Effects of Charge Density on the Recognition Properties of Molecularly Imprinted Polymeric Hydrogels

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ABSTRACT: Molecular imprinting is a technique used to synthesize polymers that display selective recognition for a given molecule of interest. In this study, the role of hydrogel electrostatic charge density on the recognition and selectivity properties of protein-imprinted hydrogels was explored, and the effect of variations of the template extraction protocol on the MIP recognition properties were also studied in depth. Using 3-methacrylamidopropyltrimethylammonium chloride (MAPTAC) as a cationic monomer and 2-acrylamido-2-methylpropanesulfonic acid (AMPS) as an anionic monomer, a number of acrylamide-based hydrogels with varying positive and negative charge densities were prepared. The 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, 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.

Introduction

Molecular imprinting[1−3] is a technique used to synthesize polymers that display selective recognition for a given molecule of interest. Molecularly imprinted polymers (MIPs) are usually prepared by the simultaneous polymerization and cross-linking of functional monomer units in the presence of the target (template) molecule. Subsequent removal of the template yields a highly cross-linked polymer matrix containing cavities that exhibit complementary size, shape, and functional group orientation. The resultant MIPs possess the ability to selectively recognize, rebind, and retain the target molecule.

Traditionally, MIPs have been prepared for the recognition of molecules that are characterized by both low molecular weight and high solubility in organic solvents, as there exist a number of complications that arise when preparing MIPs for high molecular weight molecules in polar solvents. For instance, as the molecular weight of the target molecule increases, diffusion through the densely cross-linked polymer matrix becomes increasingly difficult, resulting in complications during the template extraction and rebinding experiments. In addition, polar solvents attenuate the attractive interaction between oppositely charged groups, making the synthesis of high-affinity binding cavities using electrostatic functional groups increasingly difficult. Because of these complications, fairly little research has been performed concerning the preparation of imprinted polymers for the recognition of biomacromolecules, such as peptides and proteins, and larger biomacromolecular complexes, such as viruses[4−6] and cells[7] in aqueous media. Therefore, the need for imprinted polymers capable of selectively recognizing proteins remains high for applications in diagnostics, vaccine development, recombinant protein purification, and numerous other fields.

Mosbach et al.[8−10] performed much of the early work in the imprinting of peptides and proteins. In these publications, organic solvents were used for both synthesis of the imprinted polymers and the subsequent recognition studies. Hjerten et al.[11] pioneered the use of acrylamide-based hydrogels for the synthesis of imprinted polymers capable of selectively recognizing hemoglobin in aqueous media. This work has been extended using imprinted hydrogels in combination with electrophoresis methods to develop a procedure for the selective recognition and separation of proteins,[12] viruses,[13] and cells.[7] A number of reviews have recently been published summarizing work on the molecular imprinting of peptides and proteins.[14−16]

Relatively few studies have been conducted thus far to explore the role of electrostatic interactions in molecularly imprinted polymers. Hjerten et al.[11] 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.[17] 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 compare the recognition properties of the charged polymers to an uncharged imprinted counterpart. Kameoka et al.[18] synthesized imprinted polymer particles composed of acrylamide and either acrylic acid or *N,N*-dimethylaminopropylacrylamide as comonomers for lysozyme recognition. They found that peak lysozyme recognition occurred at specific molar amounts of charged (acrylic acid) functional groups.

The goal of the present work is twofold. First, the charge density of bovine hemoglobin (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-methacrylamidopropyltrimethylammonium chloride (MAPTAC), while the anionic monomer was 2-acrylamido-2-methylpropanesulfonic 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 ∼6.8. The absorption properties of Bhb-imprinted and nonimprinted hydrogels were measured using batch rebinding experiments, and selectivity experiments were performed using bovine cytochrome *c* as a competitive template. Cytochrome *c* was used to determine

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whether or not the gels synthesized in this study are capable of selectively recognizing Bhb on the basis of 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 rebinding 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 et al.[19] noticed that a similar situation existed in chitosan-based MIPs. They found that both Bhb-imprinted and nonimprinted 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 nonimprinted gels. They also concede that in their previously published papers[20,21] the excessively high template affinity of their MIPS and uncharacteristically low affinity of the nonimprinted polymers were most likely the result of improper template extraction experiments.[22−24] In these experiments, the MIPs were washed with SDS−HOAc solutions, but the nonimprinted (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 nonimprinted 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.

Experimental Section

Materials. Acrylamide, ammonium persulfate, bovine hemoglobin, bovine cytochrome *c*, *N,N′*-methylenebis(acrylamide), *N,N,N′,N′*-tetramethylethyldiamine, 3-methacrylamidopropyltrimethylammonium chloride, 2-acrylamido-2-methylpropanesulfonic acid, sodium dodecyl sulfate, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO) and used as received.

Synthesis. Synthesis of the neutral protein-imprinted polymer hydrogels was adapted from Reddy et al.[25] 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 of acrylamide (monomer), 6 mg of *N,N*-methylenebis(acrylamide) (cross-linker), 10 μL of 5% (v/v) *N,N,N′,N′*-tetramethylethylenediamine (TEMED, catalyst), and 12 mg of Bhb template were dissolved in 1 mL of deionized water in a microcentrifuge tube. Nitrogen was bubbled through the solution for 5 min 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 copolymerization 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. Nonimprinted, 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 1.

Table 1. Synthesis Parameters for Neutral, Positively (MAPTAC), and Negatively Charged (AMPS) Hydrogels (All Values in mg)

	acrylamide	AMPS	MAPTAC	BAAm	Bhb
neutral					
imprinted	54.0	0.0	0.0	6.0	12.0
nonimprinted	54.0	0.0	0.0	6.0	0.0
0.25% AMPS					
imprinted	53.87	0.14	0.0	6.0	12.0
nonimprinted	53.87	0.14	0.0	6.0	0.0
0.5% AMPS					
imprinted	53.73	0.27	0.0	6.0	12.0
nonimprinted	53.73	0.27	0.0	6.0	0.0
1% AMPS					
imprinted	53.46	0.54	0.0	6.0	12.0
nonimprinted	53.46	0.54	0.0	6.0	0.0
0.25% MAPTAC					
imprinted	53.87	0.0	0.14	6.0	12.0
nonimprinted	53.87	0.0	0.14	6.0	0.0
0.5% MAPTAC					
imprinted	53.73	0.0	0.27	6.0	12.0
nonimprinted	53.73	0.0	0.27	6.0	0.0

Bhb Template Extraction. Template extraction occurred in two distinct steps. First, a series of five washes were performed using deionized water. Subsequent to these water washes, a series of five washes were performed using a solution containing 10% (v/v) acetic acid and 10% SDS (w/v), referred to as SDS−HOAc hereafter.

Following synthesis and granulation, the imprinted and nonimprinted hydrogels were suspended in 2 mL of deionized water in a 15 mL polycarbonate centrifuge tube and centrifuged (Sorvall, Waltham, MA) for 5 min at 3000 rpm. This was repeated five times. Subsequent to these five water washes, the gel was washed five 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 five water washes and five 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 nonimprinted 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.

Template Rebinding. In a typical template rebinding experiment, washed and granulated Bhb-imprinted and nonimprinted 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 min. Following template association, gels were removed from the mixer and subjected to the same extraction experiments described in the Bhb template extraction section.

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 and 395 for Bhb in the SDS−HOAc solution. Similar scans performed on solutions of cytochrome *c* in deionized water and SDS−HOAc revealed peaks at 407 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. The supernatant from each step of the

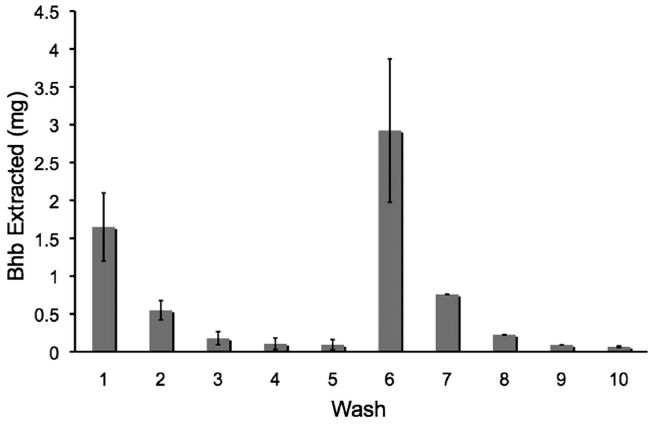

Figure 1. Wash profile for an uncharged (0% ionic groups) Bhb-imprinted hydrogel. Washes 1−5 were performed using deionized water, while washes 6−10 were performed using SDS−HOAc solution.

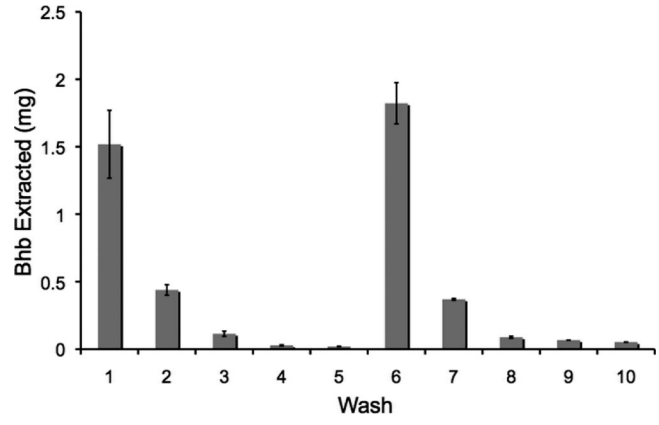

Figure 2. Bhb rebinding experiment performed on an uncharged (0% ionic groups) Bhb-imprinted polymer hydrogel. Washes 1−5 were performed using deionized water, while washes 6−10 were performed using SDS−HOAc solution.

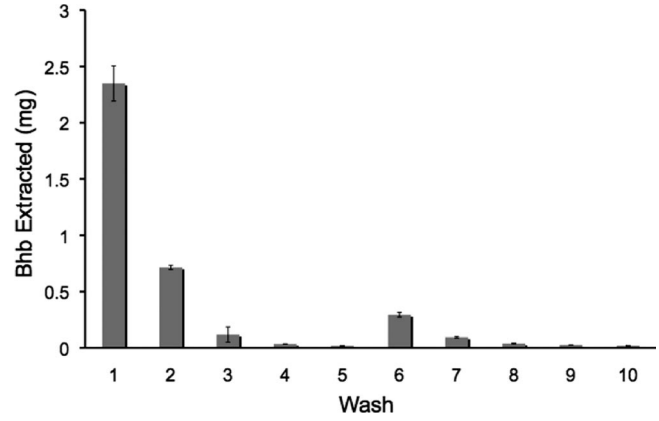

Figure 3. Binding experiment performed on a uncharged (0% ionic groups) nonimprinted hydrogel. Washes 1−5 were performed using deionized water, while washes 6−10 were performed using SDS−HOAc solution.

extraction experiment was diluted appropriately to ensure that it fell within the range of the calibration curve.

Hydrogel Swelling. Swelling experiments were designed to determine the swollen state of the hydrogels in conditions that mimic those present during template rebinding. All swelling experiments were performed using nonimprinted 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 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 min and then centrifuged for 5 min 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 eq 1.

$$\mathrm{SR} = \frac{M_f}{M_i} \tag{1}$$

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 nonimprinted counterpart. In our studies, we perform a similar comparison; however, we also compared nonimprinted 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 1 shows a typical Bhb wash profile for an acrylamide (uncharged) hydrogel imprinted with 12 mg of Bhb.

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 sties, 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.[25,26] 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 and 3 are representative of template-binding experiments performed on uncharged Bhb-imprinted and nonimprinted polymer hydrogels, respectively. The imprinted polymer hydrogel (Figure 2) 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 min association step. In other words, a fraction of Bhb does not enter the gel during the 10 min association step. Therefore, it will be present in the supernatant of wash 1.

Table 2. Summary of Rebinding Results for Uncharged Bhb-Imprinted and Nonimprinted Hydrogels

uncharged	unbound	low affinity (H_2O)	high affinity (SDS−HOAc)
imprinted	1.8 ± 0.15	0.57 ± 0.01	2.1 ± 0.31
nonimprinted	2.3 ± 0.20	0.88 ± 0.05	0.47 ± 0.03

Table 3. Results of Bhb Rebinding Experiments Performed on Negatively Charged (AMPS Containing) Bhb-Imprinted and Nonimprinted Hydrogels of Varying Charge Density[a]

	unbound	low affinity (H_2O)	high affinity (SDS−HOAc)
0.25% AMPS			
imprinted	0.90 ± 0.58	0.82 ± 0.34	3.1 ± 0.25
nonimprinted	1.6 ± 0.25	1.0 ± 0.06	2.2 ± 0.51
0.5% AMPS			
imprinted	0.51 ± 0.10	0.24 ± 0.04	2.8 ± 0.05
nonimprinted	0.80 ± 0.19	0.57 ± 0.26	2.9 ± 0.10
1.0% AMPS			
imprinted	0.021 ± 0.01	0.065 ± 0.05	4.6 ± 0.50
nonimprinted	0.023 ± 0.03	0.053 ± 0.01	4.6 ± 0.35

[a] Data represent the amount of Bhb bound within each hydrogel. All values are in mg.

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 nonimprinted hydrogel (Figure 3) 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 binding experiments on uncharged gels are summarized in Table 2.

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 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, ∼52% (3.1 mg) of the Bhb bound within the imprinted hydrogels resided within high-affinity binding sites. Comparatively, ∼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 sites increased.

Table 3 shows the data from rebinding experiments performed with Bhb on a nonimprinted 0.25% AMPS hydrogel. In these nonimprinted hydrogels, 37% (2.2 mg) of the protein bound was bound in high-affinity sites within the nonimprinted hydrogel. In addition, 17% (1.0 mg) of the Bhb remained unbound after the 10 min association step, and 27% (1.6 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 rebinding experiments conducted on hydrogels containing 0.5% AMPS functional groups are shown in Table 3. In the imprinted hydrogels, ∼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.

Table 4. Results of Bhb Rebinding Experiments Performed on Positively Charged (MAPTAC Containing) Bhb-Imprinted and Nonimprinted Hydrogels of Varying Charge Density[a]

	unbound	low affinity (H_2O)	high affinity (SDS−HOAc)
0.25% MAPTAC			
imprinted	2.2 ± 0.02	0.41 ± 0.11	2.1 ± 0.50
nonimprinted	2.3 ± 0.10	0.88 ± 0.03	1.3 ± 0.15
0.5% MAPTAC			
imprinted	0.022 ± 0.08	0.046 ± 0.01	2.5 ± 0.32
nonimprinted	0.017 ± 0.01	0.067 ± 0.07	3.0 ± 0.10

[a] Data represent the amount of Bhb bound within each hydrogel. All values are in mg.

However, comparison of this data with data from nonimprinted hydrogels containing 0.5% AMPS functional monomers (Table 3) shows that at this charge density both Bhb-imprinted and nonimprinted 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 nonimprinted hydrogels containing 1% AMPS functional monomer were synthesized to confirm the disappearance of the imprinting effect at higher AMPS charge densities. Table 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 nonimprinted hydrogels displayed a large fraction of high-affinity binding, as 77% (4.6 mg) of the total Bhb bound within the imprinted and nonimprinted 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 4 shows the results of rebinding experiments for Bhb-imprinted and nonimprinted 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 nonimprinted counterpart. While the unbound Bhb fraction is similar in both gels, the nonimprinted 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 4 also shows the data for Bhb imprinted and nonimprinted hydrogels containing 0.5% MAPTAC functional groups. The data show that the results of these binding experiments for both imprinted and nonimprinted hydrogels are nearly identical. These results suggests 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 nonimprinted counterparts. In other words, neither imprinted nor nonimprinted 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%

Table 5. Results of Selectivity Experiments Performed Using Cytochrome *c* Template on l Bhb-Imprinted and Nonimprinted Hydrogels of Varying Charge Density[a]

gel	α
uncharged	2.1
0.25% AMPS	1.1
0.25% MAPTAC	0.62

[a] Data represent the amount of cytochrome *c* extracted from each gel in mg.

MAPTAC, as these were the only samples that exhibited high-affinity for Bhb. The selectivity factor, α, which is defined as the ratio of bound Bhb to bound cytochrome *c* bound for these gels is reported in Table 5.

The data in Table 5 indicate 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 negative charge at pH 7 due to its relatively high isoelectric point of approximately 10.0−10.5. This net negative charge on the cytochrome *c* molecules should repel the negatively charged sulfonic acid groups of the imprinted hydrogel containing AMPS, resulting in lower affinity for cytochrome *c*. However, this is clearly not the case, as the selectivity experiments confirmed that the Bhb-imprinted gels absorb both Bhb and cytochrome *c* with similar affinity. Therefore, another mechanism may be responsible for the high-affinity binding in these gels, as is discussed later in the text. Gels containing 0.25% MAPTAC monomer have a slight positive charge, and therefore, they may exhibit a high affinity for the negatively charged cytochrome *c* template. Indeed, we find that Bhb-imprinted 0.25% MAPTAC gels displayed a large amount of high-affinity absorption for cytochorme *c*. It is interesting to note that the selectivity factor (α) 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 of polycarbonate centrifuge tube and centrifuged for 5 min 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 five 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 3 M 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 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 nonimprinted hydrogels under the original and modified wash conditions. Compared to the original conditions, the amount of high-affinity bound Bhb in the imprinted gel containing 0.25% AMPS monomer decreased by

Table 6. Results of Bhb Rebinding Experiments Performed on Negatively Charged (AMPS Containing) Bhb-Imprinted and Nonimprinted Hydrogels of Varying Charge Density Washed Using the Modified Wash Protocol[a]

	unbound	low affinity (H_2O)	high affinity (SDS−HOAc)
0.25% AMPS			
imprinted	1.02 ± 0.60	0.33 ± 0.014	1.46 ± 0.18
nonimprinted	1.08 ± 0.28	0.48 ± 0.25	2.43 ± 0.38
0.5% AMPS			
imprinted	0.53 ± 0.010	0.20 ± 0.021	4.26 ± 0.028
nonimprinted	1.25 ± 0.17	0.47 ± 0.049	4.03 ± 0.42
1.0% AMPS			
imprinted	0.06 ± 0.03	0.19 ± 0.04	4.72 ± 0.22
nonimprinted	0.042 ± 0.06	0.11 ± 0.014	4.99 ± 0.16

[a] Data represent the amount of Bhb bound within each hydrogel. All values are in mg.

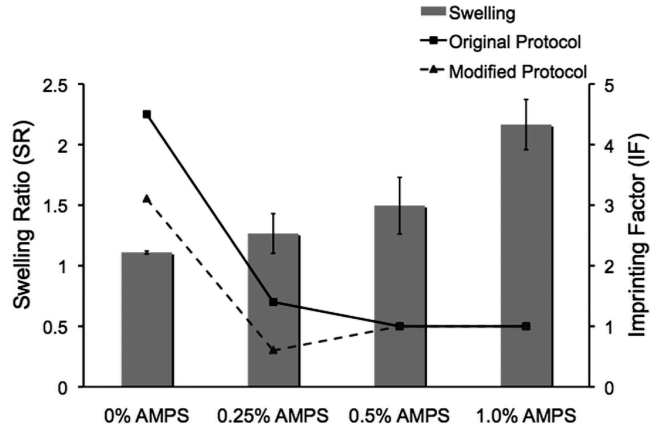

Figure 4. Swelling data and imprinting factor (IF) for gels with various amounts of negatively charged AMPS monomer. The columns represent the swelling data, while the solid and dashed lines represent the IF of gels washed under the original and modified protocol, respectively.

∼50% after the modified wash. The nonimprinted 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 nonimprinted gels consistently displayed similar high-affinity binding data, regardless of whether the modified or original binding protocol was used. The nonimprinted 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 nonimprinted 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 nonimprinted hydrogel which can be explained in terms of hydrogel swelling. Figure 4 shows the swelling data for nonimprinted 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

Table 7. Results of Bhb Rebinding Experiments Performed on Negatively Charged (MAPTAC Containing) Bhb-Imprinted and Nonimprinted Hydrogels of Varying Charge Density Washed Using the Modified Wash Protocol[a]

	unbound	low affinity (H_2O)	high affinity (SDS−HOAc)
0.25% MAPTAC			
imprinted	0.13 ± 0.064	0.28 ± 0.042	4.6 ± 0.12
nonimprinted	4.7 ± 0.28	0.92 ± 0.60	0.32 ± 0.02
0.5% MAPTAC			
imprinted	0.39 ± 0.014	0.37 ± 0.014	3.77 ± 0.01
nonimprinted	1.2 ± 1.1	0.64 ± 0.39	2.3 ± 0.22
1.0% MAPTAC			
imprinted	0.021 ± 0.0	0.060 ± 0.004	3.6 ± 0.37
nonimprinted	0.021 ± 0.0	0.69 ± 0.13	3.6 ± 0.49

[a] Data represent the amount of Bhb bound within each hydrogel. All values are in mg.

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. For Bhb-imprinted and nonimprinted 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 5).

In contrast to the gels containing negatively charged AMPS comonomers, positively charged gels containing MAPTAC monomers displayed stark differences across the original and modified wash protocols. Table 7 contains Bhb template rebinding data for gels containing varying amounts of positively charged MAPTAC monomer. The data reveal that gels containing 0.25% MAPTAC washed with the modified protocol exhibited a pronounced improvement in high-affinity Bhb binding over their nonimprinted 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 nonimprinted 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 nonimprinted 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. Because of 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 nonimprinted gels containing 1.0% MAPTAC showed similar high-affinity binding data, indicating a diminished recognition effect above 0.5% MAPTAC.

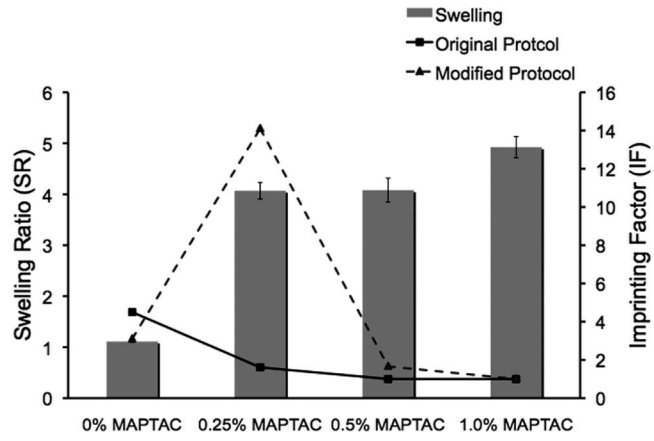

Figure 5. Swelling data and imprinting factor (IF) for gels with various amounts of positively charged MAPTAC monomer. The columns represent the swelling data, while the solid and dashed lines represent the IF of gels washed under the original and modified protocol, respectively. The peak in the dashed line at 0.25% MAPTAC represents the optimum monomer concentration for binding Bhb.

To better understand the binding properties of the gels containing various amounts of positively charged MAPTAC monomer, swelling experiments were performed on the nonimprinted gels. Figure 5 displays the results of these experiments. The swelling data reveal an abrupt increase in swelling upon the addition of 0.25% MAPTAC to neutral gels with the charged gel swelling ~400% more than the neutral gels. In addition to swelling data, Figure 4 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% 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 reveal 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 rebinding 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 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.

Table 8. Selectivity Factor (α) for Gels Containing 0.25% MAPTAC Monomer Washed under the Modified Protocol

gel	α
uncharged	2.1
0.25% MAPTAC	6.6
0.5% MAPTAC	4.6

Conclusions

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 interaction 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 effect of template extraction chemistry on the measured binding properties of MIPs is particularly interesting, as a significant number of published studies[22−24] reveal that the use of SDS−HOAc for template extraction 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 necessarily the result of any recognition properties imparted to the gel through the imprinting process.

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