Macromolecularly Imprinted Calcium Phosphate/Alginate Hybrid Polymer Microspheres with the Surface Imprinting of Bovine Serum Albumin in Inverse-Phase Suspension

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ABSTRACT: Macromolecularly imprinted calcium phosphate/alginate hybrid polymer microspheres surface imprinted with bovine serum albumin were prepared in inverse-phase suspension by introducing the intermixture solutions of bovine serum albumin (BSA) and $CaCl_2$ in sodium alginate aqueous solution where $(NH_4)_2HPO_4$ was added beforehand. Morphology of the imprinted microspheres was observed by optical microscopy. Rebinding dynamic and thermodynamic behaviors of surface molecularly imprinted calcium phosphate/alginate polymer microspheres (CP/A SMIPMs) and embedding molecularly imprinted calcium phosphate/alginate polymer microspheres (CP/A EMIPMs) were evaluated. CP/A SMIPMs exhibited significant improvement in equilibrium rebinding capacity (Q_e) and imprinting efficiency (IE), compared to CP/A EMIPMs. The surface of CP/A SMIPMs was more competent to facilitate the migration and re-assembling of proteins. Surface specific peak and interior specific peak were proposed to describe the characteristics of surface and interior specific rebinding behaviors in rebinding dynamics. The effects of $(NH_4)_2HPO_4$ addition were investigated in detail, along with the concentration of sodium alginate and $CaCl_2$ solutions to the rebinding and imprinting property of imprinted microspheres. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 2687–2693, 2008

Key words: surface imprinting; calcium phosphate/alginate hybrid polymer microspheres; bovine serum albumin; macromolecularly imprinted polymer microspheres; imprinting efficiency

INTRODUCTION

As a promising technology for creating specific binding sites within an artificial material, molecular imprinting has made great improvements in the past decades. Molecularly imprinted polymer microspheres (MIPMs) are attracting more and more attention because MIPMs exhibit preferable sphericity, better flow characteristics and higher binding capacities compared to bulk polymers.[1–3] In recent years many examples of protein macromolecular imprinting using polymer hydrogel matrix have been demonstrated, that showed enhanced molecular recognition properties on account of the soft and wet property with specific network structures.[4–10] The challenge for the imprinting of proteins is fragility and complexity of the bulky templates. One effective approach to overcome this obstacle is to encase protein templates within a preformed polymer. Nevertheless, it has some fundamental drawbacks, such as the slow rebinding kinetics arising from the inner diffusion of proteins toward the recognition sites shaded by polymer matrix and the denaturation of proteins due to the heat produced by polymerization. Besides, the complicated procedure and incomplete removal of template proteins adversely affected the imprinting of proteins. A possible solution to the problem of protein diffusion is imprinting the surfaces of polymers or inorganic solid substrates.[11–14] Because of the more accessible recognition sites, faster mass transfer and binding kinetics, which is available in terms of the imprinting of macromolecular structures, surface imprinting has received significant attention over the past few years.

To avoid denaturation of proteins in polymerization, synthesized or natural polymers with functional groups capable of interacting with proteins could be used as the matrix for protein imprinting. Zhang et al.[15,16] reported BSA imprinted calcium alginate hydrogel microspheres in a mild circumstance. To improve the performance of the hydrogel, a small quantity of hydroxyethyl cellulose was used to associate sodium alginate to form interpenetrating networks. In our previous work BSA embedding imprinted calcium phosphate/alginate hybrid polymer microspheres (CP/A EMIPMs) were prepared by assembling BSA with sodium alginate and $(NH_4)_2HPO_4$ and utilizing $CaCl_2$ as gelling agent in

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inverse-phase suspension. Rebinding tests indicated that the microspheres exhibited an obvious improvement in rebinding capacity (Q) and imprinting efficiency (IE) comparing with those microspheres prepared without calcium phosphate. However, because BSA was almost fully entrapped, some of the protein templates couldn't be removed effectively and the diffusion of proteins toward inner recognition sites was difficult.

In this article, surface macromolecularly imprinted calcium phosphate/alginate hybrid polymer microspheres (CP/A SMIPMs) were prepared in inverse-phase suspension with surface imprinting of bovine serum albumin. Rebinding behaviors of CP/A SMIPMs and EMIPMs were evaluated.

EXPERIMENTAL

Materials

Sodium alginate (SA) was purchased from Tianjin Yuanhang Chemical Reagent (Tianjin, China), chemical grade. $(NH_4)_2HPO_4$ was purchased from Tianjin Tianhe Chemical Reagent Factory (Tianjin, China), analytic grade. Bovine serum albumin (BSA) with an isoelectric point of 4.7 and molecular weight of 66,000 was obtained from Institute of Hematology Chinese Academy of Medical Science (Tianjin, China), electrophoretic grade. $CaCl_2$, Chloroform, hexane, Tris(hydroxymethyl)- methylamine, hydrochloric acid, Span85 and Tween80 were all from Kewei Chemical Reagent Company of Tianjin University (Tianjin, China). $CaCl_2$, Chloroform, hexane and Tris(hydroxymethyl)methylamine were analytical reagents. The other reagents were chemical grade, and used without further purification. Deionized water was used all through.

Preparation of CP/A SMIPMs

In a typical experiment, a homogenously mixed solution was obtained by dissolving 0.5128 g SA and 0.15 g $(NH_4)_2HPO_4$ in 20 mL deionized water under magnetic stirring. In a 100-mL beaker, 40 mL mixture of chloroform and hexane (2 : 3, v/v) containing 0.4 g Span85 and 0.4 g Tween80 was mixed adequately with stirring for 5 min. Under magnetic stirring, the pasty solution was dispersed in the continuous medium in the beaker for 15 min. Then 15 mL $CaCl_2$ solution (5%, w/v) containing 20 μM BSA was added slowly into the system and crosslinking reaction proceeded for another 50 min. The obtained CP/A SMIPMs were washed with deionized water for three times and were preserved in 30 mL 1% $CaCl_2$ solution. All of the above procedures were carried out at room temperature.

Nonimprinted calcium phosphate/alginate hybrid polymer microspheres (denoted as NIPMs) were synthesized under the same condition as CP/A SMIPMs, though in the absence of BSA.

Preparation of CP/A EMIPMs

A homogenously mixed solution was obtained by dissolving 0.5128 g SA and 0.15 g $(NH_4)_2HPO_4$ in 20 mL 20 μmol/L BSA solution under magnetic stirring. In a 100-mL beaker, 40 mL mixture of chloroform and hexane (2 : 3, v/v) containing 0.4 g Span85 and 0.4 g Tween80, was mixed adequately with stirring for 5 min. Under magnetic stirring, the pasty solution was dispersed in the continuous medium in beaker for 15 min. Then 15 mL 5% $CaCl_2$ (w/v) solution was added slowly into the system and crosslinking reaction proceeded for another 50 min. The obtained CP/A EMIPMs were washed with deionized water for three times and were preserved in 30 mL 1% $CaCl_2$ solution. All the process was carried out at room temperature.

Elution of template BSA

Approximate 4–6 g obtained BSA imprinted microspheres were placed in a beaker containing 30 mL washing solution (a mixed solution of 1% $CaCl_2$ solution and 0.5M Tris-HCl buffer with a pH of 7.54).[15] These samples were agitated in an appropriate rate for 60 h. The washing solution was renewed three times during this period. The removal process was conducted at room temperature. NIPMs were also washed according to the washing procedure of imprinted microspheres.

Morphology of CP/A SMIPMs

Morphology of freshly prepared CP/A SMIPMs was captured using optical microscopy (Axiovert 25C, Carl Zeiss, Germany) connected with a digital camera.

Rebinding experiments

Accurately weighted amount of 1.000 g wet microspheres (using filter paper to absorb the surface water) was placed in a 25-mL flasket where 15 mL of 20 μmol/L BSA was poured beforehand. The flasket was sealed by a piece of plastic film. Rebinding capacity (Q) (μmol/g) was calculated based on the difference of BSA concentration before and after rebinding, the volume of aqueous solution and the weight of the beads according to

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

where C_0 (μmol/L) is the initial BSA concentration, C_t (μmol/L) is the BSA concentration of different

time, V (L) is the volume of BSA solution, and W (g) is the weight of microspheres. The concentration of BSA in solution was evaluated by absorbance at 280 nm using an UV-1800 spectrophotometer (Hitachi, Japan) at room temperature. The detection was ended after 60 min and then equilibrium-rebinding capacity (Q_e) was obtained.

RESULTS AND DISCUSSION

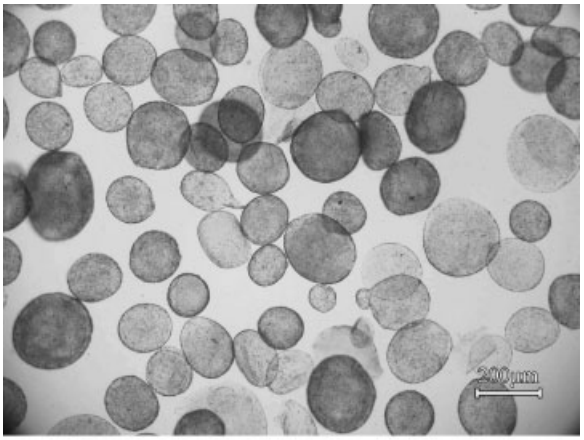
Morphology of CP/A SMIPMs

Optical micrograph of CP/A SMIPMs is presented in Figure 1. It is shown that CP/A SMIPMs were spherical and the surface of the beads was rough. In the amplified photograph of the surface [see Fig. 1(b)], a large quantity of well-distributed spiculate crystals could be observed. CP/A SMIPMs had rougher surface than calcium alginate hydrogel microspheres.[15,16]

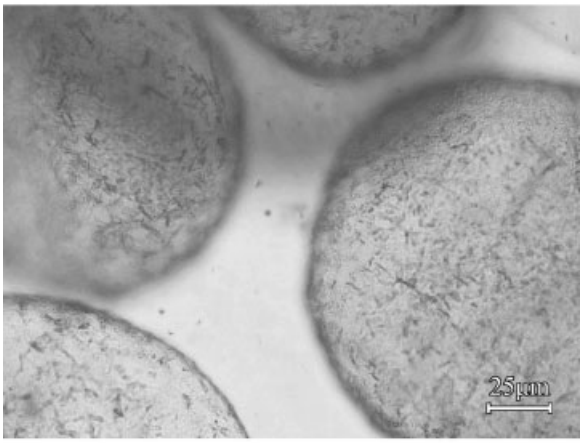
Q_e and IE of CP/A SMIPMs prepared with different amount of SA and $(NH_4)_2HPO_4$

Table I shows Q_e of CP/A SMIPMs, EMIPMs and NIPMs with different amount of SA and $(NH_4)_2HPO_4$. It is found that for the same concentration of SA, the Q_e of CP/A SMIPMs and EMIPMs varied with the mass of $(NH_4)_2HPO_4$, increasing initially but decreasing subsequently. When 0.15 g $(NH_4)_2HPO_4$ and 2.5% SA were used, the Q_e of SMIPMs was almost as two times as that of EMIPMs.

Schematic representation of preparation and rebinding process of CP/A SMIPMs is indicated in Figure 2. Most of BSA was partially entrapped or surface bound during gelling process, thus the protein could be easily removed. Besides, the surface-distributed imprinting cavities in SMIPMs facilitated the transfer of proteins. For CP/A EMIPMs, BSA was almost fully entrapped and it is very difficult to remove entirely under the same experimental conditions.[17] The BSA template couldn't be fixed when a small amount (<0.05 g) of $(NH_4)_2HPO_4$ was used.



(a)



(b)

Figure 1 Optical microscope photograph of CP/A SMIPMs in wet form (0.15 g $(NH_4)_2HPO_4$, 2.5% SA, 5% $CaCl_2$).

While $(NH_4)_2HPO_4$ increased to a certain degree, the excess calcium phosphate would hinder the transfer of proteins and occupy the imprinting cavities.

Table I demonstrates that the Q_e of both SMIPMs and EMIPMs increased with the raise of the concentration of SA solution. We attributed this fact to the increased number of imprinting cavities formed, because more hybrid composite components were

TABLE I
Q_e of CP/A SMIPMs, EMIPMs, and NIPMs with Different Amount of SA and $(NH_4)_2HPO_4$ (5% $CaCl_2$)

	SMIPMs			EMIPMs			NIPMs		
$(NH_4)_2HPO_4$ (g)	2% SA	2.5% SA	3% SA	2% SA	2.5% SA	3% SA	2% SA	2.5% SA	3% SA
0	0.0050	0.0096	0.0134	0.0071	0.0107	0.0116	0.0048	0.0072	0.0076
0.05	0.0058	0.0151	0.0199	0.0083	0.0137	0.0184	0.0054	0.0087	0.0113
0.1	0.0154	0.0270	0.0374	0.0145	0.0186	0.0238	0.0093	0.0115	0.0135
0.15	0.0280	0.0483	0.0743	0.0219	0.0240	0.0332	0.0137	0.0144	0.0211
0.2	0.0188	0.0445	0.0825	0.0172	0.0208	0.0329	0.0106	0.0133	0.0226
0.3	0.0115	0.0184	0.0415	0.0159	0.0208	0.0283	0.0114	0.0145	0.0213

Unit of Q_e: μmol/g.

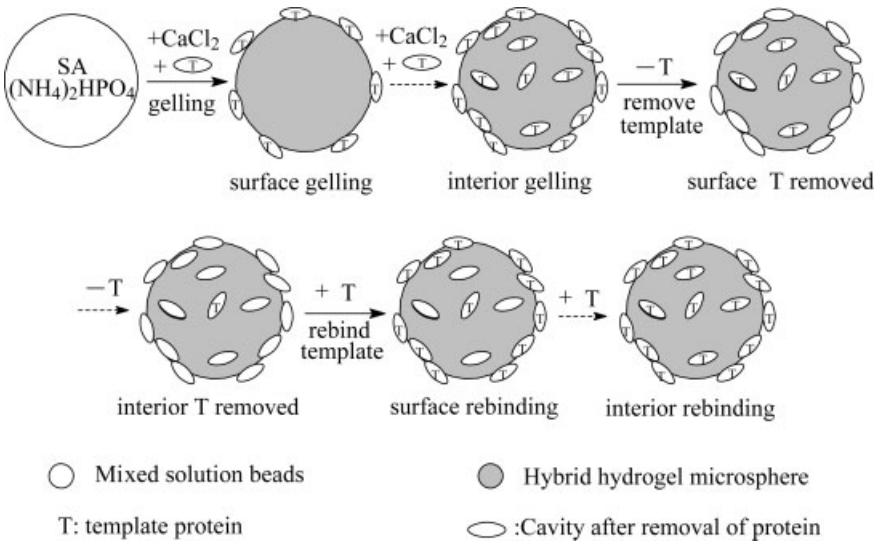


Figure 2 Schematic representation of preparation and rebinding process of CP/A SMIPMs. To prepare CP/A SMIPMs, a mixed solution of SA and $(NH_4)_2HPO_4$ is dispersed in the continuous medium to form uniformity beads. The gelling process is initiated by the mixed solution of BSA and $CaCl_2$, through which most of template protein are surface bound or partly entrapped in the hybrid polymer microspheres. Then removal of the template will leave the imprinted cavities and the template induced functional groups on the cavities. The preparation of EMIPMs is the same as SMIPMs except that BSA are mixed with SA and $(NH_4)_2HPO_4$ before gelling process, through which template protein is entrapped in hydrogel microspheres.

produced with the increase of SA. However, considering the difficulties of dispersing the high concentration of SA solution, 2.5% SA solution was used in the following experiment.

Figure 3 shows the imprinting efficiency (*IE*) of CP/A SMIPMs and EMIPMs prepared with different amount of SA and $(NH_4)_2HPO_4$. The *IE* of the microspheres was defined as follows:[15]

$$\mathrm{IE} = Q_{\mathrm{MIPMs}}/Q_{\mathrm{NIPM}}$$

Here Q_{MIPMs} is the Q_e of the imprinted microspheres and Q_{NIPM} is the Q_e of corresponding non-imprinted microspheres. It is found that the IE of CP/A SMIPMs and EMIPMs increased initially but decreased subsequently with the increase of $(NH_4)_2HPO_4$. When 0.15 g $(NH_4)_2HPO_4$ and 2.5% SA were used, the IE of SMIPMs was 3.355, as almost two times as that of EMIPMs.

Q_e and IE of CP/A SMIPMs crosslinked with different concentration of $CaCl_2$

Table II shows Q_e and IE of CP/A SMIPMs and EMIPMs crosslinked with different concentration of $CaCl_2$. The Q_e increased slowly with the raise of $CaCl_2$ in a low concentration (3–4%) but increased rapidly in a higher concentration of 4.5–5%, especially for CP/A SMIPMs. Because some of the BSA will leak along with water in gelling process, it is necessary to introduce $(NH_4)_2HPO_4$ in the SA matrix to form hybrid components which we denoted as Alg-$(CaHPO_4)_n$-Ca-Alg or Alg-$[Ca_x(PO_4)_y(OH)_z]_n$-Ca-Alg. In a low concentration of $CaCl_2$ (<4%) the few hybrid components formed are incapable of entrapping protein template effectively and creating a high number of imprinted cavities. When a concentration of 5% $CaCl_2$ solution was used both CP/A SMIPMs and EMIPMs exhibited higher Q_e. However, with the sequential increase of $CaCl_2$ there is a little increase of Q_e for SMIPMs and a slight drop of Q_e for EMIPMs.

In the case of CP/A SMIPMs this is attributed to the higher number of imprinted cavities formed with

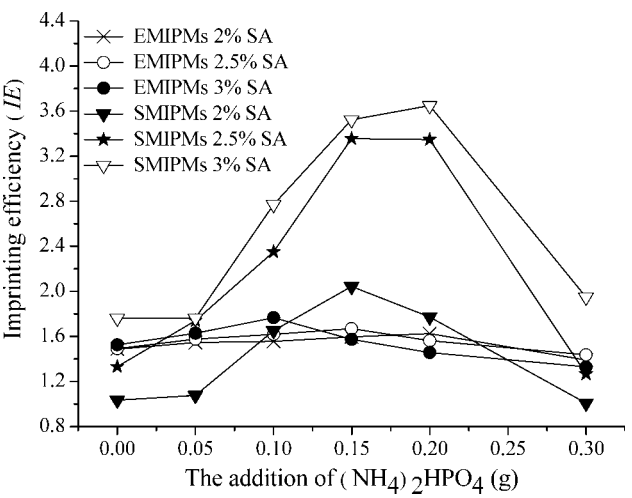


Figure 3 IE of CP/A SMIPMs and EMIPMs with different amount of SA and $(NH_4)_2HPO_4$ (5% $CaCl_2$).

TABLE II
Q_e and *IE* of CP/A SMIPMs and EMIPMs Cross-Linked with Different Concentration of $CaCl_2$

Concentration of $CaCl_2$	Q_e (μmol/g)			IE	
	SMIPMs	EMIPMs	NIPMs	SMIPMs	EMIPMs
3%	0.0162	0.0162	0.0137	1.181	1.187
3.5%	0.0177	0.0176	0.0140	1.266	1.273
4%	0.0215	0.0185	0.0141	1.528	1.312
4.5%	0.0382	0.0218	0.0143	2.673	1.526
5%	0.0480	0.0240	0.0143	3.355	1.669
6%	0.0493	0.0240	0.0147	3.365	1.636
7%	0.0513	0.0240	0.0148	3.466	1.622
10%	0.0553	0.0245	0.0154	3.590	1.591

0.15 g $(NH_4)_2HPO_4$, 2.5% SA.

the increase of $CaCl_2$ concentration, because the surface imprinted microspheres facilitated the transport of protein. For CP/A EMIPMs, it is more difficult to remove the template BSA from the hybrid polymer microspheres prepared with high concentration of $CaCl_2$ (>5%) and the excess crystals will hinder the transfer of proteins during rebinding process, although more BSA was entrapped.

It is also found from Table II that the *IE* of CP/A SMIPMs and EMIPMs varied with the concentration of $CaCl_2$ like the Q_e. When 5% $CaCl_2$ solution was used, the *IE* of CP/A SMIPMs and EMIPMs was 3.355 and 1.667, respectively. It is indicated that CP/A SMIPMs were more competent for the imprinting of proteins.

Rebinding dynamics of CP/A SMIPMs

The rebinding dynamic curves of CP/A SMIPMs, EMIPMs and NIPMs are shown in Figure 4 and it is found that the microspheres had quick rebinding rate. An abrupt increase of Q was observed in the three curves during the first 20 min. Then the Q remained constantly, and after 30 min it almost reached equilibrium. The rebinding was considered to proceed in two steps, permeating and combing in. First, the adsorbed proteins in solution permeated into molecular imprinted cavities, after which the molecules combined with the imprinted cavities. The combining process was much faster than the permeating process, so the speed of rebinding was decided basically by the speed of permeation. The speed of permeation at the second stage was much slower because with the saturation of the surface imprinting cavities the permeating molecules had to overcome the added resistance coming from the walls of the pores. CP/A SMIPMs exhibited quicker rebinding rate in comparison with EMIPMs because the surface of SMIPMs facilitated the transfer of proteins. When almost all of the molecular cavities were occupied, the rebinding would be in equilibrium. The Q_e of CP/A SMIPMs was about two times as much as that of EMIPMs, although only 3/4 amount of BSA was used for the preparation of SMIPMs.

The rebinding dynamic curves of BSA on the surface of microspheres can be described as apparent first-order kinetic:

$$-\frac{dC}{dt} = kt \tag{2}$$

Integration of the eq. (2) gives:

$$C = C_0 \exp(-kt) \tag{3}$$

So the Q with time would be:

$$Q_t = Q_e[1 - \exp(-kt)] \tag{4}$$

where t is the rebinding time (min); C_0 (μmol/L) is the initial concentration of BSA, Q_t (μmol/g) is the rebinding capacity of different time; Q_e (μmol/g) is the equilibrium rebinding capacity; k (min^{-1}) is an apparent rate constant.

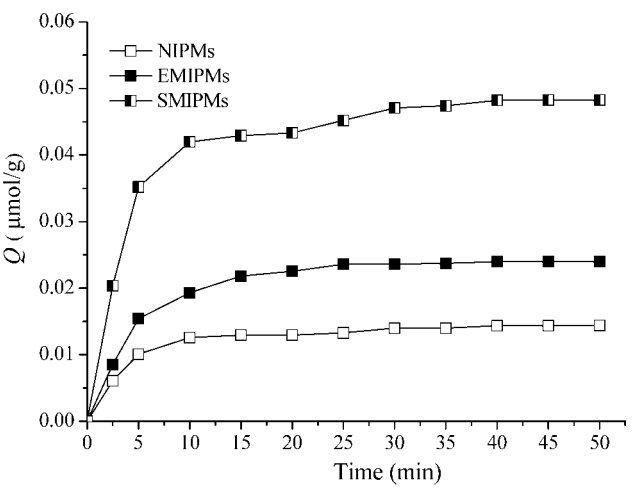


Figure 4 Rebinding kinetics curves of CP/A SMIPMs, EMIPMs, and NIPMs (0.15 g $(NH_4)_2HPO_4$, 2.5% SA, 5% $CaCl_2$).

To use the equation expediently, one can rearrange eq. (4) into the following form:

$$\ln Y = -kt,\ Y = (Q_e - Q_t)/Q_e \quad (5)$$

According to eq. (5), a plot of lnY versus t would be a straight line. From the slops of the plots, the values of k for CP/A SMIPMs, EMIPMs and NIPMs were found to be 0.1036, 0.1446, and 0.2312 min^{-1}, respectively.

Figure 5 shows the *IE* of CP/A SMIPMs and EMIPMs with a function of time. It is found that the IE of SMIPMs reached maximum at the first 5 min and the IE of EMIPMs reached maximum at about 25 min. For SMIPMs, most of BSA was partially entrapped or surface bound in gelling process. After removal of templates, imprinted cavities were situated at the surface of imprinted microspheres or in the proximity of surface, providing a complete removal of templates and an excellent accessibility to target macromolecule. For EMIPMs, BSA was entrapped in gelling process and most of the imprinted cavities distributed inside the microspheres after removal of templates, which is unfavorable for the permeation of macromolecule. The peaks of maximum IE may be the characteristic of specific binding effect. The peak at 5 min may be the characteristic of surface specific rebinding effect and is called surface specific peak, when specific rebinding is dominated by surface cavities. The peak at 25 min may be the characteristic of interior specific rebinding effect and is called interior specific peak, when specific rebinding is dominated by interior cavities.

Rebinding isotherms of CP/A SMIPMs

Figure 6 shows the rebinding isotherms of CP/A SMIPMs, EMIPMs and NIPMs. We can find that

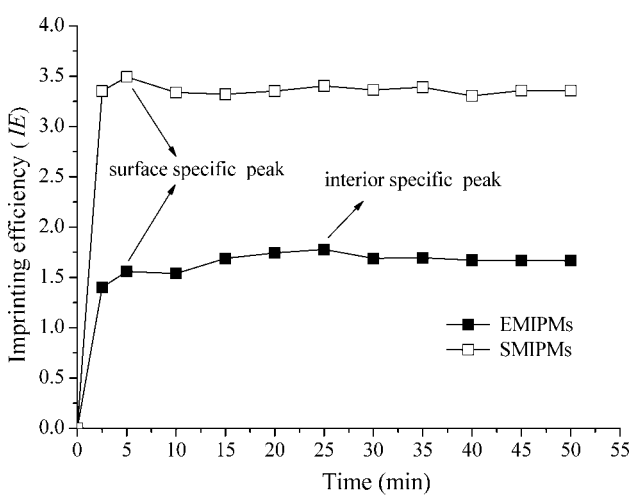


Figure 5 The IE of CP/A SMIPMs and EMIPMs with a function of time.

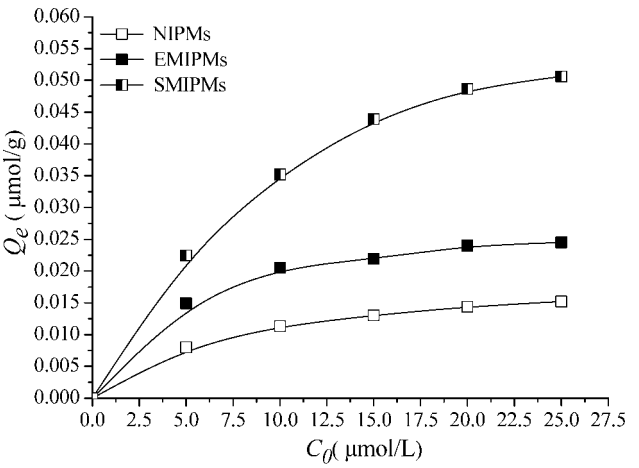


Figure 6 Rebinding isotherms of CP/A SMIPMs, EMIPMs, and NIPMs (0.15 g $(NH_4)_2HPO_4$, 2.5% SA, 5% $CaCl_2$).

when C_0 was below 15 μmol/L, the Q_e increased quickly with the increase of initial concentration of BSA, especially for SMIPMs. However, when C_0 was over 15 μmol/L, the rebinding curve became relatively flat. Both SMIPMs and EMIPMs exhibited obvious improvement in Q_e for BSA, compared with NIPMs. The results proved a higher BSA affinity for imprinted microspheres relative to nonimprinted ones. SMIPMs were more favorable for the migration and re-assembling of proteins and more competent for macromolecular imprinting.

CONCLUSIONS

BSA surface imprinted calcium phosphate/alginate hybrid polymer microspheres were prepared by inverse suspension crosslinked with $CaCl_2$, in a simple and time effective manner. Rebinding tests showed that SMIPMs exhibited an obvious improvement in Q_e and IE for BSA, compared with EMIPMs. When 0.15 g $(NH_4)_2HPO_4$, 5% $CaCl_2$ and 2.5% SA solution were used, the *Qe* of CP/A SMIPMs was as almost two times higher than the one exhibited by EMIPMs, although less template was used in preparation. CP/A SMIPMs exhibited faster rebinding rate and reached equilibrium within 30 min. We consider the surface of CP/A SMIPMs was more competent for the migration and reassembling of proteins and had more potential for macromolecular imprinting. Surface specific peak and interior specific peak were proposed to describe the characteristics of surface and interior specific rebinding effect for templates in rebinding dynamics.

References

1. Mayes, A. G.; Mosbach, K. Anal Chem 1996, 68, 3769.
2. Zhang, L. Y.; Cheng, G. X.; Fu, C. Polym Int 2002, 51, 687.

3. Pang, X. S.; Cheng, G. X. In The Protein-Imprinted Polymer Materials; Nova Science: New York, 2006; p 91.
4. Mallik, S. J.; Plunkett, S. J.; Dhel, P. K.; Johnson, R. D.; Pack, D.; Shnek, D.; Arnold, F. H. New J Chem 1994, 18, 299.
5. Lu, S. L.; Cheng, G. X.; Pang, X. S. J Appl Polym Sci 2006, 99, 2401.
6. Lu, S. L.; Cheng, G. X.; Pang, X. S. J Appl Polym Sci 2006, 100, 684.
7. Ou, S. H.; Wu, M. C.; Chou, T. C.; Liu, C. C. Anal Chim Acta 2004, 504, 163.
8. Guo, T. Y.; Xia, Y. Q.; Hao, G. J.; Song, M. D.; Zhang, B. H. Biomaterials 2004, 25, 5905.
9. Parmpi, P.; Kofinas, P. Biomaterials 2004, 25, 1969.
10. Pang, X. S.; Cheng, G. X.; Lu, S. L. Anal Bioanal Chem 2006, 384, 225.
11. Liao, J. L.; Wang, Y.; Hjerten, S. Chromatography 1996, 42, 25.
12. Glad, M.; Narrlow, O.; Sellergren, B.; Siegbahn, N.; Mosbach, K. J Chromatogr 1985, 347, 11.
13. Piletsky, S. A.; Piletska, E. V.; Chen, B.; Karim, K.; Weston, D. G.; Barrnett, P. Anal Chem 2000, 72, 4381.
14. Nicholls, I. A.; Rosengren, J. P. Bioseparation 2002, 10, 301.
15. Zhang, F. J.; Cheng, G. X.; Ying, X. G. React Funct Polym 2006, 66, 712.
16. Zhang, F. J.; Cheng, G. X.; Gao, Z.; Li, C. P. Macromol Mater Eng 2006, 291, 485.
17. Huang, J. J.; Zhao, K. Y.; Gao, N.; Cheng, G. X.; J Hebei Univ Tech 2007, 36, 21.