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Molecularly imprinted polymer grafted on polysaccharide microsphere surface by the sol–gel process for protein recognition

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Abstract

An interfacial organic–inorganic hybridization concept was applied to the preparation of a new spherical imprinted material for protein recognition. The functional biopolymer chitosan (CS), shaped as microsphere and high-density cross-linked, constituted of the polysaccharide core for surface imprinting. After the model template protein, bovine serum albumin, was covalently immobilized by forming imine bonds with the functional amine groups of CS, two kinds of organic siloxane (3-aminopropyltrimethoxysiloxane: APTMS, and tetraethoxysiloxane: TEOS) assembled and polymerized on the polysaccharide–protein surface via sol–gel process in aqueous solution at room temperature. After template removal, the protein-imprinted sol–gel surface exhibited a prevalent preference for the template protein in adsorption experiments, as compared with four contrastive proteins. Bioinformatics methods were also employed to investigate the imprinting process and the recognition effect. The influence of siloxane type, pH, siloxane/water ratio on template removal and recognition selectivity was assessed. Under optimized imprinting conditions, a large quantity of well-distributed pores was observed on the immobilized-template imprinted surface. The surface-imprinted adsorbent offered a fast kinetics for template re-adsorption and could be reused. Compared with the imprinted material prepared with free-template, material prepared with immobilized-template possessed higher adsorption capacity towards template protein. Easy preparation of the described imprinted material, high affinity and good reusability make this approach attractive and broadly applicable in biotechnology for down-stream processing and biosensor.

Keywords: Surface imprinting; Organic–inorganic; Sol–gel; Polysaccharide; Protein recognition

1. Introduction

Synthetic materials specific for protein recognition are highly desirable in biotechnology for down-stream processing and biosensor. Selective recognition elements traditionally used within affinity materials have been molecules such as antibodies and cell receptors. Despite success achieved with those biomaterials, their limited stability and expensive production preclude the application in cheap and reusable affinity adsorbents, membranes, and sensors [1,2]. Recently, the use of relatively inert molecular imprinted polymers (MIPs) for protein recognition offers potential by introducing selective specificity into low-cost, stable and easily fabricated materials [1–5].

The preparation of MIPs using molecular imprinting technology lies in the template-mediated assembly of functional monomers. Despite applications target molecules with low molecular weight [1,6–8], MIPs have now proved success in mimicking natural receptors and biological macromolecules, e.g. whole cells, viruses, and especially proteins [1–4,9]. Due to the fragility and complexity of protein molecules, specific recognition is generally achieved by the introduction of a large number of weak complementary interactions between proteins and monomers, e.g. hydrogen bonds, Van der Waals forces, ionic bonds and hydrophobic interactions [1]. Such multiply interactions result in tailored binding pockets with distinct size, shape, and three-dimensional distribution of functional groups. Currently, novel protein-imprinted polymers have been conducted via different strategies. To overcome limitations associated with bulk imprinted materials, surface imprinting seems to be the most promising way in creating protein-imprinted polymers [10–12].

The majority of imprinting technology, demonstrated to date, involves the synthesis of MIPs in organic monomer-solvent systems. Moreover, most polymerization systems lack of true water

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compatibility. However, proteins are usually sparingly soluble and aggregate in common monomer-solvent systems. To tag MIPs with specificity for proteins, the synthesis of MIPs in aqueous environment is preferable for better selectivity and binding [10–13]. Acrylamide has often been applied as a monomer to perform surface imprinting in aqueous media [10–16]. Guo et al. prepared MIPs using free hemoglobin (Hb) as the template molecule, acrylamide as the functional monomer, chitosan (CS) beads or vinyl groups modified CS beads as supporting matrices [14–18]. Specific attention must be paid to improve weak mechanical strength and long-term stability [15,16]. For example, chemical modification of CS beads with maleic anhydride was performed to introduce vinyl groups, which took part in the copolymerization between acrylamide and the cross-linker, *N*,*N*′-methylenebisacrylamide. Such protocol resulted in the chemical interaction between the imprinted gel and CS matrix. However, template proteins are susceptible to the attack of free radicals in radical polymerization [19]. General synthesis of chemically and mechanically stable MIPs for protein recognition in aqueous media, therefore, remains a significant challenge.

With the development of sol–gel technique, molecularly imprinted sol–gel materials (MISGMs) have been extensively studied for the preparation of selective adsorbents [6,8,12,20]. Considering the easy preparation of sol–gels and their compatibility with proteins and polar environments, coating molecularly imprinted silica sol–gel polymer on a supporting matrix has considerable potential for creating novel materials specific for proteins. Results suggest that proteins could catalytically hydrolyze siloxane precursors and promote silica formation. Additionally, the overall stability of proteins embedded in the sol–gels has been broadly proven to enhance with regard to temperature, pH, and chemical denaturation [21–24]. Until now, preparation of MISGMs using protein-imprinted sol–gel process on silica-based matrix has been attempted in a number of works. Chou et al. combine sol–gel and self-assembly technology for the preparation of human serum albumin imprinted film on the surface of piezoelectric quartz crystal [25]. The imprinted film gives selective recognition to the template protein. Shiomia et al. prepare Hb imprinted sol–gel material on the surface of aminopropyl silica in aqueous media. The Hb recognition occurs through shape, hydrophobicity and surface charge [12].

The supporting matrix plays an important role in surface imprinting. Incorporation of the functional biopolymer, CS, into inorganic network via sol–gel process takes on increasing importance in developing affinity matrices for proteins [26]. The reason comes from three aspects. Firstly, unique properties of CS enable covalent assembly of biomolecules in a spatially selective manner. Those properties include reactivity of primary amine groups, hydrophilicity and biocompatibility [26–30]. Secondly, polysaccharide-manipulated sol–gel process has shown catalytic effect and acceleration on siloxane polymerization and potentially relates to the biomineralization processes [22]. Thirdly, silica materials containing covalently grafted gluconamide moieties provide biocompatible environment for the entrapped protein [31]. Very recently, we have reported direct preparation of porous functional matrix based on surface coating technique and CS incorporated sol–gel process [32–34]. The obtained organic–inorganic hybrid matrices show promising in metal ion removal and protein separation.

This work attempts to present a new strategy for the preparation of spherical imprinted polymer for protein recognition. High-density cross-linked CS microsphere, formed using emulsion cross-linking method, constituted of the supporting core. Bovine serum albumin (BSA) was used as the model template and covalently immobilized on CS core by forming imine bonds. In the presence of the immobilized BSA, surface grafting of imprinted polymer to the polysaccharide core was achieved through interfacial organic–inorganic hybridization via a sol–gel process in aqueous solution at room temperature. After template removal by breakage of imine bonds, the imprinted surface showed selective recognition towards template protein in adsorption experiments, as compared with four contrastive proteins. The preparation methodology, main characteristic features were described and discussed in detail.

2. Experimental

2.1. Chemicals

CS, with 98% deacetylation and an average molecular weight of 6×10^4 g mol^{-1} (Yuhuan Biomedical Corp., China) was used to prepare the polysaccharide microsphere. Liquid paraffin, span 80, and epichlorohydrin were purchased from Qingdao Chemical Co., China. Formaldehyde (25%) were from Shanghai Bioengineering Corp., China. The following siloxanes were used: 3-aminopropyltrimethoxysiloxane (APTMS, Alfa aesar), gamma-glycidoxypropyltrimethoxysiloxane (GPTMS, Alfa aesar), methyltrimethoxysiloxane (MTMOS, Sigma–Aldrich), tetraethoxysiloxane (TEOS, Alfa aesar). BSA (Sigma) was used as the template protein. Beta-amylase, cytochrome *C*, transferrin and lysozyme, all from Kejie Biomedical Corp., China, were used as contrastive proteins. All other chemicals used were in analytical grade. Doubly deionized water (DDW) was used throughout this work.

2.2. Preparation of CS microsphere

CS solution was prepared by dissolving in 1 mol l^{-1} acetic acid aqueous solution with a 4 wt% CS concentration. Fifty milliliters transparent CS solution was added to 100 ml liquid paraffin containing 3 ml surfactant, span 80, with vigorous stirring for 1 h. The aqueous droplets in the water-in-oil (w/o) emulsion were quickly harden by adding 8 ml formaldehyde and reacted for 30 min at 60 °C. Then, pH was adjusted to be 10–11 using sodium hydroxide and 15 ml epichlorohydrin was added. The cross-linking process was allowed for another 6 h at 60 °C. The formed microsphere with active epoxy groups was shaken in 0.5 mol l^{-1} ammonia solution at 60 °C for 4 h to recover some amino groups lost in cross-linking [35]. A soxhlet extraction with *n*-hexane was used to

remove un-reacted reagents and emulsifer involved in microsphere. The final product was washed with ethanol followed by DDW.

2.3. Preparation of immobilized-template imprinted composite microsphere (I-MIP)

To form free aldehyde ends, the terminal amino groups on CS microsphere reacted with bifunctional glutaraldehyde. One gram microsphere was added to 10 ml 2.5 wt% glutaraldehyde in borate buffer solution (pH 8.2) and incubated for 2 h at room temperature with shaking. Then, modified microsphere equilibrated with 10 ml template solution, 2 mg ml^{-1} BSA in borate buffer solution (0.2 mol l^{-1}, pH 8.2), for 4 h at room temperature. Protein in solution was measured by the Bradford method [36]. The amount of immobilized BSA was calculated by the following equation:

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

C_0 and C_e represented initial and equilibration concentration of BSA, V was the volume of solution, and m was the weight of the microsphere.

After the template BSA was immobilized, the pH of the sol–gel process for the surface coating on the BSA-immobilized beads was optimized. One gram BSA-immobilized bead was respectively added to 10 ml of solution under different pH conditions. The pH of solution was adjusted using the following solutions: sodium acetate/hydrochloric acid for pH 2, sodium acetate/acetic acid for pH 4, sodium phosphate/phosphoric acid for pH 7, sodium carbonate/sodium biocarbonate for pH 9. All the solutions were controlled at 0.2 mol l^{-1}. Mixed binary siloxanes were then added and sol–gel process carried out for 12 h at room temperature with shaking. Afterwards, 10 ml of 0.5 mol l^{-1} oxalic acid was added to the obtained bead for template removal [12]. That was allowed for 8 h at room temperature with shaking and removed protein in the supernatant was measured. For the best performance, sol–gel process was finally set at pH 9. After thoroughly washed with DDW, the immobilized-template imprinted composite microsphere was obtained, which was designated as I-MIP.

For the preparation of control non-imprinted polymer, the same procedure was used only without addition of BSA. The obtained material was designated as NIP.

2.4. Preparation of free-template imprinted composite microsphere (F-MIP)

One gram initial CS microsphere was thoroughly mixed with 10 ml of 2 mg ml^{-1} BSA in sodium carbonate/sodium biocarbonate solution (pH 9). Then, mixed siloxanes were added and subsequent steps were performed in the same manner as described above. The final product, the free-template imprinted composite microsphere, was designated as F-MIP.

2.5. Characterization of the materials

The primary amino groups on CS microsphere were assayed by titration [15]. Water content of both CS microsphere and protein-imprinted composite microsphere in DDW was calculated as follows:

$$\text{water content} = \frac{W - W_d}{W} \quad (2)$$

After the microsphere was filtered, the weight of the wet sample (W) was determined after further removing the surface water by blotting with moistured filter paper. The weight of the dry sample (W_d) was determined after the microsphere was dried under vacuum.

To characterize surface morphology of the materials, scanning electron microscopy (SEM) images were obtained at 5.0 kV on a Hitachi S-4100 (Hitachi, Japan) field emission scanning electron microscope. X-ray energy dispersion spectroscopy (EDX) measurements were performed using a GENENIS 4000 energy dispersive X-ray micro analyzer (EDAX, USA) to investigate the transfer of elemental distribution on solid bead. The accelerating voltage used for the EDX analyses was 25 kV. The thermal and decomposition characteristics of the materials were determined using simultaneous thermogravimetry and differential scanning calorimeter (TG/DSC), conducted with a NETZSCH STA 409 thermogravimetric analyzer (Bruker, USA) in the temperature range of 30–800 °C at a heating rate of 20 °C min^{-1}, with air flushed at 200 ml min^{-1}. Nitrogen adsorption porosimetry measurement was performed on a Omnisorp 100CX (Coulter, USA) to provide data on surface area and pore diameter. Nitrogen isotherms were obtained in both adsorption and desorption modes. The surface areas of the supporting core and composite microsphere were determined by the BET method. Pore size distribution curves were established from the desorption branches of the isotherms using the BJH model.

2.6. Adsorption experiments

Binding kinetics of BSA to the I-MIP was performed by adding 200 μL BSA solution (100 mg ml^{-1}) to 1 g imprinted adsorbent immersed in 10 ml buffer solution. Taking a sample at regular interval to analyze BSA concentration in the supernatant until adsorption equilibrium was reached. Adsorption isotherm of BSA was determined to measure the adsorption capacity. One gram adsorbent was equilibrated with 10 ml solution of various BSA concentrations, ranged from 0.05 mg ml^{-1} to 2 mg ml^{-1}, for 60 min at room temperature. The BSA adsorptions to non-imprinted polymer (NIP), and free-template imprinted composite microsphere (F-MIP) were also determined. Molecular recognition selectivity was evaluated by differences in static equilibrium adsorption between BSA and contrastive proteins. One gram imprinted material was equilibrated with the solution containing 20 mg of the template BSA or contrastive protein for 90 min at room temperature.

In all above adsorption experiments, sodium phosphate/phosphoric acid at pH 7 was used. The protein adsorbed on

the adsorbent was determined by mass balance between liquid and solid phases, according to Eq. (1).

2.7. Desorption and reproducibility

Desorption studies were performed using 1% (v/v) acetic acid containing 5% (w/v) tween 20 to strip the re-adsorbed BSA, which was followed by thorough wash for regeneration. In test of adsorbent recycling, the imprinted material was used through four adsorption/regeneration cycles during 10 days.

3. Results and discussion

3.1. Preparation of immobilized-protein imprinted composite microsphere (I-MIP)

The surface imprinting strategy selected in present investigation relied on the use of a spherical core constituted by a polysaccharide, CS. The template protein was covalently immobilized on the supporting CS bead. Moreover, interfacial organic–inorganic hybridization via protein-imprinted sol–gel process was used for the surface coating. Schematic representation for synthesis of the protein-imprinted polymer on CS microsphere with immobilized protein template was illustrated in Fig. 1. The synthesis involved three steps. Firstly, template BSA was covalently immobilized on the polysaccharide core by forming imine bonds. The second step involved siloxanes polymerization on the polysaccharide–protein surface. It resulted in a polymeric network molded around the template. The template protein was removed in the third step. Cavities complementary to the template protein in shape, size, and functional group orientation were created.

In the developed affinity material, CS was used as integral component. Due to the reactivity of primary amine groups, CS has been exploited in many studies to covalently couple biomolecules. An analysis of the cross-linked CS microsphere gave 0.31 ± 0.01 mmol of amine content per gram wet beads (water content, $(55.4 \pm 2.1)\%$). A range of simple and standard methods could be used for coupling proteins to CS through amines. In our case, amino groups reacted with bifunctional glutaraldehyde to form aldehyde ends, which reacted spontaneously with free amino sites on protein by forming imine bonds (Schiff's bases) [37]. Without being reduced to secondary amines, imine bonds were fully reversible and could be readily broken for template removal. Shiomia et al. applied oxalic acid as an effective agent to remove the template protein, which was covalently immobilized using imine bonds [12]. In our experiments, washing with oxalic acid at 0.5 mol l^{-1} was also adopted as the template removing procedure.

Protein immobilization on aldehyde-modified surface involves amino sites exposed on protein surface, which were mainly located on the side-chain of lysine and arginine. To detect those two residues exposed on protein surface, the relative solvent accessibility to the amino acids sequence of BSA was predicted with AccPRO (http://www.igb.uci.edu/tools/scratch/) [38]. It was observed that no lysine and arginine were buried (accessibility no higher than 10%) inside the protein. Moreover, all 60 lysine and 20 arginine (76% of total 26 arginine residues) were exposed to the solvent with accessibility higher than 40%. Obviously, there were abundantly accessible amine groups on BSA surface, indicating the rational design of coupling by forming imine bonds. This circumstance also favored multipoint attachment between BSA and aldehyde-activated surface. That resulted in template protein assembling with stable spatial orientation and low-energy configuration [38]. Additionally, the "tumbling rate" and aggregation of template protein during the imprinting process were reduced. As a result, the functional groups possessed precise position around the template protein. The immobilized-protein imprinted materials possessed uniform binding sites and excellent recognition properties [16,39]. On average, 18 ± 0.42 mg of BSA was immobilized on 1 g of aldehyde-modified CS microsphere.

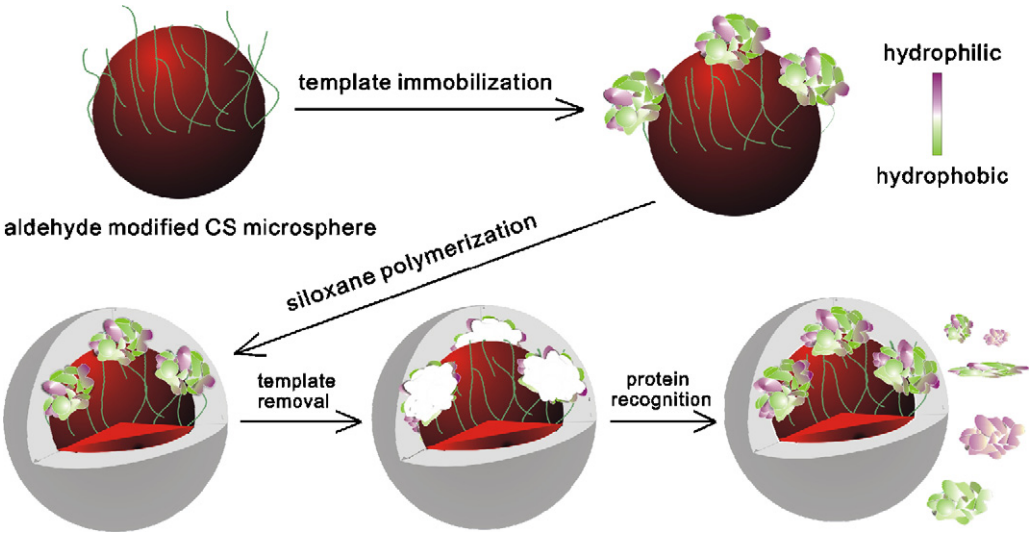

Fig. 1. Schematic representation for synthesis of the protein-imprinted polymer on CS microsphere using immobilized protein template.

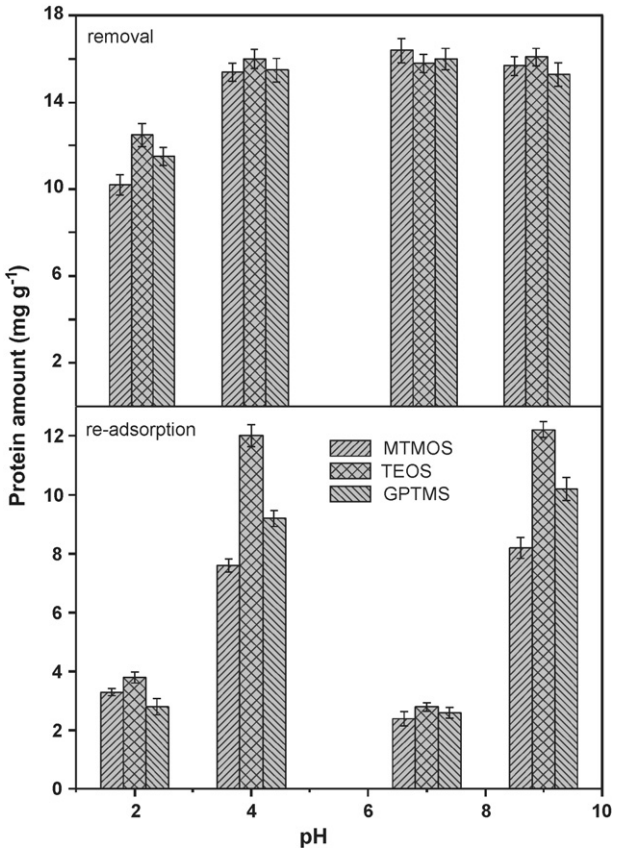

Fig. 2. Influences of siloxane type and pH of sol–gel process on removal and re-adsorption of template BSA Siloxane labeled in the figure showed the partnership of APTMS in three types of mixed binary siloxanes (mole ratio, 1:1). The siloxane/water ratio (v/v) was 1%.

3.2. Effect of starting siloxane type and pH of sol–gel process on characteristics of prepared I-MIP

Following immobilization of the template protein, siloxane monomers polymerized onto the BSA-CS surface through sol–gel process. The selectivity of imprinted sol–gel materials depended on various factors, including starting monomer type, siloxane to water ratio and pH of sol–gel process [20]. As suggested by Shiomia et al. APTMS could cover aldehyde groups [12]. In our procedure, APTMS was thus chosen as the universal monomer. Its partnership in three types of mixed binary siloxane was GPTMS, MTMOS, and TEOS, respectively. As a preliminary screening procedure to search for suitable monomers and pH, two siloxane monomers in three types of mixed binary siloxanes were all mixed at a fixed mole ratio of 1:1. The sol–gel reaction was acid (pH 2, pH 4), neutral (pH 7) or base (pH 9) catalysed, respectively (siloxane/water ratio (v/v), 1%). The influences on resulting characteristics of template removal and re-adsorption were evaluated. As seen from Fig. 2, both template removal and recognition capacity of the imprinted materials significantly depended on siloxane type, and pH of sol–gel coating process. Materials obtained using sol–gel process at pH 7 demonstrated high removal of immobilized template, whereas, re-adsorptions were lowest. As we know, siloxanes exhibited low gelation kinetics at neutral conditions.

Table 1
Characteristics of the template protein and contrast proteins

	Length[a]	MW[b]	p*I*	Number of predicted TMH[c]	Total prob. of N-in[d]
BSA	607	66	4.7	0	0.02293
Beta-amylase	546	57	6.5	1	0.99807
Cytochrome *C*	545	57	10.2	1	0.99021
Transferrin	706	76	5.9	0	0.00733
Lysozyme	211	14.6	10.8	1	0.45484

[a] The length of the protein sequence.
[b] The molecular weight (KDa) of the protein.
[c] The number of predicted transmembrane helices.
[d] The total probability that the N-term is on the cytoplasmic side of the membrane.

As a result, inefficient coating was obtained. Though immobilized template could be removed to a certain extent, materials prepared at pH 2 possessed low BSA re-adsorption capacities. It indicated inefficient template imprinting. In general, materials imprinted at pH 4 and pH 9 demonstrated easy template removal and considerable BSA re-adsorption. However, both template removal and BSA re-adsorption weakened in those six materials when MTMOS was used as the partnership of APTMS. This result suggested that MTMOS-derived sol–gel was inefficiently complementary to the template protein. Chou et al. used microcontact method for C-reactive protein (CRP) imprinting [25]. The affinity of the imprinted film towards template was found to be affected by hydrophilic/hydrophobic nature of the cover glass, which was used as a carrier for protein and functional monomer. In this regard, we also assumed that hydrophilic/hydrophobic characteristic played important role in complementary between siloxanes and proteins.

Transmembrane helices (TMHs) in BSA were predicted using TMHMM2.0 (http://www.cbs.dtu.dk/services) to evaluate hydrophobicity of the template protein [40]. It was noted that there were no predicted TMHs in BSA molecule. The total probability that the N-term was on the cytoplasmic side of the membrane (total prob. of N-in) was 0.02293 (Table 1). These results suggested that BSA was highly hydrophilic. When MTMOS was used as the co-precursor, the non-hydrolyzed hydrophobic group remained in the final sol–gel and endowed the sol–gel with a hydrophobic character. It was reported that TMOS-derived gel was fully hydrophobic when 20% MTMOS was used as co-precursor [41]. Due to the hydrophilic feature of the template, MTMOS-derived sol–gel was therefore not efficiently in template trapping and imprinting. In contrast to GPTMS, this trend was distinct considering the content of hydrophobic chain in siloxane molecule as well as the resulting sol–gel. Based on the above supposition, APTMS and TEOS were therefore chosen as the most appropriate co-precursors.

3.3. Effect of molar ratio of starting siloxane on characteristics of prepared I-MIP

The molar ratio of APTMS to TEOS was further scanned in sol–gel process at pH 4 and pH 9. Template removal and rebinding was used to evaluate the characteristics of the obtained

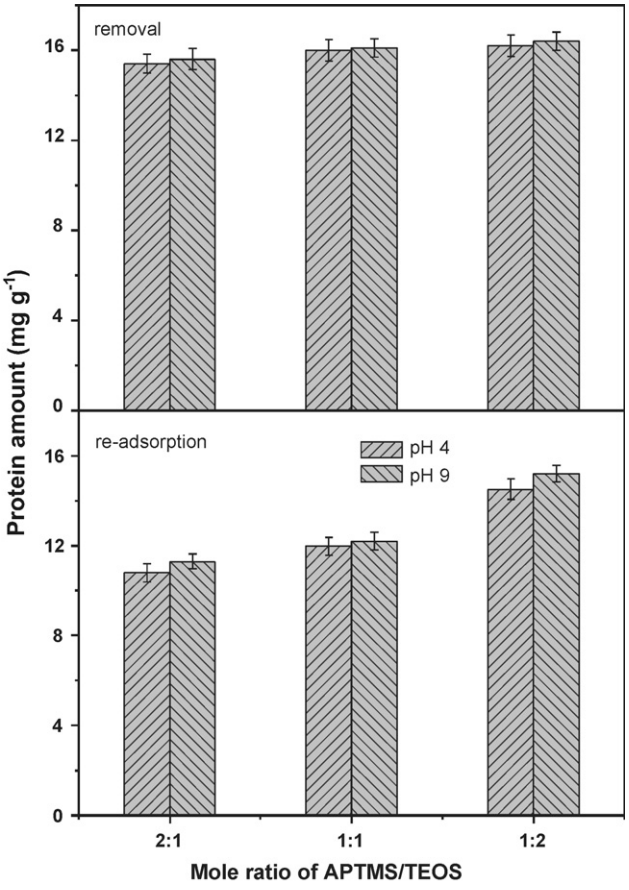

Fig. 3. Influence of the molar ratio of APTMS/TEOS on removal and re-adsorption of template BSA. Sol–gel process was performed at pH 4 and pH 9 using a siloxane/water ratio (v/v) of 1%.

materials. The molar ratios of APTMS to TEOS were 2:1, 1:1 and 1:2. The siloxane/water ratio (v/v) was fixed at 1%. As shown in Fig. 3, materials imprinted at alkaline condition demonstrated better performance than those obtained in acidic solution. It might attribute to the higher polymerization density in alkaline solution. As reported by Stancil et al. mismatched sites were less likely to form because the complementary pairs were less flexible in their motion when cross-linking density increased [42]. At a APTMS/TEOS ratio of 1:2, maximum template removal and re-adsorption capacity were observed. Therefore, sol–gel process was set at pH 9 using a APTMS/TEOS mole ratio of 1:2.

3.4. Effect of siloxane/water ratio on characteristics of prepared I-MIP

The influence of siloxane/water ratio on characteristics of template removal and re-adsorption was evaluated. The siloxane/water ratios (v/v), 0.5%, 1%, 1.5%, were scanned and result was shown in Fig. 4. High siloxane/water ratio (1.5%) led to rapid gelation in which the template BSA might be buried and thereby less removable. The remained template occupied binding sites and prevented the adsorption of other molecules. On the contrary, low siloxane/water ratio (0.5%) led to poor efficiency in coating. Though the template could be easily removed,

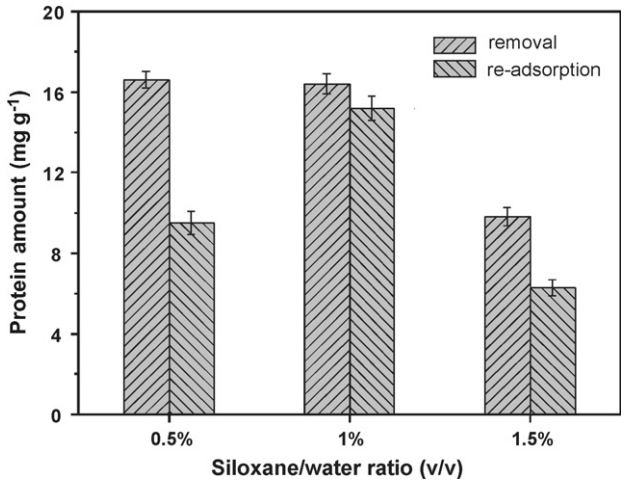

Fig. 4. Influence of siloxane/water ratio on removal and re-adsorption of template BSA. Sol–gel process was performed at pH 9 using a APTMS/TEOS mole ratio of 1:2.

re-adsorption capacity was low. At a moderate siloxane/water ratio of 1%, maximum template removal and re-adsorption capacity were observed. The transfer of elemental distribution on supporting CS microsphere and imprinted materials was investigated to validate surface grafting using X-ray energy dispersion spectroscopy (EDX). For CS microsphere, only C and O signals appeared. The appearance of Si signals in imprinted materials firmly established the existence of surface imprinting layer. Moreover, Si distribution increased as siloxane/water ratio increased, from a low value of 4.08% at low siloxane/water ratio (0.5%) to 11.15% at 1%, and the maximum of 18.17% at siloxane/water ratio of 1.5% (date not shown for brief). Finally, I-MIP prepared using polymerization between APTMS and TEOS (APTMS/TEOS mole ratio of 1:2, siloxane/water ratio (v/v) of 1%) at sodium carbonate/sodium biocarbonate solution (pH 9) was chosen for further investigation.

3.5. Characterization of the prepared I-MIP

The thermal stability of the prepared I-MIP was investigated by simultaneous thermogravimetry and differential scanning calorimetry (TG/DSC). Non-supported siloxane-condensed powder, which was prepared by casting mixed siloxanes on a clean glass plate, and the supporting CS microsphere were also tested for comparison. As shown in Fig. 5, thermogravimetric curves of CS microsphere (Fig. 5a) and non-supported siloxane-condensed powder (Fig. 5b) had two main degradation stages. The first weight loss occurred at about 100 °C, which resulted from the loss of adsorbed water. The second weight loss region might be caused by CS decomposition and structural reorganization of the polysiloxane, respectively. At the same time, those weight losses correlated with exothermic peaks in the DSC curve, indicating energy release. Due to synergic effects of CS and sol–gel layer, surface-grafted composite microsphere exhibited another two weight loss stages despite peak caused by loss of adsorbed water. As supported by previous observations, polymeric matrix formed by organic–inorganic hybridization usually

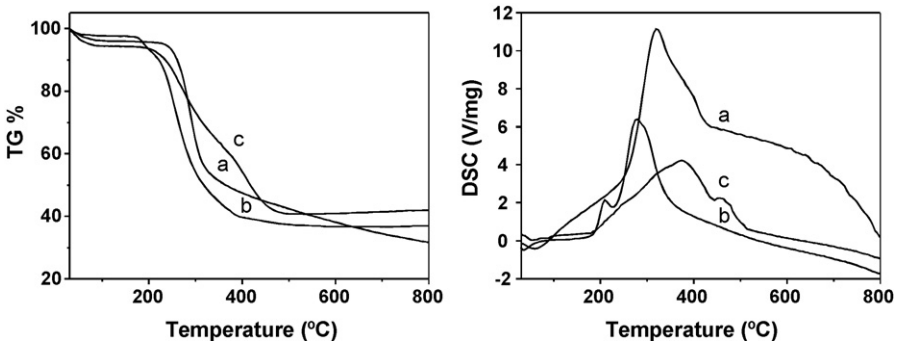

Fig. 5. The simultaneous thermogravimetry and differential scanning calorimetry analysis of CS microsphere (a), I-MIP (b) and non-supported siloxane-condensed powder (c).

showed improved thermal stability compared with single components [32,43]. In this regard, we attributed this observation to the hybrid effect between CS and polysiloxane at the core/shell interface.

Fig. 6 represented surface morphology of supporting CS microsphere and imprinted composite material. The supporting CS bead prepared via emulsion cross-linking was spherical with an average size of about 20 μm (Fig. 6a). The dense and slightly coarse microsphere possessed a porous structure. The BET surface area was 41.25 $m^2\ g^{-1}$ and average pore diameter was 8.7 nm, given by nitrogen adsorption porosimetry. The imprinted material demonstrated significantly different morphology (Fig. 6b). A large quantity of well-distributed pores was observed on the sphericial surface and they were netlike. The BET surface area was 48.65 $m^2\ g^{-1}$ and average pore diameter was 43.2 nm. The pores might possess geometrical distribution of ligands, increase specific surface area, and avoid problem of mass transfer [44].

3.6. Batch adsorption of template BSA to the prepared I-MIP

The surface-imprinted materials had good site accessibility for target protein molecules, because the protein-imprinted sites were located at, or close to, the surface. Consequently, restricted mobility associated with bulk imprinting materials was overcome. The general kinetic profile of template adsorption to I-MIP was investigated. As shown in Fig. 7, BSA possessed a fast adsorption profile in the initial step with significantly specific binding within 15 min. After about 60 min from the start, the adsorption process reached equilibrium. The adsorption of I-MIP was faster than that of F-MIP because 110 min must be

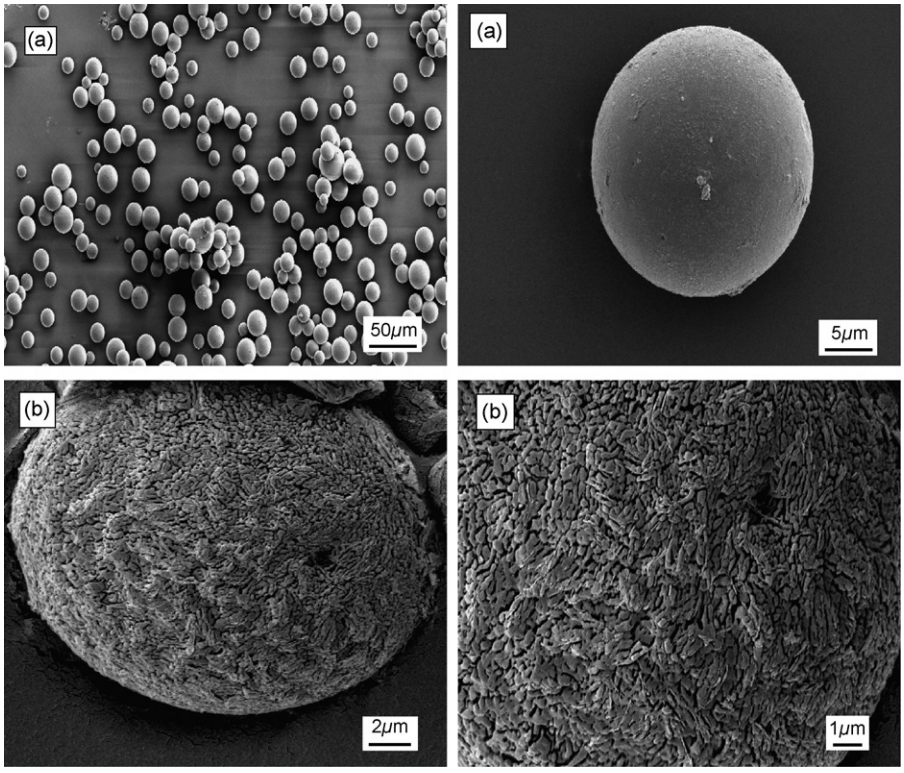

Fig. 6. SEM image (5.0 kV) of CS microsphere (a) and the immobilized-BSA imprinted material, I-MIP (b).

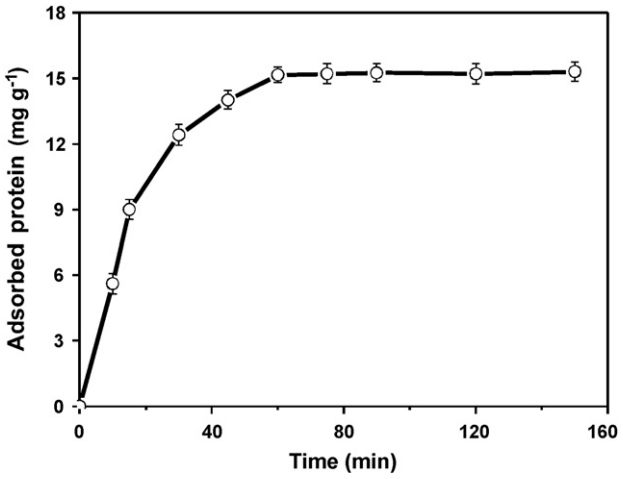

Fig. 7. Binding kinetics of BSA to the immobilized-BSA imprinted adsorbent (I-MIP).

required to reach adsorption equilibrium of F-MIP. Adsorption isotherm of BSA to I-MIP was similar to the Langmuir sorption isotherm. Data were well fitted to the linear form of the Langmuir equation expressed as following:

$$\frac{C_e}{Q} = 0.0063 + 0.0647C_e, \qquad \gamma^2 = 0.9903$$

C_e and Q represented equilibrium concentration of protein, and the amount of adsorbed protein on per gram of wet adsorbent respectively. The maximum protein adsorption, Q_{max}, was 15.5 mg g^{-1} wet bead (water content, (47.8 ± 1.9)%) and adsorption equilibrium constant (association constant) K was 1.03 L mol^{-1}.

3.7. Protein recognition selectivity of the prepared materials

Molecular recognition selectivity was evaluated by static distribution coefficient (K_D), and separation factor (α) being calculated according to static equilibrium adsorption of BSA and contrastive substrates, cytochrome C, transferrin, beta-amylase and lysozyme. Selective recognition of template and contrastive protein by immobilized-BSA imprinted (I-MIP), free-BSA imprinted (F-MIP) and non-imprinted (NIP) adsorbents were summarized in Table 2. The two template imprinted polymers, I-MIP and F-MIP, demonstrated selectivity towards template protein. However, the control non-imprinted material showed poor adsorption performance. In general, selective protein recognition was built on the assumption that molecularly imprinted polymer offered a three-dimensional microenvironment with shape-matching and complementary multiple-point interactions. In non-imprinting process, in contrast, groups were isolated from each other and distributed randomly in sol–gel network. So the multipoint interactions were much less, leading to reduced BSA adsorption (2.8 mg g^{-1} wet bead) and low selectivity [1]. Moreover, I-MIP possessed higher adsorption towards BSA (15.5 mg g^{-1} wet bead) than F-MIP did (12.6 mg g^{-1} wet bead). Compared with free-BSA imprinting process, template immobilization resulted in reduced "tumbling rate" and prevented aggregation. As a result, the uniform binding sites showed better accessibility and improved rebinding capacity.

Table 2
Selective recognition of template and contrastive proteins by BSA-imprinted (I-MIP, F-MIP) and non-imprinted adsorbent (NIP)

Adsorbent	Substrate	Q (mg g^{-1})	K_D[a]	α[b]
I-MIP	BSA	15.5	68.9	
	Transferrin	8.3	14.1	4.9
	Lysozyme	3.1	3.6	19.1
	Beta-amylase	2.1	2.3	30
	Cytochrome *C*	2.4	2.7	25.5
F-MIP	BSA	12.6	34.1	
NIP	BSA	2.8	3.3	

[a] K_D, Static distribution coefficient, K_D: C_p/C_s, where C_p and C_s represent the substrate concentration on the adsorbent and in the solution, respectively.
[b] α, Separation factor, α: K_D(BSA)/K_D(contrast protein). One gram imprinted or non-imprinted material was equilibrated with 20 ml of 1 mg ml^{-1} template BSA or contrastive protein for 90 min at room temperature. The buffer solution was sodium phosphate/phosphoric acid at pH 7.

For recognition of I-MIP, the level of selective discrimination naturally based the level of complementation between outer surface of protein and inner surface of "imprinting cavities". We speculated such complementation depended on shape-matching and multiple hydrogen bonding caused by matched hydrophilic/hydrophobic characteristics (Fig. 1). In our case, hydrophilic property of BSA made hydrophilic groups in the grafted layer spatially distribute at complementary positions. That leaded to the formation of a large quantity of hydrogen bonds between silanol groups of polysiloxane and surface polar residues of proteins [12]. The directional nature of hydrogen bond together with synergic action of multiple hydrogen bonds resulted in high degree of shape complementary. As shown in Table 2, hydrophobic proteins such as lysozyme (14.6 kDa, p*I* 10.8, 1 predicted TMH, total prob. of N-in: 0.45484), beta-amylase (57 kDa, p*I* 6.5, 1 predicted TMH, total prob of N-in: 0.99807) and cytochrome C (57 kDa, p*I* 10.2, 1 predicted TMH, total prob of N-in: 0.99021) could hardly bind to the imprinted adsorbent. Though beta-amylase and cytochrome *C* possessed similar hydrophobic property and molecular weight, however, isoelectric point was significantly different. In detail, beta-amylase was negatively charged while cytochrome *C* was positively charged in adsorption process (sodium phosphate/phosphoric acid at pH 7). However, the static distribution coefficients (K_D) for beta-amylase and cytochrome C were similar. Moreover, lysozyme and cytochrome C showed distinct K_D despite similar isoelectric point. Thus, recognition did not vitally depended on the electrostatic interaction. Transferrin (76 kDa, p*I* 5.9, 0 predicted TMH, total prob of N-in: 0.00733) had similar isoelectric point and hydrophilic property with BSA. Though it could be more efficiently adsorbed than the three hydrophobic proteins, K_D was much lower than BSA. The results might attribute to recognition based on shape-matching.

3.8. Desorption and reproducibility of the prepared I-MIP

Desorption and reproducibility properties of the prepared I-MIP were characterized. Re-adsorbed BSA could be stripped

by being washed with 1% (v/v) acetic acid containing 5% (w/v) tween 20, which was followed by regeneration through washing with DDW. In test of recycling, the imprinted adsorbent was used through four adsorption/regeneration cycles during 10 days. The capacity of I-MIP through four cycles was found to be $(91 \pm 3)\%$ of the fresh adsorbent. The sol–gel process endowed the adsorbent with physical robustness, mechanically durable network and stability in storage [42]. Additionally, the grafting of the imprinted layer through interfacial organic–inorganic hybridization also improved the stability of composite material.

4. Conclusions

The successful preparation of spherical imprinted material demonstrated the efficiency of interfacial organic–inorganic hybridization concept in surface imprinting. The imprinted polymer was conveniently grafted on polysaccharide core through immobilized-protein imprinted sol–gel process in aqueous solution at room temperature. The protein-imprinted surface possessed high affinity toward template protein. The complementation in hydrophilicity/hydrophobicity was a major factor affecting the imprinting formation and template recognition. The grafting of imprinted layer through interfacial organic–inorganic hybridization improved stability and reproducibility properties of the final material. Easy preparation of the I-MIP, its recognize ability and high stability make this methodological study and application attractive in biotechnology and biosensors.

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References

[1] B. Sellergren, Angew. Chem. Int. Ed. 39 (2000) 1031.
[2] K. Matsumoto, A. Torimaru, S. Ishitobi, T. Sakai, H. Ishikawa, K. Toko, N. Miura, T. Imato, Talanta 68 (2005) 305.
[3] A.H. Cui, A. Singh, D.L. Kaplan, Biomacromolecules 3 (2002) 1353.
[4] S.T. Wei, M. Jakusch, B. Mizaikoff, Anal. Chim. Acta 578 (2006) 50.
[5] Y. Li, H.H. Yang, Q.H. You, Z.X. Zhuang, X.R. Wang, Anal. Chem. 78 (2006) 317.
[6] Y.K. Lu, X.P. Yan, Anal. Chem. 76 (2004) 453.
[7] R. Suedee, W. Intakong, F.L. Dickert, Talanta 70 (2006) 194.
[8] G.Z. Fang, J. Tan, X.P. Yan, Anal. Chem. 77 (2005) 1734.
[9] D.M. Hawkins, D. Stevenson, S.M. Reddy, Anal. Chim. Acta 542 (2005) 61.
[10] K. Sreenivasan, Talanta 68 (2006) 1037.
[11] D. Lakshmi, B.B. Prasad, P.S. Sharma, Talanta 70 (2006) 272.
[12] T. Shiomi, M. Matsui, F. Mizukami, K. Sakaguchi, Biomaterials 26 (2005) 5564.
[13] S.A. Piletsky, H. Matuschewski, U. Schedler, A. Wilpert, E.V. Piletska, T.A. Thiele, M. Ulbricht, Macromolecules 33 (2000) 3092.
[14] S. Hjerten, J.L. Liao, K. Nakazato, Y. Wang, G. Zamaratskaia, H.X. Zhang, Chromatography 44 (1997) 227.
[15] T.Y. Guo, Y.Q. Xia, G.J. Hao, M.D. Song, B.H. Zhang, Biomaterials 25 (2004) 5905.
[16] T.Y. Guo, Y.Q. Xia, J. Wang, M.D. Song, B.H. Zhang, Biomaterials 26 (2005) 5737.
[17] G.Q. Fu, J.H. Zhao, H. Yu, L. Liu, B.L. He, React. Funct. Polym. 67 (2007) 442.
[18] T.Y. Guo, Y.Q. Xia, G.J. Hao, B.H. Zhang, G.Q. Fu, Z. Yuan, B.L. He, J.F. Kennedy, Carbohyd. Polym. 62 (2005) 214.
[19] S.H. Ou, M.C. Wu, T.C. Chou, C.C. Liu, Anal. Chim. Acta 504 (2004) 163.
[20] A.M. Siouffi, J. Chromatogr. A 1000 (2003) 801.
[21] J.E. Meegan, A. Aggeli, N. Boden, R. Brydson, A.P. Brown, L. Carrick, A.R. Brough, A. Hussain, R.J. Ansell, Adv. Funct. Mater. 14 (2004) 31.
[22] Y.A. Shchipunov, T.Y. Karpenko, Langmuir 20 (2004) 3882.
[23] A.M. Massari, I.J. Finkelstein, M.D. Fayer, J. Am. Chem. Soc. 128 (2006) 3990.
[24] A. Kumar, R.R. Pandey, B. Brantley, Talanta 69 (2006) 700.
[25] P.C. Chou, J. Rick, T.C. Chou, Anal. Chim. Acta 542 (2005) 20.
[26] J. Abdullah, M. Ahmad, L.Y. Heng, N. Karuppiah, H. Sidek, Talanta 70 (2006) 527.
[27] H. Yi, L.Q. Wu, R. Ghodssi, G.W. Rubloff, G.F. Payne, W.E. Bentley, Anal. Chem. 76 (2004) 365.
[28] Z.J. Wang, Y.H. Yang, J.S. Li, J.L. Gong, G.L. Shen, R.Q. Yu, Talanta 69 (2006) 686.
[29] H. Yi, L.Q. Wu, W.E. Bentley, R. Ghodssi, G.W. Rubloff, J.N. Culver, G.F. Payne, Biomacromolecules 6 (2005) 2882.
[30] L. Qian, X.R. Yang, Talanta 68 (2006) 721.
[31] J.A. Cruz-Aguado, Y. Chen, Z. Zhang, M.A. Brook, J.D. Brennan, Anal. Chem. 76 (2004) 4182.
[32] F. Li, X.M. Li, S.S. Zhang, J. Chromatogr. A 1129 (2006) 223.
[33] F. Li, H.Q. Jiang, S.S. Zhang, Talanta 71 (2007) 1487.
[34] F. Li, P. Du, W. Chen, S.S. Zhang, Anal. Chim. Acta 585 (2007) 211.
[35] L. Yang, W.W. Hsiao, P.J. Chen, Membr. Sci. 197 (2002) 185.
[36] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[37] M.G. Zhang, W. Gorski, J. Am. Chem. Soc. 127 (2005) 2058.
[38] M.M. El-Masry, A. De Maio, P.L. Martelli, R. Casadio, A.B. Moustafa, S. Rossi, D.G. Mita, J. Mol. Catal. B: Enzym. 16 (2001) 175.
[39] L. Betancor, A. Hidalgo, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisan, Biotechnol. Prog. 19 (2003) 763.
[40] J. Ethier, J.M. Boyd, Mol. Microbiol. 38 (2000) 891.
[41] J.T. Huang, J.Q. Zhang, J. Zhang, S.H. Zheng, J. Appl. Polym. Sci. 95 (2005) 358.
[42] K.A. Stancil, M.S. Feld, M. Kardar, J. Phys. Chem. B 109 (2005) 6636.
[43] Y.L. Liu, Y.H. Su, J.Y. Lai, Polymer 45 (2004) 6831.
[44] J.H. Smått, S. Schunk, M. Lindén, Chem. Mater. 15 (2003) 2354.