Protein-Imprinted Soft-Wet Gel Composite Microspheres with Magnetic Susceptibility. II. Characteristics

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ABSTRACT: Protein-imprinted soft-gel composite microspheres with magnetic susceptibility (MS-PIGMs) were prepared by inverse suspension polymerization using Fe_3O_4 particles as magnetically susceptible component and bovine serum albumin and lysozyme (Lyz) as templates, respectively. The average content of magnetically susceptible component (Fe_3O_4) inside MS-PIGMs was determined using thermogravimetric analyzer, and the magnetic characteristics of MS-PIGMs were measured by vibrating sample magnetometer. The results showed that the resulting MS-PIGMs had a certain magnetic response to external magnetic fields, and their average content of Fe_3O_4 was 2.08%. Their recognition specificity was investigated using BSA and Lyz as both templates and control molecules and characterized by high-performance liquid chromatography, and the mechanism of imprinting and recognition was analyzed. It was shown that the resulting BSA imprinted soft-gel composite microspheres with magnetic susceptibility (BSA-PIGMs) and Lyz imprinted soft-gel composite microspheres with magnetic susceptibility (Lyz-PIGMs). All exhibited good recognition selectivity for their templates, and the relative separation factor (β) was 4.75 and 5.88, respectively. The recognition selectivity of MS-PIGMs to their templates depended mainly on the synergic action of a large quantity of hydrogen binding being caused by complementation and very close contact of outer surface of proteins with inner surface of "imprinting cavities." © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 2401–2407, 2006

Key words: gels; molecular imprinting; molecular recognition; templates

INTRODUCTION

Molecular imprinting technique (MIT) is a manual method for preparing polymers with predetermined recognition selectivity to certain molecules.[1] These kinds of polymers were referred to as molecularly imprinted polymers (MIPs), which provided a means of creating specific recognition and combination similar to those in biological system such as antibodies to antigens, enzymes to zymolytes,[2–4] had exhibited extensive application prospects in enantiomer separation,[5–7] antibody binding mimic,[8–10] enzyme mimic,[11,12] biomimic sensor,[13,14] control of equilibrium shifting of chemical reaction,[15] byproduct removal,[16] and so on.[17–20]

The characterizations of molecular recognition selectivity of MIPs have not been a unified method yet for their broad application areas. Characterization methods of MIPs in different application areas are various, such as separation coefficient, separation degree, retention time, or binding constant were often used in characterizations of stationary phase of chromatograph and solid phase extraction; electric current, conductance, and some optical and mass parameters were generally used in characterizations of chemical sensors, while catalytic efficiency, reaction rate, and so on were usually applied in characterizing catalytic activity of MIPs in catalysis areas.

Molecular recognition selectivity is the most important parameter in characterizing MIPs because molecular recognition is the essential character of MIPs. Adsorption properties, e.g. adsorption capacity and adsorption rate are also important parameters when MIPs are used as adsorbent in separation fields because adsorption capacity reflecting the adsorption ability of adsorbent and adsorption rate illustrating the speed to reach adsorption equilibrium. To MIPs endowed with magnetically susceptible components, their magnetic responsibilities to external magnetic fields are important parameters too.[21]

In the accompanying paper,[22] protein-imprinted soft-wet gel composite microspheres with magnetic susceptibility (MS-PIGMs) have been prepared by inverse suspension polymerization (ISP) using Fe_3O_4 particles as magnetically susceptible component,

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acrylamide (AM) and *N*,*N*′-methylenebisacrylamide (BisAM) as polymeric matrix components, and bovine serum albumin (BSA, the resulting MS-PIGMs being named BSA-PIGMs) and lysozyme (Lyz, the resulting MS-PIGMs being named Lyz-PIGMs) as templates, respectively. The preparation and particle morphology of MS-PIGMs have been demonstrated in detail. In this study, the characteristics of the resulting MS-PIGMs including their magnetic characteristics, adsorption capacity, and molecular recognition selectivity were investigated and characterized in detail, and imprinting and recognition mechanism of MS-PIGMs were analyzed detailedly.

EXPERIMENTAL

Reagents

MS-PIGMs (BSA-PIGMs, Lyz-PIGMs) and nonimprinted magnetically susceptible soft-wet gel composite microspheres (non-PIGMs) were self made.[22] Bovine serum albumin (BSA) and lysozyme (Lyz) (electrophoresis purity >99%) were purchased from Chinese Academy of Medical Sciences. Acetic acid (Ace), acetone, and sodium dodecyl sulfate (SDS) were all analytical reagents. All materials were used without further purification. Double distilled water was used throughout.

Determination of single adsorption capacity

The single adsorption experiments of MS-PIGMs to their template protein were carried out using their template protein only, and the procedure was given as follows:

(1) Adding 2 μmol BSA into 100 mL distilled water with stirring, and then 20 μM BSA solution was obtained.
(2) BSA-PIGMs (1 g) after saturated swelling by distilled water were added into 5 mL BSA solution, immersing and shaking at 25°C. Taking a sample at regular intervals to analyze the concentration of BSA solution until adsorption equilibrium was reached, and then adsorption capacity of different time and static equilibrium adsorption capacity of BSA-PIGMs ($Q_{\infty 1}$) were determined according to the concentration change of the solution.

$Q_{\infty 1}$ (μmol/g) was expressed as follows:

$$Q_{\infty 1} = (C_{S0} - C_S) \times V/m \quad (1)$$

where C_{S0} is initial concentration of protein (μmol/mL), C_S is adsorption equilibrium concentration of protein (μmol/mL), V is volume of protein solution (ml), and m is the amount of MS-PIGMs (g).

In this experiment, $C_{S0} = 20 \times 10^{-3}$ μmol/mL, $V = 5$ mL, and $m = 1$ g, and so the following equation was obtained.

$$Q_{\infty 1} = 5(20 - C_S) \quad (2)$$

(3) Drawing adsorption capacity–adsorption time curve of BSA-PIGMs using adsorption time as abscissa and adsorption capacity as ordinate.
(4) the single adsorption experiments of non-PIGMs to BSA were carried out using the same procedure as BSA-PIGMs.
(5) The determination of single adsorption capacity of Lyz-PIGMs was the same as that of BSA-PIGMs, only BSA solution being replaced by Lyz solution. The single adsorption capacity experiments of non-PIGMs to Lyz were carried out using the same procedure as Lyz-PIGMs.

Determination of competitive adsorption capacity

The competitive adsorption experiments of MS-PIGMs were carried out using BSA and Lyz as both templates and control molecules. The procedure was given as follows:

(1) Adding each of BSA and Lyz 2 μmol into 100 mL distilled water with stirring, then 20 μM mixed protein solution was obtained.
(2) BSA-PIGMs (1 g) after saturated swelling by distilled water was added into 5 mL mixed protein solution, immersing and shaking at 25°C for 24 h. Static equilibrium adsorption capacity of BSA-PIGMs ($Q_{\infty 2}$) were determined according to the concentration change of the mixed protein solution.

According to eqs. (1), (2), and experiment condition, $Q_{\infty 2}$ (μmol/g) was expressed as follows:

$$Q_{\infty 2} = 5(20 - C_S) \quad (3)$$

of which, C_S had the same meaning as given in eq. (2).

(3) The determination of contrastive adsorption capacity of Lyz-PIGMs and non-PIGMs was the same as BSA-PIGMs.

Determination of molecular recognition selectivity

Molecular recognition selectivity was evaluated by static distribution coefficient (K_D), separation factor (α), and relative separation factor (β) being calculated according to static equilibrium adsorption capacity of MS-PIGMs ($Q_{\infty 2}$) obtained in the contrastive adsorption experiments of MS-PIGMs.

K_D, α, and β were defined as follows:[21]

$$K_D = C_P/C_S \quad (4)$$

where C_P is the amount of analyte absorbed by per gram of MS-PIGMs, and when adsorption reached equilibrium, C_P is equal to $Q_{\infty 2}$ (μmol/g), *i.e.*, $C_P = Q_{\infty 2}$.

According to eqs. (3) and (4), eq. (5) was obtained.

$$K_D = 5(20 - C_S)/C_S \quad (5)$$

$$\alpha = K_{D1}/K_{D2} \quad (6)$$

of which, K_{D1} and K_{D2} are the static distribution coefficient of templates and control molecules, respectively.

$$\beta = \alpha_1/\alpha_2 \quad (7)$$

where α_1 and α_2 are separation factor of MS-PIGMs and non-PIGMs.

Analysis methods

Thermoanalyzer

The average Fe_3O_4 content of MS-PIGMs was given according to the weight percentage of the residue remaining after thermal analysis from room temperature to 1300°C in static air, and it was determined by thermogravimetric analysis by using NETZSCH STA449 thermoanalyzer with a heating rate of 20°C/min.

Vibrating sample magnetometer

Magnetic characteristics of MS-PIGMs and Fe_3O_4 were measured by using LDJ-9600 vibrating sample magnetometer (VSM, America LDJ Company).

Liquid chromatography

The molecular recognition selectivity of MS-PIGMs and non-PIGMs was evaluated by chromatographic analysis of the concentration of the mixed protein using Waters liquid chromatography (Waters 600E pump; M32 chromatograph workstation; column: Waters μbondapak 300 × 3.9 mm^2; UV absorbance at 280 nm; carrier liquid: acetonitrile/H_2O = 35/65; and flow rate of carrier liquid 1.0 mL/min).

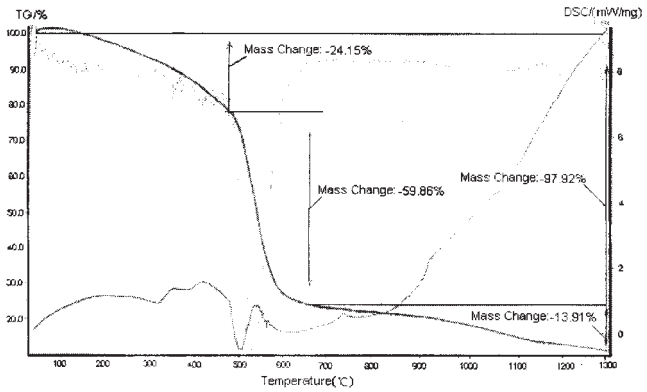

Figure 1 TG-DSC analysis picture of MS-PIGMs.

RESULTS AND DISCUSSION

Magnetic characteristics of MS-PIGMs

Fe_3o_4 content of MS-PIGMs

Magnetic characteristics of magnetic materials are related to their sorts generally, while those of magnetic composite materials are usually related to content of magnetic component inside. So, Fe_3O_4 content is very important to the magnetic responsibility of MS-PIGMPs. In general, the higher the Fe_3O_4 content, the stronger the magnetic responsibility of MS-PIGMs is. For this reason, the average Fe_3O_4 content of the resulting MS-PIGMs (The amount of Fe_3O_4 in polymerization recipe was 0.2 g, and the theoretical Fe_3O_4 content was 1.96%) was determined by thermogravimetric analysis, and thermogravimetric analyzer (TG) analysis was shown in Figure 1.

It could be seen from Figure 1 that the TG curve of MS-PIGMs was composed of three stages of mass change from room temperature to 1300°C. The first stage occurred from room temperature to at about 480°C, and the decrease of weight was 24.15%, followed by linear decrease of the second stage from about 480 to 660°C, and the weight decrease was 59.86%. Then the TG curve was suddenly smoothout in the third stage from about 660 to 1300°C. The decrease of weight was 13.91%, and so the total decrease of weight was 97.92%. Therefore, the average Fe_3O_4 content of the resulting MS-PIGMs was 2.08% (if the increase of weight produced by the oxidation of Fe_3O_4 to Fe_2O_3 in the air atmosphere, the average Fe_3O_4 content should be 2.02%). It was higher in a small degree than that of theoretical value (1.96%) according to polymerization recipe, the most probable reason was due to the experimental error.

Magnetic responsibility of MS-PIGMs

Magnetic hysteresis loop was a vital character of magnetic materials. It reflects response ability of magnetic

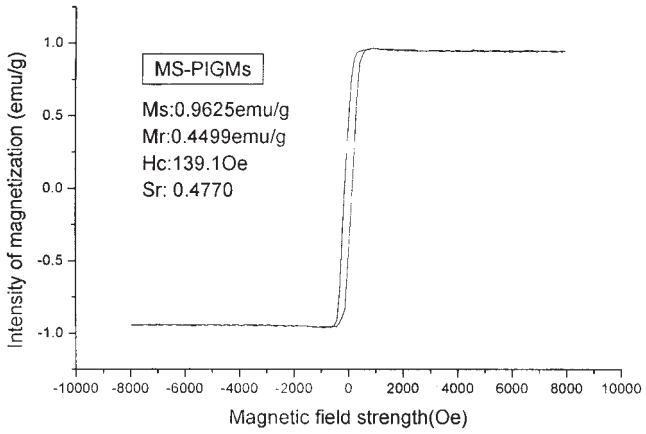

Figure 2 Magnetic hysteresis loop of MS-PIGMs.

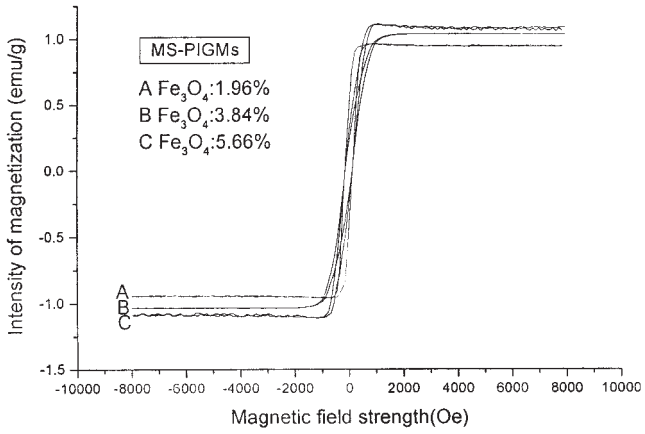

Figure 4 The magnetic hysteresis loops of MS-PIGMs with different Fe_3O_4 content.

materials to the change of external magnetic field (denoted by magnetic field strength) firstly, and it characterizes ability of magnetic materials to keep magnetic field strength when external magnetic field being removed (denoted by coercive force, H_c).

Figures 2 and 3 were show the magnetic hysteresis loop of MS-PIGMs (the average Fe_3O_4 content was 2.08%) and Fe_3O_4, respectively.

Comparing Figure 2 with Figure 3, we could see that the magnetic hysteresis loop of MS-PIGMs was similar to that of Fe_3O_4 in shape, and they were all slimly closed curve. The main magnetic characteristic parameters of the resulting MS-PIGMs were changed largely compared with Fe_3O_4 used as magnetically susceptible component, but the saturation magnetization (M_s) of MS-PIGMs (0.9625 emu/g, see Fig. 2) still indicated that MS-PIGMs possessed a certain magnetic responsibility. Magnetic remanence (M_r) of MS-PIGMs was very small (0.4499 emu/g) in favor of redispersion after the external magnetic field being removed.

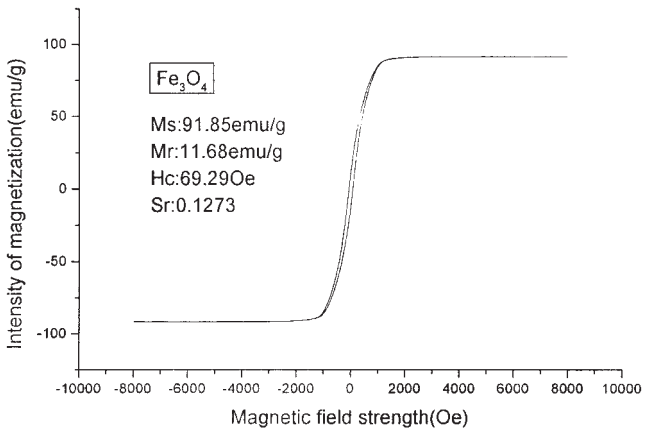

Figure 3 Magnetic hysteresis loops of Fe_3O_4 particles.

Effect of Fe_3O_4 content on magnetic responsibility of MS-PIGMs

As mentioned earlier, magnetic responsibility of magnetic composite materials are usually related to content of magnetic component inside. In general, the higher the Fe_3O_4 content, the stronger the magnetic responsibility of MS-PIGMs is. For this reason, the effect of Fe_3O_4 content on magnetic responsibility of MS-PIGMs was investigated experimentally. The comparison of magnetic hysteresis loops of MS-PIGMs with different Fe_3O_4 content was given in Figure 4, and the comparison of their major magnetic parameters was listed in Table I.

It could be seen from Figure 4 that the magnetic hysteresis loops of with different Fe_3O_4 content were the same in shape. Table I indicated that the more the Fe_3O_4 content of MS-PIGMs, the bigger the M_s was, and the stronger the magnetic responsibility. But meanwhile, the change of M_s of MS-PIGMs along with the change of Fe_3O_4 was not large, for example, Fe_3O_4 content of No. C was nearly three times compared with No. A, while M_s of No. C was increased only about 15% compared with No. A. This result indicated that the effect of Fe_3O_4 content on magnetic responsibility of MS-PIGMs was very remarkable when Fe_3O_4 content changed in a certain range. It could be still seen from Table I that M_r, H_c, and squareness ratio (S_r)

TABLE I
Comparison of Major Magnetic Parameters of MS-PIGMs with Different Fe_3O_4 Content

No.	A	B	C
Fe_3O_4 content (%)	1.96	3.84	5.66
M_s (emu/g)	0.9625	1.036	1.111
M_r (emu/g)	0.4499	0.2031	0.2730
H_c (Oe)	139.1	126.7	132.7
S_r	0.4770	0.1967	0.2517

TABLE II
The Result of Single Adsorption of MS-PIGMs

MS-PIGMs	Adsorption time (h)	C_s (10^3 μmol/mL)		$Q_{\infty 1}$ (10^3 μmol/g)	
		BSA	Lyz	BSA	Lyz
BSA-PIGMs	24	12.48	—	37.60	—
Lyz-PIGMs	24	—	11.59	—	42.05
Non-PIGMs	24	16.61	16.27	16.95	18.65

of MS-PIGMs all had some change but were not very remarkable.

Adsorption properties of MS-PIGMs

Single adsorption capacity

The experimental results of single adsorption capacity of BSA-PIGMs and Lyz-PIGMs to their templates, and non-PIGMs to BSA and Lyz are given in Table II.

It could be seen from Table II that all the adsorption capacity of BSA-PIGMs and Lyz-PIGMs were higher obviously than that of non-PIGMs. This result indicated that imprinting process increased adsorption capacity of gel microspheres evidently.

Adsorption capacity–adsorption time curve of single adsorption experiments of MS-PIGMs to their template protein was shown in Figure 5.

It could be seen from Figure 5 that the change rules of adsorption capacity–adsorption time curves of BSA-PIGMs and Lyz-PIGMs were the same, *i.e.*, the adsorption capacity increased along with adsorption time lasting, and adsorption capacity increased rapidly in the beginning, then increasing rate slowed down in later stage till reaching adsorption equilibrium. At the same time, adsorption time reaching adsorption equilibrium of BSA-PIGMs and Lyz-PIGMs had marginal difference, the former was 270 min, and the latter was 240 min.

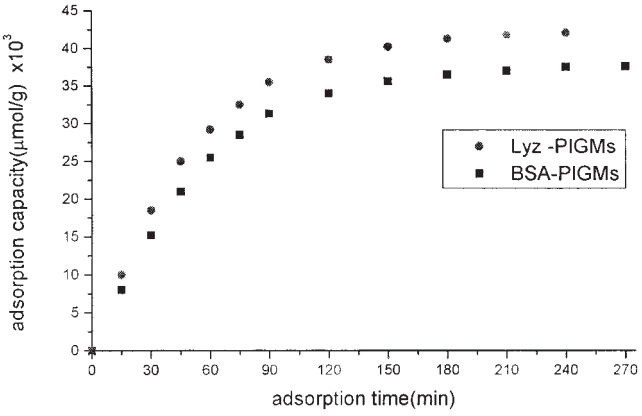

Figure 5 Adsorption capacity–adsorption time curves of MS-PIGMs.

TABLE III
The Results of Competitive Adsorption and Molecular Recognition Specificity of MS-PIGMs

MS-PIGMs	C_s (10^3 μmol/mL)	$Q_{\infty 2}$ (10^3 μmol/g)	K_D	α	β
BSA-PIGMs					
BSA	13.87	30.65	2.21	4.42	4.75
Lyz	18.17	9.15	0.50		
Lyz-PIGMs					
BSA	18.33	8.35	0.46	6.35	5.88
Lyz	12.62	36.90	2.92		
Non-PIGMs					
BSA	16.57	17.15	1.04	0.93	—
Lyz	16.35	18.25	1.12	1.08	

Competitive adsorption capacity

The experimental results of competitive adsorption capacity of BSA-PIGMs and Lyz-PIGMs to their templates and control molecules, and non-PIGMs to BSA and Lyz are given in Table III.

From Table III, we could see that the static equilibrium adsorption capacity of BSA-PIGMs to their templates (BSA) was obviously higher than to its control molecules (Lyz), and the situation of Lyz-PIGMs was the same, while the static equilibrium adsorption capacity of non-PIGMs to BSA and to Lyz had not much difference. These facts indicated that imprinting process resulted in selectivity adsorption.

3.3 Molecular recognition selectivity of MS-PIGMs

Molecular recognition selectivity of BSA-PIGMs and Lyz-PIGMs was calculated according to their static equilibrium adsorption capacity, and the calculation results are given in Table III.

It could been seen clearly by Table III that separation factor of BSA-PIGMs (α = 4.42) was much higher than that of non-PIGMs (α = 0.93), and separation factor (α = 6.35) of Lyz-PIGMs was remarkably higher than that of non-PIGMs (α = 1.08). This indicates that the resulting BSA- PIGMs and Lyz-PIGMs had high adsorption selectivity and molecular recognition specificity to their templates. It also could be seen that relative separation factor of BSA-PIGMs and Lyz-PIGMs were both high (β = 4.74 and 5.88, respectively), showing that "imprinting" did improve the adsorption selectivity of polymer gel microspheres.

Effect of templates on molecular recognition selectivity

Comparing the molecular recognition selectivity of BSA-PIGMs and Lyz-PIGMs, we could see that sepa-

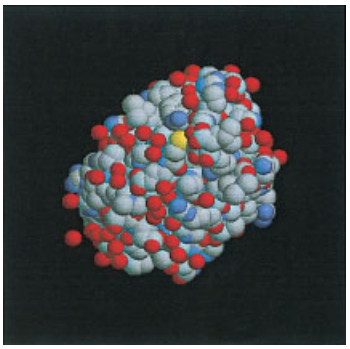

Figure 6 Space structure model of Lyz.[23] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ration factor (α) and relative separation factor (β) of Lyz-PIGMs were all higher than that of BSA-PIGMs. This indicated that the recognition selectivity of Lyz-PIGMs to their templates (Lyz) was higher than that of BSA-PIGMs to their templates (BSA). This might be caused by the difference of size between BSA molecule and Lyz molecule.

Lyz is a kind of ovoglobulin with ellipsoidal shape, its size is 4.5 nm × 3 nm × 3 nm, and relative molecular weight is 14,300~14,600. Lyz is composed of a polypeptide chain with 129 amino acid residue and 4 disulphide bridges. The molecular conformation of Lyz was successfully determined by Phillips and his colleagues (1965) using X-ray crystal structure analysis with 0.2 nm distinguishability, and its space structure model[23] was given in Figure 6.

BSA is composed of a polypeptide chain with 579 amino acid residue, and its relative molecular weight is 66,000~68,000. BSA was used usually as standard protein in determining solution concentration of protein, and was often added in enzymes to prevent the denaturation of enzyme. BSA is one of proteins being investigated penetratedly, but unfortunately nobody determined its space structure model such as Lyz could do up to now.

To MS-PIGMs, their "imprinted cavities" were in dynamic changing condition for polyacrylamide chains had high flexibility, and so some of them must be enlarged and some shrinked. To BSA-PIGMs, because Lyz was smaller than BSA in size, Lyz entering into the "BSA imprinted cavities" should be easy and thus the bonding possibility should be large, but the bonding interaction might be relative small. By contrast, to Lyz-PIGMs, BSA was larger than Lyz in size, and so BSA entering into the "Lyz imprinted cavities" should be very difficult, but it still had some change to enter the enlarged "Lyz imprinted cavities." The entrance change was small, but the bonding interaction must be large, because BSA once entered into the enlarged "Lyz imprinted cavities," it would be difficult to break loose from the jammed cavity. For the combined effect of bonding possibility and bonding interaction, the adsorption of BSA-PIGMs to Lyz was larger than that of Lyz-PIGMs to BSA, and thus Lyz-PIGMs exhibited better molecular recognition selectivity than BSA-PIGMs.

Molecular recognition mechanism of MS-PIGMs

From the preparation process of MS-PIGMs,[22] it could be seen that there were no functional monomers, which could generate relatively strong electrostatic interaction with templates, such as methacrylic acid, being added into system, and so there was no electrostatic interaction between functional monomers and templates in "imprinted cavities" of MS-PIGMs, and thus the mechanism of imprinting and recognition of proteins was obviously different from that of small molecules (such as amino acid).

All proteins had a large quantity of electric charge and nonpolar groups, and so they could be interact strongly with functional monomers, which had the same type of groups through electrostatic and hydrophobic interaction. These strong interactions usually had selectivity to the small molecular imprinting, because the number of bonding sites needed by small molecules was small (usually 2~3). But in fact, these strong interactions had no selectivity to protein, because the electriferous or nonpolar groups provided by functional monomers could interact with many kinds of proteins with different structures. So, these strong interactions should be replaced by relative weak interactions, such as hydrogen bonds, van der Waals force, and induced dipole interaction. To attain an overall strong bonding, the number of weak bonding sites must be large enough. The interactions between AM and BisAM and proteins were a large quantity of hydrogen bonds caused by amido bonds of AM and BisAM with peptide bond (amido bond) of proteins. A very close contact between proteins and polyacrylamide chains was a prerequisite for hydrogen bond that was a kind of short-range acting force. This proximity was created when AM and BisAM polymerized around the protein molecules and was enhanced by each bonding. That is to say, imprinting process resulted in synergic action of a large quantity of hydrogen bonds caused by complementation of outer surface of proteins with inner surface of "imprinting cavities." Nontemplate proteins could not create this kind of synergic action for they had no complementary surface with inner surface of "imprinting cavities" and thus could not form the very close contact and then generate the aforementioned synergic action mentioned.

CONCLUSIONS

The resulting MS-PIGMs had a certain magnetic responsibility to external magnetic fields; their magnetic

parameters were different along with the change of their average content of Fe_3O_4.

The results showed that all BSA-PIGMs exhibited a good recognition selectivity for their templates, and the relative separation factor was 4.75 and 5.88, respectively, and the change rules of adsorption capacity–adsorption time curves of BSA-PIGMs and Lyz-PIGMs were the same.

Template molecules had some effects on molecular recognition selectivity of MS-PIGMs, and the resulting MS-PIGMs imprinted by smaller template molecules exhibited better molecular recognition selectivity than by larger template molecules.

The mechanism of imprinting and recognition of MS-PIGMs to proteins was obviously different from that of small molecules. The recognition selectivity of MS-PIGMs to their templates depended on mainly the synergic action of a large quantity of hydrogen binding being caused by complementation and very close contact of outer surface of proteins with inner surface of "imprinting cavities."

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