Preparation and Characterization of Molecularly Imprinted Polymer of Bovine Serum Albumin

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ABSTRACT: Molecularly imprinted polymer beads of bovine serum albumin (BSA) were prepared via inverse phase suspension polymerization, using BSA as the template molecule, a combination of acrylamide and methacrylic acid (MAA) as double functional monomers, and *N*, *N*′-methylene bisacrylamide as the crosslinker. The effect of different monomer ratios and degrees of crosslinking were investigated. When both selectivity and physical properties of the resultant polymer beads were taken into account, the ratio of MAA in the total monomers was chosen at 40% (m/m) and the degree of crosslinking at 30% (n/n), the resultant polymer beads had good selectivity ($\alpha = 2.77$) and good physical properties. The effects of pH and temperature were studied. It turned out that the functionalization of polymers of BSA prepared via inverse-phase suspension polymerization exhibited specific recognition for BSA. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 723–728, 2012

Key words: molecularly imprinted; functionalization of polymers; molecularly recognition; inverse phase suspension polymerization; bovine serum albumin

INTRODUCTION

Molecularly imprinted polymer (MIP),[1] an artificial recognition material with high selectivity and affinity to the template molecule, has been widely used in many fields, such as separations of enantiomers and isomers,[2–4] solid phase extraction,[5–7] bionic chemistry sensors,[8,9] model enzyme catalysis,[10] clinical analysis of drugs,[11] and membrane separation technique.[12,13] Compared with other recognition materials, MIPs enjoy many advantages, e.g., simple preparation procedure, predetermined selectivity, low cost, wide application, and reusability. The synthesis and its application of MIPs, especially those that have special recognition ability to biological macromolecules such as proteins, and polypeptides, have been increasingly attracting attention in life science. So far, most researches about MIP technique involve small molecules,[14–16] the limitation of the application of MIP in biological macromolecules lies in such factors as large size, complex structure, conformational flexibility, and solubility concern of the macromolecules. First of all among them is aqueous compatibility, for exposure to polar solvents weakens the interaction between the template molecules and the functional monomers thereby resulting in a decrease in selectivity for determination. However, most applications of MIPs in environmental and biological samples are performed in aqueous medium. Therefore, the development of new strategies for the application of MIPs in aqueous medium is absolutely imperative.

There have been a few reports about molecular imprinting techniques for biological macromolecules. One of the main methods is surface–imprinted. Tan et al.[17] prepared bovine serum albumin (BSA) surface-imprinted particles by two-stage core-shell miniemulsion polymerization, and Chen and coworkers[18] prepared protein molecularly imprinted membranes on the surface of multiwalled carbon nanotubes using BSA as template molecule, still, Ju and coworkers[19] prepared a surface protein-imprinted nanowire for protein specific recognition through the combination of immobilization of template protein molecules on the pore walls and chemical polymerization of dopamine in physiological conditions. Another method involves monolithic materials. Zhang and coworkers[20] developed a series of imprinted monolithic polyacrylamide materials through one-step *in situ* polymerization in an HPLC column tube, such as silica-polyacrylamide hybrid monolithic columns for the recognition of BSA, materials made out of lysozyme and macroporous cytochrome.[21] These monolithic columns have been successfully applied to the fast separation for template proteins separation. However, relatively complex preparation steps and time-consuming procedures, as well as the adsorption capacity, can be the general flaws for these method.[22] Therefore, a straightforward and cost-effective method is always in great

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need. In this article, we adopted acrylamide and methacrylic acid (MAA) as double functional monomers via inverse phase suspension polymerization in the preparation of MIP of BSA, which aimed at improving the selectivity of MIP and adsorption capacity. The results indicated that the result polymer showed good recognition properties and fair adsorption capacity.

EXPERIMENTAL SECTION

Materials and instruments

MAA was purchased from Tianjin Chemistry Reagent Factory (Tianjin, China) and was distilled under reduced pressure before use, *N*, *N*′-methylenebisacrylamide (BA, Shanghai Reagent Factory, China), Span-80 (Tianjin Fu-Chen Chemical Reagent Factory, China) were all of chemical grade. Acrylamide (AM, Guang-Hua Chemical Reagent Factory of Shantou, China) was purified by recrystallization. Ammonium persulfate (APS, Jin-Bei Chemical Engineering CO.LED of Tianjin, China), trifluoroacetic acid (TFA, Pai-Ni Chemical Reagent Factory of Zhengzhou, China), cyclohexane and acetic acid (Xi'an Chemistry Reagent Factory, China) were of analytical grade. Acetone and disodium hydrogen phosphate were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Acetonitrile (HPLC Grade Fisher Scientific), BSA (Roche), ovalbumin, and lysozyme were purchased from Sigma. Water used in the experiment was double distilled water. All other reagents were of analytical grade, unless otherwise stated. Environmental Scanning Electron Microscope (ESEM, Quanta 200, FEI Corporation, America) was used to analyze the surface morphologies.

Synthesis of MIP

The MIP beads were prepared via an inverse phase suspension polymerization method. A total of 80 mL of cyclohexane and 0.800 g of Span80 were added to a 100-mL three-necked flask equipped with a mechanical stirrer and a nitrogen inlet. The mixture was stirred under nitrogen atmosphere until the surfactant was uniformly dispersed. Following this step, 0.78 g AM (11 mmol), 0.7710 g BA (5 mmol), 0.1528 g NaOH, 0.48 mL MAA (5.6 mmol), and 1 mL BSA (2 mg/mL) were dissolved in 24 mL double-distilled water under nitrogen atmosphere. The reaction was initiated by the addition of 0.5 mL of APS and 1 mL of promoter TMEDA under stirring (500 rpm) at 20°C lasting for 2 h. The polymer beads formed were washed alternately with acetone and double-distilled water to remove the unreacted monomers and other impurities and were left dry at room temperature. To remove the template BSA, the obtained MIP beads were washed with 10% (v/v) acetic acid for 24 h and sequentially with double-distilled water until neutral pH was achieved. Other MIP beads were synthesized under the same conditions except that different monomer ratios and degrees of crosslinking were used.

The nonimprinted polymer (NIP) beads were prepared using the same procedures but without the addition of the template molecule. The NIP beads were washed in the same procedure as in the preparation of the MIP beads described above.

Analysis ESEM

The lyophilized imprinted polymer beads and NIP beads were mounted on metal stubs after the gold sputtering treatment, and then, their surface morphologies were analyzed by ESEM with an accelerating voltage at 20 kV.

Adsorption experiments

In static adsorption experiment, 10 mg of the polymer bead obtained under the variables investigated was dispersed in 10 mL of 1.0 mg/mL of BSA solution and was then kept in the thermostatic water bath at 20°C for 24 h. In the investigation of the effect of temperature and acidity, the experimental procedures were the same except that the condition being investigated varied. In the following step, the mixtures were centrifuged and the protein concentrations in the supernatant were determined by UV absorption.

In dynamic adsorption experiment, the MIP beads were dispersed in a certain volume of BSA solution with the initial concentration of 1.0 mg/mL. At different time intervals, the mixtures were centrifuged and the supernatant solutions were collected, concentrations of which were determined using the UV-vis spectrophotometer.

The experimental data were expressed in terms of adsorption capacity per unit mass (g) of the polymer beads, and the adsorption capacity (denoted as Q) was calculated according to the following formula[23]

$$Q = (C_0 - C_s) \times V/m \quad (1)$$

Where C_0 (mg/mL) is the initial concentration of protein in the solution, C_s (mg/mL) is the protein concentration of the supernatant, V (mL) is the volume of the initial protein solution, and m (g) is the mass of the polymer beads.

The imprinting factor (α) and selectivity factor (β) were used to evaluate the separation selectivity of MIP beads amid various other proteins, and α was calculated as follows[24]

$$\alpha = Q_{MIP}/Q_{NIP} \quad (2)$$

TABLE I
The Effect on Adsorption Capacity of Polymer Beads Obtained under Different Conditions

Polymer	AM (mmol)	MAA (mmol)	BA (mmol)	Q (mg/g)	α
MIPAM	16.6		0.300	180	2.00
NIPAM	16.6		0.300	90.0	
MIPMAA		16.6	0.300	260	2.60
NIPMAA		16.6	0.300	100	
MIP (AM-*co*-10%MAA)	15.2	1.40	0.300	250	1.79
NIP (AM-*co*-10%MAA)	15.2	1.40	0.300	140	
MIP (AM-*co*-20%MAA)	13.8	2.80	0.300	420	1.50
NIP (AM-*co*-20%MAA)	13.8	2.80	0.300	280	
MIP (AM-*co*-30%MAA)	12.4	4.20	0.300	750	4.41
NIP (AM-*co*-30%MAA)	12.4	4.20	0.300	170	
MIP (AM-*co*-40%MAA)	11.0	5.60	0.300	750	5.36
NIP (AM-*co*-40%MAA)	11.0	5.60	0.300	140	
MIP (AM-*co*-50%MAA)	9.60	7.00	0.300	820	3.73
NIP (AM-*co*-50%MAA)	9.60	7.00	0.300	220	
MIP (AM-*co*-60%MAA)	8.20	8.40	0.300	450	2.05
NIP (AM-*co*-60%MAA)	8.20	8.40	0.300	220	

BSA concentration: 1.0 mg/mL, pH = 4.8, 20°C.

Where Q_{MIP} and Q_{NIP} are protein adsorption capacity of MIP beads and NIP beads, respectively.β was calculated as follows :

$$\beta = \alpha_{\text{template protein}}/\alpha_{\text{competitor protein}} \quad (3)$$

where $\alpha_{\text{template protein}}$ and $\alpha_{\text{competitor protein}}$ are imprinting factor of the template protein and the competitor protein, respectively.

RESULTS AND DISCUSSION

Choice of monomers and optimization of monomer ratio

In the preparation of MIP via an inverse phase suspension polymerization, the effect of different functional monomers on the recognition capacity to the template molecule were investigated, including AM, MAA and the combination of the AM and MAA. The effect on adsorption of monomer ratio of the polymer beads to BSA was investigated by varying monomer molar ratios while keeping the total number of moles of the monomers constant.

As is shown in Table I, the combination of AM and MAA as functional monomer enhanced both the rebinding capacity and the imprinting factor to a greater extent than using the two individually. Thus, the combination of AM and MAA was chosen as functional monomers. The adsorption results obtained under different ratios of polymer beads to the template molecule are displayed in Table I. The adsorption capacity of MIP beads to BSA increased rapidly with the increasing ratio of MAA up to 40% in the total monomers, and then exhibited a gentle climbing, thereafter decreased gradually. The selectivity of the polymer beads to BSA, however, reached its maximum (α = 5.36) while the ratio of MAA was at 40%. As a compromise between adsorption capacity and selectivity, we chose the ratio of MAA in the total monomers at 40% in the following experiments. Another disadvantage resulting from MAA with ratios higher than 40% lay in the increasing difficult in the process of washing.

Effect of degree of crosslinking on adsorption capacity

The effects of degree of crosslinking on the adsorption capacity of the polymer beads to BSA were studied, and the results are shown in Table II.

As is shown in Table II, the ideal adsorption capacity of polymer beads to BSA as well as selectivity was obtained when the degree of crosslinking was in the range (20%–50%) or at less than 5%. More or less beyond this range, the adsorption capacity decreased substantially. Adequate rigidity is another indispensible consideration, while the rigidity of beads increased along with the increasing degree of crosslinking, which was attributed to the increased density and strength of the polymer beads. A degree of crosslinking was chosen at 30% when taking both adsorption capacity and rigidity into account.

Analysis ESEM

The ESEM microphotographs of the two different polymer beads obtained are shown in Figure 1.

As is shown in Figure 1, the morphologies of the imprinted polymer beads and NIP beads were quite different from each other. The surface of the imprinted polymer beads had both regular and compact holes, while the surface of the NIP beads was irregular and loose. It was assumed[25] that the

TABLE II
The Effect of Degree of Crosslinking on the Adsorption Capacity of the Polymer Beads to BSA

Polymer	AM (mmol)	MAA (mmol)	BA% (n/n)	Q (mg/g)	α
MIP (AM-*co*-40%MAA)	11.0	5.60	1.80	750	5.36
NIP (AM-*co*-40%MAA)	11.0	5.60	1.80	140	
MIP (AM-*co*-40%MAA)	11.0	5.60	7.80	300	3.75
NIP (AM-*co*-40%MAA)	11.0	5.60	7.80	80.0	
MIP (AM-*co*-40%MAA)	11.0	5.60	20.0	220	2.44
NIP (AM-*co*-40%MAA)	11.0	5.60	20.0	90.0	
MIP (AM-*co*-40%MAA)	11.0	5.60	30.0	360	2.77
NIP (AM-*co*-40%MAA)	11.0	5.60	30.0	130	
MIP (AM-*co*-40%MAA)	11.0	5.60	50.0	220	2.44
NIP (AM-*co*-40%MAA)	11.0	5.60	50.0	90.0	

BSA concentration: 1.0 mg/mL, pH = 4.8, 20°C.

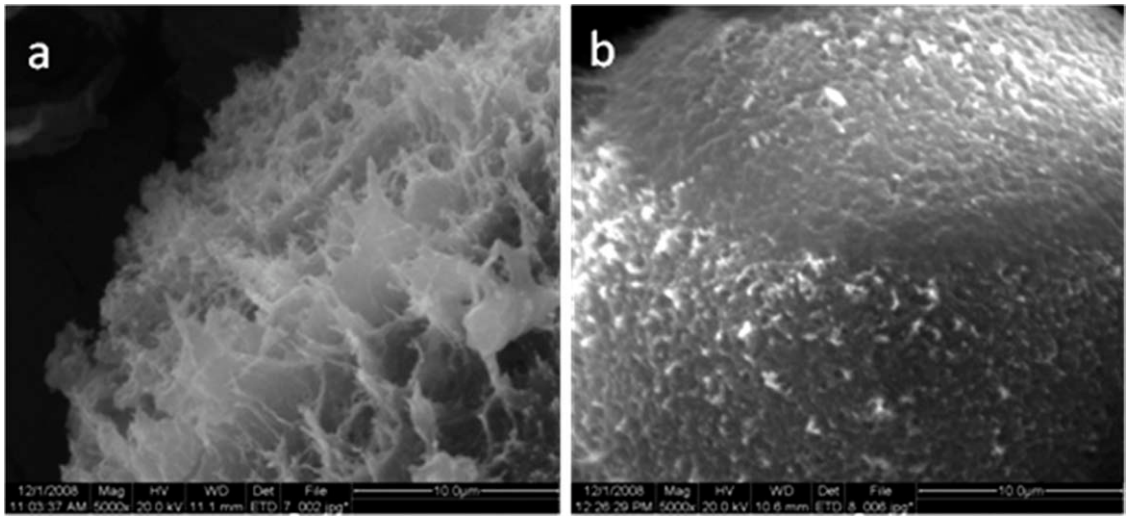

Figure 1 ESEM microphotographs of polymer beads (×5000): (a) NIP (AM-40%MAA) and (b) MIP (AM-40%MAA).

surface of the imprinted polymer beads became regular and compact after being imprinted with BSA, the further evidence is still needed.

Adsorption specificity

To verify the selective recognition of the BSA imprinted polymer beads, ovalbumin (Ob) and lysozyme (Lys) were selected as competitive proteins which are different from BSA in whether mass or charge. The adsorption capacities of the polymer beads to these proteins in the optimized synthetic conditions were determined through a static method. The results are displayed in Table III.

As revealed in Table III, the BSA-MIP beads exhibited good adsorption selectivity for the template protein. The adsorption capacity of BSA-MIP beads toward BSA was much greater than that toward Ob or Lys. The following fact held the clue to these phenomenon, although Ob and Lys were small enough to get into the imprinting cavities, the recognition sites of the imprinting cavities were not complementary to Ob and Lys in structure; therefore, they two were less likely to be adsorbed onto the BSA-MIP beads. When it came to the comparison of adsorption specificity, as might be expected, the NIP beads adsorbed template molecules much less than that of MIP beads because the NIP beads had not specific recognition sites generated by template protein. The physical adsorption was the primary cause for the adsorption of the NIP beads.

Adsorption kinetics

The dynamic adsorption of the MIP beads to BSA was assessed with an initial protein at a concentration of 1.0 mg/mL, and the profile is shown in Figure 2.

It can be seen that the adsorption capacity showed a rapid increase at the beginning, and increased slightly along, at last exhibited a plateau about 7 h later. This was because the surface of the MIP beads had more adsorption sites for BSA and less mass transfer resistance at the beginning of adsorption process, which allowed for these imprinted cavities to capture the imprinted molecules thereby increasing the adsorption rate. However, with the advance of time, the number of adsorption sites decreased, imprinted cavities of the surface were gradually occupied by the imprinted molecules, and mass transfer resistance of BSA to the deeper imprinted cavities increased accordingly, adsorption rate reduced and the increasing of adsorption capacity slowed down.

Effect of acidity

As is shown in Figure 3, the adsorption capacity of the MIP beads to BSA was found at its maximum when the pH was set at 4.6. So the polymer beads were synthesized at the same pH so that BSA had the most suitable conformation to match the recognition cavities of the polymer beads, which substantially facilitate the bonding between the template and the polymer. However, there was no specific cavity in the NIP beads, so the bonding force

TABLE III
Comparison of Binding Specificity of MIP Beads and NIP Beads to Different Proteins

Protein	Q_{MIP} (mg/g)	Q_{NIP} (mg/g)	α	β
BSA	360	140	2.77	
Ob	141	143	0.99	2.80
Lys	230	250	0.92	3.01

Protein concentration: 1.0 mg/mL, pH = 4.8, 20°C.

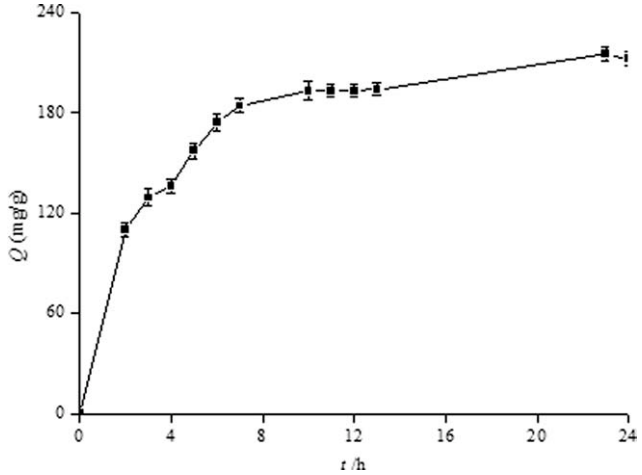

Figure 2 The dynamic curves for the adsorption of BSA to the MIP beads. BSA concentration: 1.0 mg/mL, pH = 4.8, 20°C.

between the NIP and BSA was mainly electrostatic interaction between the amino group and carboxylic group, what is more, there was no so such specificity as in MIP beads.

Effect of temperature

The effect of temperature on the adsorption ability is shown in Figure 4.

As displayed in Figure 4, the adsorption capacity increased with increasing temperature, which revealed that the adsorption process of the MIP beads to the template molecule was an endothermic process.

The experimental data were analyzed according to the classical adsorption isothermal equation, i.e., the Freundlich equation[24]

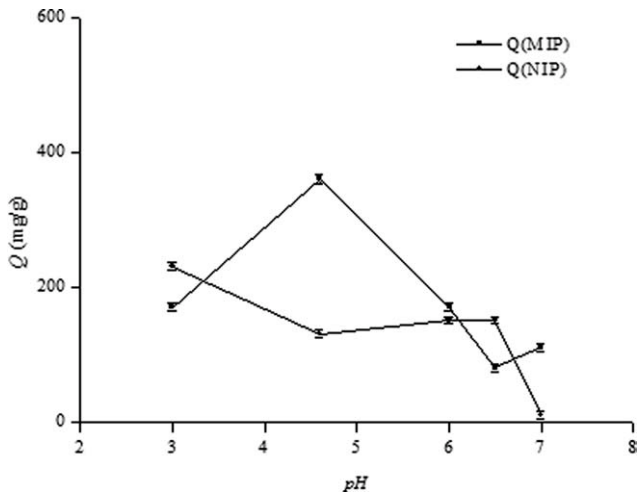

Figure 3 Adsorption of the MIP beads and the NIP beads to BSA under different pH. BSA concentration: 1.0 mg/mL, 20°C.

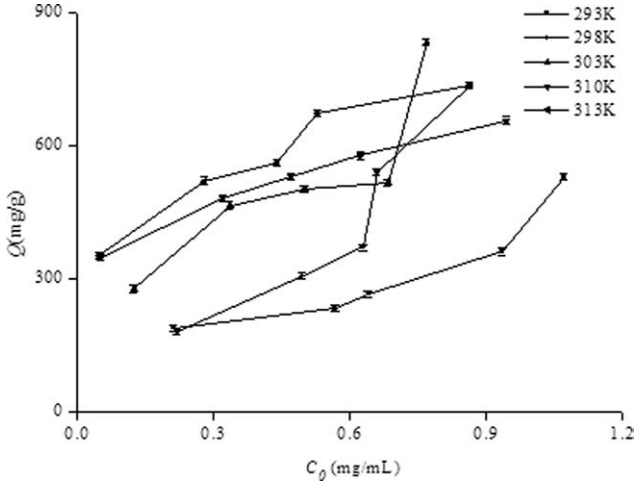

Figure 4 Adsorption isotherms of the MIP beads to BSA under different temperature.

$$\ln(Qe) = \ln(K_f) + 1/n \ln(C_e) \quad (4)$$

The results are shown in Table IV.

As shown in Table IV, the correlation coefficient R was all greater than 0.86, indicating that the adsorption of the MIP beads to the template was the "preferential adsorption."

The adsorption enthalpy change (ΔH), free energy change(ΔG), and entropy change(ΔS) of the MIP beads to the template were calculated according to the following formulas[26]

$$\ln(1/C_0) = -(\Delta H/RT) + \ln(K_0) \quad (5)$$

$$\Delta G = -nRT \quad (6)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (7)$$

The thermodynamic data of the MIP beads to the template molecule at different adsorption capacities are shown in Table V.

As shown in Table V, the isometric adsorption enthalpy change of the MIP beads to BSA was positive, suggesting that the adsorption was an endothermic process. In general, the heat of adsorption from physical process was less than that from chemical process. The value of the former was in the range of

TABLE IV
Freundlich Parameters for the Adsorption of the MIP Beads to BSA

T (K)	The fitting equation	K_F	N	R
293	$\ln Q = 0.555 \ln C_e + 5.97$	390	1.80	0.876
298	$\ln Q = 0.976 \ln C_e + 6.58$	717	1.02	0.944
303	$\ln Q = 0.490 \ln C_e + 6.63$	758	2.04	0.918
310	$\ln Q = 0.211 \ln C_e + 6.46$	636	4.74	0.990
313	$\ln Q = 0.253 \ln C_e + 6.61$	743	3.96	0.982

TABLE V
Thermodynamic Parameters for the Adsorption of the MIP Beads to BSA

		−ΔG (KJ/mol)					ΔS (J/mol·K)				
Q (mg/g)	ΔH (KJ/mol)	293K	298K	303K	310K	313K	293K	298K	303K	310K	313K
200	182						636	618	617	626	614
300	132	4.39	2.54	5.14	12.2	10.40	467	453	454	467	456
400	97.1						347	334	337	353	343

8.37–62.8 KJ/mol while that of the latter was 125.6–418.68 KJ/mol.[27] Therefore, the adsorption process of BSA was mainly a chemical adsorption process. The adsorption free energy change (ΔG) was negative, and the entropy change (ΔS) was positive, revealing that the adsorption could occur.

CONCLUSIONS

MIP beads of BSA, which had definite recognition ability and fairly good adsorption properties, were prepared using AM and MAA as double functional monomers. The inverse phase suspension polymerization adopted was not only simple but also practical in tackling aqueous compatibility of imprinting proteins. Negative ΔG and positive ΔS demonstrated that the adsorption can take place spontaneously. Positive ΔH indicated that the adsorption of the MIP beads to BSA was an endothermic process.

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