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"Induced fit" recognition of proteins by surface imprinted silica with "soft" recognition sites

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ABSTRACT

Molecular imprinting is a useful method to make enzyme mimics for protein recognition. Classic protein imprinting involves entrapping proteins within polysiloxane or polyacrylamide, but due to the rigidity of the recognition sites and the limited interaction between proteins and small molecule monomers, they often have unsatisfactory capacity, poor reproducibility and low specificity. In this report, "soft" and flexible recognition sites that can allow "induced fit" of the target proteins were created by a novel surface imprinting technique. When about 25% of the template proteins were removed, the unremoved proteins created "soft" and flexible loops that can lock into place upon protein rebinding, which provides additional favorable interactions between the rebind proteins and the imprinted sites. The adsorption capacity of the surface imprinted silica is 24.8×10^{-7} mol/g. The "soft" recognition sites can distinguish target hemoglobin from other proteins such as bovine serum albumin, Cytochrome C and RNase A.

1. Introduction

Molecular imprinting is a well-established and facile technique to produce synthetic polymers bearing recognition sites that can selectively adsorb target molecules [1,2]. The imprinting of small molecules in organic solvents has demonstrated great potential. However, the imprinting of proteins into polysiloxane or polyacrylamide polymers in buffer solutions has suffered from unsatisfactory reproducibility and low specificity. In order to improve the specificity, Wulff and Knorr reported the use of functionalized monomers containing ureas and amidines, which can form stronger bidentate "stoichiometric–non-covalent" hydrogen-bonded complexes with carboxylic and phosphoric acid templates. [3] However, these monomers have apparently not yet been utilized for the imprinting of proteins. An "epitope" approach developed by Rachkov [4] is now attractive, where the template used for polymer development is not the whole protein but a short representative peptide of the protein, just as an epitope represents an antigen in immunology. Through this method, the selective recognition of oxytocin, a nonapeptide, was demonstrated by a polymer imprinted with a tetrapeptide, YPLG [5].

Biorecognition is not a static "lock-and-key" process but involves an "induced fit" interaction. In order to mimic the "induced fit" in molecular imprinting, Umeno used a polymer-coated DNA strand for protein recognition. [6,7] They synthesized poly (*N*-isopropyl acrylamide) terminated with psoralen and combined it with a DNA strand to form a conjugate. The conjugated polymer coating of the strand blocked the proteins trying to interact with the DNA. This blocking effect was then used to imprint the binding site of EcoR1 (a restriction endonuclease) by incubating the DNA with the endonuclease before conjugation with the polymer, which reserved a binding pocket within the flexible DNA strand that is specific for EcoR1. Schrader et al. developed copolymers imprinted with water soluble protein that incorporate a variety of functionalized monomers. [8] The functionality of these monomers was chosen to provide specific interactions with the amino-acid side chains on target proteins. The copolymerization was shown to be statistical—*i.e.*, the different monomers did not form blocks within the polymer. This method is distinct in that the flexible copolymers are not crosslinked upon interaction with the target protein, and the conformations that bind the protein are not locked into place. The copolymers are not truly "imprinted" and the recognition is entirely due to induced fit. In addition, the co-monomers have to be complementary to the surface residues of target protein. So the copolymer is produced by statistical copolymerization of different monomers, such a procedure is difficult to control, and the polymerization procedure must be designed based on different target. In Ying's report, a BSA-imprinted hydrogel may change its conformation to fit BSA when it is approaching [9]. Protein will also adapt to modified conformation of the imprints and finally be recognized. And the recognition between BSA and hydrogel involves induced fit. However, they did not provide any data that related to the selectivity.

To imprint a protein, metal chelating ligands have been used as monomer [10,11]. Liu et al. prepared porcine serum albumin

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(PSA) molecular imprinted polymer (MIP) using metal coordination during surface imprinting. The metal ions served as recognition elements for the rebinding of target proteins together with the imprinted cavities [12]. However, the imprinted site is rigid and heterogeneous and during the recognition the metal ions may leak or cause protein denaturation. Additionally, until now it is regarded that all template proteins should be removed to get more recognition sites. However, some template proteins can be too tightly bound to be removed, and the unremoved proteins may actually have bioactivity, spatial configuration and recognition ability, just like the immobilized proteins in molecular biochromatography [13]. In molecular biochromatography, the proteins are immobilized on the surface of the stationary phase to rapidly probe drug–protein binding based on the biological interactions between the bioactive compounds and the proteins, enzymes and antibodies.

In this work, we create "soft" and flexible recognition sites for the "induced fit" recognition of hemoglobin (Hb) using a new surface imprinting approach. The template Hb was immobilized on the surface of silica by metal coordination. We chose nonporous silica instead of large pore silica as the solid support to ensure that proteins can be immobilized only on the silica surface. In contrast to classic surface imprinting, the template Hb and the coordinated metal ions were removed sub-stoichiometrically after the polymerization. The unremoved proteins together with the imprinted cavities created well-defined "soft" pockets to recognize the target protein *via* "induced fit".

2. Experimental section

2.1. Materials

3-Glycidoxypropyltrimethoxysilane (GLYMO, 97%) was purchased from Acros Organics (Geel, Belgium). Propyltrimethoxysilane (PTMS, 97%), aminopropyltrimethoxysilane (APTMS, 97%) and iminodiacetic acid (IDA, 98%) were supplied by Aladdin (Shanghai, China). Nonporous silica (1.7 μm) was obtained from Nano-Mirco Co. (Jiangsu, China). Hemoglobin (from bovine blood, M_r 64.5, pI 6.8–7.2), bovine serum albumin [Fraction v] (from bovine blood, M_r 66, pI 6.5–7.5), Cytochrome C (from horse heart, M_r 12.7, pI 10), RNase A (from bovine pancreas, M_r 13.7, pI 4.6) were supplied by Sigma (St. Louis, MO, USA). Ethylene diamine tetraacetic acid (EDTA), copper sulfate, phosphoric acid (H_3PO_4) and sodium dihydrogenphosphate (NaH_2PO_4) of analytical grade were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Water used throughout the experiments was produced by a Milli-R04 purification system (Millipore, Germany).

2.2. Instrumentation

A HITACHI S-4800 scanning electron microscope (HITACHI Co., Japan) and a JEOL JEM-1200EX transmission electron microscope (JEOL, Japan) were used to inspect the morphology of bare silica SIS and NIS and Elemental analyses. All chromatographic measurements were performed using a Shimazu Prominence LC-M20A series HPLC (Kyoto, Japan) with an Agilent 300SB-C8 (250 mm × 4.6 mm, 5 μm, 300 Å) column (USA).

2.3. Preparation of hemoglobin surface imprinted silica

GLYMO groups were introduced according to the literature [12]. Dry silica beads (1 g) were added to a mixture of dry toluene (50 mL) and GLYMO silane (2 mL). The mixture was heated to reflux at 110 °C for 17 h, cooled to room temperature, filtered and washed with acetone and ethanol for three times, respectively. To introduce the IDA functional groups, the silica–epoxy (100 mg) was mixed with the IDA solution (0.75 M, 25 mL, containing 0.34 M NaCl and 2 M Na_2CO_3) and the mixture was stirred for 17 h at 70 °C, then $CuSO_4$ (0.1 M, 25 mL) was added to the activated silica (100 mg) to immobilize Cu^{2+} on the silica surface.

After washing the product repeatedly with water, the hemoglobin (Hb) solution (5 mg/mL with 100 mM PBS, 2 mL, pH=8.0) was mixed with the modified silica and oscillated for 15 min at room temperature to form protein-immobilized silica beads by metal coordination. The beads were washed by PBS solution (100 mM, 2 mL, pH=7.0) for three times, then 1 mL PBS solution containing 10 μL PTMS and 10 μL APTMS were added, and the polymerization was carried out at room temperature for 24 h with stirring to form the imprinted layer on the silica surface.

The surface imprinted silica (SIS) was washed by PBS solution for three times to remove the after polymerization. EDTA (100 mM, 25 mL) was added to remove Cu^{2+} by oscillation at room temperature for 12 h. Then formic acid (20%, 25 mL) was added to partially remove the template Hb. The particles were then washed by PBS solution immediately for three times and stored in PBS solution at 4 °C to preserve protein activity.

The corresponding non-imprinted silica (NIS) was prepared in the same way without adding Hb.

2.4. Adsorption experiments

Protein concentrations were determined by HPLC using a linear gradient of water/acetonitrile (both containing 0.1% trifluoroacetic acid) from 80:20 to 20:80 in 20 min. The UV detection wavelength was 280 nm, the flow rate was 1.0 mL/min and the injection volume was 10 μL.

Protein solutions (in 100 mM PBS, pH=7.0) of known concentrations were mixed with the SIS particles and incubated for 2 h at room temperature. After incubation, the samples were centrifuged at 5000 rpm for 3 min at room temperature, and the concentrations of the residual protein in the solution were determined by HPLC. The adsorption capacity (Q, mg of protein/g of particles) was calculated according to the following equation: $Q=(C_0-C_f)V/m$, 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 particle mass in each rebinding mixture. The amount of Hb bound to SIS was calculated by subtracting the amount of free Hb in the supernatant from the amount of Hb initially added. The template removed ratio is the bounded Hb divided by the removed Hb.

Adsorption isotherms were recorded to determine the adsorption constants of SIS for Hb. The Hb solutions (0.02–4.0 mg/mL, 1.0 mL) in PBS (100 mM, pH=7.0) were incubated with 2.0 mg/mL SIS particles for 2 h at room temperature. The adsorption kinetics of Hb toward SIS was investigated by changing the adsorption time from 0 to 90 min at a constant Hb concentration of 0.4 mg/mL.

3. Results and discussion

SEM and TEM were employed to investigate the morphology of the surface imprinted silica (SIS). From the SEM images (Fig. 1), it can be seen that the surface of bare silica is smooth. After polymerization on the surface, the surface of SIS and the non-imprinted silica (NIS) became rough. The average thickness of the SIS polymer layer was determined to be 8 nm by TEM. Because of imprinting, nano-sized holes can be observed on the SIS surface. The specific surface area is 1.9, 18.7 and 34.9 $m^2\,g^{-1}$ for bare silica, SIS and NIS, respectively. The pore volume for SIS and NIS is 0.1 and 0.2 cc g^{-1}, respectively, but undetectable for bare silica. The elemental composition of the SIS surface was analyzed by energy dispersive spectrometry. Compare with bare silica (Si 32.1,

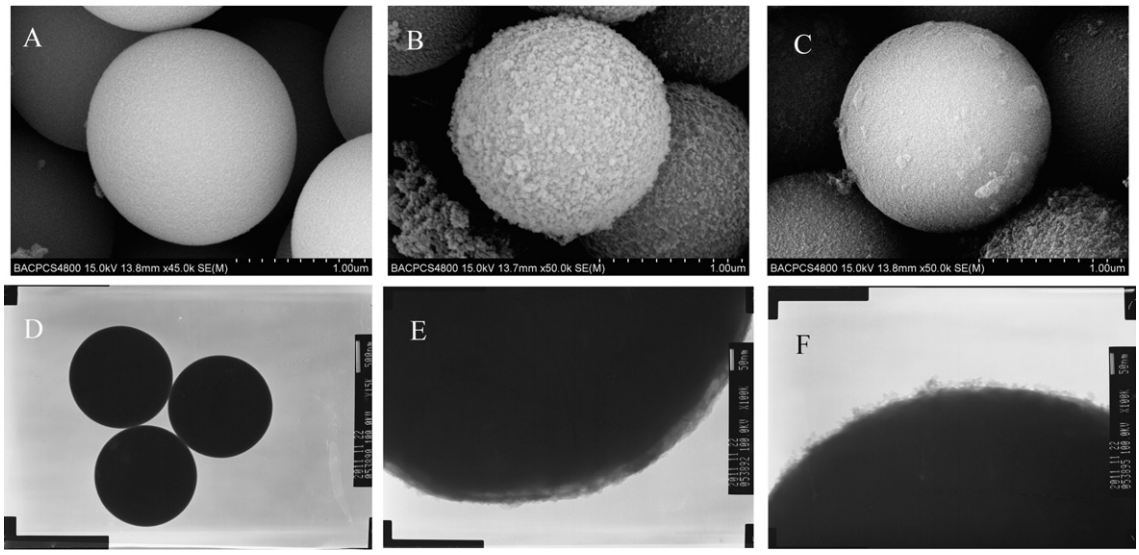

Fig. 1. SEM and TEM of (A, D) bare silica, (B, E) SIS, and (C, F) NIS, respectively.

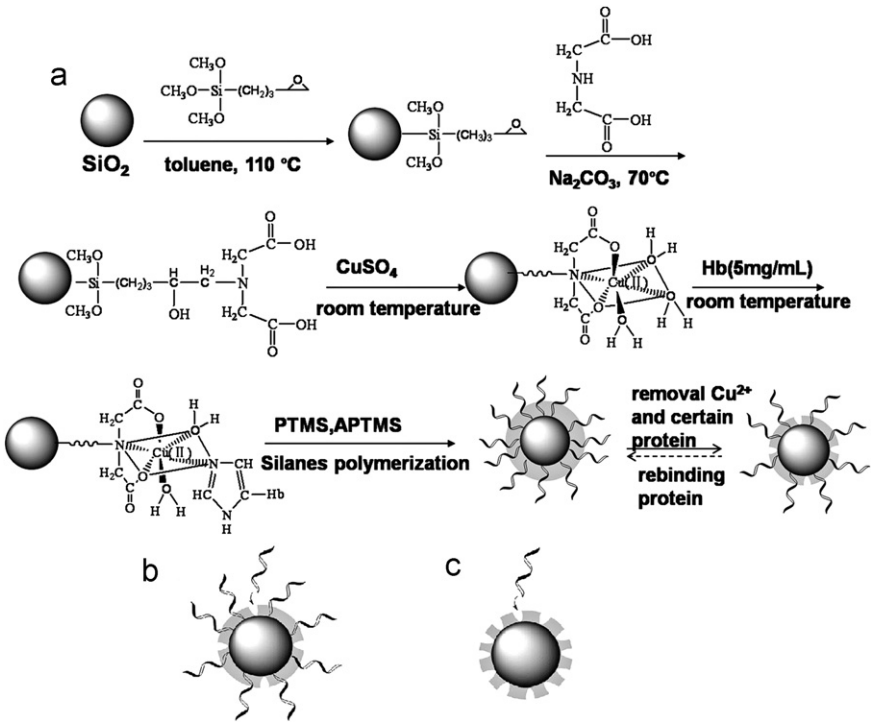

Fig. 2. (a) Preparation of SIS with soft and flexible recognition sites. (b) Induced fit recognition of proteins by SIS. (c) Recognition of proteins by classic MIPs.

O 67.9, C 0), SIS (Si 15.0, O 61.7, C 23.3) and NIS (Si 20.2, O 68.1, C 11.7) show higher content of C and lower content of Si, which indicates the successful polymerization. Because of unremoved hemoglobin on the surface, SIS has higher content of C than NIS.

There are several challenging issues in classic protein imprinting such as entrapment of macromolecular templates within the polymers, unfavorable kinetics of adsorption and desorption, heterogeneous binding sites, denaturation of biomacromolecules, *etc.* [10,14,15]. To solve these problems, surface imprinting on a solid matrix has been developed [16–22], which can improve mass transfer and reduce permanent entrapment of the template [12,23]. Usually, to prepare a surface imprinted silica (SIS), the template protein is first covalently immobilized on the silica surface and then removed after polymerization [14,17], but not all of the proteins can be removed and extensive template washing can corrupt the imprinting sites because extremely acidic conditions can destroy the silica matrices.

The SIS for Hb was prepared as depicted in Fig. 2a. The imprinted silica using immobilized Hb was proved to be superior than the imprinted silica formed using free Hb [17]. The Hb was immobilized according to the literature [12]. Nonporous silica was employed to make sure that Hb can be immobilized only on the surface of the silica matrix. On average, 56 mg of Hb was immobilized on 1 g nonporous silica. EDTA [24] and formic acid were used to sub-stoichiometrically remove the template. The SISs were washed by EDTA for 0.5, 1 and 3 h to remove Cu^{2+} and

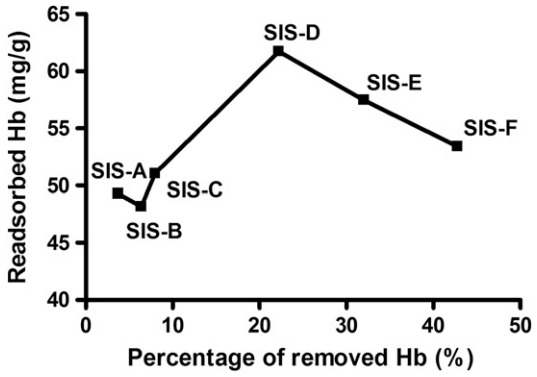

Fig. 3. The percentage of the removed template Hb and the re-adsorbed Hb of SIS A–F. Experimental conditions: 1.0 mL of 1.0 mg/mL Hb in PBS solution (100 mM, pH=7.0) incubated with 10.0 mg of the SIS for 2 h at room temperature.

afford SIS-A, SIS-B and SIS-C, respectively, which were further washed by 20% formic acid for another 5 min, 30 min and 6 h to give SIS-D, SIS-E and SIS-F, respectively.

For classic MIPs, proteins were recognized by the interactions between the proteins and the monomers within the rigid imprinted cavities [14,23] (Fig. 2c). For our SISs, proteins were recognized by both their inclusion in the cavities and their multiple interactions with the unremoved proteins on the silica surface (Fig. 2b). The unremoved proteins surrounding the imprinted sites actually formed soft pockets that can "induced fit" the target proteins. With higher removal of the template Hb (from SIS-A to SIS-F) (Fig. 3), the re-adsorbed Hb increased from SIS-A to SIS-D, which can be attributed to the exposure of more imprinted cavities. However, adsorption of Hb decreased from SIS-D to SIS-F due to the lack of flexible protein ligands that participate in the recognition. When 22% of the template was removed (SIS-D), the recognition sites on the silica surface had the highest recognition ability. Upon binding proteins, the unremoved proteins surrounding the imprinted sites undergo a number of small conformation changes to form a complex with the rebinding protein. Unlike small molecule template, the proteins present a large number of potential recognition sites over a relatively large surface area. The recognition process depends on the appropriate geometric organization of the unremoved proteins (including hydrophilic domains) to have the host molecules match the reciprocal functionality in the guests.

It has been noticed that the flexibility of the imprinted sites affects the recognition. The flexibility of MIPs is usually adjusted by the amount of crosslinker employed. Classic work with small molecules has demonstrated that no imprinting is observed below a critical threshold of crosslinker concentration. However, Schrader's work demonstrates that good recognition of proteins is possible in the complete absence of crosslinker, as the target protein can undergo an induced fit upon complex formation where both the number and strength of favorable binding interactions are maximal [9]. Schrader's protein-imprinted copolymers that incorporate a variety of functionalized monomers are distinct in that the flexible copolymers are not truly "imprinted" and recognition is entirely due to induced fit. Their copolymers with hydrophobic residues display a compact nature resembling a protein, which is resulted from the burial of dodecyl tails in its inner core and the exposure of the polar bisphosphonate head groups into the bulk solvent. A prearranged rigid binding site is not a requirement for selective recognition. According to Ying's investigation, BSA-imprinted hydrogel changes its conformation to fit BSA when it is approaching [8]. Protein will also adapt to modified conformation of the imprints and finally be recognized. Compared with Schrader's functionalized copolymers and Ying's hydrogel, in this work, the unremoved proteins on the silica surface

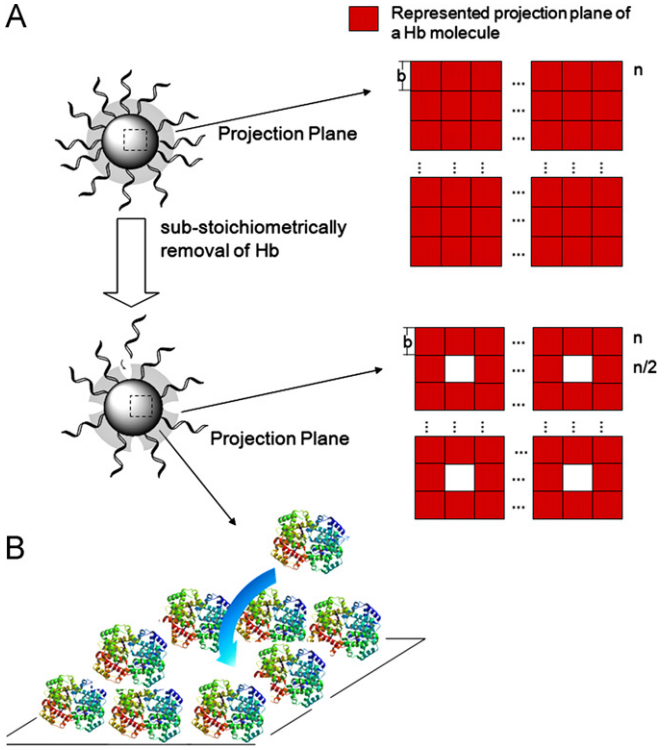

Fig. 4. Recognition of protein by a soft recognition site on the SIS. (A) Before template removal. (B) After template removal. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

are a key factor in producing efficient protein imprints and generating the induced fit of the target proteins. The recognition is carried out by a synergistic effect between the unremoved protein and imprinted cavities. Besides, the induced fit is provided by unremoved protein, which is more flexible than hydrogel and linear polymer,

Assuming that the Hb was uniformly distributed on the silica surface, each red box in Fig. 4 represents a Hb molecule, while white boxes represent the cavities after Hb removal (Fig. 4b) and b is defined as the side of the square that one Hb molecule occupies. The value of b^2 is 3.5×10^{-17} m^2 (35 nm^2) as can be calculated by the specific surface area, the molecular weight of Hb and the numbers of Hb molecules immobilized on 1 g silica. Therefore, b is 5.9 nm, which just fits the size of the Hb molecule ($5.5 \times 5.5 \times 7.0$ nm^3). According to the above calculation, Hb molecules were uniformly and closely immobilized on the silica surface next to each other, as shown in Fig. 4a. The imprinted cavities were uniformly distributed when the template Hb was removed sub-stoichiometrically (25%) and were surrounded by the unremoved Hb. According to our investigation, the best recognition would be achieved when the imprinted cavities were surrounded by unremoved Hb, as shown in Fig. 4b. It can be concluded according to Fig. 4 that the percentage of white boxes is 25%, *i.e.*, the percentage of removed Hb was 25%, which is close to the previous experiment results that the highest recognition ability was achieved when 22% of the template was removed. Soft "induced fit" recognition sites were created, which composed of rigid imprinted cavities and the flexible unremoved proteins surrounding them, the recognition site is also more homogeneous than classic protein MIPs.

The adsorption binding kinetics was determined for both SIS and NIS (Fig. 5). Because all of the imprinted cavities were located on the surface of SIS, the recognition sites were accessible for target proteins. The adsorption of Hb on SIS reached equilibrium within 20 min, which was faster than most protein MIPs reported by Gai [18], Kan [19] and Li [25]. It indicated that our SIS has a good site accessibility toward the target protein molecules.

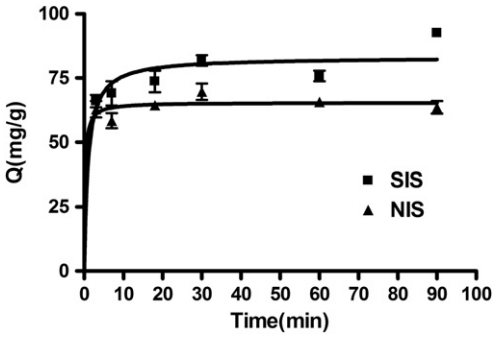

Fig. 5. Adsorption kinetic curves of SISs and NISs. Experimental conditions: 1.0 mL of 0.4 mg/mL Hb solution, incubated with 2.0 mg of the particles for certain hours at room temperature.

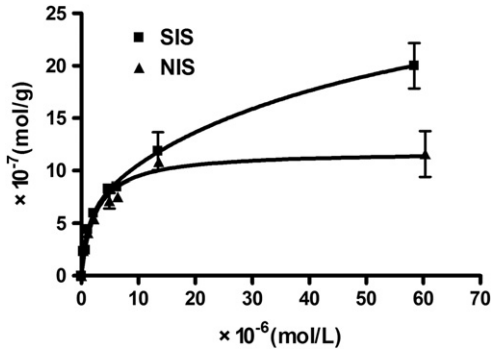

Fig. 6. Adsorption isotherms of Hb on SISs and NISs. Experimental conditions: 1.0 mL of 0.02–4 mg/mL Hb solution, incubated with 2.0 mg of the particles for 2 h at room temperature.

In contrast, the functional groups were distributed randomly on the surface of NIS, and there are no flexible protein ligands that can interact with the target, which resulted in the slower and non-specific adsorption of Hb on NIS. SIS adsorbed more Hb than NIS due to the imprinting effect.

The adsorption isotherms were used to determine the adsorption capacity and the adsorption constants of SIS for Hb (Fig. 6). The Hb solutions (0.02–4.0 mg/mL) prepared in PBS (100 mM, pH 8.0) were incubated with SIS particles (2.0 mg/mL) for 2 h at room temperature. After the equilibration, curve fitting were carried out by adsorption equation as follows:

$$Q = Q_{\max 1} \times \frac{C_e}{\left(\frac{1}{K_1} + C_e\right)} \quad (1)$$

$$Q = Q_{\max 1} \times \frac{C_e}{\left(\frac{1}{K_1} + C_e\right)} + Q_{\max 2} \times \frac{C_e}{\left(\frac{1}{K_2} + C_e\right)} \quad (2)$$

where Q and Q_{max} are the experimental protein adsorption and the theoretical maximum adsorption capacity of SIS (mol/g), respectively, C_e is the protein concentration at equilibrium, and K is the equilibrium constant for adsorption. According to the regression results, SIS has two kinds of binding sites with different affinity ($Q_{max1}=7.6\times10^{-7}$ mol/g, $K_1=9\times10^5$ L/mol and $Q_{max2}=24.8\times10^{-7}$ mol/g, $K_2=1.8\times10^4$ L/mol), while NIS has only one kind of binding site with low affinity ($Q_{max}=11.8\times10^{-7}$ mol/g and $K=2.5\times10^5$ L/mol). The K_1 and Q_{max1} of SIS are the adsorption constant and the adsorption capacity of the nonspecific adsorption, and K_2 and Q_{max2} are those of the specific adsorptions.

The selectivity of different SIS (SIS-A, SIS-D and SIS-F) for a mixture of proteins was investigated. BSA (v) (M_r 66, pI 6.5–7.5), Cyt C (M_r 12.7, pI 10) and RNase A (M_r 13.7, pI 4.6), which differ in molecular weight and isoelectric point, were used as the competitors. As shown in Fig. 7, the amounts of Hb (M_r 64.5, pI 6.8–7.2) adsorbed on SIS were more than those of the competitors. There was no adsorption of RNase A and Cyt C on SIS-A, D and F. BSA has similar pI and molecular weight with Hb and was adsorbed on the SIS at a minor amount. The selectivity factors of SIS-A, D and F for BSA, calculated as Q_{Hb} divided by Q_{BSA}, were 4.3, 5.0 and 4.7, respectively. The results confirm the successful formation of imprinted cavities on the SIS. Among the SISs tested, SIS-D with 22% template removal showed the best selectivity for Hb, which is in agreement with Fig. 3.

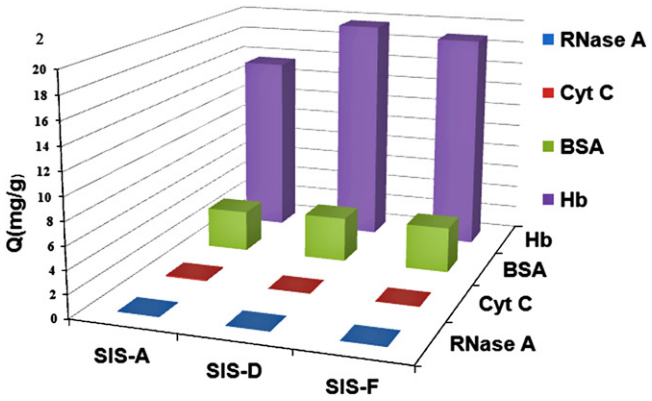

Fig. 7. Competitive adsorption of four proteins on SIS A (3.7% Hb removed), D (22% Hb removed) and F (44% Hb removed). Experimental conditions: 1.0 mL of 0.25 mg/mL Hb, BSA, Cyt C, RNase A in PBS solution.

Table 1
Adsorption results of SIS-D and other reported MIPs.

Adsorption equilibrium time	Q_{max} ($\times10^{-7}$ mol/g)	Selectivity parameter (β_{min})*	Particle size (μm)
1 h	1.6	2	0.15
6 h	8.3	1.5	0.12
1 min	3.0	2	0.4
30 min	25	5	1.5

* β_{min} is the minimum ratio of Q (template protein) to each Q (competitor proteins).

For classic acrylate MIPs [12,18,19,23], proteins were recognized by the rigid and inflexible recognition sites. A reciprocal hydrogen bond acceptor or donor group positioned within an imprinted cavity, corresponding to a suitable region on the surface of a protein, would offer excellent binding as it can use the directional properties of a hydrogen bond to its advantage. However, the selectivity may be unsatisfactory because only part of the protein is recognized. In this work, the SIS with "soft" recognition sites had better selectivity (Table 1) because the unremoved proteins surrounding the imprinted cavities created a flexible pocket that can receive the target proteins through induced fit. Compared with most other MIPs reported in the literatures [18,19,26], our SIS has better adsorption kinetics and selectivity, although the adsorption rate of PSA MIPs prepared by Liu [12] is faster because of the metal coordination during recognition. Compared with the other MIPs listed in Table 1, our SIS has the biggest particle size, which suggests that it has the smallest surface area and should have poorer capacity. However, our SIS has much higher Q_{max}, which suggests the advantage of this new surface imprinting technique.

4. Conclusions

By sub-stoichiometrically removing the template proteins, "soft" recognition sites which can "induced fit" target proteins can be

created. The surface imprinted silica with "soft" recognition sites, which contain imprinted cavities and the nearby unremoved proteins, are superior in binding capacity and mass transfer compared with classic protein MIPs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.07.067.

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