Novel surface modified molecularly imprinted polymer using acryloyl-β-cyclodextrin and acrylamide as monomers for selective recognition of lysozyme in aqueous solution

Wei Zhang, Lei Qin, Xi-Wen He, Wen-You Li, Yu-Kui Zhang

A novel protein imprinted polymer for selective recognition of lysozyme was obtained. Acryloyl-β-cyclodextrin, which offered hydrophilic exterior and hydrophobic cavity that were allowed to self-assemble with the template protein through hydrogen interaction and hydrophobic interaction, was synthesized and used as the functional monomer. Polymerization was carried out in the presence of acrylamide as an assistant monomer, which resulted in a new type of protein imprinted polymer. Langmuir adsorption model was employed to describe the isotherms, and maximum adsorption capacity was evaluated. The performance of such imprinted polymer was further demonstrated by high-performance liquid chromatography, and the results showed that the column packed with the lysozyme imprinted silica beads could effectively separate lysozyme from the mixture of lysozyme–cytochrome c, lysozyme–bovine serum albumin, lysozyme–avidin or lysozyme–methylated bovine serum albumin, which showed its high selectivity.
gel used in our previous work was replaced by the silica beads, and the spherical beads are preferred over irregular particles according to the standard chromatogram theory. The strategy of the present work was further developed by the preparation of protein molecularly imprinted polymer using acryloyl-β-CD and AA as monomers, so that the MIP was targeted to recognize the sequence of protein, or to recognize the shape of protein [27]. As shown in Fig. 1, the large number of possible bonds can be created between the protein and the functional monomers, and the spatial arrangement of the complementary functional entities of the network, together with the shape image correspond to the imprinted molecules. To the best of our knowledge, no reports to date have been published on the use of acryloyl-β-CD together with the conventional monomer AA for protein imprinting.

In this work, lysozyme (Lyz), as the template protein, was covalently immobilized on the surface of silica beads, and the MIP was formed on the surface of silica beads. And when the template protein was removed, complementary binding sites were thus created on the surface of silica beads. To test the selectivity of imprinted particles, a series of adsorption studies were conducted and chromatographic method was chosen because of its convectively confirming imprinting effect. The results demonstrated that the MIP was capable of selective recognition of the template protein.
2. Experimental

2.1. Materials

Bovine serum albumin (BSA, molecular weight (MW) 67 kDa, isoelectric point (pI) 4.9), cytochrome c (Cyt, MW 12.4 kDa, pI 10.2) and lysozyme (Lyz, MW 14.4 kDa, pI 11) used in this study were purchased from Lanji of Shanghai Science and Technology Development Company (Shanghai, China). Methylated bovine serum albumin (MBSA, MW 68 kDa, pI 8.5) and avidin (Avi, MW 66 kDa, pI 10) were obtained from Sigma–Aldrich Co. (St. Louis, USA). Silica beads (40–60 μm, Tianjin Chromatography Technology Company, Tianjin, China) were activated with acid before being silanized. 3-Methacryloyloxypropyl trimethoxysilane (WD-70) and 3-aminopropyl trimethoxysilane (WD-56) were purchased from Chemical Factory of Wuhan University (Wuhan, China). β-Cyclodextrin (β-CD) was from the Institute of Tianjin JingKe Fine Chemicals (Tianjin, China). AA and N,N-methylenebisacrylamide (MBAA) were purchased from Chemistry Reagent Factory of Chinese Qianjin (Tianjin, China). Potassium persulfate, m-nitrophenol, acetic acid (HAC) and sodium dodecyl sulfate (SDS) were obtained from the Institute of Tianjin Guangfu Fine Chemicals (Tianjin, China).

2.2. Synthesis of the acryloyl-β-cyclodextrin

The acryloyl-β-cyclodextrin was synthesized as described by the group of Shun-ichi Nozakura and Makoto Komiyama [28,29]. Briefly, it was synthesized through the ester-exchange reaction of m-nitrophenyl acrylate with β-CD in water, and the m-nitrophenyl acrylate was synthesized by using acryoyl chloride and m-nitrophenol.

2.3. Introduction of vinyl and amido groups on the surface of silica beads

Dried silica beads (10 g) were dispersed in toluene (100 ml) in a three-necked round-bottomed flask equipped with a magnetic stirrer, a reflux condenser and a nitrogen gas inlet, and then 10 ml of silane coupling agents (VWD 70: VWD 56 = 1:1) was added. The mixture was stirred and purged with nitrogen at 70 °C for 12 h. Then the silica beads were filtered out and washed successively with toluene, acetone and ether. The modified silica beads (Fig. 1) were dried under vacuum at 70 °C for 6 h.

2.4. Aldehyde functionalization

The silanization modified silica beads (5 g) prepared above were soaked in 20 ml phosphate buffer solution (PBS, pH 6.2, 0.01 mol/l) containing glutaraldehyde with concentration of 0.2 mol/l. This mixture was stirred at room temperature for 12 h. The resulting silica beads (Fig. 1) were washed with doubly distilled water repeatedly.

2.5. Preparation of imprinted and non-imprinted polymers

Lyz (80 mg) was added to a 10-ml centrifuge tube containing 5 ml of buffer (PBS, pH 7.0, 0.01 mol/l) and 1.0 g aldehyde-modified silica beads, and the mixture was incubated for 1 h at room temperature with the aim to covalently bind the protein. Then 200 mg of acryloyl-β-CD, 400 mg of AA and 20 mg of MBAA were added to this solution, which was incubated 3 h under shaking for pre-polymerization. After the mixture was purged with nitrogen to remove oxygen, the polymerization was initiated by addition of 30 mg of potassium persulfate and 40 mg of sodium hydrogen sulfite. Polymerization was continued for 24 h and the MIP was obtained (Fig. 1). To remove the template Lyz, the obtained MIP was washed with 10% (v/v) SDS-5% (v/v) HAc containing 5% (w/v) oxalic acid [12,14], ethanol, and doubly distilled water in order.

The non-imprinted polymer (NIP) was prepared using the same procedure, but without addition of template molecule. The NIP was washed in a similar manner as the MIP.

2.6. Characterization of particles

UV-2450 UV–vis spectrophotometer was from Shimadzu (Kyoto, Japan). The surface morphology of the particles was studied using a Quanta 200 scanning electron microscope (FEI, Eindhoven, The Netherlands). Vario EL elemental analyzer (Elementar, Hanau, Germany) was employed to investigate the surface elemental composition of the particles. Nitrogen adsorption–desorption analysis was carried out at 77 K on a Micromeritics TriStar 3000 porosimeter (Norcross, GA, USA).

2.7. Adsorption experiments

The adsorption experiments involve adsorption dynamic, specific adsorption and adsorption isotherm. In these experiments, a unit mass of polymer was dispersed in certain volume of protein solution. The mixtures were agitated in a shaken bed. At different time intervals, the mixtures were centrifuged and the supernatant solutions were collected, concentrations of which were determined using the UV–vis spectrophotometer. The amount of protein adsorbed onto the polymer was determined according to the following formula:

\[ Q = \left( C_0 - C_\infty \right) V / m \]

where \( C_0 \) (mg/ml) and \( C_\infty \) (mg/ml) represent the initial and final protein solution concentration, respectively. \( V \) (ml) is the sample’s volume and \( m \) (g) is the mass of the polymer. The tests were carried out in triplicate.

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

\[ \alpha = Q_{\text{MIP}} / Q_{\text{NIP}} \]

where \( Q_{\text{MIP}} \) and \( Q_{\text{NIP}} \) are the adsorption capacity of the template or the analogue on MIP and NIP, respectively.

The selectivity factor (β) is defined as

\[ \beta = \alpha_{\text{tem}} / \alpha_{\text{ana}} \]

where \( \alpha_{\text{tem}} \) is the imprinting factor toward the template molecule and \( \alpha_{\text{ana}} \) is the imprinting factor toward the analogue.

2.8. Chromatographic measurement

HPLC measurement was carried out with the LC-20AD solution system from Shimadzu (Kyoto, Japan). HPLC conditions employed for this work were as follows: mobile phase, 0.3 mol/l NaCl solution; 20 μl of 1.0 mg/ml of analytes in PBS (0.01 mol/l, pH 7.0) were injected for analysis; flow rate, 0.1 ml/min; room temperature; UV detection, at 278 nm.

3. Results and discussion

3.1. Characterization studies

3.1.1. Scanning electron micrograph

SEM was employed to capture the detailed morphology of the silica beads, the modified silica beads, the Lyz-MIP beads and the NIP beads (Fig. 2). Silica beads with a highly rigid matrix.
and hydrophilic surface were ideal support for imprinting various organic or biological molecules. In this work, silica beads were obtained from commercial sources, and the active hydroxyl groups on silica beads were much less. To meet the reaction demand, silica beads were activated with acid before being silanized, which showed spherical shapes and smooth surface (Fig. 2a and b). After the silica beads were modified with silane coupling agents, it exhibited a rough surface and many micropores distributed on the support (Fig. 2c). As can be seen in Fig. 2d that after the support was treated with glutaraldehyde, the pores in Fig. 2c were disappeared and many small particles were suited on the surface of the support. The morphological feature of the MIP beads showed some macropores (Fig. 2e), which benefited for the adsorption of template protein. The image of the NIP beads (Fig. 2f) showed a rough surface containing many micropores that were small and could not be seen clearly.

### 3.1.2. Elemental analysis

The silica beads were modified with a series of chemical reagents. To ascertain each modification, elemental analysis was employed. The results were shown in Table 1. After the silanization treatment, the nitrogen atomic composition was from 0.00 to 0.56%, which suggested that amine groups were successfully introduced onto the surfaces of the silica beads. After aldehyde treatment, the

<table>
<thead>
<tr>
<th>Bead type</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica beads</td>
<td>1.90</td>
<td>0.32</td>
<td>0</td>
</tr>
<tr>
<td>Silanization treatment</td>
<td>3.51</td>
<td>0.93</td>
<td>0.56</td>
</tr>
<tr>
<td>Aldehyde treatment</td>
<td>4.50</td>
<td>1.57</td>
<td>0.49</td>
</tr>
<tr>
<td>MIP</td>
<td>9.88</td>
<td>1.91</td>
<td>2.08</td>
</tr>
<tr>
<td>NIP</td>
<td>7.93</td>
<td>1.73</td>
<td>1.50</td>
</tr>
</tbody>
</table>
carbon composition was from 3.51 to 4.50%. After polymerization (MIP), it can be seen that the nitrogen composition was from 0.49 to 2.08%. And the nitrogen composition of the NIP was from 0.49 to 1.50%. The increase of nitrogen composition (MIP or NIP) was attributed to the formation of polymer on the surface of the silica beads. It can be seen that the elemental analysis of the MIP was different from that of the NIP. The differences showed that the template Lyz was not able to be completely extracted from the MIP [19]. According to the experimental results, 45.9 mg of template Lyz was immobilized on every gram of the silica beads and 18% of the template Lyz was remained on the packing after washing of the MIP.

### 3.2. Adsorption dynamics of polymers

Adsorption dynamic studies were carried out for the MIP and NIP particles to investigate the adsorption process (Fig. 3). It can be seen from Fig. 3 that specific surface areas, total pore volume and mean diameter of the MIP were not distinctly different from those of the NIP [13]. Therefore, the difference of adsorption between the MIP and NIP in the subsequent study could not be attributed to the morphological difference of the MIP beads and NIP beads, but to the imprinting effect.

### 3.3. Adsorption isotherm

The porosity and surface area of the polymers were determined by nitrogen sorption measurements (Table 2). It can be seen from Table 2 that specific surface areas, total pore volume and mean diameter of the MIP were not distinctly different from those of the NIP [13]. Therefore, the difference of adsorption between the MIP and NIP in the subsequent study could not be attributed to the morphological difference of the MIP beads and NIP beads, but to the imprinting effect.

### 3.4. Adsorption specificity

To investigate the binding specificity property of the polymers, this experiment was carried out for the binding of Cyt, BSA, MBSA, Avi or Lyz (Fig. 6 and Table 4). The MIP and the NIP were exposed to the solution of Cyt, BSA, MBSA, Avi or Lyz.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Specific surface area (m²/g)</th>
<th>Total pore volume (cm³/g)</th>
<th>Mean diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz-MIP</td>
<td>72.91</td>
<td>0.22</td>
<td>12.11</td>
</tr>
<tr>
<td>NIP</td>
<td>87.21</td>
<td>0.28</td>
<td>13.03</td>
</tr>
</tbody>
</table>

### Table 2

Comparison of MIP and NIP from nitrogen sorption measurements.

Fig. 3. Adsorption dynamic of Lyz on MIP (■) and NIP (▲). Experimental conditions: $V = 5.0$ ml; $C = 0.5$ mg/ml; the mass of polymer: 50.0 mg.

Fig. 4. Adsorption isotherm of Lyz on MIP (■) and NIP (▲). Experimental conditions: $V = 5.0$ ml; $C = 0.1–0.8$ mg/ml; the mass of polymer: 50.0 mg; adsorption time: 14 h.

### Table 3

Theoretical maximum adsorption capacity ($Q_{\text{max}}$) and Langmuir adsorption equilibrium constant ($b$) from the Langmuir model.

<table>
<thead>
<tr>
<th>Model</th>
<th>Samples</th>
<th>$Q_{\text{max}}$ (mg/g)</th>
<th>$b$ (ml/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Langmuir</td>
<td>MIP</td>
<td>76.7</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>NIP</td>
<td>10.7</td>
<td>11.6</td>
</tr>
</tbody>
</table>
Fig. 5. Adsorption isotherm of Lyz on MIP (■) and NIP (▲), linearized according to the Langmuir model.

Fig. 6. Selective adsorption of Cyt, BSA, MBSA, Avi or Lyz on the MIP and NIP. Experimental conditions: \( V = 5.0 \text{ ml} \); the concentration of each protein solution was 0.5 mg/ml; the mass of polymer: 50.0 mg; adsorption time: 14 h.

It can be seen from Fig. 6 and Table 4 that the Lyz-MIP exhibited good adsorption selectivity for the protein template. The adsorption capacity of Lyz-MIP binding Lyz was much higher than that of Cyt. In binding process, many specific recognition sites respect to template protein were generated on the surface of MIP, so the template protein was strongly bound to the polymer. As the competitive protein, although Cyt was small enough to get into the imprinting cavities, the recognition sites of the imprinting cavities were not complementary to the Cyt, so it had less chance to be adsorbed on the Lyz-MIP. For the proteins of BSA, MBSA and Avi, the molecular volumes of the proteins were larger than that of the Lyz, the imprinting cavities were not complementary to them. So the adsorption capacity of the MIP to the three proteins was much lower than that of template Lyz. In contrast, the NIP adsorbed template much less than that of MIP since NIP had not generated specific recognition sites due to the absence of template protein. Therefore, the physical adsorption was the primary factor for the NIP.

Table 4

<table>
<thead>
<tr>
<th>Proteins</th>
<th>( Q_{MIP} ) (mg/g)</th>
<th>( Q_{NIP} ) (mg/g)</th>
<th>( \alpha )</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz</td>
<td>44.6</td>
<td>9.80</td>
<td>4.55</td>
<td>2.22</td>
</tr>
<tr>
<td>Cyt</td>
<td>10.8</td>
<td>9.60</td>
<td>1.12</td>
<td>0.92</td>
</tr>
<tr>
<td>BSA</td>
<td>8.47</td>
<td>7.68</td>
<td>1.10</td>
<td>1.07</td>
</tr>
<tr>
<td>Avi</td>
<td>8.77</td>
<td>8.22</td>
<td>1.08</td>
<td>4.25</td>
</tr>
<tr>
<td>MBSA</td>
<td>11.4</td>
<td>10.5</td>
<td>1.09</td>
<td>4.17</td>
</tr>
</tbody>
</table>

3.5. Chromatographic analysis

3.5.1. Imprinting effect of the MIP

In the HPLC studies, the prepared MIP and NIP were packed into stainless steel column (100 mm × 4.6 mm I.D.) to evaluate their characteristics. The imprinting effect of MIP was investigated and the column packed with NIP was analyzed at the same condition (Fig. 7).

It can be seen from Fig. 7 that the retention time \( t \) was in the order of \( t_{Lyz} > t_{Cyt} > t_{MBSA} > t_{Avi} > t_{BSA} \) on the MIP column (Fig. 7a) and the retention time toward the five proteins was in the same place on the NIP column (Fig. 7b).

3.5.2. Column separation properties

In this test, our aim is to demonstrate the feasibility of the imprinted polymer that can selectively recognize template protein from mixtures (Fig. 8). Binary protein solution of Lyz-Cyt (1.0 mg/ml)-Cyt (1.0 mg/ml)-BSA (1.0 mg/ml)-Avi (1.0 mg/ml) were prepared. 0.3 mol/l NaCl was used as mobile phase and 20.0 \( \mu \)l of protein mixture was applied. The chromatographic separation conditions had been investigated and optimized.

It can be seen from Fig. 8 that the binary protein mixture of Lyz-Cyt, Lyz-BSA, Lyz-Avi or Lyz-MBSA was separated. The imprinting column discriminated proteins by the synergistic effects of shape complementarity and multiple weak interactions (e.g. hydrophobic interaction and hydrogen bond interaction) provided by the functional monomers. In the case of BSA, the molecular volume was larger than that of Lyz so that it had less chance to...
entirely slip into the imprinting cavities of Lyz and to interact with the functional groups. Therefore, Lyz-MIP column could separate the mixture of Lyz and BSA. For the MBSA and Avi, the pI of the two proteins was similar to Lyz, but the molecular volumes of two proteins were larger than that of the Lyz. So the mixture of Lyz-MBSA or Lyz-Avi was separated by imprinting effect. The molecular volume and pI of Cyt and Lyz are quite similar. Though Cyt was small enough to get into the imprinting cavities, the recognition sites of the imprinting cavities were not complementary to the Cyt. Consequently, the Cyt could not form entirely multiple weak interactions with the functional groups, so the retention time of Cyt was shorter than that of the template protein.

4. Conclusion

In summary, a unique kind of protein imprinted polymer was successfully synthesized by using acryloyl-β-CD and AA as monomers, and applied in aqueous media. A series of adsorption experiments of the imprinted polymer showed high selective binding for Lyz. Acryloyl-β-CD and AA cooperated together could improve the selective recognition ability of the MIP. Column packed with the Lyz-MIP can separate Lyz from the mixture of Cyt-Lyz, BSA-Lyz, Lyz-Avi or Lyz-MBSA. The present imprinting protocol is a promising tool for the protein recognition.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 20875049), the National Basic Research Program of China (973 Program) (nos. 2007CB914100 and 2006CB705703).

References