Molecular imprinting is a technique used to synthesize polymers that display selective recognition for a given template molecule of interest. In this study, the role of hydrogel electrostatic charge density on the recognition properties of protein-imprinted hydrogels was explored. Using 3-methacrylamidopropyl trimethylammonium chloride (MAPTAC) as a positively charged monomer and 2-acrylamido-2-methylpropane sulfonic acid (AMPS) as a negatively charged monomer, a number of acrylamide-based polyelectrolyte hydrogels with varying positive and negative charge densities were prepared. The imprinted hydrogels were synthesized in the presence of the target molecule bovine hemoglobin (Bhb). The ability of the hydrogels to selectively recognize Bhb was examined using a competitive template molecule, cytochrome c. The Bhb imprinted gels exhibited template recognition properties that were dependent on both the monomer charge density and on whether the chosen monomer carried a positive or
negative charge.

In addition to polyelectrolye hydrogels, polyampholyte hydrogels containing both positively and negatively charged monomers were also synthesized. The simultaneous presence of two oppositely charged monomers in the pre-polymerization mixture resulted in imprinted hydrogels with cavities that contain highly specific functional group orientation. The polyampholyte hydrogels exhibited decreased swelling when compared to their polyelectrolyte counterparts, due to the shielding of repulsive interactions between oppositely charge monomers. This decreased swelling resulted in greater template recognition, but lower selectivity, when compared to their polyelectrolyte counterparts.

In addition, we found that common agents used in template extraction may be responsible for the specific and selective binding properties exhibited by molecularly imprinted polymers in many published studies, and the effect of variations of the template extraction protocol on the MIP recognition properties were also studied in depth.
MOLECULARLY IMPRINTED POLYMERS
FOR THE SELECTIVE RECOGNITION OF PROTEINS

by

Daniel S. Janiak

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
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Dedication

To my parents. I love you and owe you everything.
Acknowledgments

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Chapter 1

Introduction

1.1 Significance

Molecularly imprinted polymers (MIPs) [71, 67, 76, 64, 50] are synthetic materials produced by the cross-linking of functional monomers or polymers in the presence of a template molecule. The template is subsequently removed, leaving cavities possessing size, shape, and functional group orientation which are complementary to 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 preferentially bind in specific locations to only the target molecule and not to similar molecules. Figure 1.1 provides a schematic of the molecular imprinting process. While MIPs have been prepared for a large number of target molecules and applications [27, 62, 11, 61] over the years, the majority of the template molecules studied have been characterized by their low molecular weight and insolubility in aqueous systems.

Much of the pioneering work in the molecular imprinting of peptides and proteins was conducted by Mosbach [3, 38, 63, 4, 2] using amino acid derivatives as template molecules to create stationary phases for chromatographic applications. The MIPs synthesized in these early studies
were tested for their ability to selectively recognize their respective target molecules in organic solvents. While these experiments laid the groundwork for the synthesis of artificial receptors capable of recognizing peptides and proteins, most molecules of biological importance are water soluble, and many natural recognition events such as antigen-antibody binding occur in aqueous media. Therefore, there exists a strong need to create artificial receptors which are capable of recognizing peptides and proteins in aqueous media in order to create materials and devices capable of mimicking natural processes. For instance, MIPs which can selectively recognize specific proteins or peptide sequences in aqueous media have the potential to be used as substrates in medical diagnostic applications. In addition, aqueous MIPs could be used as the solid phase for chromatographic analysis and purification of peptides and proteins. Finally, the synthesis of MIPs which exhibit high affinity, selectivity, and binding capacity may provide

Figure 1.1: Schematic of the molecular imprinting process.
researchers with a low-cost, easily obtainable method for studying the fundamental interactions which occur during biological recognition processes. The goal of this research is to understand the role of electrostatic charge density on the recognition and selectivity properties of protein-imprinted hydrogels. The results of this project will aid in the development of rationally designed MIPs with higher specificity and selectivity than those currently available.

1.1.1 Background

There are a number of different strategies for creating polymeric receptors targeting peptides and proteins. Functional groups forming strong template interactions with the target molecules are commonly used. Examples of this include electrostatic and metal-chelating groups [47, 26]. In addition, shape interactions between the template molecule and the synthetic receptor can be exploited. Weak interactions such as hydrophobic and hydrogen bonding have also been employed in numerous cases [56, 10, 51]. Some notable recent accomplishments are the detection of peptides using a molecularly imprinted piezoelectric sensor [44], the synthesis of MIPs in the presence of a cloned bacterial protein which are capable of selectively recognizing the authentic protein when incubated in cell extract [79], the development of MIPs with enzyme-like properties [17], and the synthesis of MIPs in the presence of proteins within chromatography columns provid-
ing a monolithic bed for the selective recognition of the imprinted protein [60]. The following sections outline a number of the approaches used to create MIPs capable of recognizing biological macromolecules.

1.1.2 The Epitope Approach

The process of molecular imprinting of macromolecules such as peptides and proteins is complicated by a number of factors. Well defined recognition sites are more easily formed by the imprinting of small molecules with rigid structures, therefore imprinting small sequences of amino acids (dipeptides, tripeptides, etc.) should be fairly straightforward. Proteins, however, are inherently capable of assuming a large number of conformations depending on a number of factors, including but not limited to, solution temperature, pH and ionic strength. Steric factors also make molecular recognition of proteins difficult, as it is extremely difficult for their large structures to move freely through highly cross-linked polymer networks. One proposed method of reducing the complications associated with the imprinting of proteins and peptides has been termed the epitope approach [57, 58, 52] to molecular imprinting. Traditionally, an epitope refers to the small active site located within the larger protein structure on an antigen, which combines with the antigen-binding site on an antibody or lymphocyte receptor [1]. Figure 1.2 shows a schematic of the epitope approach to molecular imprinting. In this technique, a small sequence of
amino acids from the larger protein target molecule is used to create the imprint. When a protein containing this specific amino acid sequence is allowed to incubate in the presence of the MIP, the entire protein can thus be recognized and bound. The epitope approach has been successfully used to bind oxytocin by imprinting the Tyr-Pro-Leu-Gly amino acid sequence [57]. In this study, synthesis of the MIP was performed in an organic environment, but subsequent rebinding experiments were performed using chromatographic methods in both aqueous-rich and aqueous-poor mobile phases. In the aqueous-poor mobile phase, hydrogen bonds and ionic interactions are the dominating factor in creating selective recognition sites. In the aqueous rich phase, ionic and hydrophobic interactions provide the dominant binding interaction.

1.1.3 Surface Imprinting

Confining the recognition sites of MIPs to surfaces is another technique which has been developed to circumvent the steric problems associated with the imprinting of biological macromolecules in aqueous systems. In a classic example of surface imprinting [65], protein imprinted surfaces were created by first adsorbing a layer of protein onto a mica substrate, followed by the deposition of disaccharide molecules onto the protein layer. Upon dehydration, hydrogen bonds were formed between hydroxyl groups on the sugars and functional groups on the protein molecules. Radio-
Figure 1.2: Schematic of the epitope approach utilized for molecular imprinting of proteins and peptides. (a) Target molecule (b) Epitope (Imprinted) portion of target molecule (c) Imprinted polymer before template removal (d) Imprinted polymer after template removal, with cavities that display shape which is complementary to the imprinted portion (epitope) of the target molecule. (e) MIP subsequent to template re-binding. The MIP selectively recognizes the imprinted (epitope) portion of the target molecule, thus, the entire target molecules is successfully bound.
frequency (RF) glow discharge plasma, a common plasma deposition technique used to create controlled thin films of fluoropolymers, was then used to deposit hexafluoropropylene onto the protein-dissacharide complexes. Upon removal of the mica substrate and target protein, the hydroxyl groups of the disaccharide molecules which were spatially complementary to the templated proteins were available as binding sites. In another example of MIPs in which the recognition sites have been confined to the surface, Sellergren used hierarchical imprinting in an attempt to create well structured MIPs with homogeneous morphology. The template molecule is attached to the surface of a porous solid. Polymerization occurs within the pores of a silica bead and subsequent to polymerization, the silica is etched away leaving a porous structure with sites complementary to the structure of the template molecule. The mobile phase in these experiments consisted of a combination of water, acetonitrile, and acetic acid. Solid phase synthesis products were also used directly as a template [72]. In one case, the surface of microtiter plates was coated with 3-aminophenylboronic acid and polymerized in the presence of various protein templates. All plates showed selectivity for their respective templates [10]. Molecularly imprinted polymer layers were formed around silica beads in the presence of glucose oxidase under conditions which support electrostatic interaction between monomer precursors and the template molecules [48].
Figure 1.3: Schematic of the hierarchical approach to molecular imprinting. (a) The target peptide or protein, bound to a sacrificial substrate. (b) Cross-linking and functional monomers. (c) Functional monomers associated with the target peptide or protein (d) MIP subsequent to removal of the sacrificial substrate and removal of the template molecules.
1.1.4 Recognition Mechanisms

In addition to overcoming the steric and conformational problems associated with molecular imprinting in aqueous media, the exact mechanism by which recognition occurs has also proved difficult to determine. The addition of functional monomers capable of participating in electrostatic interactions with the templated protein was shown, in early studies, to weaken the adsorption characteristics of the MIP, suggesting that electrostatic interactions did not play a large role in the recognition of peptides and proteins within this particular system [42]. On the other hand, molecularly imprinted polymers synthesized for the selective recognition of N-terminal histidine peptides in aqueous solutions most certainly exploit template-receptor complexation through electrostatic interaction [26]. There is strong evidence to suggest that the cooperative interaction between hydrogen bond formation and hydrophobic interaction is the most dominant form of template-receptor complexation in aqueous MIPs. Hydrogen bonds are highly directional, and many artificial receptors rely on the high directionality of hydrogen bonds and shape specificity of target molecules to create receptors capable of strong interaction and molecular recognition [65, 5]. The role of hydrophobic and hydrogen bonding effects in the recognition of peptides has been studied by chromatographic analysis of MIPs imprinted with various peptide sequences in aqueous-organic mixtures [51]. It was observed that the separation factors of these poly-
mers were significantly lower in acetonitrile-water mixtures than in pure acetonitrile. In addition, it was shown that water significantly lowers the effectiveness of hydrogen bonds between the template and the receptor, as the increased water content weakens the hydrogen bonding interaction between the template and the receptor [57, 58, 39]. Hydrogen bonds are a major factor in the formation of strong template-receptor interactions in non-polar media [51]. Other studies on MIP hydrogels suggest that a combination of hydrogen bonding and electrostatic effects allow for the selective recognition of target molecules in aqueous media [14]. It has been proposed that binding in aqueous media can be enhanced by exploiting cooperative interaction effects and selecting proper hydrophobic microenvirmonments to create better receptors [5]. In one of the most striking examples of molecular recognition by the cooperative action of hydrophobic and electrostatic interactions, β-cyclodrextin, a cyclic oligosaccharide was acrylated and co-polymerized with an electrostatic functional polymer, 2-acryoylamido-2,2-dimethylpropane sulfonic acid, in the presence of D-phenylalanine as the template molecule. β-cyclodrextin has a hydrophobic core which can bind aromatics groups on the side chains of peptides and protein molecules. While β-cyclodrextin has an inherent selectivity for L-phenylalanine, this natural selectivity is reversed upon imprinting with D-phenylalanine. This MIP was tested in mixtures of acetonitrile and water at various ratios. As is expected, the hydrophobic effect is the dominant factor in imprinting in aqueous-rich mixtures while electrostatic
effects dominate template-receptor complexation in aqueous-poor media [55, 56].

1.1.5 Microenvironment

In addition to the number of interactions which are possible between the template and the MIP receptor, the microenvironment surrounding the binding site can have a large role in determining how effective the MIP will be in recognizing its target molecule. For this reason, it is extremely difficult to make a quantitative comparison between MIPs that have been synthesized and tested under a number of different conditions. Ionic strength effects were studied in a system in which ionic interactions were the dominant force associated with template-receptor-recognition. A number of buffer systems were used to observe the effect of ionic strength and buffer composition on the binding capacity of the MIPs. All buffers examined resulted in a decrease in binding capacity of the MIP hydrogels. Interestingly, the absence of a buffer, with a 60 mM KCl solution showed the largest inhibition of MIP binding capacity. It was therefore concluded that the ionic strength of the binding solution, not the buffer composition itself, plays a large role in determining the effectiveness of the MIP [26]. The inhibition of MIP binding capacity in these experiments is most likely the result of a change in polymer conformation and/or variations in the microenvironment surrounding the template binding sites caused by changes in salt.
concentration. For example charge screening effects may effectively block the ionic interaction between the template at high salt concentration, as the salt dissociates into highly mobile anions and cations which can shield sites on both the target and the template. The effect of buffer composition and ionic strength has not been studied in other systems; however, it is highly possible that these two variables will have a large effect on MIP systems, even those which do not use ionic interactions as their primary recognition mechanism. This is because proteins can adopt a wide number of conformations depending on their environment.

1.1.6 Composition

The structure and composition of MIPs used for aqueous imprinting can also have a large effect on its ability to recognize its target molecule. Re-binding experiments conducted with MIP hydrogels imprinted in the presence of bovine serum albumin (BSA) were shown to have a strong dependence on the initial BSA concentration used during synthesis. This is most likely related to the fact that high template concentration during synthesis will result in a larger number of available binding sites upon subsequent imprinting experiments. Studies of lysozyme imprinted silica beads revealed that the amount of lysozyme adsorbed onto the beads depended on the composition of functional monomers used during preparation of the particles. This dependence suggests that there exists some
optimum distance between charged groups on the synthetic receptor which will correspond to a maximum in the complexation between receptor and template. The zeta-potential of the lysozyme molecules and the imprinted silica beads was also studied, and results revealed a coincidence between the zeta-potential of the template and that of the imprinted polymer beads at the value where the maximum amount of specifically adsorbed lysozyme was observed. This yields the possibility that zeta-potential matching may play an important role in the design of synthetic receptors [33]. Cross-linking concentration is also a critical factor in creating synthetic receptors with high affinity for their target molecules [26, 73].

1.1.7 Template Removal

Although commonly overlooked, the removal of target molecules from MIPs subsequent to re-binding experiments is a critical factor in the imprinting capability of the MIP. While very little work has focused solely on the study of template removal strategies, the methods which have been studied have revealed a number of important details. Template removal from polyacrylamide hydrogels synthesized in the presence of bovine hemoglobin was achieved with an aqueous solution consisting of varying ratios of sodium dodecyl sulfate (SDS) and acetic acid (HOAc) [30]. A qualitative model for the interaction between the SDS-HOAc solution and target protein within the cavity is presented and an optimum SDS/HOAc
ratio of 10% (weight/volume) SDS and 10% (volume/volume) HOAc is discovered. Interestingly, it was found that while increasing the amount of SDS and HOAC in the wash solution improves template removal, the re-binding effect is clearly compromised. In another MIP system, a solution consisting of acetic acid and Tween 20 was used prior to conducting template re-binding experiments [10]. It is quite possible that the combination of an acidic solution with a detergent is the most effective method of template removal for MIPs imprinted with peptide or proteins. Another obvious choice for template removal in these systems would be the use of proteolytic enzymes (proteases) which are capable of cleaving peptide bonds and subsequently destroying the structure of the imprinted protein or peptide [46]. The peptide fragments could then be removed from the cavity by simply rinsing with buffer solution. The protease trypsin, for example, was investigated as a template removal agent. However, after washing of an MIP with a trypsin solution, the re-binding efficiency suffered as cleaved protein fragments blocked binding sites within the imprint cavity [30].

1.1.8 Electrostatic Charge Density

Relatively few studies have been conducted thus far to explore the role of electrostatic interactions in molecularly imprinted polymers. Hjerten et al. [42] have explored the role of weakly ionizable acidic groups on the
recognition properties of imprinted polymers with human hemoglobin as the template, and found that the adsorption characteristics of the MIPs are weakened when charged groups were introduced into the polymer matrix. Zheng et al. [34] synthesized amphoteric, acrylamide based imprinted polymers with bovine serum albumin (BSA) and chicken egg lysozyme as templates. Using high-pressure liquid chromatography (HPLC), they concluded that the cooperative effects of multiple oppositely charged functional moieties enhanced the binding properties of the MIPs. However, they did not include data that compares the recognition properties of the charged polymers to an uncharged imprinted counterpart. Kameoka et al. [33] synthesized imprinted polymer particles composed of acrylamide and either acrylic acid or N,N-dimethylaminopropylacrylamide as co-monomers for lysozyme recognition. They found that peak lysozyme recognition occurred at specific molar amounts of charged (acrylic acid) functional groups.

The overall goal of this research is to understand the role that charge density plays in influencing the recognition properties of protein-imprinted hydrogels. Protein-imprinted hydrogels were synthesized at various charge densities, and the recognition properties of these hydrogels were measured using batch template rebinding experiments. The hydrogels were tested for their selectivity towards the template using a competitive protein, Cytochrome c. The first goal of this dissertation was to study the effects of charge density on the recognition properties of polyelectrolyte protein-
imprinted hydrogels. The hydrogel charge density was varied by introducing varying amounts of positively or negatively charged monomers into neutral hydrogels. The second goal was to study the effect of charge density on the recognition properties of hydrogels containing a combination of positively and negatively charged monomers (polyampholyte hydrogels). The third goal was to determine the effect that the template removal process had on the recognition properties of the protein-imprinted hydrogels.
Chapter 2

Protein Imprinted Polyelectrolyte Hydrogels

2.1 Introduction

The goal of this research is to study how charge density variations within protein imprinted polymeric hydrogels effect their ability to recognize bovine hemoglobin (Bhb) template molecules. To accomplish this goal, the charge density of Bhb imprinted hydrogels is systematically varied by incorporating small amounts of negatively and positively charged monomers to study its effect on template recognition, selectivity and specificity. The cationic monomer used in the synthesis of the MIP gels in this study was 3-methacrylamidopropyl trimethylammonium chloride (MAP-TAC), while the anionic monomer was 2-acrylamido-2-methylpropane sulfonic acid (AMPS). The template, Bhb, is an oxygen transport protein with a molecular weight of 64,500 g/mol and an isoelectric point (pI) of approximately 6.8. The absorption properties of Bhb-imprinted and non-imprinted hydrogels were measured using batch re-binding experiments and selectivity experiments were performed using bovine cytochrome C as a competitive template. Cytochrome C was used to determine whether or not the gels synthesized in this study are capable of selectively recognizing Bhb on the basis of both molecular weight and isoelectric point.
Cytochrome C has a lower molecular weight (12,384 g/mol) and higher isoelectric point (pI = 10-10.5) than Bhb.

As the template re-binding experiments were being conducted, it became evident that the wash used to extract protein from the imprinted hydrogels, which consisted of sodium dodecyl sulfate (SDS) and acetic acid (HOAc), was partly responsible for the specific recognition properties displayed by the hydrogels. Recently, Fu [19] noticed that a similar situation existed in chitosan-based MIPs. They found that both Bhb-imprinted and non-imprinted beads based on chitosan and acrylamide showed excessively high-affinity for the Bhb template after washing with a solution containing SDS and HOAc. In other words, there was no difference in the binding properties of Bhb-imprinted gels compared to non-imprinted gels. They also conceded that in their previously published papers [20, 21] the excessively high template affinity of their MIPS and uncharacteristically low affinity of the non-imprinted polymers was most likely the result of improper template extraction experiments [23, 24, 77]. In these experiments, the MIPs were washed with SDS-HOAc solutions, but the non-imprinted (control) polymers were not. This inadequate control resulted in unusually high selectivity and specificity. We suspect that a number of published studies related to protein-imprinted polymers suffer from the same shortfalls as those outlined by Fu’s publications.

Therefore, the second goal of this work was to determine how imprinted and non-imprinted gels containing varying amounts of charged
monomer behave under two distinct wash protocols. The first protocol involves washing the gels with an aqueous solution containing sodium dodecyl sulfate (SDS) and acetic acid (HOAc). As it is believed that SDS entrapped within the gels is the main impetus behind the high-affinity binding reported in previous studies, the second protocol that we developed involves an additional step to ensure the removal of any SDS that remains bound in the gel after washing.

2.2 Materials and Methods

2.2.1 Materials

Acrylamide, ammonium persulfate, bovine hemoglobin, bovine cytochrome C, N,N-methylenebisacrylamide, N,N,N,N-tetramethylethyldiamine, 3-methacrylamidopropyl trimethylammonium chloride, 2-acrylamido-2-methylpropane sulfonic acid, sodium dodecyl sulfate, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO) and used as received.

2.2.2 Synthesis

Stock solutions of the AMPS and MAPTAC monomers were prepared by dissolving a specified amount of monomer into deionized water and titrating with 1 M NaOH or 1 M HCl to pH 7. In a typical imprinted polymer synthesis, 54 mg acrylamide (monomer), 6 mg N,N-methylenebisacrylamide (BAAm, cross-linker), 10 µl of 5% (v/v) N,N,N,N-
tetramethylethylenediamine (TEMED, catalyst) and 12 mg Bhb template were dissolved in 1 ml of deionized water in a microcentrifuge tube. Nitrogen was bubbled through the solution for 5 minutes to purge any oxygen that is capable of inhibiting the formation of free radicals. Subsequent to nitrogen bubbling, 10 µl of 10% (w/v) ammonium persulfate (APS, initiator) was added to the solution. Free radical cross-linking co-polymerization occurred overnight, producing gels that were then removed from the microcentrifuge tubes and granulated by passing through a 75 µm sieve (Fisher Scientific, Pittsburgh, PA) prior to washing. Non-imprinted, neutral hydrogels were prepared in the same manner, in the absence of the template molecule Bhb. All polymer gels in this work were prepared as described above, with a small percentage (0.25-1.0%) of the uncharged acrylamide monomer being replaced with an anionic (AMPS) or cationic (MAPTAC) monomer to allow for systematic variation of polymer charge density. The synthesis parameters for all hydrogels prepared in this study are given in Table 2.1.

2.2.3 Bhb Template Extraction

Template extraction occurred in two distinct steps. First, a series of five washes was performed using deionized water. Subsequent to these water washes, a series of five washes was performed using a solution containing 10% (v/v) acetic acid and 10% SDS (w/v), referred to as SDS-HOAc
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<th></th>
<th>Acrylamide</th>
<th>AMPS</th>
<th>MAPTAC</th>
<th>BAAm</th>
<th>Bhb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neutral</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>54.0</td>
<td>0.0</td>
<td>0.0</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>54.0</td>
<td>0.0</td>
<td>0.0</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>0.25% AMPS</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Imprinted</td>
<td>53.87</td>
<td>0.14</td>
<td>0.0</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.87</td>
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<td>0.0</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>0.5% AMPS</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.73</td>
<td>0.27</td>
<td>0.0</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.73</td>
<td>0.27</td>
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<td>0.0</td>
</tr>
<tr>
<td><strong>1.0% AMPS</strong></td>
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<tr>
<td><strong>0.25% MAPTAC</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.87</td>
<td>0.0</td>
<td>0.14</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.87</td>
<td>0.0</td>
<td>0.14</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>0.5% MAPTAC</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.73</td>
<td>0.0</td>
<td>0.27</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.73</td>
<td>0.0</td>
<td>0.27</td>
<td>6.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 2.1: Synthesis parameters for neutral, positively (MAPTAC) and negatively charged (AMPS) hydrogels. All values are in mg.
Following synthesis and granulation, the imprinted and non-imprinted hydrogels were suspended in 2 ml of deionized water in a 15 ml polycarbonate centrifuge tube and centrifuged (Sorvall, Waltham, MA) for 5 minutes at 3000 rpm. This was repeated 5 times. Subsequent to these five water washes, the gel was washed 5 times in the same manner using the SDS-HOAc solution. The supernatants from the water and SDS-HOAc washes were collected for analysis. Following these washes, all gels were equilibrated by washing repeatedly in an excess of deionized water. A total of 5 water washes and 5 SDS-HOAc were found to be sufficient, as the amount of Bhb detected in the wash supernatants was found to be below the detectable limits of the UV-Vis spectrophotometer.

In addition to the original protocol described above, a modified protocol was developed to study the binding properties of Bhb imprinted and non-imprinted hydrogels under varying extraction conditions. The modified protocol is nearly identical to the original protocol, the only difference being the addition of a triplicate wash with 2 ml of 3 M NaCl prior to equilibration with deionized water. The goal of these NaCl washes was to remove any SDS remaining within the gel subsequent to the SDS-HOAc washes.
2.2.4 Template Rebinding

In a typical template rebinding experiment, washed and granulated Bhb-imprinted and non-imprinted hydrogels were loaded with 2 ml of a deionized water solution containing 3 mg/ml of Bhb. The gels were placed on a Labquake mixer (Barnstead International, Dubuque, IA) and allowed to associate with the template for 10 minutes. Following template association, gels were removed from the mixer and subjected to the same extraction experiments described in the Bhb template extraction section.

2.2.5 UV-Vis Analysis

Analysis of the supernatants collected from the washing and rebinding experiments were performed using UV-Vis measurements on a Perkin-Elmer Lambda 25 spectrophotometer. A calibration curve was prepared by performing absorbance measurements on deionized water solutions containing known amounts of Bhb. A similar curve was prepared for solutions of Bhb in SDS-HOAc. Spectral scans (190-900 nm) performed on these solutions revealed peaks at 404 nm for Bhb in water (Figure 2.1) and 395 for Bhb in the SDS-HOAc solution (Figure 2.2). Similar scans performed on solutions of cytochrome C in deionized water and SDS-HOAc revealed peaks at 407 nm and 397 nm respectively. Calibration curves that correlate the concentration of Bhb and cytochrome C to the measured value of UV-Vis absorbance were generated using the peak absorbance values.
An example of a calibration curve derived from spectral scans performed on a solution of Bhb in water is shown in Figure 2.3. The supernatant from each step of the extraction experiment was diluted appropriately to ensure that it fell within the range of the calibration curve.

Figure 2.1: UV-Vis scan of Bhb in water at various concentrations. The intensity of the peaks decreases as the concentration of Bhb is lowered.

2.2.6 Hydrogel Swelling

Swelling experiments were designed to determine the swollen state of the hydrogels in conditions that mimic those present during template re-binding. All swelling experiments were performed using non-imprinted hydrogels. Hydrogels of varying charge density were synthesized, granulated, and weighed to determine the initial mass of the gels, \( M_i \). The gels were then washed using the original protocol described above. Subsequent
Figure 2.2: UV-Vis scan of Bhb in SDS-HOAc at various concentrations. The intensity of the peaks decreases as the concentration of Bhb is lowered.

Figure 2.3: Calibration curve for Bhb in water.
to washing, the gels were then placed in 15 ml centrifuge tubes along with 2 ml of deionized water. The gels were swollen in these tubes for 10 minutes and then centrifuged for 5 minutes at 3000 rpm. The deionized water supernatant was removed and the gels were weighed again to determine the final weight, $M_f$. The swelling ratio (SR) was determined by dividing the final mass by the initial mass as shown in Equation 2.1.

$$SR = \frac{M_f}{M_i}$$

(2.1)

2.3 Results and Discussion

Characterization of molecularly imprinted polymers is usually performed through comparison of the absorption/binding characteristics of an imprinted polymer relative to the absorption/binding characteristics of the non-imprinted counterpart. In our studies, we perform a similar comparison, however, we also compared non-imprinted and imprinted charged polymers relative to their uncharged counterparts to determine the role of charge density in the recognition properties of MIPs. Charge density refers to the ratio of the mass of charged monomers to the total mass of monomers within each gel.

The template extraction experiment is separated into two steps, which allows us to determine the amount of Bhb bound with high and low-affinity in the hydrogels. We believe that is extremely critical to determine the
amount of template bound with low and high-affinity within the gels as low-affinity binding sites will be of little interest in commercial applications. Figure 2.4 shows a typical Bhb wash profile for an acrylamide (uncharged) hydrogel imprinted with 12 mg of Bhb.

Figure 2.4: Wash profile for an uncharged (0% ionic groups) Bhb-imprinted hydrogel. Washes (1-5) were performed using deionized water, while washes (6-10) were performed using SDS-HOAc solution.

Washes 1-5 represent the amount of Bhb bound (2.6 mg) in low-affinity sites, as this fraction of Bhb was removed using only deionized water, requiring no harsh solvents or high ionic strength solutions. Washes 6-10 represent the amount of Bhb bound (4.1 mg) in high-affinity sites, as this fraction of protein is only removed during washes performed using the harsh SDS-HOAc solution. The Bhb fraction removed during these washes contributes to the formation of high-affinity imprinted sites that
can selectively recognize Bhb in subsequent template binding experiments. It is interesting to note that a substantial fraction, (45%) 5.4 mg of the imprinted protein remains within the hydrogel, even after extraction cycles using the harsh removal solvent were performed. Similar results were noted in previous studies involving free-radical cross-linking copolymerization of neutral, anionic, and cationic monomers in the presence of protein molecules [53]. It is likely that functional groups on the protein are susceptible to attack from free radicals, introducing the possibility of covalent bond formation between functional monomers within the gel and the template Bhb molecules. Breaking of these bonds would be extremely difficult, even under the harsh extraction conditions, and thus we expect a fraction of the template molecule to remain embedded within the hydrogel.

Figures 2.5 and 2.6 are representative of template-binding experiments performed on uncharged Bhb-imprinted and non-imprinted polymer hydrogels, respectively. The imprinted polymer hydrogel (Figure 2.5) clearly demonstrates that the molecular imprinting process has a marked effect on the absorption properties of the gel.

In wash 1, a significant amount of protein (1.5 mg) is detected in the supernatant. It is important to make the distinction that the Bhb detected in wash 1 during rebinding experiments is not necessarily bound with low-affinity; rather, it represents the unbound fraction of Bhb left in solution upon termination of the 10-minute association step. In other words, a fraction of Bhb does not enter the gel during the 10-minute association
Figure 2.5: Hb re-binding experiment performed on an uncharged (0% ionic groups) Hb-imprinted polymer hydrogel. Washes (1-5) were performed using deionized water, while washes (6-10) were performed using SDS-HOAc solution.
step. Therefore, it will be present in the supernatant of wash 1.

Figure 2.6: Binding experiment performed on a uncharged (0% ionic groups) non-imprinted hydrogel. Washes (1-5) were performed using deionized water, while washes (6-10) were performed using SDS-HOAc solution.

Performing a summation of the Bhb extracted in washes 2-5 reveals that only a small fraction of Bhb (0.57 mg) was detected in the wash supernatant, indicating that a large fraction of Bhb remains within the imprinted hydrogels after washing them with water. A large fraction (2.1 mg) of Bhb was detected in wash cycles 6-10, indicating that most of the Bhb within this hydrogel was bound in high-affinity sites, as it was only removed using the harsh SDS-HOAc wash. The non-imprinted hydrogel (Figure 2.6) shows the opposite effect, as most of the Bhb was either unbound (2.3 mg) or extracted during washes 2-5 (0.88 mg), indicative of low-affinity interaction between the polymer hydrogel and the Bhb molecules. Results for
Table 2.2: Summary of re-binding results for uncharged Bhb-imprinted and non-imprinted hydrogels.

<table>
<thead>
<tr>
<th></th>
<th>Unbound</th>
<th>Low-Affinity (H₂O)</th>
<th>High-Affinity (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncharged</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>1.8 ± 0.15</td>
<td>0.57 ± 0.01</td>
<td>2.1 ± 0.31</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>2.3 ± 0.20</td>
<td>0.88 ± 0.05</td>
<td>0.47 ± 0.003</td>
</tr>
</tbody>
</table>

An increase in the negative charge density of the hydrogels was achieved by copolymerizing a specified amount of the negatively charged AMPS monomer with uncharged acrylamide. We expected that increasing the charge density would encourage the formation of high-affinity binding sites, by allowing for the association of positive charges on the protein surface with negative charges on the AMPS functional groups. Table 2.3 shows the results of rebinding experiments performed on Bhb-imprinted hydrogels containing varying amounts of AMPS monomer.

Within the gels containing 0.25% AMPS functional monomer, approximately 52% (3.1 mg) of the Bhb bound within the imprinted hydrogels resided within high-affinity binding sites. Comparatively, approximately 35% (2.1 mg) was bound in high-affinity binding sites in the uncharged acrylamide gels. Thus, as the charge density of the imprinted hydrogels was increased from 0% to 0.25%, the fraction of Bhb bound in high-affinity
<table>
<thead>
<tr>
<th></th>
<th>Unbound</th>
<th>Low-Affinity (H₂O)</th>
<th>High-Affinity (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.25% AMPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.90 ± 0.58</td>
<td>0.82 ± 0.34</td>
<td>3.1 ± 0.25</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>1.6 ± 0.25</td>
<td>1.0 ± 0.06</td>
<td>2.2 ± 0.51</td>
</tr>
<tr>
<td><strong>0.50% AMPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.51 ± 0.10</td>
<td>0.24 ± 0.04</td>
<td>2.8 ± 0.05</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>0.80 ± 0.19</td>
<td>0.57 ± 0.26</td>
<td>2.9 ± 0.10</td>
</tr>
<tr>
<td><strong>1.0% AMPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.021 ± 0.01</td>
<td>0.065 ± 0.05</td>
<td>4.6 ± 0.50</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>1.6 ± 0.25</td>
<td>1.0 ± 0.06</td>
<td>2.2 ± 0.51</td>
</tr>
</tbody>
</table>

Table 2.3: Results of Bhb re-binding experiments performed on negatively charged (AMPS containing) Bhb-imprinted and non-imprinted hydrogels of varying charge density. Data represents the amount of Bhb bound within each hydrogel. All values are in mg.
sites increased.

Table 2.3 shows the data from re-binding experiments performed with BHb on a non-imprinted 0.25% AMPS hydrogel. In these non-imprinted hydrogels, 37% (2.2 mg) of the protein bound was bound in high-affinity sites within the non-imprinted hydrogel. In addition, 27% (1.6 mg) of the BHb remained unbound after the 10 minute association step, and 17% (1.0 mg) was bound in low-affinity sites. Both these values represent slight increases from the imprinted gel, suggesting a transition to low-affinity binding as the charge density of the gels increases.

The data from re-binding experiments conducted on hydrogels containing 0.5% AMPS functional groups are shown in Table 2.3. In the imprinted hydrogels, approximately 4% (0.24 mg) is bound in low-affinity sites, while a much larger fraction 47% (2.8 mg) is bound in high-affinity sites within the hydrogel. Thus, this imprinted hydrogel appears to display optimum performance. However, comparison of this data with data from non-imprinted hydrogels containing 0.5% AMPS functional monomers (Table 2.3) shows that at this charge density both BHb-imprinted and non-imprinted hydrogels behave nearly identically. In other words, the imprinting effect found in BHb imprinted hydrogels containing 0% and 0.25% AMPS functional monomer was eliminated as the amount of AMPS groups within imprinted hydrogels was increased to 0.5% of the total monomer concentration. BHb-imprinted and non-imprinted hydrogels containing 1% AMPS functional monomer were synthesized to confirm the disappearance
of the imprinting effect at higher AMPS charge densities. Table 2.3 clearly shows that there was very little low-affinity binding in either hydrogel, as is indicated by the extremely small fraction of protein removed during the deionized water extraction cycles. Both the Bhb-imprinted and non-imprinted hydrogels displayed a large fraction of high-affinity binding, as 77% (4.6 mg) of the total Bhb bound within the imprinted and non-imprinted resided in high-affinity binding sites.

In addition to analyzing the effects of the anionic AMPS functional group on the absorption properties of Bhb-imprinted polymers, we also looked at the effects of a cationic MAPTAC group on the MIPs recognition properties. All experiments conducted using the MAPTAC functional monomer were performed in an identical manner to the experiments using AMPS. Table 2.4 shows the results of re-binding experiments for Bhb-imprinted and non-imprinted polymer hydrogels containing 0.25% MAPTAC functional monomers.

The Bhb-imprinted hydrogel containing 0.25% MAPTAC functional groups is clearly superior in its Bhb recognition properties when compared with its non-imprinted counterpart. While the unbound Bhb fraction is similar in both gels, the non-imprinted gel had 15% (0.88 mg) of the total Bhb bound in low-affinity sites and 22% (1.3 mg) bound in high-affinity sites. The imprinted gel has only 7% (0.41 mg) bound in low-affinity sites while 35% (2.1 mg) is bound in high-affinity sites.

Table 2.4 also shows the data for Bhb imprinted and non-imprinted
<table>
<thead>
<tr>
<th></th>
<th>Unbound</th>
<th>Low-Affinity (H₂O)</th>
<th>High-Affinity (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.25% MAPTAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>2.2 ± 0.02</td>
<td>0.41 ± 0.11</td>
<td>2.1 ± 0.50</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>2.3 ± 0.10</td>
<td>0.88 ± 0.03</td>
<td>1.3 ± 0.15</td>
</tr>
<tr>
<td><strong>0.50% MAPTAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.022 ± 0.08</td>
<td>0.046 ± 0.01</td>
<td>2.5 ± 0.32</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>0.017 ± 0.01</td>
<td>0.067 ± 0.07</td>
<td>3.0 ± 0.10</td>
</tr>
</tbody>
</table>

Table 2.4: Results of Bhb re-binding experiments performed on positively charged (MAPTAC containing) Bhb-imprinted and non-imprinted hydrogels of varying charge density. Data represents the amount of Bhb bound within each hydrogel. All values are in milligrams.
hydrogels containing 0.5% MAPTAC functional groups. The data shows that the results of these binding experiments for both imprinted and non-imprinted hydrogels are nearly identical. These results suggest that unlike the anionic AMPS hydrogels, in which the imprinting effect was not entirely eliminated until a charge density of 1% was reached, the imprinting effect in hydrogels containing the cationic MAPTAC monomer vanished at a charge density of 0.5% MAPTAC.

To test the selectivity of the Bhb-imprinted hydrogels in this study, template-rebinding experiments were performed by loading Bhb imprinted gels with cytochrome C. It is expected that Bhb imprinted gels, which exhibit an imprinting effect will have an affinity for cytochrome C that is equal to that of their non-imprinted counterparts. In other words, neither imprinted nor non-imprinted gels should have a preference for the cytochrome C template. Selectivity experiments were performed on uncharged gels and gels containing 0.25% AMPS and 0.25% MAPTAC, as these were the only samples that exhibited high-affinity for Bhb. The selectivity factor, \( \alpha \), which is defined as the ratio of bound Bhb to bound cytochrome C bound for these gels is reported in Table 2.5.

The data in Table 2.5 indicates that uncharged gels have approximately twice the affinity for Bhb than cytochrome C. Unlike the uncharged hydrogels, the negative (AMPS containing) hydrogels both exhibited only slight selectivity for the Bhb template. Cytochrome C has a net positive charge at pH 7, due to its relatively high isoelectric point of approximately
Gel & \( \alpha \)  
\hline
Uncharged & 2.1  
0.25% AMPS & 1.1  
0.25% MAPTAC & 0.62  
\hline

Table 2.5: Results of selectivity experiments performed using cytochrome C template on Bhb-imprinted and non-imprinted hydrogels of varying charge density. This data represents the amount of cytochrome C extracted from each gel in milligrams.

10.0 - 10.5. This net positive charge on the cytochrome C molecules should attract the negatively charged sulfonic acid groups of the imprinted hydrogel containing AMPS, resulting in higher affinity for cytochrome C. The selectivity experiments confirmed that the Bhb-imprinted gels absorb both Bhb and cytochrome C with similar affinity. Gels containing 0.25% MAPTAC monomer have a slight positive charge and therefore, they should repel the positively charged cytochrome C template. Instead, we find that Bhb-imprinted 0.25% MAPTAC gels displayed a large amount of high-affinity absorption for cytochrome C. It is interesting to note that the selectivity factor \( \alpha \) of these gels is lower than unity. In other words, Bhb-imprinted gels containing 0.25% MAPTAC actually had a higher preference for cytochrome C than for the original Bhb template. Possible reasons for this unexpected behavior are discussed in the following discussion.
In addition to the results obtained above, a separate set of experiments was performed using a new, modified wash protocol as follows. Granulated hydrogels were suspended in 2 ml of deionized water in a 15 ml polycarbonate centrifuge tube and centrifuged for 5 minutes at 3000 rpm. Aliquots were taken from the wash and analyzed using UV-Vis spectroscopy to determine the amount of Bhb extracted from the gels. This was repeated 5 times. Subsequent to these five water washes, the gel was washed five times in the same manner using the SDS-HOAc solution with all supernatant fractions being collected for analysis. Following the SDS-HOAc wash series, all gels were washed in triplicate using 2 ml of a 3M NaCl solution. The purpose of this additional NaCl is to ensure the removal of any excess SDS molecules that may be bound in the gel subsequent to the SDS-HOAc washes. The hydrogels were then washed excessively with deionized water to remove any residual SDS, HOAc, or NaCl molecules that may have been absorbed. The data from a series of gels washed using this modified protocol are shown in Table 2.6.

Comparison between the data obtained from the modified wash protocol and that of the original protocol reveals only slight differences in the amount of unbound and low-affinity bound Bhb. Therefore, SDS does not play a significant role in the low-affinity binding sites present in the gel. There was, however, a clear differentiation between the high-affinity interactions in Bhb-imprinted and non-imprinted hydrogels under the original and modified wash conditions. Compared to the original conditions, the
Table 2.6: Results of Bhb re-binding experiments performed on negatively charged (AMPS containing) Bhb-imprinted and non-imprinted hydrogels of varying charge density washed using the modified wash protocol. Data represents the amount of Bhb bound within each hydrogel. All values are in mg.

<table>
<thead>
<tr>
<th>AMPS</th>
<th>Unbound</th>
<th>Low-Affinity (H₂O)</th>
<th>High-Affinity (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% AMPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>1.02 ± 0.60</td>
<td>0.33 ± 0.014</td>
<td>1.46 ± 0.18</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>1.08 ± 0.28</td>
<td>0.48 ± 0.25</td>
<td>2.43 ± 0.38</td>
</tr>
<tr>
<td>0.50% AMPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.53 ± 0.010</td>
<td>0.20 ± 0.021</td>
<td>4.26 ± 0.028</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>1.25 ± 0.17</td>
<td>0.47 ± 0.049</td>
<td>4.03 ± 0.42</td>
</tr>
<tr>
<td>1.0% AMPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.06 ± 0.03</td>
<td>0.19 ± 0.04</td>
<td>4.72 ± 0.22</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>0.042 ± 0.06</td>
<td>0.11 ± 0.014</td>
<td>4.99 ± 0.16</td>
</tr>
</tbody>
</table>
amount of high-affinity bound Bhb in the imprinted gel containing 0.25% AMPS monomer decreased by approximately 50% after the modified wash. The non-imprinted gel containing 0.25% AMPS monomer showed slightly increased high-affinity binding, although this is not believed to be significant as it is within the experimental margin of error. The decrease in high-affinity binding of gels containing 0.25% AMPS can be explained in the following manner. Bhb imprinted gels washed using the original protocol and modified protocol always contain a fraction of Bhb protein that cannot be extracted. During the SDS-HOAc wash series, SDS molecules are bound to the permanently entrapped proteins, and a fraction of this SDS remains bound within the Bhb imprinted hydrogel even after the deionized water equilibration steps are completed. When the rebinding experiments are performed, Bhb interacts with the SDS bound in the imprinted gel, resulting in high-affinity binding. The non-imprinted gels consistently displayed similar high-affinity binding data, regardless of whether the modified or original binding protocol was used. The non-imprinted gel does not initially contain Bhb, and therefore, no substrate exists within these gels to promote high-affinity binding of SDS. The absence of SDS within the non-imprinted gel resulted in binding affinity that was independent of the wash protocol used.

As the amount of negatively charged AMPS groups is increased, there is a dramatic decrease in the high-affinity binding properties of the Bhb imprinted and non-imprinted hydrogel which can be explained in terms of
hydrogel swelling. Figure 2.7 shows the swelling data for non-imprinted hydrogels containing varying amounts of negatively charged AMPS functional groups.

As the amount of negatively charged AMPS groups increases from 0% to 0.25%, the gel swells 14% more, resulting in similar template rebinding behavior between neutral and negatively charged gels. The hydrogels containing 0.5% AMPS and those containing 1.0% AMPS swelled 36% and 95% more than neutral hydrogels, respectively. Bhb-imprinted and non-imprinted gels containing 0.5% AMPS and 1.0% AMPS, no detectable difference was detected between the high-affinity binding properties of the gels. Therefore, any recognition properties imparted to the gel through the molecular imprinting process ceases to exist as the charge density increases beyond 0.5% AMPS.

Gels containing 0.5% AMPS and 1.0% AMPS swelled significantly, enabling them to absorb a considerable amount of SDS molecules. These SDS molecules remained within the gel after equilibration with deionized water and NaCl. The high SDS content in these gels resulted in higher apparent specific binding, increased binding capacity, and diminished imprinting factor. In fact, physically entrapped SDS molecules can also cause an imprinting factor to decrease below unity, as is indicated by the results of selectivity experiments performed on 0.25% MAPTAC gels (Table 2.5).

In contrast to the gels containing negatively charged AMPS monomers, positively charged gels containing MAPTAC monomers displayed stark dif-
Figure 2.7: Swelling data and imprinting factor (IF) for gels with various amounts of negatively charged AMPS monomer. The columns represent the swelling data, while the solid and dashed lines represent the IF of gels washed under the original and modified protocol, respectively.
ferences across the original and modified wash protocols. Table 2.7 contains Bhb template re-binding data for gels containing varying amounts of positively charged MAPTAC monomer. The data reveals that gels containing 0.25% MAPTAC washed with the modified protocol exhibited a pronounced improvement in high-affinity Bhb binding over their non-imprinted counterparts. After being washed using the modified protocol, imprinted gels containing 0.25% MAPTAC exhibited a decrease in the unbound Bhb fraction and a large increase in the amount of Bhb bound in high-affinity sites. The non-imprinted gels containing 0.25% MAPTAC displayed a significant decrease in the amount of Bhb bound in high-affinity sites and an increase in the unbound fraction. The low-affinity binding profiles for gels containing 0.25% MAPTAC remained relatively constant across both wash protocols. Upon increasing the charge density to 0.5% MAPTAC, Bhb-imprinted gels washed under the modified protocol showed a slight increase in the amount of Bhb bound in high-affinity sites, while non-imprinted gels showed a slight decrease in high-affinity binding accompanied by an increase in the unbound fraction of Bhb. This resulted in the emergence of an imprinting effect in 0.5% MAPTAC gels which was not originally present in 0.5% MAPTAC gels washed under the original protocol. Due to the emergence of this effect, gels containing 1.0% MAPTAC were synthesized in order to study whether this effect persisted at higher charge densities. Bhb-imprinted and non-imprinted gels containing 1.0% MAPTAC showed similar high-affinity binding data, indicating a dimin-
ished recognition effect above 0.5% MAPTAC.

To better understand the binding properties of the gels containing various amounts of positively charged MAPTAC monomer, swelling experiments were performed on the non-imprinted gels. Figure 2.8 displays the results of these experiments. The swelling data reveals an abrupt increase in swelling upon the addition of 0.25% MAPTAC to neutral gels with the charged gel swelling approximately 400% more than the neutral gels. In addition to swelling data, Figure 2.8 also shows the variation of imprinting factor (IF) as a function of increasing charge density.

There is a marked difference between the behavior of the MAPTAC gels washed using the two different protocols. When MAPTAC gels were washed using the original protocol, the imprinting factor (IF) decreased as the charge density increased from 0% to 1.0%. This decrease in IF also corresponded to an increase in swelling as the charge density increased. The decrease in IF as charge density and swelling ratio increase can be understood in terms of imprinted cavity deformation. As the charge density increases, the shape of the imprinted cavities is distorted as a result of increased swelling. Highly swollen gels result in severe binding cavity distortion that leads to indiscriminant binding of Bhb, regardless of whether the gel has been imprinted in or not. In gels washed using the modified protocol the situation becomes more complicated. As the swelling and charge density increase, MAPTAC containing gels washed with the modified protocol exhibit a pronounced increase in imprinting factor at 0.25%.
<table>
<thead>
<tr>
<th>Charge Density</th>
<th>Unbound</th>
<th>Low-Affinity (H₂O)</th>
<th>High-Affinity (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.25% MAPTAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.13 ± 0.064</td>
<td>0.28 ± 0.042</td>
<td>4.6 ± 0.12</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>4.7 ± 0.28</td>
<td>0.92 ± 0.60</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td><strong>0.50% MAPTAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.39 ± 0.014</td>
<td>0.37 ± 0.014</td>
<td>3.77 ± 0.01</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>1.2 ± 1.1</td>
<td>0.64 ± 0.39</td>
<td>2.3 ± 0.22</td>
</tr>
<tr>
<td><strong>1.0% MAPTAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>0.021 ± 0.0</td>
<td>0.060 ± 0.004</td>
<td>3.6 ± 0.37</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>0.021 ± 0.0</td>
<td>0.69 ± 0.13</td>
<td>3.6 ± 0.49</td>
</tr>
</tbody>
</table>

Table 2.7: Results of Bhb re-binding experiments performed on negatively charged (MAPTAC containing) Bhb-imprinted and non-imprinted hydrogels of varying charge density washed using the modified wash protocol. Data represents the amount of Bhb bound within each hydrogel. All values are in mg.
Figure 2.8: Swelling data and imprinting factor (IF) for gels with various amounts of positively charged MAPTAC monomer. The columns represent the swelling data, while the solid and dashed lines represent the IF of gels washed under the original and modified protocol, respectively. The peak in the dashed line at 0.25% MAPTAC represents the optimum monomer concentration for binding Bhb.
Table 2.8: Selectivity factor ($\alpha$) for gels containing MAPTAC monomer washed under the modified protocol.

<table>
<thead>
<tr>
<th>Gel</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharged</td>
<td>2.1</td>
</tr>
<tr>
<td>0.25% MAPTAC</td>
<td>6.6</td>
</tr>
<tr>
<td>0.5% MAPTAC</td>
<td>4.6</td>
</tr>
</tbody>
</table>

MAPTAC. As the charge density continues increase, the imprinting factor decreases until it reaches a terminal value of 1 at a charge density of 1.0% MAPTAC. There is a large increase in imprinting factor for gels containing 0.25% MAPTAC washed under the modified protocol. The data reveals that gels containing 0.25% MAPTAC may reside at an optimum concentration of positively charged monomer to bind the target protein Bhb. In an attempt to verify the presence of this high imprinting factor and ensure that this data point was not an anomaly, five additional re-binding experiments were performed on gels washed under the modified protocol. Gels containing 0.25% MAPTAC consistently exhibited this high imprinting factor. The selectivity of gels containing 0.25% MAPTAC and 0.5% MAPTAC washed under the modified protocols is shown in Table 2.8.

The selectivity of neutral gels is included for comparison purpose. As expected, the gel containing 0.25% MAPTAC exhibits the highest selectivity, providing further confirmation that the optimum charge density exists.
at this point.

2.4 Summary

Protein imprinted hydrogels are highly complex systems, and therefore, there are numerous explanations for the results outlined above. The binding experiments performed in this work were conducted at pH 6.8, which is nearly equal to the isoelectric point of the Bhb template. Under these conditions the Bhb template will display both positive and negative charges on its surface while maintaining a net charge of zero under the template binding conditions studied. Offsets (positive or negative) in the charge density of the imprinted hydrogels should provide increased recognition, as the strength of the electrostatic between functional groups on the Bhb template and the complementary groups on the imprinted gels is increased. However, our experiments showed decreasing recognition with increasing charge density in case of negatively charged AMPS containing gels, while gels containing positively charged MAPTAC moieties displayed maximum selectivity and recognition properties at a charge density of 0.25%. Our results indicate that there is a complex interplay between hydrogel swelling, electrostatic interaction between the hydrogels and the template, and cavity shape recognition mechanisms that occur within imprinted polymers. The data also reveals that it may be possible to locate optimum imprinting conditions, although the correlation between hydrogel
charge density and template chemistry has yet to be determined. The ef-
fect of template extraction chemistry on the measured binding properties
of MIPs is particularly interesting, as a significant number of published
studies [23, 24, 77] reveal that the use of SDS-HOAc for template extrac-
tion is widespread. The mechanism presented within this paper reveals
that the high template binding affinity measured in many studies may be
the result of the effect of the wash chemistry on the gels, and not neces-
sarily the result of any recognition properties imparted to the gel through
the imprinting process.
Chapter 3

Protein Imprinted Polyampholyte Hydrogels

3.1 Introduction

Polyampholytes are polymers that contain both positive and negative charges along their backbone. The presence of both positively and negatively charged monomers with a protein imprinted hydrogel should enable increased template molecule recognition for two reasons. First, the simultaneous presence of two oppositely charged monomers in the prepolymerization mixture should result in imprinted hydrogels with cavities that contain highly specific functional group orientation. The Bhb protein contains a distribution of positively and negatively charged functional groups on its surface, and therefore an imprinted hydrogel containing both positively and negatively charged monomers should result in a more accurate complementary structure. Secondly, the polyampholyte hydrogels should exhibit decreased swelling when compared to their polyelectrolyte counterparts. Repulsive interaction between oppositely charge monomers are shielded within polyampholyte hydrogels, resulting in decreased swelling and a lower degree of cavity deformation.
3.2 Materials and Methods

3.2.1 Synthesis

The synthesis of protein imprinted polyampholyte hydrogels was conducted in a similar manner to the synthesis of polyelectrolyte hydrogels as discussed in Chapter 1. The total charge density of all polyampholyte hydrogels was fixed at 0.25%. A typical synthesis for a net neutral polyampholyte hydrogel containing 50% AMPS monomer and 50% MAPTAC monomers was performed as follows. Stock solutions of the AMPS and MAPTAC monomers were prepared by dissolving a specified amount of monomer into deionized water and titrating with 1 M NaOH or 1 M HCl to pH 7. 53.86 mg acrylamide (monomer), 0.07 mg AMPS (monomer), 0.07 mg (MAPTAC) monomer, 6 mg N,N-methylenebisacrylamide (cross-linker), 10 µl of 5% (v/v) N,N,N,N-tetramethylethylenediamine (TEMED, catalyst) and 12 mg Bhb template were dissolved in 1 ml of deionized water in a microcentrifuge tube. Nitrogen was bubbled through the solution for 5 minutes to purge any oxygen that is capable of inhibiting the formation of free radicals. Subsequent to nitrogen bubbling, 10 µl of 10% (w/v) ammonium persulfate (APS, initiator) was added to the solution. Free radical cross-linking co-polymerization occurred overnight, producing gels that were then removed from the microcentrifuge tubes and granulated by passing through a 75 µm sieve (Fisher Scientific, Pittsburgh, PA) prior to washing. Non-imprinted, neutral hydrogels were prepared in the same
Table 3.1: Synthesis parameters for neutral, positively (MAPTAC) and negatively charged (AMPS) hydrogels. All values are in mg.

<table>
<thead>
<tr>
<th>AMPS/MAPTAC</th>
<th>Acrylamide</th>
<th>AMPS</th>
<th>MAPTAC</th>
<th>BAAm</th>
<th>Bhb</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%/50%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.86</td>
<td>0.07</td>
<td>0.07</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.86</td>
<td>0.07</td>
<td>0.07</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td>25%/75%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.86</td>
<td>0.04</td>
<td>0.1</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.86</td>
<td>0.04</td>
<td>0.1</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td>75%/25%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imprinted</td>
<td>53.86</td>
<td>0.1</td>
<td>0.04</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Non-Imprinted</td>
<td>53.86</td>
<td>0.1</td>
<td>0.04</td>
<td>6.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

3.2.2 Bhb Template Extraction

Template extraction experiments were identical to the experiments performed on protein imprinted polyelectrolyte hydrogels discussed in Chap-
ter 1 and were performed in the following manner. First, a series of five washes was performed using deionized water. Subsequent to these water washes, a series of five washes was performed using a solution containing 10% (v/v) acetic acid and 10% SDS (w/v), referred to as SDS-HOAc hereafter. Finally, the gels were washed with 2 ml of 3 M NaCl prior to equilibration with deionized water. The goal of these NaCl washes was to remove any SDS remaining within the gel subsequent to the SDS-HOAc washes.

The effects of the template removal protocol on the binding properties of protein imprinted and non-imprinted polyampholyte hydrogels were studied in order to determine whether or not these gels behave in a similarly to their polyelectrolyte counterparts. Specifically, a study was performed to determine the effects of SDS on the binding affinity of the polampholyte gels. The study was conducted in the following manner. A number of non-imprinted gels, each containing 50% AMPS and 50% were synthesized according to the experimental procedure outlined in the section above. The resultant gels were washed with either a 10% SDS solution, a 10% HOAc solution, or the SDS-HOAc solution normally used for template extraction. Subsequent to washing the gels, a typical re-binding experiment was conducted where the gels were exposed to a 2 ml solution containing 6 mg Bhb and allowed to associate for 10 minutes. The gels were then washed five times with water and aliquots were taken from each wash and analyzed using UV-Vis at 404 nm to determine the concentration of Bhb within each
wash. Using the three components of the template removal solution separately, the effect that each of the components has on the binding properties of the gels can be determined. A dependence (increase or decrease) of low-affinity binding on the composition of the template removal solution would be indicative of a binding affinity that is subject to modulation through simple variation of the template removal protocol.

3.3 Results and Discussion

Figure 3.1 shows the results of a template re-bind experiment performed on a non-imprinted, net neutral polyampholyte hydrogel containing 50% AMPS and 50% MAPTAC that has been washed solely with HOAc. The figure clearly shows that after a large fraction (4.6 mg) of the Bhb template is removed after five washes with deionized water have been completed. These results indicate that HOAc itself has little effect on the template recognition properties of the polyampholyte hydrogels.

The results of a Bhb re-bind experiment performed on a non-imprinted, net neutral polyampholyte hydrogel containing 50% AMPS and 50% MAP-TAC that was washed solely with SDS is shown in Figure 3.2. The gel washed only with SDS exhibits a much lower unbound Bhb fraction (1.6 mg) compared in comparison to the unbound fraction (2.8 mg) of the similar gel that was washed only with HOAc. The decrease in the unbound fraction of Bhb in supernatant of the gel washed with SDS indicates that,
Figure 3.1: Binding experiment performed on a net neutral (50% AMPS - 50% MAPTAC), non-imprinted polyampholyte hydrogel washed with a 10% HOAc solution. Washes (1-5) were performed using deionized water. After template association occurs, a large fraction of the 6 mg Bhb available resides within the gel. Therefore, similar to their polyelectrolyte counterparts, SDS molecules diffuse into the polyampholyte hydrogel matrix during the template extraction step and remain trapped within the matrix, acting as a high-affinity sink for the Bhb template. Figure 3.3 contains the results of a Bhb template rebinding experiment performed on a non-imprinted polyampholyte hydrogel containing 50% AMPS and 50% MAPTAC that was washed with the SDS-HOAc solution. Approximately 1.4 mg Bhb was unbound following the template association. Therefore, the polyampholyte gels washed with SDS and SDS-HOAc behaved in a similar fashion to the polyelectrolyte gels of Chapter 1, absorbing a considerable
fraction of Bhb with relatively high-affinity when compared with the gel washed with HOAc only. These results indicate that SDS alone, not HOAc or the SDS-HOAc combination, is responsible for the high-affinity binding exhibited by both protein imprinted and non-imprinted polyampholyte hydrogels. In an effort to remove any excess SDS and prevent non-specific binding caused by SDS entrapped within the polymer matrix, all protein imprinted and non-imprinted hydrogels were washed using the modified protocol discussed in Chapter 1. Specifically, an additional wash with 3 M NaCl was added subsequent to the SDS-HOAc wash.

Figure 3.2: Binding experiment performed on a net neutral (50% AMPS - 50% MAPTAC), non-imprinted hydrogel washed with a 10% SDS solution. Washes (1-5) were performed using deionized water.
Figure 3.3: Binding experiment performed on a net neutral (50% AMPS - 50% MAPTAC), non-imprinted hydrogel washed with a solution containing 10% SDS and 10% HOAc. Washes (1-5) were performed using deionized water.
3.3.1 Positively Charged Polyampholytes

Figure 3.4 shows the results of a Bhb template re-binding experiment performed on an imprinted polyampholyte hydrogel containing 25% AMPS and 75% MAPTAC. The gel shows excellent recognition properties, as approximately 4.6 mg (77%) of the Bhb template resided in high-affinity binding sites subsequent to the template association step. In comparison, an insignificant amount (1%) of the Bhb template remained in low-affinity binding sites. The results indicate that the inclusion of both positive and negatively charged monomers in the hydrogel matrix results in an increase of the recognition properties of the MIP hydrogel. Figure 3.5 shows the Bhb template re-binding for an identical, non-imprinted polyampholyte hydrogel. Notice that the re-binding profile is nearly the opposite of the re-binding profile for imprinted polyampholyte hydrogel.

3.3.2 Negatively Charged Polyampholytes

The negatively charged polyampholytes, those containing 25% MAPTAC and 75% AMPS displayed similar behavior to their positively charged counterparts. Figure 3.6 shows data from Bhb template re-binding studies performed on negatively charged, imprinted polyampholyte hydrogels. The imprinted hydrogels displayed template recognition properties that were nearly identical to those displayed by the positively charged polyampholyte hydrogels. Approximately 4.8 mg (80%) of the Bhb tem-
Figure 3.4: Bhb template rebinding data for positively charged, Bhb imprinted polyampholyte hydrogels containing 25% AMPS and 75% MAP-TAC.

Figure 3.5: Bhb template rebinding data for positively charged, non-imprinted polyampholyte hydrogels containing 25% AMPS and 75% MAP-TAC.
plate was bound in high-affinity binding sites subsequent to the template association step. In addition, a small amount (1%) of the Bhb template was present in low-affinity binding sites, indicating a strong preference for the Bhb template by the imprinted gels. The non-imprinted gels (Figure 3.7) showed slight differences from the behavior of the positively charged polyampholyte hydrogels. The non-imprinted, negatively charged polyampholyte gels exhibited slightly higher high-affinity binding (0.54 mg) than the non-imprinted positively charged gels (0.31 mg), although this difference does not appear to be significant.

Figure 3.6: Bhb template rebinding data for negatively charged, Bhb imprinted polyampholyte hydrogels containing 25% MAPTAC and 75% AMPS.
Figure 3.7: Bhb template rebinding data for negatively charged, non-imprinted polyampholyte hydrogels containing 25% MAPTAC and 75% AMPS.

3.3.3 Neutral Polyampholytes

Net neutral polyampholytes, those containing equal amounts of positive and negative charge, should undergo only a minimal amount of cavity deformation. The equal number of positive and negatively charged monomers should effectively screen one another and therefore limit swelling. The results of Bhb template rebinding experiments performed on net neutral, Bhb imprinted polyampholyte hydrogels containing 50% AMPS and 50% MAPTAC are show in Figure 3.8. The gels exhibit excellent template recognition properties, with 3.6 mg Bhb bound in high-affinity sites and only 0.4 mg Bhb bound in low affinity sites. The binding experiment performed on non-imprinted gels, show in Figure 3.9, revealed that
non-imprinted net neutral hydrogels exhibited template affinity that was nearly opposite of their imprinted counterparts. Nearly 5.2 mg of Bhb was either unbound or bound in low affinity sites subsequent to the template association step, while only 0.2 mg was bound in high affinity sites.

Figure 3.8: Bhb template rebinding data for net neutral, Bhb imprinted polyampholyte hydrogels containing 50% MAPTAC and 50% AMPS.

3.3.4 Selectivity

The selectivity of the polyampholyte hydrogels was measured against a competitive template, cytochrome c. The selectivity is defined as the ratio of bound Bhb to bound Cytochrome in the imprinted hydrogels. Cytochrome c has a lower molecular weight and higher isoelectric point than the Bhb template, therefore, it can be used to assess the recognition properties of the hydrogels in relation to template size and isoelectric point.
Selectivity experiments were conducted by loading Bhb imprinted and non-imprinted hydrogels with cytochrome c. Ideally, the Bhb imprinted polyampholyte hydrogels should exhibit low affinity for cytochrome c and higher affinity for the Bhb template.

As Table 3.2 shows, the selectivity exhibited by the polyampholyte gels for Bhb was moderate. The positively charged polyampholyte gels exhibited slightly higher selectivity than the negatively charged gels, although the difference was minor. Overall, the polyampholyte hydrogels showed a lower selectivity than gels containing only positive or negatively charged monomers.
<table>
<thead>
<tr>
<th>Gel</th>
<th>( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% MAPTAC - 75% AMPS</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>25% AMPS - 75% MAPTAC</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>50% AMPS - 50% MAPTAC</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.2: Selectivity factor \((\alpha)\) for polyampholyte gels washed under the modified protocol.

3.3.5 Swelling

To gain a better understanding of the recognition properties of the polyampholyte hydrogels, swelling experiments were performed on non-imprinted polyampholyte hydrogels. The swelling data (Figure 3.10) reveals that polyampholyte hydrogels containing a net negative charge showed increased swelling compared to those gels containing a net positive charge. In comparison with many of the polyelectrolye hydrogels, the swelling ratio of all polyampholyte hydrogels is considerably lower. In fact, only the polyelectrolye hydrogels containing 0.25% AMPS, and the uncharged polyelectrolye hydrogels (100% Acrylamide) exhibited lower swelling ratios. The lower swelling ratios exhibited by the polyampholyte hydrogels should result in higher affinity for the Bhb template, due to the fact that lower swelling ratios generally correspond to decreased cavity deformation. The polyampholyte hydrogels did, in general, exhibit a higher binding affinity.
for the Bhb template as well as an increased imprinting factor.

Figure 3.10: Swelling ratio (SR) for polyampholyte hydrogels.

3.4 Summary

The results of the experiments performed on polyampholyte hydrogels indicate that swelling can be controlled to eliminate excess cavity deformation through the simultaneous inclusion of positively and negatively charged co-monomers within the hydrogel. While this decreased swelling results in an increased imprinting factor, the selectivity of the hydrogels is significantly decreased when compared with gels containing only positively or negatively charged monomers. The high specificity arises from the decreased swelling of the polyampholyte hydrogels compared to their polyelectrolyte counterparts. Decreased swelling results in decreased cavity deformation, and therefore the cavity retains its size, shape, and functional
group orientation upon Bhb template re-binding, resulting in higher template affinity and increased imprinting factors. The low selectivity is a result of the presence of the AMPS monomer. The positive charge of the competitive cytochrome c template at neutral pH is attracted to the negatively charged AMPS monomer. The polyampholyte hydrogels all contain AMPS, and therefore, the cytochrome c is attracted to negatively charged regions of the polyampholyte hydrogels, resulting in decreased selectivity.
Chapter 4

Future Work

4.1 Overview

Despite the recent advancements made in the synthesis and characterization of MIPs which can selectively recognize proteins and peptides in aqueous media, there is still an enormous amount of work that needs to be conducted in the field. Hydrogen bonds, hydrophobic and electrostatic interactions are the dominant mechanisms through which template-receptor complexation occurs in aqueous systems. However, the exact recognition mechanism, and the way in which the aforementioned forces cooperate to recognize target molecules in many MIP systems is largely unknown. In addition, many fundamental properties of aqueous MIPs, such as the dependence of MIP affinity on cross-linker concentration, functional monomer concentration, solvent, buffer composition, ionic strength, pH, and temperature have yet to be systematically studied. Finally, the recognition binding of larger macromolecular complexes, such as viruses [7, 17] and cells, has gone largely unstudied. These factors combined with the increasing interest in creating materials which are capable of mimicking biological processes, ensure that the study of MIPs capable of the selective recognition of peptides, proteins, and other biological macromolecules in aqueous
media will receive considerable attention in years to come.

The recognition properties of molecularly imprinted hydrogels should be studied using a mixture of proteins in solution elucidate a greater understanding of the specificity and selectivity. This is currently a very difficult task, as the current method for measuring the amount of protein bound within the hydrogels involves concentration measurements using a UV/Vis spectrophotometer. The presence of two competitive proteins in solution simultaneously makes them nearly indistinguishable, and therefore the binding properties would be extremely difficult to elucidate. This difficulty may be overcome by carefully selecting proteins that have UV-Vis peaks at easily distinguishable wavelengths, or by tagging the proteins with fluorescent dyes such as rhodamine.

Protein imprinted hydrogels should be synthesized with monomers other than those used in this study. Acrylic acid, for example, could be used in place of the AMPS monomer, to determine how the binding properties of the hydrogels change when a weak acid is used in place of a strong one. Similarly, 2-dimethylaminoethyl methacrylate (DMAEMA) could be used in place of MAPTAC. In addition, derivatives of acrylamide such as an N-(tert-butyl)acrylamide, which contain hydrophobic moieties, could be added to the hydrogels to determine their effect on imprinting. There exists a large library of acrylamide co-monomers available which are susceptible to free radical polymerization, and therefore, numerous combinations exist to optimize selectivity and specificity within hydrogels.
Bibliography


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