Effect of Electrostatic Functional Monomers on Lysozyme Recognition by Molecularly Imprinted Hydrogel

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ABSTRACT: Electrostatic functional monomers (EFMs) play an important role in noncovalent molecular imprinting due to their formed complexes with the complementary segment(s) of the template molecule. In this work using UV difference spectroscopy, interaction saturations of methacrylic-acid and 2-dimethyl-amino-ethyl-methacrylate in complex formation with lysozyme (Lyz) surface was found to occur at molar ratios to Lyz of 400 and 100, respectively. Based on these results and the estimated free to total EFMs ratios, four sets of imprinted/nonimprinted hydrogel samples were synthesized alongside the two sets based on lysozyme surface charges and equal EFMs. Comparisons showed that the highest absorption capacity of 59 mg/g and imprinting effect of 1.58 correspond to samples with EFM/Lyz ratios at saturation and minimum free to total EFM/Lyz ratios, respectively. Minimization of free monomers is hence important in recognition of proteins by avoiding the nonspecific binding. This can have generic application for specific separation of other macromolecules. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41366.

INTRODUCTION

Since 1990 when molecular imprinting emerged as a novel method for specific molecule recognition, it has been exploited in a range of applications such as small molecules sensors, selective adsorption of biomolecules, and macromolecules, such as proteins in pharmaceutical, and biotechnology industries. In this method, during the polymerization process a particular molecule (i.e., template) is molded in the polymer matrix which is subsequently extracted from the polymer. The void created in the polymer matrix is utilized to isolate or identify specific molecules from a mixture.[1]

Although molecular imprinting method has been successfully used to separate low molecular mass (less than 1500 Da) biomolecules,[2–5] imprinting of macromolecules such as proteins has been associated with great challenges because of their size, complexity, structural variety under different polymerization medium and conditions, insolubility in organic solvents and their diffusional restrictions.[6–13] Additionally, the polar solvents (i.e., aqueous), used to synthesize MIPs (by proteins) adversely affect the specific recognition of molecules since hydrogen bonding to functional monomers becomes frail due to the competition with water molecules. Therefore, protein recognition can be improved by providing more controlling interactions, for example, much powerful electrostatic interactions (by positive and negative charges) in preference to hydrogen bonding. Electrostatic functional monomers (EFMs) such as methacrylic acid (MAA) and 2-dimethyl amino ethyl methacrylate (DMA) have been used by many researchers to reinforce the bonding between the template molecules and monomers in aqueous phase.[14–22] However, in the previous studies regardless of the protein surface charge distribution, equal amounts of EFMs are used alongside the neutral-hydrophilic monomer, acrylamide (AAm).[14,17–21]

Conferred that the charged fraction of protein surface could affect the appropriate amounts of EFMs, Bergmann considered a relation between the EFMs and their paired amino acids.[15] He observed that the type and amount of amino acids in lysozyme could indicate the required type and amount of EFMs in molecular imprinting.[16] Verheyen, however, doubted the existence of this relation as it had not yet been confirmed.[10]

To study the effect of EFMs on molecular imprinting of proteins, their interaction with the complementary amino acids on the exterior surface of protein during the complex formation phase is an important issue. These interactions can be determined by UV difference spectroscopy or complexation method, previously applied by Anderson et al.[23] It is based on the

measurement of ultraviolet/visible spectrum absorbance difference between the complex and its constructors. Later, Svenson-Anderson evaluated the complex formation between a dipeptide and methacrylic acid.[24] This technique has been successfully used to study complex formation for acrylamide/lysozyme by Liu et al.[25] and bovine hemoglobin (BHb)/acrylamide (AAm) by Gai.[26]

Another main topic in protein imprinting is the determination of free electrostatic monomers or extra monomers. Protein surface is covered with abundant –COOH and –NH$_2$ groups, which can bind to extra electrostatic monomers via hydrogen bonding. In other words, these unordered extra monomers can interact to every protein and eventually lead to nonspecific adsorption.[14–21]

As a useful protein with various applications, we considered lysozyme (MW of about 14,300 Da) as a polyfunctional template consisting of about 129 amino acids providing a net positive charge for this molecule. It has an isoelectric point of 10.4; however, due to the different amino acids with varied isoelectric points in lysozyme; it has local positive and negative charges on its surface at the neutral pH. Lysozyme has two significant amino acids, aspartic acid (pI = 4.5) and glutamic acid (pI = 3.7) that lose their protons at the neutral pH, resulting in a negative charge. This protein also has three positively charged amino acids, lysine (pI = 9.74), histidine (pI = 7.59), and arginine (pI = 10.76). Among hydrophilic amino acids on the surface of lysozyme, asparagine (Asn), and glutamine (Gln) may become slightly positively charged.

In this work, aiming at specific recognition of lysozyme via imprinted hydrogels, initially the point charges of lysozyme surface were determined using Cn3D software and then the molar saturation ratios of electrostatic functional monomers to lysozyme were separately found out by measuring the UV absorption difference between the components of complex (Lyz and monomers) and the generated complex at a given wavelength. Using results obtained via difference absorption, the ratios of free electrostatic monomers (not taking part in complexation) to the total monomers used were theoretically calculated and the effect of the free EFMs on lysozyme imprinting and imprinting effect was studied.

MATERIALS AND METHODS

Chemicals
Acrylamide (AAm), N,N'-methylenebisacrylamide (MBA), 2–dimethylaminoethyl methacrylate, methacrylic acid, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), sodium hydrogen phosphate buffer and other buffers were prepared from Merck and lysozyme (Chicken egg white) was obtained from Sigma-Aldrich. Other chemicals were of analytical grade.

Analyses
Surface Functional Groups of Lysozyme. Since the surface properties of lysozyme can specify the appropriate type and amounts of EFMs, the structural information of lysozyme (PDB code: 1LYZ) was acquired from the National Center for Biotechnology Information (NCBI) site using Cn3D software.[27] This included the composition of positively and negatively charged as well as hydrophilic amino acids.

Complex Formation by UV Difference Spectroscopy. To determine the saturated interaction of lysozyme by each EFM, the ultraviolet absorption difference (ΔA) was calculated by separate measurement of the absorption of each complex constituents as well as the absorbance of mixture containing the complex for various EFM/Lyz ratios via spectrometry according to eq. (1).

$$\Delta A = A_t - A_x$$
$$A_t = A_{Lys} + A_{MAA}(\text{or } A_{DMA}) \tag{1}$$

where A_t is the total absorbance of lysozyme and MAA (or DMA) and A_x is the absorbance of "lysozyme-monomer" mixture at an appropriate wavelength. Plotting ΔA versus EFM/Lyz ratio and finding the ratio above which ΔA levels off, gives the saturated interaction ratio.

An appropriate wavelength should be specified for the UV difference spectroscopy study. The ultraviolet absorption spectrum of solutions was therefore recorded using a Shimadzu single-beam spectrophotometer, using appropriate dilution at various wavelengths ranging 190–290 nm. Additionally, the absorptions of the components of the solutions were separately measured in order to calculate the difference absorbance required in eq. (1).

Ratio of Free/Total EFMs After Complex Formation. During the pre-polymerization phase when complex formation occurs, a number of EFMs do not take part in complex formation process and after polymerization phase, these free monomers constitute the nonspecific binding sites within polymer structure and hence diminish lysozyme recognition. To determine the ratio of free to total electrostatic functional monomers, the absorption of ultraviolet spectra can be utilized.[28] Assuming the reaction given in eq. (2) for complex formation of EFM with lysozyme:

$$L + nM \rightleftharpoons LM_n \tag{2}$$

where L, M, LM_n, and n represent lysozyme, EFM, complexes created and the number of each complexed EFM, respectively, eqs. (3)–(6) can be used to calculate the molar concentration of the complex[28]:

$$[M]_t = [M]_f + n[LM_n] \tag{3}$$

$$A_x = \varepsilon_1[LM_n] + \varepsilon[L]_f + \varepsilon_2[M]_f \tag{4}$$

$$A_t = \varepsilon[L]_t + \varepsilon_2[M]_t \tag{5}$$

$$[LM_n] = \frac{\Delta A}{n\varepsilon_2 + \varepsilon - \varepsilon_1} = k\Delta A \tag{6}$$

in which k is a constant value, brackets represent molar concentrations, ε, ε_1, and ε_2 represent the Extinction coefficient for lysozyme, formed complexes and EFM at a definite wavelength and the subscripts f and t designate free and total, respectively. If lysozyme is fully complexed with EFMs, ΔA reaches a maximum value of ΔA_∞ and eq. (6) can be rewritten as given in eq. (7):

$$[L]_t = [LM_n]_\infty = \frac{\Delta A_\infty}{n\varepsilon_2 + \varepsilon - \varepsilon_1} = k.\Delta A_\infty \tag{7}$$

Combining eqs. (6) and (7), eq. (8) can be derived:

$$[LM_n] = \frac{[L]_t}{\Delta A_\infty} . \Delta A = k . \Delta A \qquad (8)$$

Defining θ as the fraction of complexed lysozyme (at a specified amount of total lysozyme and EFM concentrations) and knowing that the maximum interaction between lysozyme and EFMs occurs at ΔA_∞, the ratio of bounded EFMs to the total lysozyme can be calculated by eq. (9):

$$\theta = \frac{\Delta A}{\Delta A_\infty} = \frac{[LM_n]}{[L]_t} \qquad (9)$$

For a given concentration of lysozyme and EFM, the ratio of free/total EFMs (θ_1) can be obtained using eq. (10):

$$\theta_1 = \frac{[M]_f}{[M]_t} = \frac{[M]_t - n[LM_n]}{[M]_t} = 1 - n \frac{[LM_n]}{[M]_t} \qquad (10)$$

Substituting θ from eq. (9) into eq. (10) results in eq. (11):

$$\theta_1 = 1 - \frac{n.\theta.[L]_t}{[M]_t} \qquad (11)$$

A plot of θ_1 versus EFM/Lyz ratio can be used to interpret the data (i.e., adsorption capacity and imprinting effect) obtained for the synthesized imprinted samples. Additionally, the results of complex formation and free/total EFMs ratio can facilitate the selection of EFM levels in designing the synthesis experiments.

Determination of Experimental Levels of EFMs/Lyz. Six levels of EMFs/Lyz ratios were used to synthesize imprinted samples. The highest value corresponded to the saturation state of EFMs obtained by difference absorbency. To obtain the lowest level, a plot of θ_1 versus EFM/Lyz ratio was used and the ratio at which minimum value of θ_1 occurred was selected. In this study, the minimum level corresponded to the one-eighth of the saturated level and therefore to consider two middle points, half and quarter saturation levels were also selected. Two other samples were made based on 1: equal molar ratio of EMFs/Lyz according to literature[14,17–21] and 2: the surface charges on lysozyme suggested by Bergmann.[16]

Adsorption Capacity and Imprinting Effect of Synthesized Hydrogels. Before assessing the adsorption capacity of each synthesized hydrogel, 50 μL of sodium phosphate buffer (pH: 7.0, 50 mM) was added to 80 mg of the powdered sample to provide a well-swelled and equilibrated hydrogel. The hydrogel was then conditioned with 8 mL of lysozyme solution (0.5 mg/mL) in sodium phosphate buffer (pH: 7.0, 50 mM) for 8 h on a shaker at 150 rpm and at ambient temperature. After centrifugation at 15,000 rpm, the supernatant was tested for the remaining lysozyme using a UV–vis spectrophotometer (CECIL-Bio Quest) at a wavelength of 280 nm. Lysozyme concentration at equilibrium (C_p') was then obtained using lysozyme standard curve. Subsequently, binding capacity of lysozyme (Q, mg/g) defined as the ratio of absorbed protein (mg) to the dry mass of hydrogel powder (g) was calculated for both imprinted and nonimprinted samples according to eq. (12):

$$Q = \frac{V.(C_p - C_p')}{W} \qquad (12)$$

where C_p is the initial concentration of lysozyme solution (mg/mL), V is the solution volume (mL), and W is the dry weight of hydrogel powder (mg). Imprinting effect (IE) defined as the ratio of adsorption capacities of imprinted to nonimprinted samples was also determined.

Experiments

Saturated Interaction of Lysozyme with EFMs. The effect of EFM/Lyz ratio on complex formation was investigated for MAA and DMA. Experiments were performed in 5 mL glass vials by addition of 1 mL of 70 μM lysozyme solution in sodium phosphate buffer (pH: 7.0, 50 mM). To prepare "MAA-lysozyme" complex at various MAA/Lyz molar ratios of 12.5–800, MAA solutions at concentrations of 55.6, 27.6, 13.9, 6.95, 3.48, 1.74, and 0.87 mM in buffer 7 (as above) were prepared and of which 1 mL was added to each vial containing lysozyme, respectively.

Preparation of "DMA-lysozyme" solution was similar to "MAA-lysozyme" except from addition of DMA solutions at desired concentrations to lysozyme solutions into the vials. After mixing, vials were left for 15 min to equilibrate the solution. It should also be mentioned that despite the reported hydrolysis of ester in DMA (17 h half-life at pH 7.4 and 37°C), after polymerization it was quite stable even at extreme condition (80°C, pH 1 and 7).[16,29] There was therefore no concern on DMA hydrolysis as the polymerization process took place for less than 20 min at about 25°C.

Synthesis of Imprinted and Nonimprinted Hydrogels. Three sets of experiments were carried out using AAm as a neutral, hydrophilic, and structural monomer as well as MAA and DMA as powerful electrostatic functional monomers carrying negative and positive charges, respectively. In the first set of experiments, complex formation of lysozyme with EFMs was examined at various molar ratios of EFMs/Lyz. Second, six different molecularly imprinted hydrogels alongside their corresponding nonimprinted samples were synthesized as given in Determination of Experimental Levels of EFMs/Lyz section and last, the adsorption capacities of these synthesized hydrogels were assessed.

Hydrogel matrices were prepared via free radical polymerization[19] using a constant amount of lysozyme. The amounts of AAm and EFMs used as independent variables in synthesizing the samples are given in Table I, according to different bases. These hydrogels were formed by copolymerization of AAm, EFMs, and MBA as crosslinker (CR).

To prepare the hydrogels, 80 mg of lysozyme in sodium phosphate buffer solution (pH: 7.0, 50 mM) was firstly added to each 10 mL vial. Desired amounts of EFMs (MAA and DMA) and AAm were then added into the vials according to Table I such that the overall sample volume became 8 mL. The samples were left for 15 min to allow complex formation. Afterwards, MBA, ammonium persulfate (APS: 10% W/W of total monomers) and TEMED (TEMED/APS = 1.4 V/W) were added into vials as crosslinker, initiator, and accelerator, respectively. The samples were then left for 12 h at room temperature for completion of the reaction. Similarly, nonimprinted samples were prepared exactly in the same manner as the imprinted polymers (MIPs) except from the Lyz that was not used in the formulation. To provide structurally and morphologically similar samples, their total monomer concentration (%T = mass of total

Table I. Design of Synthesis Experiments for Imprinted and Nonimprinted Hydrogels

Sample	Basis	Molar ratio of monomers/Lyz[a]			MAA/DMA (molar ratio)
		AAm	DMA	MAA	
A	Equal EFM/Lyz molar ratio	955 (378)	955(835)	955 (457)	1
B	Surface charge	4011 (1586)	50 (44)	90 (43)	1.8
C	Minimum ratio of free to total EFMs	4130 (1633)	12.5 (11)	50 (24)	4
D	EFMs/Lyz at 1/4 complex saturation	4041 (1598)	25 (22)	100 (48)	4
E	EFMs/Lyz at 1/2 complex saturation	3864 (1528)	50 (44)	200 (96)	4
F	EFMs/Lyz at complex saturation	3510 (1388)	100 (88)	400 (192)	4

All samples contained 160 mg of MBA. Imprinted samples were prepared in presence of 80 mg Lyz.
[a] The values in the parentheses indicate mass (mg) of the monomers used.

monomers/volume of mixture) and their crosslinker content ($\%C$ = mass of crosslinker/mass of total monomers) were kept constant at 21 and 9.5%, respectively.

All samples were dried in an oven (55–60°C) for 48 h until constant weight. Subsequently, the dried samples were separately ground by a mill (Oscillating Mill-MM400) and sieved to provide particles with size smaller than 75 μm (200 meshes). Imprinted samples were repeatedly washed with NaCl solution (1M) and distilled water to extract the template molecule (lysozyme). Nonimprinted samples were similarly washed to provide equally treated samples. About 2 g of each imprinted sample (A-F in Table I) and the corresponding nonimprinted sample were prepared and stored for further use.

RESULTS AND DISCUSSION

In this section, amino acid pattern of lysozyme surface obtained by Cn3D software is firstly demonstrated. The saturation values of EFMs obtained by absorption difference spectroscopy are then contrasted with surface charges on lysozyme. Finally, the adsorption capacities and imprinting effects of the samples synthesized based on experimental design are discussed.

Surface Structural Study of Lysozyme

The results of Cn3D software given as surface charges of lysozyme and its amino acids distribution are displayed in Table II. Taking into account that 29% of amino acids on lysozyme surface are hydrophobic, the molar percentages of positively and negatively charged as well as hydrophilic amino acids on the surface of lysozyme molecule can be calculated as 21, 11, and 39%, respectively.

Based on the distribution of hydrophilic as well as positively and negatively charged amino acids on the surface of lysozyme, it can be concluded that using electrostatic functional monomers can enhance the imprinting effect. This can be as a result of more powerful complexes which can be formed by such monomers compared with the uncharged or nonelectrostatic monomers. However, to make this finding applicable, the number of required electrostatic monomers must also be specified. Bergmann[15,16] simply suggested using an amount of EFM equal to the quantity of the amino acid with an opposite charge. Sample B in Table I is fabricated by this inspiration.

EFM-Lyz Interaction

To monitor the template-monomer interaction, the effect of EFM/Lyz ratio on the shift of UV absorption-band was investigated.[30] Figures 1 and 2 show that the wavelengths of maximum absorbance shift by few values for both DMA and MMA. Although the UV absorption increases with EFM/Lyz ratio, beyond a specific ratio no further shift in the absorption band is detected. This confirms that the interactions between the EFMs (MAA or DMA) and lysozyme are saturated at certain amounts of these functional monomers. The spectrum valley of DMA-Lyz complex in Figure 1 shows a red shift from 251 to 260 nm while Figure 2 shows a blue shift from 251 to 243 nm for MAA-Lyz complex and this remains constant at MAA/Lyz ratio of above 400.

EFM-Lysozyme Complex Formation

Since a specified wavelength is required to measure the difference absorbency, the effect of wavelength on the saturation spectra for EFMs and lysozyme was first investigated. It was found that 235 nm is an appropriate wavelength for UV difference spectrometry. Having specified the appropriate wavelength, saturation concentrations of EFMs were identified. UV difference absorbance spectra at the selected wavelength are illustrated in Figure 3. By raising the molar ratio of MAA/Lyz, increases can be observed in the measured difference; however, the difference levels off at above a ratio of 400 : 1. This means that one mole of lysozyme molecule will be saturated by 400 mole of MAA. For DMA-lysozyme complex, lysozyme

Table II. Number of Positively and Negatively Charged and Hydrophilic Amino Acids on the Exterior Surface of Lysozyme Molecule

Charged amino acids		Hydrophilic amino acids	
Name	Number (charge)	Name	Number
Histidine	1 (+)	Tyrosine	3
Arginine	11 (+)	Glutamine	5
Lysine	6 (+)	Asparagine	14
Glutamic acid	2 (−)	Threonine	6
Aspartic acid	7 (−)	Serine	8
Total	27	Total	36
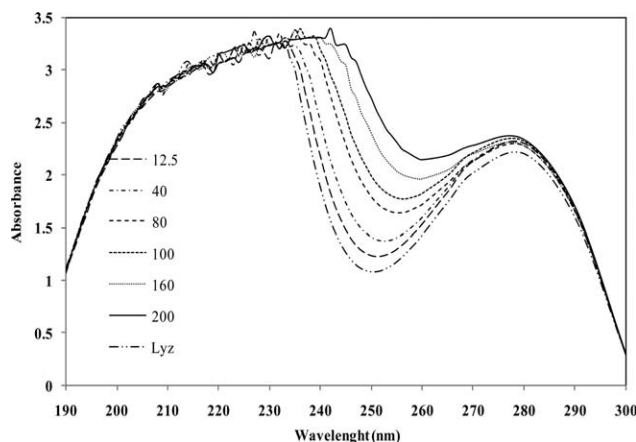
Figure 1. Absorption spectra at various molar ratios of DMA/lysozyme.

Figure 3. Effect of EFM/Lyz molar ratio on complex formation with lysozyme (ΔAbsorbance at 235 nm) and free/total EFM ratio (θ_1) for DMA and MMA.
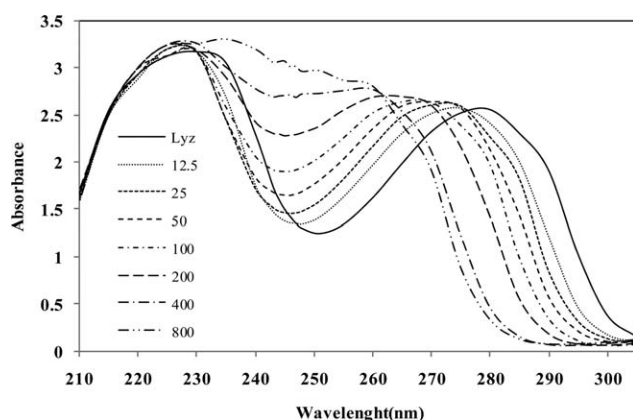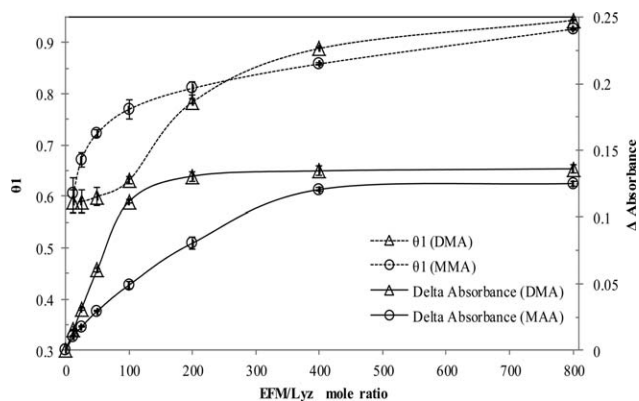
saturation occurs at 100 mole of DMA. Based on these findings a MAA/DMA ratio of 4 was used in synthesizing the four samples C-F as identified in Table I. The upper levels of EFMs in Table I (sample F) were designed based on these saturation values.

Effect of EFM/Lyz Ratio on Free/Total EFM Ratio

Since presence of free functional monomers diminishes the separation specificity, the ratios of free/total EFM (θ_1) was examined at various EFM/Lyz ratios, using Eq. (11). To calculate θ_1 using Eq. (11), n representing the number of amino acids on the surface of lysozyme interacting with EFMs by hydrogen and/or electrostatic bonding, needs to be determined. According to Table II, lysozyme contains 9 negatively charged and 36 hydrophilic amino acids on its surface, therefore the number of required DMA monomers (n) for DMA-Lyz complex becomes 45. Taking into account the 18 positively charged and 36 hydrophilic amino acids per lysozyme molecule, formation of MAA-Lyz complex needs 54 MAA monomers. Using these n values, θ_1 was plotted versus the molar ratios of EFM/Lyz for MAA and DMA as given in Figure 3. The minimum values of θ_1 for MAA and DMA occurred at 25 and 50 molar ratios of EFM/Lyz, respectively.

Adsorption Capacities and Imprinting Effect of Samples

Adsorption capacity and imprinting effect of the synthesized samples (according to Table I) are shown in Figure 4. Sample A was based on equal molar ratio of functional monomers to lysozyme while the surface charges on lysozyme suggested by Bergmann were the basis for Sample B. Results obtained from the surface analysis of lysozyme, that is, 21% positively and 11% negatively charged amino acids on lysozyme surface, were used to provide this sample having an approximate ratio of MAA/DMA equal to 1.8. Based on the complex formation results and the saturation levels of 400 and 100 moles obtained for MAA and DMA per mole of lysozyme, respectively, a MAA/DMA molar ratio of 4 was used in all other samples (Samples C–F). Samples F (the highest amounts of EFMs) and C (the lowest amounts of EFMs) correspond to the saturation levels of EFMs in complex formation with lysozyme and the minimum free/total EFM ratio (θ_1), respectively. The two other samples (D and E) were selected in between.

Imprinted Samples A, D, E, and F showed nearly similar high capacities, with the highest value of 59 mg/g for Sample F. The lowest adsorption capacity of 34.5 mg/g corresponds to sample C containing MAA and DMA to lysozyme ratios of 50 and 12.5, respectively. As can be seen apart from Sample C, the adsorption capacity of the nonimprinted samples are relatively close to
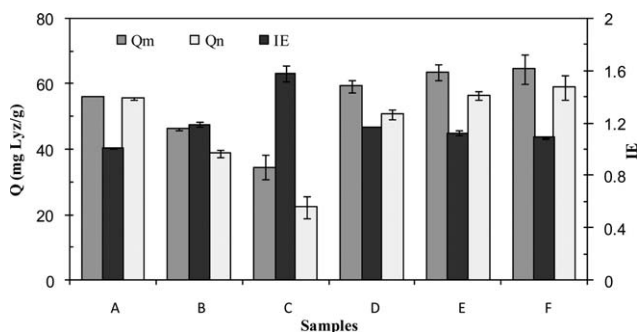

Figure 2. Absorption spectra at various molar ratios of MAA/lysozyme.

Figure 4. Lysozyme adsorption capacities and imprinting effects of the synthesized samples given in Table I. Q_m, Q_n, and IE represent the adsorption capacity of imprinted and nonimprinted polymers and imprinting effect, respectively.

Table III. Percentages of the Three Functional Monomers (FMs) Obtained via Various Methods

Method	Percentages of FMs		
	AAm	DMA	MAA
UV difference spectroscopy	28.6	14.3	57.1
Surface charges	57.1	14.3	28.6
Corrected surface interactions	28.6	14.3	57.1

their corresponding imprinted samples showing a low separation specificity. To quantitatively examine the specificity, imprinting effects were also illustrated in Figure 4. Interestingly, it was observed that the highest imprinting effect of 1.58 is related to Sample C, which exhibited the lowest Lyz adsorption capacity.

As can be seen in Figure 4, Sample B containing a MAA to DMA ratio of 1.8, does not lead to the highest imprinting effect. This can be attributed to the fact that it ignores the possible interactions between MAA and other hydrophilic amino acids, which may play an important role in the complex formation. Therefore, the contribution of functional monomers in complex formation was determined by UV difference spectroscopy as 200, 100, and 400 moles of AAm, DMA, and MAA per mole of lysozyme, respectively. Accordingly, a MAA to DMA ratio of 4 was selected, which resulted in a superior imprinting effect. These results showed that the hydrogen bond between MAA monomers and the hydrophilic amino acids of lysozyme surface should be considered in addition to the electrostatic interactions between MAA and the charged amino acids. Among hydrophilic amino acids on the surface of lysozyme given in Table II, asparagine (Asn), and glutamine (Gln) containing $-CH_2ONH_2$ and $-C_2H_4CONH_2$ groups may become slightly positively charged. Therefore, the number of complexes formed by MAA increases by 19 as 14 Asn and 5 Gln exist on the surface of lysozyme. This necessitates the exploitation of a corrected surface charge to avoid the deviations from the reality. Table III compares the percentages of the three functional monomers obtained via UV difference spectroscopy and surface charge methods with those based on the corrected surface charges and the corresponding interactions. It can be seen that the results obtained from the UV difference spectroscopy method are unlike those obtained by surface charges, while the corrected surface interactions show comparable results.

Although in most studies an equal amount of these electrostatic and hydrophilic functional monomers has been utilized,[14,17–21] our results showed the lowest IE of 1 for Sample A, as prepared at this molar ratio. This can be due to the fact that during the polymerization process most of EFMs (MAA and DMA) act as free monomers dispersed randomly in polymer matrix leading to similar structures of imprinted and nonimprinted polymers. Furthermore, investigation of the effect of θ_1 as another key parameter on the IE, showed that by increasing θ_1 the imprinting effect decreases, and vice versa. In other words, it was demonstrated that the ratio of free to total EFM more profoundly affect the imprinting effect compared with the saturation of

lysozyme surface by that monomer. Comparison of the number of free and complexed DMA (MAA) molecules shows that out of the total 25 molecules, 15 DMA (16 MAA) remain free at a low molar ratio of 25, while this becomes 356 molecules for DMA (344 for MAA) at a high molar ratio of 400. Therefore, to enhance the imprinting effect, minimum ratios of total EFM/Lyz needs to be applied to reduce the free EFMs.

CONCLUSIONS

In this study, selection of proper amounts of electrostatic functional monomers and the adverse effect of free (or extra) electrostatic monomers in prepolymerization mixture were investigated by complexation or UV difference spectroscopy method. Experimental results showed that although adsorption capacity increases with the amount of electrostatic monomers (MAA and DMA), reductions are observed in imprinting effect due to the accumulation of free monomers resulting in increased sites of nonspecific binding. This seems to be an inherent limitation of such system. Although lysozyme saturation analysis can be used to find the proper ratio of MAA/DMA, the analysis of free to total EFM ratio should be exploited to determine the amounts of EFMs enhancing the imprinting effect. Furthermore, results confirmed the hypothesis of electrostatic monomers interaction with other hydrophilic amino acids on the surface of lysozyme. Therefore, the corrected surface interaction was proposed for determination of EFM/Lyz ratios resulting in similar ratios of MAA/DMA to those obtained by lysozyme saturation analysis.

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