Surface-modified polystyrene beads as photografting imprinted polymer matrix for chromatographic separation of proteins

Lei Qin a, Xi-Wen He a, Wei Zhang a, Wen-You Li a,*, Yu-Kui Zhang a,b,∗∗

a Department of Chemistry, Nankai University, Tianjin 300071, China
b National Chromatographic Research and Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, China

1. Introduction

Molecular imprinting has been proved to be an important tool for the rapid fabrication of organic polymeric and inorganic network-structured materials that selectively bind a template molecule [1,2]. Commonly this technique has demonstrated great potential when targeting small molecules of molecular weight <1500, while extending the technique to biological macromolecules such as proteins, whole cells and viruses has been proven difficult. When imprinting proteins, there are a number of inherent problems to address that are related to the molecular size, complexity, conformational flexibility, solubility and sensitivity to environment [3].

Though protein imprinting is proved to be one of the most challenging tasks, the field is progressing rapidly. The first success in protein imprinting was achieved by the group of Gald et al. [4]. A number of molecular imprinting polymers (MIPs) selective for proteins have now been reported using methodologies including sol–gel [5,6], metal chelating [7,8], hybrid-DNA [3], epitope approach [9,10] and acrylate chemistry [11–14] through three-dimensional [5,11] or two-dimensional [14–18] imprinting. Recently, several new methods have been adopted to prepare protein MIPs [19] and accelerated the process of protein molecular imprinting technology. Among the above methods, polyacrylamide imprinting of proteins has been proven useful for the separation of proteins in chromatographic mode [11,20]. However, so far, the protein imprinting materials used as stationary phase of HPLC to separate proteins are rare [15].

Surface grafting on supports has been recently proposed as a new surface imprinting technology for the synthesis of molecularly imprinted polymer with a particular morphology and better accessibility to the specific binding sites [21]. Unfortunately, solution polymerization and resulting gelation are difficult to be avoided in traditional surface initiated radical polymerization. The extra solution polymer would lead to the heterogeneous recognition sites and peak tailing when used as stationary phase [22]. One solution to this shortcoming is to use the iniferter (initiator transfer agent terminator) which is one kind of surface initiated living-radical polymerization (SIP) initiators. The iniferter traditionally has been used in a dissolved form, but here polymerization in solution could be avoided by attaching the iniferter on support surface before it decomposes into active radical and non-active radical, where

∗ Corresponding author at: Department of Chemistry, Nankai University, Tianjin 300071, China. Tel.: +86 22 2349 4962; fax: +86 22 2350 2458.
** Corresponding author. Tel.: +86 411 84379560; fax: +86 411 84379560.
E-mail addresses: wyli@nankai.edu.cn (W.-Y. Li), ykzhang@dicp.ac.cn (Y.-K. Zhang).
the active radical bound to the support surface initiates polymerization while the non-active radical in solution mainly reacts with growing radical to form a “dormant species”. Compared with the traditional radical polymerization, this polymerization process can be well controlled by iniferter due to the avoidance of the adverse reactions such as radical coupling or disproportionation action [23]. Iniferter was also found important applications in the manufacturing of micropatterned or biocompatible surfaces [23]. However, the use of this concept for the preparation of protein molecularly imprinted polymer has no precedent.

Lysozyme is considered as an important index in the diagnosis of various diseases including tuberculosis and fungal meningitis. In this paper, lysozyme was chosen as template protein and mesoporous chloromethylated polystyrene (MCP) containing iniferter was used as a rigid support for grafting the polyacrylamide gel to produce lysozyme-MIP beads. The lysozyme-MIP beads were applied to separate lysozyme from competitive proteins only using environment-friendly aqueous mobile phase.

2. Experimental

2.1. Materials

Bovine hemoglobin (Hb, pl (isoelectric point) 6.9, MW 66.0 kDa) and cytochrome c (from bovine heart, pl 9.8, MW 12.3 kDa) were obtained from Sigma (St Louis, MO, USA). Lysozyme (pl 11.2, MW 13.4 kDa), bovine serum albumin (BSA, pl 4.9, MW 68.0 kDa) and ovalbumin (pl 4.7, MW 43.0 kDa) were purchased from DingGuo Biotech (Beijing, China). MCP beads (40–50 µm) with chlorine substitution of 2.8 mmol/g were purchased from Nankai Hecheng S&T (Tianjin, China). Acrylamide (AAm), N,N′-methylenebisacrylamide (MBAA), potassium persulfate (KPS) and hydrogen sulfite sodium (NaHSO3) were purchased from KeMiOu Chemical (Tianjin, China). Diethyldithiocarbamate sodium (DEDTC) and sodium dodecyl sulfate (SDS) were purchased from Institute of GuangFu Fine Chemicals (Tianjin, China). Phosphate buffer solution (PBS, 10 mM Na2HPO4 and 10 mM NaH2PO4, pH 6.2) was used as working medium. The
other chemicals and solvents were of analytical grade or better.

2.2. Preparation of MIP beads

2.2.1. Modification of MCP with iniferter

The iniferter was introduced by the reaction of the MCP-bound chloromethyl group with DEDTC. Briefly, 1.36 g of DEDTC was dissolved in 12 ml of dry ethanol, and then it was dropped into dry ethanol (20 ml) containing MCP beads (4.5 g). The resulting suspension was heated at 60 °C under stirring for 12, 24, 36 and 48 h, respectively. The MCP beads modified with iniferter (modified-MCP beads) were successively washed with double distilled water and methanol, and then dried at 40 °C under vacuum overnight.

2.2.2. Preparation of lysozyme-MIP beads

The modified-MCP beads were subsequently used for the photografting of imprinting polymer films. The general preparing procedure was depicted as Fig. 1. 2.0 g of modified-MCP beads was suspended to the prepolymerization mixture consisting of lysozyme (90 mg), AAm (2.8 g) and MBAA (0.30 g) in 40 ml of PBS (10 mM, pH 6.2). Thereafter, the mixture was purged with nitrogen and then sealed. Polymerization reaction was initiated by ultraviolet irradiation in an ice water bath. The subsequent grafting polymerization was performed for certain hours to control the thickness of the polymer films. After the polymerization, the lysozyme-MIP beads were washed by repeated centrifugation and re-suspension with double distilled water, solution of acetic acid (10%, v/v) containing SDS (10%, w/v) and double distilled water to elute the template, and then dried prior to be packed into the HPLC column. The traditional lysozyme MIP (lysozyme-MIPt) was prepared using the same prepolymerization recipe as that of the MIP beads but without the addition of the template.

2.3. Characterization techniques

Fourier transform infrared (FT-IR) spectra of MCP beads, modified-MCP beads and lysozyme-MIP beads were recorded with an Avatar 360 instrument (Nicolet, Waltham, MA, USA) in the range of 4000–500 cm⁻¹ using KBr pellets.

Elemental analyses were performed with Vario EL elemental analyzer (Elementar, Hanau, Germany) after carefully drying of the samples.

Nitrogen adsorption–desorption analysis was done at 77 K on a Micromeritics TriStar 3000 porosimeter (Norcross, GA, USA). Prior to the measurements, 100–150 mg of the samples was heated at 60 °C for at least 10 h. The surface areas (S) were evaluated using the BET method. The total pore volumes (Vp) and the average pore diameter (dp) were evaluated using the BJH theory.

Scanning electron microscopy (SEM) of the imprinted beads was studied with a Quanta 200 (FEI, Eindhoven, The Netherlands). The particle size distribution was reflected by SEM. The averages were calculated by the following formula [26]:

\[
D_n = \frac{\sum_{i=1}^{k} n_i D_i}{\sum_{i=1}^{k} n_i}
\]

where \( D_n \) is the number-average diameter, \( D_i \) is the weight-average diameter, \( N \) is the total number of the measured particles, and \( n_i \) is the particle diameters of the determined microspheres. When the value of \( U \) ranges between 1.0 and 1.1, it means the polymer microspheres are mono-dispersed. The value of \( U \) is much closer to 1.0, the mono-dispersity of the polymer microspheres much better.

2.3.1. Film thickness calculations

The calculation of the film thickness \( d \) (nm) was performed as follows [25]

\[
d = \frac{mN MW}{\rho S D_n} \times 10^3
\]

where \( mN \) is the weight of nitrogen of the grafted polymer per gram of MCP beads, \( MW \) is the average molecular weight of the grafted polymer assuming stoichiometric incorporation of reactive monomer, \( M_N \) is the average molecular weight of the nitrogen fraction of the grafted polymer, \( \rho \) (g/ml) is the average density of monomers, and \( S \) (m²/g) is the specific surface area of the MCP beads.

2.3.2. HPLC measurements and evaluation

The HPLC measurements were carried out on a Shimadzu LC-20AD (Kyoto, Japan). The lysozyme imprinting and non-imprinting beads were packed into stainless steel columns (100 mm × 4.6 mm i.d.), respectively. 100 μl of 1 mg/ml solution of single protein or a protein mixture was injected. The protein mixture contained lysozyme and one of the competitive proteins (Hb, BSA, ovalbumin or cytochrome c).

![Fig. 2. SEM microphotographs of the lysozyme-MIP beads.](image-url)
The capacity factors ($k_t$ and $k_c$) and the separation factor ($\alpha$) were calculated using the following formulas:

$$k_t = \frac{(t_t - t_0)}{t_0}, \quad k_c = \frac{(t_c - t_0)}{t_0}, \quad \alpha = k_t/k_c,$$

where $t_t$ and $t_c$ are the retention times of the template protein and the competitive protein, respectively, and $t_0$ is the retention time of the void marker, thiourea.

The chromatographic imprinting factor ($CIF$) for the template is calculated from the capacity factors obtained on MIP column and NIP column ($CIF = k_{MIP}/k_{NIP}$).

The resolution ($R_h$) is calculated by the following equation:

$$R_h = \frac{2(W_t - W_c)}{W_t + W_c}$$

where $W_t$ and $W_c$ are the peak widths of the template and the competitive protein, respectively.

### 3. Results and discussion

#### 3.1. The advantage of using iniferter

An advantage of using immobilized iniferter is the stability of the dithiocarbamate radical. Since the dithiocarbamate radical is unlikely to initiate new chains, propagation in solution is minimal. The effect of the SIP on the imprinting beads was well-illustrated in SEM of the lysozyme-MIP beads (Fig. 2). The grafting polymer using SIP appeared homogeneous with no agglomeration and no visible non-grafted polymer in the polymerization solution. The polydispersity index ($U$) of 1.036 was calculated from Fig. 2, which indicated that the lysozyme-MIP beads were of mono-dispersion size. Besides, the pore size distribution of the lysozyme-MIP beads was homogeneous (Fig. 3) through the SIP.

Moreover, the iniferter allowed an efficient control of the grafting process, so the film thickness was tunable by controlling the ultraviolet irradiation time (Table 1). The thickness of the film affected the chromatographic selectivity of the MIP column, which will be discussed below. Of particular interest was the use of a hydrophobic MCP bead surface to graft a hydrophilic polyacrylamide imprinted polymer. Some reports have indicated that such interfaces can stabilize imprinted site, even when using polar, water-containing monomer/template systems [27].

#### 3.2. Modification time of MCP beads with iniferter

In the grafting of polymers from the modified-MCP beads, the density of initiator groups is an important factor controlling the density and homogeneity of the grafted layer. The reaction time of the chloromethyl groups with DEDTC could influence the density of initiator groups (Table 2). It can be seen from Table 2 that the %N of modified-MCP beads increased with the increase of the reaction time. When the reaction time was over 24 h, the %N of modified-MCP beads increased slowly. So the time chosen as the reaction time of modification was 24 h.

#### 3.3. Characteristic studies

The IR spectra of MCP beads, modified-MCP beads, and lysozyme-MIP beads were shown in Fig. 4. It was notable that the modified-MCP beads showed new peaks at 1414.44, 1531.30 and 1205.11 cm$^{-1}$ corresponding to thiocarbonyl group of the iniferter, which suggested that the iniferter had been modified to the MCP beads. The presence of the iniferter was also checked by elemental analysis (Table 2, reduction of %C and appearance of %N). The strong peak at 1654.56 cm$^{-1}$ of lysozyme-MIP beads was attributed to the carbonyl groups of the polyacrylamide. The IR results indicated that imprinted polymers have been grafted from the modified-MCP beads. It was further confirmed by elemental analysis of the beads before (C% = 76.8%, N% = 1.65%) and after (C% = 60.5%, N% = 5.91%) polymerization.

The MCP beads were mesoporous as judged from the nitrogen sorption data ($S = 59.2 \text{ m}^2/\text{g}$; $d_p = 28.5 \text{ nm}$; $V_p = 0.377 \text{ ml/g}$). Specific surface area, total pore volume and average pore diameter of lysozyme-MIP beads and NIP beads were different (Table 1). The pore diameter of lysozyme-MIP beads (30.2 nm) was possibly in the range of diffusion of lysozyme molecule whose molecular size is close to that of thiourea.

### Table 1

<table>
<thead>
<tr>
<th>Bead type</th>
<th>$S$ (m$^2$/g)</th>
<th>$V_p$ (ml/g)</th>
<th>$d_p$ (nm)</th>
<th>$d^s$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP beads</td>
<td>59.2</td>
<td>0.377</td>
<td>28.5</td>
<td></td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>39.3</td>
<td>0.343</td>
<td>32.2</td>
<td></td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>41.3</td>
<td>0.332</td>
<td>32.0</td>
<td>4.70</td>
</tr>
<tr>
<td>Lysozyme-MIP beads$^{abc}$</td>
<td>39.7</td>
<td>0.255</td>
<td>30.2</td>
<td>5.62</td>
</tr>
<tr>
<td>Lysozyme-MIP beads$^{abc}$</td>
<td>33.8</td>
<td>0.217</td>
<td>29.0</td>
<td>7.10</td>
</tr>
<tr>
<td>NIP beads</td>
<td>28.4</td>
<td>0.198</td>
<td>20.6</td>
<td></td>
</tr>
</tbody>
</table>

- $^a$ The film thickness ($d$) was calculated from formula.
- $^b$ It is the reaction time of the chloromethyl groups with DEDTC.
- $^c$ It is the reaction time of the polymerization under ultraviolet irradiation.

### Table 2

<table>
<thead>
<tr>
<th>Bead type</th>
<th>%C</th>
<th>%N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP beads</td>
<td>79.9</td>
<td>0.00</td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>78.5</td>
<td>1.39</td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>76.8</td>
<td>1.65</td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>76.0</td>
<td>1.68</td>
</tr>
<tr>
<td>Modified MCP beads$^{abc}$</td>
<td>74.3</td>
<td>1.70</td>
</tr>
<tr>
<td>Lysozyme-MIP beads$^{abc}$</td>
<td>60.5</td>
<td>2.91</td>
</tr>
</tbody>
</table>

* It is the reaction time of the chloromethyl groups with DEDTC.
* It is the reaction time of the polymerization under ultraviolet irradiation.
3.0 nm $\times$ 3.0 nm $\times$ 4.5 nm [28]. On the basis of the data, it was concluded that lysozyme-MIP beads have effective pore volume and dimensions for lysozyme molecules.

The surface morphology of the beads was observed by SEM (Fig. 5). From Fig. 5a, it can be seen that the surface of modified-MCP beads was rugged after being modified with the iniferter. In the SEM photograph of lysozyme-MIP beads (Fig. 5b), a large quantity of well-distributed pores could be observed and they were netlike. This porous structure on the surface of lysozyme-MIP beads facilitated the mass transfer rate of releasing and rebinding template protein. Due to the imprinting effect, the surface morphology of lysozyme-MIP beads and NIP beads were different (Fig. 5b and c), which was consistent with nitrogen sorption data (Table 1). However, in the case of lysozyme-MIPt beads (Fig. 5d), the grafting imprinted polymer was not homogeneous, which was the shortcoming of the traditional polymerization.

3.4. Chromatographic analysis

3.4.1. Imprinting effect of lysozyme-MIP beads

The retention behavior of lysozyme on lysozyme-MIP column, lysozyme-MIPt column and the corresponding NIP column were analyzed in the same condition (Fig. 6). It can be found that the retention capacity of lysozyme-MIP column to the template was higher than that of NIP column (Table 3). The lysozyme-MIP column had a good imprinting effect (CIF = 12.8). These data indicated that molecular imprinting process had resulted in the formation and preservation of a microenvironment based on shape memory size and positions of functional groups of the template on the surface of the lysozyme-MIP beads [29]. For NIP beads, however, the non-specific adsorption had dominant effect due to the lack of imprinting process and there were no suitable cavities for the recognition of lysozyme.

Although the lysozyme-MIPt column had imprinting effect in some degree with the CIF of 2.64 (Fig. 6c), it was not better than lysozyme-MIP column (Fig. 6a and b). And both the lysozyme-MIPt column and NIPt column showed peaks tailing in binding the template. Lysozyme-MIP beads provided better chromatographic property mainly due to the narrow size distributions and homogeneous pore size.

3.4.2. Recognition specificity of the imprinting column

The selectivity of the imprinting column was tested by using Hb, BSA, ovalbumin or cytochrome c as competitive protein which is different in mass and charge from lysozyme. In the SIP, the grafted layer thickness increased with the increase of the reaction time. After 4 h polymerization, the lysozyme-MIP column had a good resolution (Fig. 7). The lysozyme-MIP beads prepared using shorter polymerization time (2 h) exhibited only partial resolution and weak retention of the template. Longer grafting time (6 h) increased the number of recognition site and resulted in strong retention of the template, but the $R_s$ did not follow this trend. Over thickness of the imprinting layer did not benefit for the mass transfer efficiency of the protein. The capacity factor of lysozyme-MIP column for lysozyme was much higher than that for Hb, BSA, ovalbumin or cytochrome c (Table 3). The lysozyme-MIP column exhibited...
## Table 3
Chromatographic properties of the lysozyme-MIP column and the NIP column to the single protein.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Lysozyme-MIP column</th>
<th>NIP column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention time (min)</td>
<td>Capacity factor (k)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>9.21</td>
<td>1.53</td>
</tr>
<tr>
<td>Hb</td>
<td>3.98</td>
<td>0.09</td>
</tr>
<tr>
<td>BSA</td>
<td>3.88</td>
<td>0.06</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>3.97</td>
<td>0.09</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>4.02</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min.

### Fig. 6
Chromatograms of lysozyme on (a) the lysozyme-MIP column, (b) the NIP column and (c) the lysozyme-MIPt column (… ) and NIP column (—). Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min. Peak identification: (1) lysozyme.

### Fig. 7
Chromatograms of the mixture of lysozyme and cytochrome c on the lysozyme-MIP column prepared in different hours. Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min. Peak identification: (1) lysozyme and (2) cytochrome c.

### Table 4
Chromatographic properties of the lysozyme-MIP column to the protein mixture.

<table>
<thead>
<tr>
<th>Protein mixture</th>
<th>Lysozyme-MIP column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capacity factor (k)</td>
</tr>
<tr>
<td>1</td>
<td>Hb</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
</tr>
<tr>
<td>2</td>
<td>BSA</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
</tr>
<tr>
<td>3</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
</tr>
<tr>
<td>4</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
</tr>
</tbody>
</table>

Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min.

As known, the peptide bonds of the protein could form hydrogen bonds with AAm even in polar solution [30]. Multiple-point hydrogen bonds interaction between the template protein and AAm can form strong interactions [14]. The imprinting column discriminated proteins by the synergistic effects of shape complementarity and multiple weak interactions provided by the functional monomers.

In the cases of Hb, BSA or ovalbumin, the molecular volume was larger than that of lysozyme so that they had less chance to entirely slip into the imprinting cavities created by lysozyme and to interact good recognition for template from a protein mixture (Table 4 and Fig. 8). Concerning lysozyme-MIPt column, no selectivity could be demonstrated for the template.
Fig. 8. Chromatograms of the mixture of lysozyme with one of Hb, BSA, ovalbumin or cytochrome c on the lysozyme-MIP column. Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min. Peak identification: (1) lysozyme; (2) Hb; (3) BSA; (4) ovalbumin and (5) cytochrome c.

Table 5
Reproducibility of the lysozyme-MIP column for the mixture of lysozyme and cytochrome c.

<table>
<thead>
<tr>
<th>Protein mixture</th>
<th>Resolution ($R_s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First time</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.82</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Column: 100 mm × 4.6 mm (i.d.); sample amount: 100 μl (1 mg/ml); detection wavelength: 280 nm; mobile phase: PBS (10 mM, pH 6.2); flow rate: 0.2 ml/min.

with the functional groups. Therefore, lysozyme-MIP column could separate the mixture of lysozyme with one of Hb, BSA or ovalbumin. The molecular volume and pI of cytochrome c and lysozyme are quite similar. Though cytochrome c was small enough to get into the imprinting cavities, the recognition sites of the imprinting cavities were not complementary to the cytochrome c. Consequently, the cytochrome c could not form entirely multiple weak interactions with the functional groups, so the retention time of cytochrome c was shorter than that of the template protein. Concerning the imprinted composites, the high selectivity to lysozyme was successfully demonstrated.

In contrast, for NIP column, it was difficult to form multiple hydrogen bonds and strong interaction between the template and NIP-beads, which led to the weak retention capacity of lysozyme, and so as for the competitive proteins. The NIP column did not display any protein recognition ability and retained the five kinds of proteins nearly close to the void fraction (Table 3).

3.5. Reusability and stability of lysozyme-MIP column

Using the mixture of lysozyme and cytochrome c as example, five times of selective separation experiments were performed on the same lysozyme-MIP column (Table 5). The results showed that the lysozyme-MIP column was very stable and maintained the separation capacity at almost constant value.

4. Conclusions

In the present study, this new approach significantly broadens the scope of protein imprinting through surface initiated living-radical polymerization and demonstrates that iniferter-modified support materials can be used to prepare protein molecularly imprinted polymer. It is a new stationary phase for the separation of bioactive compounds only using aqueous mobile phase. MIP beads prepared by SIP exhibited a better imprinting effect than traditional MIP beads prepared by traditionally initiated radical polymerization. Through using SIP, the thickness of imprinting polymer layer could be rationally controlled to obtain good selectivity. The lysozyme-MIP column exhibited a pronounced imprinting effect and was capable of separating the template from competitive proteins, whereas the NIP column had no selective properties.

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