 DOI: 10.1002/masy.200900105

The Rebinding Properties of Bovine Serum Albumin Imprinted Calcium Alginate/Phosphate Hybrid Microspheres Via the Adjustment of pH Values and Salt Concentration

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Summary: Bovine serum albumin imprinted calcium phosphate/alginate hydrogel microspheres were prepared with sodium alginate (SA), $(NH_4)_2HPO_4$, and using $CaCl_2$ as gelling agent, bovine serum albumin (BSA) as template in inverse suspension. The optimized rebinding properties of BSA imprinted hydrogel microspheres were investigated by controlling pH value and ionic concentration from the viewpoint of adjusting the process of gelling, removing template and rebinding. The optimized pH values for the imprinting of BSA in gelling, removing template and rebinding process was 4.1, 8.3 and 4.8, respectively. The effect of NaCl concentration on the BSA rebinding was also determined. We provided a strategy to get the optimized imprinting efficiency by altering pH value and ionic concentration in a weakly ionic cross-linked hydrogel system on the process of protein's imprinting.

Keywords: bovine serum albumin; calcium phosphate/alginate hydrogel microspheres; swelling; imprinting efficiency; pH value; molecular imprinting

Introduction

Recently there has been increased research in protein imprinting using hydrogel.[1–4] Hydrogels behave better than rigid materials at macromolecular imprinting on account of the soft and wet property with polymer network structures.[5] The delicate structures exhibit oscillatory variety such as swelling in response to pH, temperature, ionic concentration etc. Similarly, proteins have a flexible structure and self-assembly performance, which probably occur to a certain degree of aggregation, deformation or structural transformation due to the slight change of environmental conditions, such as pH,[6] ionic concentration[7] and temperature etc. These responses would be of value in controlling specific binding process of proteins and hydrogels in molecular imprinting.

Tanaka and coworkers[8] described sensitive hydrogels with stimuli-sensitive recognition very similar to recognition in proteins. Pang et al.[2] imprinted bovine serum albumin (BSA) in a MAA/acryl amid matrix. The affinity toward the BSA template was measured in terms of specific adsorption capacity (i.e., the difference between the binding capacities of the imprinting and non-imprinted polymers). At the optimum, the specific adsorption was about 40% higher than for the poly(acryl amide) matrices. Ohad Kimhi et al[9] reported the interactions between lysozyme-imprinted hydrogel and their template protein using adsorption measurements, competitive adsorption experi-

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ments, and isothermal titration calorimetry (ITC). Adsorption isotherms and competitive adsorption studies detected better affinity and higher capacity of the imprinted polymer toward the template protein. Analysis of ITC data identified major differences in the binding enthalpy of lysozyme when the imprinted and the non-imprinted polymers were compared. Daniel M. Hawkins et al.[10] developed bovine haemoglobin imprinted polyacrylamide hydrogels and explored in detail a variety of template removal strategies. The hydrogel MIP exhibited specific selectivity to the template molecule over structurally similar proteins. Gokhan Demirel et al.[11] prepared pH/temperature sensitive imprinted ionic poly (N-tert-butylacrylamide-coacrylamide/maleic acid) hydrogels for BSA. Hydrogels imprinted with BSA showed higher adsorption capacity and specificity for BSA than non-imprinted hydrogels. Adsorption studies showed that other stimuli, such as pH, temperature and initial BSA concentration also influenced the BSA adsorption capacity of both non-imprinted and imprinted hydrogels.

Since the configuration and property of proteins and hydrogels along with their interactions can be adjusted by altering the environmental conditions, a strategy could be provided to design hydrogels with switch-like behavior in its affinity for a protein corresponding to the gelling, removing template and rebinding processes. The imprinting effects of protein imprinted polymer hydrogel could be optimized by the change of environmental conditions, such as pH, ionic concentration etc. The adjustment of hydrogels includes the condition of gelling and the degree of swelling of the hydrogel etc. The adjustment for proteins includes the conformation, aggregation and charge properties of proteins etc. Thus a few minor adjustments of environmental conditions will possibly alter the imprinting properties of protein imprinted hydrogel microspheres.[12]

In order to obtain an optimized specific rebinding, the intermolecular interaction of protein and hydrogels in three processes is quite different. In gelling process, it is necessary to get more molecular pre-assembly of proteins and hydrogel polymers through which the adjustment of the protein's shape and charge distribution. The hydrogels should have right strength to maintain activity of proteins. The experimental conditions should favor the collapse of the hydrogel and benefit the interaction between hydrogel and the template protein. In the process of removing template, it is crucial to minimize or eliminate the interactions between template proteins and hydrogel polymers. The conditions should favor the swelling of the gel and reduce the interaction between proteins and hydrogels as far as possible. Besides, the template proteins had better exhibit volume shrinkage. In rebinding process, it is vital to get more specific interaction of proteins and hydrogel polymers. Proper adjustment of protein's configuration and appropriate swelling or shrinking of the matrix is necessary to facilitate the migration of protein.

However, few works have been done about protein imprinting in gels in the viewpoint of adjusting the interaction between proteins and imprinting hydrogel matrix during the processes. The traditional preparation process of polymerization was complicated and time consuming, so it is very difficult to investigate the effect of environmental conditions on the adsorption and imprinting efficiency of imprinted hydrogels. In our previous work, BSA-imprinted calcium phosphate/alginate polymer microspheres (CP/A MIPMs) were prepared by assembling BSA with SA and $(NH_4)_2HPO_4$ and utilizing $CaCl_2$ as gelling agent.[13,14] Adsorption tests indicated that the imprinted microspheres exhibited an obvious improvement in rebinding capacity comparing with non-imprinted microspheres. The different rebinding properties of BSA surface imprinted microspheres (SMIPMs) and BSA embedding imprinted microspheres (EMIPMs) were preliminarily investigated. The imprinting efficiency (*IE*), which was defined as the ratio of the rebinding amount of imprinted polymers to

non-imprinted ones, was often utilized to estimate the specific rebinding properties of imprinted materials.[2,11] The aim of present work is to research the appropriate experimental conditions from the viewpoint of adjusting the process of gelling, removing template and rebinding in order to get the optimal *IE*. The rebinding properties of BSA imprinted hydrogels were discussed in detail according to the relationship between the swelling ratio of hydrogels and pH values along with the interaction of them.

Experimental Part

Materials

Sodium alginate (SA) was purchased from Tianjin Yuanhang Chemical Reagent Co., Ltd. $(NH_4)_2HPO_4$ was purchased from the Tianjin Tianhe Chemical Reagent Factory. BSA ($M_w = 66000$, pI = 4.8) was obtained from the Institute of Hematology, Chinese Academy of Medical Science, electrophoretic grade. $CaCl_2$, Chloroform, hexane, Tris(hydroxymethyl)-methylamine, hydrochloric acid were all from Kewei Chemical Reagent Company of Tianjin University (Tianjin, China). $CaCl_2$, Chloroform, hexane and Tris(hydroxymethyl)methylamine were analytical reagents.

Preparation of CP/A EMIPMs

To prepare CP/A EMIPMs (BSA embedded molecular imprinted calcium phosphate/alginate polymer microspheres), 0.5128 g SA and 0.15 g $(NH_4)_2HPO_4$ were dissolved in 20 mL 20 μmol/L BSA solution (pH 3.6-8.6). In a 100 mL beaker, a 40 mL mixture of chloroform and hexane (2:3, v/v) containing a certain amount of Span 85 and Tween 80 was mixed adequately with stirring.[13] The pasty solution was dispersed in the continuous medium in the beaker. 15 min later, 20 mL 5% (w/v) $CaCl_2$ solution was added slowly into the system and the crosslinking reaction proceeded for another 50 min. The obtained microspheres were washed fully with deionized water to dispose of waste.

In order to remove BSA, the obtained microspheres were placed in a beaker containing 30 mL elution solution — a mixture of 1% (w/v) $CaCl_2$ solution and Tris-HCl buffer (0.05 M Tris with a certain pH). The samples were agitated gently for 60 h, during which the wash buffer was renewed every 12 h. All of the above processes were carried out at room temperature.

Non-imprinted calcium phosphate/alginate polymer microspheres (CP/A NIPMs) were prepared according to the same recipe except no protein was added, and were washed according to the wash process of CP/A EMIPMs.

Preparation of CP/A SMIPMs

The preparation of CP/A SMIPMs (BSA surface molecular imprinted calcium phosphate/alginate polymer microspheres) followed the same process as described elsewhere.[14] Briefly, 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 (pH 3.6-8.6, adjusted by HCl and NaOH solutions). In a beaker, a 40 mL mixture of chloroform and hexane (2:3, v/v) containing a certain amount of Span 85 and Tween 80 was mixed adequately. In the crosslinking reaction, 20 mL mixture solution containing 5% (w/v) $CaCl_2$ and 20 μmol/L BSA (pH 3.6-8.6) was added, while the other steps were the same as for CP/A SMIPMs.

Equilibrium Swelling Ratio of CP/A Microspheres

The equilibrium swelling ratio (*ESR*) of CP/A hybrid microspheres was measured by calculating the weight of the wet and dried beads according to the reference.[13] The *ESR* of CP/A microspheres under different conditions was calculated as:

$$ESR = \frac{W_w - W_d}{W_d}, \quad (1)$$

where W_w and W_d represent the mass of wet and dried microspheres, respectively.

Particle Size and Distribution of BSA

The particle size and distribution of BSA were analyzed by laser particle size analyzer

(BI-90 Plus, Brookhaven Instruments) at 25 °C.

BSA Rebinding

An accurately weighed amount of 1.000 g wet microspheres was placed in a 25 mL flask with 15 mL of 20 μmol/L protein solution with a certain pH and salt concentration. The rebinding capacity (Q) (μmol/g) was calculated according to

$$Q = (C_0 - C_t)V/W, \quad (2)$$

where C_0 (μmol/L) is the initial protein 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 polymer 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 rebinding capacity (Q_e) was obtained. The imprinting efficiency (IE) of the microspheres was defined as follows:

$$IE = Q_{MIPMs}/Q_{NIPMs}, \quad (3)$$

where Q_{MIPMs} and Q_{NIPMs} are the Q_e of the imprinted and corresponding non-imprinted microspheres.

Result and Discussions

Preparation and Characterization of BSA Imprinted CP/A Microspheres

The schematic representation of preparation and rebinding process of CP/A SMIPMs was described in literature.[14] 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 hydrogel microspheres. Then removal of the template will leave the imprinted cavities and the template induced functional groups on the cavities. Schematic representation of preparation and rebinding of CP/A EMIPMs is listed in Figure 1. BSA are mixed with SA and $(NH_4)_2HPO_4$ before gelling process and template protein is entrapped in hydrogel microspheres.

The optical micrographs of CP/A SMIPMs and EMIPMs are presented in Figure 2. It is shown that the BSA imprinted CP/A beads was rough and a large quantity of well-distributed spiculate crystals could be observed. There was no obvious morphology difference for CP/A EMIPMs and SMIPMs. The amplified

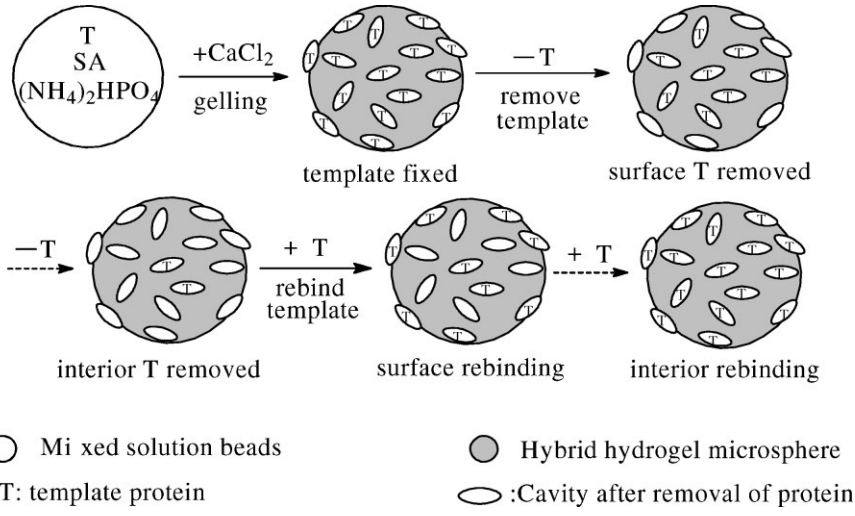


Figure 1.
Schematic representation of preparation and rebinding of CP/A EMIPMs.

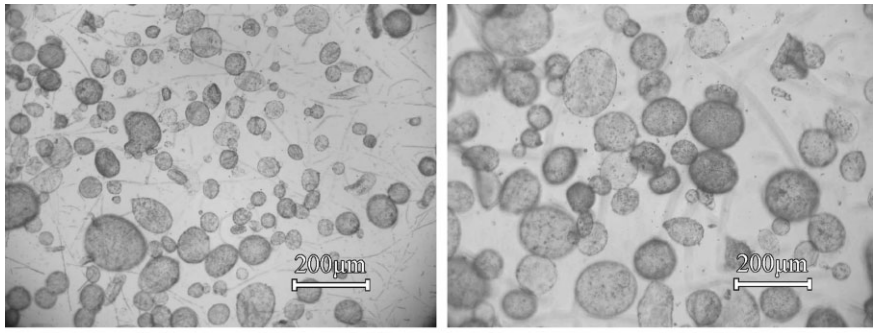


Figure 2.
Optical microscope photograph of CP/A EMIPMs and SMIPMs in wet form.

photograph of the surface microspheres was fully described in our previously published paper by SEM, TEM and ESEM.[13] The diameter of the wet BSA-imprinted CP/A microspheres were passed through sieves of different mesh sizes and the microspheres with a diameter in the range 20–200 μm were collected for later rebinding experiment.

In order to prove that the hybrid component made the matrix more stable, the same amount (0.5g) of calcium alginate and calcium phosphate/alginate microspheres were placed respectively in two beakers filled with 30ml aqueous solution with pH = 3.5 and five pieces of zeolite. Both beakers were oscillated for 24h at 25 °C with an oscillation rate of 150 r/min. Then the microspheres were collected and observed by optical microscopy. Figure 3 shows the morphology of calcium alginate and calcium phosphate/alginate microspheres under the same oscillation treatment. It is found that calcium phosphate/alginate microspheres maintained their spherical shape, while many calcium alginate beads were damaged after oscillating.

The IR spectrum of calcium alginate (CA), calcium phosphate (CP) and calcium phosphate/alginate (CP/A) was described in our previous published paper, which demonstrated that some hybrid components were produced in the CP/A hydrogel.[13]

ESR of CP/A Microspheres Under Different pH Values and Ionic Concentrations

Figure 4 shows the *ESR* of CP/A microspheres under different pH values before wash, after wash and after rebinding. There was little difference for the *ESR* of microspheres prepared with different pH values

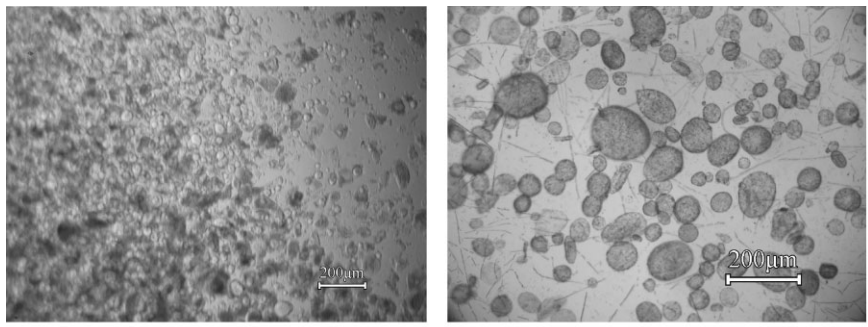


Figure 3.
Micrograph of calcium alginate and calcium phosphate/alginate after oscillation.

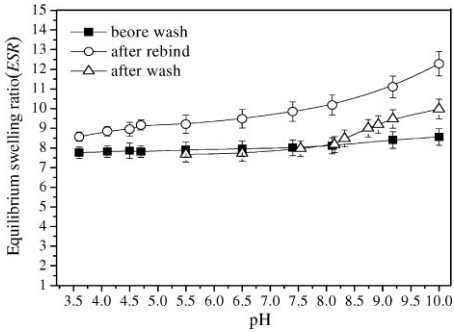


Figure 4.
The equilibrium swelling ratio (*ESR*) of CP/A microspheres under different pH values before wash, after wash and after rebinding.

before wash. The *ESR* increased with the increased pH value of Tris-HCl buffers, and in more alkaline regions it increased more rapidly. Under all pH conditions after rebinding, the *ESR* (samples were washed with pH 7.54 Tris-HCl buffers) increased. There was no visible difference in the *ESR* before and after wash with pH 7.54 Tris-HCl buffers. This is mainly attributed to the carboxylic acid groups of the alginate, which transform into the ionized form (COO^-) as the pH value of the aqueous solution increases. The electrostatic repulsion between the ionized groups causes the hydrogels to swell.[15] All the tests showed that alginate-based hydrogel exhibited pH-sensitive swelling property.

Figure 5 shows the *ESR* of CP/A microspheres under different NaCl and $CaCl_2$ concentration after wash. It is found that in NaCl solutions the *ESR* increased rapidly with the increase of NaCl in a lower concentration, and then reached equilibrium with the NaCl concentration of 0.2 mol/L. In $CaCl_2$ solutions the trend was reversed. In NaCl solutions, the calcium ions were partly replaced by sodium ions and caused increased swelling. $CaCl_2$ solution can prevent the hydrogel from swelling too much. The hydrogel did not get dissolved in the medium of varying pH and NaCl concentration through ion-exchange process like calcium alginate beads as some literatures described.[16] It was the hybrid component that made the matrix more stable.

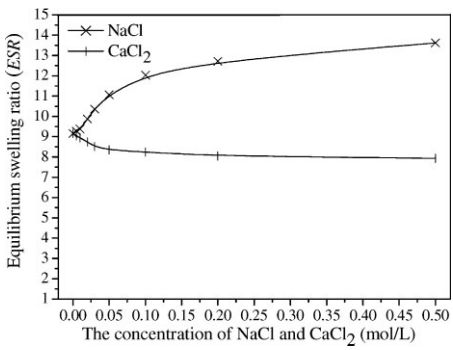


Figure 5.
The *ESR* under different NaCl and $CaCl_2$ concentrations after washed in pH 8.32 Tris-HCl buffer. The swelling test was performed in pH 4.8 aqueous solutions.

Effect of pH Values on the Q_e of CP/A NIPMs, EMIPMs and SMIPMs in the Process of Gelling

Figure 6 shows the Q_e of CP/A NIPMs, EMIPMs and SMIPMs gelated in different pH values of BSA aqueous solution or water. The maximum Q_e for imprinted microspheres was observed when the pH value of BSA solution in gelling was 4.1. More acidic and more basic pH solutions caused significantly lower Q_e. The imprinted microspheres exhibited higher Q_e than non-imprinted ones. Proteins have positive charge under their isoelectric points and have negative charge over the isoelectric points. When the pH value is

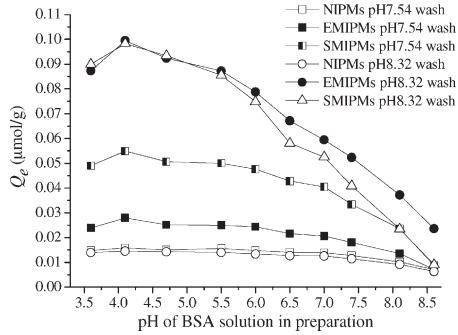


Figure 6.
The Q_e of CP/A NIPMs, EMIPMs and SMIPMs under different pH values of BSA aqueous solution in gelling. The rebinding test was performed in pH 4.8 BSA aqueous solutions.

over the isoelectric point (4.8) of BSA, the interactions between BSA and negatively charged SA could be reduced. A lower pH value than the isoelectric point could benefit the interactions between BSA and SA.

Effect of pH Values on the Q_e of CP/A NIPMs, EMIPMs and SMIPMs in Washing Process

It is reported that the rate of BSA diffusion out of alginate microspheres increased with the increase of pH, due to the increased negative charge on the protein.[17] The washing buffer with a higher pH value may be convenient to get rid of BSA from the microspheres considering the swelling of calcium alginate gels and the decreased interactions between BSA and the matrix in a high pH. Figure 7 shows the Q_e of CP/A NIPMs, EMIPMs and SMIPMs under different pH values of Tris-HCl buffer. It is found that with the increase of pH values of Tris-HCl buffer the Q_e of imprinted microspheres increased rapidly first and then reached maximum at the pH of 8.32-8.84. With the continue increase of pH value of Tris-HCl buffer, the Q_e of imprinted microspheres decreased. For non-imprinted microspheres, the Q_e exhibited slightly decreased with the increase of pH value of Tris-HCl buffer.

In the process of gelling, BSA templates were entrapped (mainly for EMIPMs) or partially entrapped (mainly for SMIPMs). In order to remove BSA, it is necessary to break the interaction between BSA and the matrix. As discussed previously, in a pH over the isoelectric point of BSA, the interaction between BSA and the matrix could be reduced. On the other hand, the appropriate swelling of the hydrogel may favor the migration of protein and more protein templates were removed, which generated more imprinted cavities. Figure 4 showed that the *ESR* of the matrix increased distinctly after washed with pH 8.32 Tris-HCl buffer, but there was no visible difference for the *ESR* after washed with pH 7.54 Tris-HCl buffer. For the EMIPMs, BSA was almost fully entrapped in gelling process and it was very difficult to remove adequately under a lower swelling condition washed by the buffer with a pH of 7.54. For the SMIPMs, BSA was partially entrapped or surface bound and the template protein could be more easily removed.

Effect of pH on the Q_e of CP/A NIPMs, EMIPMs and SMIPMs in Rebinding Process

Figure 8 shows the Q_e of CP/A NIPMs, EMIPMs and SMIPMs under different pH values of BSA aqueous solution in rebinding process. The maxima Q_e of CP/A polymer microspheres were observed at pH 4.8, just at the isoelectric point of BSA (4.8). The decrease in Q_e at more

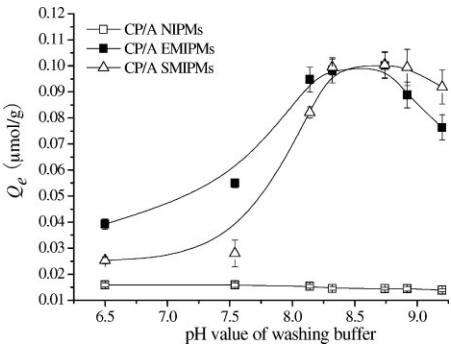


Figure 7.
The *Qe* of CP/A NIPMs, EMIPMs and SMIPMs under different pH values of BSA aqueous solution in gelling. The rebinding test was performed in pH 4.8 BSA aqueous solutions.

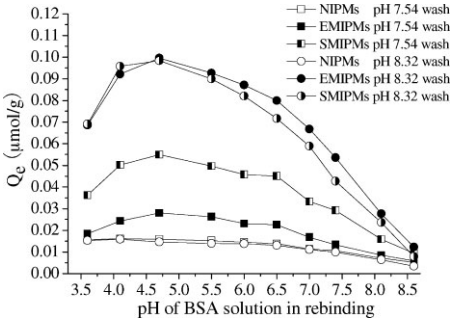


Figure 8.
The Q_e of CA/P SMIPMs, EMIPMs and NIPMs under different pH values of BSA aqueous solution in rebinding. The imprinted hybrid microspheres were gelated in pH 4.1 BSA aqueous solutions and NIPMs were gelated in pH 4.1 water.

basic pH values can be attributed to the decrease of possible electrostatic interactions between BSA and the negatively charged matrix. When the pH value is over the isoelectric point of BSA, proteins have negative charges, thus potentially reducing the interactions between BSA and the negatively charged matrix. On the other hand, near the pKa value of alginate, the alginic acid gel was formed and the migration of protein was blocked, and parts of the imprinted sites and cavities could be destroyed. Thus, the overall result of these factors is that Q_e reached the maxima values in media at pH 4.8.

Effect of pH on the *IE* of CP/A EMIPMs and SMIPMs in the Process of Gelling, Removing Template and Rebinding

The effect of pH value on the swelling of hydrogel and the interaction of BSA and the hydrogel had been discussed briefly in the above description. The effect of pH values on the aggregation, deformation or structural transformation of proteins was even more significant for protein imprinting.[18] As a result of aggregate formation for proteins, effective diameters can be enhanced and the configuration may be unacceptable for imprinting. The relationship between pH values and effective diameters (*ED*) of BSA is shown in Figure 9. The *ED*, which was reported as the average of quintuple measurements, would be an approximate indication of actual size of the aggregation of BSA.

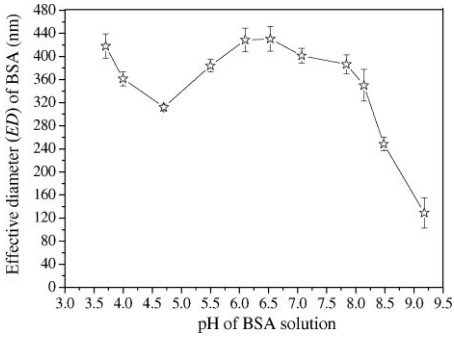


Figure 9.
Effective diameters (*ED*) of BSA in under different pH values.

When the pH values varied from 3.5 to 6.0, the minimum *ED* of BSA was observed at pH 4.7. The *ED* of BSA decreased with the increase of pH value when pH was higher than 6.0.

Figure 10 shows the *IE* of imprinted CP/A hydrogels under different pH values of BSA aqueous solution in the three processes. One can find the relationship between *IE* and *ESR* of the hydrogels, along with the treated effective diameter (*TED* =*ED*/35) of BSA. There was little difference for the *ED* of BSA and *ESR* of polymer microspheres gelated in the pH value range of 4.1-7.0. In the gelling process, a lower pH value (4.1) was necessary to get a fine assembly of the charged BSA and SA, through which the unique details of the protein's shape and charge distribution were memorized. In such a pH values, the template BSA possessed a less *ED*, the imprinting matrix exhibited a less *ESR*. The *IE* of BSA-imprinted CP/A microspheres reached maximum at pH 4.1 in gelling. Interesting is that with the increase of pH value of Tris-HCl buffer from 7.54 to 8.32, the maximal *IE* of CP/A SMIPMs increased from 3.468 to 6.75, and EMIPMs increased

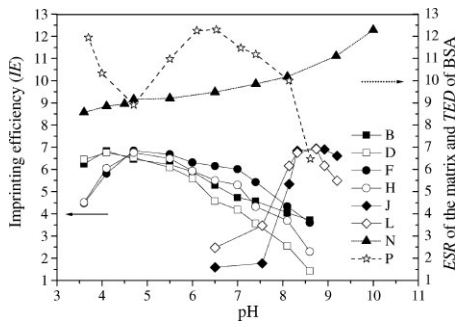


Figure 10.
The *IE* of CP/A SMIPMs and EMIPMs under different pH values of BSA aqueous solution in the process of gelling, removing template and rebinding B: EMIPMs, 8.32 wash 4.7adsorb, pH vary in gelling D: SMIPMs, 8.32 wash 4.7adsorb, pH vary in gelling F: EMIPMs, 4.1 gelate 8.32 wash, pH vary in rebinding H: SMIPMs, 4.1 gelate 8.32 wash, pH vary in rebinding J: EMIPMs, 4.1 gelate 4.7 adsorb, pH vary in washing L: SMIPMs, 4.1 gelate 4.7 adsorb, pH vary in washing N: *ESR* after rebinding (short dot line) P: treated effective diameter (*TED*) of BSA (short dot line, *TED*=*ED*/35).

from 1.766 to 6.83 respectively. In the next section it will be discussed in detail.

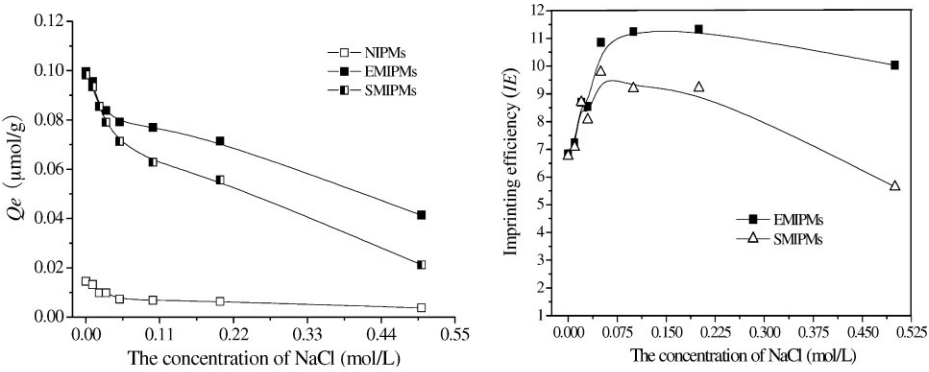
If a hydrogel is made to swell it becomes unfavorable for the rebinding of target molecules.[19] It is necessary to minimize the interaction of BSA and the matrix for the removal of template protein. A pH 8.32 Tris-HCl buffer was optimized in view of the proper swelling of the hydrogel and the reduced interaction between BSA and the matrix. The *ESR* of hydrogel before and after washed was 7.809 and 8.478, respectively. In such a pH value the *ED* of BSA was only 83% of that in pH 4.1. The small *ED* of template protein favored the diffusion of BSA. It was really an effective way to remove macromolecular template from a hydrogel via controlling its swell conditions.

SMIPMs represented higher *IE* than EMIPMs at under the pH of 8.14, and when the pH was over 8.84, the trend reversed. For EMIPMs, BSA was almost fully entrapped in gelling process and it was very difficult to remove adequately under a lower swelling condition washed by the buffer with a pH of 7.54. For SMIPMs, BSA was partially entrapped or surface bound and the template protein could be more easily removed. With the increased *ESR* of the matrix, on the one hand, BSA was removed more easily; on the other hand, the imprinted sites and cavities could be destroyed or deformed. The overall result of the two factors is that the *IE* of the hydrogel reached maximum when the pH of Tris-HCl buffer was 8.32.

In rebinding process, appropriate swelling of the matrix is needed to favor the migration of protein. However, if the matrix swells too much, it will lead to disrupted imprinting sites and deformed cavities. The *ESR* of polymer microspheres after rebinding at pH 4.8 BSA solution was 9.161. The *ED* of BSA at pH 4.7 was about 311.85 nm, reaching the minimum in a pH value range from 3.5 to 6.0. In such an *ESR* there was almost no frustration for the migration of proteins and the dominant effect during the rebinding process was the interaction between BSA and the matrix. The maximum *IE* was observed at the isoelectric point of BSA (4.8). There was no significant distinction for the *IE* of SMIPMs and EMIPMs at pH 5.0-6.5, especially for those washed with pH 7.54 Tris-HCl buffer. In fact the pH value reached about 5.5-6.2 after sodium alginate was dissolved, sc. the gelling process was performed in neutral medium. The gelling process created imprinting cavities, which could preferentially rebind template proteins. When the pH of BSA aqueous solution was close to the pH value of gelling, conformational memory of the hydrogel and BSA favored the rebinding. At other pH values, variation of the charge on protein molecules and conformational changes of BSA and hydrogel caused a decrease in *IE*. Although the complicated interaction is still need to investigate, we summarized the optimized pH values for the imprinting of proteins with a hydrogel considering the gelling, removing template and rebinding processes.

Effect of Ionic Concentration in Rebinding Process

Figure 11(a) shows the Q_e of BSA-imprinted and non-imprinted CP/A microspheres as a function of NaCl concentration in the BSA aqueous solution. For all samples the Q_e decreased with the increase of NaCl concentration, especially for the NaCl concentration range of 0-0.1mol/L. The Q_e of SMIPMs decreased more sharply than EMIPMs and NIPMs with the increasing NaCl concentration from 0.1 to 0.5mol/L. The distinct decrease of Q_e could be explained in two ways: (I) Salt ions interacted with BSA via charge-charge interactions and masked the rebinding between BSA the matrix. (II) The calcium ions could be replaced by sodium ions and resulted in excessive swelling of the matrix, which destroyed imprinted cavities and was unfavorable for the rebinding of proteins. EMIPMs can maintain more imprinted sites and cavities than SMIPMs in a swelling condition, so a higher Q_e was observed for EMIPMs. No significant decrease of Q_e was observed for the NIPMs with the increasing NaCl concentration from 0.1 to 0.5 mol/L.



(a) the *Qe* of CP/A NIPMs, EMIPMs and SMIPMs (b) the *IE* of CP/A SMIPMs and EMIPMs

Figure 11.
The *Qe* and *IE* of BSA imprinted and non-imprinted CP/A microspheres under different NaCl concentration in rebinding. The imprinted hybrid microspheres were gelated in pH 4.1 BSA aqueous solutions and NIPMs were gelated in pH 4.1 water. Samples were washed with pH 8.32 Tris-HCl buffer and rebinding test was performed in pH 4.8 BSA aqueous solution.

It is found in Figure 11(b) that the *IE* of both imprinted microspheres increased initially with the increase of NaCl concentration range of 0-0.05 mol/L, and then decreased with the sequential increase of NaCl concentration range of 0.1-0.5 mol/L. The *IE* of SMIPMs decreased more sharply than EMIPMs with the increasing NaCl concentration from 0.05 to 0.5 mol/L, indicating that the EMIPMs were more stable and could maintain more imprinted sites and cavities. SMIPMs exhibited more spiculate peaks than EMIPMs, indicating that the surface imprinted CP/A microspheres were more sensitive to the alteration of NaCl concentration. The reason why the *IE* of both imprinted microspheres reached the maxima is under investigating.

Rebinding Dynamics

The rebinding dynamic curves of CP/A NIPMs, EMIPMs and SMIPMs are shown in Figure 12 and it is found that the microspheres had quick rebinding rate. The SMIPMs exhibited quicker rebinding rate in comparison with EMIPMs during the first 240 min because the surface of SMIPMs facilitated the transfer of proteins. The Q_e of CP/A NIPMs, EMIPMs and SMIPMs was 0.01455, 0.09952 and 0.0983 μmol/g respectively.

Rebinding Isotherms

Figure 13 shows the rebinding isotherms of CP/A EMIPMs, SMIPMs and NIPMs. One can find that the Q increased with the increase of the initial concentration of BSA. The shape of rebinding curves was similar to the Langmuir rebinding curve. The Langmuir isotherms equation can be described as:

$$C_s/Q_e = C_s/Q_0 + 1/(KQ_0), \quad (4)$$

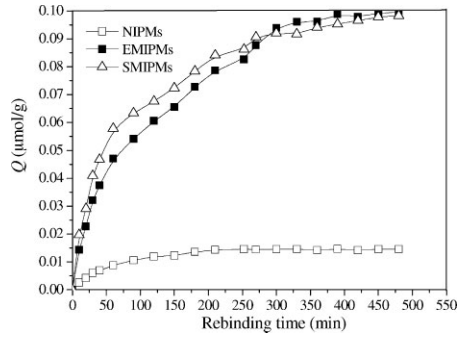


Figure 12.
The rebinding dynamic curves of CP/A NIPMs, EMIPMs and SMIPMs. The imprinted microspheres were gelated in pH 4.1 BSA aqueous solutions and NIPMs were gelated in pH 4.1 water. Samples were washed with pH 8.32 Tris-HCl buffer and rebinding test was performed in pH 4.8 BSA aqueous solution.

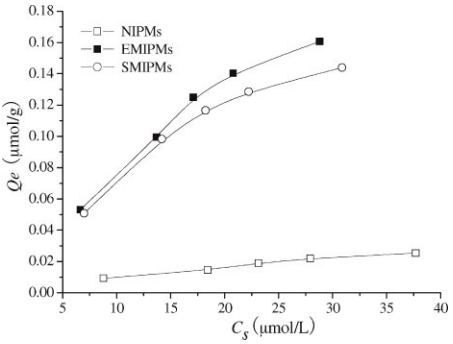


Figure 13.
Rebinding isotherms of CP/A EMIPMs, SMIPMs and NIPMs.

where *Cs* (μmol/L) is the equilibrium protein concentration, Q_0 (μmol/g) is the saturated rebinding capacity and *K* (L/μmol) is rebinding constant. From the curve of Cs/Q_e versus *Cs* the obtained saturated rebinding capacity Q_0 for CP/A NIPMs, EMIPMs and SMIPMs were 0.05967, 0.42081 and 0.30367 μmol/g, respectively. The values of K for NIPMs, EMIPMs and SMIPMs were 0.01969, 0.02285 and 0.03159, respectively. The higher Q_0 of imprinted beads was attributed to the complexation interaction and complementary cavities between BSA molecules and the imprinted microspheres.[13] The results proved a higher BSA affinity for the imprinted microspheres relative to non-imprinted ones.

Even the rebinding behavior of templet protein onto the imprinted hydrogel is so complicated and it is a prerequisite to obtain the optimal conditions for the best rebinding of protein on its corresponding imprinted materials. In our later research the selective rebinding or recognization properties will be done in a proper way in a mixed protein solutions.

Conclusion

Bovine serum albumin imprinted calcium phosphate/alginate hydrogel microspheres were prepared with sodium alginate (SA), $(NH_4)_2HPO_4$, and using $CaCl_2$ as gelling agent. Equilibrium swelling and rebinding properties of the two macromolecularly imprinted polymer microspheres for BSA aqueous solutions were evaluated in different pH values and ionic concentrations. The relationship between the specific rebinding properties of BSA-MIPMs and the pH values of BSA aqueous solutions was investigated during the processes of gelling, removing template and rebinding. The optimized pH values for the imprinting of BSA in gelling, removing template and rebinding process was 4.1, 8.3 and 4.8, respectively. A strategy was provided to gain the optimum specific rebinding properties, namely the optimal imprinting efficiency, by designing or adjusting of the structure and property of hydrogel polymers and proteins in the gelling, removing and rebinding process. Rebinding isotherms of the phosphate/alginate hydrogel microspheres proved a greater bovine serum albumin affinity for imprinted microspheres relative to nonimprinted ones.

Acknowledgements: The authors thank the National Natural Science Foundation of China (No. 20774604), the project of Science and Technical Development of China (2007AA03Z533), the special program for key research of Chinese national basic research program (2008CB417202) and Tianjin basic applied and forefront technology research projects (09JCZDJC23200) for supporting this research work.

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