Preparation of Bovine Serum Albumin-Imprinted Calcium Polyacrylate/Alginate Hybrid Microspheres Via Ca^{2+} Crosslinking

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Received 24 June 2008; accepted 20 December 2008
DOI 10.1002/app.29936
Published online 2 April 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Macromolecularly imprinted calcium polyacrylate/alginate hybrid polymer microspheres with the imprinting of bovine serum albumin were prepared with sodium alginate and sodium polyacrylate by using $CaCl_2$ as a gelling agent in inverse-phase suspension. Infrared spectrum and conductance titration demonstrated that hybrid components were produced in these microspheres. Rebinding isotherms of the microspheres proved a greater bovine serum albumin affinity for imprinted microspheres relative to nonimprinted ones. Selectivity tests indicated that calcium polyacrylate/alginate hybrid polymer microspheres exhibited good recognition properties for the template protein with the separation factor over 5.30 comparing to the competitive proteins with a similar M_w. The influence of cross-linker $CaCl_2$ concentration on the imprinting efficiency (IE) and rebinding capacity was researched. It was found that the cross-linking density and imprinting efficiency of the hybrid hydrogel could be improved simultaneously. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 1133–1140, 2009

Key words: calcium polyacrylate/alginate (CPA/A); hybrid polymer microspheres; sodium alginate; sodium polyacrylate; bovine serum albumin; molecular imprinting

INTRODUCTION

As a promising technology of creating highly specific binding sites within an artificial material, molecular imprinting has developed rapidly for the specific recognition of small molecules.[1–6] However, the imprinting of biological macromolecules such as proteins has met with many difficulties.[7] Recently, there has been increased research in protein imprinting with polymer hydrogels.[8–11] Polyacrylamide hydrogel often has been applied in protein molecular imprinting.[10,11] Nevertheless, the use of conventional synthesized hydrogel has some fundamental drawbacks, such as the deformation or denaturation of proteins caused by the heat produced by polymerization. Besides, bulk polymers prepared by conventional polymerization method need to be smashed, ground, and sieved to get particles, during which large quantities of polymer particles were wasted.

To avoid the deformation or denaturation of proteins in the polymerization process, Zhang et al.[12] prepared bovine serum albumin (BSA)-imprinted calcium alginate hydrogel microspheres in a mild circumstance. Hydroxyethyl cellulose was used to associate sodium alginate (SA) to form interpenetrating networks, which reinforced the mechanical strength of calcium alginate microspheres and increased the imprinting efficiency of them. However, the effort was met with many limitations, including incomplete template removal and small binding capacity arising from the inner diffusion of proteins toward the matrix cross-linked by glutaraldehyde. In our previous work, BSA-imprinted calcium phosphate/alginate hybrid composite microspheres were prepared by assembling BSA with SA and $(NH_4)_2HPO_4$ and using $CaCl_2$ as a gelling agent in inverse-phase suspension.[13,14] Rebinding tests indicated that the microspheres exhibited an obvious improvement in rebinding capacity and imprinting efficiency compared with those microspheres prepared without calcium phosphate.

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Contract grant sponsor: National Natural Science Foundation of China; contract grant number: 20574051.
Contract grant sponsor: Special Program for Key Research of Chinese National Basic Research Program; contract grant number: 2008CB417202.
Contract grant sponsor: Tianjin Colleges Science and Technology Development Fund; contract grant number: 2006ZD40.
Contract grant sponsor: Science and Technology Development Project of Tianjin; contract grant number: 06TXTJJC14400.

Sodium polyacrylate (SPA) is an anionic polyelectrolyte and also bears an important feature of gelling in the presence of divalent cations, such as Ca^{2+}. In this study, we reported another hybrid polymer microsphere for imprinting proteins. The microspheres were prepared with SA and SPA by the use of $CaCl_2$ as a gelling agent in inverse-phase suspension. The hybrid polymer was characterized by conductance titration and Fourier transform infrared (FTIR) spectroscopy. The morphology of BSA-imprinted calcium polyacrylate/alginate hybrid polymer microspheres (CPA/A MIPMs) was observed by optical microscopy and scanning electron microscopy (SEM). Rebinding and recognition properties of imprinted and nonimprinted microspheres were studied.

EXPERIMENTAL

Materials

Sodium alginate (SA, $M_w = 218{,}000$) was purchased from Tianjin Yuanhang Chemical Reagent (Tianjin, China). Electrophoretic-grade BSA, bovine serum hemoglobin (Hb), lysozyme (Lyz), and γ-globulin (Glo) were obtained from the Institute of Hematology, Chinese Academy of Medical Science. Electrophoretic-grade egg albumin (EAB) was obtained from Sigma (St. Louis, MO). Sodium polyacrylate (SPA) was prepared with the solution polymerization method via free radical reaction mechanism with the M_w of 1.12×10^5. $CaCl_2$, NaOH, castor oil, butyl acetate, Tris(hydroxymethyl)-methylamine, hydrochloric acid, and ethylic cellulose were all from Kewei Chemical Reagent Company of Tianjin University (Tianjin, China), were chemical grade, and were used without further purification. Deionized water was used throughout all experiments.

Preparation of imprinted and nonimprinted CPA/A hybrid polymer microspheres

We obtained a homogenous mixed solution by dissolving 0.01 g of SPA and 0.5128 g of SA in 20 mL of 20 μmol/L BSA solution (pH 4.8). In a beaker, a 40 mL mixture of castor oil and butyl acetate (3 : 2 v:v) containing 0.04 g of ethylic cellulose was adequately stirred. The pasty solution was dispersed in the continuous medium; 5 min later, 20 mL of 5% $CaCl_2$ (w : v) solution was added slowly into the system and the gelling reaction proceeded for another 50 min. The microspheres were washed fully with deionized water to dispose of waste. To remove BSA, the microspheres were placed in a beaker containing 30 mL of elution solution—a mixture of 1% $CaCl_2$ solution and Tris-HCl buffer (0.05*M* Tris with a pH of 8.32). The samples were agitated gently for 60 h at 25°C, during which the washing buffer was renewed every 24 h, producing CPA/A MIPMs. Nonimprinted CPA/A NIPMs were synthesized and washed under the same condition of MIPMs, in the absence of BSA.

Morphology of the imprinted hybrid polymer microspheres

The morphology of CPA/A hybrid polymer microspheres (imprinted and nonimprinted) in the wet form was characterized with the use of optical microscopy (Axiovert 25C) connected with a digital camera. A scanning electron microscope (SEM, model S3500N; Hitachi, Berkshire, UK) was used to observe the surface amplification of CPA/A MIPMs.

IR spectra

CPA and CA were prepared by the addition of sufficient $CaCl_2$ solutions into SPA and SA aqueous solutions, respectively. The products were fully washed with deionized water, dried, and ground to powder. Then the powder was mixed with KBr and analyzed by an infrared spectrometer (FTS 6000; Bio-Rad, Cambridge, MA).

Conductance titration

A mixed solution was obtained by dissolving 0.0513 g of SA and 0.030 g of SPA in 150 mL of deionized water under magnetic stirring. Then, 0.050 mL of $CaCl_2$ solution (titrant, 0.091 mol/L) was dropped into the mixed solution with stirring. The conductance of the mixed solution was tested using a conductometer (DDS-307) at 25°C.

Mechanical stability of CPA/A hybrid microspheres

The same amount of CA and CPA/A microspheres were placed respectively in two beakers filled with 30 mL of 0.5 mol/L NaOH aqueous solution and three pieces of zeolite. Both beakers were oscillated for 24 h at 25°C with an oscillation rate of 150 r/min. Then the microspheres were collected and observed by optical microscopy to check the cracked beads.

Rebinding experiments

An accurately weighed amount of 1.00 g imprinted or nonimprinted wet microspheres (using filter paper to absorb the surface water) was placed in a flask with 15 mL of 20 μmol/L protein solution (pH 4.8). The rebinding capacity (Q) (μmol/g) was calculated according to

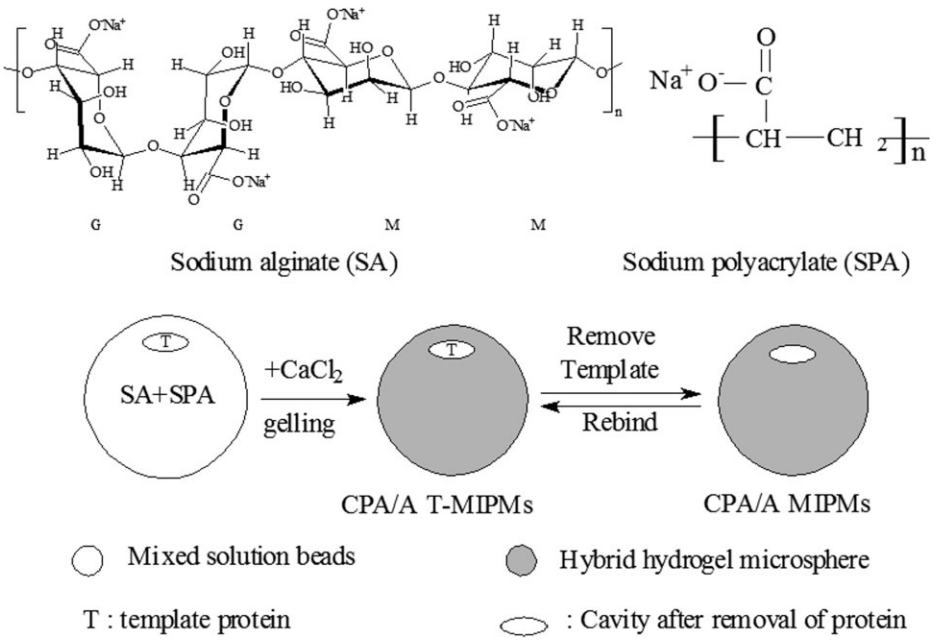

Figure 1 Schematic representation of preparation and rebinding process of CPA/A MIPMs.

$$Q = (C_0 - C_t)V/W, \quad (1)$$

where C_0 (μmol/L) is the initial BSA concentration, C_t (μmol/L) is the protein concentration at a different time, V (L) is the volume of the protein solution, and W (g) is the weight of the microspheres. The concentration of protein in the solution was evaluated by absorbance at 280 nm using a UV-1800 spectrophotometer. The detection continued until the change in concentration of the solution was undetectable, and the equilibrium adsorption capacity (Q_e) was obtained.

RESULTS AND DISCUSSION

Preparation of BSA-imprinted CPA/A hybrid polymer microspheres

Figure 1 shows the schematic representation of preparation and rebinding process of CPA/A MIPMs. First, a mixed solution of BSA, SPA, and SA is dispersed in continuous medium to form uniformity beads. The formation of interactions between the template and functional polymer may involve one or more of the following interactions: (A) hydrogen bond, (B) electrostatic interactions, or (C) hydrophobic or van der Waals interactions. Each were formed with complementary functional groups or structural elements of the template. The gelling process is initiated in the presence of $CaCl_2$, through which the template was fixed to an insoluble matrix. Then removal of the template will leave imprinted cavities and template induced functional groups on the cavities. The template molecules may be preferential and selectively rebound by the hybrid polymer.

Morphology and mechanical strength of BSA-imprinted CPA/A microspheres

An optical micrograph of CPA/A MIPMs in wet form is presented in Figure 2(a). The diameter of microspheres ranged from 30 to 100 μm, with an average diameter of 68.2 μm. In the SEM photograph [Fig. 2(b)], rumple structures could be observed on the surfaces of the microspheres because the water evaporation makes the pores close and leads to the formation of rumples. Figure 3 shows the morphology of CA and CPA/A microspheres under the same oscillation treatment. We found that CPA/A hybrid microspheres maintained their spherical shape, whereas many CA beads were damaged after oscillating for 24 h in 0.5 mol/L NaOH solution. CPA/A microspheres exhibited higher machine intensity than calcium alginate beads and survived in alkali solutions.

IR spectrum characterization

Figure 4 shows the FTIR spectrum of CA, calcium polyacrylate (CPA) and CPA/A along with the structure units of them. The strong peaks at $\sim$ 1680 and $\sim$ 1416 cm^{-1} were assigned to the asymmetric and symmetric $-COO^-$ stretching vibration of the salified carboxyl group, respectively. In calcium alginate gels, hydrogen bridges between carboxyl groups are organized in zones of fusion joining the adjacent polysaccharide chains and the chains

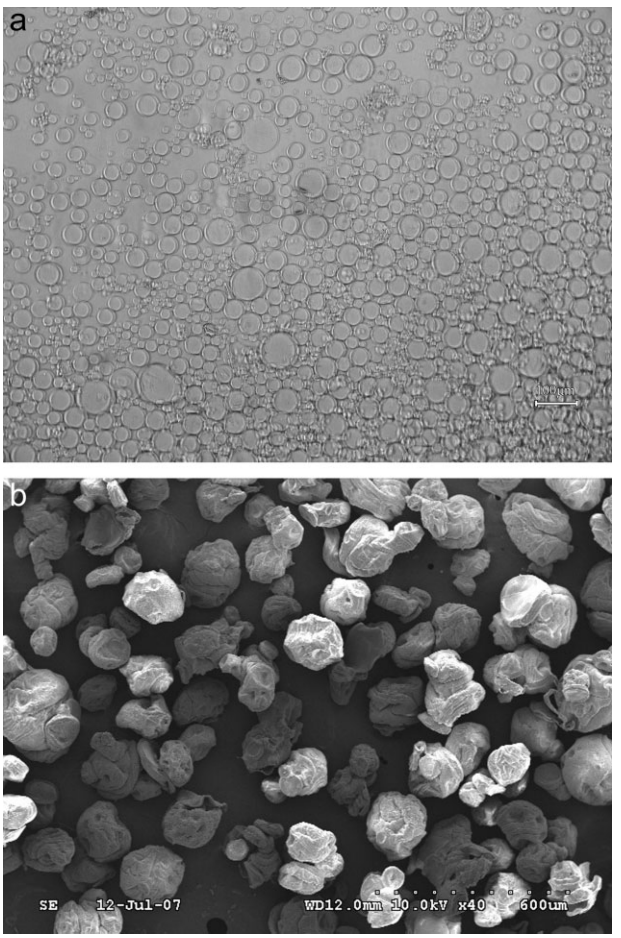

Figure 2 Micrograph of CPA/A MIPMs. (a) Optical micrograph in wet form; (b) SEM photograph in dried form.

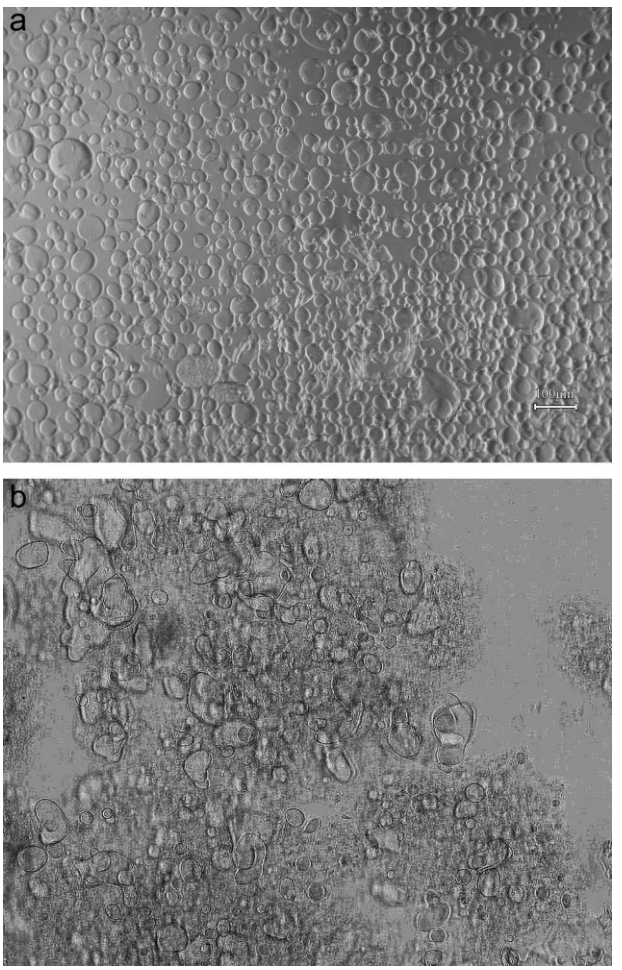

Figure 3 Micrograph of CPA/A NIPMs and calcium alginate microspheres after oscillation. (a) CPA/A NIPMs (b) CA microspheres.

aggregate due to the formation of multiple bonds with calcium ions.[15] Such a pattern resulted from the formation of coordinate bonds between the metallic ions and carboxyl and hydroxyl groups of the paranosic cycles of L-glucuronic acid. Compared with the IR spectrum of CA and CPA, new bands appear in CPA/A at 1152 cm^{-1}. The band at 1152 cm^{-1} was the secondary hydroxyl group stretch (characteristic peak of —CH—OH in CA), because the cross-linking of Ca^{2+} with —COO^- in SA and SPA influenced the secondary hydroxyl group. These results demonstrate that CPA/A was not simply the physical mixture of CPA and CA, and some hybrid components were produced via Ca^{2+} cross-linking.

Conductance titration

The quantitative description of the gelling process was determined by conductance titration. Figure 5 shows the increase rate of conductance with the amount of $CaCl_2$ solution ($\Delta\sigma/\Delta v$) vs. the volume of $CaCl_2$ solution. It is found that the gelling process of SA (structure unit $NaC_6H_7O_6$) can be divided into two stages, which correspond to the reaction of D-manuronic linkages (pKa = 3.38) and L-glucoside linkages (pKa = 3.65), respectively. There was no difference in $\Delta\sigma/\Delta v$ between deionized water and

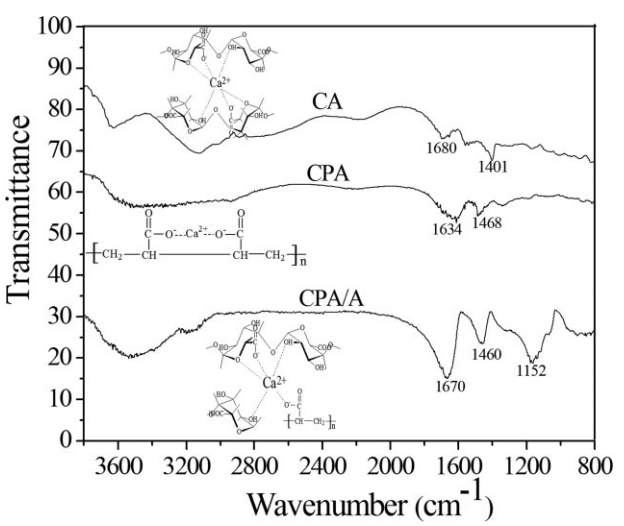

Figure 4 FTIR spectrum of CA, CPA, and CPA/A.

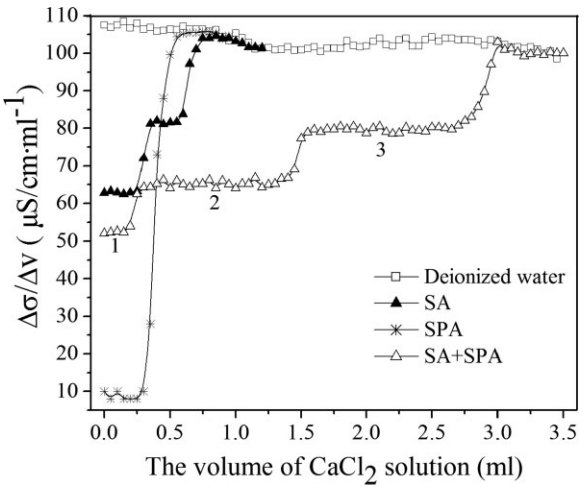

Figure 5 Conductance titration curves of SA, SPA, and SPA+SA solution titrated with 1% $CaCl_2$ solution.

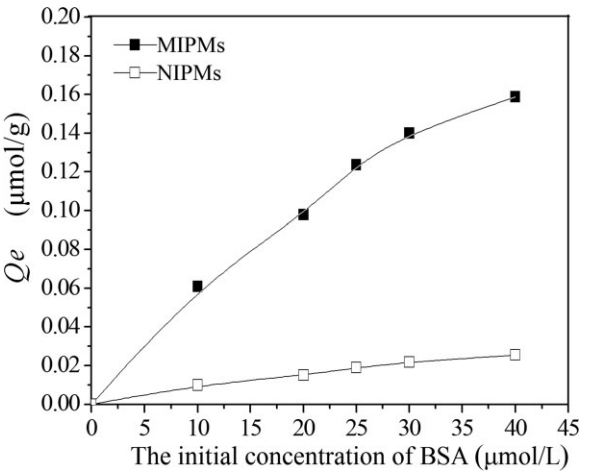

Figure 6 Rebinding isotherms of CPA/A MIPMs and NIPMs.

SA solution when Ca^{2+} was excessive. At titration terminal point, 0.0693 mmol $CaCl_2$ was consumed and the molar ratio of $CaCl_2$ to $NaC_6H_7O_6$ was 0.281. By contrast, the gelling process of SPA (structure unit $CH_2CHCOONa$) was simple. At titration terminal point, the molar ratio of $CaCl_2$ to $CH_2CHCOONa$ was 0.171 with the consumption of 0.0546 mmol $CaCl_2$.

When $CaCl_2$ solution was added dropwise into the mixture of SA and SPA, the gelling process was remarkably complicated and three stages (**1**–**3**) were observed. Stage **1** may be the reflection of gelling process of SPA, and stage **2** may be the gelling process of SPA and D-manuronic linkages of SA. Stage **3** reflected the gelling course of SPA and L-glucoside linkages. At titration terminal point, SA and SPA consumed 0.275 mmol $CaCl_2$. It is found that the $CaCl_2$ consumed was not equal to the summation of $CaCl_2$ reacting solely with SA and SPA, another 0.151 mmol $CaCl_2$ was consumed. It is speculated that the following reactions happen like this:

$$Ca^{2+} + CH_2CHCOO^- + C_6H_7O_6^- \rightarrow CH_2CHCOO{-}Ca{-}C_6H_7O_6$$

Neither of carboxyl group in SA or in SPA is easy to react with Ca^{2+} in a very low concentration, when macromolecules are in a stretched condition. In the mixed solution of SA and SPA, linear SPA will diffuse into the neighborhood of carboxyl group in SA, facilitating the cross-linking of Ca^{2+}. In this way more carboxyl groups were cross-linked and the mechanical strength of hydrogel was improved remarkably.

Rebinding isotherms of CPA/A MIPMs

As shown in Figure 6, the Q_e of CPA/A MIPMs and NIPMs increased with an increasing initial concentration of BSA. Compared with Alg/HEC microspheres with an interpenetrating network,[12] CPA/A MIPMs were more favorable for the migration and re-assembling of protein and exhibited higher Q_e. Besides, the carboxyl group (COO^-) in CPA/A MIPMs improved the pH-sensitive swelling property of the hybrid polymer microspheres, which would be of value in controlling specific binding of proteins to their recognition materials and preparing hydrogels exhibiting enzyme-like behavior.[16]

The shape of the rebinding curves was similar to the Langmuir adsorption curve, and was described as follows:

$$C_s/Q_e = C_s/Q_0 + 1/(KQ_0) \quad (2)$$

where C_s (μmol/L) is the equilibrium protein concentration, Q_0 (μmol/g) is the saturated rebinding capacity, and K (L/μmol) is the rebinding constant.

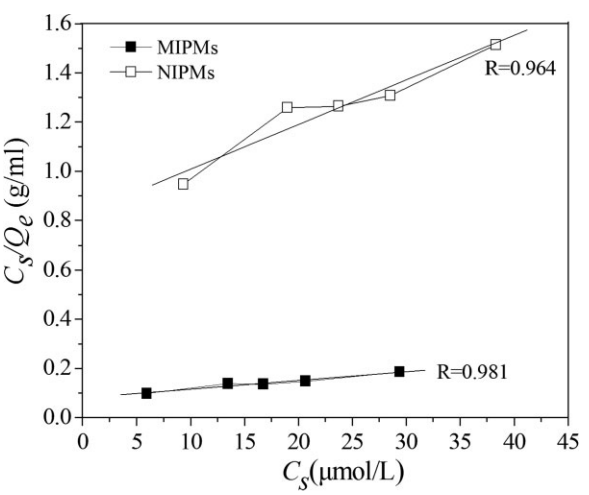

Figure 7 Curve of C_s/Q_e *vs.* C_s for CPA/A MIPMs and NIPMs.

TABLE I
Q_e and IE of CPA/A Hybrid Polymer Microspheres Cross-Linked with Different Concentration of $CaCl_2$

$CaCl_2$ concentration	3%	4%	5%	6%	7%	8%
Q_e[a] (NIMs)	45.88	42.89	39.7	32.27	28.13	25.81
Q_e (MIPMs)	96.21	128.82	146.2	121.99	107.49	98.64
IE[b]	2.097	3.003	3.683	3.78	3.821	3.824

[a] Unit of Q_e: 10^{-3} μmol/g.
[b] IE = Q_{MIPMs}/Q_{NIPMs}.

Figure 7 is the curves of C_s/Q_e versus C_s. We can see that C_s/Q_e and C_s had good linear relation in the concentration of 10–40 μmol/L ($R > 0.96$). The values of Q_0 for CPA/A MIPMs and NIPMs were 283 and 55.1 × 10^{-3} μmol/g, respectively. The values of K for CPA/A MIPMs and NIPMs were 0.0444 and 0.0220, respectively. CPA/A MIPMs exhibited obvious improvement in saturated adsorption capacities for BSA compared with NIPMs, demonstrating a higher BSA affinity for the imprinted microspheres relative to the nonimprinted ones.

Q_e and IE of CPA/A MIPMs cross-linked with different concentration of $CaCl_2$

Table I shows the Q_e and IE of CPA/A MIPMs cross-linked with different concentrations of $CaCl_2$. The Q_e increased slowly with increasing $CaCl_2$ concentration in the range of 3–5%, and decreased in a greater concentration of 5–8%. Because some of the BSA will leak along with water in gelling process, SPA was introduced into the SA matrix to form hybrid components and improved the mechanical strength of the hydrogel. A low concentration of $CaCl_2$ (<4%) was incapable of entrapping protein template effectively and creating plenty of imprinted cavities. When a concentration of 5% $CaCl_2$ solution was used, the Q_e of CPA/A MIPMs reached its maximum. However, with further increases of $CaCl_2$ concentration, there was a slight drop of Q_e for CPA/A MIPMs and NIPMs.

The increase of Q_e for MIPMs is attributed to the more imprinted cavities formed with the increase of hybrid components in a greater $CaCl_2$ concentration. At the same time, for large proteins, highly cross-linked hydrogel will limit the access of proteins toward the imprinted sites. Therefore, the concentration of $CaCl_2$ has to be optimized, and we use 5% $CaCl_2$ solution as cross-linker. For CPA/A NIPMs, no template protein was used in gelling process; therefore, the Q_e decreased with the increase of $CaCl_2$ concentration.

IE often has been used to describe the imprinting effect of MIPMs.[10] It is shown in Table I that the IE of CPA/A MIPMs increased with the increase of $CaCl_2$ concentration and reached equilibrium when $CaCl_2$ concentration was 7%. In traditional protein imprinting by polymerization, the IE reached maximum and then began to decrease with the increase of cross-linking density.[10,11] The ionic cross-linked hybrid hydrogel reported here can improve the cross-linking density and imprinting efficiency of MIPMs simultaneously.

Recognition specificity of BSA-imprinted CPA/A microspheres

The molecular recognition selectivity of molecular imprinted microspheres can be evaluated by the static distribution coefficient K_D, the separation factor α and the relative separation factor β, which are defined in Refs. 10 and 11:

$$K_D = C_P/C_S \tag{3}$$

where C_P (μmol/g) is the concentration of the target molecule on microspheres, and C_S (μmol/ml) is the concentration in the solution. Here, C_P is equal to Q_e.

$$\alpha = K_{D1}/K_{D2} \tag{4}$$

where K_{D1} and K_{D2} are the static distribution coefficients of the template and the other molecules, respectively.

$$\beta = \alpha_{MIP}/\alpha_{NIP} \tag{5}$$

where α_{MIP} and α_{NIP} are the separation factor of imprinted and nonimprinted microspheres, respectively.

The selectivity testing of BSA-imprinted CPA/A hybrid polymer microspheres was conducted under equilibrium binding conditions with the use of Lyz, EBA, Hb, and Glo as contrastive molecules. The molecular weight (M_w) and isoelectric point (pI) of BSA and contrastive proteins are listed in Table II.

TABLE II
M_w and Isoelectric Point (pI) of BSA and Contrasting Proteins

Protein	Lyz	EBA	BSA	Hb	Glo
M_w	14400	45000	67000	68000	160000
pI	10.7	4.7	4.8	6.7	6.8

TABLE III
K_D, α, and β Value of BSA-Imprinted or -Nonimprinted CPA/A Hybrid Polymer Microspheres

Proteins adsorbed	CPA/A MIPMs			CPA/A NIPMs			
	Q_e[a]	K_D	α	Q_e	K_D	α	β
Lyz	86.9	6.120	1.177	28.5	1.575	0.509	2.312
EBA	24.6	1.337	5.388	15.9	0.840	0.954	5.648
BSA	97.3	7.204	–	15.2	0.801	–	–
Hb	24.0	1.301	5.535	14.7	0.773	1.037	5.338
Glo	12.8	0.669	10.776	8.4	0.432	1.854	5.812

[a] Unit of Q_e: 10^{-3} μmol/g.

Table III shows the K_D, α, and β values of BSA-imprinted or nonimprinted CPA/A microspheres. The K_D of BSA onto BSA-imprinted microspheres was much greater than that of contrastive proteins and the separation factors (α) were greater than 5.3 except Lyz, indicating that BSA-imprinted CPA/A microspheres exhibited good recognition selectivity for the template protein. In contrast, nonimprinted microspheres showed lower K_D and α value for all proteins. The higher BSA affinity of the imprinted microspheres is attributed to the generation of BSA high affinity binding sites and complementary cavities in the matrix during gelling process. It is found that the separation factor (α) of BSA-imprinted and nonimprinted microspheres increased with the increase of the contrastive proteins' M_w.

As we mentioned in the section "Preparation of BSA-imprinted CPA/A hybrid polymer microspheres," the imprinting process of CPA/A microspheres created a microenvironment based on the complementary cavities and combined interactions of BSA and the hydrogel. In rebinding process, BSA-imprinted CPA/A microspheres favored the adsorption of BSA or its analog, leading to a high K_D. The poor match between BSA-imprinted hydrogel and the competitive proteins will result in a lower K_D and α value.

The adsorption of protein was considered to proceed in two steps, permeating and combing in. First, the adsorbed protein molecules in solution permeated into imprinted cavities, after which the molecules combined with the functional groups arranged in the cavities. A small protein like Lyz was easier to diffuse into the imprinted matrix than a large protein. However, as a basic protein, Lyz had more favorable interactions with the CPA/A hydrogel. Therefore, the K_D and α value of Lyz was much greater for both imprinted and nonimprinted microspheres. Larger proteins such as Glo could be excluded from the binding cavities created in imprinting process, which led to a lower K_D and higher α. We also found that the relative separation factor (β) of BSA-imprinted was greater than 2.3, indicating that the "imprinting" process did improve the adsorption selectivity of CPA/A hybrid polymer microspheres.

We tried to use the hydrogel as a column to evaluate the real selectivity of the imprinted microspheres. However, it is likely to be a problem when CPA/A microspheres is used as stationary phase in chromatography or solid extraction, considering the increase of column pressure in HPMC caused by swelling of the hydrogel. In our present research, we mainly focused on the preparation of the BSA-imprinted hybrid hydrogel. Even the static rebinding behavior of proteins onto the imprinted hydrogel is so complicated and it is a prerequisite to obtain the optimal conditions for the static rebinding of protein on its corresponding imprinted materials. In our further research the rebinding and recognition properties of imprinted hydrogel microspheres in different conditions will be illuminated in detail.

CONCLUSIONS

BSA-imprinted calcium polyacrylate/alginate hybrid polymer microspheres were prepared with SA and SPA by the use of $CaCl_2$ as a gelling agent in an inverse-phase suspension. IR spectrum and conductance titration analysis indicated that hybrid components were produced in these microspheres. Rebinding isotherms of imprinted microspheres proved a greater BSA affinity relative to nonimprinted ones. Selectivity tests indicated that the imprinted microspheres exhibit good recognition properties for the template protein comparing to the proteins with a similar M_w. The cross-linking density and imprinting efficiency of the hydrogel can be improved simultaneously. And the ionic cross-linked hybrid hydrogel proved to be useful for imprinting other proteins.

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