Preparation of Molecularly Imprinted Fe_3O_4/P(St-DVB) Composite Beads with Magnetic Susceptibility and their Characteristics of Molecular Recognition for Amino Acid

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ABSTRACT: Tyrosine and phenylalanine imprinted Fe_3O_4/P(St-DVB) composite beads with magnetic susceptibility were prepared by suspension polymerization using Fe_3O_4 as the magnetically susceptible component, methacrylic acid and acrylamide as functional monomers, styrene and divinylbenzene as polymeric matrix components, stearic acid as porogen, and poly(ethylene glycol) 4000 as dispersant. Scanning electron microscopy examination of the composite beads showed macropores on the surface of spherical beads. The diameters of the composite beads and the macropores were in the ranges $\sim$ 400–450 and 4–20 μm, respectively. The average content of Fe_3O_4 inside the composite beads was 3.78%, and Fe_3O_4 was unevenly distributed. The mechanism of macropore formation and the concept of "intellectual cavity" of molecularly imprinted composite beads were proposed. The recognition selectivity of the composite beads was investigated using tyrosine and phenylalanine as both templates and comparative molecules. Tyrosine-imprinted composite beads exhibited a good recognition selectivity for tyrosine, and the separation factor was up to 3.67. In contrast, phenylalanine-imprinted composite beads had little recognition selectivity for phenylalanine and the separation factor was only 1.12. It was confirmed that the three-site interaction between tyrosine and functional monomers was stronger than the two-site interaction between phenylalanine and functional monomers. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 3790–3796, 2003

Key words: composites; molecular recognition; templates; polymerization; macroporous polymers

INTRODUCTION

Molecular imprinting is a new method of molecular recognition that has been studied extensively and used for many purposes, such as, enantiomer separation,[1–3] antibody binding mimic,[4–6] enzyme mimic,[7] biosensor mimic,[8,9] control of equilibrium shifting of chemical reaction,[10] and byproduct removal.[11] Molecular imprinting polymers (MIPs) prepared by this technique are becoming one of the most potentially interesting materials in the third millennium.[12]

MIPs were prepared originally by bulk polymerization[1,13] followed by mechanical grinding of the monolithic block generated to give small particles. The polymerization processes of these two methods were simple, but their workup processes were complicated and time consuming. The particles were usually irregular in shape with wide size distributions, and the yield of useful particles was typically $\sim$ 20%.[14] As an alternative, MIPs particles can be prepared directly, for example, by suspension polymerization,[15,16] in the form of spherical beads of controlled diameter. The spherical beads can be used directly after production,

merely the templates have to be removed via extraction. Moreover, these MIPs have many advantageous properties, such as, their regular shape, large specific surface area, and strong adsorption capacity, and their size can be adjusted according to the requirement. The chemical and mechanical stabilities of MIP particles are very good because they are resistant to impact, high temperature, high pressure, acid and alkali conditions, as well as many kinds of organic solvents. The spherical beads prepared by such molecular imprinting techniques are referred to as spherical molecularly imprinted polymers (SMIPs).[17]

When magnetically susceptible components, such as, Fe, Co, or Ni or their oxides, are encapsulated inside the polymeric beads, the composite beads have magnetically susceptible characteristics. As such, the beads can easily and conveniently be separated from the system in which they are located by external magnetic fields, and hence they are called "Dynabeads".[18] When SMIPs encapsulate the magnetically susceptible components just mentioned, they should be easily separated by external magnetic fields after they finish their "active" adsorption and recognition.

In the present paper, molecularly imprinted polymeric composite beads with magnetic susceptibility (MS-SMIPs) were prepared by suspension polymerization according to the principle of molecular im-

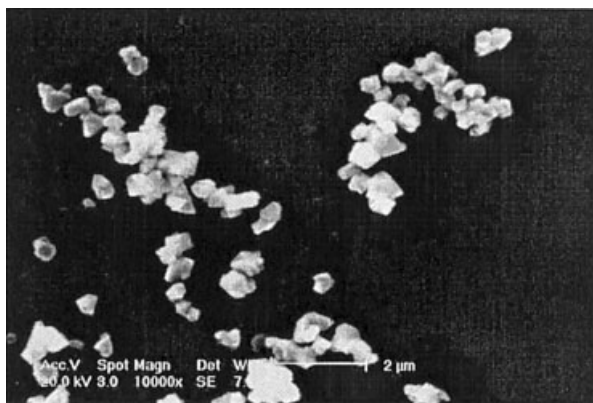*Correspondence to:* G. Cheng (gxcheng@tju.edu.cn).

Figure 1 SEM photograph of Fe_3O_4.

printing technique and "particle design". Then, the molecular recognition selectivity of MS-SMIPs for their templates was investigated using tyrosine and phenylalanine as both templates and comparative molecules.

EXPERIMENTAL

Reagents

Fe_3O_4 was obtained from Institute of Chemistry and Metallurgy, Chinese Academy of Sciences (see Figure 1 for morphology and size). Tyrosine (Tyr) and phenylalanine (Phe) were from Beijing Xinjingke Biotechnology Company Ltd.(see structures in Figures 2a and 2b, respectively). Styrene (St) was purchased from Tianjin No.1 Chemical Reagent Factory and was purified by distillation under reduced pressure before use. Divinylbenzene (DVB), methacrylic acid (MAA), acrylamide (MA), benzene, 2,2'-azobisisobutyronitrile (AIBN), poly(ethylene glycol) 4000 (PEG4000), stearic acid (SA), acetic acid, and NaOH were all analytical reagents and were used without further purification. Double-distilled water was used throughout.

Preparation of MS-SMIPs

Suspension polymerizations were carried out in a 250-mL three-necked round-bottomed flask equipped with a reflex condenser, nitrogen inlet, and stirrer. The flask was immersed in a thermostated water bath at the reaction temperature. The stirrer speed was maintained at 300 rpm. The polymerization recipes are given in Table I. A typical procedure is as follows: The required amount of PEG4000 was dissolved in a certain amount distilled water in a 250-mL beaker, and then ground magnetite Fe_3O_4 was added and disposed by ultrasonic dispersion for 30 min. Then this mixture was transferred into the flask. AIBN was added to the mixture of St, DVB, and SA in benzene. When AIBN and SA were all dissolved, the two mix-

tures were poured into the flask also, and the stirrer was started. Tyr (or Phe) was added into the remaining distilled water, and MAA and AM were added after Tyr (or Phe) was dissolved. This mixture was then stirred for 30 min and then transferred into the flask. The temperature of the water bath was increased, and the reaction system was purged with nitrogen for 10 min prior to reaching the reaction temperature. The reaction lasted for 24 h at 70°C. After the completion of polymerization, the temperature of the water bath was raised to 85°C and the reaction system was exhausted to maintain a vacuum for 30 min and remove benzene and unreacted monomers. The MS-SMIPs were then obtained by cooling the resultant of reaction to room temperature and filtering through a filter screen. The non-imprinted magnetically susceptible composite beads were prepared in the same manner in the absence of templates.

Elution of porogen and templates

The resulting MS-SMIPs were transferred into a 500-mL beaker after being washed three times with distilled water. Next, 150 mL of distilled water was added and the solution was heated to 70°C with stirring. This step was followed by the dropwise addition of 10 mL of 1 M NaOH. The temperature was kept at 70°C for 30 min and then decreased. The solution was filtered and the resulting MS-SMIPs were washed three times with distilled water. Next, the MS-SMIPs were immersed with 100 mL of 1% acetic acid solution for 24 h. This step was followed by repeated filtering and washing MS-SMIPs with distilled water until the washing water was of neutral pH. Finally, the MS-SMIPs were vacuum dried in an oven at 80°C for 2 h to a constant weight.

Molecular recognition of MS-SMIPs for amino acid

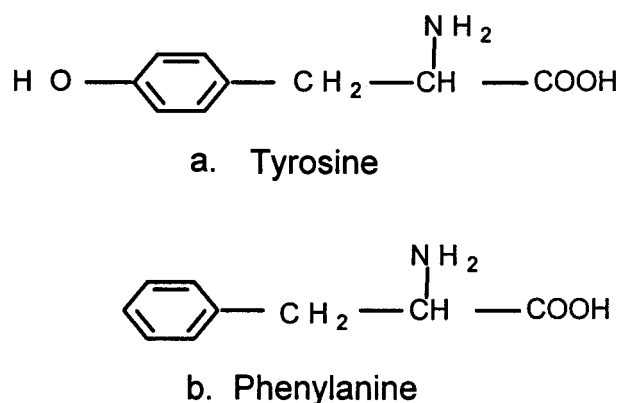These experiments were carried out using Tyr and Phe as both templates and comparative molecules. First, a

Figure 2 Structure of (a) Tyr and (b) Phe.

TABLE I
Recipes for the Preparation of MS-SMIPs by Suspension Polymerization

No.	Fe$_3$O$_4$ (g)	Tyr (mmol)	Phe (mmol)	MAA (mmol)	AM (mmol)	St (g)	DVB (g)	PEG (g)	SA (mmol)	Ben (ml)	AIBN (g)	H$_2$O (mL)
Tyr-IPS	0.8	2	—	20	20	15	5	20	2	5	0.2	150
Phe-IPS	0.8	—	2	20	20	15	5	20	2	5	0.2	150
Non-IPS	0.8	—	—	20	20	15	5	20	2	5	0.2	150

2.5 mmol/L mixed amino acid solution was prepared by adding 0.25 mmol each of Tyr and Phe to 100 mL of distilled water and stirring.

To determine selective adsorption and molecular recognition, 1 g of MS-SMIPs was added to 3 mL of mixed amino acid solution, and the flask was immersed in a water bath at room temperature and shaken for 24 h. A sample of the solution was taken, and the concentration of the mixed amino acid solution was determined and the adsorption capacity and recognition selectivity of MS-SMIPs were determined according to the concentration change of the solution.

Scanning electron microscopy

The surface morphology and the size of MS-SMIPs were characterized with Hitachi S-3000N scanning electron microscope (SEM).

Energy spectrum

The distribution of encapsulated Fe$_3$O$_4$ inside MS-SMIPs was studied with the SEM, which was equipped with an EDAX-PHOENIX energy spectrum probe.

Thermoanalyzer

The weight percentage of the residue remaining after thermal analysis from room temperature to 1300°C in static air was given as the average Fe$_3$O$_4$ content of MS-SMIPs. This value was determined by thermogravimetric analysis (TGA) with a NETZSCH STA449 thermoanalyzer at a heating rate of 20°C/min.

Liquid chromatography

The molecular recognition selectivities of MS-SMIPs and non-imprinted composite beads were evaluated by chromatographic analysis of the concentration of the mixed amino acid with an HP-100 liquid chromatograph (C$_{18}$ column, 250 × 4.6 mm; UV absorbance, 254 nm; flow rate of carrier liquid, 0.7mL/min).

RESULTS AND DISCUSSION

Size and morphology of MS-SMIPs

The SEM photograph of the resulting MS-SMIPs shown in Figure 3a demonstrates that MS-SMIPs are spherical and monodispersed, and their diameters are in the range ∼ 400–450 μm. In the amplified SEM photograph of one MS-SMIP (Fig. 3b), many well-distributed macropores, with diameters in the range 4–20 μm, are evident on the surface. It was well known that the pores are of benefit because they increase the specific surface area and adsorption capacity of MIPS and improve the mass transfer rate for releasing and rebinding the templates. So, it is of importance to make MIPS with a large quantity of pores on their surface. For this reason, porogens often are used to obtain pores. In the present study, SA, a
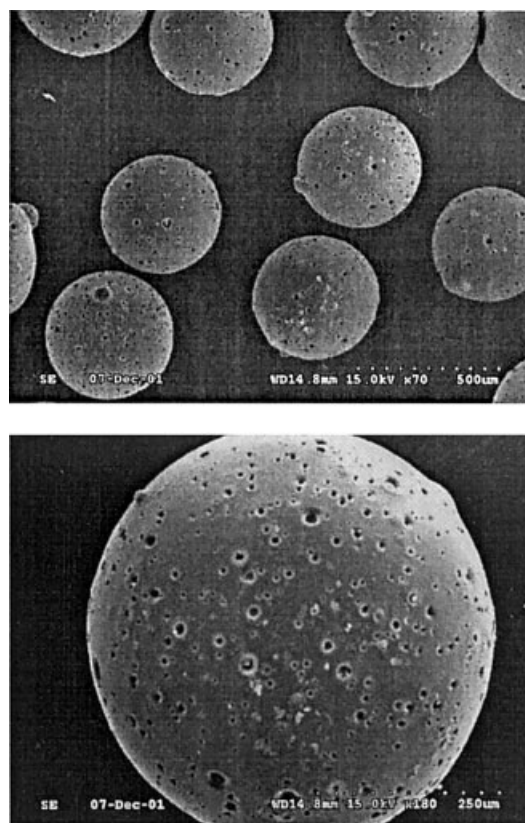

Figure 3 (a) SEM photograph of MS-SIMPS and (b) amplification of the SEM photograph of MS-SIMPS.
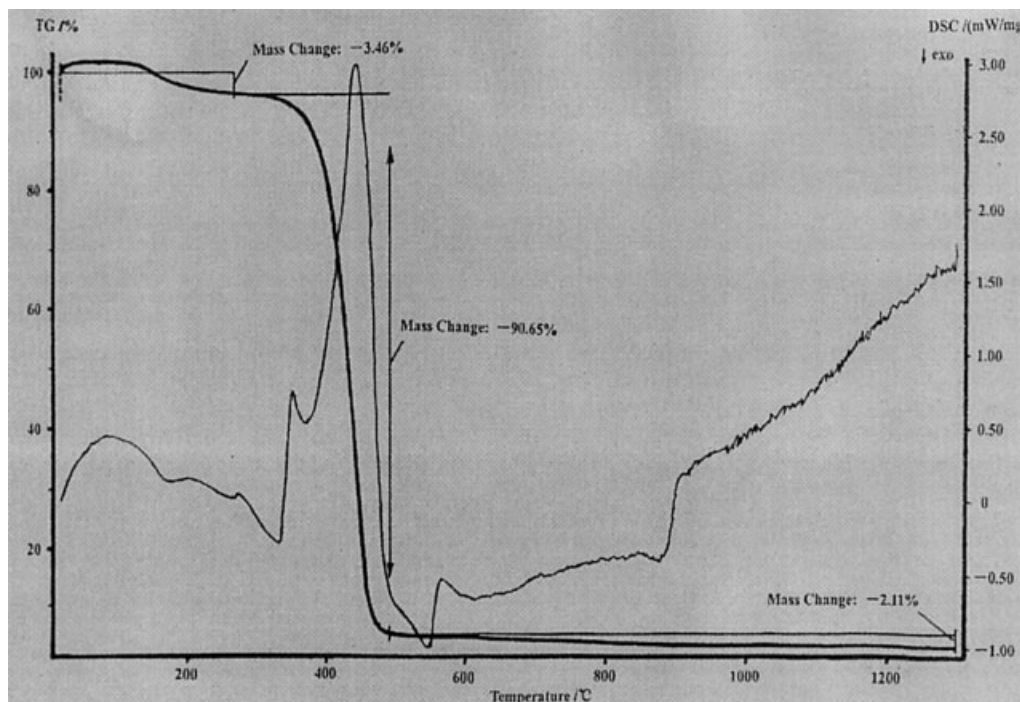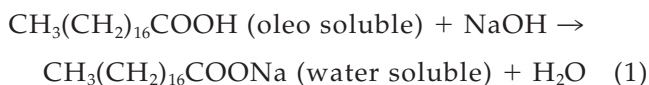
Figure 4 TG and DSC results for MS-SMIPS.

kind of saturated fatty acid, was used as the porogen. It was easy to be remove from MIPS by reacting with alkali to form a salt, which would therefore transfer into an aqueous phase from an organic phase. Furthermore, SA is a saturated acid so it doesn't inhibit the polymerization. The pore formation mechanism was analyzed as follows: During the beginning of the polymerization, SA was dissolved in benzene and therefore dispersed equally well with St and DVB. As a consequence, the organic phase of the suspension polymerization was homogeneous. As the polymerization proceeded, polymers were gradually produced, and the solubility of SA in the organic phase decreased slowly. With this decrease, microphase separation occurred between SA and polymers, and two phases formed in the organic phase of the suspension polymerization. The polymeric matrix was the continuous phase and SA was the disperse phase. Then, SA located on the surface of MS-SMIPs was removed by reacting with NaOH to form sodium stearate:

$$CH_3(CH_2)_{16}COOH \text{ (oleo soluble)} + NaOH \rightarrow$$

$$CH_3(CH_2)_{16}COONa \text{ (water soluble)} + H_2O \quad (1)$$

Therefore, SA transferred into the aqueous phase from the organic phase, and the "position" that SA once "occupied" on the surface of MS-SMIPs was thus "vacated", and an abundance of macropores could therefore form on the surface. In addition, the SA molecule has a hydrophilic group (i.e., —COOH), so it

has a tendency to locate on the surface of beads for the benefit of the formation of surface pores.

Content of Fe_3O_4 inside MS-SMIPs

The resulting MS-SMIPs could be collected together by the magnetic attraction of a permanent magnet and redispersed into the system they located when the magnet was removed. This result proves that the resulting MS-SMIPs are not permanently magnetized themselves and only temporarily exhibit a magnetic orientation in the presence of a magnetic field. This temporary magnetic orientation resulted from the magnetically susceptible component (i.e., Fe_3O_4) inside the MS-SMIPs, so Fe_3O_4 content is very important for the magnetic susceptibility of MS-SMIPs. In general, the higher the Fe_3O_4 content, the stronger is the magnetic susceptibility of MS-SMIPs. A TG-DSC photograph of MS-SMIPs is shown in Figure 4. The TG curve shows that there are three stages of mass change from room temperature to 1300°C. The first stage, in which the decrease in weight was 3.46%, occurred at ~ 100–365°C. In the second stage, from ~ 365 to 490°C, there was a linear decrease in weight to 90.65%. Then the TG curve suddenly leveled off in the third stage from ~ 490 to 1300°C. In this stage, the decrease of weight was 2.11%, so the total decrease of weight was 96.22%. Therefore, the average Fe_3O_4 content was 3.78%(ignoring the increase of weight produced by the oxidation of Fe_3O_4 to Fe_2O_3 in the air atmosphere).
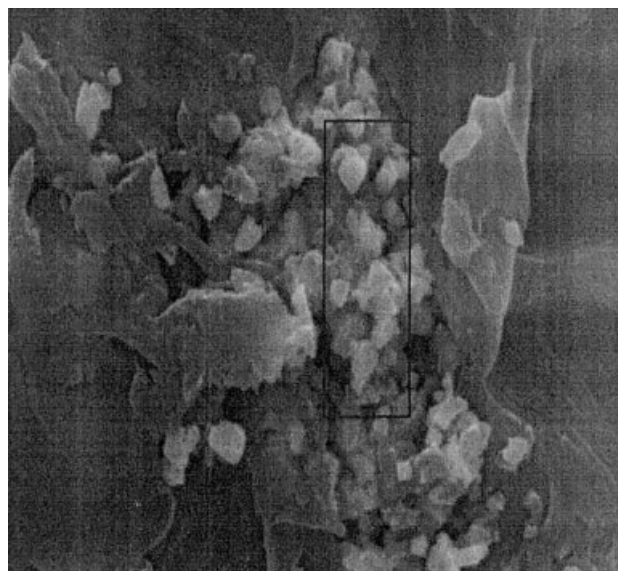
Figure 5 SEM photograph of a section of MS-SMIPS.

Distribution of Fe_3O_4 inside MS-SMIPs

The SEM photograph of a section of MS-SMIPs, shown in Figure 5, and the energy spectrum photograph of that section, shown in Figure 6, illustrate that Fe_3O_4 in the selected region is relatively concentrated and the distribution of Fe_3O_4 inside MS-SMIPs is uneven. These results suggest that the monomer droplets incorporated many aggregates of Fe_3O_4 particles to act as cores to perform the polymerization. PEG4000, added as a stabilizer to change the Fe_3O_4 particle surfaces from hydrophilic to hydrophobic, also appears to act as a dispersant of the polymerization suspension that is transferred onto the surface of the monomer droplets during the course of polymerization. This transfer improved the stability of composite beads, but decreased the stability of Fe_3O_4 particles and caused them to coalesce.
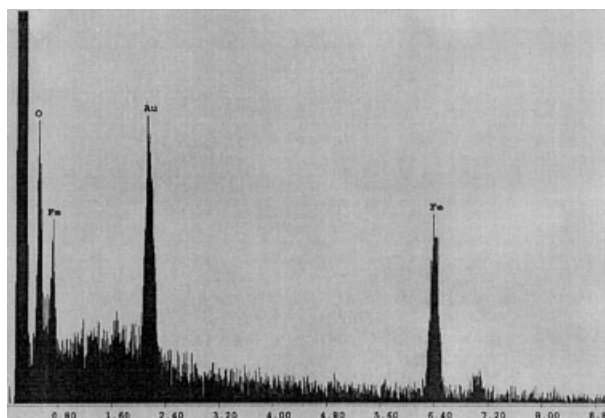

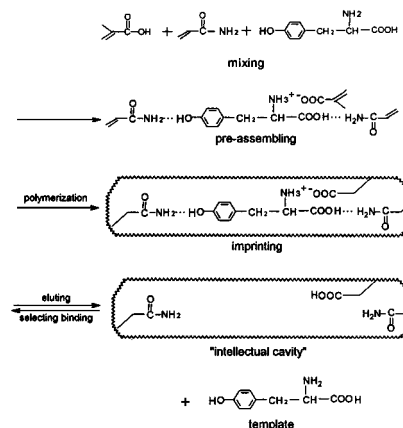Figure 6 The energy spectrum of a selected region from Figure 5.

Figure 7 Schematic representation of imprinting and eluting of the templates of MS-SMIPS.

Imprinting mechanism of MS-SMIPs to templates

The imprinting and eluting process of MS-SMIPs, using Tyr as an example, is described in detail in Figure 7. This scheme distinctly illustrates the imprinting mechanism of MS-SMIPs to their templates. Obviously, the molecular imprinting process includes the mixing and the pre-assembling of the functional monomers with templates, the polymerization of the functional monomers with the polymeric matrix components, and the eluting process of templates from the surface of MS-SMIPs. We have confirmed that the pre-assembling process is crucial in the whole process, because it determines the arrangement of functional groups. In particular, the selected templates were water soluble and were imprinted into composite beads in the aqueous phase, so that the pre-assembling process can decrease the loss of templates and increase the degree of imprinting. Therefore, an overall increase in the adsorption capacity of MS-SMIPs can be achieved. This process was carried out here by the formation of an ionic bond between the —COOH group of MAA and the —NH₂ group of Tyr, and a hydrogen bond between the —CONH₂ group of AM and the —COOH and —OH groups of Tyr.

Molecular recognition selectivity of MS-SMIPs

The molecular recognition selectivity of MIPS can be evaluated by the static distribution coefficient K_D and separation factor α. The parameter K_D reflects the adsorption capacity of MIPS and is defined as follows:

$$K_D = C_P/C_S \qquad (2)$$

where C_P is the amount of analyte absorbed by per gram of MIPS (mmol/g) and C_S is the initial concentration of analyte solution (mmol/mL). The parameter α indicates the recognition selectivity of MIPS for templates and is defined as follows:

TABLE II
Results of Selective Recognition of Analytes by MS-SMIPs[a]

No.	C_S (mmol/mL) $\times 10^3$	C_{P1} (mmol/g) $\times 10^3$	C_{P2} (mmol/g) $\times 10^3$	K_{D1} (g/mL)	K_{D2} (g/mL)	α
Tyr-IPS	2.5	1.65	0.45	0.66	0.18	3.67
Phe-IPS	2.5	0.96	0.84	0.38	0.34	1.12
Non-IPS	2.5	0.30	0.33	0.12	0.13	0.92

[a] Where C_{P1} and C_{P2}, for Tyr-IPS and Phe-IPS, are the amounts of their templates and comparative molecules adsorbed per gram of Tyr-IPS or Phe-IPS, respectively. For Non-IPS, C_{P1} and C_{P2} are the amount of Tyr and Phe adsorbed, respectively, per gram of Non-IPS.

$$\alpha = K_{D1}/K_{D2} \qquad (3)$$

where K_{D1} and K_{D2} are the static distribution coefficient of templates and comparative molecules, respectively. Usually, the larger the value of α, the better the recognition selectivity; for example, when α is $\lesssim 1.0$, MIPS are regarded as having no selectivity.

The molecular recognition selectivity of the two MS-SMIPs and non-imprinted composite beads was evaluated by using the same analyte (2.5 mmol/L each of Tyr and Phe) and using Tyr and Phe as both templates and comparative molecules to compare their adsorption capacity to templates with to comparative molecules. The results were shown in Table II, where Tyr-IPS and Phe-IPS are MS-SMIPs prepared with Tyr and Phe as templates, respectively, Non-IPS is non-imprinted composite beads, and the adsorption capacity and recognition selectivity of two MS-SMIPs and Non-IPS are expressed by K_D and α, respectively. It is evident that Tyr-IPS had a large adsorption capacity and exhibits a good selectivity for its templates (Tyr), with a separation factor of up to 3.67. In contrast, Phe-IPS has little recognition selectivity for its templates (Phe), although the adsorption capacity of Phe-IPS is larger than that of Non-IPS and the separation factor is similar to that of Non-IPS.

Mechanism of molecular recognition

According to the principle of molecular imprinting, the cavities with fixed size, shape, and arrangement of functional groups complemented to the templates would remain after removing the templates from MS-SMIPs. We propose that this type of cavity be named "intellectual cavity" because it not only has functional groups arranged regularly in space but has "memory" and can "recognize" its "old tenant". Because Tyr has three groups (i.e., —NH$_2$, —COOH, and —OH), it can interact with MAA and AM to form a three-site interaction (binding site), as illustrated in Figure 8a. In contrast, Phe has two groups (i.e., —NH$_2$ and —COOH), so it can form only two binding sites with MAA and AM (see Figure 8b), and the two binding sites interacting with Phe and Tyr would be about the same. Therefore, it can be inferred that there is a difference in the recognition selectivity between Tyr-

IPS and Phe-IPS for their respective templates because of their "intellectual cavities". Tyr-IPS has —CONH$_2$ in its "intellectual cavity" and can therefore form a hydrogen bond with the —OH of Tyr, but cannot form a hydrogen bond with Phe because Phe has no —OH. So, when Tyr and Phe enter into the "intellectual cavity" of Tyr-IPS, the "intellectual cavity" displays stronger affinity for Tyr than for Phe. The "intellectual cavity" of Phe-IPS has only two functional groups, —COOH and —CONH$_2$. However, because Tyr and Phe both have —NH$_2$ and —COOH groups, they can both complement with —COOH and —CONH$_2$ to form a binding site, and Phe-IPS shows almost the same affinity as does Tyr-IPS to Tyr and Phe.

The comparison of the "intellectual cavity" of Tyr-IPS with that of Phe-IPS led to the conclusion that the hydrogen binding site produced by —CONH$_2$ with —OH plays a decisive role in the course of molecular recognition of Tyr-IPS for Tyr. The main reason why the recognition selectivity of Tyr-IPS is higher than that of Phe-IPS is the interaction of three binding sites is stronger than that of two binding sites.

CONCLUSIONS

Amino acid-imprinted Fe$_3$O$_4$/P(St-DVB) composite beads (MS-SMIPs) with magnetic susceptibility were prepared by suspension polymerization, using tyrosine and phenylalanine as templates, Fe$_3$O$_4$ as the
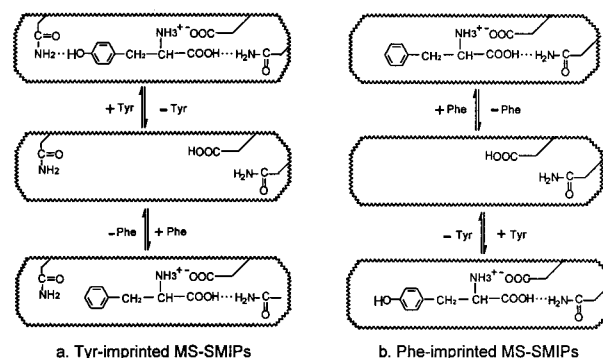

Figure 8 Illustration of the interaction of MS-SMIPS with the templates.

magnetically susceptible component, methacrylic acid and acrylamide as functional monomers, and poly-(ethylene glycol) 4000 as dispersant. The resulting MS-SMIPs were spherical and monodispersed with macropores on their surface. The diameters of the spherical beads were in the range 400–450 μm, and the macropore diameters were in the range 4–20 μm. The average Fe_3O_4 content of MS-SMIPs, which was unevenly distributed inside the MS-SMIPs, was 3.78%.

The mechanism of macropore formation involved elaboration of saturated stearic acid (SA). The macropores were the result of microphase separation of SA with a polymeric matrix; that is, SA first "occupied a position" and then "vacated" the position.

The concept of "intellectual cavity" of MS-SMIPs was proposed. The mechanism of molecular imprinting and molecular recognition of tyrosine and phenylalanine were described in detail, and the molecular recognition selectivity of MS-SMIPs for their templates were investigated. It was shown that tyrosine-imprinted MS-SMIPs exhibited a good recognition selectivity for tyrosine. In contrast, phenylalanine-imprinted MS-SMIPs had little recognition selectivity for phenylalanine, although their adsorption capacity was larger than that of non-imprinted magnetically susceptible composite beads. It was confirmed that the three-site interaction between tyrosine and functional monomers was stronger than the two-site interaction between phenylalanine and functional monomers, which probably led to the selectivity differences.

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