Optimization of Virus Imprinting Methods To Improve Selectivity and Reduce Nonspecific Binding

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Received August 2, 2007; Revised Manuscript Received September 25, 2007

Molecular imprinting is a technique that creates synthetic materials containing highly specific receptor sites that have an affinity for a target molecule. When large particles such as viruses are imprinted, special consideration must be taken to ensure the formation of complementary cavities. Factors that influence imprint formation, include uniformity of the precross-linked mixture and release of the virus template after cross-linking. In this study, tobacco mosaic virus (TMV) was used as a model virus. Polymer−virus aggregates formed when poly(allylamine hydrochloride) (PAA) was mixed with TMV at low polymer concentrations (<0.0001% w/v), but such aggregates were prevented at high polymer concentrations (>25% w/v). Various wash protocols were compared for their ability to remove the virus template from the cross-linked molecularly imprinted polymer (MIP), with sodium hydroxide (1 M) exhibiting the best performance. On the basis of these results, optimized MIPs targeted for TMV virus were synthesized, exhibiting a high affinity to TMV (imprinting factor of 2.3) and low affinity to tobacco necrosis virus, the nontarget virus.

Introduction

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. One popular method to synthesize molecularly imprinted polymers (MIPs) involves cross-linking functional polymer chains in the presence of a template.[1−3] The polymer chains associate with the template through their respective functional groups. A cross-linker is then added to covalently connect polymer chains using the functional groups that have not associated with the template. Upon reaction of polymer, template, and cross-linker, the template is trapped within the three-dimensional polymer network matrix. The noncovalent 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. For large templates like viruses, we refer to the term "molecular imprinting" only as a method to produce high-affinity polymers for these specific viruses and do not imply that imprinting has been achieved at the molecular level.

There are many factors that need to be considered when developing a procedure for MIP synthesis. One is the uniformity of the prepolymerized MIP mixture. If the individual reagents (such as polymer and template) aggregate when mixed together, then the resulting three-dimensional polymer will contain cavities complementary to such aggregate formations, leading to increased nonspecific binding to the target molecule, and loss in selectivity.[4] Another factor is the ease of release of the template to create a complementary cavity in the cross-linked polymer. 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 cross-linked hydrogel, then a recognition site will not be created to rebind the target molecule.[5] Methods must be developed to prevent aggregate formation prior to polymerization and cross-linking of the MIP and to maximize the removal of the template to complete the molecular imprinting process. 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 better understanding the interactions of the virus template with the polymer matrix and optimizing the method of template removal from the cross-linked polymer through appropriate wash protocols.

The Kofinas group has first reported virus MIPs synthesized to selectively bind TMV. This work involved the use of poly(allylamine hydrochloride) (PAA) cross-linked with epichlorohydrin (EPI) in the presence of TMV virus template to create the MIP.[3] However, the resulting hydrogel appeared cloudy and is thought to contain polymer−virus aggregation. To avoid this problem, the cross-linker was changed to ethylene glycol diglycidyl ether (EGDE). EGDE has shown to be a more effective cross-linker compared to EPI because of its improved

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10.1021/bm7008526 CCC: $37.00
Published on Web 11/14/2007

solubility in water. It was observed that the polymer and virus form an inhomogeneous 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 cross-linked MIP. Moreover, the amount of virus extracted from the MIP in different wash solutions was compared to determine the optimal conditions for template removal and thus better understand the conditions needed to successfully remove the templated virus from the imprinted polymer matrix. On the basis of the results, an optimized procedure for the synthesis of TMV MIPs was developed. Binding tests using the TMV imprinted hydrogels in solutions of TMV (target virus) or tobacco necrosis virus (TNV), a nontarget virus, were used to determine the effects of the optimized procedure on viral affinity.

Experimental Section

Materials. Poly(allylamine hydrochloride) ($M_w = 15000$), allylamine (>99%), ascorbic acid, ethylene glycol diglycidyl ether (50%, technical grade), magnesium chloride hexahydrate (reagent plus ≥99%), potassium chloride (99.0−100.5%, ACS reagent), sodium acetate (molecular biology tested), sodium bicarbonate (99.7–100.3%, ACS reagent), sodium hydroxide (99.998%), sodium phosphate dibasic (ultra, ≥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 (hydroxymethyl)aminomethane (molecular biology grade), polyethylene glycol ($M_w = 8000$, molecular biology grade), potassium chloride (ACS grade), and sucrose (ultracentrifugation grade) were purchased from Fisher Chemicals (Suwanee, GA). Calcium chloride (purified), chloroform (ACS grade), hydrochloric acid (ACS reagent), potassium carbonate (anhydrous, grandular, ACS reagent), sodium chloride (A.C.S reagent), and sodium sulfate (ACS 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.

Virus Purification and Fluorescent Labeling. Wild-type TMV from infected *Nicotaina tobacium* were isolated and purified as described by Gooding and Hebert.[6] Virions were further purified by centrifugation at 22000 rpm in a Beckman SW28 rotor for 2 h 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 30000 rpm in a Beckman TI70 rotor for 2 h 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 cm/mg. Wild-type TNV was isolated and purified using a similar procedure. For this virus, the centrifugation of the sucrose density gradient was conducted at 25000 rpm for 2 h. Virus concentrations for TNV were determined by absorbance at 260 nm, using an extinction coefficient of 5 cm/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.[7] Once the modified TMV virions were labeled with Texas Red, they were purified using the same purification procedure as the wild-type TMV.

Virus Aggregation Studies. One milligram of TMV was placed in a 1.5 mL microcentrifuge tube containing poly(allylamine hydrochloride) in water at different concentrations (% w/v) ranging from 5 to 35%. The pH was adjusted to 7 using 10 M NaOH prior to addition of the virus. The total volume was adjusted to 1 mL using water. 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 mL of the solution on a glass slide and observing through an Olympus BX60 optical microscope. One milligram of TMV was also mixed with allylamine (1% v/v and 20% v/v, pH 7). Aggregate formation was visually observed.

Virus MIP Synthesis. A 0.7 mL portion of 50% (w/v) PAA was placed in a 2 mL microcentrifuge tube. Ten microliters of 10 M NaOH was added to the polymer solution with mixing. One milligram of fluorescent labeled TMV was then added to the polymer mixture, and the solution was mixed. The total volume was adjusted to 1 mL using H_2O prior to cross-linking. Then 0.15 mL of ethylene glycol diglycidyl ether (EGDE) was added with mixing and the polymer mixture was allowed to cure for 24 h.

Template Removal from Virus MIP. Wash solutions of H_2O, 1 M NaCl, 6 M urea, and 1 M NaOH were investigated for their ability to remove the viral template from the polymer matrix. The same procedure is used for all the types of wash solutions: Four TMV MIPs were synthesized, cut using a scalpel, and placed in a 50 mL plastic tube. Fifty milliliters of 1 M NaOH was added to the tube, and the tube was rotated for 6 and 12 h time intervals. After each time interval, 1 mL of the wash solution was collected and 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 was calculated by placing fluorescently labeled TMV in each wash solution (final concentration of 0.04− 0.2 mg/mL) and measuring the fluorescence. After five wash cycles, the gels were further washed with H_2O 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 a Bio-Rad ChemiDoc XRS imaging system under ultraviolet light.

Wild-Type TMV MIP Synthesis. Wild-type TMV was used as the viral template. A 0.7 mL portion of 50% (w/v) PAA was placed in a 2 mL microcentrifuge tube. Ten microliters of 10 M NaOH was added to the polymer solution with mixing. Wild-type TMV of various amounts ranging from 0.01 to 1.5 mg was then added to the polymer mixture with mixing. Then 0.15 mL of ethylene glycol diglycidyl ether (EGDE) was added with mixing and the polymer mixture was allowed to cure for 24 h. The total volume was adjusted to 1 mL using H_2O prior to cross-linking. The MIP solution was then placed on a Petri dish and allowed to cure for 24 h at room temperature. Once cured, the MIPs were cut into 2 mm by 2 mm squares and placed in a 50 mL plastic tube. Fifty milliliters of 1 M NaOH solution was added to the tube and rotated for a 6 h interval using a Barnstead International Labquake shaker. The wash solution was discarded, new solution was added, and the cycle was repeated five more times. After the MIPs were washed with 1 M NaOH, the gels were then washed with 50 mL of deionized water for 4 h intervals until the pH of the wash solution became 7. The cross-linked polymers were removed from the solution and dried at 55 °C in an oven. The drying temperature has been observed to have no effect on the appearance or mecahnical integrity of the gels.

Virus Binding Experiment. Fifteen milligrams 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 h time interval. After the tubes were rotated, 0.1 mL of the virus solution was analyzed using a Perkin-Elmer Lambda 25 UV/vis spectrophotometer. To determine the concentration of TNV in solution after conducting the binding test, the Pierce bicinchoninic acid (BCA) protein assay kit from Thermo Scientific was used. After the binding test was performed, a 50 μL aliquot was taken and mixed with 1 mL of the BCA reagent and incubated in a water bath at 37 °C for 30 min. After incubation, the sample was cooled to room temperature and then analyzed for absorbance using a UV/vis spectrophotometer at 562 nm.

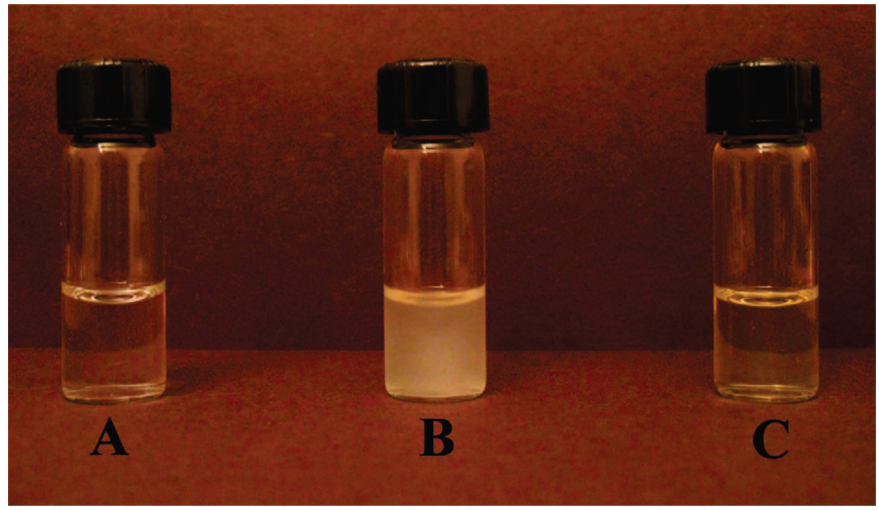

Figure 1. Virus aggregation as a function of polymer concentration: (A) 1 mg/mL TMV in H_2O; (B) 1 mg/mL TMV in 1% (w/v) PAA solution; (C) 1 mg/mL TMV in 35% (w/v) PAA in H_2O.

Results and Discussion

Aggregate Formation in the Polymer−Virus Solution. TMV was chosen as the model virus because it is well studied and characterized.[8] PAA was chosen because it is water soluble and contains primary amine groups with a pK_a of 9.67.[9] At a neutral pH, TMV and PAA have an overall negative and positive charge, respectively. EGDE was chosen for its ability to cross-link 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 visualized in Figure 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.

Aggregation has also been reported 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.[10] 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

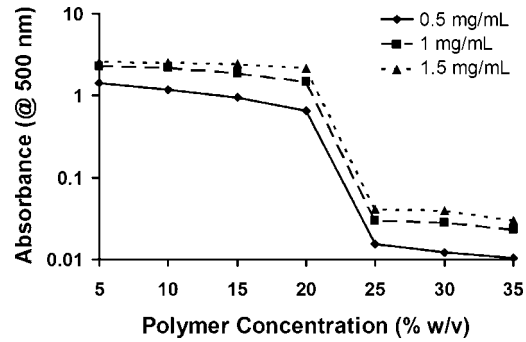

Figure 2. Aggregation of TMV and PAA as a function of % (w/v) PAA (pH 7). TMV concentrations of 0.5 mg/mL, 1 mg/mL, and 1.5 mg/mL were investigated.

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 no polymer chain was able to associate with multiple protein molecules, thus preventing aggregate formation and any observed turbidity in the solution.

Similar to the BSA/PAA system, strong noncovalent 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 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. All three tested TMV concentrations showed aggregation at 20% but no aggregation at 25%. An aliquot of the polymer−virus solution was placed on a glass slide and examined using an optical microscope. From the micrograph (Figure 3), large aggregates can be seen which were as long as 50 mm and as wide as 10 mm. A polymer concentration of 15% (w/v) was also examined (Figure 3). At this concentration, smaller and dispersed aggregates can be seen. The measured turbidity at 15% (w/v) polymer concentration was lower than that 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 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

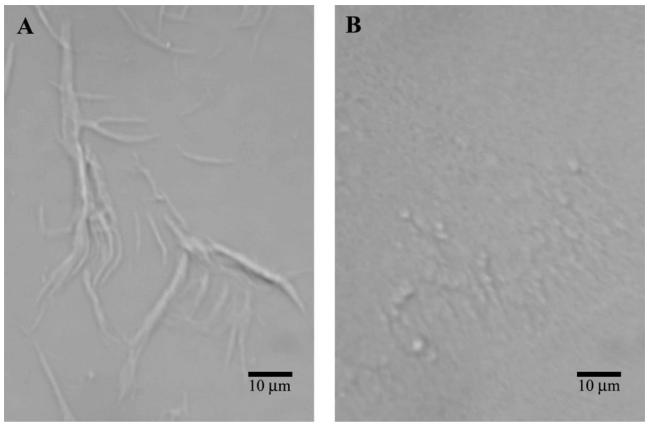

Figure 3. Optical micrographs of virus aggregation at (A) 5% (w/v) and (B) 15% (w/v) PAA concentrations. Concentration of TMV was 1 mg/mL, and solution pH = 7.

for tethering to occur. Instead, what is believed to be happening is a neutralization of charge. A solution containing only TMV virus appears clear. This may be due to the overall negative charge of the virions repelling each other and dispersing them in solution. When PAA polymer 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 thus neutralizing their charge. This charge neutralization will reduce the repulsive forces between virions, resulting in aggregation, and is 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, dispersement of PAA/TMV aggregates in the solution, and decreased turbidity until the solution becomes clear again.

The synthesis of the MIP hydrogel should occur at a polymer concentration where virus 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 cross-linked 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 rebind to single targeted virus particles. A PAA concentration of 25% or higher should be chosen for all imprinting procedures to ensure that virus aggregation is not occurring during the process of imprinting.

Bach et al.[11] have reported that in water the TMV virus particle has 1000 negative charges. On the basis of the information on PAA from SigmaAldrich (MW = 15000, monomer unit MW = 93.548, DPI = 160) each polymer chain has 160 positive charges. Therefore, the maximum turbidity should occur when the ratio of polymer chain to virus particles is 6.25:1. For our experimental conditions, a minimum polymer concentration of 25% is needed to obtain a clear solution. For 1 mg of TMV, there are 1.528×10^{13} particles, or 1.528×10^{16} negative charges. At 25% w/v PAA, there are 1.00367×10^{19} chains, or 1.6059×10^{21} positive charges. Therefore, to prevent aggregation, a ratio of polymer chains to virus particles should be 656825:1, which corresponds to a charge ratio of 105064:1.

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 H_2O, 1 M NaCl, 6 M urea, or 1 M NaOH. The extraction of the virus from the MIP was quantified by the amount of fluorescence in the solution after the wash was performed. The measured fluorescence was related to the amount of TMV present in solution using a calibration curve of fluorescence versus TMV concentration for each solution. The percent template removed was calculated by dividing the amount of virus extracted by the initial amount of virus that was imprinted. The ability of the various wash solutions to remove the virus from the cross-linked MIPs is summarized in Table 1.

Table 1. Percentage of TMV Extracted from TMV-Imprinted Polymers after Washing with H_2O, 1 M NaCl, 6 M Urea, and 1 M NaOH and Cummulative TMV Removal Amount after Five Wash Cycles

wash solution	% TMV template removed	
	first wash	fifth wash (final)
H_2O	0.03	0.12
NaCl	0.06	0.22
6 M urea	12.09	21.16
1 M NaOH	41.15	82.40

Virus MIPs washed with H_2O resulted in the lowest amount of template extracted. This is an indication that the polymer matrix was associating with the virus through its amine groups and not by just 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 of the salt, as evidenced by only a low amount of viral template extracted. A solution composed of 6 M urea has been known to denature TMV.[12,13] However, this solution was only able to remove a moderate amount of templated virus. A 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 is known to rapidly degrade.[14] The second reason why NaOH was successful in virus extraction from the MIP was that high pH conditions (pH > pK_a of PAA) lead to the amine groups of the PAA becoming uncharged. The pH values of H_2O, NaCl, and urea were 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. The fluorescence intensity emitting from TMV MIPs after being washed with H_2O, NaCl, urea, and NaOH can be visually observed in Figure 4. Both the MIPs washed with 6 M urea and 1 M NaOH emitted less red fluorescence than those washed with H_2O and 1 M NaCl, with the 1 M NaOH emitting the least fluorescence out of the four washed hydrogels. Hydrochloric acid (HCl) was also investigated 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 hydrogel at these conditions.

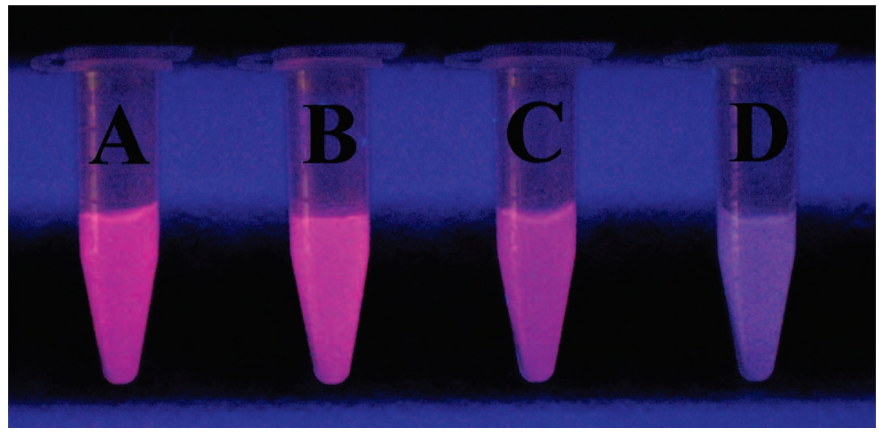

Figure 4. Fluorescence of TMV-MIPs after washing with (A) H_2O, (B) 1 M NaCl, (C) 6 M urea, and (D) 1 M NaOH.

Table 2. Percentage of Viral Template Removed from TMV MIPs Using 6 and 12 h Wash Cycles

rotation time (h)	% TMV template removed per cycle (overall)							
	first	second	third	fourth	fifth	sixth	seventh	eighth
6	44.0 (−)	15.2 (59.2)	7.9 (67.1)	5.3 (72.3)	4.8 (77.1)	4.4 (81.5)	4.2 (85.7)	4.0 (89.6)
12	47.4 (−)	13.5 (60.9)	7.3 (68.2)	5.7 (73.9)				

Table 3. Binding Capacity (mg of TMV/g of polymer) of TMV MIPs Varying Initial Polymer Concentration and Initial Amount of TMV Imprinted, from 0.5 to 1.5 mg/mL

amt 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

As reported by Choi et al.,[14] it is expected that all of the TMV RNA is degraded and removed by the 1 M NaOH washing. After determination that a wash solution of 1 M NaOH was the most effective 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. Two exposure time cycles, 6 and 12 h, were investigated. 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 and placed in a plastic tube and 50 mL of 1 M NaOH was added. The tube was rotated for 6 or 12 h time intervals, and after each time interval the wash solution was analyzed for fluorescence. A calibration curve relating the amount of fluorescently labeled TMV (0.2− 0.04 mg/mL) when placed in 1 M NaOH and the fluorescence intensity emitted was constructed and used to determine the amount of viral template extracted from the TMV MIP into the wash solution. The results are summarized in Table 2.

A calibration curve relating the fluorescence intensity of the 1 M NaOH wash solution to the amount of fluorescently labeled TMV was created and used to determine the amount of virus extracted from the TMV imprinted hydrogels. By comparison of 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 to 12 h only increased the percentage of template removal of the first wash by 3.4%. After 48 h, the 6 h wash cycle was able to remove more virus template than the 12 h wash cycle. Therefore, a 6 h wash cycle performed better at removing the viral template from the MIP. The results of the 6 h wash cycle were further investigated. After the sixth wash cycle, the amount of virus removed from the MIP decreases by ~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 small amount of mechanical breakage of the MIPs during the wash cycles. It is desirable to reduce mechanical breakage because this may destroy the complementary cavities and increase nonspecific binding. Such mechanical breakage may be creating small hydrogel pieces which may still contain fluorescent TMV (trapped inside the matrix) that may pass through a 0.22 μm filter. Such solution will have flurorescent TMV which will show up as fluorescence. Therefore, six wash cycles may be the optimal amount of washing that is needed to efficiently remove the viral template from the TMV imprinted polymer. Six hour wash intervals are more efficient, since the same approximate amount of virus is removed in half the time.

Virus Binding Studies. On the basis of these results, an optimized protocol to ensure homogeneity during MIP synthesis was developed. The pre-MIP mixture was composed of PAA (>25%), EGDE, and TMV (<1.5 mg/mL). To extract the viral template from the cross-linked hydrogel, a wash solution consisting of 1 M NaOH was used. Six wash cycles were used, with each cycle being 6 h long. TMV MIPs imprinted with wild-type TMV of various amounts (from 0.5 to 1.5 mg/mL) were synthesized. MIPs were synthesized composed of 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 h. The results are summarized in Table 3.

The imprinting factor is calculated from the ratio of virus binding capacity of the imprinted polymer with that of the nonimprinted (control) polymer. Virus binding capacity is referring to the amount of binding of the MIP to either TMV or TNV measured in milligram of virus per gram of polymer. On the basis of the data of Table 3, the highest imprinting factor can be found 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@DV

Table 4. Binding Capacity (mg of TMV/g of polymer) of TMV MIPs Varying Initial Polymer Concentration and Initial Amount of TMV Imprinted, from 0.1 to 0.4 mg/mL

amt 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

low amounts of TMV initially templated, low template loaded MIPs were further investigated by synthesizing TMV MIPs initially imprinted with TMV, ranging from 0.1 to 0.4 mg/mL. These results are presented in Table 4. The error in determining binding capacities for Tables 3 and 4 has been estimated to be 25%.

The TMV virus binding capacity exhibited by the MIPs composed of 25% PAA showed no trend, ranging in values between 1.23 and 1.79 mg of TMV/g of polymer. However, the virus binding capacity exhibited by the MIPs composed of 35% PAA seems to increase as the amount of virus initially imprinted increased from 0.1 to 0.3 mg/mL and then levels off between 0.3 and 0.4 mg/mL. The highest binding capacity of 2.73 mg of TMV/g of polymer was seen by the MIP imprinted with 0.4 mg/mL. The imprinting factor for this MIP (35% PAA, 15% EGDE, and 0.4 mg/mL TMV) was 2.31. This particular MIP showed the highest imprinting factor when comparing the experimental results of Tables 3 and 4.

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 nontarget virus. Tobacco necrosis virus (TNV) was thus used when conducting the binding test. TMV MIPs were placed in a solution containing 0.1 mg/mL of nontarget TNV in 0.1 M phosphate buffer, pH 7, and mixed for 6 h. The results of the binding test comparing TMV MIPs and nonimprinted MIPs in solutions of TMV or TNV viruses are summarized in Figure 5.

When the results of the binding capacities of TMV MIPs synthesized using PAA as the polymer and EPI as the cross-linker are compared, two improvements can be seen over what has been previously reported.[3] The imprinting factor has increased from 2.1 to 2.3, and the nonspecific binding (binding of the nonimprinted control polymer) has decreased from 4.22 to 1.18. These results demonstrate that with optimized procedures, TMV MIPs with better selectivity can be achieved. The nontarget virus, TNV, showed a much lower affinity relative to the target TMV, indicating 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 nontarget viruses.

Conclusions

The MIP prepolymerization 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

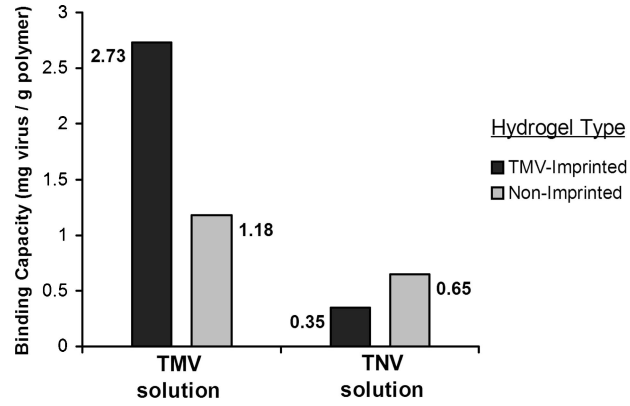

Figure 5. Binding capacity of TMV MIPs, initially imprinted with 0.4 mg/mL TMV, placed in 0.1 mg/mL solution of TMV or TNV and mixed for 6 h.

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 a virus particle is completely enveloped by polymer chains and no polymer chain interacts with two or more viruses. At this point (minimum 25% PAA (w/v)), 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 up to 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 cross-linked 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 cross-linked MIP (82.40%) when compared to other wash solutions, because the high pH of the wash solution caused both the degradation of TMV virions and 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 result 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 were further investigated. When the percent of template removed as well as time needed to complete the washes was considered, washing the virus imprinted hydrogels using 6 h wash cycles for six cycles proved to be most efficient at removing the virus, with 81.50% template removed.

With 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 tests were performed on TMV MIPs to determine binding capacity and imprinting factor. The highest imprinting factor of 2.3 resulted from an MIP composed of 35% PAA at pH 7, 15% EGDE, and 0.4 mg/mL TMV. But more importantly, the nonspecific binding, or binding of the nonimprinted (control) polymer was very low, with a binding capacity of 1.18. TNV, the nontarget icosahedral virus, showed a lower affinty to rodlike TMV MIPs. A better comparison would have been the measurement of the binding

specificity of a rodlike virus. Our results suggest that the process of molecular imprinting can create shape-selective cavities within an imprinted hydrogel that can bind to a larger amount of target virus than nontargeted viruses, and by using optimized procedures, TMV MIPs with better shape selectivity can be achieved.

Acknowledgment. This material is based upon work supported by the United States Department of Agriculture National Research Initiative Competitive Grants Program (USDA-NRICGP) Grant No. 2005-35603-16278.

References and Notes

(1) Wizeman, W. J.; Kofinas, P. Molecularly imprinted polymer hydrogels displaying isomerically resolved glucose binding. *Biomaterials* **2001**, *22*, 1485–1491.
(2) Parmpi, P.; Kofinas, P. Biomimetic glucose recognition using molecularly imprinted polymer hydrogels. *Biomaterials* **2004**, *25*, 1969–1973.
(3) Bolisay, L. D.; Culver, J. N.; Kofinas, P. Molecularly imprinted polymers for tobacco mosaic virus recognition. *Biomaterials* **2006**, *27*, 4165–4168.
(4) Yungerman, I.; Srebnik, S. Factors contributing to binding-site imperfections in imprinted polymers. *Chem. Mater.* **2006**, *18*, 657–663.
(5) Hawkins, D. M.; Stevenson, D.; Reddy, S. M. Investigation of protein imprinting in hydrogel-based molecularly imprinted polymers (HydroMIPs). *Anal. Chim. Acta* **2005**, *542*, 61–65.
(6) Gooding, G. V.; Hebert, T. T. A simple technique for purification of tobacco mosaic virus in large quantities. *Phytopathology* **1967**, *57*, 1285–1286.
(7) Yi, H. M.; Nisar, S.; Lee, S. Y.; Powers, M. A.; Bentley, W. E.; Payne, G. F.; Ghodssi, R.; Rubloff, G. W.; Harris, M. T.; Culver, J. N. Patterned assembly of genetically modified viral nanotemplates via nucleic acid hybridization. *Nano Lett.* **2005**, *5*, 1931–1936.
(8) Culver, J. N. Tobacco mosaic virus assembly and disassembly: Determinants in pathogenicity and resistance. *Annu. Rev. Phytopathol.* **2002**, *40*, 287–308.
(9) Kobayashi, S.; Tokunoh, M.; Saegusa, T.; Mashio, F. Poly(Allylamine) - chelating properties and resins for uranium recovery from seawater. *Macromolecules* **1985**, *18*, 2357–2361.
(10) Ball, V.; Winterhalter, M.; Schwinte, P.; Lavalle, P.; Voegel, J. C.; Schaaf, P. Complexation mechanism of bovine serum albumin and poly(allylamine hydrochloride). *J. Phys. Chem. B* **2002**, *106*, 2357–2364.
(11) Bach, G. A.; Hollingsworth, A. D.; Koch, D. L. Electrophoretic mobility of rigid rodlike particles in dilute aqueous and glycerol suspensions: Comparison between theory and experiment. *J. Colloid Interface Sci.* **2002**, *251*, 208–213.
(12) Buzzell, A. Action of urea on tobacco mosaic virus. *J. Am. Chem. Soc.* **1960**, *82*, 1636–1641.
(13) Buzzell, A. Action of urea on tobacco mosaic virus 0.2. Bonds between protein subunits. *Biophys. J.* **1962**, *2*, 223–233.
(14) Choi, C. W.; Park, S. H.; Choi, J. K.; Ryu, K. H.; Park, W. M. Chemical degradation of tobacco mosaic virus followed by infectivity assay, reverse transcription-polymerase chain reaction and gel electrophoresis. *ActaVirol* **2000**, *44*, 145–149.

BM7008526