

ABSTRACT

Title of Dissertation: MOLECULARLY IMPRINTED POLYMERS
 FOR RECOGNITION OF TOBACCO VIRUSES

Linden D. Bolisay, Doctor of Philosophy, July 20, 2007

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The goal of this research is to elucidate the mechanism of virus recognition in molecularly imprinted polymers (MIPs) using already utilized techniques. The clinical relevance of this study relates to the development of a virus imprinted MIP, which would apply to the identification, classification, and removal of viruses. The separation of viruses and virus-like particles from various media represents an enormous challenge to the fields of medicine, healthcare, and biotechnology.

Since virus MIPs must function in aqueous environments, our approach employs a more flexible non-covalent imprinting method which starts from a readily available polymer and utilizes an aqueous environment for both MIP synthesis and testing. Crosslinked polymers imprinted against *Tobacco mosaic virus*

(TMV) via non-covalent interactions were synthesized using poly (allylamine hydrochloride) (PAA), epichlorohydrin (EPI), and TMV. The TMV imprinted polymer exhibited an increase affinity to the target virus compared to the control polymer and demonstrated a preferential affinity (imprinting factor of 2.1), based on shape, to the target virus compared to a non-target virus, *Tobacco necrosis virus* (TNV). In contrast, there was no significant increase in binding of the control polymer to either target or non-target virus.

Once it was determined that virus imprinted polymers can be successfully synthesized having preferential binding to a targeted virus, the synthesis procedure was optimized to obtain better binding characteristics to the targeted virus. Efforts were made to avoid polymer-template aggregation in the MIP pre-polymerization mixture, and determine a proper wash solution by the ability to remove the templated virus from the crosslinked polymer. TMV imprinted hydrogels were synthesized using an optimized procedure and binding test performed on these MIPs to determine binding capacity, and more importantly, imprinting factor. The highest imprinting factor of 2.3 resulted from the MIP composed of 35 % PAA at pH 7, 15 %, ethylene glycol diglycidyl ether (EGDE), and 0.4 mg/mL TMV. The TMV imprinted hydrogels exhibited a lower binding capacity to TNV than when exposed to TMV. These results show that using optimized procedures, TMV MIPs with better shape selectivity can be achieved.

MOLECULARLY IMPRINTED POLYMERS
FOR RECOGNITION OF TOBACCO VIRUSES

by

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DEDICATION

To my Mom and Dad, for their endless support throughout my life.

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First and foremost, I would like to thank my father, Manuel Pascual Bolisay, and mother, Rosalinda De Venecia Bolisay. Without their unconditional support throughout my entire life, none of my accomplishments would have been possible. Words cannot explain how grateful I am to have them as my parents. I love you both, and I see this Ph.D. degree as something "we" have earned.

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Chapter 1

Introduction

1.1 Significance

Molecular imprinting is a technique that creates synthetic materials containing highly specific receptor sites that have an affinity for a target molecule. Three-dimensional cavities are created within a polymeric matrix that is complementary to the size, shape, and functional group orientation of the target molecule. The size and shape of the cavity allow the target molecule or similar molecules to occupy the cavity space, while the functional group orientation within the cavity will bind in specific locations complementary to only the target molecule and not to similar molecules. The result is molecular imprinted polymers (MIPs) that can mimic the recognition and binding capabilities of natural biomolecules like antibodies and enzymes. MIPs have several advantages over biomolecules, such as synthesis, stability, and reusability. MIPs can be seen applied in a wide

range of technologies such as catalysis, separation and purification, drug delivery, and detection [1, 2, 3, 4, 5, 6, 7]. There remains an important need across many applications for materials that display selective and high affinity binding of biological analytes.

MIPs synthesized in this work would be applied to the removal of viruses. This is currently a very difficult task, but the need is widespread in diverse sectors such as human and animal health, crop protection, biopharmaceuticals, and biological warfare. For example, biopharmaceutical products need to be virus-free. These MIPs, when placed into a packed column and used as a purification stage, will act as virus-specific sponges and selectively capture the targeted virus while allowing other non-targeted molecules to pass through. Research in virus MIP have been done in the past [5, 6]. However, their applications are very different than the virus MIP system used in this work. Such MIP systems consist of two-dimensional surface imprinting of viruses on a crosslinked polymer surface that is attached to a sensor. When the virus template is imprinted and removed, these sensors are able to bind small amounts of the target virus on the polymer surface. Two-dimensional surface imprinting is designed to detect the presence of small amounts of viruses whereas three-dimensional imprinting can be used to bind and extract large amounts of virus from a given solution. For example, two-dimensional imprinting can be used to detect the presence of hepatitis virus in blood, whereas three-dimensional imprinting can be used to extract hepatitis virus from the

blood. Currently no work has been published on the synthesis of MIPs for large virus extraction.

The majority of work with molecular imprinting use organic monomers as their starting material and synthesis of these MIPs are carried out in organic solutions. This may be a problem with templates that are naturally found in aqueous solutions because interactions in an organic environment can be different than in an aqueous environment. Using aqueous polymer chains as the starting material and conducting the synthesis of MIPs in an aqueous environment may lead to better selectivity and binding of aqueous target molecules. A detailed study of the gel's morphology and parameters that influence imprint formation and target virus recognition is needed before MIPs can be commercially synthesized, with control of the polymer morphology, virus specificity, and virus binding capacity. The goal of this research is to synthesize an MIP with an affinity to a targeted virus and to understand the factors that influence the selectivity and specificity of the MIP. The results from this project will help develop new and more efficient methods of selecting and containing target molecules.

1.2 Background

The concept of molecular imprinting was started by Linus Pauling in the 1940's, in an attempt to explain antibody formation [8]. In his theory, an antigen serves as a template around which an antibody would bind to form a mold. Although it

was proven later that his theory was incorrect, he did start the concept of what is now called molecular imprinting. The first studies of molecular imprinting were performed by a student of Pauling's, Frank Dickey [9, 10]. Attempting to prove the Pauling's theory, Dickey successfully synthesized silica gels made in the presence of either methyl orange, ethyl orange, propyl orange, or butyl orange, and exhibited selective binding of the MIPs to their respective targeted dye molecule. Since this initial discovery, there has been a variety of work aimed at the development and refinement of molecular imprinting procedures.

Molecular imprinting is a simple, but elegant, principle of using elements of the target molecule to create its own recognition site. The first step is to create a highly crosslinked polymeric matrix around a template molecule. This template can be the targeted molecule, or a very close structural analogue. The template serves as a model around which the matrix conforms resulting in a cavity of matching size and shape, and functional groups alignment complementary to itself. The second step is the removal of the template molecule from the polymeric matrix. This is usually done by cutting or grinding the MIP into small units, then subjecting them to a series of washes to diffuse the template out of the matrix. These washes, ranging from organic solvents, acidic and basic solutions, to salt solutions, are used to break and inhibit the association of the template with the polymeric matrix and thus diffuse the template out. The result is a synthetic material that contains cavities that are able to selectively recognize

and bind to a target molecule. A schematic of the molecular imprinting process can be seen in Figure 1.1.

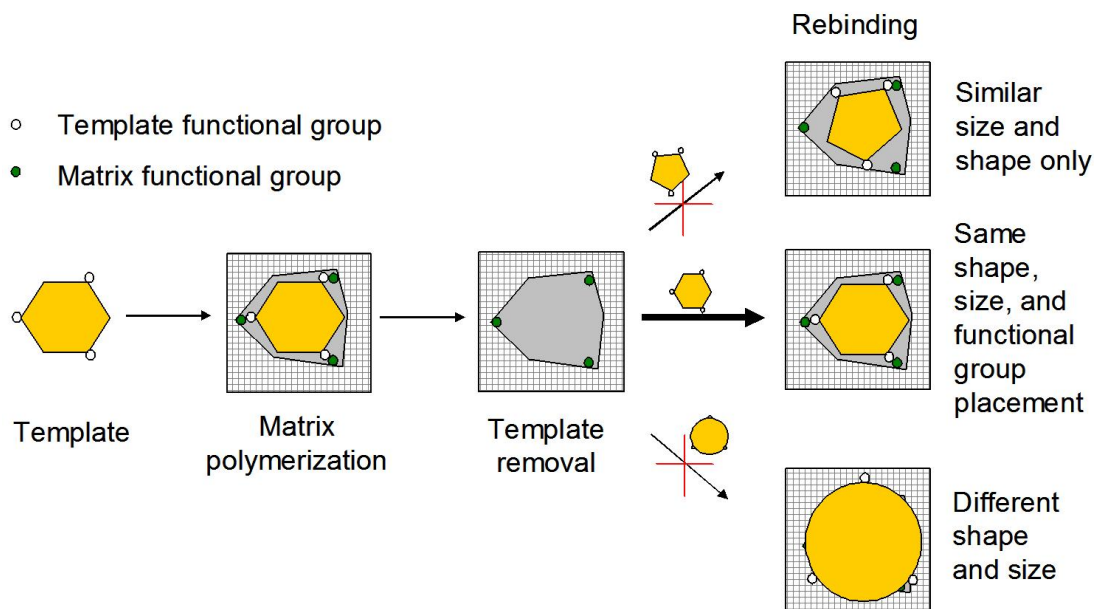


Figure 1.1: Schematic of the imprinting process.

1.3 Mechanism of molecular imprinting

Currently, the majority of MIP research uses functional monomers as the starting material [11, 12, 13]. During MIP formation, monomer units associate with the template through their respective functional groups. Usually the monomer has two functional groups, one group to interact with the template and the other group to covalently bind to other monomers. Monomers are then polymerized around the template and held in place by covalently binding with crosslinking monomers. Once the template is removed through wash steps, cavities are created

which are complementary to the shape, size, and functional group orientation of the template. The reactions are conducted in an organic solvent. A modification of this method is to use a functional monomer that is both able to associate with the template as well as crosslink with other monomers [14]. This simplifies the amount of variables needed to be considered when synthesizing a MIP such as ratio of functional monomer to crosslinker and ratio of functional monomer to template. An alternative way to make MIPs is to crosslink functional polymer chains in the presence of a template [15]. The template associates with polymer chains through their respective functional groups. A crosslinker is then added to covalently connect two polymer chains together using the remaining functional groups that have not associated with the template. Upon reaction of polymer, template, and crosslinker, the imprinted template is trapped within the three-dimensional polymer network matrix. The non-covalent association of template to the polymer matrix is disrupted using a wash to remove the template, resulting in a three-dimensional polymeric matrix containing cavities complementary to the template. A schematic of both imprinting processes can be seen in Figure 1.2. This method is currently being used in the Kofinas research group [15, 16]. Most MIP synthesis experiments are conducted in an organic solvent, especially those that use functional monomers. A problem with switching from organic to aqueous environments is that the interactions between the template and the MIP can be very different between the two systems. Removing all traces of organic molecules

used in the synthesis process, which can be deadly when exposed to living organisms, can be a difficult task. Conducting the entire synthesis and testing of MIPs in an aqueous solution will prevent these problems from occurring.

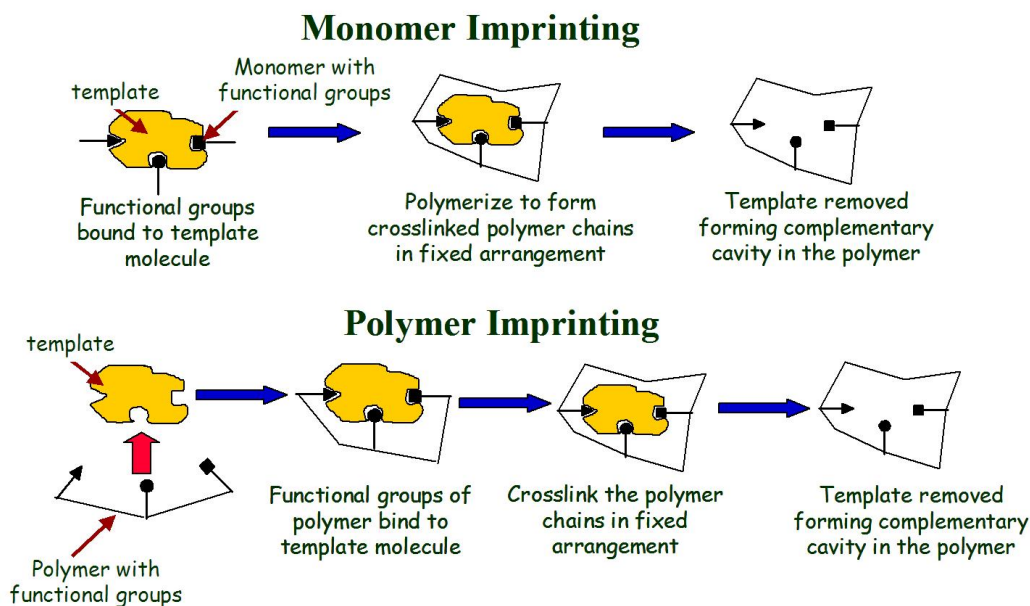


Figure 1.2: Comparison of the imprinting process using functional monomers and polymers.

Two distinct approaches have been used in creating MIPs. Early research in MIPs used reversible covalent binding during the synthesis of MIPs [18, 19]. Prior to matrix formation, the functional groups of the monomer and template are covalently linked. After the MIP is synthesized, the covalent bond is broken and the template is washed out of the matrix. During rebinding, the target molecule is again covalently linked to the matrix within the binding sites. The advantage of using reversible covalent binding is that the functional groups of the polymer

matrix will only be within the cavity and arrange nearly perfect around the template after the template is removed. The disadvantage is that a small number of different template-monomer complexes can be created, limiting the number of molecules that can be targeted by MIPs. The alternate, and more recent approach, uses non-covalent interactions during the synthesis of MIPs [12, 20, 21]. Throughout the entire MIP synthesis, washing, and rebinding, the non-covalent bond of the template and polymer matrix are formed, disrupted, and reformed again. The advantage of using non-covalent interactions is that more molecules can be targeted by MIPs. The disadvantage of this approach is that functional groups of the polymer matrix are not always located within the cavities, leading to higher non-specific binding. Both imprinting schematics utilizing covalent and non-covalent association can be seen in Figure 1.3. An uncommon approach combines the advantages of both covalent and non-covalent imprinting strategies [22, 23]. In this strategy, monomers and template are covalently linked. After polymerization, the bond is cleaved resulting in functional groups of the matrix capable of non-covalent association (for example, an ester bond between the matrix and the template is converted into hydroxyl groups). However, the number of different template-monomer complexes that can be created is still low, and the interactions between the template and matrix may not be the same in the covalent and non-covalent forms. To date, MIPs utilizing the non-covalent approach are more widely used due to the ability to imprint a wide range of compounds.

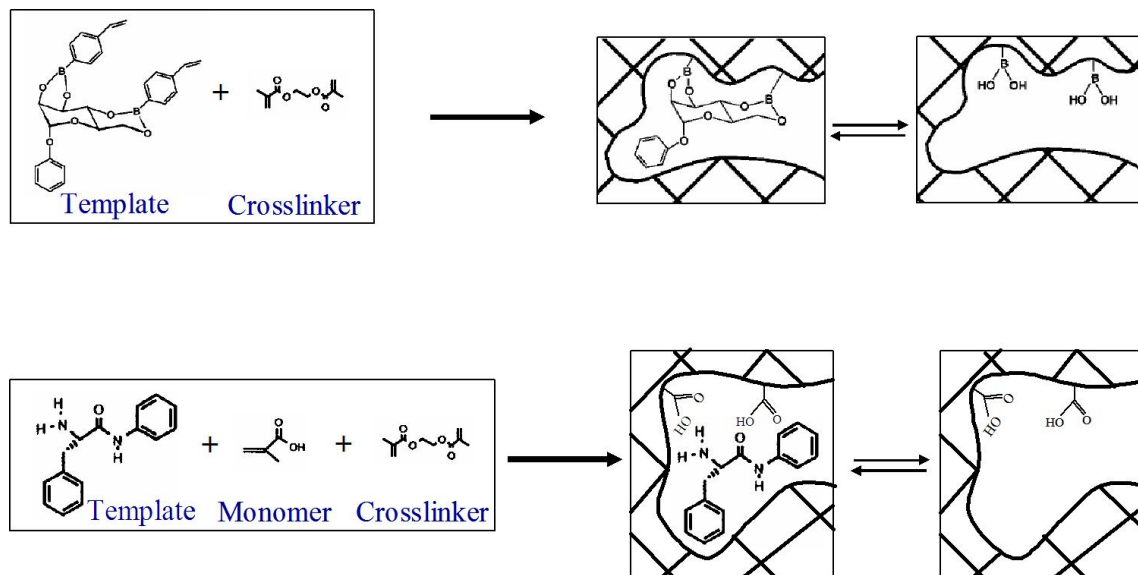


Figure 1.3: Comparison of the imprinting process using covalent and non-covalent association between functional groups.

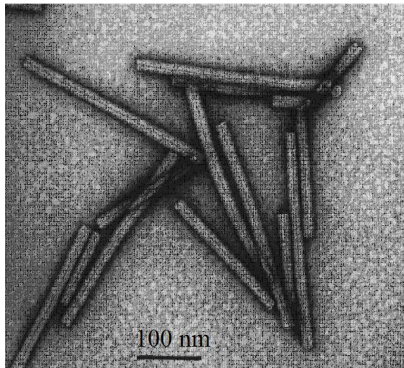
The materials used for molecular imprinting play a role in the release of the template and uptake of the target molecule. The resulting MIP needs to have cavities within the matrix that are able to form a tight fit with the target molecule, while at the same time maintain that shape when the cavity is not occupied. This can be controlled by optimizing the molecular weight between crosslinks. If the molecular weight between crosslinks is too small, cavities will not be formed that conform to the shape and size of the template. In addition, the template will have a difficult time diffusing out of the polymer matrix. If the molecular weight is too large, the cavities will not be structurally stable and able to maintain their complementary shape once the template is removed.

Molecular imprinted polymers (MIPs) have several advantages over their natural analogues such as antibodies and enzymes:

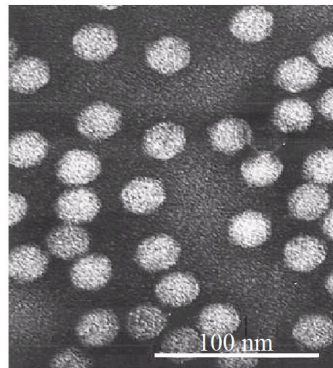
1. The synthesis is relatively easy compared to the production of biomolecules. Components needed for MIP synthesis (monomers, polymers, crosslinkers, wash solutions) can be easily obtained by purchasing them from chemical companies, and the synthesis can last about 24 hours. Harvesting antibodies or enzymes from animals and the subsequent purification processes can be quite long and complicated.
2. MIPs can be stored at room temperature in a dry state for long periods of time without a decrease in performance. Biomolecules need to be stored in a controlled environment to maintain their effectiveness and can be quite expensive.
3. Biomolecules can only be used in very specific conditions, usually at room temperature, at neutral pH, and in aqueous environments. MIPs can maintain stability in a wide range of conditions, including organic solvents, high temperature, and pH.
4. Biomolecules can only be reused for a limited number of cycles before their performance decreases. MIPs can be reused for many cycles as long as the target molecule is removed prior to the next cycle of use.

1.4 Model viruses used in this work

Tobacco mosaic virus (TMV) has been a well studied and characterized virus, and is used extensively in this work [24]. This virus infects a wide range of plants including many vegetables, flowers, and weeds. TMV can only be transmitted through mechanical means to uninfected plants, usually by contaminated hands or clothing from working with infected plants. The infected plants show a mosaic-like pattern on the leaves. They have stunted growth and leaves, flowers, and fruits become damaged. TMV is non-enveloped, rod shaped, and is 300 nm in length and 18 nm in diameter. The virus is composed of a single 5130 kb RNA strand surrounded by 2130 protein subunits (153 amino acids per subunit) arranged in a right hand helix. Surface proteins are mostly glutamic acid, aspartic acid, lysine, and arginine, with an overall isoelectric point of 3.5. At a neutral pH, the virus particle has an overall negative charge. *Tobacco necrosis virus* (TNV) will also be used extensively in this work [25]. This virus also infects a wide range of plants, including tobacco, beans, and cucumber, causing necrosis in their leaves. TNV is non-enveloped, icosahedral shaped, and is 24 nm in diameter. The virus is composed of a single 3.8 kb RNA strand inside a virus shell consisting of 180 protein subunits (each subunit containing 267-272 amino acid residues). The overall isoelectric point is 4.5, giving the virus particle an overall negative charge at a neutral pH. Pictures of the two viruses can be seen in Figure 1.4.



Tobacco Mosaic Virus



Tobacco Necrosis Virus

Figure 1.4: Transmission electron micrographs of TMV and TNV.

MIPs show promise in revolutionizing and making the field of separations and purifications more efficient. Nonetheless, studies need to be conducted to understand and optimize these MIPs to successfully replace their natural counterparts. The work presented in this dissertation will shed some light into this investigation of MIPs and take one step forward to creating such artificial antibodies.

1.5 Summary

The overall goal of this dissertation is to elucidate the mechanism of virus recognition in molecularly imprinted polymers (MIPs). A non-covalent approach involving crosslinkable polymer chains as the starting material was utilized to imprint viruses. TMV was used as the model virus for imprinting. Binding test with TMV and TNV (two tobacco viruses of different shapes) were used to determine the influence of cavity shape on selectivity of the MIPs.

The first goal of this dissertation was to synthesize a molecularly imprinted polymer that will recognize and selectively bind TNV. The second goal was to understand the interactions of the virus template with the functional polymeric chains prior to crosslinking, and to optimize the method of template removal from the crosslinked polymer through appropriate wash protocols. The third goal was to utilize the optimized protocol and synthesize a TMV imprinted polymeric hydrogel with better specificity and selectivity properties.

Chapter 2

Synthesis of virus imprinted polymers for the recognition of Tobacco Mosaic Virus

2.1 Introduction

The goal of this research is to synthesize MIPs with an affinity to a targeted virus. We discuss the non-covalent synthesis and binding capacity of molecularly imprinted polymers, using poly (allylamine hydrochloride) (PAA) as the polymer matrix and TMV as the template. PAA was chosen because it is soluble in water and has a high amount of amine groups that can associate with the viral template. In its crosslinked form, the polymer possesses low toxicity [15, 16, 17]. The virus that was targeted was *Tobacco mosaic virus* (TMV). Batch experiments using TMV imprinted hydrogels and non-imprinted polymer (NIP) hydrogels were conducted in aqueous solutions of TMV to determine the binding capacities of each polymer to the target virus. *Tobacco necrosis virus* (TNV) was also used

in batch experiments to compare the binding capacity of a non-target molecule. These TMV MIPs show increased binding to the target virus, TMV, compared to the non-target virus, TNV.

2.2 Materials and Methods

2.2.1 Materials

Poly(allylamine hydrochloride) (Mw 15,000), NaOH (99.998%), Epichlorohydrin (99%), and ascorbic acid were purchased from Sigma-Aldrich (Milwaukee, WI). Sodium chloride (A.C.S. grade), sodium phosphate (dibasic, anhydrous, enzyme grade), ethylenediaminetetraacetic acid (disodium salt, electrophoresis grade), polyethylene glycol (Mw=8000, molecular biology grade), potassium chloride (A.C.S. grade), and sucrose (ultracentrifugation grade) were purchased from Fisher Chemicals (Suwanee, GA). Chloroform (A.C.S. grade) was purchased from J.T. Baker (Phillipsburg, NJ). Ethyl alcohol (200 proof) was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY). All chemicals were used as received. Distilled, deionized water was obtained using the Millipore Super-Q water system. Deionized water was obtained using the Millipore Elix-5 water system.

2.2.2 Virus Purification

Wildtype TMV from infected *Nicotiana tabacum* were isolated and purified as described by Gooding and Hebert [27]. Virions were further purified by centrifugation at 22,000 rpm in a Beckman SW28 rotor for 2 hours in a 10 to 40% sucrose density gradient at 4 °C. The white band corresponding to the virus layer was extracted, and pelleted by centrifugation at 30,000 rpm in a Beckman TI70 rotor for 2 hours in a solution of water at 4 °C. The pellet was resuspended in water and analyzed for virus concentration using a Perkinelmer Lambda 25 UV/Vis spectrophotometer. Virus concentrations for TMV were determined by measuring absorbance at 260 nm, corrected for light scattering at 325 nm, using an extinction coefficient of 3.01 cm²/mg. Wildtype TNV was isolated and purified using a similar procedure. For this virus, the centrifugation of the sucrose density gradient was conducted at 25,000 rpm for 2 hours. Virus concentrations for TNV were determined by absorbance at 260 nm, using an extinction coefficient of 5 cm²/mg.

2.2.3 Hydrogel Synthesis and Template Extraction

To determine if virus imprinting is possible, initial experiments were conducted using imprinting procedures already developed for targeting glucose with minor modifications[15]. First 0.5 mL of 50 % (w/v) PAA was placed in a microcentrifuge tube. Next, 0.3 mL of 1 M sodium hydroxide (NaOH) was added to the

solution and mixed using a vortex machine to increase the pH to 9. At pH 9, the structure of the plant viruses are stable and are negatively charged. The amine groups on the polymer chains are positively charged. Then 1 mg of TMV was added to the solution and mixed to disperse the virus particles. 0.0103 mL of epichlorohydrin (EPI) was then added to the mixture and mixed using a Vortex-Genie (Scientific Industries, Inc.) set at "8" for about 4 hours to evenly disperse the crosslinker. The final volume was 1 mL and the ratio of polymer to virus was 250 mg to 1 mg. The mixture was allowed to cure in a microcentrifuge tube for 5 days.

2.2.4 Template Extraction

The hydrogel was cut and placed in a 50 mL plastic tube filled with 50 mL of 70 % ethanol (EtOH). The tubes were rotated for 24 hours using a Barnstead International Labquake Shaker. The EtOH solution was discarded, and 50 mL of 1 mM of sodium chloride (NaCl) was added to the tube and placed in boiling water for 1 hour (mixing the tubes using a Vortex every 15 minutes). The NaCl solution was discarded, and 1 M NaCl was added to the tube and again placed in boiling water for 1 hour (mixing the tubes using a Vortex every 15 minutes). Next, cut hydrogel was transferred to a 600 mL beaker, filled with 500 mL of deionized water, and washed for 3 days, changing the water every 8 hours to remove the EtOH and NaCl from the hydrogels. Finally, the washed hydrogel

was then dried in a 55 °C oven.

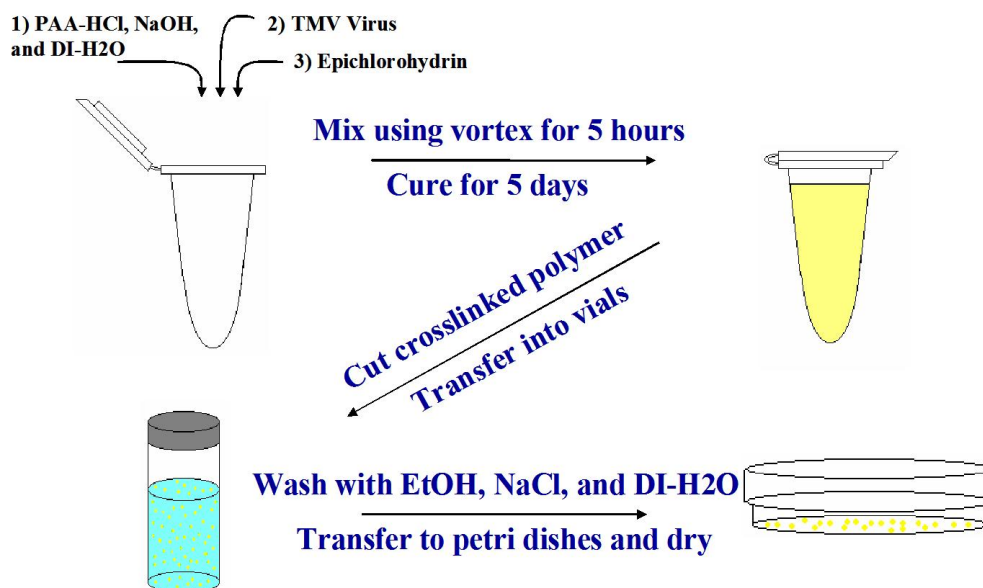


Figure 2.1: Schematic of virus imprinting procedure.

2.2.5 Virus Binding Test

Once the washed and dried virus imprinted polymer hydrogel was obtained, it was analyzed for its binding capacity. A schematic of this procedure is shown in Figure 4. 50 mg of dried polymer hydrogel was placed in a microcentrifuge tube. Next, 1 mL of 1 mg/mL TMV was added to the microcentrifuge tube and mixed using a vortex for 4 hours. The supernatant was collected and filtered using a 0.45 μm filter to remove the hydrogel while still allowing virus particles to flow through. The filtered supernatant was analyzed using a Lambda 25 UV/Vis spectrophotometer (Perkinelmer Inc.), measuring absorbance at 260 nm,

280 nm, and 325 nm. A schematic of the binding test can be seen in Figure 2.2. Equation 2.1 was used to calculate the concentration of TMV in solution and equation 2.2 was used to calculate the concentration of TNV in solution.

$$[\text{TMV}] = \frac{\text{Abs @ 260 nm} - (\text{Abs @ 325} \cdot 2.44)}{3} \cdot \text{Dilution Factor} \quad (2.1)$$

$$[\text{TNV}] = \frac{\text{Abs @ 260 nm}}{5} \cdot \text{Dilution Factor} \quad (2.2)$$

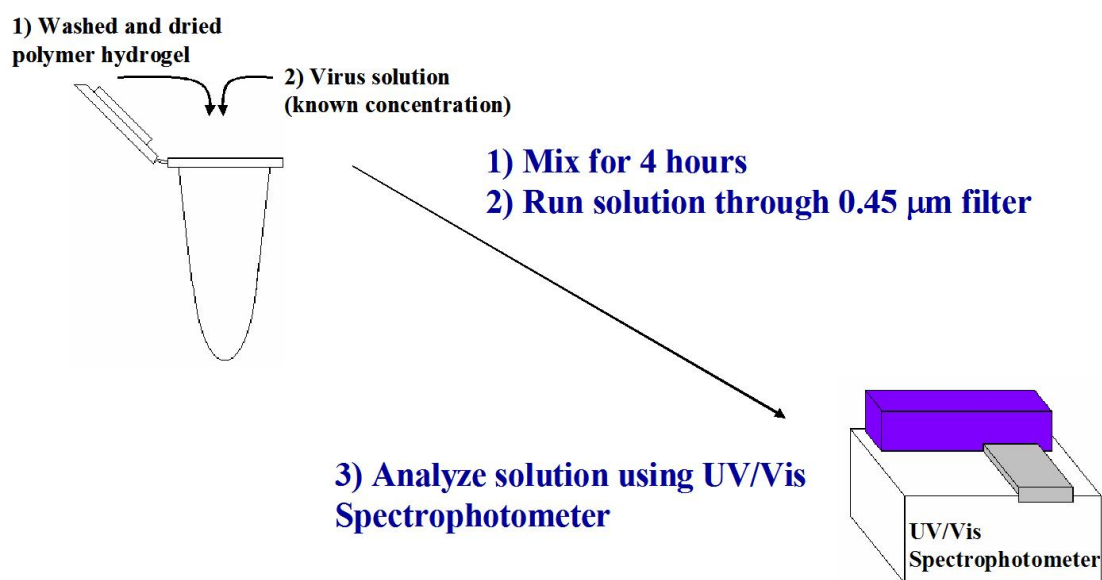


Figure 2.2: Schematic of binding test procedure.

2.3 Results and Discussion

The synthesis of MIPs involves three steps, as seen shown Figure 2.3. The first step involves the association of functional groups between the template molecule and monomer units on the polymer chains. This association can be by covalent or non-covalent. In our case, polymer chains are used to non-covalently interact with the template. Next, polymer chains are frozen in place by a crosslinking reaction, resulting in a polymeric network molded around the template. The last step involves the removal of the template from the polymeric network, resulting in cavities that have the shape, size, and functional group orientation that is complementary to the target molecule.

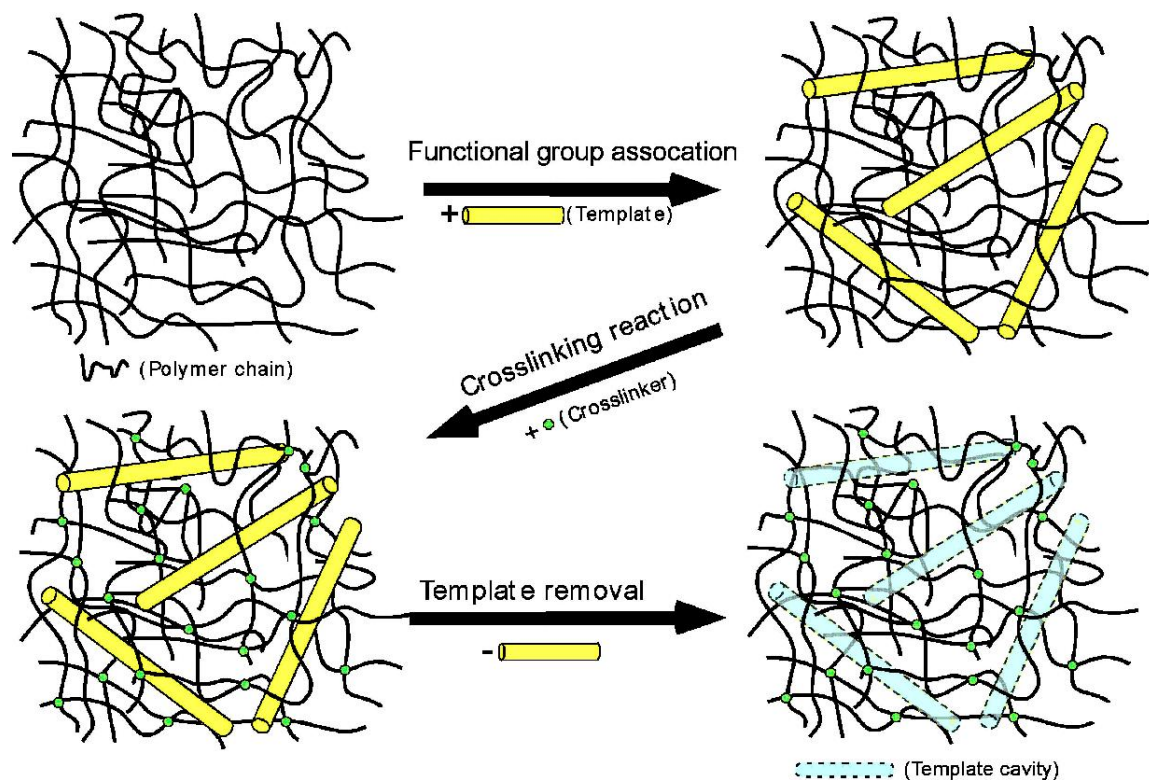


Figure 2.3: Schematic of molecular imprinting procedure with TMV.

Figure 2.4 shows the binding capacity of TMV imprinted crosslinked polymers. Two crosslinked polymers, one imprinted with TMV and a control sample that was non-imprinted were examined for their TMV and TNV binding capabilities. The expectation was that the TMV imprinted gel, having cylindrical cavities, would preferentially bind the virus of the same shape (TMV) compared to another tobacco virus of different shape (TNV). Experiments were performed in triplicate. As shown in Figure 2.4, the TMV imprinted polymer displayed a 2.1 fold increase in affinity to the TMV virus compared to the non imprinted control polymer. The TMV imprinted polymers, on the other hand, did not exhibit any

significant change in TNV affinity compared to the non-imprinted control. These experiments thus demonstrated that a virus imprinted polymer can be synthesized which is selective to a specific virus based on shape (geometrical) factors (cylindrical TMV virus versus spherical TNV virus). The experimental results overall indicate the ability of the crosslinked polymer gel matrix to conformingly map viral surface features, retain these features when the gel is swollen, and specifically and selectively capture a virus based on these features.

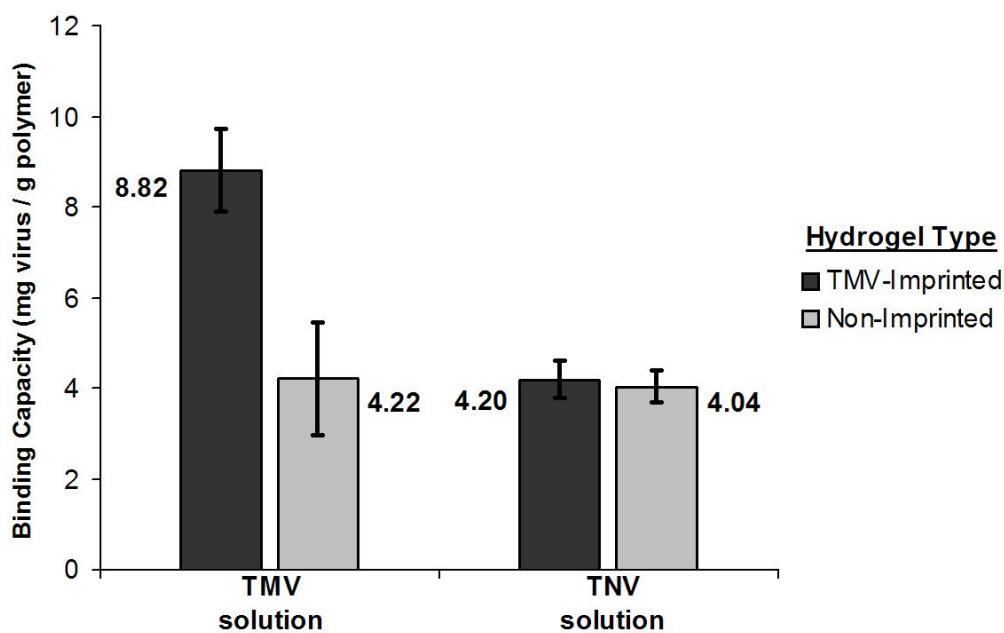


Figure 2.4: TMV Binding capacity of TMV- Molecularly Imprinted Polymers compared to non-imprinted control polymers

2.4 Conclusions

Crosslinked polymers imprinted against TMV via non-covalent interactions were synthesized. The TMV imprinted polymer exhibited an increase affinity to the target virus compared to the control polymer. Furthermore, the TMV imprinted polymer demonstrated a preferential affinity, based on shape, to the target virus compared to the non-target virus. In contrast, there was no significant increase in binding of the control polymer to either target or non-target virus. The mechanism of virus imprinting involves the creation of cavities of similar shape to the original virus particle, providing a spatially defined site for binding of the templated virus. The experiments presented in this chapter have been published in the journal *Biomaterials* [28].

Chapter 3

Optimization of virus imprinting technique to improve selectivity and reduce non-specific binding

3.1 Introduction

Molecularly imprinted polymers (MIPs) can be synthesized by crosslinking functional polymer chains in the presence of a template [15, 16, 28]. Polymers synthesized using this technique of molecular imprinting demonstrate high affinity towards a templated target molecule. The polymer chains associate with the template through their respective functional groups. A crosslinker is then added to covalently connect two polymer chains using the functional groups that have not associated with the template. Upon reaction of polymer, template, and crosslinker, the imprinted template is trapped within the three-dimensional

polymer network matrix. The non-covalent association of template to the polymer matrix is disrupted using a wash to remove the template, resulting in a three-dimensional polymeric matrix containing cavities complementary to the imprinted template.

There are many factors that need to be considered when developing a procedure for MIP synthesis. One is the uniformity of the pre-polymerized MIP mixture. If the individual reagents (such as polymer and template) aggregate when mixed together, the resulting three-dimensional polymer will contain cavities complementary to such aggregate formations, leading to increased non-specific binding to the target molecule, and loss in selectivity [29]. Another factor is the ease of release of the template to create a complimentary cavity. The ability of MIPs to selectively bind to the target molecule is derived from the vacated complementary cavities. If the wash solution is not successful in removing the template from the crosslinked gel, then a recognition site will not be created to rebind the target molecule [30]. Steps must be developed to ensure the prevention of aggregate formation prior to polymerization and crosslinking of the MIP and to maximize the removal of the template to complete the molecular imprinting process has been completed. In this study, methodologies that avoid template aggregation and enhance template removal were investigated for their effect on virus binding and specificity. We used *Tobacco mosaic virus* (TMV) as a model virus to determine the optimum conditions required for efficient imprinting.

The separation of viruses and virus-like particles from various media represents an enormous challenge to the fields of medicine, healthcare, and biotechnology. The removal of virus-like particles from cell-culture media and cell debris is an extremely inefficient process that results in increased development times for vaccines, medical diagnostics, and gene therapy treatments. It is possible to produce virus specific MIPs with superior affinity and selectivity capabilities by understanding the interactions of the virus template with the polymer matrix, and optimizing the method of template removal from the crosslinked polymer through appropriate wash protocols. We refer to the term "molecular imprinting" as a method to produce high-affinity polymers for specifically viruses, and do not imply that imprinting has been achieved at the molecular level. Virus MIPs were synthesized to selectively bind TMV. Previous work involved the use of poly (allylamine hydrochloride) (PAA) crosslinked with epichlorohydrin (EPI) in the presence of TMV template to create the virus MIP [28]. However, the resulting hydrogel appeared cloudy and is thought to contain polymer-virus aggregation. To avoid this problem, the crosslinker was changed to ethylene glycol diglycidyl ether (EGDE). EGDE has shown to be a better crosslinker compared to EPI because of its improved solubility in water. It was observed that the polymer and virus form a non-homogeneous mixture at an extended range of polymer concentrations. Virus template aggregation studies were performed to gain knowledge on how to ensure a homogeneous mixture prior to the formation

of the MIP. The amount of virus extracted from the MIP in different wash solutions was compared to determine the optimal conditions for template removal, and thus understand the conditions needed to successfully remove the templated virus from the imprinted polymer matrix. Based on the results, an optimized procedure for the synthesis of TMV MIPs was developed. Binding test using the TMV imprinted hydrogels in solutions of TMV (targeted virus) or *Tobacco necrosis virus* (TNV), a non-targeted virus, were used to determine the effects of the optimized procedure on viral affinity.

3.2 Materials and Methods

3.2.1 Materials

Poly (allylamine hydrochloride) ($M_w = 15,000$), allylamine ($> 99\%$), ascorbic acid, ethylene glycol diglycidyl ether (50 %, technical grade), magnesium chloride hexahydrate (reagent plus $\geq 99\%$), potassium chloride (99.0 - 100.5 %, A.C.S. reagent), sodium acetate (molecular biology tested), sodium bicarbonate (99.7 - 100.3 %, A.C.S. reagent), sodium hydroxide (99.998 %), sodium phosphate dibasic (ultra, $\geq 99.5\%$), sodium phosphate monobasic monohydrate (ultra, for molecular biology) and urea (SigmaUltra) were purchased from Sigma-Aldrich (Milwaukee, WI). Sodium phosphate (dibasic, anhydrous, enzyme grade), ethylenediaminetetraacetic acid (disodium salt, electrophoresis grade), tris (hy-

droxymethyl) aminomethane (molecular biology grade), polyethylene glycol (Mw = 8000, molecular biology grade), potassium chloride (A.C.S. grade), and sucrose (ultracentrifugation grade) were purchased from Fisher Chemicals (Suwanee, GA). Calcium chloride (purified), chloroform (A.C.S. grade), hydrochloric acid (A.C.S. reagent), potassium carbonate (anhydrous, granular, A.C.S. reagent), sodium chloride (A.C.S. reagent), and sodium sulfate (A.C.S. reagent) were purchased from J.T. Baker (Phillipsburg, NJ). Ethyl alcohol (95 %) was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY). Texas red C2 maleimide was purchased from Invitrogen (Carlsbad, CA). All chemicals were used as received. Deionized water was obtained using the Barnstead NANOpure DIamond water system.

3.2.2 Virus purification and fluorescent labeling

Wildtype TMV from infected *Nicotiana tabacum* were isolated and purified as described by Gooding and Hebert [27]. Virions were further purified by centrifugation at 22,000 rpm in a Beckman SW28 rotor for 2 hours in a 10 % to 40 % sucrose density gradient at 4 °C. The white band corresponding to the virus layer was extracted, and pelleted by centrifugation at 30,000 rpm in a Beckman TI70 rotor for 2 hours in a solution of water at 4 °C. The pellet was resuspended in water and analyzed for virus concentration using a Perkinelmer Lambda 25 UV/Vis spectrophotometer. Virus concentrations for TMV were determined by

measuring absorbance at 260 nm, corrected for light scattering at 325 nm, using an extinction coefficient of 3.01 cm²/mg. Wildtype TNV was isolated and purified using a similar procedure. For this virus, the centrifugation of the sucrose density gradient was conducted at 25,000 rpm for 2 hours. Virus concentrations for TNV were determined by absorbance at 260 nm, using an extinction coefficient of 5 cm²/mg. Genetic manipulation of the TMV coat protein and fluorescence labeling using Texas Red C2 maleimide were conducted using the procedure described by Yi et al. [31]. Once the modified TMV virions were labeled with Texas Red, they were purified using the same purification procedure as the wildtype TMV.

3.2.3 Virus Aggregation Studies

1 mg of TMV was placed in a 1.5 mL microcentrifuge tube containing PAA in water at different concentrations (% w/v) ranging from 5 % to 35 %. The pH was adjusted to 7 using 10 M sodium hydroxide (NaOH) prior to addition of the virus. The total volume was adjusted to 1 mL using water (H₂O). The solution was mixed using a vortex and then analyzed for absorbance at 500 nm using a UV/Vis spectrophotometer. Samples at 5 %, 15 %, and 30 % PAA were further examined by placing 5 μ L of the solution on a glass slide and observing through an Olympus BX60 optical microscope. 1 mg of TMV was also mixed with allylamine (1 % v/v and 20 % v/v, pH 7). Aggregate formation was visually observed.

3.2.4 Fluorescent Virus MIP Synthesis

0.7 mL of 50 % (w/v) PAA was placed in a 2 mL microcentrifuge tube. 10 mL of 10 M NaOH added to the polymer solution and mixed. 1 mg of fluorescent labeled TMV was then added to the polymer mixture and mixed. The total volume was adjusted to 1 mL using H₂O prior to crosslinking. Then 0.15 mL of EGDE was added to the polymer mixture, mixed, and allowed to cure for 24 hours.

3.2.5 Template Removal Study from Virus MIP

H₂O, 1 M sodium chloride (NaCl), 6 M urea, and 1 M NaOH, were used as wash solutions. For each wash solution, an MIP was synthesized and cut in the microcentrifuge tube using a scalpel. For this study, the MIPs were synthesized using 1/5th the original proportions (total volume of 0.2 mL) in a 2 mL microcentrifuge tube. Once cured, the hydrogels were cut using a scalpel, and 1.6 mL of a wash solution was added to the tube and rotated for 24 hours using a Barnstead International Labquake Shaker. The wash solutions were collected and filtered using a 0.45 μ m syringe filter. 0.2 mL of the filtered wash solution was placed in a black 96-well plate and analyzed for fluorescence using Molecular Devices SpectraMax M5 Microplate Reader. After 5 wash cycles, the gels were further washed with H₂O to remove the wash solution from the hydrogels. The MIPs were placed in 0.5 mL microcentrifuge tubes, and the fluorescence intensity within the hydrogel

was examined using Bio-Rad ChemiDoc XRS imaging system under ultraviolet light. Calibration curves relating fluorescence to the amount of TMV in solution was calculated by adding different amounts of fluorescently labeled TMV to each wash solution, with a final concentration ranging from 0.11 to 0.02 mg/mL, then measuring the fluorescence intensity from a 0.2 mL aliquot. The curves were used to determine the percent of template removed from the TMV MIP using different wash solutions.

Once the optimal wash solution of 1 M NaOH was determined, four MIPs were synthesized using 1/5 the original proportions (total volume of 0.2 mL) in a 2 mL microcentrifuge tube. Once cured, the hydrogels were cut using a scalpel, and 1.6 mL of a wash solution was added to the tube and rotated for 3, 6, 12, and 24 hr time intervals. After each time interval, 1 mL of wash solution was collected, filtered with a 0.45 μm syringe filter, and 0.2 mL of filtrate was placed in a black 96-well plate and analyzed for fluorescence.

Once the optimum wash cycle of 6 hours was determined, an MIP was again synthesized using the original proportions (total volume of 1 mL) and cut into a 50 mL plastic tube tube using a scalpel. Next, 50 mL of 1 M NaOH was added to the tube and rotated using the optimal time determined in previous experiments. After each time interval, 1 mL of wash solution was collected, filtered using a 0.45 μm syringe filter, and 0.2 mL of filtrate was placed in a black 96-well plate and analyzed for fluorescence. A calibration curve relating fluorescence to the amount

of TMV in solution was calculated by adding different amounts of fluorescently labeled TMV to 1 M NaOH, with a final concentration ranging from 0.02 to 0.004 mg/mL, then measuring the fluorescence intensity from a 0.2 mL aliquot. The curve was used to determine the percent of template removed from the TMV MIP using 1 M NaOH.

3.2.6 Wildtype TMV MIP synthesis

MIPs were synthesized using the optimal conditions for homogeneity and template removal. Wildtype TMV was used as the viral template. 0.7 mL of 50 % (w/v) PAA was placed in a 2 mL microcentrifuge tube. 10 μ L of 10 M NaOH added to the polymer solution and mixed. Wildtype TMV of various amounts ranging from 0.01 mg to 1.5 mg was then added to the polymer mixture and mixed. Then 0.15 mL of ethylene glycol diglycidyl ether (EGDE) was added to the polymer mixture, mixed, and allowed to cure for 24 hours. The total volume was adjusted to 1 mL using H₂O prior to crosslinking. The MIP solution was then placed on a petri dish and allowed to cure for 24 hours at room temperature. Once cured, the MIPs were cut into 2 mm by 2 mm squares and placed in a 50 mL plastic tube. 50 mL of 1 M NaOH solution was added to the tube and rotated for 6 hour intervals using a Barnstead International Labquake Shaker. The wash solution was discarded, new solution was added, and the cycle was repeated 6 times. After washing the MIPs with 1 M NaOH, the gels were then washed with

50 mL of H₂O for 4 hour intervals until the pH of the wash solution became 7. The crosslinked polymers were removed from the solution and dried at 55 °C in an oven.

3.2.7 PAA/EGDE hydrogel swelling studies

Non-imprinted MIPs were used to investigate the swelling behavior of the polymer hydrogels in different solutions. 7 mL of 50 % (w/v) PAA was placed in a 15 mL plastic tube. 0.1 mL of 10 M NaOH added to the polymer solution and mixed. Then 1.5 mL of EGDE was added to the polymer mixture, mixed, and allowed to cure for 24 hours. The total volume was adjusted to 10 mL using H₂O prior to crosslinking. The MIP solution was then placed on a petri dish and allowed to cure for 24 hours at room temperature. Once cured, the MIPs were cut into 0.75 mm disks, weighed, and placed in a 2 mL microcentrifuge tube. 1.6 mL of a solution was added to each tube, and after 24 hours the hydrogel disks were weighed. The solutions used were 0.1 M of magnesium chloride (MgCl₂), sodium chloride (NaCl), sodium sulfate (Na₂SO₄), phosphate (PO₄) buffer, potassium chloride (KCl), sodium acetate (NaC₂H₃O₂), sodium bicarbonate (NaHCO₃), calcium chloride (CaCl₂), and potassium carbonate (K₂CO₃).

3.2.8 Virus binding test

15 mg of dried polymer hydrogel was placed in a 2 mL microcentrifuge tube. Next, 1.8 mL of 0.1 mg/mL TMV or TNV solution in 0.1 M phosphate buffer, pH 7, was added to the microcentrifuge. The tubes were then rotated for a 6 hours time interval. After, 0.1 mL of the virus solution was analyzed using a Perkinelmer Lambda 25 UV/Vis Spectrophotometer.

To determine the concentration of TNV in solution, the Pierce Bicinchoninic acid (BCA) protein assay kit from Thermo Scientific was used. After the binding test was performed, a 50 μ L aliquot was taken and mixed with 1 mL of the BCA reagent and incubated in a water bath at 37 °C for 30 minutes. After incubating, the sample was cooled to room temperature, then analyzed for absorbance using a UV/Vis spectrophotometer at 562 nm.

3.3 Results

3.3.1 Aggregate formation in the polymer-virus solution

TMV was chosen as the model virus because it is well studied and characterized [24]. PAA was chosen because it is water soluble, and contains primary amine groups with a pKa of 9.67 [32]. At a neutral pH, TMV and PAA have an overall negative and positive charge respectively. EGDE was chosen for its ability to crosslink amine groups at a neutral pH and for its high solubility in water.

A solution containing 1 mg of TMV in 1 mL of water (pH 7) is a clear solution. However, once a small amount of PAA was added (0.0001 % w/v of PAA in water, pH 7), aggregates formed which were observed as an increase in turbidity of the solution. As the polymer concentration increased to 30 %, the turbidity decreased and the solution became clear again. The turbidity of the solution at different polymer concentrations can be seen in Figure 3.1. Aggregation was not observed when 1 mg of TMV was added to a solution of allylamine at pH 7, the monomer unit of PAA. This suggests that the formation of aggregates is due to the presence of high molecular weight PAA polymer chains.

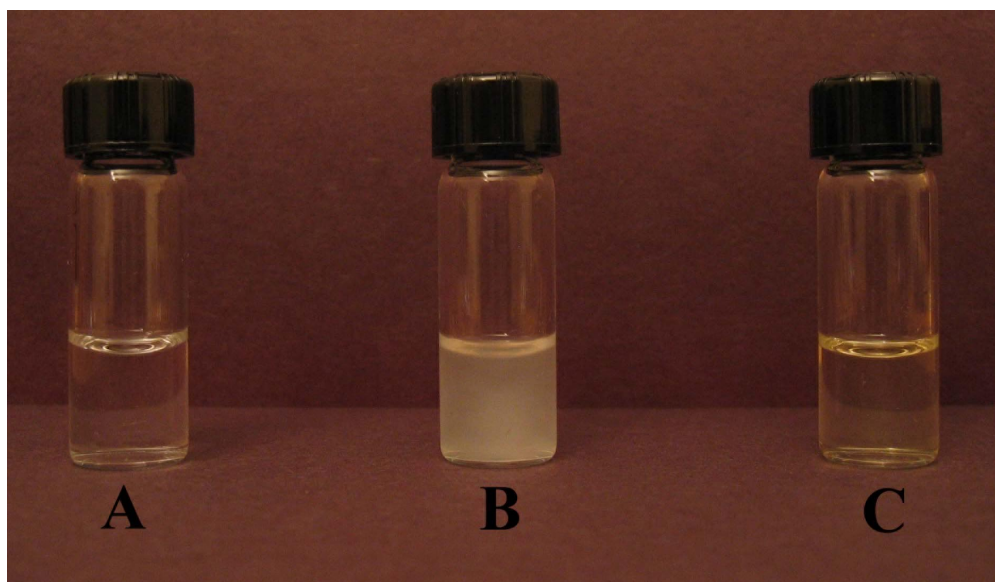


Figure 3.1: Virus aggregation as a function of polymer concentration. A) 1 mg/mL TMV in water. B) 1 mg/mL TMV in 1 % (w/v) PAA solution. C) 1 mg/mL TMV in 35 % (w/v) PAA solution.

Virus aggregation has also been observed in various protein/polymer systems, such as the bovine serum albumin (BSA) and the PAA system at pH 7.4 studied by Ball et al. [33]. At this pH, all the amine groups of the PAA chain ($pK_a = 9.67$) are positively charged, while BSA is negatively charged ($pI = 4.7$). Ball et al. observed that as PAA is added to a buffered solution of BSA, aggregation appeared and increased up to a maximum before it decreased and eventually disappeared. Aggregation was quantified by the amount of absorbance at 500 nm. When a small amount of PAA chains was initially added to a solution of BSA molecules, each polymer chain bound to multiple protein molecules to neutralize their net charges. The binding of a single chain to multiple protein molecules increased the protein concentration at a particular point, and resulted in the formation of aggregates and increased turbidity of the solution. At this point, only a small number of protein molecules were associated. As the PAA concentration was increased, more BSA molecules were bound together and the aggregate size became larger until all protein molecules were associated with polymer chains. This corresponded to the maximum observed turbidity. Increasing the amount of PAA even further led to chains competing for a BSA molecule, resulting in decreased aggregate size and turbidity of the solution. Eventually, the amount of PAA chains was so large that one BSA molecule was completely surrounded by multiple PAA chains and that no polymer chain was able to associated with multiple protein molecules, thus preventing aggregate formation and any observed

turbidity in the solution.

Similar to the BSA/PAA system, strong non-covalent interactions occurred in our system between the virus particles and PAA polymer chains due to their opposite charges at pH 7. In our experiments, TMV was added to various concentrations of PAA and analyzed for polymer-virus aggregation similar to methods described by Ball et al. Figure 3.2 shows the turbidity of the polymer solution as a function of PAA concentration. At a polymer concentration of 5 % (w/v), there were large amounts of aggregation observed in the solution as indicated by a high absorbance at 500 nm. An aliquot of the polymer-virus solution was placed on a glass slide and examined using an optical microscope. From the micrograph (Figure 3.3), large aggregates can be seen which were as long as 50 μ m and as wide as 10 μ m. A polymer concentration of 15 % (w/v) was also examined (Figure 3.3). At this concentration, smaller and dispersed aggregates can be seen. The measured turbidity at 15 % (w/v) polymer concentration was lower than at 5 % (w/v), indicating that the maximum turbidity occurred at a lower concentration.

In the case of BSA/PAA, Ball et al. hypothesized that the formation of aggregates were caused by PAA chains tethering BSA molecules together. However, in the case of TMV/PAA, the size difference between virus particles and polymer chains may be too large for tethering to occur. Instead, what is believed to be occurring is a neutralization of charge. A solution containing only TMV appears

clear. This may be due to the overall negative charge of the virions repelling each other and dispersing them in solution. When PAA is added to the solution, the positively charged groups on PAA and the negatively charged groups of TMV will interact and bind to each other and neutralize their charge. This neutralization will reduce the repulsive forces between virions, resulting in the formation of aggregation and observed as an increase in turbidity. As the amount of positively charged PAA is added, more TMV is neutralized. The maximum turbidity in the solution will be observed when enough PAA is added to neutralize all the negative charges of the virus particles. Adding more PAA will result in an excess of positively charged polymers interacting with TMV, leading to an increase in repulsive forces, dispersion of PAA/TMV aggregation in the solution, and decreased turbidity until the solution becomes clear again.

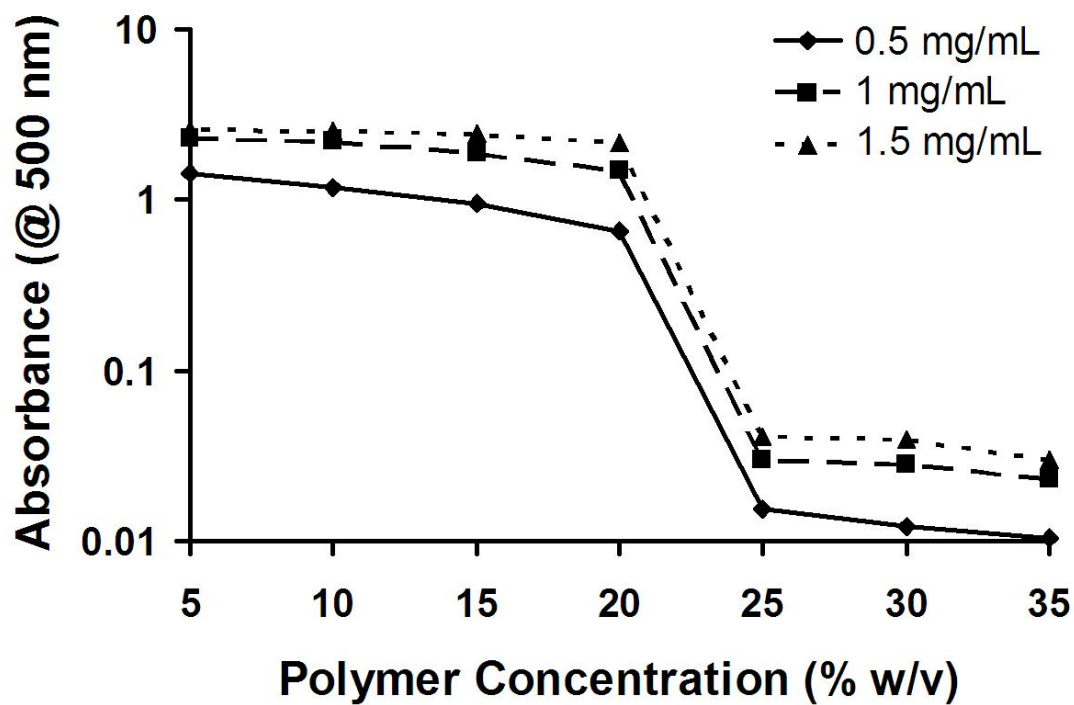


Figure 3.2: Aggregation of TMV and PAA as a function of % (w/v) PAA (pH 7).

TMV concentrations of 0.5 mg/mL, 1 mg/mL, and 1.5 mg/mL were investigated.

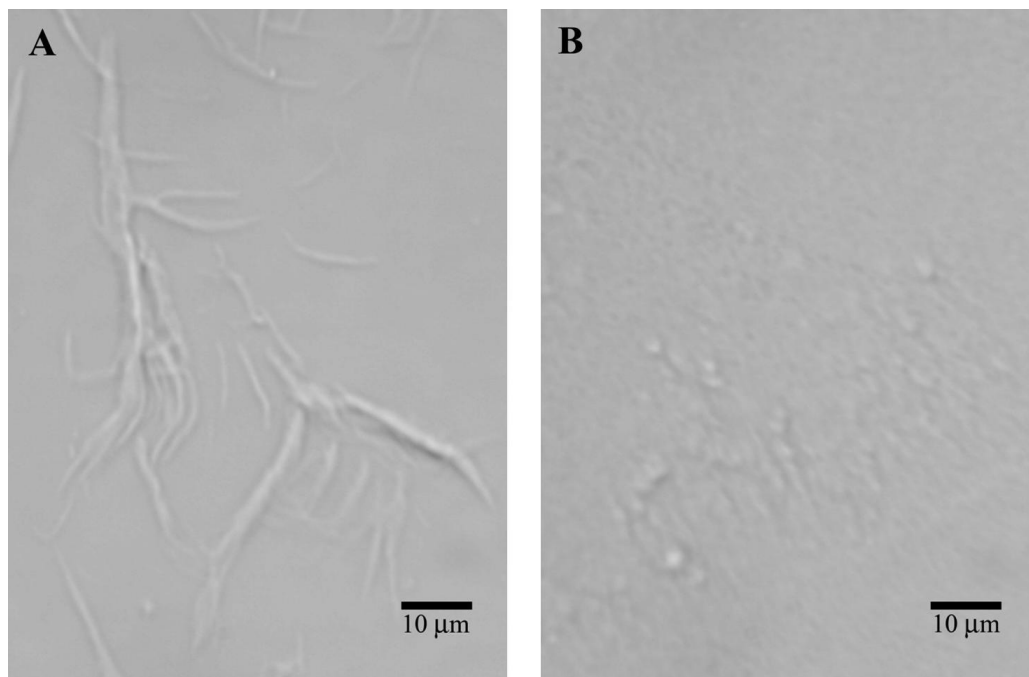


Figure 3.3: Optical micrograph of virus aggregation at A) 5 % (w/v) and B) 15 % (w/v). Concentration of TMV was 1 mg/mL, and solution pH = 7.

The synthesis of the MIP hydrogel should occur at a polymer concentration where template aggregation is absent, in order to create isolated and well dispersed cavities within the polymer matrix that are complementary to the shape, size, and functional group arrangement of the virus template. If the formation of the crosslinked hydrogel were to occur at a lower polymer concentration, the resulting cavities would be in the shape of virus aggregates and the virus MIP would lose its ability to selectively bind to a single targeted virus particles. A PAA concentration of higher than 25 % should be chosen for all imprinting procedures to ensure that virus aggregation was not occurring during imprinting.

3.3.2 Virus removal studies

Virus MIPs were synthesized using 1-cys modified TMV labeled with fluorescent Texas Red to allow the quantification of the virus amounts within the polymer. Once the hydrogels were formed, they were immersed in either H₂O, NaCl (1 M), urea (6 M), or NaOH (1 M). The purpose of the H₂O wash was to determine whether the polymer matrix bound the virus or just surrounded the template. NaCl was chosen as a way to extract TMV from the MIP by disrupting the associations between the functional groups through the introduction of positive and negative charged ions. Urea was chosen because it is commonly used to denature proteins, and a concentration of 6 M has been shown to degrade TMV [34, 35]. NaOH was used to denature TMV as well as neutralize the positive charge of the polymer matrix. The extraction of the virus from the MIP was quantified by the amount of fluorescence in the solution after the wash was performed. The fluorescence was related to the amount of TMV present in solution using a calibration curve of fluorescence versus TMV concentration in each wash solution. Different amounts of fluorescently labeled TMV were added to 0.2 mL of each wash solution, with a final concentration ranging from 0.11 to 0.02 mg/mL. The fluorescence was measured for each solution, and a calibration curve was determined. The percent of TMV extracted from the TMV MIPs from each wash was determined by comparing the fluorescence of the wash with the calibration curve for each solution. The calibration curve relating the concentration of fluorescently

labeled TMV with the fluorescence of the solution is shown in Figure 3.4. The ability of the wash solutions to remove the virus from the MIPs is summarized in Table 3.1. The amount of red fluorescence emitted from each gel is proportional to the amount of Texas Red labeled TMV present in the polymer matrix.

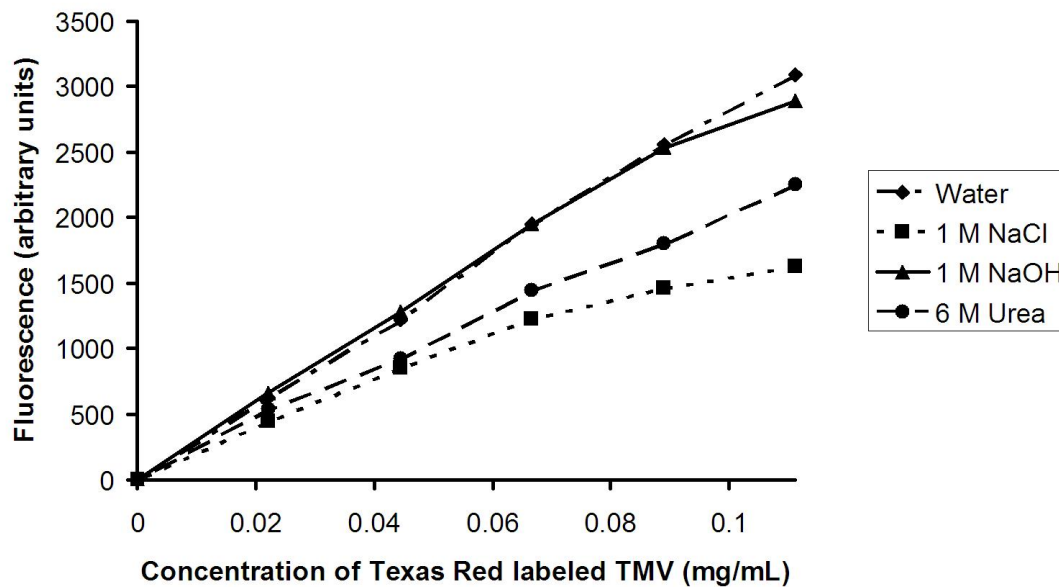


Figure 3.4: Fluorescence of solution versus concentration of Texas Red labeled TMV in different solutions.

Wash solution	Wash cycles	
	(% TMV template removed)	
	1 st wash	5 th wash (Final)
H ₂ O	0.03	0.12
NaCl	0.06	0.22
6M Urea	12.09	21.16
1M NaOH	41.15	82.40

Table 3.1: Percent of TMV extracted from TMV MIPs after washing with H₂O, 1 M NaCl, 6 M Urea, and 1 M NaOH.

Virus MIPs washed with H₂O resulted in the lowest amount of template extracted. This indicated that the polymer matrix was associating with the virus through its amine groups and not just by surrounding the template. Charged ions are also capable of disrupting the association between the template and MIP by substituting each functional group of the TMV protein coat with that of the corresponding charged ion, thus eluting the virus from the polymer matrix. NaCl has large amounts of dissociated ions in water, but at a 1 M concentration, the association between the functional groups of the virus template and polymer matrix may be too strong to be disrupted by the charged ions, as shown by only a low amount of viral template extracted. A solution composed of 6 M Urea

has been known to denature TMV [34, 35]. However, this solution was only able to remove a moderate amount of templated virus. The solution of 1 M NaOH was able to remove the most virus template within the MIP compared to all other wash solutions. There may be two reasons why NaOH was more successful in virus removal. First is its ability to denature the template virus into small protein subunits. TMV in high alkaline conditions has been reported to result in the degradation of virions [36]. The second reason why NaOH was successful in virus extraction from the MIP was that high pH conditions ($\text{pH} > \text{pK}_a$ of PAA) lead to the amine groups of the PAA becoming uncharged. The pH of H_2O , NaCl, and Urea were both approximately 7, while the pH of NaOH was approximately 12. A high solution pH results in the disruption of the functional group association between the virus template and the polymer matrix and leads to the release of the viral subunits from the matrix into the surrounding wash solution. Hydrochloric acid (HCl) was also examined for its ability to extract the templated virus from the imprinted polymer matrix. However, when fluorescent labeled TMV was added to a solution of HCl (1 M or higher concentration), the formation of small aggregates was observed, which made it difficult to accurately determine the amount of TMV extracted from the gel at these conditions. The fluorescence intensity emitting from TMV MIPs after being washed with H_2O , NaCl, urea, and NaOH can be seen in Figure 3.5.

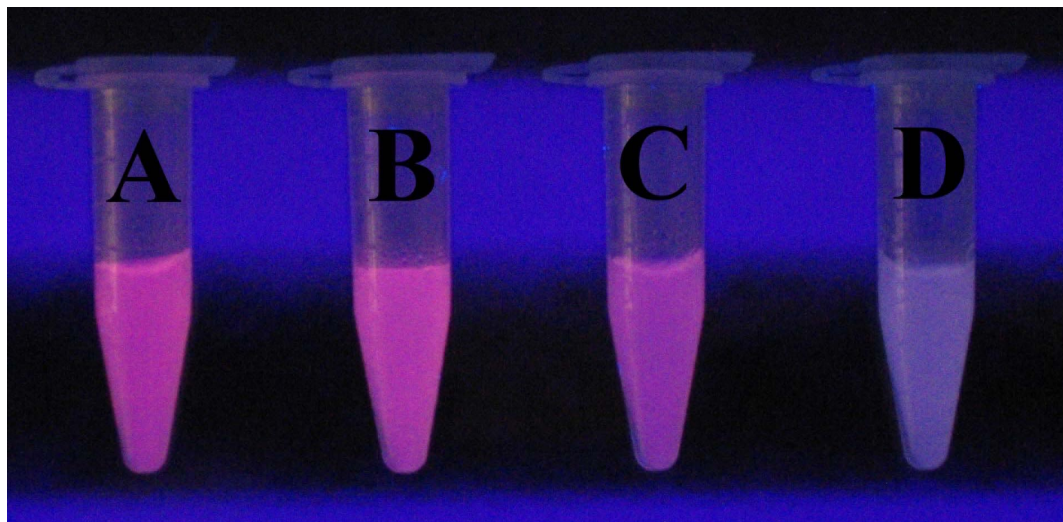


Figure 3.5: Fluorescence of TMV-MIPs after washing with A) H₂O, B) 1 M NaCl, C) 6 M urea, and D) 1 M NaOH.

Both the MIPs washed with 6 M urea and 1 M NaOH emitted less red fluorescence than those washed with H₂O and 1 M NaCl, with the 1 M NaOH emitting the least out of the four washed hydrogels. Hydrochloric acid (HCl) was also examined for its ability to extract the templated virus from the imprinted polymer matrix. However, when fluorescent labeled TMV was added to a solution of HCl (1 M or higher concentration), the formation of small aggregates was observed, which made it difficult to accurately determine the amount of TMV extracted from the gel at these conditions.

The breakdown of TMV into protein subunits can lead to easier diffusion out of the polymer matrix and into the wash solution. However, if the association between the template and the polymer matrix is not disrupted, the protein

subunits will still be bound within the MIP, and the complementary cavity will still be occupied. Disruption of virus-MIP functional group association and the disassembly of the virus are required for optimal template removal.

After determining that a wash solution of 1 M NaOH was the best at removing the virus from TMV imprinted hydrogels when compared to the other wash solutions, an experiment was conducted to determine the optimal exposure time needed to efficiently remove the virus. Four MIPs were synthesized, and after curing, each MIP was cut using a scalpel and washed with 1 M NaOH . The tubes were rotated for different durations. After each wash cycle, the wash solution was analyzed for fluorescence. The results can be summarized in Table 3.2.

Rotation time (hrs)	Fluorescence intensity of wash solution, 200 μ L (arbitrary units)		
	1 st	2 nd	Total
3	2044	1559	3063
6	2620	1439	4059
12	2907	1298	4205
24	2837	1161	3998

Table 3.2: Fluorescence of wash solution (0.2 mL aliquot) after washing TMV MIPs in 1 M NaOH for 3 hour, 6 hour, 12 hour, and 24 hour time intervals.

The amount of fluorescence in the wash solution is related to the amount of TMV that has been removed from the TMV imprinted hydrogel after washing the crosslinked hydrogels. Hydrogels were washed for a particular time cycle twice, and the fluorescence of the first wash and the total fluorescence of the two washes are reported. From Table 3.2, the 6 hour wash cycle proved to be the optimal exposure time when compared to the other wash periods. The maximum amount of TMV extracted from one wash cycle was shown at the 12 hour wash (2907 units for the initial cycle and 4205 units for total). Exposing the TMV MIPs for one 3 hours in 1 M NaOH only extracted 52.5 % of the maximum TMV possible and 69.5 % for both cycles combined. One 6 hour time period removed 90.1 % of the maximum TMV possible, and two washes removed 96.5 %. Washing the TMV imprinted polymers for 12 hours or longer makes no difference in the amount of TMV removed from a single wash cycle. When both the total amount of TMV removed from the MIP and the exposure time of the wash solution are considered, then the 6 hour wash cycle proves to be the most efficient in removing virus from the TMV imprinted hydrogel.

The amount of virus extracted from a TMV imprinted hydrogel by washing the MIPs in 1 M NaOH for 6 hour as well as 12 hour wash cycles was examined. MIPs were synthesized consisting of 35 % w/v PAA at pH 7, 15 % v/v EGDE, and 1 mg of fluorescent labeled TMV. Once the MIPs was synthesized, they were cut, placed in a plastic tube, and 50 mL of 1 M NaOH was added. The tube

was rotated for 6 hour or 12 hour time intervals, and after each time interval the wash solution was analyzed for fluorescence. The results can be summarized in Table 3.3.

Rotation time (hrs)	Wash cycle							
	% TMV template removed per cycle (overall)							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th
6	44.0	15.2	7.9	5.3	4.8	4.4	4.2	4.0
	(-)	(59.2)	(67.1)	(72.3)	(77.1)	(81.5)	(85.7)	(89.6)
12	47.4	13.5	7.3	5.7				
	(-)	(60.9)	(68.2)	(73.9)				

Table 3.3: Percentage of viral template removed from TMV MIPs using 6 hour and 12 hour wash cycles.

A calibration curve relating the fluorescence intensity of the 1 M NaOH wash solution and the amount of fluorescently labeled TMV was created and used to determine the amount of virus extracted from the TMV imprinted hydrogels. By comparing the results of the fluorescence intensity from the washes with the calibration curve, the percentage of viral template removed from the TMV MIPs can be determined. Increasing the wash time from 6 hours to 12 hours only increased the percentage of template removal of the first wash by 3.4 %. After 48

hours, the 6 hour wash cycle was able to remove more template than the 12 hour wash cycle. Therefore, a 6 hour wash cycle performed better at removing the viral template from the MIP. The results of the 6 hour wash cycle is examined further. After the 6th wash cycle, the amount of virus removed from the MIP decreases 4 %, and remains at this level of extraction after subsequent wash cycles. The percentage of template removal remaining at 4 % and not decreasing to zero may be due to a small amount of mechanical breakage of the MIPs during the wash cycles. Therefore, 6 wash cycles may be the optimal amount of washing that is needed to efficiently remove the viral template from the TMV imprinted polymer.

3.3.3 PAA/EGDE crosslinked hydrogel swelling studies

Hydrophobic polymer networks are capable of swelling in water. The swollen state of hydrogels (PAA) is a consequence of the balance between cohesive and dispersive forces acting on the hydrated chains and the hydrophilic nature of these materials is due to the presence of polar groups along the polymer chains [37, 38]. When PAA is exposed to water, the water molecules will occupy the space within the polymer matrix in order to minimize the dispersive forces between the positive amine, $-\text{NH}_3^+$, functional groups. This results in the hydrogel taking in water, increasing the weight, and thus swelling of the material.

If the binding test is conducted by placing the imprinted hydrogel in water,

the cavities will be too large to specifically bind to the target virus. The optimal amount of swelling of the hydrogel should be similar to that immediately after the MIP is synthesized (after curing but before washing). One way to reduce the swelling of the hydrogel in water is to add counter ions in the solution during the binding test. Ions will associate with the charged functional groups of the polymer matrix, reducing the repulsive forces, and therefore lowering the swelling ratio.

To investigate the effects of different ionic solutions to the swelling of PAA hydrogels, non-imprinted MIPs were synthesized and cut into disks and weighed. They were then exposed to different ionic solutions (0.1 M) for 24 hours, then reweighed. The increase in weight due to swelling was determined by comparing the weights of the hydrogel disks after synthesis and after exposure to the solution. The results of this experiment are shown in Figure 3.6.

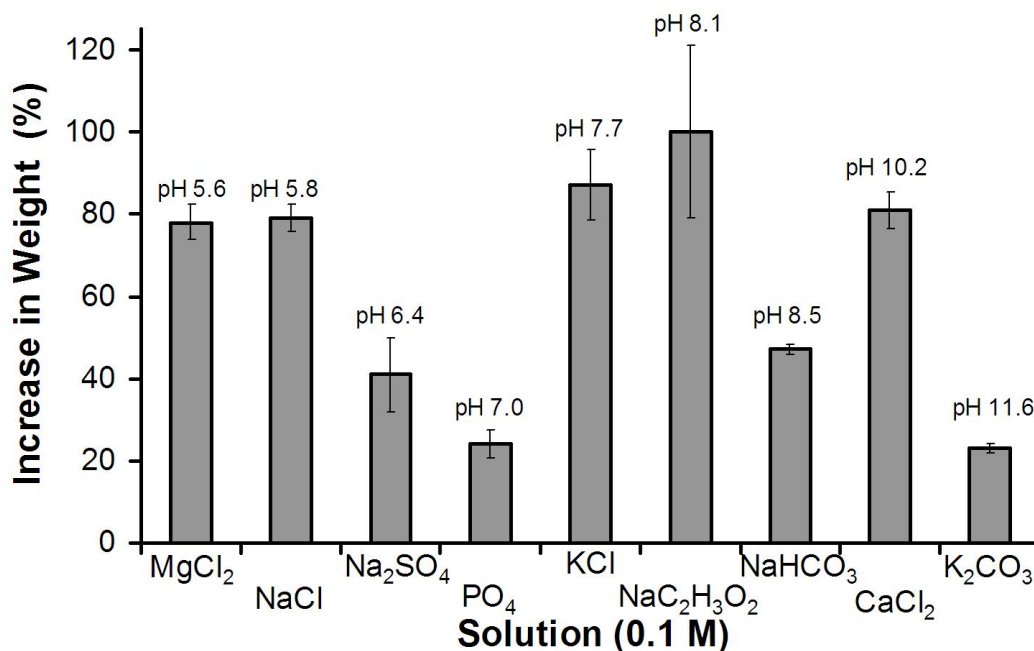


Figure 3.6: The swelling ratio of 35 % PAA crosslinked with 15 % EGDE hydrogels in different 0.1 M ionic solutions.

From the results of the swelling experiments, it can be seen that the solutions consisting of phosphate (PO_4) buffer and potassium carbonate (K_2CO_3) exhibited the lowest hydrogel weight increase, of 24 % and 23 % respectively, when compared to the other solutions. However, another condition that needs to be considered is the pH of the solution. TMV is stable between pH 4 - 10, and the most stable at pH 7. If TMV is exposed to pH conditions outside this range, the virus disassembles. Therefore the PO_4 solution outperforms K_2CO_3 in terms of low solution uptake of the polymer hydrogel as well as stability of the virus.

The reason why the phosphate buffer was able to reduce the swelling of the

PAA hydrogel is most likely due to the electronegativity of the ion. Phosphate has a -3 charge per ion. Therefore it is more effective at binding to the positive groups of the polymer matrix and reducing the dispersive forces between amine molecules. As a result of these observations, binding test were performed using viruses in 0.1 M phosphate buffer.

3.3.4 Virus binding studies

Based on the previous results, an optimized protocol to ensure homogeneity was developed. The pre-MIP mixture was composed of PAA ($\geq 25\%$), EGDE, and TMV (≤ 1.5 mg/mL). To extract the viral template, a wash solution composed of 1 M NaOH was used. Six wash cycles, with each cycle 6 hours long was used. During binding test, viral solutions of 0.1 M phosphate buffer (pH 7) was used.

TMV MIPs imprinted with wildtype TMV of various amounts (from 0.5 mg/mL to 1.5 mg/mL) were synthesized. MIPs were synthesized which were composed 15 % EGDE and either 35 % PAA or 25 % PAA at pH 7. After the viral template was washed out, TMV MIPs were placed in a solution containing 0.1 mg/mL of TMV in 0.1 M phosphate buffer, pH 7, and mixed for 6 hours. The results can be summarized in Table 3.4.

Amount of TMV initially imprinted (mg/mL)	TMV imprinted hydrogels, 15 % (v/v) EGDE, different PAA concentration	
	35 % (w/v)	25 % (w/v)
0 (control)	1.18	1.36
0.5	1.97	1.96
1	1.87	1.91
1.5	1.87	1.50

Table 3.4: Binding capacity (mg TMV/g polymer) of TMV MIPs varying initial polymer concentration and initial amount of TMV imprinted, from 0.5 mg/mL to 1.5 mg/mL.

The imprinting factor is calculated from the ratio of binding capacity of the imprinted polymer with that of the non-imprinted (control) polymer. Based on Table 3.4, the highest imprinting factor can be seen in each set at the MIP with the lowest amount of TMV imprinted (0.5 mg/mL). As the amount of TMV initially imprinted increased, the imprinting factor decreased. This may be due to residual TMV within the MIPs that may have not been removed by the 1 M NaOH wash, resulting in a decrease in available binding sites for TMV to bind. Since the imprinting factors for both the 35 % PAA and 25 % PAA MIPs increase at low amounts of TMV initially templated, low template loaded MIPs was further

investigated by synthesizing TMV MIPs initially imprinted with TMV, ranging from 0.1 mg/mL to 0.4 mg/mL. The results are presented in Table 3.5.

Amount of TMV initially imprinted (mg/mL)	TMV imprinted hydrogels, 15 % (v/v) EGDE, different PAA concentration	
	35 % (w/v)	25 % (w/v)
0 (control)	1.18	1.36
0.1	2.19	1.79
0.2	2.44	1.32
0.3	2.71	1.23
0.4	2.73	1.60

Table 3.5: Binding capacity (mg TMV/g polymer) of TMV MIPs varying initial polymer concentration and initial amount of TMV imprinted, from 0.1 mg/mL to 0.4 mg/mL.

The binding capacity exhibited by the MIPs composed of 25 % PAA seems to not exhibit any trend, ranging between 1.23 and 1.79. However, the binding capacity exhibited by the MIPs composed of 35 % PAA seems to increase as the amount of virus initially imprinted increased from 0.1 mg/mL to 0.3 mg/mL, then leveling off between 0.3 mg/mL and 0.4 mg/mL. The highest binding capacity of 2.73 mg TMV / g polymer was seen by the MIP imprinted with 0.4 mg/mL.

The theoretical binding capacity, which is the maximum amount of virus the TMV MIP can bind assuming completion of reaction and removal of all virus template, was calculated to be 2.32 mg TMV / g polymer. This number was determined by dividing the amount of virus initially imprinted polymer by the amount of polymer used for imprinting, then corrected by adding the amount of non-specific binding (the binding capacity of the control MIP). The observed binding capacity of 2.73 was 17 % higher than the theoretical binding capacity. A completely enclosed cavity is not needed to bind to TMV. During the mixing process, TMV MIPs break into small pieces, exposing cavities on the surface. These cavities may be half of the original enclosed cavity, but may still bind to the target virus. The binding of TMV to the imprinted hydrogels is believed to occur mostly on the surface. The TMV virions may not be able to travel through the matrix and bind to a complementary cavity. Surface binding may be the reason why the observed binding capacity is higher than theoretically expected.

The imprinting factor of this MIP (35 % PAA, 15 % EGDE, and 0.4 mg/mL TMV) was 2.31. This factor is calculated by dividing the binding capacity of the imprinted polymer by the binding capacity of the non-imprinted polymer. This particular MIP showed the highest imprinting factor when comparing the results of Table 3.4 and Table 3.5.

The TMV MIP consisting of 35 % PAA, 15 % EGDE, and 0.4 mg/mL TMV was further examined for its ability to bind to a non-targeted virus. *Tobacco*

necrosis virus (TNV) was used when conducting the binding test. TMV MIPs were placed in a solution containing 0.1 mg/mL of TNV in 0.1 M phosphate buffer, pH 7, and mixed for 6 hours. The binding capacity of the TMV imprinted hydrogel to TNV was 0.35 mg TNV / g polymer, while the non-imprinted (control) hydrogel was 0.65 mg TNV / g polymer. Both TMV imprinted and non-imprinted (control) hydrogels show low binding capacity to TNV when compared to the binding capacity of TMV shown in Table 3.5. TNV seems to naturally have a low affinity to the PAA/EGDE matrix when compared to TMV as evidenced in the binding capacity of TNV and TMV to the control polymer. However, the TMV MIP exhibits a lower binding capacity to TNV than the non-imprinted (control) hydrogel. The difference between the control and TMV imprinted MIPs are the cavities within the imprinted hydrogels which are not present within the control. These cavities are complementary to TMV (rod shaped approximately 300 nm in length and 18 nm in diameter). Because these cavities are specific to TMV, they may be reducing the ability for TNV to bind (spherical shaped and 24 nm in diameter). TNV does not have the same shape and size than these cavities. If the TNV is able to enter the cavity, the non-complementary shape may result in low amount of functional group associations between the polymer matrix and the non-targeted virus. This may be the reason why the TNV virus binds higher to the control hydrogel when compared to the TMV imprinted hydrogel.

When comparing the results of the binding capacities of Figure 2.4 and Ta-

ble 3.5, two improvements can be seen. First, the the imprinting factor has increased from 2.1 to 2.3. The second is that the non-specific binding (binding of the non-imprinted or control polymer) has decreased from 4.22 to 1.18. These results shows that using optimized procedures, TMV MIPs with better selectivity can be achieved. The TMV imprinted hydrogels exhibit a lower binding capacity to TNV than when exposed to TMV. This may be due to cavities within the imprinted hydrogels that are complementary to TMV. The TNV does not have the same shape as the binding cavities, thus having a lower affinity to the hydrogels than TMV. These results can be summarized in Figure 3.7. These results show that the process of molecular imprinting can create shape selective cavities within an imprinted hydrogel that can selectively bind to a larger amount of target virus than non-targeted viruses.

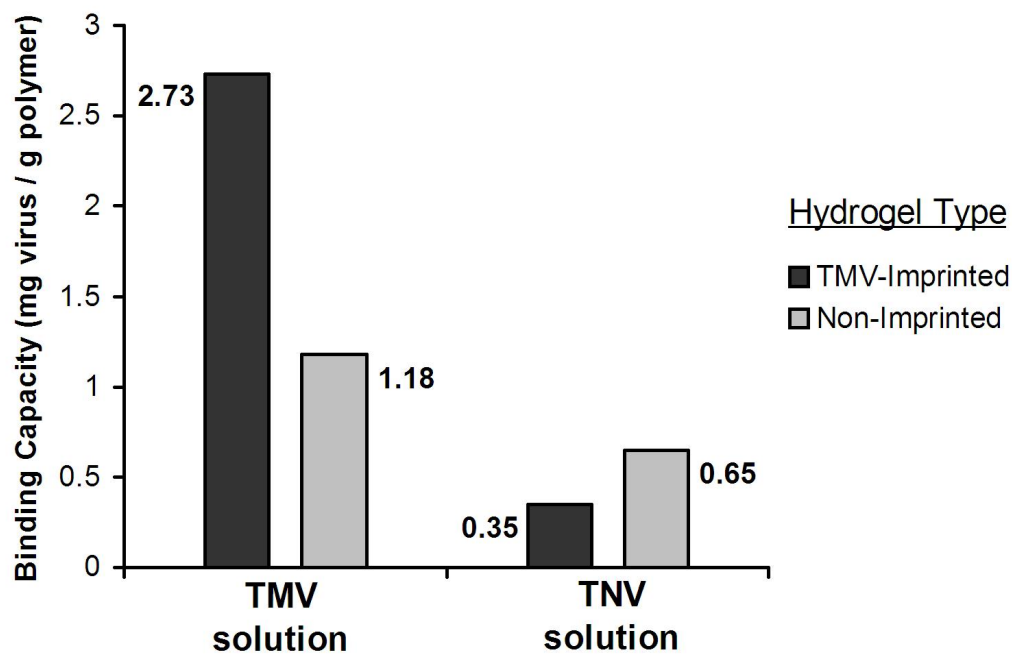


Figure 3.7: Binding capacity of TMV MIPs, initially imprinted with 0.4 mg/mL TMV, placed in 0.1 mg/mL solution of TMV or TNV and mixed for 6 hours.

3.4 Conclusions

The MIP pre-polymerization mixture must be free of aggregation in order to ensure the formation of cavities within the polymer matrix that are complementary to the virus template. Initially, when small amounts of PAA chains are added to a solution containing TMV, the positively charged chains will neutralize their net charge by binding multiple negatively charged virus particles. This process will tether virus rods together, effectively increasing the concentration of TMV at a particular point, thus causing aggregation. The size and amount of aggregate

formation will increase as more polymer chains are added to the system until all virus particles are associated with polymer chains. Adding more PAA to the system will result in multiple polymer chains binding to a single virus particle resulting in less aggregation, until one virus particle is completely enveloped by polymer chains and that no chain interacts with two or more viruses. At this point, no aggregation is present in the system. A minimum concentration of 0.0001 % PAA (w/v) and 1 mg of TMV (1 mL total volume) results in aggregate formation in the system, which persists until a polymer concentration of 30 % (w/v). It is at this high polymer concentration that MIP synthesis should occur to ensure the optimal formation of cavities complementary to TMV virus particles.

The proper wash solution must be chosen for its ability to successfully remove the templated virus from the crosslinked polymer. If the templated virus is not removed from within the polymer matrix, there will be no cavities formed able to rebind to the target virus, resulting in loss of selectivity and affinity. A wash solution of 1 M NaOH removed the highest amount of viral template TMV from the crosslinked MIP (82.40 %) when compared to other wash solutions because the high pH of the wash solution caused both the degradation of TMV virions as well as the neutralization of the positively charged polymer amine groups binding to the overall negative charge on the virus structure. Both viral degradation and neutralization of the amine functional group results in the efficient release of the

virus template into the wash solution. The optimization of the amount of virus template removed using different wash protocols provides a simple and robust method to create virus MIPs with superior affinity and selectivity capabilities. TMV MIPs washed with 1 M NaOH was further investigated. When considering the percent of template removed as well as time needed, washing the virus imprinted hydrogels using 6 hour wash cycles for 6 cycles proved to be most efficient at removing the virus, with 81.50 % template removed.

Using these results to optimize the procedure for MIP synthesis, TMV imprinted hydrogels were synthesized with varying polymer concentration as well as initial amount of TMV imprinted. Once washed and dried, binding test were performed on TMV MIPs to determine binding capacity, and more importantly, imprinting factor. The highest imprinting factor of 2.3 resulted from the MIP composed of 35 % PAA at pH 7, 15 % EGDE, and 0.4 mg/mL TMV. But more importantly, the non-specific binding, or binding of the non-imprinted (control) polymer was very low, with a binding capacity of 1.18. The TMV imprinted hydrogels exhibit a lower binding capacity to TNV (0.35) than when exposed to TMV. This may be due to cavities within the imprinted hydrogels that are complementary to TMV. TNV does not have the same shape and size than these cavities. If the TNV is able to enter the cavity, the non-complementary shape may result in low amount of functional group associations between the polymer matrix and the non-targeted virus. These results show that the process of molec-

ular imprinting can create shape selective cavities within an imprinted hydrogel that can bind to a larger amount of target virus than non-targeted viruses, and by using optimized procedures, TMV MIPs with better shape selectivity can be achieved.

Chapter 4

Future work

There are many opportunities to advance the study of molecularly imprinted polymers and viruses beyond this dissertation. It is hoped that the suggestions contained in this section will lead to productive applications of the concepts advanced in this writing.

The binding characteristics of the TMV MIPs should be studied when exposing the imprinted hydrogel to a viral mixture such as TMV and TNV in solution. This has shown to be a difficult task since current techniques to determine concentration of the virus in solution has employed a UV/VIs spectrophotometer (measuring the absorbance of the solution at 260 nm, 280 nm, and 325 nm). If two viruses are present in the solution, there is no way to distinguish the concentration of each virus. One suggestion to this problem is to use fluorescently labeled viruses. TMV has already been genetically modified to contain an extra cysteine residue which is located on the outside of the coat protein and exposed

to the solution. Utilizing this modification, the fluorescent label, Texas Red maleimide, can be covalently attached to this residue, thus fluorescently labeling the virus. Hopefully, this same procedure can be done with TNV, but using a different fluorescent label that does not overlap in the excitation wavelength of Texas Red maleimide. Once this is done, binding test can be conducted using the two fluorescently labeled virus in the same solution, and the concentration of each virus can be determined within the solution as well as within the hydrogel.

The binding isotherm of the TMV imprinted hydrogels to the targeted and non-targeted viruses is an important characteristic of these MIPs. This can be determined by exposing the virus MIP to the virus solution for different periods of time and determining the binding capacity at each point. However, this may be difficult because TMV has shown to degrade when binding test are performed for longer than 6 hours. Degradation of TMV can be observed by the decrease in purity of the viral solution. A binding test was conducted using 35 % PAA, 15 % EGDE TMV MIPs ranging from 0.4 mg/mL to 0.1 mg/mL of TMV initially imprinted, and exposing them to a 0.1 mg/mL solution of TMV for 24 hours. Aliquots were taken every 6 hours and analyzed for purity. The purity was averaged for all samples, and the results shown in Figure 4.1.

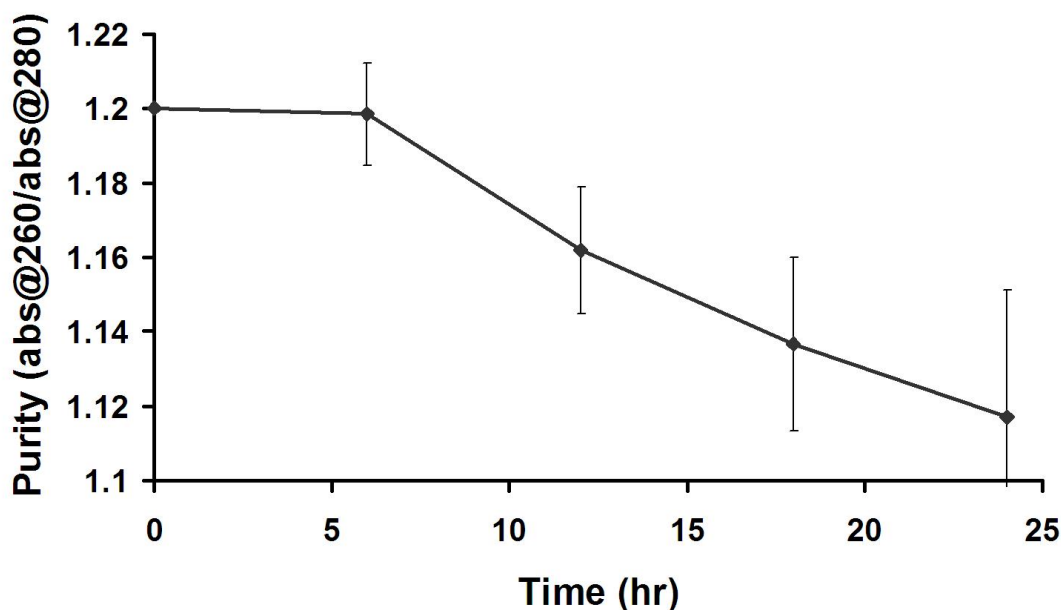


Figure 4.1: Purity of TMV virus solution during a 24 hour TMV MIP binding test.

As shown in Figure 4.1, the purity begins to decrease after 6 hours. At that point, it is very difficult to determine whether the virus is bound to the imprinted hydrogel, or degrading in the solution. One way to prevent degradation is to perform binding tests in low temperature conditions. Binding tests can be performed on the MIPs to determine if they perform better at low temperatures.

A TNV imprinted hydrogel can be synthesized to determine if the virus imprinting procedure can be used to create MIPs that are specific to different viruses. The same binding procedures can be used to determine the binding capacity of the imprinted hydrogel to targeted and non-targeted viruses.

Atomic force microscopy (AFM) can be used to visualize the complementary cavities within the virus imprinted hydrogel. Initial experiments have already been performed using TMV imprinted hydrogel which have not been washed (TMV is still present within the MIP). Once the hydrogel was synthesized, it was cut and analyzed using the AFM. The results can be seen in Figure 4.1.

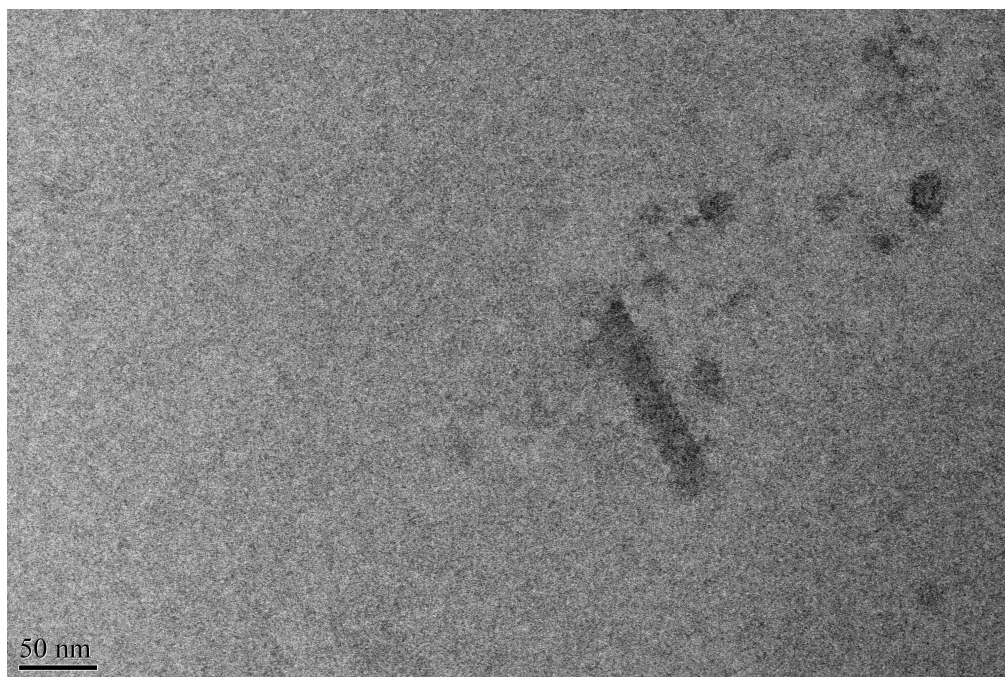


Table 4.1: An AFM image of a TMV imprinted hydrogel with TMV still present within the unwashed MIP (courtesy of Xin Zhang).

Figure 4.1 shows an image of the TMV MIP using AFM on tapping mode. The dark color is a result of a different density of material. The dark rod shape in the middle of the figure may be part of a TMV rod-shaped particle. Although it is not 300 nm in length, it is approximately 18 nm in diameter. Because TMV

rods are in a random orientation within the imprinted hydrogel, the virus has to be within the plane of the blade when the MIP is cut, in order to get a dark rod that is 300 nm in length. This AFM shows promise in visually observing a cavity within a virus imprinted hydrogel.

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