collected supernatants, all solutions were cleared with a 0.8 µm nitrocellulose filter, except particles synthesized with 152 µl of ethanol. For the high ethanol MIPs, nanoparticles were captured separately by two different pore size filters. A 0.8 µm nitrocellulose collected a fraction of the particles, while the remainder were captured by a 0.45 µm membrane; figure 26 shows the immobilization of nanoparticles by nitrocellulose paper. Because ethanol is a protein denaturant and yet it is required to increase particle centrifugation, the optimal ethanol volume was fixed at

63 µl. While ethanol could be removed entirely, recuperating particles after multiple washes increased sample losses to unacceptable quantities.

Figure 25. Duplicate test of ethanol influence on color. From left to right, increasing alcohol concentrations.

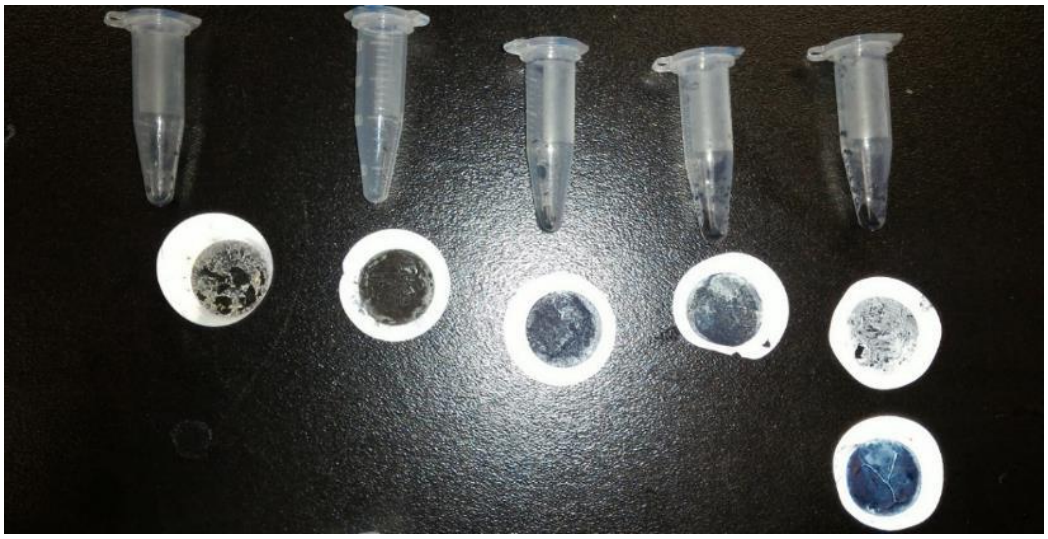

Figure 26. Ethanol influence on color and particle size. From left to right, increasing alcohol concentrations.

For ink as a variable, increasing amounts of ink resulted in higher concentrations of nanoparticles, as indicated by the darker color of the supernatant after centrifugation and filtering with an 8 µm filter, see figure 27 below. For the centrifuged particles, the color intensity increased as more ink was used. No change in particle color could be observed after 31 µl of ink. For particles developed with red ink, particles behaved similarly, where black particles remained suspended in

solution for a slightly longer period over the red particles. The absence of ink in particles resulted in fewer and unstable particles in suspension, which were difficult to distinguish from the solution.

Finally, pseudo ink particles had gel like particles with visible swelling, see figure 28. These particles could not be centrifuged as easily as black ink particles and sample losses were high. Furthermore, nanoparticles could not be observed in pseudo ink solutions. Thus, ink's role for particle synthesis was determined necessary for the formation of denser particles. Since there were no significant differences after 31 µl of ink, this volume was determined as optimal.

Figure 27. Duplicate test of ink influence on color and particle size. From left to right, increasing ink concentrations.

Figure 28. Duplicate test of pseudo ink influence on color and particle size. From left to right, increasing ink concentrations.

3.3.3 Particle Morphology

Since the process is not an ideal Stöber synthesis, particles were not spherical. In fact, particles had a morphology caused by the aggregation and agglomeration of primary particles; as explained in the literature review. Because of this, particle shape varies wildly. Figures 29 to 31 show MIPs produced for the multi-variable test.

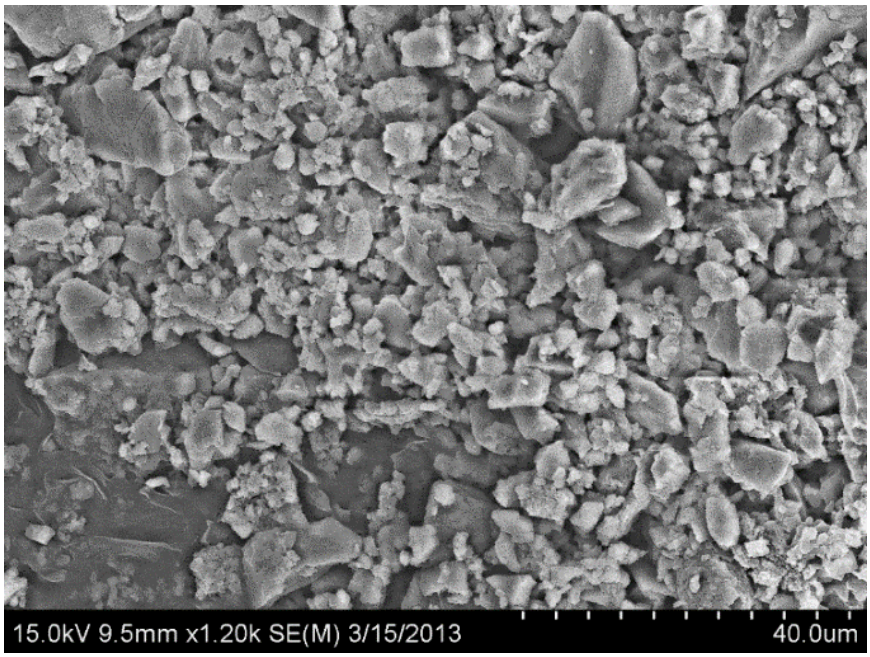

Figure 29. Scanning electron Microscopy (SEM) image of black MIPs, 1200X.

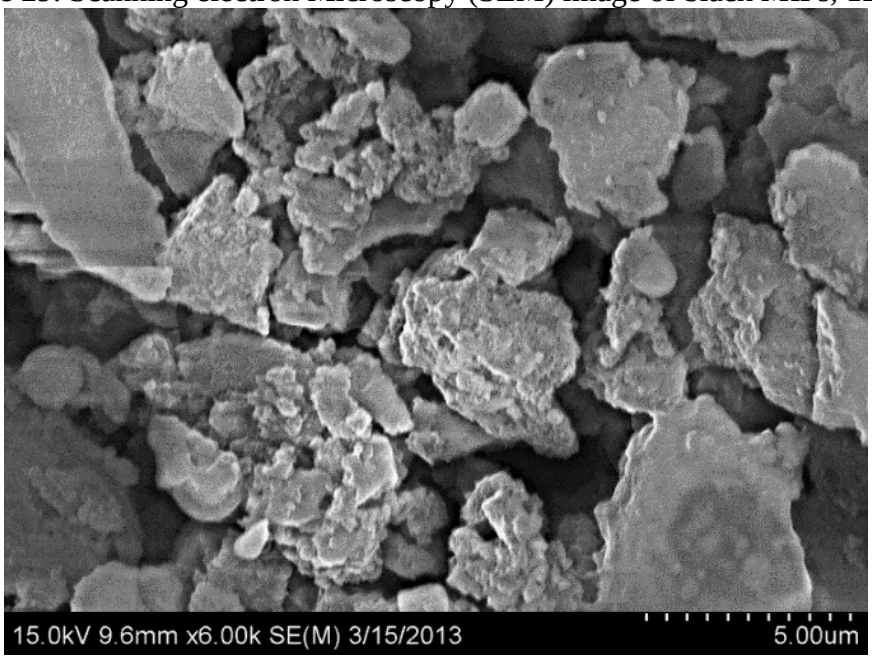

Figure 30. SEM image of black MIPs, 6000X.

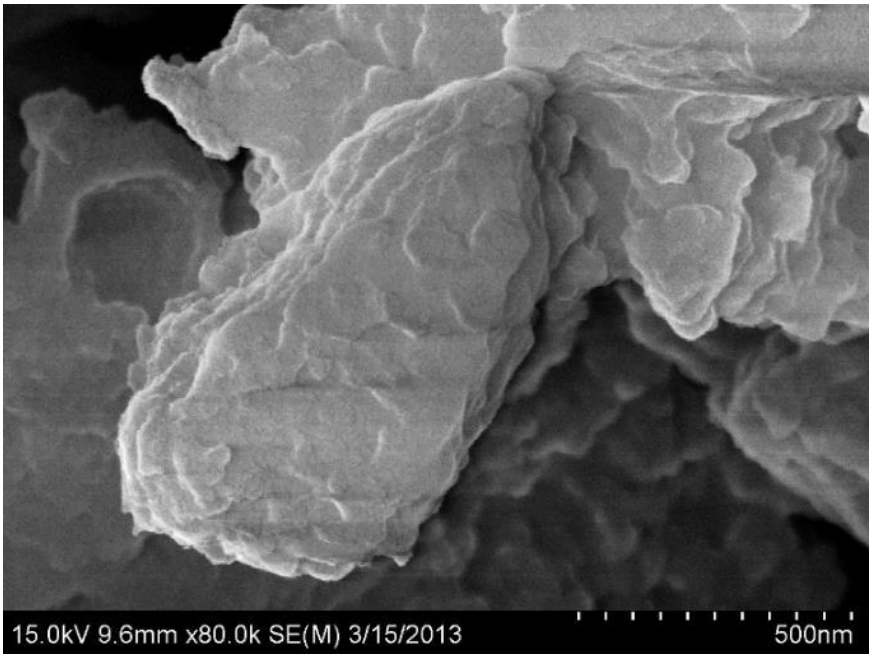

Figure 31. SEM image of black MIPs, 80,000X.

Particle morphology of water variable showed interesting results, in which the volume of water changed the way particles formed. The morphology varied from spherical, fibrous, plate, and a combination of two structures. For general molecular imprinting, the shape is not as relevant as the strength and the quantities of recognition sites. Nonetheless, the shape can influence particle behavior in solutions in agglutination assays. The shape differences due to increasing water volumes are shown in figure 32 to 36 below. Interestingly, figure 37 shows rough spherical MIPs which may be advantageous for their use in agglutination and particle immobilization assays. Thus, SEM images further support an optimal TEOS:water ratio of 4.

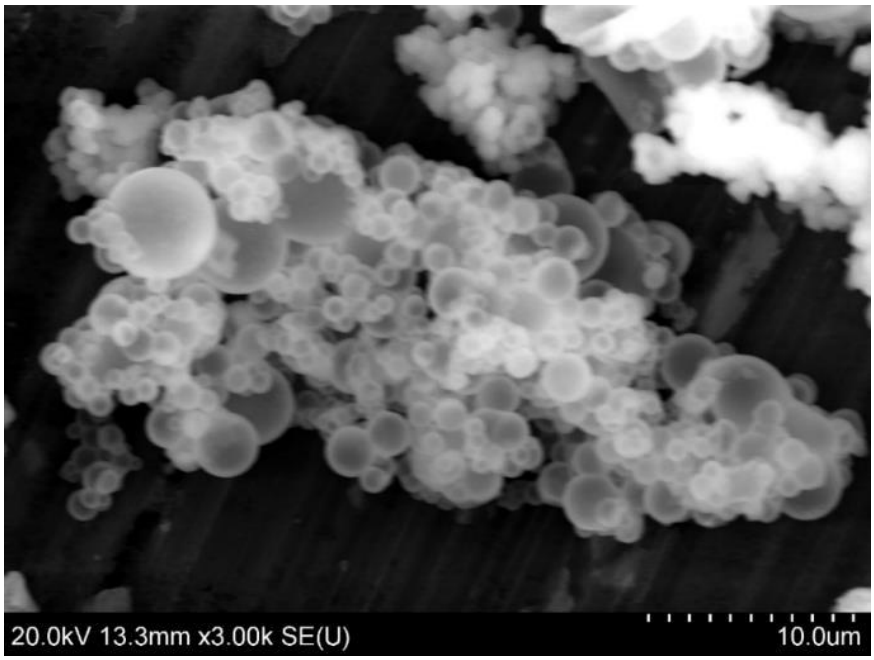

Figure 32. SEM image of spherical MIPs with 1:1 water:teos ratio, 3,000X.

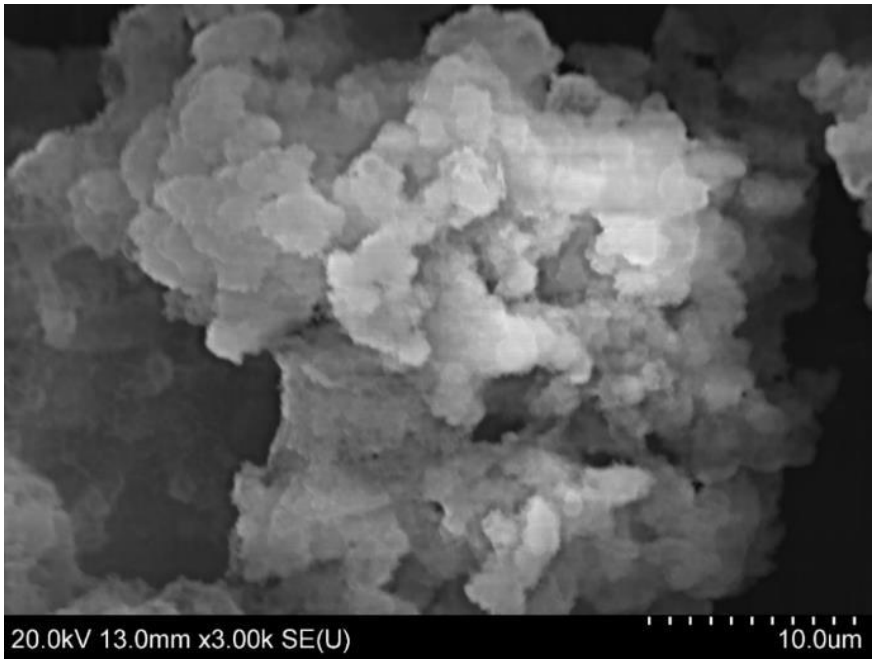

Figure 33. SEM image of plate MIPs with 2:1 water:teos ratio, 3,000X.

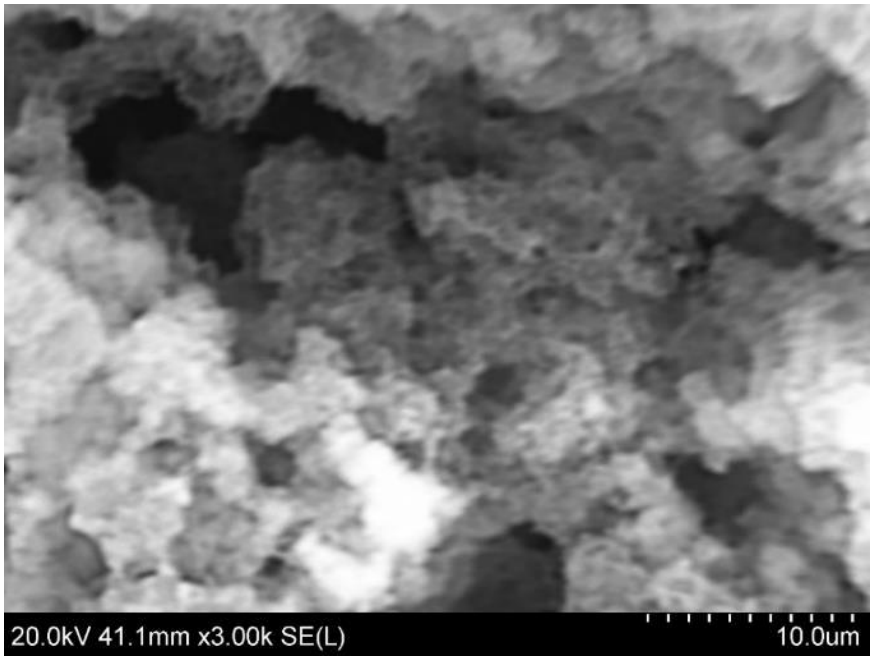

Figure 34. SEM image of fibrous MIPs with 3:1 water:teos ratio, 3,000X.

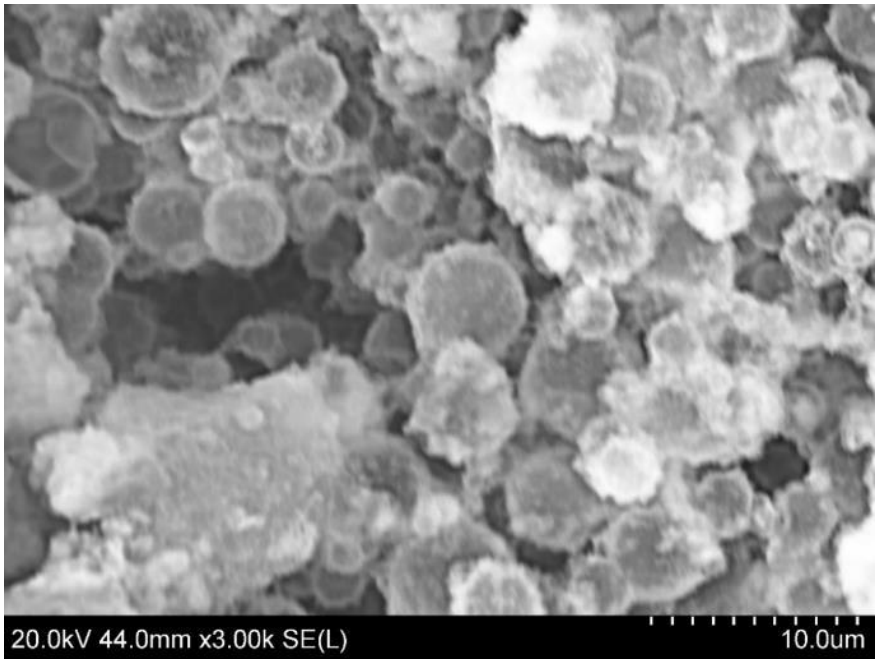

Figure 35. SEM image of rough spherical MIPs with 4:1 water:teos ratio, 3,000X.

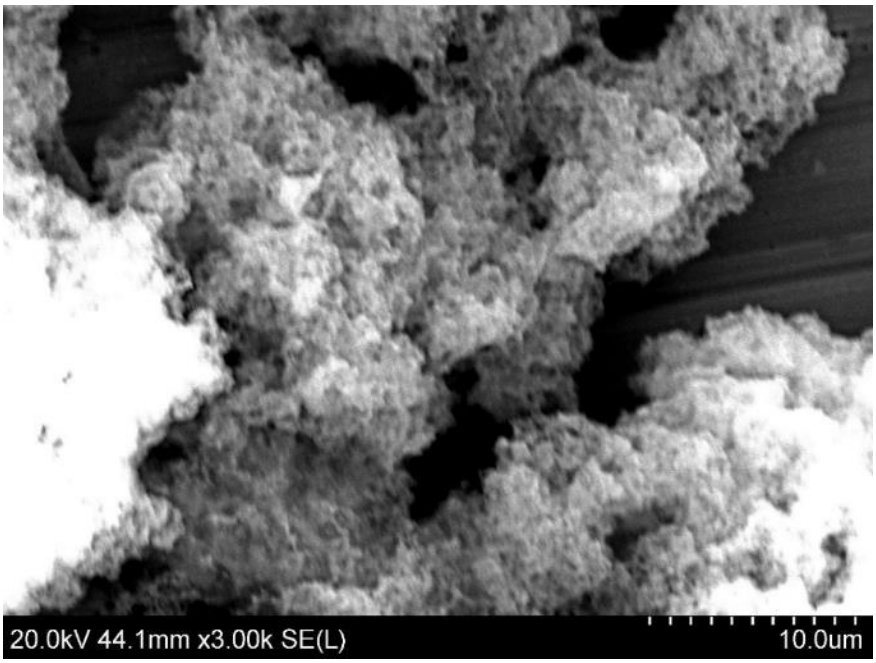

Figure 36. SEM image of fibrous MIPs with 1:1 water:teos ratio, 3,000X.

3.3.4 Molecular Imprinting with Ink

The differences between black, red, and white MIPs were apparent, where black MIPs had the greatest performance. In black particles, positive samples precipitated within a minute, while negative samples remained fully dispersed for greater than 5 minutes. Red particles, visually orange, behaved similarly in which positive sample precipitated within less than a minute, while negatives samples precipitated within 3 minutes. Thus, carbon black does not play a critical role in molecular imprinting by itself. Nonetheless, MIPs cannot be used without ink as immunoprecipitation assay. It is possible that the effect of the dispersant, used in inks, may maintain particles dispersed until template recognition causes immunoprecipitation. Although red particles precipitated faster than black particles, the precipitation rate may be adjusted by increasing concentrations of ink synthesis. Figure 37 below, shows a comparison of MIPs developed with different color particles. Unfortunately, white particles precipitated equally and had no distinguishable recognition between positive and negative samples.

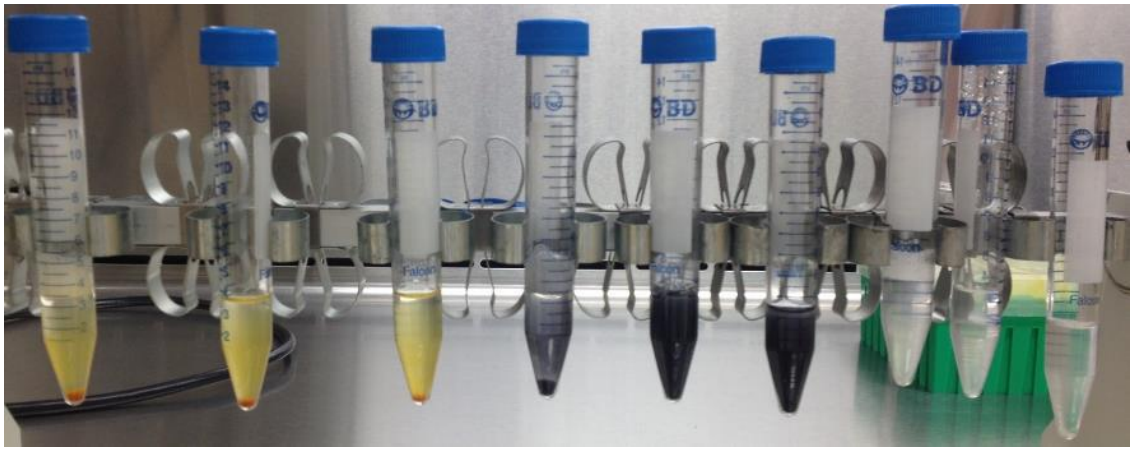

Figure 37. Red, black, and white molecularly imprinted particles. From left to right, WNVA positive sample, BSA false positive sample, blank negative sample.

3.4 Conclusion

To better understand the roles of reagents during MIP synthesis, a multi-variable test was carried. During the washing steps, centrifuge tubes with MIPs 10 and 13 cracked and released some of the material. Centrifuge tubes reacted with TEOS, and particles had to be remade. To prevent this from reoccurring, TEOS was added to solution 2 after the addition of water. Once the reagent order was revised, no additional tubes cracked. Furthermore, we found that ethanol must be present during molecular imprinting, despite this being a strong protein denaturant. Finally, when MIPs were stored in dilute concentrations, they degraded. Thus, MIPs need to be stored dry.

After determining reagent volumes, our most significant finding was the possibility of improving molecular imprinting. Since synthesis of silica particles requires an acid or basic pH to occur, a neutral pH could not be used. Coincidentally, when the active monomer was added, the synthesis solution reached a pH close to neutral. Despite this, silica particles nucleation had already occurred and the growth and aggregation of particles continued even after the pH was changed. Because of this, physiological pH matching can be obtained by the careful use of active monomers and the addition of acid or base to fine tune the pH. Finally, agglutination assays will be discontinued for future tests, as they are subject to misinterpretation and may not be specific. This can occur when control particles precipitate faster than MIPs with template.

Chapter 4. MIP Synthesis in Physiological PH

4.1 Introduction

There is a significant demand for robust and stable receptor molecules that can mimic biological molecules, such as antibodies [10]. Relying only on natural recognition molecules have greatly limited the uses and capabilities of many aspects of health sciences due to product expense and stability. This is especially important in low resourced areas where the lack of resources and limited cold-chain makes antibody based diagnostics very difficult to implement.

In low resource settings, the absence of diagnostic tests, due to their costs and insufficient infrastructure, has led to diseases being treated by their clinical symptoms and the local prevalence of the disease. Currently, the use of antibodies in lateral flow assays have limited their applications to a few diseases. Furthermore, antibodies suffer from slow, difficult, and expensive manufacturing, and temperature requirements can be difficult to maintain during transit to medically underserved areas [7], [8]. Because of this, an alternative biorecognition molecule is necessary. To fabricate robust immunoassays, molecular imprinting has been proposed [16], [61], [85].

While the basic reagent concentrations for MIPs has been established in chapter 3, improvements to the synthesis environment are required to produce reliable and repeatable molecular imprints. For an effective MIP recognition site to develop, it must closely resemble the template's conformational structure at physiological pH. If a proper steric complementarity is obtained, strong and stable short range interactions are possible. If a conformational structure variation occurs during synthesis, due to incorrect pH or any other denaturing condition, binding sites will develop specificity against the denatured protein; thus, recognition of template may not be possible. Because of this, pH control is critical.

Furthermore, pH control plays a major role in the development of ionic complementarity with the template. When pH is not monitored, the template's amino acids can change their ionic charges if the synthesis pH becomes acid or basic. It is possible for an amino acid, within the same

protein, to become positively, neutral, and even negatively charged depending on the solution pH. For example, at pH 7 a protein template might have 17 negative and 60 positive amino acids, whereas, at pH 5 the charges might be reversed. When synthesis pH distorts the template, MIPs will form steric complementarity for the incorrect conformation of the template. Thus, MIPs fail, or their effectiveness is reduced.

In the previous chapter, primary reagents were set. Nonetheless, natural antibodies have negative and positive electrostatic interactions, as well as hydrophobic attractions. Our initial molecular imprinting formula only used a positive active monomer; thus, hydrophobic and negative monomers are now added. We will investigate physiological pH enforcement while using an updated molecular imprinting formula which includes negative and hydrophobic interactions.

Particularly, pH enforcement required changes in the order of the reactives used during particle synthesis. The changes were necessary to simplify the pH enforcement of the MIP synthesis. The addition of active monomers is before pH neutralization; thus particle nucleation is favored, but molecular imprinting cannot occur without a template. After adding active monomers, pH neutralization was accomplished by adding hydrochloric acid or ammonium hydroxide as necessary. Also, time becomes relevant as it influences the monomer's activation during the acidic conditions, where a short TEOS activation prevents particle formation and a long TEOS activation can form particles before molecular imprinting can occur. Hence, the optimization of monomers order and times of reactions needs to be tested.

To further refine MIPs, the temperature must be considered. Temperature is likely to influence imprints in template recognition in a similar manner as it changes the antibody antigen interactions. In antibodies, the use of low temperatures increases short range interactions during recognition. Thus is should be possible that binding strength in MIPs can be increased by carrying tests under refrigeration. Also, the speed of recognition can be accelerated by room temperature testing where the increased entropy allows antibody antigen to encounter long range interactions faster. To better understand MIPs, a temperature test must be developed for when particles are

being synthesized under low temperature, to promote steric complementarity, and room temperature, to promote long range interactions with active monomers. Furthermore, after particle synthesis, binding temperatures need to be tested as well. This is necessary as to determine if the synthesis conditions must match the same testing conditions under MIPs recognition.

4.2 Materials and Methods

4.2.1 Materials

Tetraethyl orthosilicate (TEOS), 3-aminopropyl triethoxysilane (APS), and phosphate buffered saline as tablets were obtained from Sigma-Aldrich (St. Louis, MO), reagent grade and used as is. Hydrochloric acid (37%), anhydrous acetic acid, and ethanol were acquired from Fisher Scientific (Pittsburgh, PA), reagent grade. Succinic anhydride, octyl trichlorosilane (OTS), ammonium hydroxide, sodium hydroxide were obtained from Sigma-Aldrich. Polymer grafted carbon black was obtained by collecting ink from HP 33 cartridges (HP, Palo Alto, CA). According to literature, black ink is composed of 15 nm carbon black particles [121], [122] grafted with 2-Pyrrolidone [123].

Ultrapure water was obtained from a Milli-Q Millipore unit with a water quality of at least 18.2 MΩ. Elution buffer was made with 0.15 M NaCl and 0.5 % acetic acid. WNVA were obtained from hyper immune mouse ascitic fluid (HMAF) [118] and prepared as described in section 4.1.2. Bovine serum albumin (BSA) and bovine gamma globulin (BGG) standard ampules were obtained from Thermo Scientific (Waltham, MA). For confocal imaging, affinity purified WNVA, BSA, and BGG were dyed using ATTO 495 NHS ester following manufacturer's recommendations at a protein concentration of 100 µg/ml. Biomate 3 UV-Vis spectrophotometer was used for protein determination with BCA and micro BCA kits from Fisher Scientific.

4.2.2 Preparation of cAPS

The negatively charged monomer was developed in house, there were no reagents available for purchase. Carboxybutyl 3-amidepropyl triethoxysilane (cAPS) was synthesized by reacting 5

ml of APS with 2.137 grams of succinic anhydride. The solution was then left in a rotisserie overnight. Afterward, the solution was centrifuged for 5 minutes at 3200 g's to separate unreacted succinic anhydride.

4.2.3 Preparation of West Nile antibodies

WNVA was prepared in mice as hyper immune mouse ascitic fluid (HMAF) as described in the article [118]. WNVA was then purified by affinity gel goat antibody to mouse IgG column from MP Biomedicals (Santa Ana, CA). Briefly, the affinity column was equilibrated with a 2X PBS buffer solution. Afterward, 1.0 ml of serum containing WNVA was added. The column was then rinsed with 10 ml of PBS. Finally, the antibody was eluted from the column with elution buffer; the sample was collected. Eluted antibodies were then desalted with a Hitrap 5 ml desalting column (General Electric Healthcare Life Sciences, Wauwatosa, WI), previously equilibrated in 1X PBS buffer.

4.2.4 Preparation of fluorescently labeled proteins

For confocal imaging, affinity purified WNVA, BSA, and BGG were dyed using ATTO 495 NHS ester at a protein concentration of 120 µg/ml. Briefly, each protein was desalted in separate Hitrap columns, previously equilibrated in 0.1 M bicarbonate buffer at a pH of 8.3. Afterward, 75 µl of ATTO 495 NHS was added to 1.5 ml of each protein and reacted for one hour in a rotisserie. Finally, the proteins were desalted with 1X PBS using Hitrap columns; labeled protein concentrations were approximately 100 µg/ml.

4.2.5 Synthesis of Imprinted Silica Particles

Imprinted silica particles were prepared by a modified sol-gel method as described in [25–28]. Synthesis of imprinted particles was a four-step procedure, where the order of reagents was imperative. Briefly, for every 146 µl of TEOS, we used the following volumetric amounts of active monomers: 45 µl of APS, 11 µl of cAPS, and 16 µl of OTS. First, the active monomers were mixed in a centrifuge tube labeled as vial 1. Then, 365 µl of 0.03 M NaCl, 146 µl of TEOS, the

full contents of vial 1, and 15 µl of black ink were added and mixed by pipetting in a new centrifuge tube labeled as vial 2. Afterward, a pH electrode was used to measure the solution's pH. The measurement was possible by inserting the electrode's tip in the centrifuge tube and inverting the tube to cover the electrode's tip. The solution's pH was then adjusted as necessary by adding HCl or NH4OH until a pH of 7.3 with a tolerance of ±0.5 was achieved.

Once pH was adjusted, 441 µl of purified WNVA, as template, was added at a concentration of 200 µg/ml to the MIP solution. The solution was then pipette mixed and left overnight in a rotisserie. As a comparative, molecularly imprinted particles were synthesized as above except the solution's pH was not adjusted. As a negative control, non-imprinted particles (NIPs) were synthesized under identical conditions except 1X PBS buffer was used instead of the template.

Unreacted monomers were removed by centrifuging all particles at 3200 g's for 5 minutes; the supernatant was discarded. Then, the template was removed by adding 1.2 ml of elution buffer to particles and resuspending them in a vortex mixer. If large particles were noted, these were disrupted by sonication for 1 minute in a Branson 2510 sonicator. Afterward, particles were centrifuged, and the elution process was repeated once more. Any remaining template was removed by resuspending particles in 1.2 ml of a 50% v/v mixture of glacial acetic acid and methanol for one hour. The particles were then centrifuged, and the supernatant was discarded. Afterward, the particles were washed in 1X PBS in triplicate and resuspended in a final volume of 400 µl. No trace of protein could be detected in the last 1X PBS supernatant collected; a micro BCA kit was used for detecting proteins. Finally, particles were stored as is at room temperature for later use. After particle washing, a total of 7 ± 2.45 mg of micro particles were collected per batch.

4.2.6 HPLC Chromatography and Western Blot

To determine the effect that pH control has in molecular imprinting, particles were loaded to empty HPLC columns, and WNVA were added. The performance of pH enforced MIPs were

compared against non-pH enforced MIPs and non-imprinted particles (NIPs). Studies were carried using a LabAlliance series 3 pump, a Rheodyne injector model 9725i, a GE Tricorn 10/50 column, a UV-VIS model 500 detector with a rise time of 0.3 seconds and 0.01 AU sensitivity, and an SRI model 333 integrator for data collection.

A total of 2 MIP and 2 NIP columns were produced by transferring particles to an empty GE Tricorn 10/50 HPLC column. The particles were then immobilized inside the column with silica gel by adding 500 µl of TEOS, followed by 100 µl of APS, 25 µl of cAPS, 200 µl of ink, and 700 µl of ethanol. Afterward, the solution was strongly mixed for a minute using a pipette. In approximately 45 minutes, solutions gelled in the HPLC columns encapsulating the particles. Finally, columns were packed by flowing 2X PBS at a maximum flow rate of 1 ml/min and a maximum column backpressure of 150 psi. If the backpressure was exceeded, column repacking was needed. Column flow rates varied from 0.6 ml/min to 1 ml/min.

Packed columns were then equilibrated with 2X PBS until the absorbance at 280 nm stabilized. After equilibration, 60 µl of WNVA serum was loaded to a 20 µl loop in the sample injector. The sample was then injected at a flow rate of 0.5 ml/min. The first elution peak was recorded, and the sample was discarded; the sample is composed of rejected protein from the column. After column re-equilibration, the mobile phase was changed to elution buffer. Shortly thereafter, new peaks were recorded, and samples were collected for later analysis with western blot. To prevent protein denaturation, collected samples were desalted using 1X PBS buffer with a Hitrap desalting column.

Western blot was carried with the samples collected and compared against WNVA serum, and affinity purified WNVA using a 10% SDS-page acrylamide gel, polymerized for 30 minutes. All samples were treated with 10 mM 2-mercaptoethanol and 10% (w/v) SDS at boiling temperature for 10 minutes. The volumes per well were calculated based on a total 20 µg of protein loaded per well. After blotting, the gel was transferred to a nitrocellulose paper and developed with Oriole fluorescent gel stain. The following sample volumes were used:

- 10 µl of molecular weight marker in well 1
- 5 µl of WNVA serum in well 2, as full protein content control
- 20 µl of affinity purified WNVA in well 3, as the positive control
- 45 µl of sample collected from non pH control column peak 1 in well 4
- 45 µl of sample collected from non pH control column peak 2 in well 5
- 40 µl of sample collected from pH control column in well 6

4.2.7 ELISA

To verify if MIPs retained active antibody, MIPs were encapsulated in silica gel and loaded with WNVA in 2 ml centrifuge tubes. A total of 2 MIP and 1 NIP tubes were made. Each tube was loaded with 1.2 ml of 1X PBS and 300 µl of either MIPs or NIPs. Afterward, 60 µl of WNVA was added to each centrifuge tube. The particles were then resuspended using a vortex mixer, and the columns were incubated for 3 hours in a rotisserie at room temperature. After incubation, the tubes were centrifuged for 5 minutes at 3200 g's, and the eluent was discarded. To reduce nonspecific binding, particles were then washed with a total of 6 ml of 1X PBS. Particles were then centrifuged, and 800 µl of elution buffer was added to detach retained antibody. The elution process was repeated in duplicate, and both supernatants were separately collected in centrifuge tubes; protein presence was then verified individually using 50 µl of the sample with a BCA kit.

Afterward, the supernatants from the MIP and the NIP tubes were desalted using a Hitrap column previously equilibrated with 1X PBS. To understand the importance of desalting antibodies with 1X PBS, the MIP's second supernatant sample was not desalted. Finally, the collected elutions were analyzed with an enzyme linked immunosorbent assay (ELISA) for WNVA. Affinity purified WNVA and 1X PBS were used as positive and negative controls.

4.2.8 Confocal Imaging

To determine if MIPs specifically absorb WNVA, MIPs were loaded with fluorescently labeled WNVA, BSA, or BGG. Furthermore, the temperature influence during synthesis and

rebinding was measured. To do so, MIPs were prepared at 27°C (RT), 4°C (CT), and at -10°C (FT). For template recognition, we tested two binding conditions 4°C, and 27°C. As positive tests, MIPs were loaded with fluorescently labeled WNVA. Negative controls MIPs were loaded with either fluorescently labeled BSA or BGG. The negative comparative controls were made with NIPs incubated with fluorescently labeled WNVA, BSA, or BGG.

The study was carried with 30 µl of pH matched MIPs or NIPs pipetted to a 0.6 ml centrifuge tube and 20 µl of the appropriate fluorescently labeled protein. Samples were then gently mixed with a pipette and vials were left overnight in a rotisserie to reach equilibrium. Samples were then centrifuged, the supernatant discarded and particles were washed twice with 300 µl of 0.03 M NaCl. Particles were then centrifuged and resuspended in 100 µl of 0.03M NaCl. Finally, 5 µl of each sample was loaded to a microscope glass slide and viewed under the confocal microscope.

Confocal and standard brightfield images were obtained using an inverted Nikon Ti-U microscope, and a Nikon C1 confocal system equipped with NIS elements and EZ-C1 software. The images were analyzed using Fiji imaging software [28]. For brightfield images, all particles were counted using the particle analysis tool. For confocal images, a histogram was generated, and pixels above the signal noise were counted. To generate an average image fluorescence intensity (AFI), confocal image's pixels were multiplied by their signal intensity, added together, and divided by total black pixels in the brightfield image.

4.3 Results and Discussion

The addition of carboxylate group to APS was confirmed with a Thermo Nicolet Nexus FTIR. APS trace showed distinct amine peaks at 3300-3500 cm^{-1} but none at 1710-1780 cm^{-1}, whereas cAPS had peaks at both regions; thus carboxylation of APS was confirmed. Figure 38 below shows a blue and orange trace obtained from APS and cAPS respectively.

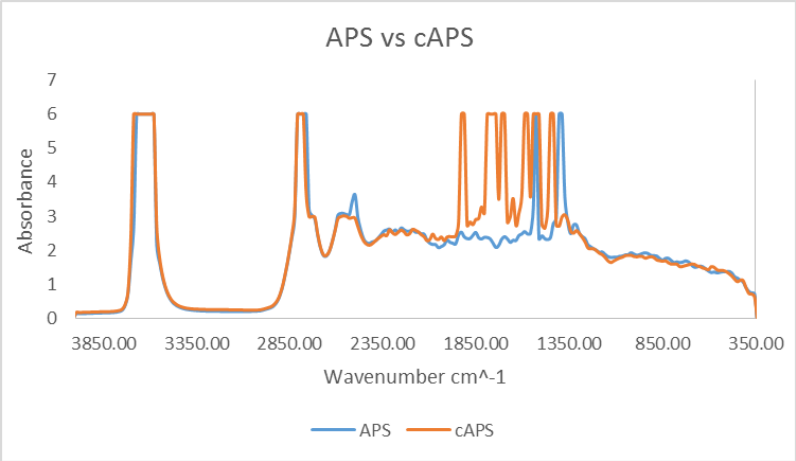

Figure 38. FTIR trace of APS as blue line and cAPS as orange line.

4.3.1 Physiological pH Control on MIP

Particularly, HPLC traces showed one peak immediately after sample injection for all columns; thus it was unbound protein, image shown below. No peaks were then later detected for the NIP column; thus, no protein was retained by the column. Shortly thereafter, the pH controlled column showed a small peak at an elution volume of 3.00 ml. The elution contained no measurable protein by micro BCA. Therefore the peak was attributed to the mobile phase change to elution buffer; the sample was discarded. At an elution volume of 3.75 ml, both MIP columns showed peaks, where the strongest came from the pH matched column; both samples were collected. Finally, the non pH control column showed an additional peak at an elution volume of 5.7 ml; the sample was collected. Figure 39 shows HPLC traces obtained from control NIPs, pH controlled MIPs, and non pH control MIPs.

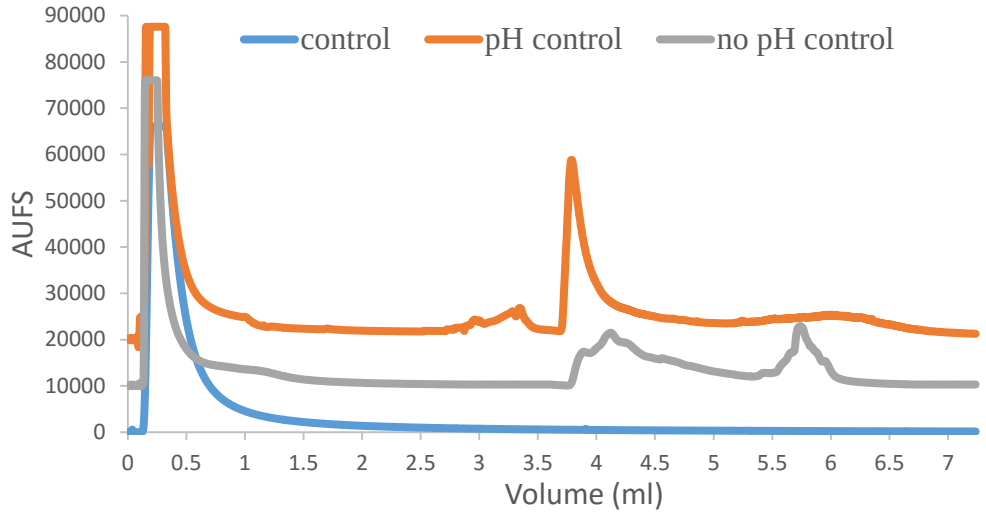

Figure 39. HPLC binding traces

Western blot, figure 40 below, revealed that purified antibody presented bands at 50 and 25 kDa, which corresponds to the heavy and light chain of an IgG antibody. All the elutions from MIP columns exhibited a common band at 50 kDa and 25 kDa; thus, all imprints successfully captured WNVA. However, non pH controlled column presented a band at a molecular weight of 70 kDa, which did not match any of the purified antibody bands. Thus, the non pH imprinted particles most likely absorbed proteins from serum nonspecifically.

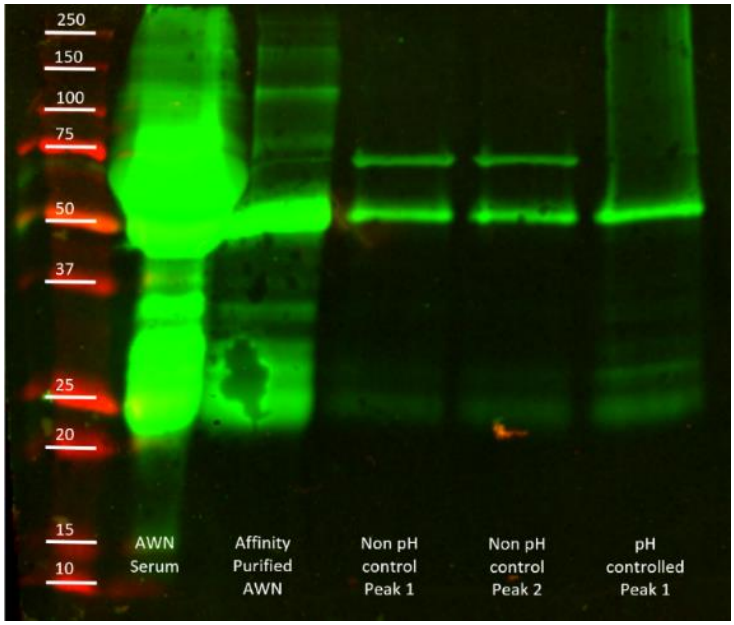

Figure 40. Western Blot. Well 1 shows M.W. marker, well 2 shows WNVA in serum, well 3 shows affinity purified WNVA, well 4 and 5 show non pH control peak 1 and 2 respectively, well 6 shows pH controlled peak 1.

4.3.2 Antibody Preservation

Positive ELISA reading, as determined by negative and positive controls, was set to an optical density (O.D.) cutoff value of 0.07 and a maximum of 2.93. ELISA cutoff was determined by the average of 6 negatives samples plus 3 times the standard deviation (XD + 3SD). The supernatant from the centrifuge tubes was used to determine if antibody remained active after purification with MIPs. A positive reading from supernatants was found from both MIP samples. Where MIP sample 1 had the highest antibody activity with an O.D. of 2.7 and MIP sample, 2 had an O.D. of 0.324. The lower reading from sample 2 was due to the antibody being left in the acidic elution buffer, thus partially inhibiting antibodies in it. Finally, the NIP sample had WNVA with an O.D. of 0.25, where washing was not sufficient to remove all nonspecific bound proteins from the particles.

4.3.3 MIP performance

Images of MIPs and NIPs exposed to WNVA are shown in figure 41. The higher brightness of MIPs with WNVA indicate preferential binding to MIPs against NIPs. Furthermore, image 42 compares MIPs in BSA and BGG, where green fluorescence of MIPs was barely observable; thus MIPs absorb nonspecifically marginal amounts of protein.

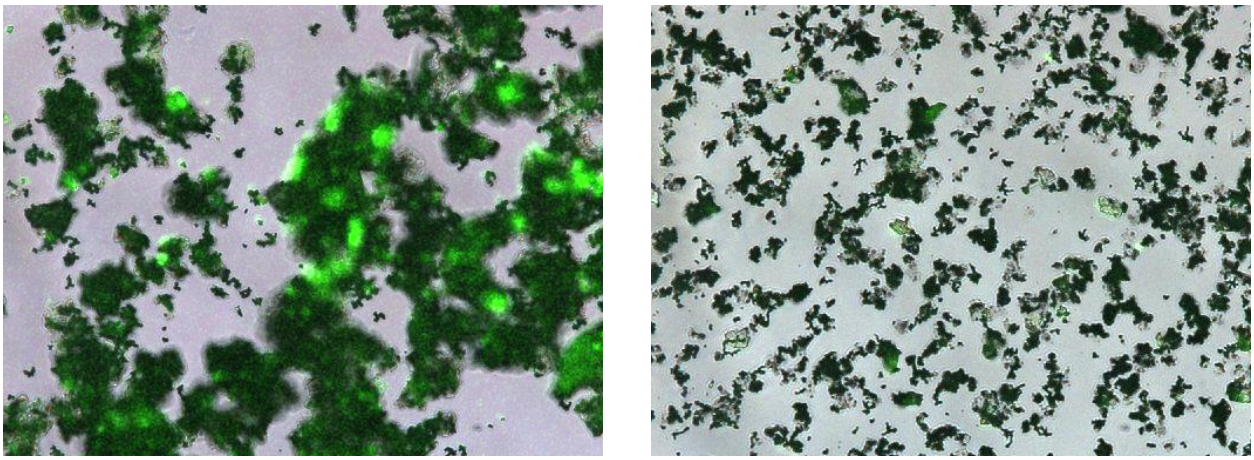

Figure 41. A) MIPs with WNVA, B) NIPs with WNVA

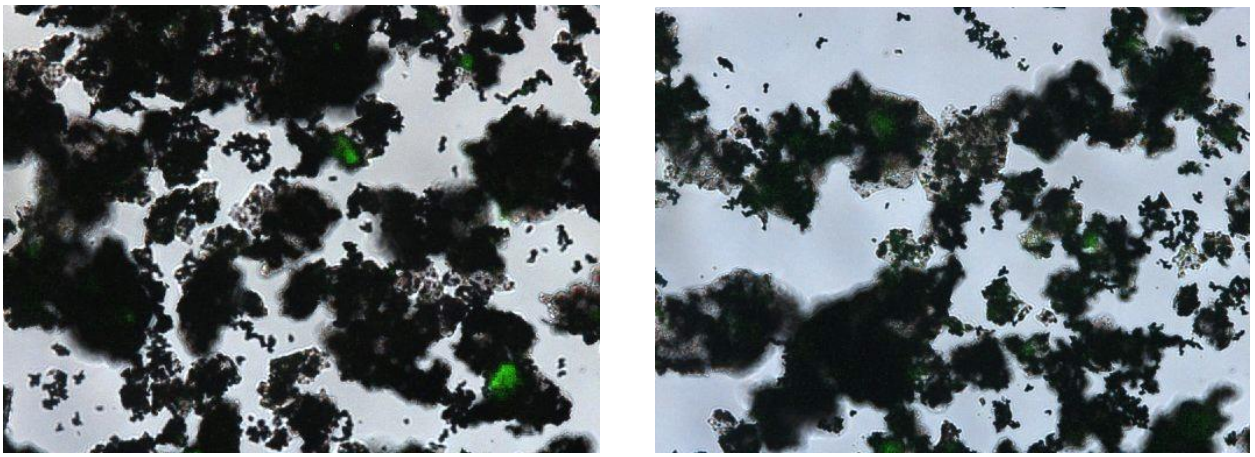

Figure 42. A) MIPs in BSA, B) MIPs in BGG

The average intensity of green fluorescence in MIPs bound to WNVA was 11 times higher than of NIPs. MIPs absorption of WNVA was 23 times higher than that of BSA or BGG. Figure 43 compares the average green fluorescent intensity of MIPs and NIPs with WNVA, BSA or BGG. There was no statistically significant difference between any of the controls in MIPs or NIPs, while the confidence interval was greater than 99.8 % for any control and MIPs in the presence of WNVA.

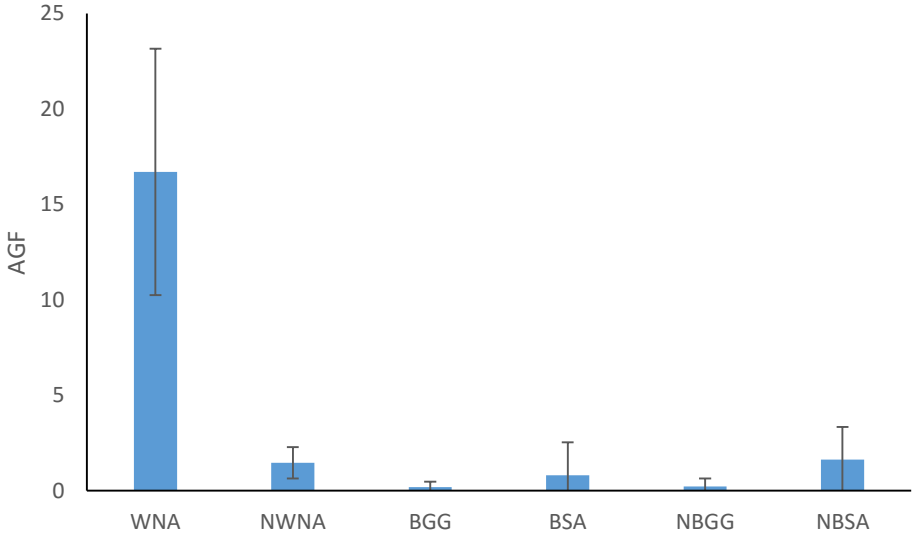

Figure 43. MIPs average intensity of WNVA vs controls.

Synthesis temperature studies showed better performance at room temperature synthesis, while synthesis at below 0º celsius showed no difference from room temperature; this was most

likely due to the reaction continued once the solution was brought back to room temperature. Figure 44 shows a room temperature average intensity of 19.4 A.U. with an SD of 4.8, while cold temperature had the worst performance at 10.95 A.U. with an SD of 3.3. Unfortunately, the confidence value was low at 86% since the test only had duplicate data. Because of this, data is significant but not statistically.

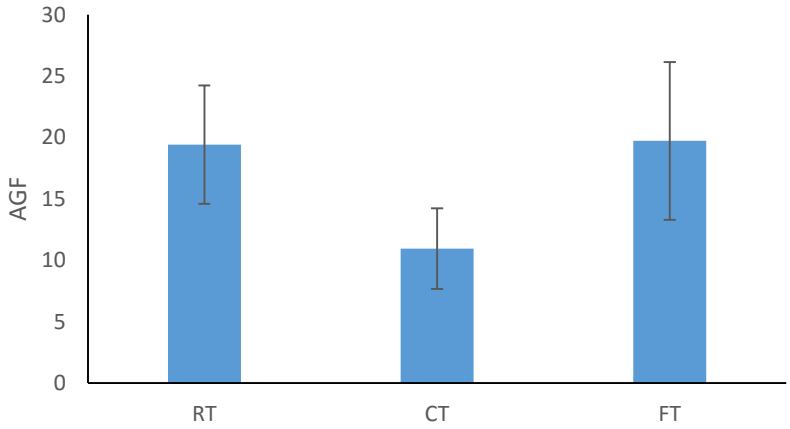

Figure 44. Synthesis temperature comparison by average intensity.

Rebinding of template showed improved performance when carried at cold temperatures with an average intensity of 21.5 A.U. with an SD of 5.2, while room temperature had an average intensity of 11.8 A.U. with an SD of 3.0 A.U, the confidence interval was 96%; data is shown below in figure 45. The increased binding of template correlates well with antibodies, where they show improved antigen affinities when used at 4-7º C.

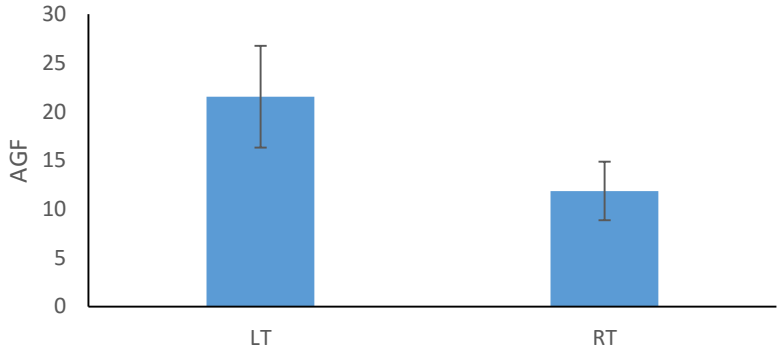

Figure 45. Binding temperature comparison by average intensity.

4.4 Conclusion

To improve our molecular imprinting formula, physiological pH matching was applied. Although synthesis of particles is not favored at neutral solutions, it was possible to create MIPs by the neutralization of the pH after a brief period of monomer activation in an acidic environment. Once pH was neutralized template was added, and MIP synthesis continued. Before pH matching, protein absorbance was low, and imprinting was not always successful. By devising a method that allowed synthesis of particles at physiological pH, higher capture ratios and imprinting success greatly increased.

During pH matching, adjusting the solution's pH proved difficult as no buffer was used. Thus, the solution became highly basic or acidic at times. If solution exceeded the tolerance values 7.4 ±0.4, particles had to be discarded. Improving pH adjustment requires the use of a strong buffer to increase the repeatability of molecular imprinting formula. Currently, our imprinting success rate is approximately 70% due to errors during pH adjustment.

Finally, fluorescently tagged West Nile antibodies showed 11 times higher fluorescence on pH matched MIPs when compared to NIPs with the same template. Also, MIP particles were 23 times more selective for West Nile antibody than BSA or BGG. MIP optimizations included synthesis at room temperature and template rebinding at 4-7ºC. MIPs showed high specificity but low sensitivity, due to the requirement of sample washing after incubation, in which most non-specifically bound proteins were removed at the cost of some sample loss.

Chapter 5. Complementary Charge Matched MIPs

5.1 Introduction

MIPs, when used in solvents, are capable of strongly recognizing molecules via short range interactions. The recognition of antigen occurs with ease as there is no need to displace hydration layers and there is no ionic competition. Whereas, in aqueous solutions, long range interactions are reduced due to ionic competition, and water molecules mask recognition sites. Short range interactions cannot occur until the displacement of water molecules is achieved; hence a stable bind is not possible. Particularly, natural antibodies are capable of displacing hydration layers through their use of long range interaction; thus, they play a crucial role in aqueous molecular imprinting. It is hypothesized that due to weak long range interactions MIPs have not been widely successful.

To optimize long range interactions, ionic and hydrophobic monomer charges can be counter matched to the charges of the template. When active monomer ratios are not optimized, stereo complementarity might not be achieved due to the charge imbalance during MIP synthesis. To select active monomers ratios, crystallographic data was downloaded from the molecular modeling database (MMDB) from the National Center for Biotechnology Information (NCBI). The crystal structure of WNVA was downloaded and rendered with Cn3D [124] (database identifier: 3N9G). All hydrophobic and charged amino acids on the surface of the Fab fragment of WNVA were counted and countered matched with the respective active monomers.

Since the use of MIPs is intended for low resource areas, a portable POC device is necessary to access as many medically underserved areas as possible. Nonetheless, it is not possible to develop a device that matches the ASSURED criteria initially. Nonetheless, a minimum viable product can be developed for use in low resource hospitals. By reducing design criteria, a product can be developed in a timely and efficient manner. MIPs can later be developed into portable POC devices, once the technology further improves. Particularly, it is convenient to develop a straightforward and familiar MIP assay which can be used in common health

laboratories in low resources. By simplifying the assay process, the required training and expenses are reduced. Particularly, our testing methods for the evaluation of MIPs are not compatible with standard diagnostic methods. Thus, an ELISA like assay was developed.

5.2 Materials and Methods

5.2.1 Materials

Tetraethyl orthosilicate (TEOS), 3-aminopropyl triethoxysilane (APS), and phosphate buffered saline as tablets were obtained from Sigma-Aldrich (St. Louis, MO), reagent grade and used as is. Hydrochloric acid (37%), anhydrous acetic acid, and ethanol were acquired from Fisher Scientific (Pittsburgh, PA), reagent grade. Succinic anhydride, octyl trichlorosilane (OTS), ammonium hydroxide, sodium hydroxide were obtained from Sigma-Aldrich. Polymer grafted carbon black was obtained by collecting ink from HP 33 cartridges (HP, Palo Alto, CA). According to literature, carbon black are 15 nm carbon particles[121], [122] grafted with 2-Pyrrolidone [123]. 4-(2-Hydroxyethyl)piperazin-1-ylethanesulphonic acid (HEPES) was purchased from VWR, reagent grade. Green UV dye was purchased from CARQUEST, catalog # 376CS which is comprised of a dinapthoperylene-9, 18-dione, dodecyl derivative.

WNVA was prepared in mice as hyper immune mouse ascitic fluid (HMAF) [118] and prepared as described in section 4.1.2. Bovine serum albumin (BSA) and bovine gamma globulin (BGG) standard ampules were obtained from Thermo Scientific (Waltham, MA). Polymer grafted carbon black was obtained by collecting ink from HP 33 cartridges (HP, Palo Alto, CA). Ultrapure water was obtained from a Milli-Q Millipore unit with a water quality of 18.2 MΩ. Elution buffer was made in DI water with 0.15 M NaCl and 0.5 % acetic acid with a pH of 3.5. HEPES buffer was made with 25 mM of HEPES and 125 mM of NaCl at a pH of 7.4 in DI water. Thermo scientific Orion 3 Star and 2 Star benchtop pH meters were used to determine pH in buffers and vial 1 respectively. Biomate 3 UV-Vis spectrophotometer was used for protein determination with BCA and micro BCA kits from Fisher Scientific.

5.2.2 Preparation of cAPS

Previously made carboxybutyl 3-amidepropyl triethoxysilane (cAPS) was used as prepared in section 5.1.2, with the addition of diluting the reagent to a final volume of 15 ml with ethanol. Dilution facilitated its use with pipettes. The solution was then left in a rotisserie overnight.

5.2.3 Buffer optimizations and active monomer titrations

Previously, the adjustment of pH proved difficult due to the absence of a buffer. To estimate the required buffer capacity and improve the pH stability of the synthesis, APS and cAPS monomers were individually titrated in PBS and HEPES buffers with acid or base as required. Afterwards, 200 µl of cAPS was added to 587 µl of HEPES buffer. The solution was then slowly titrated with APS until it reached a pH above 9. This study estimates whether acid or base will be required for neutralizing the synthesis pH with a given active monomer formulation.

5.2.4 Synthesis of charged matched molecular imprinted particles

Following previous optimizations, molecular imprinting was carried out in HEPES buffer, rather than 0.03 M NaCl solution. Furthermore, active monomer ratios were updated to counter match charges present on WNVA. Counter matching was achieved by downloading crystallographic data available from the molecular modeling database (MMDB) from the National Center for Biotechnology Information (NCBI). Particularly, the Fab crystal structure of West Nile antibodies [124] was downloaded and rendered using Cn3D. All hydrophobic and charged amino acids on the surface of the Fab fragment of WNVA were counted and countered matched with active monomers.

Figure 46 shows the Fab WNVA crystal structure, where a total of 303 neutral, 42 positive, 28 negative, and 39 (I, L, V) hydrophobic amino acids were counted. For positive and negatively charged amino acids, ratios were determined with a charged monomer: amino acid of 1:1 ratio. While, hydrophobic amino acids to hydrophobic monomer ratio needs to be determined experimentally, as not all hydrophobic amino acids are exposed in WNVA. Finally, neutral amino

acids were counted, and total molecular weight (M.W.) was determined. Afterward, the total M.W. was divided by the neutral amino acid count, thus obtaining an average molecular weight per neutral amino acid. Then, the M.W. of TEOS is divided by the average M.W. of neutral amino acids and the TEOS:amino acid ratio of 1:1.77 was determined.

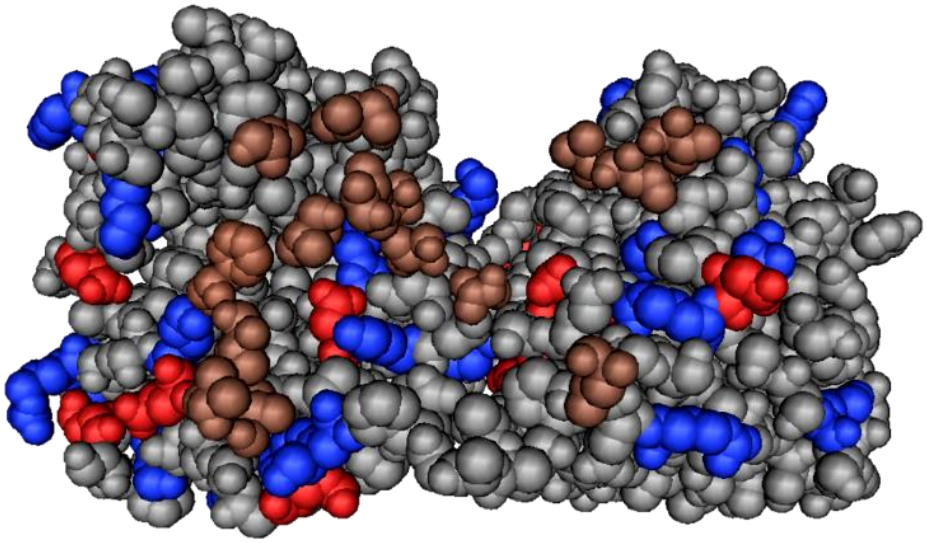

Figure 46. Crystallographic rendering of Fab section of WNVA. Amino acid charge as colored red negative, blue positive, and brown hydrophobic [124].

With the positive and negative amino acid ratios determined, imprinted silica particles were prepared by a modified sol-gel method. Synthesis of imprinted particles was a four-step procedure, where the order of reagents was imperative. Briefly, for every 146 µl of TEOS, we used the following volumetric amounts of active monomers: 7.5 µl of APS, 36 µl of cAPS, and 32 µl of OTS. First, the active monomers were mixed in a centrifuge tube labeled as vial 1. Then, 365 µl of 0.03 M NaCl, 146 µl of TEOS, the full contents of vial 1, and 31 µl of black ink were added and mixed by pipetting in a new centrifuge tube labeled as vial 2. Afterward, a pH electrode was used to measure the solution's pH. The measurement was possible by inserting the electrode's tip in the centrifuge tube and inverting the tube to cover the electrode's tip. The solution's pH was then adjusted as necessary by adding HCl or NH4OH until a pH of 7.35 with a tolerance of ±0.25 was achieved.

Once pH was adjusted, 441 µl of purified WNVA, as the template, was added at a concentration of 200 µg/ml to the MIP solution. The solution was then pipette mixed and left overnight in a rotisserie. As a comparative, molecularly imprinted particles were synthesized as above except the solution's pH was not adjusted. As a negative control, non-imprinted particles (NIPs) were synthesized under identical conditions except 1X PBS buffer was used instead of the template.

Unreacted monomers were removed by centrifuging all particles at 3200 g's for 5 minutes; the supernatant was discarded. Then, the template was removed by adding 1.2 ml of elution buffer to particles and resuspending them in a vortex mixer. If large particles were noted, these were disrupted by sonication for 1 minute in a Branson 2510 sonicator. Afterward, particles were centrifuged, and the elution process was repeated once more. Any remaining template was removed by resuspending particles in 1.2 ml of a 50% v/v mixture of glacial acetic acid and methanol for one hour. The particles were then centrifuged, and the supernatant was discarded. Afterward, the particles were washed in 1X PBS in triplicate and resuspended in a final volume of 400 µl. No trace of protein could be detected in the last 1X PBS supernatant collected; a micro BCA kit was used for detecting proteins. Finally, particles were stored as is at room temperature for later use. After particle washing, a total of 7 ± 2.45 mg of micro particles were collected per batch.

5.2.5 OTS ratio determination

With the updated APS and cAPS monomer ratios, it was necessary to manually test the required hydrophobic ratio. Testing was required as not all hydrophobic monomers are exposed in WNVA. Thus, the volume of OTS used was adjusted by 20% increments, from 20% to 100%. The following volumes were used: 32 µl, 25 µl, 19 µl, 12 µl, and 6.5 µl; a total of 15 particles were tested. Particles were then incubated with 100 µl of WNVA at a concentration of 146 µg/ml, for a total of 4 hours in a rotisserie. Afterward, the supernatant was collected, and total bound protein

to MIPs was calculated. Particles with the highest protein absorption were used to update the synthesis formula. For all other tests, MIPs were synthesized with the optimal OTS ratio.

5.2.6 Characterization

To visualize MIPs, 10 µl of particles were pipetted directly to a 12 mm carbon conductive tab. Particles were then lightly coated with 80:20 gold/palladium target in a sputter coater. Images were obtained using a Hitachi S-4800 SEM. Zeta potential was calculated with a ZS90 Malvern zetasizer, were a 50 µl sample was suspended in 1X PBS and diluted as needed by the equipment in a folded capillary cell (DTS1070), determined by total particle count. A total of 6 MIPs, produced in different batches, were compared. Each particle test was compared with at least 12 runs, as determined automatically by the equipment.

5.2.7 Binding Isotherm

Vials of approximately 315 µg of MIPs, in 240 µl of 0.03 M NaCl solution were loaded with nominal amounts of antibody as follows: 1.2, 2.4, 3.6, 4.8, 6.1, 12.6, 19, 25.2, 30, 48, 70, and 95 µg of antibody per mg of MIP. The exact antibody concentration was determined by micro-BCA, while the particle concentrations were calculated by UV-Vis (610nm). A calibration curve was obtained by measuring the absorbance of precisely weighted particles. The vials were then left overnight in a rotisserie to reach equilibrium. Particles were then centrifuged at 3200 RCF for 5 minutes, the supernatant was collected and a micro BCA assay conducted. The centrifugation did not eliminate all particles from the suspension, and therefore absorbance readings at 610 nm of control MIPs/NIPs without antibody were obtained, and those baseline absorbances were subtracted from data. The free ligand concentration was calculated as the difference of the protein concentration in the supernatant after equilibrium and the baseline absorbance of virgin particles, normalized by volume of the samples. The bound ligand concentration was determined by the difference of the total antibody mass added to the particles less the free ligand mass, normalized by the mass of particles.

5.2.8 Particle immunoassay study

To determine if MIPs can be used as a fluorescent immunoassay, a high protein bind 96 well plate was loaded with antigen. Briefly, the 96 well plate was loaded with 10 µg of purified WNVA in 100 µl of HEPES buffer in 9 wells, as positive test. Afterward, 10 µg of BSA was added to 100 µl of HEPES buffer in 9 wells, as negative test. The process was repeated with 10 µg of BGG in 9 wells, as false positive test. The well plate was then left overnight in an incubator at 37°C.

Concurrently, MIPs were prepared as previously described but with the addition of 50 µl of dinapthoperylene-9, 18-dione, dodecyl derivative, as green UV dye, to vial 2. After synthesis, a total of 3 MIP solutions were made. Solution 1 was made by suspending 200 µl of MIPs in 800 µl of HEPES buffer. Solution 2 was made by suspending 200 µl of MIPs in an 800 µl solution of 0.01% (w/v) Tween 20 in HEPES buffer. Finally solution 3 was made by suspending 200 µl of MIPs in an 800 µl solution of 0.005% BSA (w/v) in HEPES buffer. The solutions were then left incubating in their centrifuge tubes.

After the 96 well plate was incubated overnight, it was washed with 200 µl of 0.01 % (w/v) Tween 20 in HEPES buffer a total of 3 times, per well. Once the well plate was washed, MIPs in solution 1 were added to 3 wells containing BSA, 3 wells containing BGG, and 3 wells containing purified WNVA. The process was then repeated for MIPs in solution 2 and 3 in their respective wells. The 96 well plate was then incubated again for 2 hours and washed 5 times with 0.01 % (w/v) Tween 20 in 1X HEPES. Finally, the well plate was viewed in an Olympus IX71 inverted microscope with a 4X objective and a UV exciter filter-green barrier filter block. A total of 27 images were obtained, particles were counted using color threshold and analyze particle filters using Fiji software.

5.3 Results and Discussion

5.3.1 Formulation updates

In figure 47, the titration of cAPS with APS showed a linear rise from a pH of 3.6 to 6; afterwards, pH increased exponentially. Thus, depending on the APS/cAPS ratio required for a given template, synthesis pH will vary from 3.6 to 9. Particularly, our charge matched formula for WNVA requires an APS/cAPS ratio of 0.21. Hence, synthesis of MIPs will occur at an approximate pH of 6.8, requiring adjustment with NH4OH.

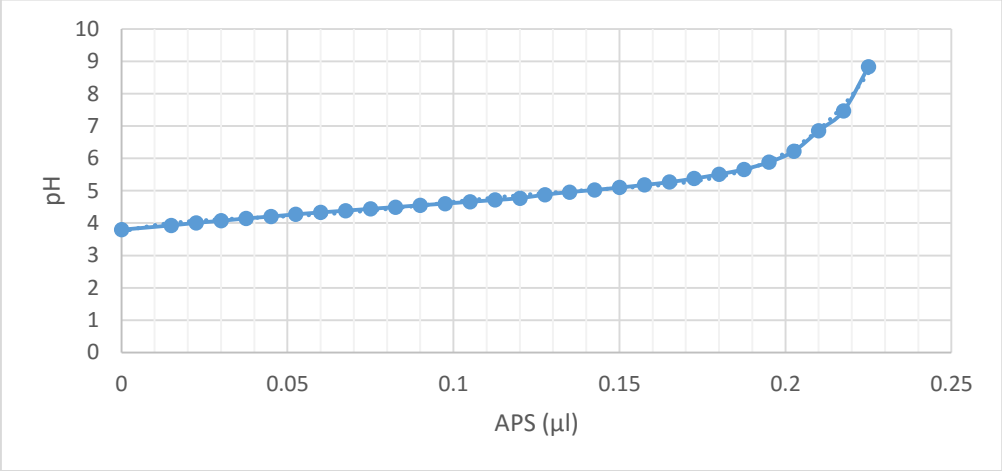

Figure 47. Titration of cAPS with APS, per µl of cAPS.

The hydrophobic amino acid to hydrophobic monomer ratio is plotted in figure 48. MIPs captured more WNVA when 60% of hydrophobic monomers were used. Thus, a 5 aminoacid to 3 monomer ratio was used. The OTS volume was adjusted to 19 µl. From this point forward, all particles were synthesized using this volume.

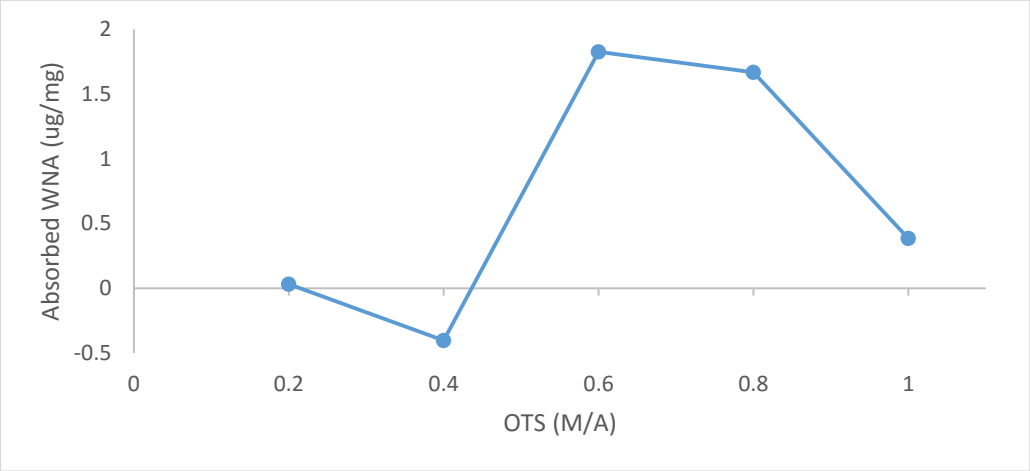

Figure 48. OTS ratio of OTS monomers to hydrophobic aminoacids.

Figures 49 and 50 show the titration of active monomers in DI water, where pH increased exponentially at 6, for cAPS. This clarified the reason why pH adjustment is difficult at 7.4. Finally, the titration of cAPS and APS with NaOH and HCl respectively in PBS, demonstrated that PBS buffer has not enough buffer capacity, even when the concentration was doubled. However, when both monomers were titrated with 2X HEPES, buffer demonstrated sufficient buffer capacity for the adjustment of pH at 7.4. Thus, the pH tolerance of synthesis MIPs was reduced to 7.35 ± 0.25 from a previous tolerance of 7.4 ± 0.4.

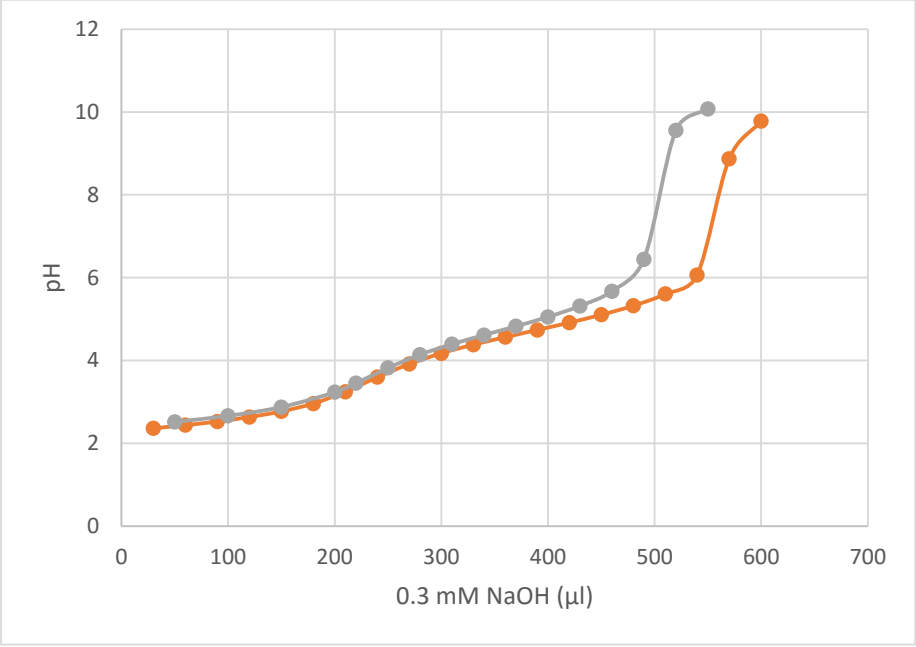

Figure 49. Titration of cAPS in DI water

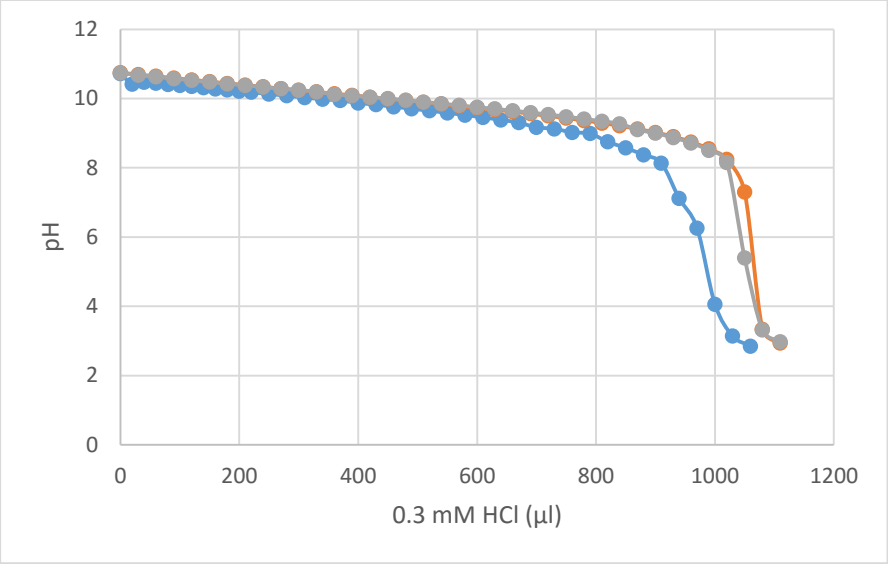

Figure 50. Titration of APS in DI water.

5.3.2 Particle characterization

The zeta potential of MIPs is shown in figure 51, where the full report is available in appendix A.1. Particularly, MIPs had a broad size and charge distribution, where the average zeta potential was -14.9 mV with an SD value of 15.6 mV.

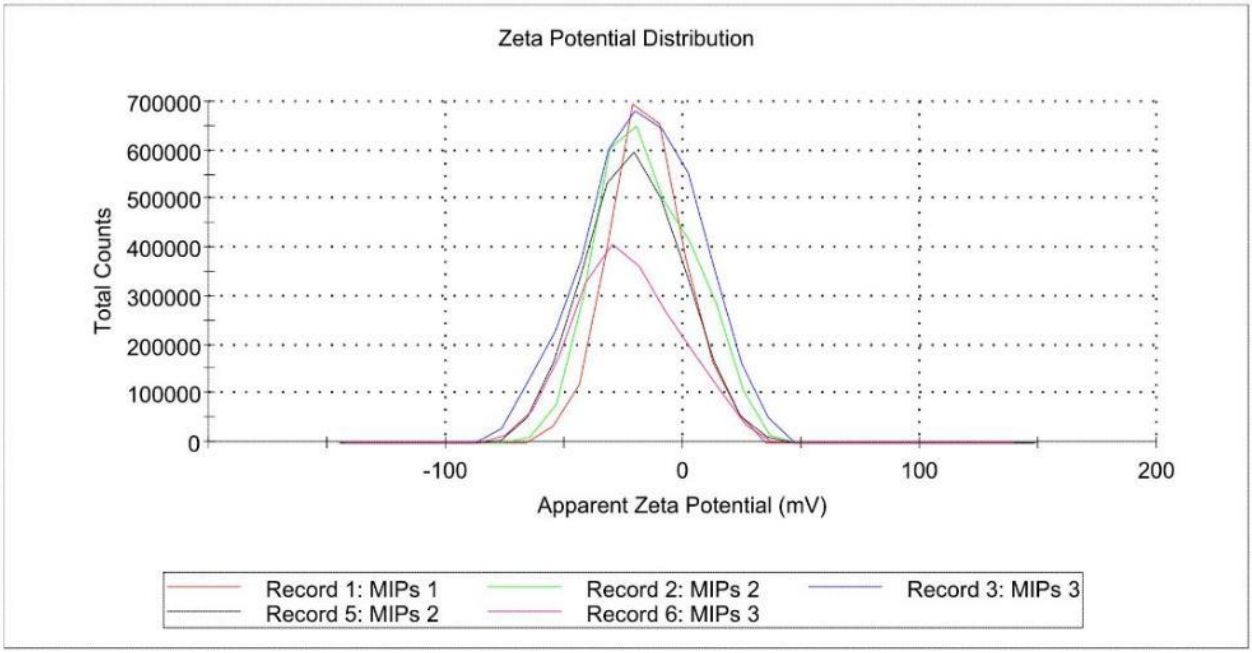

Figure 51. Zeta potential distribution profile.

Since MIP synthesis has been considerably updated, new SEM images were taken. Figure 52 shows the MIPs primary particle size at approximately 4 nm; deviation is not possible to calculate as particle size is at the limit of detection of the equipment. The average size of the aggregated particles was approximately 400 ± 200 nm as shown in figures 53. Finally, the circularity of MIPs showed improvement from previous formulations.

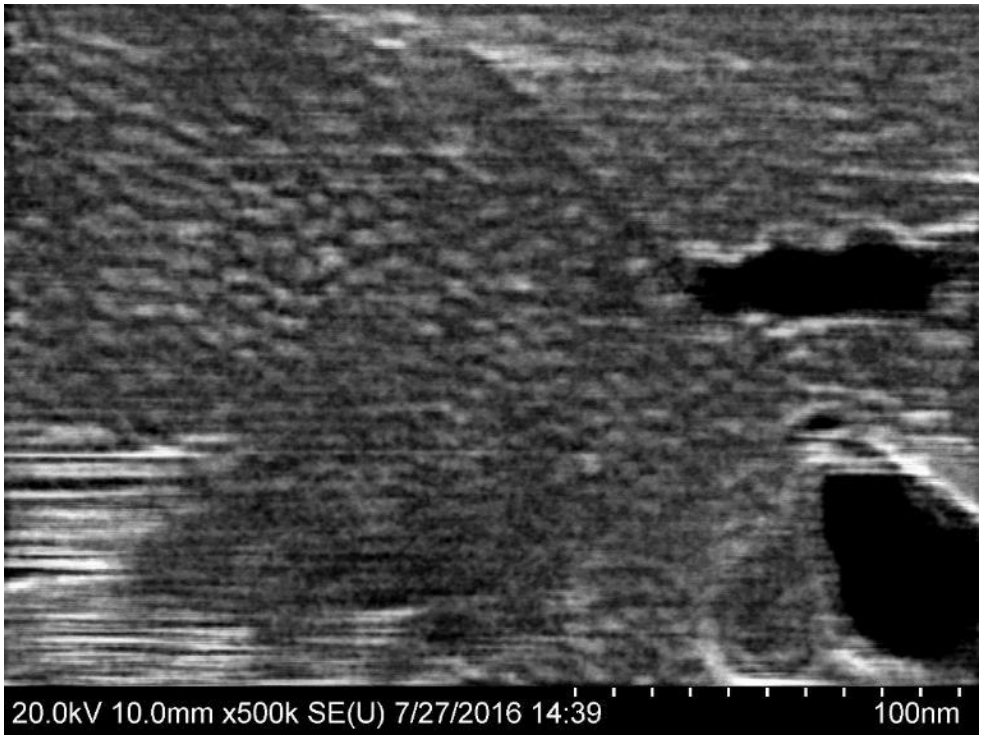

Figure 52. SEM image of MIPs at 500,000X.

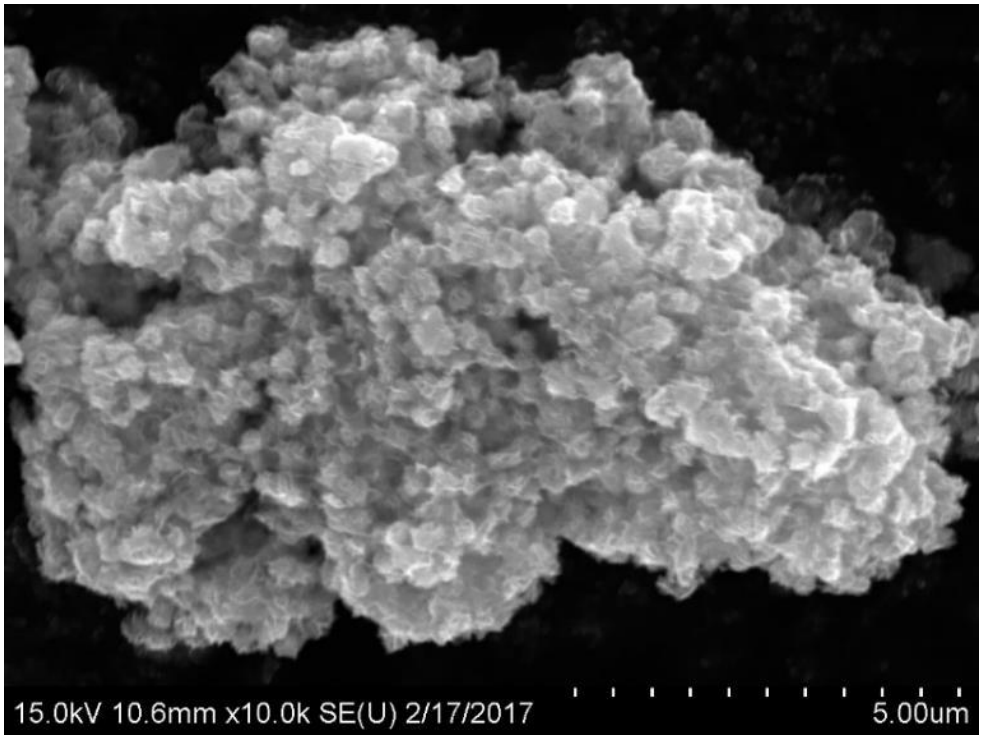

Figure 53. SEM image of MIPS at 10,000X.

5.3.3 Binding Isotherm

For the binding isotherms, a total of 96 samples were obtained. Results are shown in figure 54. Where NIPs prepared with and without pH control, absorbed about the same amount of WNVA or 28 μg/mg. MIPs prepared without pH control adsorbed approximately 48 μg antibody per mg MIP, a statistically different amount. MIPs prepared with pH control adsorbed approximately 80 μg antibody per mg MIP. Hence, 160% more binding sites are available using the pH controlled process, when compared to non pH MIPs. PH controlled MIPs bound 52 μg/mg of West Nile specifically, with a nonspecific binding capacity of 28 μg/mg. Because of this, MIPs most likely will require blocking before their use. A dissociation constant of Kd=57.45 μM was calculated. Although it is difficult to compare the dissociation constants of MIPS to that of antibodies, which range from micro molar to nano molar, partly due to the large particle size distribution of the MIPs.

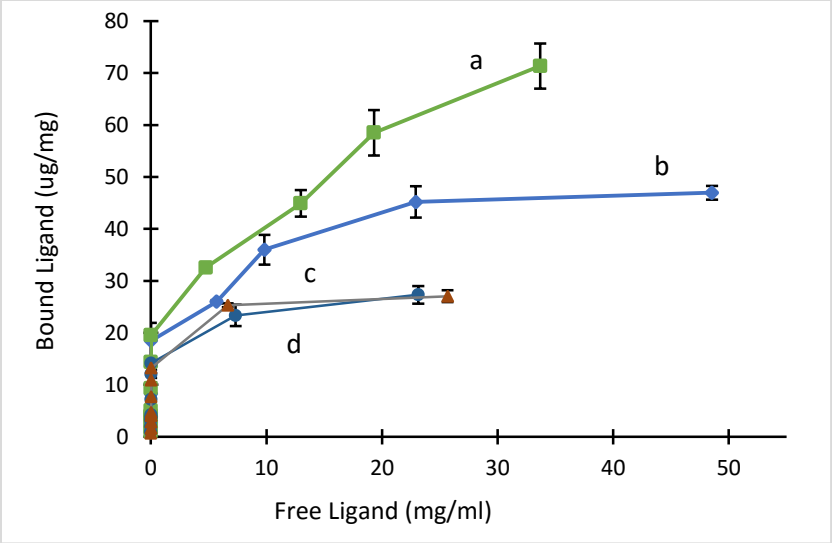

Figure 54. Binding isotherm of a) pH controlled MIPs, b) non pH controlled MIPs, c) pH controlled NIPs, d) non pH controlled NIPs

5.3.4 Fluorescently Labeled MIPs as Immunoassay

The objective of fluorescently labeled MIPs was to determine if they could be used similarly as an ELISA test. Particularly, in the 96 well plate, positive wells typically retained higher number of fluorescent MIPs whereas negative wells barely bound any particles. Interestingly, blocking MIPs with tween 20 dramatically increased background signal, essentially eliminating the recognition capabilities of MIPs. Specifically, tween 20 blocked particles had a p value of 0.38. Most importantly, BSA blocked MIPs showed a similar average of bound particles in positive samples over unblocked MIPs, with a p value of 0.33. However, BSA blocked particles had narrower deviation standards, where BSA blocked MIPs had a p value of 0.0019 against negative controls, while unblocked MIPs had a p value of 0.05. Finally, the average MIPs selectivity factor was 9.5. Figure 55, shows bound BSA blocked MIPs in a positive and negative well. The results of the 96 well plate are summarized in figure 56.

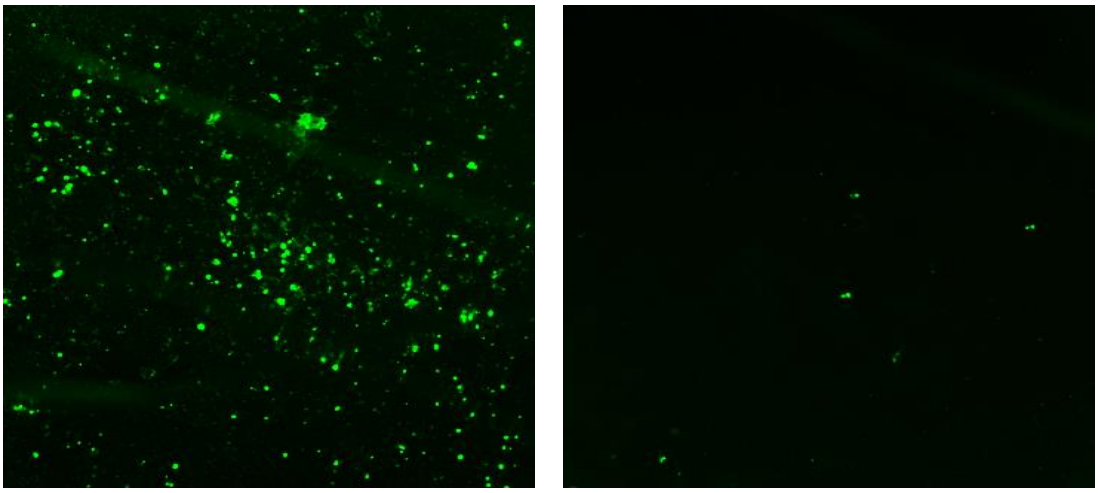

Figure 55. Fluorescently labeled MIPs. Left, positive WNVA sample. Right, negative BSA sample

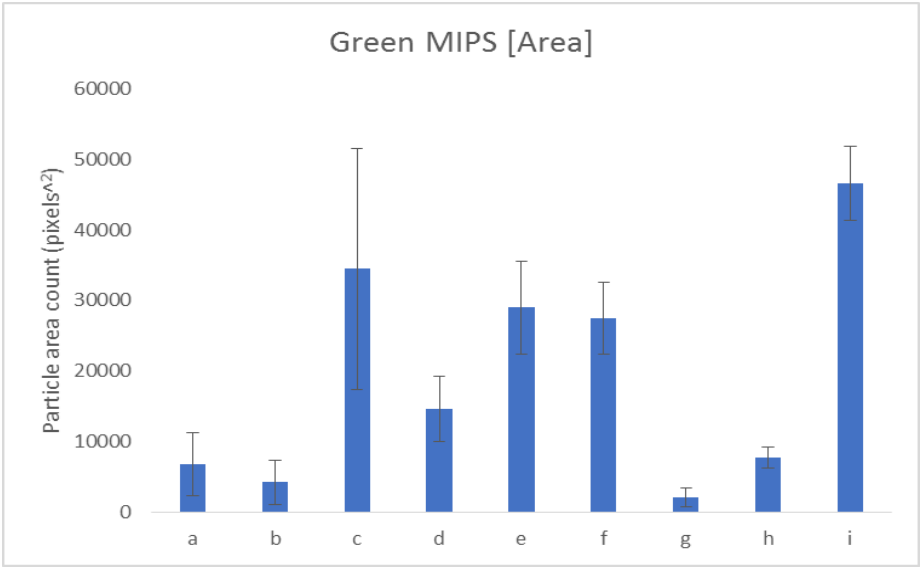

Figure 56. Fluorescently labeled MIPs immunoassay, total particles area count. Wells a-c where incubated with unblocked MIPs in wells loaded with a) BSA, b) BGG, and c) WNVA. Wells d-f were incubated with tween 20 blocked MIPs in wells loaded with d) BSA, e) BGG, and f) WNVA. Wells g-i were incubated with BSA blocked MIPs in wells loaded with g) BSA, h) BGG, and i) WNVA.

5.4 Conclusion

To improve macromolecular imprinting, MIP synthesis was updated with HEPES buffer, and active monomers charge matching was added. With pH and charge matching, MIPs were developed against WNVA with 160% more binding sites against standard molecular imprinting with a specific binging capacity of 52 µg of template per mg of particles. This increase in

imprinting was due to greater preservation of the antibody template and better ionic complementarity, which is necessary for long range interactions.

The development of a fluorescently labeled assay demonstrated MIPs potential as immunoassays, where BSA blocked MIPs can be used instead of primary and secondary antibodies. The identification of a positive test was as simple as looking at a microscope. MIPs behaved reliably, and the differences between positive and negative results were evident. Because fluorescent MIPs worked similarly as an ELISA test, they may be utilized in low resource labs that are already equipped with ELISA equipment. However, the cross reactivity with other flaviviruses, such as dengue, needs to be determined in MIPs. Thus, West Nile molecular imprints have the potential of being used for sera converted patients in the identification of West Nile infections as early as 10 days post infection, when antibody titers are high.

By reducing the cost of WNV diagnostics, doctors could screen more patients with symptoms associated with WNV, reducing the amount of undiagnosed infections. Furthermore, whenever the symptoms are mild, consumers could purchase a diagnostic kit to verify and prevent the spread of WNV infections. While lower sensitivity is expected using MIPs, their robustness and affordability can deliver better diagnostic value than current technologies for WNV infections, when compared to cost prohibitive gold standard tests. However, the use of molecularly imprinted diagnostics is not intended as a standalone test, where a positive test will require sample corroboration with an additional gold standard assay if disease confirmation is required.

In medically underserved areas, MIPs can be used to initiate a specific treatment for severe infections, such as WNV, while a standard test confirms the disease. Finally, MIPs can be used to screen for WNV in recently diseased birds to increase the specificity of the DYCAST system, though detection of virus is needed rather than a seroconversion assay. This requires new WNV MIPs to be developed.

Chapter 6. Conclusions

During this research, we developed synthetic recognition molecules, which selectively recognized West Nile antibodies, a 150 kDa macromolecule. We achieved macromolecular imprinting by reviewing how antigen-antibody interactions are formed. MIPs were synthesized at physiological pH, with counter charge matching against WNVA. Charge matching was achieved by downloading the template's crystallographic data, which then was used to fine tune monomer ratios in the synthesis of MIPs. By developing artificial recognition molecules, animal dependence can be reduced; see appendix A.2.

Major improvements in MIPs increased the reliability and repeatability of the formula, which was the most common issue at the beginning of the research. During molecular imprinting in chapter 4, without pH and charge matching, MIPs behaved erratically. Particle precipitation rates changed constantly and imprinting occur rarely. It was not uncommon to get control particles precipitate faster than positive test particles, though stepped behavior in positive samples was present in most of the successfully imprinted particles. Because of the instability of agglutination assays, subsequent chapters evaluated MIPs in different manners. While precipitation of particles was discarded for this dissertation, the method merits further development as it provides with the simplest diagnostic assay available.

Chapter 5 increased the reliability and repeatability of MIPs, where usually 3 out 4 synthesized particles were successful. MIP optimizations included synthesis at room temperature and template rebinding at 4-7ºC. Most importantly, MIPs were capable of selectively recognizing WNVA against other proteins. Particularly, MIPs showed good specificity against BSA and BGG, but suffered low sensitivity of WNVA. This was due to the requirement of sample washing after incubation, in which most non-specifically bound proteins were removed at the cost of some sample loss. This suggested that MIPs may have an affinity strength lower than the affinity of common antibodies. Thus, chapter 6 required a binding isotherm study.

Chapter 6 answered remaining questions and further formula refinements were made, such as the use of 2X HEPES. By using spectrophotometry, MIPs dissociation constant was calculated at a Kd=57.45 μM. It is difficult, however, to compare the dissociation constants of MIPs to that of natural antibodies, which range from micro molar to nano molar. The comparison is difficult due to the large particle size distribution of MIPs and the heterogeneity of the binding sites per particle. Nonetheless, charge matched molecular imprinting had a 100% success rate and the signal intensity variability is that of the standard deviation shown in the binding isotherm and the ELISA like assay.

Finally, a potential method for using MIPs in standard laboratories was developed; though further research is needed to identify its sensitivity, limit of detection, selectivity, and cross reactivity. By using MIP particles in high bind well plates, identification of pathogen is possible when particles are bound to the bottom of the plate in positive samples. Currently, the test requires fluorescence to enhance the particles visibility, but further protocol refinements will allow the use of colored particles which can be seen with simple microscopes. Although still in preliminary testing, it may be possible to use black MIPs with standard microscopy, see appendix A.2.

We have successfully developed a stable MIP formula that is able to recognize WNVA selectively and repeatedly. We are confident that in order to synthesize macromolecular imprints, synthesis at physiological pH and charge matching are necessary. Furthermore, by downloading the respective crystallographic data, we were able to imprint anti-CD4, with an imprint ratio of 7.85, and BSA, imprint ratio not yet available; see appendix A.3 and A.4

With additional research, MIPs can be used to develop new diagnostic tools, which could be delivered at cost effective prices that meets or exceeds the ASSURED criteria. It is possible to develop MIPs as single use kits for individual testing and as reusable bio-sensors for humanitarian aid in medically underserved areas with extremely limited resources. MIPs can also be used for disease monitoring of animals. Despite MIPs advantages, such as the ease of manufacturing, these

are not intended to replace regular antibodies. They merely complement and expand upon current applications.

Once MIP technologies are further developed, they could be used as therapeutics. Anti-viral MIPs could be used to reduce viral loads of persistent viruses; thus reducing infectiousness and severity of the disease [125]. While, anti-antibody MIPs could be used for the treatment of autoimmune diseases, where MIPs would neutralize self-recognizing antibodies in diseases such as lupus and multiple sclerosis, all with the advantage of little to no immune response. Preliminary data indicates that silica MIPs did not induce an immune response in mice, even when adjuvants were administered; see appendix A.5. Current challenges are the particle size and distribution. Where large particles could clog arteries and small particles would diffuse from the blood stream too quickly. Hence, a safe and optimal size of particles needs to be determined before use.

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Appendix

A.1 Zeta potential report

Zeta Potential Report
v2.3

Malvern

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Sample Details

Sample Name: MIPs 1
SOP Name: zeta potential MIPBolandgroup.sop
General Notes: Dr boland group

File Name: julior.dts
Record Number: 1
Date and Time: Wednesday, August 24, 2016 4:5...
Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (mPa.s): 0.8872
Dispersant Dielectric Constant: 78.5

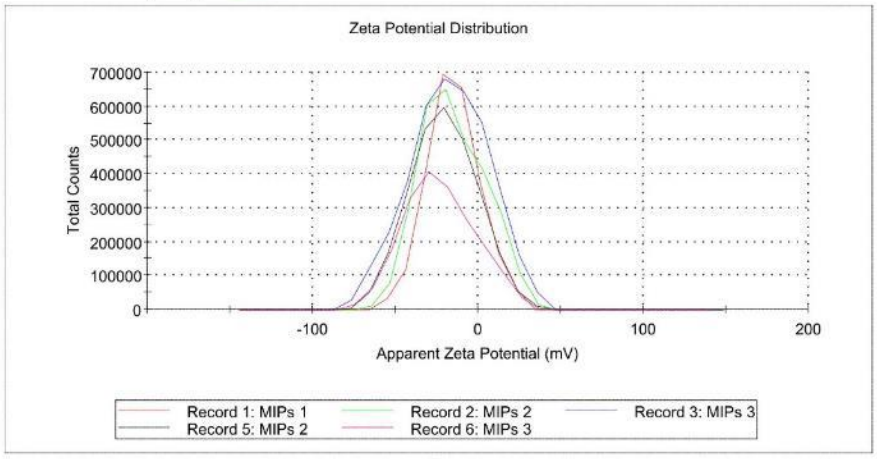
System

Temperature (°C): 25.0
Count Rate (kcps): 285.6
Cell Description: Clear disposable zeta cell
Zeta Runs: 12
Measurement Position (mm): 2.00
Attenuator: 9

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -14.9	**Peak 1:**	-14.9	100.0	15.9
Zeta Deviation (mV): 15.9	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 17.3	**Peak 3:**	0.00	0.0	0.00

Result quality : Good



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Zetasizer Ver. 7.02
Serial Number : MAL1048514

File name: 737mip.dts
Record Number: 1
24 Aug 2016 5:03:06 PM

Figure A1. Zetasizer full potential report

A.2 Molecular Imprinting Analogies

The production of natural antibodies require the use of animal models, where antigen is injected and an antibody response is made. Depending on the process, antibodies can be collected straight from animals or be further developed in vitro. Molecular imprinting has similarities with this process, where the animal model is substituted by a solution and the antigen is added directly. After synthesis, the molecularly imprinted particles are produced.

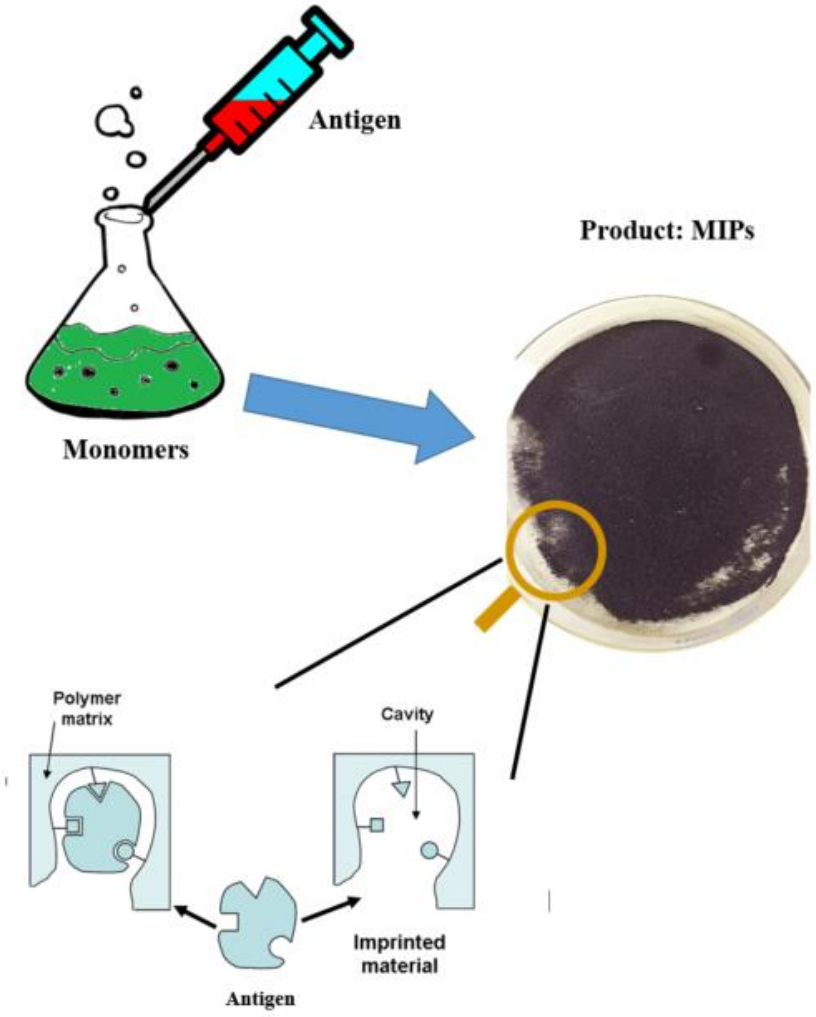

Figure A2. Simplified representation of molecular imprinting process.

A.3 BSA MIPs

MIPs were produced with BSA, then later used in a 96 well plate incubated with 20 µg of BSA as positive sample and 20 µg of BGG as negative. NIPs where tested identically.

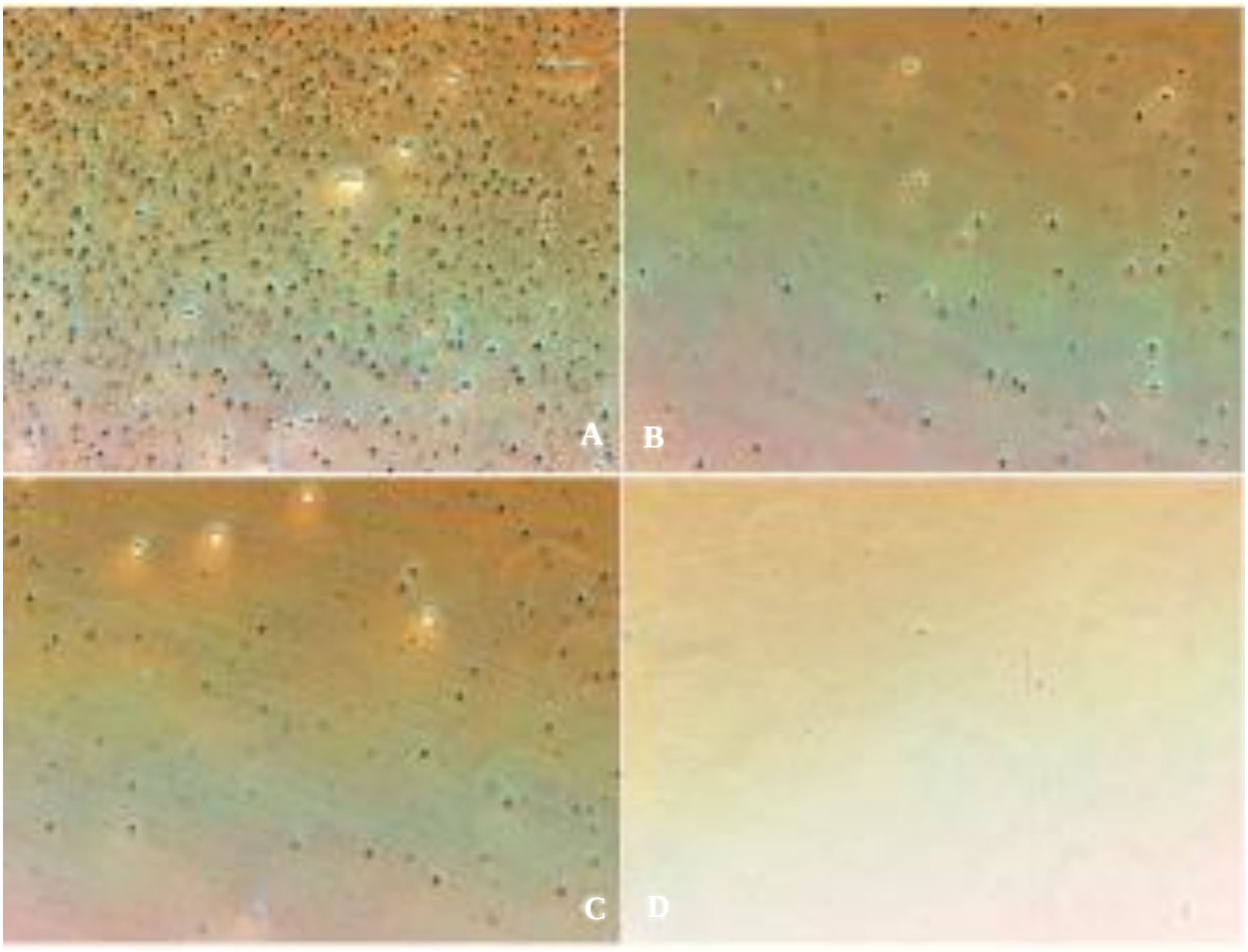

Figure A3. BSA MIPs bound to positive well (A), NIPs in BSA well (B), MIPs in BGG well (C), NIPs in BGG well (D).

A.4 CD4 MIPs

MIPs were produced with anti-CD4 as template. Afterwards, FITC labeled anti-CD4 was used as the binding molecule. The imprint ratio was measured at 7.85.

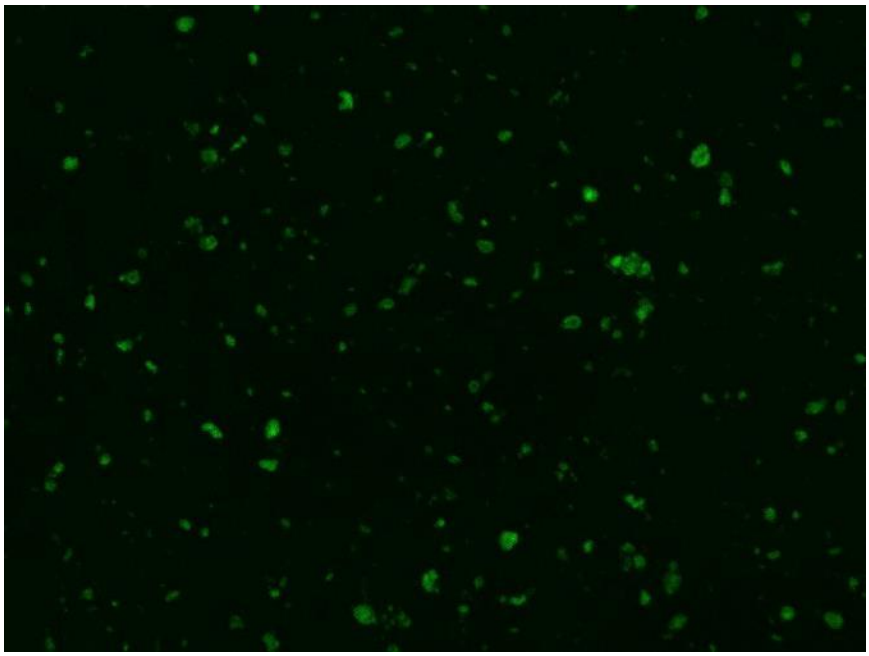

Figure A4. CD4 positive MIPs

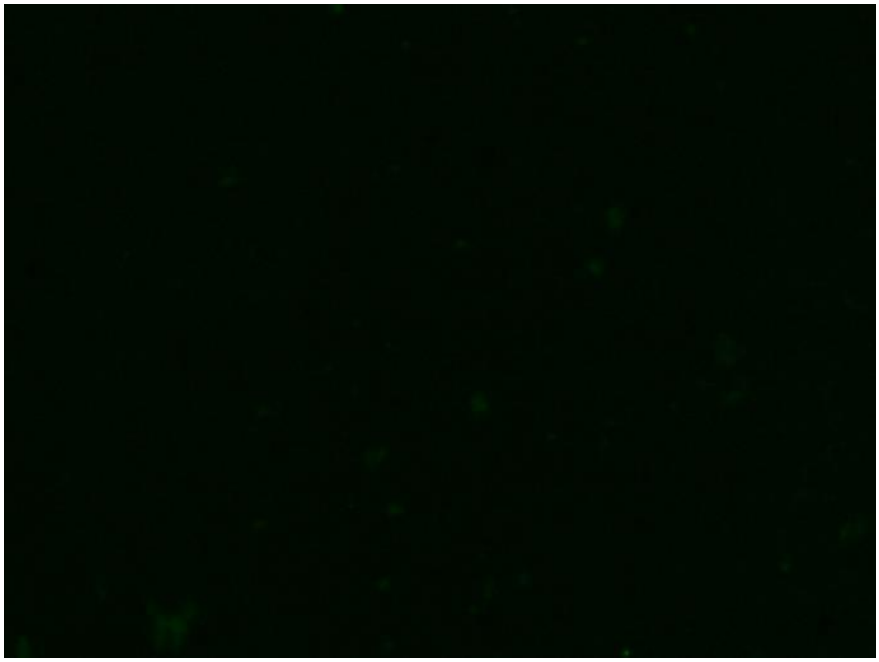

Figure A5. CD4 positive NIPs

A.5 Mice immune response to MIPs

Mice immune response to WNV MIPs. A total of 5 mice were used as controls, where 200 µl of saline solution was injected. Group 1 constituted of 5 mice and 200 µl of MIPs was injected per mice. Group 2 constituted of 5 mice and 200 µl of MIPs and 200 µl of adjuvant was injected per mice. Group 3 constituted of 5 mice and 100 µl of inactivated WNV was injected per mice. Group 4 constituted of 5 mice where 100 µl of inactivated WNV and 200 µl of MIPs was injected per mice.

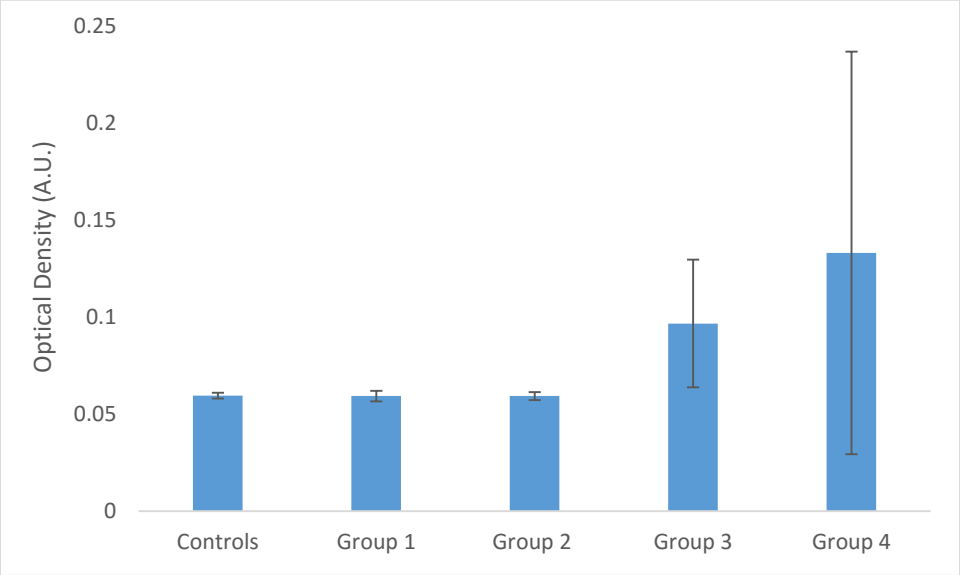

Figure A6. Mice immune response to MIPs, adjuvant, and attenuated virus.

Vita

Julio Rincon received his bachelor's degree in mechanical engineering from the University of Texas at El Paso. He then pursued his master's degree in biomedical interdisciplinary sciences, where he collaborated with the design of a skin printer which is currently licensed to Tevido Biodevices. Afterwards, he obtained his Ph.D. in biomedical engineering, where he developed molecularly imprinted polymers which are capable of selectively recognizing macromolecules, such as West Nile antibodies. Julio has presented this project at more than 10 national and international conferences and participated on the national NSF I-Corps program. Shortly after, Julio Rincon and co-founders launched MIPTek Inc., a biostartup company with a technology license of the molecularly imprinted polymers developed in the university. The founder has been awarded the MCA Innovation fund, where MIPTek Inc. will have access to advanced research facilities and funding.

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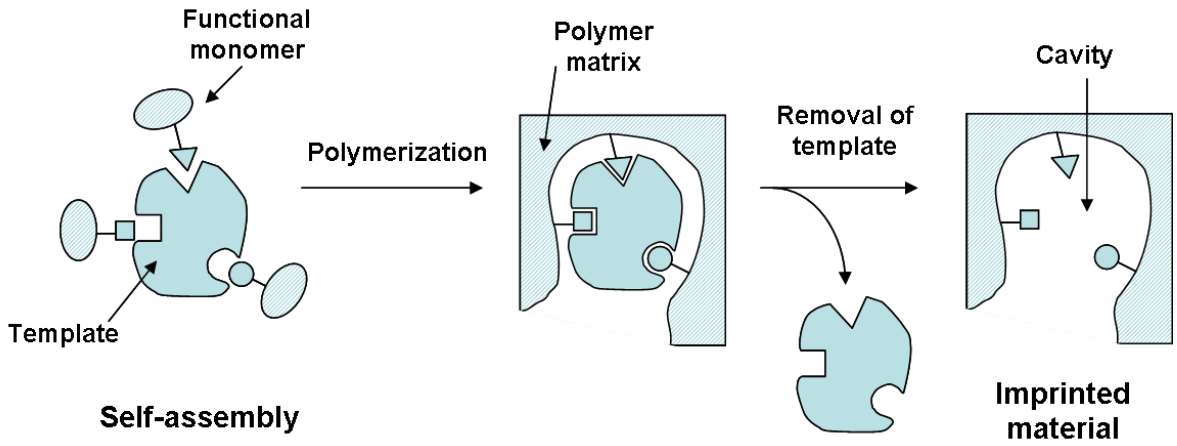

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