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Imprinted Polymer Hydrogels for the Separation of Viruses

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Summary: The goal of this research is to elucidate the mechanism of virus recognition in molecularly imprinted polymers (MIPs) using already utilized techniques. 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. Virus MIPs against tobacco mosaic virus (TMV) were synthesized. 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 cross-linked polymer.

Keywords: hydrogels; molecular imprinting; networks; swelling; virus

Introduction

Molecularly imprinted polymers (MIPs) can be synthesized by cross-linking functional polymer chains in the presence of a template.[1–4] Polymers synthesized using this technique demonstrate high affinity towards a templated target molecule. The polymer chains associate with the template through their respective functional groups. A cross-linker is then added to covalently connect the polymer chains in pairs, using the functional groups that have not associated with the template. Upon reaction of polymer, template, and cross-linker, 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.

In this investigation, we used *tobacco mosaic virus* (TMV) as a model virus to determine the optimum conditions required for efficient imprinting. MIPs have several advantages over biomolecules, such as synthesis, stability, and reusability. MIPs can be seen applied in a wide range of technologies including catalysis, separation and purification, drug delivery, and detection.[5–11] 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. When placed into a packed column and used as a purification stage, these MIPs, would act as virus-specific ''sponges'' and selectively capture the targeted virus while allowing other non-targeted molecules to pass through.

Experimental Part

The TMV-imprinted polymer hydrogels were prepared using well-established synthetic techniques.[3,4] In particular, polyallylamine (PAA) was cross-linked with

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ethylene glycol diglycidyl ether (EGDE) in the presence of the templated virus. 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, therefore, to 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 tests 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. Non-imprinted MIPs were used to investigate the swelling behavior of the polymer hydrogels in different solutions. The solutions used were 0.1 M of magnesium chloride ($MgCl_2$), sodium chloride (NaCl), sodium sulfate (Na_2SO_4), phosphate (PO_4) buffer, potassium chloride (KCl), sodium acetate (CH_3COONa), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), and potasium carbonate (K_2CO_3).

Results and Discussion

When PAA is added to a TMV solution, the positively charged groups on PAA and the negatively charged groups of TMV would interact and bind to each other, neutralizing their charge. This neutralization would reduce the repulsive forces between virions, resulting in aggregate formation, manifested by an increase in turbidity. As the amount of added positively charged PAA is increased, more TMV is neutralized. The maximum turbidity in the solution would be observed when enough PAA is added to neutralize all the negative charges of the virus particles. Further addition of PAA would result in an excess of positively charged polymers interacting with TMV, leading to an increase in the repulsive forces, dispersion of the PAA/TMV aggregates, full clarification of the solution, and elimination of the turbidity.

We determined that the synthesis of the MIP hydrogels should take place 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 individual reagents (such as polymer and template) aggregate when mixed together, the resulting three-dimensional polymer would contain cavities complementary to such aggregates, leading to increased non-specific binding to the target molecule, and loss in selectivity. 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 bind to a single targeted virus particle. A PAA concentration of higher than 25% was found to be optimal for all imprinting procedures to ensure that virus aggregation would not occur during imprinting.

When PAA hydrogels are exposed to water, the water molecules will occupy the space within the polymer matrix in order to minimize the electrostatic repulsive forces between the positively charged amine, $-NH_3^+$, functional groups. This results in the hydrogel taking in water, increasing the weight, and, therefore, the swelling of the material.

If the virus 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.

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To investigate the effects of different ionic solutions to the swelling of the 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, and reweighed. The increase in weight 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 1.

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 most stable at pH 7. If TMV is exposed to pH conditions outside this range, the virus disassembles. Thus, 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 anion. Phosphate has a charge of -3 per ion. Thus, it is more effective at binding to the positively charged groups of the polymer matrix and reducing the electrostatic repulsive forces between the polyamine chains. As a result of these observations, binding tests were performed using viruses in 0.1 M phosphate buffers. Based on these results, an optimized protocol to ensure homogeneity was developed. The pre-MIP mixture was composed of PAA (>25%), EGDE, and TMV ($< 1.5\,mg\,mL^{-1}$). To extract the viral template, a wash solution composed of 1 M NaOH was used. Six wash cycles, with each cycle lasting 6 hours, were used. During the binding test, viral solutions in 0.1 M phosphate buffers (pH 7) were used.

The binding characteristics of the TMV MIPs should be studied by exposing the imprinted hydrogel to viral mixtures containing TMV and some other virus such as TNV. This has been shown to be a difficult task since current techniques to determine concentration of the virus in solution have employed UV/vis spectroscopy (measuring the absorbance of the solution at 260 nm, 280 nm, and 325 nm). If two viruses are present in the same solution, there is no way to distinguish the concentration of each virus using this method. One suggested solution 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. Hope-

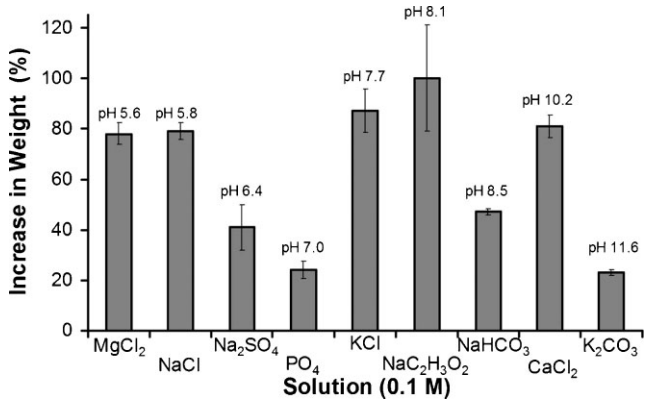

Figure 1.
Swelling ratio of 35% PAA cross-linked with 15% EGDE hydrogels in different 0.1 M ionic solutions.

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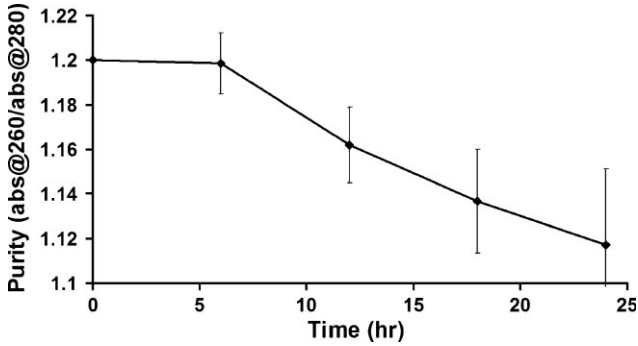

Figure 2.
Purity of TMV virus solution during a 24 hour TMV MIP binding test.

fully, 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 tests can be conducted using the two fluorescently labeled viruses 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 tests are performed for periods 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 $0.4\,mg\,mL^{-1}$ to $0.1\,mg\,mL^{-1}$ of TMV initially imprinted, and exposing them to a $0.1\,mg\,mL^{-1}$ 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 2.

As shown in Figure 2, 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.

Conclusion

Optimized methods for virus imprinting were developed using cationic hydrogels. It was found that care must be taken to ensure the prevention of 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. Methodologies that avoid template aggregation and enhance template removal were investigated for their effect on virus binding and specificity.

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