Superparamagnetic lysozyme surface-imprinted polymer prepared by atom transfer radical polymerization and its application for protein separation

Qing-Qing Gai, Feng Qu, Zong-Jian Liu, Rong-Ji Dai, Yu-Kui Zhang

Abstract

Molecular imprinting as a promising and facile separation technique has received much attention because of their high selectivity for target molecules. In this study, the superparamagnetic lysozyme surface-imprinted polymer was prepared by a novel fabrication protocol, the grafting of the imprinted polymer on magnetic particles in aqueous media was done by atom transfer radical polymerization (ATRP), and the properties of the imprinted polymer were characterized in detail. Its high selective adsorption and recognition to lysozyme demonstrated the separation ability of the magnetic imprinted material to template molecule, and it has been used for quick and direct separation of lysozyme from the mixture of standard proteins and real egg white samples under an external magnetic field. Furthermore, the elution of lysozyme from the imprinted material was achieved by PEG/sulphate aqueous two-phase system, which caused lysozyme not only desorption from the imprinted materials but also redistribution in the top and bottom phase of aqueous two-phase system. The aqueous two-phase system exhibited some of the extraction and enrichment effect to desorbed lysozyme. Our results showed that ATRP is a promising method for the protein molecularly imprinted polymer preparation.

1. Introduction

Molecular imprinting is a promising and facile separation technique to produce molecule-specific recognition sites in synthetic polymers that selectively bind template molecules [1–3]. To date, this technique demonstrated great potential in imprinting small molecules of molecular weight <1500 in organic solvents, while it was very limited to biological macromolecules such as proteins since imprinting proteins face challenges of large molecular size, complexity, conformational flexibility, solubility, and sensitivity to environment. Nonetheless, the molecules imprinting of proteins has been given more and more consideration for its potential application for selective protein separation, and some progress has been made recently [4,5].

At the beginning, strategies for imprinting protein were carried out using bulking imprinting, where molecularly imprinted polymer (MIP) prepared tended to be sharp-edged, irregular MIP bits and recognition sites within the polymer bulk, which hindered diffusion of template proteins and was not suitable for practical separation application [6]. To overcome these problems in bulking imprinting, surface imprinting on supports has been used for the synthesis of MIP with better accessibility to the specific binding sites. The new reported method provided by Qin et al. [7] avoided solution polymerization and resulting gelation with traditional radical polymerization, but it needed to be initiated by ultraviolet radiations which can cause template protein conformational change. The atom transfer radical polymerization (ATRP) has been proposed as a new surface imprinting technology [8–10], which is a new class of controlled living radical polymerization [11]. Compared with the present radical polymerization, ATRP possesses the mild reaction conditions and can be initiated at room temperature without heating and ultraviolet radiations. Moreover, ATRP can be carried out in an aqueous solution, which can be well controlled by ATRP reagent due to the avoidance of the adverse reactions such as radical coupling or disproportionation action. These advantages make ATRP very suitable for imprinting proteins. Till now, except the imprinting of some small molecules [12–14], there is no precedent for the preparation of protein molecularly imprinted polymer by ATRP.

In preparation of MIP, the supports usually used are silica beads [15], chitosan beads [16], and polymer particles [7]. In recent years, the magnetic nanoparticles as supports have attracted more and more interest in biological application since they have good bio-compatible properties, and, importantly, they can be easily isolated from samples by using an external magnetic field without the need of complicated centrifugation steps or filtration. Some scholars [17–20] have prepared protein molecularly imprinted polymers coated magnetic nanoparticles as affinity adsorbents to recognize...
and separate the template protein. When magnetic components are encapsulated into MIP, the magnetic MIP will have both magnetically susceptible property and selectivity to the target protein. The materials can be used not only as column packing but also as fast magnetic absorbent for target protein, which make the separation process easier, faster and more efficient. The combination of the magnetic absorbent for target protein, which make the separation and extraction capability to target proteins.

Commonly, reported elution solutions for protein removal from MIP were inorganic salt [21] or some special solution such as acetic acid/SDS [22]. Aqueous two-phase solution, which was bio-compatible and integrative of extraction and separation [23], has been exploited in proteomics in recent years [24,25]. Our previous work has used it for selective separation and enrichment of proteins in real human saliva and plasma sample [26]. In this work, PEG–sulphate mixture solution, a typical type of conventional aqueous two-phase solution was proposed as a new elution solution for adsorbed target lysozyme (Lyz), which has both desorption and extraction capability to target proteins.

To our knowledge, the application of ATRP in the protein molecular imprinting and the combination of superparamagnetic imprinted polymer with aqueous two-phase solution have not been reported till now, which might be a beneficial attempt for target proteins fast separation and enrichment in proteomics.

2. Experimental

2.1. Materials

Lyz and bovine serum albumin (BSA) were purchased from Amresco (Solon, OH, USA). Bovine hemoglobin (BHB), trypsin inhibitor (TI) and myoglobin (Mb) were obtained from Sigma (St. Louis, MO, USA). Cytochrome C (Cyt C) was supplied by Roche (Basel, Switzerland). N-Isopropylacrylamide (NIPAAm) was supplied by Acros Organics (Morris Plains, NJ, USA). Acrylamide (AAm) and N,N-methylenebisacrylamide (MBAA) were provided by Sigma–Aldrich (Tokyo, Japan). 3-Aminopropyltriethoxysilane (APTES) and N,N’,N”-pentamethyl diethylenetriamine (PMDETA) were obtained from Alfa Aesar (MA, USA). 2-Bromoisobutyryl bromide was purchased from Ouhe Technology Co. Ltd. (Beijing, China) and used without further purification. CuCl was purchased from Fine Chemicals (Beijing, China) and purified in acetic acid under the protection of nitrogen. Tetrahydrofuran (THF) and triethylamine were dried over CaH2 and distilled before use. HPLC grade acetonitrile was obtained from Fisher Chem. Alert Co. (NJ, USA). All other reagents were of analytical grade.

2.2. Apparatus

Morphological observation of the polymer particles was performed with a transmission electron microscopy (TEM; JEOL, JEM-2010, Japan). Fourier transform infrared (FT-IR) spectra of the polymer in KBr were recorded with a FT/IR spectrum BX (PerkinElmer, USA) instrument. The thermogravimetric analysis (TGA) was carried out using a Q5000IR (TA Instruments, USA) apparatus, the magnetic properties were determined with a vibrating sample magnetometer (VSM; LakeShore, 7307, USA), and the transition temperatures were measured by thermal analysis using differential scanning calorimetry (DSC; TA Instruments, Q2000, USA). All chromatographic measurements were performed by Shimadzu Prominence LC-20A series HPLC (Kyoto, Japan) and an Agela Technologies Venusil XBP C8 (250 mm × 4.6 mm, 5 μm, 300 Å) column (Tianjin, China).

2.3. Preparation of superparamagnetic Lyz surface-imprinted polymeric particles

Fe3O4 nanoparticles were synthesized according to the literature [27] and dispersed in alcohol (100 mL) and distilled water (1 mL) by sonication for 15 min, followed by the addition of APTES (1.5 mL). The mixture was reacted for 12 h at 40 °C under a continuous mechanical stirring. The resultant silica–Fe3O4 composites (designated Fe3O4@SiO2) were collected by an external magnetic field, washed three times with alcohol and distilled water, respectively, and then dried to powder in the vacuum. The Fe3O4@SiO2 (3.0 g) obtained was added to a mixture of THF (30 mL) and triethylamine (1 mL) in a three-neck round-bottom flask (100 mL). After the reaction mixture was bubbled three times with high-purity nitrogen for 20 min in an ice bath, 2-bromoisobutyryl bromide (1 mL) was added dropwise to start the reaction. The mixture was vibrated at room temperature for 12 h under the protection of nitrogen to obtain Fe3O4@initiator. It was collected and washed with alcohol, acetone, and distilled water by a magnet, and then dried.

The superparamagnetic Lyz surface-imprinted polymer (designated Fe3O4@Lyz–MIP) was synthesized by ATRP procedure (Fig. 1) as follows: Fe3O4@initiator (1.0 g) as the initiator, NIPAAm (0.4000 g, 3.44 mmol) as functional monomer, AAm (0.0100 g, 0.1400 mmol) as assistant monomer, MBAA (0.0053 g, 0.0878 mmol) as the cross-linker, and template Lyz (0.1900 g, 0.007 mmol) were dissolved in 30 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 to the flask under the protection of nitrogen. The reaction proceeded at room temperature for 12 h with vigorous vibration. The resulting product was washed by repeated magnetic separation and re-suspension with the detailed elution procedures as follows: the product was firstly washed three times with distilled water to remove any unreacted substance, and then immersed into 10% (v/v) acetic acid–10% (w/v) SDS solution with oscillating for 12 h (this step was repeated three times) to remove template, followed by extensive washing with distilled water until the washing water was neutral. Finally, Fe3O4@Lyz–MIP with specific sites was separated by magnetic field and dried in vacuum oven. Correspondingly, Fe3O4@NIP was generated in the same way without adding Lyz.

2.4. Determination of protein concentration and adsorption capacity

All protein concentrations in aqueous solution and aqueous two-phase system were 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% TFA. Linear gradient is from 37.5% A to 62.5% A in 20 min, UV wavelength 214 nm, flow-rate 1.0 mL/min and injection volume 10 μL.

The polymer was first allowed to swell in PBS (10 mmol/L, pH 7.0) until equilibrium. The wet state polymer then was added to the protein solution. After incubation, the polymer was isolated by an external magnetic field and the residual concentration of Lyz in the solution was determined by HPLC. The amount of adsorbed protein on polymer was 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:

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

(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.

3. Results and discussion

3.1. The characterization of Fe$_3$O$_4$@Lyz-MIP

Fig. 1 illustrates the method developed for the fixation of an ATRP initiator onto Fe$_3$O$_4$ nanoparticles and the subsequent growth of MIP layers from particles via surface initiated ATRP. The grafting polymer using ATRP appeared homogeneous with no agglomeration and no visible non-grafted polymer in the polymerization solution. These might help to avoid the broad binding site heterogeneity and the relatively low affinity and selectivity of MIP [14].

TEM was employed to observe the morphological features of Fe$_3$O$_4$@Lyz-MIP. From the TEM images (Fig. 2a, b and c), the magnetic support particles, Fe$_3$O$_4$@SiO$_2$ and Fe$_3$O$_4$@Lyz-MIP appeared to be spherical in shape. A polymer shell with defined shape and configuration was readily observed on Fe$_3$O$_4$@SiO$_2$ surface with light contrast (Fig. 2d and e). The polymer shell had an average thickness of about 15 nm. The particle size of Fe$_3$O$_4$@Lyz-MIP as estimated was about 120 nm. These values were basically consistent with that obtained in similar work by Lu et al. [13].

The FT-IR spectra of Fe$_3$O$_4$, Fe$_3$O$_4$@initiator, Fe$_3$O$_4$@Lyz-MIP and NIPAAm monomer were shown in Fig. 3A. It was notable that the Fe$_3$O$_4$@initiator showed a new peak at 1602.68 cm$^{-1}$ corresponding to carbonyl group of the ATRP initiator, which suggested that the initiator had been modified to Fe$_3$O$_4$. The strong peaks at 1634.85 and 1539.22 cm$^{-1}$ of Fe$_3$O$_4$@Lyz-MIP were attributed to the characteristic peaks of the polyNIPAAm (PNIPAAm), which were consistent with those of the NIPAAm (the amide I band, 1640 cm$^{-1}$, C=O stretching and amide II band, 1550 cm$^{-1}$, N–H bending). The IR results proved that imprinted polymer coating have been successfully grafted from Fe$_3$O$_4$@ initiator. It was further confirmed by DSC analysis shown in Fig. 3B. As the temperature increased, Fe$_3$O$_4$@Lyz-MIP presented an endothermal peak with a lower critical solution temperature (LCST) of 38.0 °C, while Fe$_3$O$_4$@ initiator did not exhibit it (Fig. 3B).
TGA was employed to further estimate grafting yield of imprinted polymer coating and quantify the amount of Fe₃O₄ encapsulated in the magnetic particles. The TGA graphs were shown in Fig. 3C. If the mass retention of Fe₃O₄@initiator at 800 °C is used as the reference, there exists ~15.2 wt% difference in the weight retentions at 800 °C between Fe₃O₄@initiator and Fe₃O₄@Lyz-MIP, probably comparable to 29.0 wt% of PNIPAAm grafted from silica nanoparticles by ATRP [28]. It indicated that the method employed for MIP coating in this work was excellently effective. The grafting yield of MIP coating to Fe₃O₄@initiator was ~15.2 wt%. The remaining mass was attributed to the more thermally resistant Fe₃O₄ magnetite, thus giving a magnetite encapsulation efficiency of ~79.4 wt%. It approached the level of theoretical Fe₃O₄ content (roughly 70.0 wt%) according to polymerization recipe. The obtained amount of Fe₃O₄ was considerably high and satisfactory as compared to the previous research reports (below 20.0 wt%) [17,19].

In general, Fe₃O₄ content is directly proportional to the magnetic responsibility of Fe₃O₄@Lyz-MIP. It is of utmost importance that the material should possess sufficient magnetic property for potential magnetic separation in practical application. Therefore, VSM analysis had been employed to characterize the magnetic properties of Fe₃O₄@initiator and Fe₃O₄@Lyz-MIP. Fig. 3D shows the two magnetization curves had a similar general shape, being symmetrical about the origin, confirming that the samples were superparamagnetic. The saturation magnetization (Ms) values obtained were 64.03 and 56.83 emu/g for Fe₃O₄@initiator and Fe₃O₄@Lyz-MIP, respectively, which are a measure for the maximum magnetic strength of the materials. The saturation magnetization of Fe₃O₄@Lyz-MIP had little change in comparison with Fe₃O₄@initiator. The decrease was expected because MIP coating had shielded the magnetite, thus somewhat reducing its magnetic response toward the external magnetic field. However, the magnetic responsibility of Fe₃O₄@Lyz-MIP was enough effective that it can be easily and quickly separated from the suspension, which is very favorable for the magnetic separation of proteins on a large scale.

3.2. Adsorption of Fe₃O₄@Lyz-MIP to template Lyz

The investigation of adsorption capacity and adsorption rate of MIP as separation materials are very important to template protein separation in liquid chromatography and solid phase extraction, which determined the potential application in real samples.

3.2.1. Influence of temperature on the adsorption capacity

From DSC results above, it was found that the polymer prepared using NIPAAm as major monomer was thermosensitive. The effect of temperature on the adsorption of Lyz to polymer particles was investigated. Fig. 4 shows that the maximum adsorption capacity of Lyz to Fe₃O₄@Lyz-MIP was around 33 °C, while adsorption capacity of Lyz to Fe₃O₄@NIP slightly increased with increasing temperature. We inferred that at 33 °C the cavities of Fe₃O₄@Lyz-MIP came to the state of imprinting. The shape of the cavity and the distribution of the functional groups should be accurately accorded with the protein at this state [29], thus, a highest affinity with the imprinting
factor (α) of 2.42 was obtained. It was indicated that the temperature played an important role in the adsorption of template to thermosensitive imprinted polymers. It can be used as a temperature gate to control the uptake and release of target molecules. The related work is in the process and will be discussed in other paper. Considering the stability of the protein, the experiments below were carried out at room temperature.

3.2.2. Adsorption rate

A binding kinetic study had been carried out for Fe₃O₄@Lyz-MIP and Fe₃O₄@NIP to determine the rate of the adsorption separation process. This is an important consideration in the practical application of the polymer particles in a separation process. The studies were performed using an initial Lyz concentration of 0.2 mg/mL. The results have shown in Fig. 5. During the initial adsorption process of Fe₃O₄@Lyz-MIP, the presence of a large amount of empty, high-affinity binding sites on the surface of the polymer particles enabled template Lyz to easily bind to them with less resistance, therefore, the adsorption kinetic of Fe₃O₄@Lyz-MIP showed a rapid increase in the first 6 h, achieving 90% of the equilibrium adsorption capacity. After that, with time increase, when adsorbed Lyz occupied most of the binding sites, the adsorption rate slowed down and eventually reached adsorption equilibrium. This is the kinetic typical for most of the adsorption processes. Considering poor diffusion properties due to the large size of the protein macromolecule, the obtained adsorption kinetics was favorable. As Fe₃O₄@NIP lacked of the imprinting process, the functional groups were distributed randomly, which resulted in the low adsorption ability of Lyz, hence, nonspecific adsorption of Lyz was observed. Therefore, Fe₃O₄@Lyz-MIP adsorbed more the template Lyz than Fe₃O₄@NIP due to the imprinting effect.

3.2.3. Maximum adsorption capacity

Further studies were carried out to determine the adsorption constants between Lyz and Fe₃O₄@Lyz-MIP. 1.0 mL 0.05–0.4 mg/mL Lyz solutions were prepared in PBS (10 mmol/L, pH 7.0) incubated with 5.0 mg of the polymer particles for 12 h at room temperature. After the adsorption experiments, the data obtained were linearized by Langmuir adsorption equation as follows:

\[
\frac{C_e}{Q} = \frac{C_e}{Q_{\text{max}}} + \frac{1}{KQ_{\text{max}}}
\]

where \(Q\) and \(Q_{\text{max}}\) are the experimental adsorption capacity to the template protein and the theoretical maximum adsorption capacity of polymer (mol/g), respectively, \(C_e\) is the concentration of protein in equilibrium solution (mol/L), and \(K\) is the Langmuir adsorption equilibrium constant for the template protein to the polymer (L/mol). When Lyz was bound to Fe₃O₄@Lyz-MIP, it was observed that the Scatchard plot was a single straight line, which indicated the binding sites of imprinted magnetic particles were identical (Fig. 6). Fig. 6 also shows the binding parameters of Lyz with Fe₃O₄@NIP. The calculated Langmuir adsorption equilibrium constant (K) and the adsorption capacity (Q_{\text{max}}) corresponding to the curves have been summarized in Table 1. As can be seen, the K and Q_{\text{max}} of Fe₃O₄@Lyz-MIP are higher than those of Fe₃O₄@NIP. These results indicated that Fe₃O₄@Lyz-MIP had a high affinity for Lyz over Fe₃O₄@NIP. Also, it was affirmed that adequate cavities with specific recognition sites for Lyz could be formed in the

<table>
<thead>
<tr>
<th>Polymer particles</th>
<th>(K \times 10^5) (L/mol)</th>
<th>(Q_{\text{max}} \times 10^{-2}) (mol/g)</th>
<th>(K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₃O₄@Lyz-MIP</td>
<td>8.2</td>
<td>8.3</td>
<td>0.9829</td>
</tr>
<tr>
<td>Fe₃O₄@NIP</td>
<td>7.0</td>
<td>4.1</td>
<td>0.9910</td>
</tr>
</tbody>
</table>
imprinted polymer coating during polymerization in the presence of Lyz as the template, which was the fundamental premise of imprinted polymer as affinity materials for protein separation.

3.3. Selectivity of Fe$_3$O$_4$@Lyz-MIP

The selective adsorption of MIP to template protein accounts for its high-affinity and anti-interference capability against other proteins, which is very significant to target protein separation from mixed samples.

3.3.1. Selectivity comparison of Fe$_3$O$_4$@Lyz-MIP to template Lyz and other proteins

Six types of proteins with different molecular weights and isoelectric points were involved to test the selectivity of the imprinted polymer particles. Fig. 7 shows the adsorption capacities of Fe$_3$O$_4$@Lyz-MIP and Fe$_3$O$_4$@NIP for different proteins with protein concentration of 0.2 mg/mL in PBS (pH 7.0). In the case of Fe$_3$O$_4$@Lyz-MIP, the sizes of BSA (MW = 66.0 kDa), BHb (MW = 64.5 kDa), TI (MW = 20.1 kDa) and Mb (MW = 16.9 kDa) were all larger than that of the template Lyz (MW = 13.4 kDa), so the access of large proteins to the imprinted sites might be limited by steric hindrance of polymer network. Also, the larger protein displayed less adsorption capacity, in other words, adsorption capacity of Fe$_3$O$_4$@Lyz-MIP for single protein decreased in the order of Mb > TI > BHb > BSA, as BSA, the largest one of them, had lowest adsorption capacity. This was expected since the degree of cross-linking of polymer in our work was not suitable for larger protein to access. However, although Cyt C (MW = 12.3 kDa) was similar to the template Lyz in molecular weight, the spatial arrangement of effective groups on its surface was different from Lyz, and Cyt C was not complementary in shape as compared to the recognition sites present in the cavities for Lyz. So, Fe$_3$O$_4$@Lyz-MIP only had significant adsorption specificity toward the template Lyz, which showed that the shape memory effect was the major factor affecting the imprinting formation and template recognition. On the other hand, six proteins divided into three groups of proteins with close isoelectric point (Lyz 11.2/Cyt C 12.3, Mb 7.0/BHb 6.9 and BSA 4.9/TI 4.2) had different adsorption capacity at pH 7.0. The results indicated that adsorption of proteins to Fe$_3$O$_4$@Lyz-MIP did not depend on electrostatic interaction. Also, the magnetic polymer particles were made hydrophilic by coating with most of hydrophilic groups. It was inferred that the type of interaction involved between the template Lyz and Fe$_3$O$_4$@Lyz-MIP could be hydrogen bonding, excluding other non-covalent interaction such as hydrophobic and ion interaction. So the synergistic effect of shape complementarity and hydrogen bonding interaction was significantly essential for molecular affinity for the template Lyz in an aqueous environment. In addition, the differences of the adsorption capacities between Fe$_3$O$_4$@Lyz-MIP and Fe$_3$O$_4$@NIP were negligible for all the proteins except template Lyz, the proteins adsorption to Fe$_3$O$_4$@NIP was due to nonspecific interactions affected by unpredictably various factors. Based on the amount of protein adsorbed ($Q$), an imprinting efficiency of 2.07 for template Lyz was achieved, consistent with that reported by Tan and Tong [17]. Table 2 shows the imprinting factor ($\alpha$) and the selectivity factor ($\beta$) of Fe$_3$O$_4$@Lyz-MIP for different proteins.

3.3.2. Selectivity of Fe$_3$O$_4$@Lyz-MIP to template Lyz in the protein mixture

The selectivity of Fe$_3$O$_4$@Lyz-MIP to the mixture of proteins was further investigated. BSA, Mb and Cyt C, three types of proteins with different molecular weights were used as the competitors. Most of the proteins adsorbed by Fe$_3$O$_4$@Lyz-MIP were the template Lyz (Fig. 8). The adsorption capacities for the three competing proteins were lower as compared to Lyz, which acted in accordance with the trends in single adsorption by steric hindrance. It was obvious that the polymer recognized the template Lyz preferentially. This is a proof of the successful formation of imprinted cavities and the importance of shape memory effect on Fe$_3$O$_4$@Lyz-MIP. However, the amount of Lyz bound to Fe$_3$O$_4$@Lyz-MIP was lower than that of the above single test. It was also found that the adsorption

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**Table 2**

Selectivity of Fe$_3$O$_4$@Lyz-MIP using Lyz as template protein.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lyz</th>
<th>BSA</th>
<th>Mb</th>
<th>BHb</th>
<th>TI</th>
<th>Cyt C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>2.07</td>
<td>0.83</td>
<td>0.97</td>
<td>1.03</td>
<td>1.08</td>
<td>1.31</td>
</tr>
<tr>
<td>$\beta$</td>
<td>2.41</td>
<td>2.06</td>
<td>1.94</td>
<td>1.85</td>
<td>1.52</td>
<td>1.24</td>
</tr>
</tbody>
</table>

$\delta$ denoted a single adsorption experiment; $\delta$ denoted a competitive adsorption experiment.

$\beta=\alpha_{\text{template}}/\alpha_{\text{nontemplate}}$, where $\alpha_{\text{template}}$ and $\alpha_{\text{nontemplate}}$ were the imprinting factor of template protein and nontemplate protein, respectively.

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**Fig. 7.** Adsorption capacities for the template protein and other proteins. Experimental conditions: 1.0 mL of 0.2 mg/mL protein incubated with 5.0 mg of the polymer particles for 12 h at room temperature.

**Fig. 8.** Competitive adsorption of Lyz with other proteins. Experimental conditions: 1.0 mL of 0.4 mg/mL proteins, incubated with 20.0 mg of the polymer particles for 12 h at room temperature.
for the template protein would be suppressed in the presence of competing proteins [18].

By contrast, the four proteins in mixture were adsorbed by Fe3O4@NIP and there was not so much discrimination against the proteins. Fe3O4@NIP did not have high adsorption ability for the four proteins, which was consistent with the single adsorption data. However, the amount of Lyz bound to Fe3O4@Lyz-MIP was much larger than that of Fe3O4@NIP with the imprinting factor of 1.93, closed to the value in the single adsorption data. These results illustrated that Fe3O4@Lyz-MIP showed high selectivity to the template Lyz in the presence of the other three protein types and was imparted into the molecular recognition property, which can be used to selectively separate Lyz from mixture of proteins. The values of α and β were calculated as presented earlier and were tabulated in Table 2.

### 3.4. Application of Fe3O4@Lyz-MIP for Lyz separation from egg white and PEG/sulphate aqueous two-phase for Lyz elution

To further demonstrate the applicability and separation effectiveness of Fe3O4@Lyz-MIP in real sample, chicken egg white, in which the concentration of Lyz is ∼3.5% from all proteins [30], was used as a Lyz source. The chromatograms of standard Lyz and a 20-fold diluted egg white were shown in Fig. 9a and b. When Lyz (peak 1) in 20-fold diluted egg white was separated by Fe3O4@Lyz-MIP using an external magnetic field, the chromatogram of supernatant showed that it (peak 1) decreased obviously in Fig. 9c. After Fe3O4@Lyz-MIP was treated with PBS (10 mmol/L, pH 7.0) to wash out the nonspecifically adsorbed proteins, 15%PEG 4000/10% (NH4)2SO4 was added to elute the specifically adsorbed Lyz. PEG/Na2SO4 aqueous two-phase caused Lyz redistribution in bottom phase (Fig. 9d) and top phase (Fig. 9e). The peak with a retention time of about 8 min in bottom phase and top phase was in accordance with Lyz standard (peak 1) in Fig. 9a. The results manifested that 15%PEG 4000/10% sulphate can elute the target protein from the polymer. It might be because that hydrogen bonding interaction between target protein and binding sites was disrupted by strong ionic formation. Further, the aqueous two-phase extraction brought the peak area of Lyz in bottom phase and top phase to be enlarged, which should attribute to its enrichment ability [26]. All other proteins component (peak 2) in the egg white sample, such as ovalbumin, ovotransferrin and ovomucoid were not found in the bottom phase or top phase, which meant they did not interfere with the binding of Lyz to Fe3O4@Lyz-MIP, and also prepared Fe3O4@Lyz-MIP having high selectivity toward the target protein in real sample has been validated. This molecularly imprinted polymer with superparamagnetic property could rapidly and easily separate target protein from complex biological samples without tedious sample treatment procedures, and would be useful for its potential in practical applications.

### 4. Conclusions

Lyz surface-imprinted polymer grafting on Fe3O4 magnetic particles has been successfully synthesized through atomic transfer radical polymerization in aqueous media. The novel fabricating protocol possesses the mild reaction conditions without heating and ultraviolet radiations and avoids the adverse reactions comparing with traditionally initiated radical polymerization. The obtained imprinted polymer was endowed higher specific recognition and selectivity to template Lyz when it was in the mixture of standard proteins and real samples. The magnetic supports coated by imprinted polymer have superparamagnetic susceptibility, which can make the separation process be completed in a short time period by using an external magnetic field. Certainly, according to the preparation procedure, other supports such as silica particles and polystyrene particles, can also be used in the preparation of protein surface-imprinted polymer. So, the new imprinting approach described above will provide new opportunities for template proteins imprinting. For target protein elution from imprinted polymer, the aqueous two-phase solution provided a potential of both elution and enrichment for target protein. This work is the combination of affinity separation, magnetic separation, solid–liquid extraction and liquid–liquid extraction, and will be also an application of the integration of superparamagnetic imprinted polymer with aqueous two-phase solution for separation and enrichment of low abundance proteins in proteomics.

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