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The preparation of bovine serum albumin surface-imprinted superparamagnetic polymer with the assistance of basic functional monomer and its application for protein separation

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ABSTRACT

Currently, small proteins imprinting are more reported since large proteins molecular imprinting faces challenge due to their bulk size and complex structure. In this work, bovine serum albumin (BSA) surface-imprinted magnetic polymer was successfully synthesized based on atomic transfer radical polymerization (ATRP) method in the presence of common monomer (*N*-isopropylacrylamide) with the assistant of basic functional monomer (*N*-[3-(dimethylamino)propyl]-methacrylamide), which provides a achievable attempt for imprinting larger target proteins based on the ATPR with the mild reaction conditions. The BSA-imprinted polymer exhibited higher adsorption capacity and selectivity to BSA over the non-imprinted polymer. Competitive adsorption tests indicated the BSA-imprinted polymer had better selective adsorption and recognition properties to BSA in the mixture. The obtained BSA-imprinted polymer was applied to bovine serum, which also showed selectivity to BSA. In addition, a conventional aqueous two-phase solution of PEG/sulphate was used as elution for adsorbed BSA, which was compared with common NaCl elution.

1. Introduction

Molecular imprinting is a technique which creates molecular recognition sites that are chemically and sterically complementary to the predetermined target molecules in a synthetic polymer [1,2]. Over the past decades, molecularly imprinted polymer (MIP) has been successfully applied to a variety of small molecules [3,4]. Recent years, much attention has been paid to the imprinting of biomacromolecules and especially protein for its potential applications as biomaterials in separation, purification, biosensor, and mimicking enzyme and antibody [5–11]. However, the large molecular size, complexity, conformational flexibility, solubility, and numbers of functional groups of the protein make its imprinting face great challenges. In order to achieve the effective imprinting, some methods have been developed, for example the surface imprinting [5,12,13] and epitope imprinting [14,15]. However, most of the current reports are concerned with small protein imprinting such as lysozyme (Lyz, MW 13.4 kDa) [5,9–11,16,17], ribonuclease A (MW 13.7 kDa) [18] and cytochrome C (Cyt C, MW 12.3 kDa) [19,20]. Based on our previous experiments in protein imprinting, large protein imprinting is more difficult because of its greater size and more complex structure.

Since the magnetic separation has the merits of ease, high efficiency and low cost, which can be used in direct purification of the crude product from a mixture without any pretreatment such as centrifugation and filtration, it has attracted more and more interest in biological application in recent years. When magnetic separation was integrated with imprinted polymer, the resulting magnetically imprinted polymer can not only selectively recognize the target proteins in complex matrix but also can be easily collected and separated by an external magnetic field [18,21–23]. In our previous work [24], the atom transfer radical polymerization (ATRP) has been first developed as a new imprinting technology on magnetic supports to prepare small protein Lyz surface-imprinted polymer. ATRP is a new class of controlled living radical polymerization, possesses the mild reaction conditions without heating and ultraviolet radiations and avoids the adverse reactions comparing with traditionally initiated radical polymerization. The imprinted polymer has high adsorption capacity and selectivity to template Lyz. However, following the same ATPR procedure, using the same *N*-isopropylacrylamide (NIPAAm) monomer and magnetic supports, we failed in the imprinting of bovine serum albumin (BSA), and the conformational memory against BSA was not observed. Albumin, which accounts for approximately 50% of the serum pro-

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teins, is considered as one of the high abundant proteins. Selectively recognizing and capturing albumin are very attractive for serum separation and purification. Since BSA (MW 66.0 kDa) is nearly five times larger than Lyz (MW 13.4 kDa), it possesses larger molecular size and more flexible conformational transitions in imprinting process, the hydrogen bonds existing between BSA and the functional monomer (NIPAAm) are very weak, which causes the difficulty of stable BSA-monomer complexes formation in imprinting process. In a few successful larger protein imprinted polymer cases, the especial functional monomers [25–28] were used in the process of imprinting BSA, which forced the strong interaction with BSA, or BSA [21] and bovine hemoglobin (MW 64.5 kDa) [29] were directly covalently grafted on the surface of supports. Due to the additional strong interactions with larger proteins or their direct fixation on the supports, the larger protein-monomer complexes formed were more stable, which was helpful to form imprinting cavities and recognition sites in imprinting process.

In this work, the presented method focused on the assistant electrostatic interaction between acid target and basic monomer, the superparamagnetic BSA surface-imprinted polymer was successfully prepared based on ATRP in the assistance of basic monomers *N*-[3-(dimethylamino)-propyl]-methacrylamide (DMAPMA). The addition of the basic functional monomer made BSA surface-imprinting available due to their strong electrostatic interaction with template BSA. The adsorption properties of BSA imprinted polymer were investigated, and its selectivity to template BSA and other standard proteins with different molecular weights and isoelectric points were compared. The obtained BSA imprinted polymer was applied to real bovine serum, which showed high selectivity to BSA. In addition, a conventional aqueous two-phase solution of PEG/sulphate was used as elution for adsorbed BSA, which was compared with NaCl elution.

2. Experimental

2.1. Materials

Lyz and BSA were purchased from Amresco (Solon, OH, USA). Human serum albumin (HSA) and myloglobin (Mb) were obtained from Sigma (St. Louis, MO, USA). Cyt C was supplied by Roche (Basel, Switzerland). Ovalbumin (OVA) was purchased from Dingguo Biotech (Beijing, China). Bovine serum was produced by Xinte Biotechnology Co. Ltd. (Guangzhou, China).

NIPAAm was supplied by Acros Organics (Morris Plains, NJ, USA). Acrylamide (AAm) and *N*, *N*′-methylenebisacrylamide (MBAA) were provided by Sigma–Aldrich (Tokyo, Japan). DMAPMA was purchased from Tokyo Chemical industry Co. Ltd. (Tokyo, Japan). *N*,*N*,*N*′,*N*′,*N*″-pentamethyl diethyenetriamine (PMDETA) was obtained from Alfa Aesar (MA, USA). 2-Bromoisobutyryl bromide were purchased from Ouhe Technology Co. Ltd. (Beijing, China) and used without further purification.

Fe_3O_4 nanoparticles were synthesized according to the literature [30]. CuCl was purchased from Fine Chemicals (Beijing, China) and purified in acetic acid under the protection of nitrogen. HPLC grade acetonitrile was obtained from Fisher Chem. Alert Co. (NJ, USA). All other reagents were of analytical grade.

2.2. Apparatus

The product morphology was observed with field-emission scanning electron microscope (FESEM; JEOL, JSM-7100F, Japan). Shimadzu UV–vis UV-1750 spectrophotometer (Kyoto, Japan) was used for the detection of the template BSA elution from the imprinted polymer. Proteins identification was performed by Shimadzu Prominence LC-20A series HPLC (Kyoto, Japan) and with an Agilent C_8 (250 mm × 4.6 mm, 5 μm, 300 Å) column. Protein identification by SDS–PAGE was performed by Kaiyuan gel electrophoresis (Beijing, China).

2.3. Preparation of superparamagnetic BSA surface-imprinted polymer

Fe_3O_4@initiator was prepared according to the literature [24] as follow. Amino-functionalized Fe_3O_4 nanoparticle (Fe_3O_4@NH_2) (3.0 g) was added to a mixture of tetrahydrofuran (30 mL) and triethylamine (1 mL). The resulting reaction mixture was bubbled three times with high-purity nitrogen in an ice bath, and then 2-bromoisobutyryl bromide (1 mL) as initiator was added dropwise to initiate the reaction. The reaction lasted for 12 h at room temperature under the protection of nitrogen. The resultant Fe_3O_4@initiator was collected by a magnet, then washed with alcohol, acetone and distilled water, and dried finally.

The superparamagnetic BSA surface-imprinted polymer (designated Fe_3O_4@BSA-MIP) was synthesized by ATRP procedure (Fig. 1) as follows: Fe_3O_4@initiator (0.2 g) as the initiator, NIPAAm (0.4000 g, 3.44 mmol) as functional monomer, DMAPMA (30 μL, 0.1674 mmol) and AAm (0.0100 g, 0.1400 mmol) as assistant monomer, MBAA (0.0053 g, 0.0878 mmol) as the cross-linker, and template BSA (0.0500 g, 0.00076 mmol) were dispersed in 20 mL of phosphate buffer solution (PBS, 10 mmol/L, pH 7.0) in a three-neck round-bottom flask for prepolymerization at room temperature. Then PMDETA (30 μL, 0.1429 mmol) was added, the air was exchanged with nitrogen three times. CuCl (15 mg, 0.1516 mmol) was quickly transferred into the flask under the protection of nitrogen. The reaction proceeded at room temperature for 12 h with vigorous vibration. The product was extensively washed by the following procedure: distilled water, NaCl (1.0 mol/L), distilled water in sequence three times to elute the template. The complete removal of BSA from the imprinted polymer was confirmed by a UV–vis spectrophotometer at 280 nm. Finally, Fe_3O_4@BSA-MIP with specific sites was separated by magnetic field, dispersed in water and kept at room temperature for further characterization and adsorption studies. Correspondingly, Fe_3O_4@NIP was generated in the same way without adding BSA.

2.4. Adsorption experiments

The wet state polymer was first balanced in PBS (10 mmol/L, pH 7.0) and then was added to the protein solution (pH 7.0). After incubation, the polymer was isolated by an external magnetic field. The residual concentration of BSA in the solution was determined by HPLC. The amount of adsorbed protein by the polymer can be determined by the difference in concentration before and after the adsorption.

The adsorption capacity (Q, mg of protein/g of polymer) is calculated according to the equation as follows:

$$Q = \frac{(C_0 - C_f)V}{m} \quad (1)$$

where C_0 (mg/mL) is the initial protein concentration, C_f (mg/mL) is the final protein concentration, V (mL) is the total volume of the adsorption mixture, and m is the mass of polymer in each rebinding mixture.

The specific recognition property of MIP is evaluated by imprinting factor (α), which is defined as

$$a = \frac{Q_{MIP}}{Q_{NIP}} \quad (2)$$

where Q_{MIP} and Q_{NIP} are the adsorption capacity of the template or the non template on MIP and NIP, respectively.

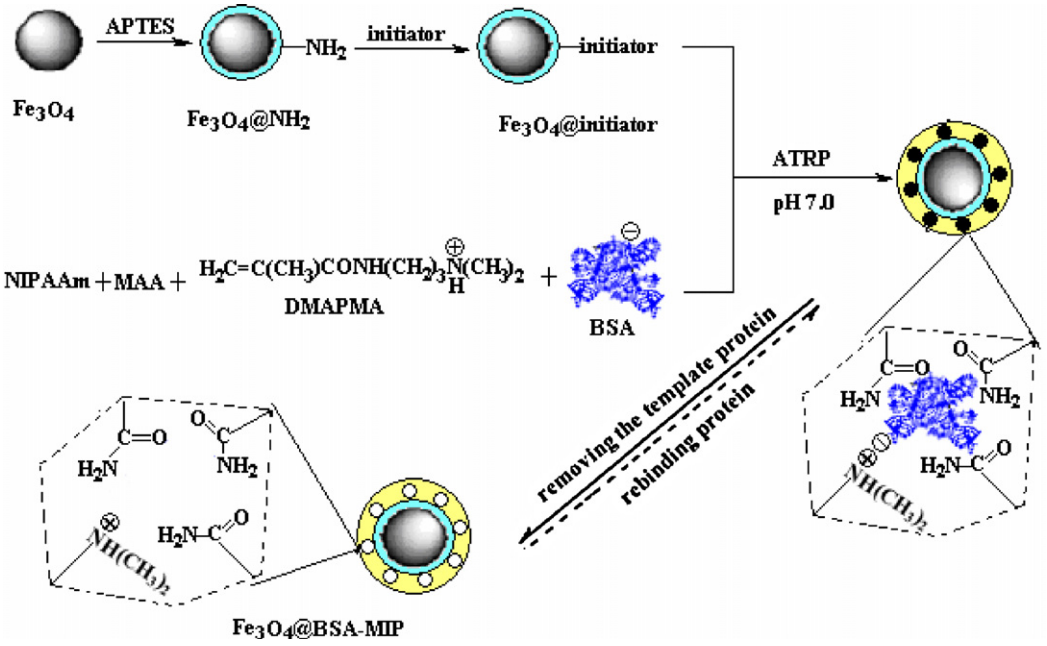

Fig. 1. Schematic representation for synthesis of the superparamagnetic BSA surface-imprinted polymer.

The selectivity factor (β) is defined as

$$\beta = \frac{\alpha_{tem}}{\alpha_{non}} \quad (3)$$

where α_{tem} and α_{non} were the imprinting factor of template protein and non template protein, respectively.

2.5. Elution of protein adsorbed

Aqueous two-phase solution (15%PEG4000/10% $(NH_4)_2SO_4$) was prepared as follows: PEG4000 (0.1875 g) and 40% $(NH_4)_2SO_4$ (0.3125 g) were dissolved in water (0.75 g), followed by shaking and centrifugation to form two phases.

After each adsorption, the imprinted polymer was treated with PBS (10 mmol/L, pH 7.0) to wash out the nonspecifically adsorbed proteins, and then the elution solution (two aqueous phase solution (15%PEG4000/10% $(NH_4)_2SO_4$) or 1 mol/L NaCl) was added to elute the specifically adsorbed proteins. The imprinted polymer was isolated by an external magnetic field, and the supernatant was collected for analysis. Additionally, when two aqueous phase solution (15%PEG4000/10% $(NH_4)_2SO_4$) was used to elute the specifically adsorbed proteins, the desorbed proteins (BSA) were contained in bottom phase. So, the supernatant in bottom phase was collected for analysis.

2.6. Proteins analysis

Protein concentration was mainly determined by HPLC. Gradient elution program was as follow: solution A: 80 vol.% acetonitrile and 20 vol.% distilled water with 0.1 vol.% trifluoroacetic acid (TFA), solution B: distilled water containing 0.1 vol.% TFA. Linear gradient was from 37.5% A to 62.5% A in 20 min, UV wavelength 214 nm, column temperature 35 °C, flow-rate 1.0 mL/min and injection volume 10 μL.

The mixture of BSA and OVA identification were performed by SDS–PAGE using 14.0% polyacrylamide separating gel and 5% stacking gel.

3. Results and discussion

3.1. The use of basic function monomer in imprinting process and the characteristics of imprinted polymer

ATRP reaction for protein imprinting has been successfully used to prepare Lyz-imprinted polymer [24]. Since template Lyz has relatively small molecular size, enough multiple hydrogen bonding interaction with the monomer NIPAAm to produce imprinted sites in polymer are achievable. The prepared Lyz-imprinted polymer demonstrated high selective adsorption and recognition to Lyz, which was used for separation of Lyz from real egg white samples. However, the imprinting of BSA failed under the same experimental condition. We speculate that BSA possesses more flexible conformational transitions in imprinting process due to its larger molecular size, the hydrogen bonds existing between monomer NIPAAm and BSA are too weak to form stable BSA-monomer complexes. In order to assist the imprinting of acid protein BSA (*pI* 4.7), electrostatic interaction between monomer and BSA is considered, which is supposed to benefit the formation of imprinted sites in polymer. Therefore, DMAPMA, a kind of basic functional monomer, was chosen as additive monomer in BSA imprinting process, the preparation of BSA-imprinting polymer refers to Section 2.3.

According to the infrared spectra and magnetic properties results in our reported work [24], the protein imprinted polymer can be synthesized by the ATRP and its layer thickness was about 15 nm. Fig. 2 shows the FESEM images of the morphological features of Fe_3O_4@MIP-BSA and Fe_3O_4@NIP, both of them appeared to be spherical in shape with no significant morphological differences, which was consistent with that reported by Tan [18,21].

3.2. Adsorption properties of Fe_3O_4@BSA-MIP to template BSA

3.2.1. Adsorption dynamics

For an important consideration in practical application of polymer particles in fast magnetic separation, the binding kinetics of Fe_3O_4@BSA-MIP and Fe_3O_4@NIP was compared, which determined the rate of the adsorption separation process. Fe_3O_4@BSA-MIP showed a rapid increase in the first 2 h, achieving 90% of the equilibrium adsorption capacity (Fig. 3a). At the beginning of adsorption

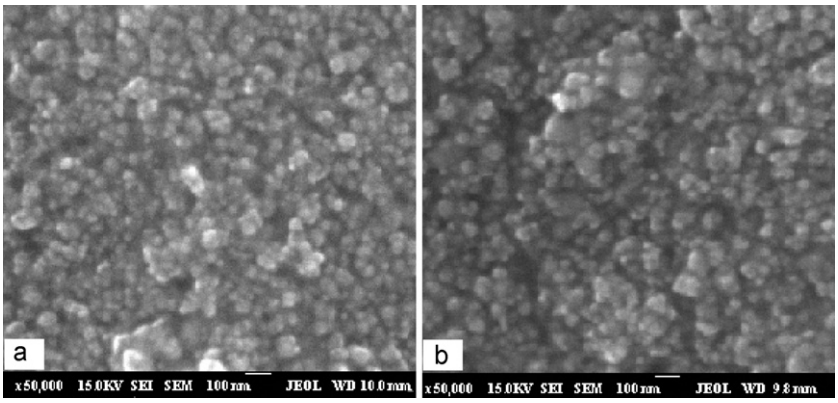

Fig. 2. FESEM images of Fe_3O_4@MIP-BSA (a) and Fe_3O_4@NIP (b).

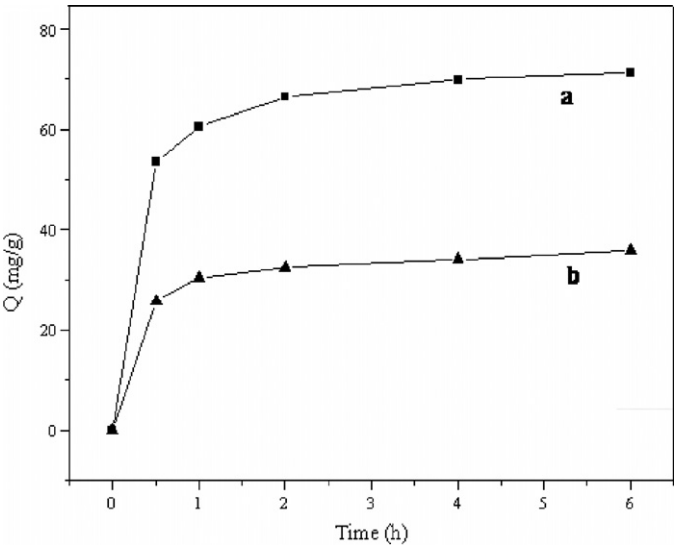

Fig. 3. The adsorption kinetic curves of BSA on Fe_3O_4@MIP-BSA (a) and Fe_3O_4@NIP (b). Experimental conditions: 1.0 mL BSA solution (0.2 mg/mL pH 7.0) was incubated with 2.0 mg polymer at room temperature.

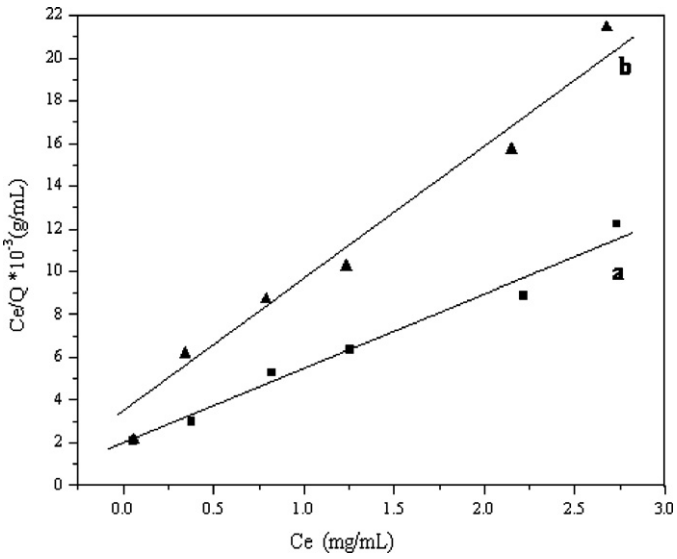

Fig. 4. Scatchard plot of the adsorption assay of BSA on Fe_3O_4@BSA-MIP (a) and Fe_3O_4@NIP (b).

process, Fe_3O_4@BSA-MIP possessed a large amount of empty binding sites on the surface, which enabled template BSA easily bind to them with less resistance. With time increase, adsorbed BSA occupied most of the binding sites and the adsorption rate slowed down and reached adsorption equilibrium eventually. Compared with the adsorption rate of Fe_3O_4@Lyz-MIP, which achieved 90% of the equilibrium adsorption capacity in the first 6 h [24], the adsorption rate of Fe_3O_4@BSA-MIP for template BSA was faster and favorable. The increased adsorption rate was caused by the addition of monomer DMAPMA in polymerization process, since it possessed tertiary amine groups. In adsorption solution (pH 7.0), the tertiary amine groups in imprinted cavities attracted negatively charged template BSA, which induced the rapid matching of proteins and recognition sites, so the adsorption equilibrium rate was accelerated. Therefore, the addition of basic monomer helped the imprinting of larger acidic BSA template.

Since the formation of Fe_3O_4@NIP lacked the BSA imprinting process, the functional groups were randomly distributed, and the low adsorption capacity to BSA was attributed to nonspecific adsorption (Fig. 3b). So, Fe_3O_4@BSA-MIP adsorbed more template BSA than Fe_3O_4@NIP due to the imprinting effect.

3.2.2. Adsorption isotherm

The binding parameters between BSA and Fe_3O_4@BSA-MIP were investigated by the effect of initial BSA concentration on adsorption capacity (Fig. 4). 0.1–3.0 mg/mL BSA solutions (1.0 mL) prepared in PBS (10 mmol/L, pH 7.0) were incubated with 2.0 mg of the polymer for 12 h at room temperature. The data obtained were linearized by Langmuir adsorption equation as follows:

$$\frac{C_e}{Q} = \frac{C_e}{Q_{max}} + \frac{1}{KQ_{max}} \quad (4)$$

where Q and Q_{max} are the experimental adsorption capacity to the template protein and the theoretical maximum adsorption capacity of polymer (mg/g), respectively, C_e is the concentration of protein in equilibrium solution (mg/mL), and K is the Langmuir adsorption equilibrium constant for the template protein to the polymer (mL/mg). According to Fig. 4, the calculated Langmuir adsorption equilibrium constant (K) and the binding capacity (Q_{max}) are summarized in Table 1. The K and Q_{max} of Fe_3O_4@BSA-MIP are

Table 1
K and Q_{max} calculated from the curves of the Scatchard plot.

Polymer	K (mL/mg)	Q_{max} (mg/g)	R
Fe_3O_4@BSA-MIP	1.41	273.6	0.9902
Fe_3O_4@NIP	1.29	150.6	0.9876

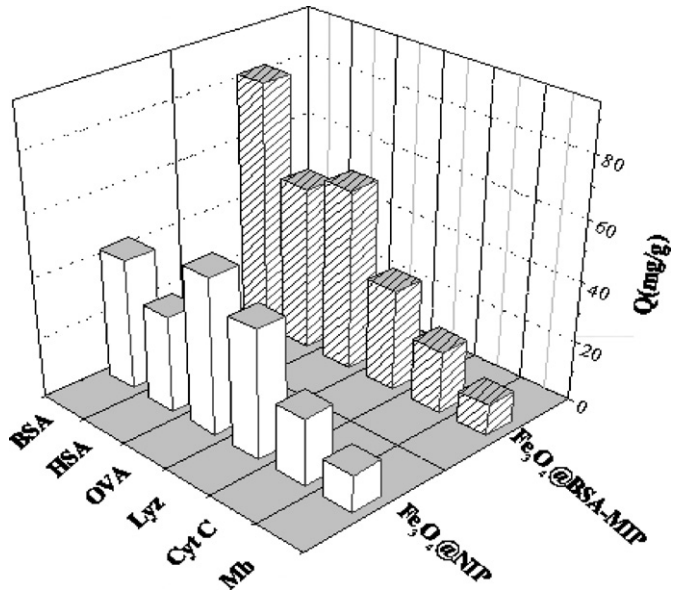

Fig. 5. The adsorption capacity of BSA and competing proteins on Fe_3O_4@BSA-MIP and Fe_3O_4@NIP. Experiment conditions: 1.0 mL protein solution (0.2 mg/mL pH 7.0) was incubated with 2.0 mg polymer for 12 h at room temperature.

higher than those of Fe_3O_4@NIP, which showed Fe_3O_4@BSA-MIP has relatively high selectivity and adsorption capacity for BSA over Fe_3O_4@NIP, and the adequate cavities with specific recognition sites for BSA could be formed during polymerization process with basic monomer. Compared with the imprinting of Lyz [24], Fe_3O_4@BSA-MIP to template BSA had higher Q_{max} due to the electrostatic attraction between BSA and the tertiary amine group of polymer chains. The adsorption selectivity of Fe_3O_4@BSA-MIP to template BSA indicated the success of BSA imprinting and the assistant effect of basic monomer DMAPMA.

3.3. Selectivity of Fe_3O_4@BSA-MIP

3.3.1. Selectivity comparison to template BSA and non-template proteins

Six proteins with different molecular weights and isoelectric points were used to test the selectivity of the BSA imprinted polymer. Fig. 5 shows that the adsorption capacities of Fe_3O_4@BSA-MIP and Fe_3O_4@NIP for different proteins. Since the size and surface charge of proteins Lyz (MW 13.4 kDa, *pI* 11.2), Cyt c (MW 12.3 kDa, *pI* 12.3) and Mb (MW 16.9 kDa, *pI* 7.0) did not match the BSA imprinted cavity in Fe_3O_4@BSA-MIP, so the adsorption comparison of them on Fe_3O_4@BSA-MIP and Fe_3O_4@NIP did not show apparent difference.

Furthermore, even though OVA (MW 43.0 kDa, *pI* 4.7) has the close isoelectric point to BSA (MW 66.0 kDa, *pI* 4.9), it has smaller size, which causes its adsorption on Fe_3O_4@BSA-MIP and Fe_3O_4@NIP very close. This indicated Fe_3O_4@BSA-MIP had nearly no selective adsorption to OVA. The results showed the predominance of molecular size, which dominated the selectivity of imprinting polymer to template BSA.

HSA, the homologous protein of BSA, has the close molecular size and isoelectric point, however, the adsorption capacity of Fe_3O_4@BSA-MIP to HSA was lower than that to BSA, which manifested further that the shape memory effect was the major factor affecting the imprinting formation and template recognition.

Among the six proteins, Fe_3O_4@BSA-MIP has the largest adsorption to template BSA, then the HSA, homologous protein with same size. For other four proteins, due to the size and charge not matching with imprinted cavities, there were not selectivity difference between Fe_3O_4@BSA-MIP and Fe_3O_4@NIP. Therefore, the differences of the adsorption capacities between Fe_3O_4@BSA-MIP and Fe_3O_4@NIP were negligible for all the proteins except template BSA and HAS, which were concordant with those reported by Zhao and coworkers [28].

Table 2
Selectivity of Fe_3O_4@BSA-MIP to BSA and competing protein.

Parameter	BSA	HSA	OVA	Lyz	Cyt C	Mb
α_s	1.91	1.71	1.15	0.78	0.92	0.84
β_s	–	1.12	1.66	2.45	2.08	2.27
$\alpha_{c(0.5:1:1)}$	1.43	–	–	0.81	–	1.35
$\alpha_{c(2:1:1)}$	1.58	–	–	0.73	–	1.12
$\alpha_{c(10:1:1)}$	2.11	–	–	0.56	–	1.00

$_S$ denoted a single adsorption experiment; $_C$ denoted a competitive adsorption experiment.
$\alpha_{(0.5:1:1)}$, $\alpha_{(2:1:1)}$ and $\alpha_{(10:1:1)}$ were the imprinting factors when BSA concentration ratios to Lyz and Mb were 0.5:1:1, 2:1:1 and 10:1:1 in competitive adsorption experiments.

Based on the amount of protein adsorbed (Q), an imprinting efficiency of 1.91 for template BSA was achieved, which was basically consistent with that of Lyz [24] reported by us and that of ribonuclease A (MW 13.7 kDa, *pI* 9.5) by Tan with other method [18]. Therefore, the presented BSA imprinting method has the similar effect as for small proteins, which manifests that larger protein imprinting like BSA is achievable. Table 2 shows the imprinting factor (α) and the selectivity factor (β) of Fe_3O_4@BSA-MIP to six proteins.

3.3.2. Selectivity to template BSA in protein mixture

The selectivity of Fe_3O_4@BSA-MIP to BSA in the mixture of BSA, Lyz and Mb and in the mixture of BSA and OVA was further investigated, and the three non-template proteins were used as the competitors (Figs. 6 and 7). When BSA concentration in the mixture was only half of Lyz and Mb, the adsorption of Fe_3O_4@BSA-MIP to BSA was still larger than that of Fe_3O_4@NIP (Fig. 6). With higher concentration ratio of BSA to Lyz and Mb (1:1:1, 2:1:1, 5:1:1 and 10:1:1), the selective adsorption to BSA increased, which was attributed to the template BSA could match the BSA imprinted cavities in Fe_3O_4@BSA-MIP. However, there was little change in

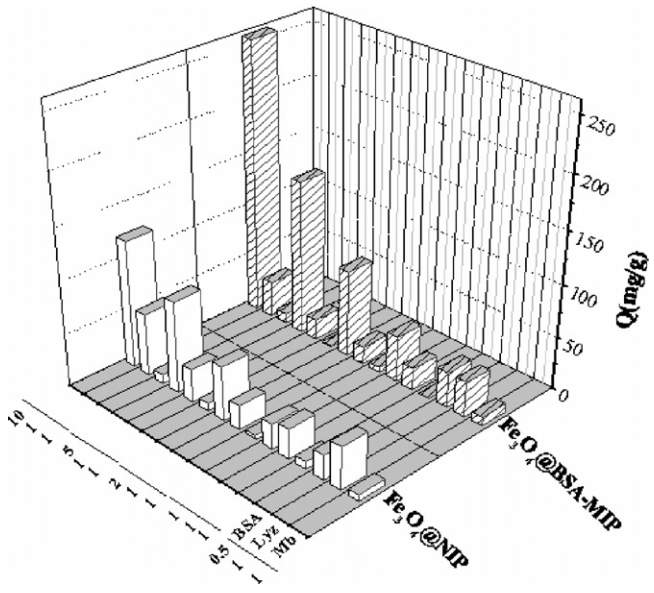

Fig. 6. Competitive adsorption of BSA with Lyz and Mb on Fe_3O_4@BSA-MIP. Experimental conditions: 1.0 mL mixed proteins solution (pH 7.0) was incubated with 2.0 mg of the polymer for 12 h at room temperature. The concentrations of Lyz and Mb always kept 0.2 mg/mL, BSA concentrations increased from 0.1 to 2.0 mg/mL.

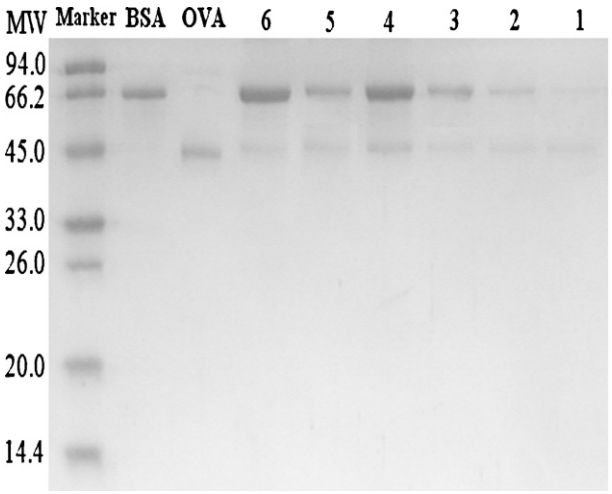

Fig. 7. SDS–PAGE analysis of BSA and OVA mixture adsorbed on Fe_3O_4@BSA-MIP and Fe_3O_4@NIP. 15 μL BSA (0.2 mg/mL, 1.0 mg/mL, and 2.0 mg/mL) and 15 μL OVA (0.2 mg/mL) were mixed respectively. Lane 1, 3 and 5 represented the BSA and OVA eluted from Fe_3O_4@NIP. Lane 2, 4 and 6 represented them eluted from Fe_3O_4@BSA-MIP. Lane 1 and 2 indicated 1:1 of BSA and OVA; lane 3 and 4 indicated 5:1 of BSA and OVA; lane 5 and 6 indicated 10:1 of BSA and OVA.

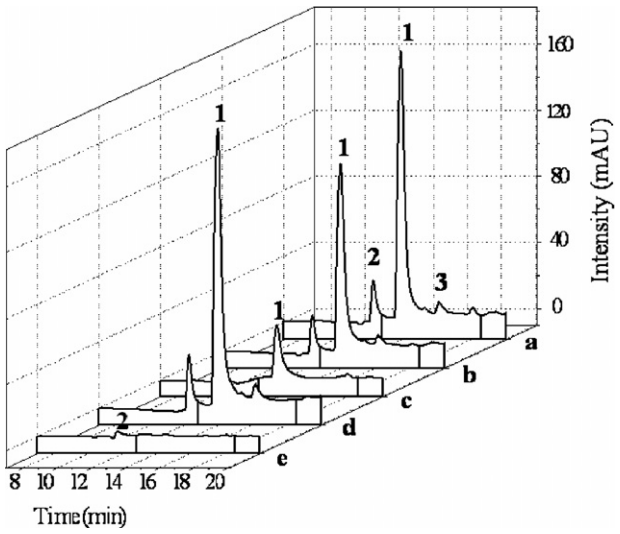

Fig. 9. Chromatograms of bovine serum adsorbed by Fe_3O_4@BSA-MIP and eluted by aqueous two-phase solutions. The 20-fold dilution of bovine serum (a); the 20-fold dilution of bovine serum after Fe_3O_4@BSA-MIP adsorption (b); the proteins eluted by PEG/sulphate from Fe_3O_4@BSA-MIP which was enriched in bottom phase (c); the remained serum proteins distributed in bottom (d) and in top phase (e), respectively. Peak 1, high abundant BSA; peak 2 and 3, unknown proteins.

adsorption capacities of Fe_3O_4@BSA-MIP to Lyz and Mb, which means the nonspecific binding of Lyz and Mb on Fe_3O_4@BSA-MIP cavities. For Fe_3O_4@NIP, the nonspecific binding for Lyz and Mb was similar. Even though the nonspecific adsorption to BSA increased with its concentration increase, it was obviously lower than the specific adsorption on Fe_3O_4@BSA-MIP.

The similar result was obtained for mixture of BSA and OVA from the SDS–PAGE analysis (Fig. 7). The amount of BSA eluted from Fe_3O_4@BSA-MIP (lane 2, 4 and 6) was always larger than that from Fe_3O_4@NIP (lane 1, 3 and 5). With the increase concentration ratio of BSA to OVA (1:1, 5:1 and 10:1, corresponding to line 2, 4 and 6), the adsorption of BSA increased, which indicated Fe_3O_4@BSA-MIP had selective recognition to BSA in protein mixture solution. For OVA, the acid protein, due to electrostatic adsorption with tertiary amine groups in polymer chains, its amount eluted from Fe_3O_4@BSA-MIP (lane 2, 4 and 6) and Fe_3O_4@NIP (lane 1, 3 and 5) were nearly same, but evidently less than the amount of BSA eluted from Fe_3O_4@MIP, which indicated the nonspecific adsorption of OVA on the polymer.

In terms of the imprinting factor (α) in Table 2, when the concentrations of competing proteins Lyz and Mb kept constant in competitive adsorption experiments, higher concentration template BSA made its corresponding α increasing from 1.43 to 2.11, but α values of the two competing proteins Lyz and Mb decreasing from 0.81 to 0.56 and 1.35 to 1.00. The result is a good complementary to that conclusion "when the concentrations of template proteins kept unchanged and the amount of competing protein increased, their imprinting factor were primarily invariable" [31].

Above results are a proof of the successful formation of imprinted cavities on Fe_3O_4@BSA-MIP with the assistance of basic monomer, and also the importance of shape memory effect and electrostatic interaction. It is also illustrated that with an increasing concentration of BSA, Fe_3O_4@BSA-MIP had the higher recognition selectivity to template BSA in competitive adsorption and had no obvious co-adsorption to other competing protein.

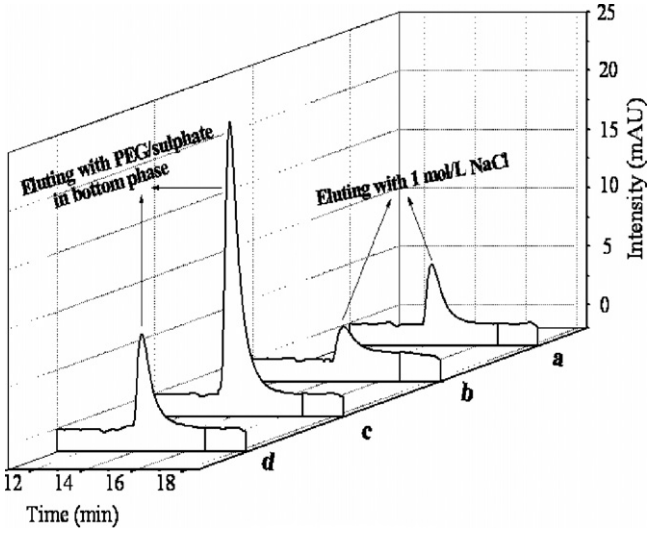

Fig. 8. Chromatograms of BSA eluted from Fe_3O_4@BSA-MIP (a) and Fe_3O_4@NIP (b) by NaCl, and elued from Fe_3O_4@BSA-MIP (c) and Fe_3O_4@NIP (d) by PEG/sulphate.

3.3.3. *Desorption of BSA by aqueous two-phase system*

The adsorbed BSA on imprinted polymer was usually eluted by inorganic salt solution [32]. Here, the ability to elute adsorbed BSA by aqueous two-phase system (PEG/sulphate) and NaCl were compared (Fig. 8). The results manifested that both PEG/sulphate, a salt-rich system, and NaCl could elute the target protein from the imprinted polymer, which attributed to the disruption of hydrogen bonding and electrostatic interaction between target protein and imprinted polymer binding sites when the high ionic strength solution was used. However, the desorbed BSA by PEG/sulphate system was totally allocated in the bottom phase. So, the BSA concentration desorbed by PEG/sulphate (Fig. 8c) was significantly larger than that desorbed by NaCl (Fig. 8a), which was attributed to the effect of aqueous two-phase extraction with the enrichment factor of two [24]. Therefore, the aqueous two-phase system (PEG/sulphate) is capable of desorption and enrichment to target protein.

The non-specific adsorption of BSA on Fe_3O_4@NIP was also observed, which could be eluted by both NaCl (Fig. 8b) and PEG/sulphate (Fig. 8d), and the aqueous two-phase system had also enrichment effect.

3.3.4. Application of Fe_3O_4@BSA-MIP for bovine serum sample

The synthesized Fe_3O_4@BSA-MIP was applied to bovine serum separation. The diluted bovine serum showed the large amount of BSA (peak 1 in Fig. 9a) and other moderate high amount proteins (peak 2 and peak 3 in Fig. 9a). After adsorption separation by some amount of Fe_3O_4@BSA-MIP using an external magnetic field, the remained BSA showed decrease (peak 1 in Fig. 9b) and other two unknown proteins did not change. Using PEG/sulphate to elute the BSA absorbed on Fe_3O_4@BSA-MIP, the apparent BSA peak was observed in bottom phase (peak 1 in Fig. 9c), which manifested Fe_3O_4@BSA-MIP had selective adsorption to BSA in bovine serum sample and less co-adsorption to others.

After adsorption, two aqueous phase (PEG/sulphate) was added in the remained bovine serum, proteins enrichment in bottom were showed obviously. Peak 1, 2 and 3 in Fig. 9d were apparently larger than those in Fig. 9b. However, only a small peak 2 in top phase was observed (Fig. 9e). It indicated that most of BSA and other two unknown proteins were distributed in salt-rich bottom phase. For complete BSA adsorption from serum, the enriched BSA in bottom phase could be adsorbed repeatedly by magnetic Fe_3O_4@BSA-MIP.

4. Conclusions

BSA surface-imprinted magnetic polymer was successfully synthesized based on ATRP method in the presence of monomer (NIPAAm) with the assistant of basic functional monomer (DMAPMA), which provides an attempt for imprinting larger target proteins. The predominance of molecular size and shape memory effect are the major factors affecting the imprinting formation and template recognition. The imprinted BSA polymer exhibits significant recognition property and selectivity to target BSA in standard protein mixture and real serum sample. The aqueous two-phase solution of PEG/sulphate has better elution and enrichment for target protein compared with conventional NaCl solution. The easy preparation, magnetic property and high selectivity of imprinted BSA polymer exhibit its potential application in BSA rapid separation and purification.

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References

[1] G. Wulff, Angew. Chem. Int. Ed. Engl. 34 (1995) 1812.
[2] K. Mosbach, Trends Biochem. Sci. 19 (1994) 9.
[3] J.L. Urraca, A.J. Hall, M.C. Moreno-Bondi, B. Sellergren, Angew. Chem. Int. Ed. 45 (2006) 5158.
[4] S.G. Hu, L. Li, X.W. He, J. Chromatogr. A 1062 (2005) 31.
[5] B.J. Gao, H.Y. Fu, Y.B. Li, R.K. Du, J. Chromatogr. B 878 (2010) 1731.
[6] Z.A. Lin, F. Yang, X.W. He, X.M. Zhao, Y.K. Zhang, J. Chromatogr. A 1216 (2009) 8612.
[7] J. Pan, X.H. Xue, J.H. Wang, H.M. Xie, Z.Y. Wu, Polymer 50 (2009) 2365.
[8] K.E. Kirat, M. Bartkowski, K. Haupt, Biosens. Bioelectron. 24 (2009) 2618.
[9] L. Qin, X.W. He, W. Zhang, W.Y. Li, Y.K. Zhang, J. Chromatogr. A 1216 (2009) 807.
[10] L. Qin, X.W. He, W. Zhang, W.Y. Li, Y.K. Zhang, Anal. Chem. 81 (2009) 7206.
[11] S. Yu, A.Q. Luo, D. Biswal, J.Z. Hilt, D.A. Puleo, Talanta 83 (2010) 156.
[12] M.S. Zhang, J.R. Huang, P. Yu, X. Chen, Talanta 81 (2010) 162.
[13] W.H. Zhou, C.H. Lu, X.C. Guo, F.R. Chen, H.H. Yang, X.R. Wang, J. Mater. Chem. 20 (2010) 880.
[14] H. Nishino, C.S. Huang, K.J. Shea, Angew. Chem. Int. Ed. 45 (2006) 2392.
[15] D.F. Tai, C.Y. Lin, T.Z. Wu, L.K. Chen, Anal. Chem. 77 (2005) 5140.
[16] W. Zhang, L. Qin, X.W. He, W.Y. Li, Y.K. Zhang, J. Chromatogr. A 1216 (2009) 4560.
[17] O.Y. Tov, S. Luvitch, H. Bianco-Peled, J. Sep. Sci. 33 (2010) 1673.
[18] C.J. Tan, Y.W. Tong, Anal. Chem. 79 (2007) 299.
[19] J. Rick, T.C. Chou, Anal. Chim. Acta 542 (2005) 26.
[20] J.X. Liu, Q.L. Deng, K.G. Yang, L.H. Zhang, Z. Liang, Y.K. Zhang, J. Sep. Sci. 33 (2010) 2757.
[21] C.J. Tan, H.G. Chua, K.H. Ker, Y.W. Tong, Anal. Chem. 80 (2008) 683.
[22] S. Lu, G. Cheng, X. Pang, J. Appl. Polym. 99 (2006) 2401.
[23] Z. Ma, Y. Guan, H. Liu, J. Polym. Sci. Polym. Chem. 43 (2005) 3433.
[24] Q.Q. Gai, F. Qu, Z.J. Liu, R.J. Dai, Y.K. Zhang, J. Chromatogr. A 1217 (2010) 5035.
[25] F. Bonini, S. Piletsky, A.P.F. Turner, A. Speghini, A. Bossi, Biosens. Bioelectron. 22 (2007) 2322.
[26] C.H. Hu, T.C. Chou, Microchem. J. 91 (2009) 53.
[27] Z.D. Hua, S. Zhou, M.P. Zhao, Biosens. Bioelectron. 25 (2009) 615.
[28] Z.D. Hua, Z.Y. Chen, Y.Z. Li, M.P. Zhao, Langmuir 24 (2008) 5773.
[29] X.W. Kan, Q. Zhao, D.L. Shao, Z.R. Geng, Z.L. Wang, J.J. Zhu, J. Phys. Chem. B 114 (2010) 3999.
[30] X. Wang, L.Y. Wang, X.W. He, Y.K. Zhang, L.X. Chen, Talanta 78 (2009) 327.
[31] Q.Q. Gai, F. Qu, Y.K. Zhang, Sep. Sci. Technol. 45 (2010) 2494.
[32] M.J. Guo, Z. Zhao, Y.G. Fan, C.H. Wang, L.Q. Shi, J.J. Xia, Y. Long, H.F. Mi, Biomaterials 27 (2006) 4381.