Specific Rebinding Property of Protein Macromolecularly Imprinted Polymer Microspheres Based on Calcium Alginate Hydrogel via Gas Jetting-Dropping Method

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ABSTRACT: Alginate hydrogel polymer microspheres are usually prepared by inverse suspending method. The main problem is the remained suspending medium and surfactant on the beads. Syringe dropping or extruding method avoids using of the organic solvents. However, it is difficult to control the particle dimension and produce in large quantity. We have developed a new method called nitrogen gas jetting-dropping (GJD) method for the preparation of bovine serum albumin (BSA) imprinted polymer microspheres based on calcium alginate hydrogel. The microspheres can be produced continuously without using any hydrophobic medium or surfactant. The particle diameter is controlled by adjusting pressure and needle dimension. BSA-imprinted alginate microspheres were prepared by the GJD method and the rebinding tests were investigated. The equilibrium rebinding capacity ranged from 0.01 to 0.014 μmol/g. The imprinting efficiency towards BSA was from 1.46 to 2.17 with the greatest separation factor of 1.523 against a contrastive template ovalbumin (OVA). © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 2331–2339, 2010

Key words: specific rebinding property; protein; molecularly imprinted polymer; alginate; microspheres

INTRODUCTION

Protein imprinting and rebinding techniques have received much attention in separation, purification,[1,2] Biosensor,[3] and diagnoses.[4] Considering hydrogel as a kind of highly flexible matrix with loose mesh, protein imprinted hydrogels are becoming popular. The preparation and characterization of a soft-wet hydrogel microspheres imprinted with bovine serum albumin (BSA) have been reported possessing higher rebinding efficiency towards the template.[5] Natural polysaccharides like alginate[6] and agarose[7] are more acceptable as imprinted matrices because of their massive water content, moderate gelating condition, and good biocompatibility.

Recent researches on preparing protein imprinted hydrogel microspheres are mainly focused on gelling and polymerizing in a suspending medium. One of the most commonly used methods is the inverse phase suspending gelating or polymerizing approach (IPSG and IPSP). Mixture of protein and polymer sol is dispersed into an inert and hydrophobic substance under mechanical or magnetic stirring. Beads with more uniform dimension and better sphericity can be obtained by adjusting surfactant content and stirring speed.[8–11] The chosen suspending mediums are alkane or natural oil like *n*-hexane,[8,9] caster oil,[10] and soybean salad oil.[11] Although this method has the advantage of convenience and mass production, the remained oil and surfactant on the microspheres are not easily removed completely and probably affect the eluting and rebinding of templates. These remained organic substances will also contaminate the beads and protein adsorbate, leading to inaccuracy of tests.[8–11]

An effective way to avoid the oil and surfactant is to form microspheres in aqueous medium. Considering the cationic gelating property of alginate, dropping and spraying methods are applicable in some of the preparations. Dropping and liquid jet method was applied in preparing Mucoadhesive beads, bacteria capsules, drug releasing carriers, etc.[12–15] The main problems of these methods are the low producing efficiency and relatively large particle diameters (from 1 to 2.5 mm).

To produce beads continuously and in large quantity, many modifications have been made in the original dropping approaches. An air jet flow will help in producing beads at a speed less than 200 mL/h per needle in the size of 1–5 mm.[16] A simple dropping machine is scaled up by attaching several needles at the bottom of a vessel.[17] Beads can also be produced in great quantity by centrifugal force instead of gravity force. The liquid is jetted or thrown from the rotating disk containing the mixed

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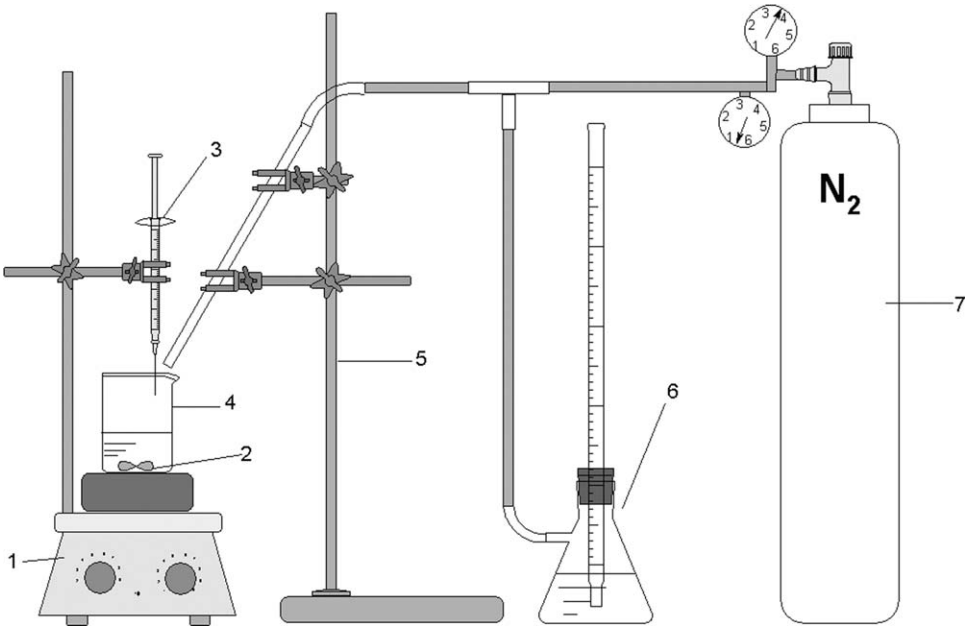

Figure 1 The illustration of the nitrogen gas jeting–dropping (GJD) experimental setting (1) magnetic stirrer; (2) stirring bar; (3) syringe; (4) beaker; (5) barometer; (6) pressure meter; (7) N_2.

solution. A disk rotating at 2000 rpm can produce 6 L/h of beads with a diameter of 1–3 mm.[18]

As for controlling the size of the beads, the mechanical force and the needle shape effects on beads diameter have been reported. Levy et al.[19] found the size of a liquid drop is larger than 1 mm when falling from a needle only by the gravity force. The size of a liquid drop can be reduced with a superposed air stream, which flows in the concentric annulus pulling down the liquid drop formed at the inner needle end. Dorian[17] has reduced the size of spherical alginate gels to 10 to 120 μm by a compressed concentric air stream blowing coaxially. The bead size can also be reduced to 25–300 μm by beveling the end of the needle at an angle of about 15–45°.

Although some of preparations above can provide the beads with small (or rather, controllable) diameters and other methods may produce in large amount, the preparation with both mass production and controllable diameter has not been reported.[20–23] In this study, BSA-imprinted polymer microspheres (BIPM) based on alginate hydrogel are prepared by means of a new developed approach called gas jetting-dropping (GJD) method. The microspheres can be produced continuously at a rate of 600 mL/h and with controllable particle diameter. The rebinding property and selectivity are discussed.

EXPERIMENTAL

Materials

Sodium alginate (SA, M_n = 35,000, M_w = 218,000) was purchased from Beijing Xudong Chemical Reagent Factory, China. BSA (66,000 Da, electrostatic point pI = 4.7, electrophoretic grade) was obtained from Fluka Chemical Co., Germany. Ovalbumin (OVA, 45,000 Da, electrostatic point pI = 4.7, electrophoretic grade) was from Sigma. Tris(hydroxymethyl) aminomethane (Tris, analytical grade) was from Institute of Biological Engineering Chinese Academy of Medical Science, China. Calcium Chloride Anhydrous was from Tianjin No.2 Chemical Reagent Factory, China. The reagents above were used as received. Alginate solution and crosslinking agent ($CaCl_2$) were freshly prepared in deionized water. Injecting syringe 29G (0.33 mm × 8 mm) was provided by BRAUN Co., Germany. Compressed nitrogen (analysis grade) was used as the injecting gas.

Preparation of the BSA-imprinted microspheres

The experimental setting of the GJD method[24] is shown in Figure 1. BSA was first dissolved in deionized water to obtain a solution of 20 μmol/L. Then a mixture of BSA and alginate was prepared by dissolving sodium alginate into the protein solution under slight stirring. The final concentration of alginate is 2% (w/v). One milliliter of this BSA-alginate solution is introduced in the syringe and extruded through a 0.33 mm-diameter needle at a dropping rate of 1 mL/min. A compressed nitrogen stream was used to blow off the alginate droplets at a controlled size through a tube pointing exactly at the needle end in an angle of about 30°. The pressure of the nitrogen stream was measured by the barometer. The sol droplets were gelated in 10% (w/v) $CaCl_2$

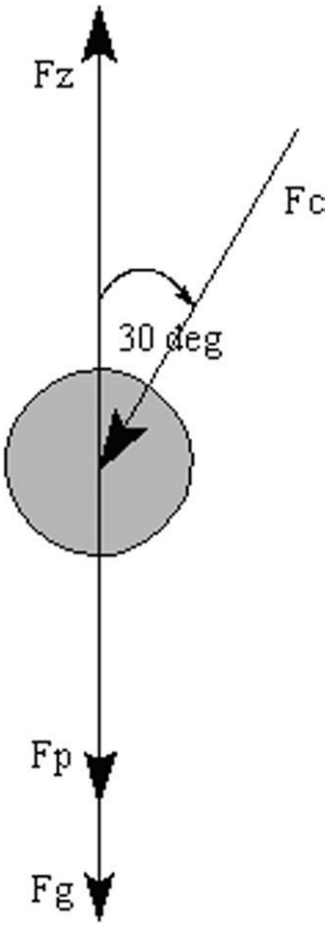

Figure 2 The force schematic of the droplet on the needle end.

solution and were then allowed to harden for 30 min during slight stirring.

BSA templates in the microspheres were removed by immersing in eluting solution with Tris-HCl (Tris 0.05 mol/L) and $CaCl_2$ (2% w/w). The solution was adjusted to pH = 7.5, in which the hydrogel was brought to a swollen state. Samples were slightly shaken every 2 h to ensure thoroughly eluting of the templates until no BSA is detectable in the supernatant liquor.

Morphology of the microspheres

Inverse optical microscopy (Carl Zeiss Axiovert 25C, Germany) was used to observe the size and surface morphology of imprinted microspheres in hydrogel state.

Rebinding tests

Rebinding in different BSA concentrations

A series of BSA solutions ranging from 10 to 22 μmol/L in concentration were prepared and adjusted to pH = 4.7 with dilute hydrochloric acid. 1.5 g of imprinted microspheres were put in the BSA solutions under 25°C. The concentration was measured by UV spectrophotometer (Ultra U-1800, Hitachi) and the rebinding capacity was calculated as follows:

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

Wherein the Q is the rebinding quantity of protein on the imprinted microspheres; C_0 and C_t are the concentration of BSA solution before and after rebinding; V is the volume of the BSA solution; W is the weight of microspheres. The imprinting efficiency (IE) was calculated as follows:

$$\mathrm{IE} = Q/Q_N \quad (2)$$

Wherein IE is the imprinting efficiency; Q_N is the quantity of protein bound on the nonimprinted microspheres.

The rebinding isotherms

Protein solution was prepared with 10 μm/L BSA (pH = 4.7) and 0.2 mol/kg $CaCl_2C_t$ of BSA was measured at specific time intervals. The rebinding quantity and imprinting efficiency were also calculated according to Formula (1) and (2).

RESULTS AND DISCUSSION

Morphology of the microspheres

The preparations of alginate microspheres in aqueous medium were generally performed by liquid jet or extruding[13–15] and then gelated in crosslinking agents. Mass production of microspheres can be conducted by concentric drop method[16] and needle array.[17] It was also found that the bead diameter could be reduced by beveling the needle into specific shape[17] or exerting compressed concentric air stream.[21] In this work, an external compressed nitrogen stream was applied as a driving force to control the dropping speed and droplet dimension. The force schematic is shown in Figure 2, in which F_g, F_p, and F_c are the droplet gravity, propelling force, and the driving force of the nitrogen stream.

It is suggested in numbers of practices that a stream with an inclination of 30° provides the most stable shaping and appropriate falling orbit. The total forces in equilibrium condition can be calculated as follows:

$$F_g + F_p + F_c \cos 30^\circ = F_z = \pi a \gamma \quad (3)$$

Wherein a is the inner diameter of the needle and γ the surface tension of alginate sol. Alginate

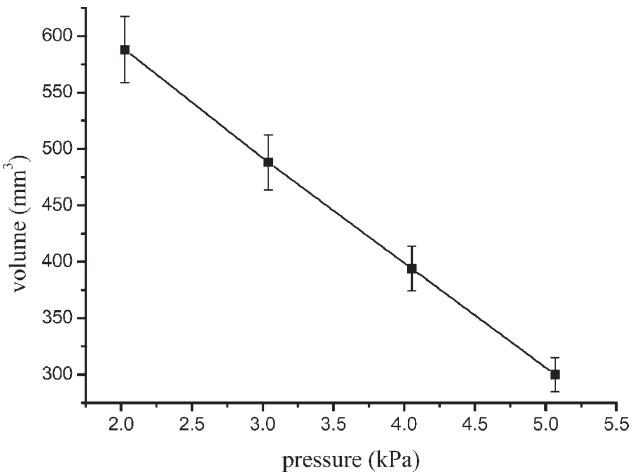

Figure 3 Volume of microspheres prepared under nitrogen streams of different pressure.

solution is extruded from the syringe at about 1 mL/min and hydrogel microspheres are produced continuously at a rate of 0.78 g/min. The diameter and volume of microspheres are affected by the pressure of nitrogen. The volume of microspheres prepared under different pressure is shown in Figure 3. When the pressure increases, the volume decreases at a liner rate. The optical micrographs of the samples are shown in Figure 4.

The diameter of the alginate microspheres is decreased as the nitrogen stream pressure changing from 2.027 to 5.066 kPa (about 20 to 50 cm H_2O). Compared with the beads dropped only by the gravity [Fig. 1(A)], those prepared by GJD method have a much smaller dimension. As the gas stream flows with higher pressure, the droplet gravity (F_g) is reduced according to formula 3. The liquid falls at smaller dimension as a result of the forces equilibrium. However, the pressure cannot be increased any more because the gas stream turbulence and $CaCl_2$ solution splashing leads to irregular shape and broad distribution of beads. Besides, some of the microspheres prepared by GJD approach are not as spherical as those by dropping method, especially the smaller ones formed in higher jetting pressures. The reason is the remarkable viscosity of alginate solution. When the droplets are leaving the needle end at a smaller size, they are actually stretched by the gas flow and more likely to drop in the gelling agent in an elliptical shape. This problem can be solved by diluting the alginate sol and lengthen the falling arch.

The sample with relatively uniform diameter is prepared under the pressure of 2.027 kPa, as indicated by the smaller SD value (standard deviation value) and polydispersity index (PDI) in Table I. The histogram is also given as an overall illustration of the microspheres' diameters prepared in different condition (Fig. 5). As the injecting pressure increases, samples were prepared with smaller diameter but less uniformity.

Rebinding property

The imprinting efficiency (IE) of the BSA-imprinted microspheres

The rebinding experiments are performed by immersing exactly weighted microspheres (1.500 g) in 20 mL 20 μmol/L BSA solution (pH = 4.7) and allowed to rebind for about 300 s. The rebinding properties of both of the imprinted and nonimprinted microspheres are measured so as to calculate the imprinting efficiency. The rebinding quantity of the imprinted microspheres is the sum of specific recognition and the nonspecific adsorption, while as for the nonimprinted ones the rebinding quantity is only concerned with nonspecific adsorption. To estimate the imprinting effect, the imprinting efficiency (IE) of the beads is calculated as formula 2.

The rebinding quantities and imprinting efficiency are plotted against time as shown in Figure 6(a). The imprinted microspheres possess higher rebinding quantity than the nonimprinted ones. The imprinting efficiency (IE) increases most at particular period, during which the imprints are considered to perform the best. The primary kinetics coefficient k is calculated as:

$$k = d(\ln Q)/dt \tag{4}$$

It can be seen in Figure 6(b) that the rebinding speed of imprinted sample is higher than the nonimprinted one during the first 100 s till approaching the rebinding equilibrium.

Macromolecular imprinting in hydrogel matrices usually involves multipoint rebinding among specifically arranged sites and segment or region interactions between the polysaccharide chains.[9,10] In such soft and wet material like alginate hydrogel containing water more than 90%, the chains folding and assembling configurations are changing all the time as water and solute exchanges. Certain configuration provides the most appropriate arrangement for the specific sites and regions and therefore leads to the highest imprinting efficiency (IE).

Rebinding property of different diameter microspheres

Microspheres with different diameters are found to perform different when rebinding. The smallest microspheres rebind with the highest speed and reach a higher level of rebinding quantity (Q). Oppositely the Q of larger ones increases much slower [Fig. 7(a)]. It is also suggested in Figure 7(b) that smaller beads possess higher rebinding coefficient k in the beginning 50 s and the reaction is brought to

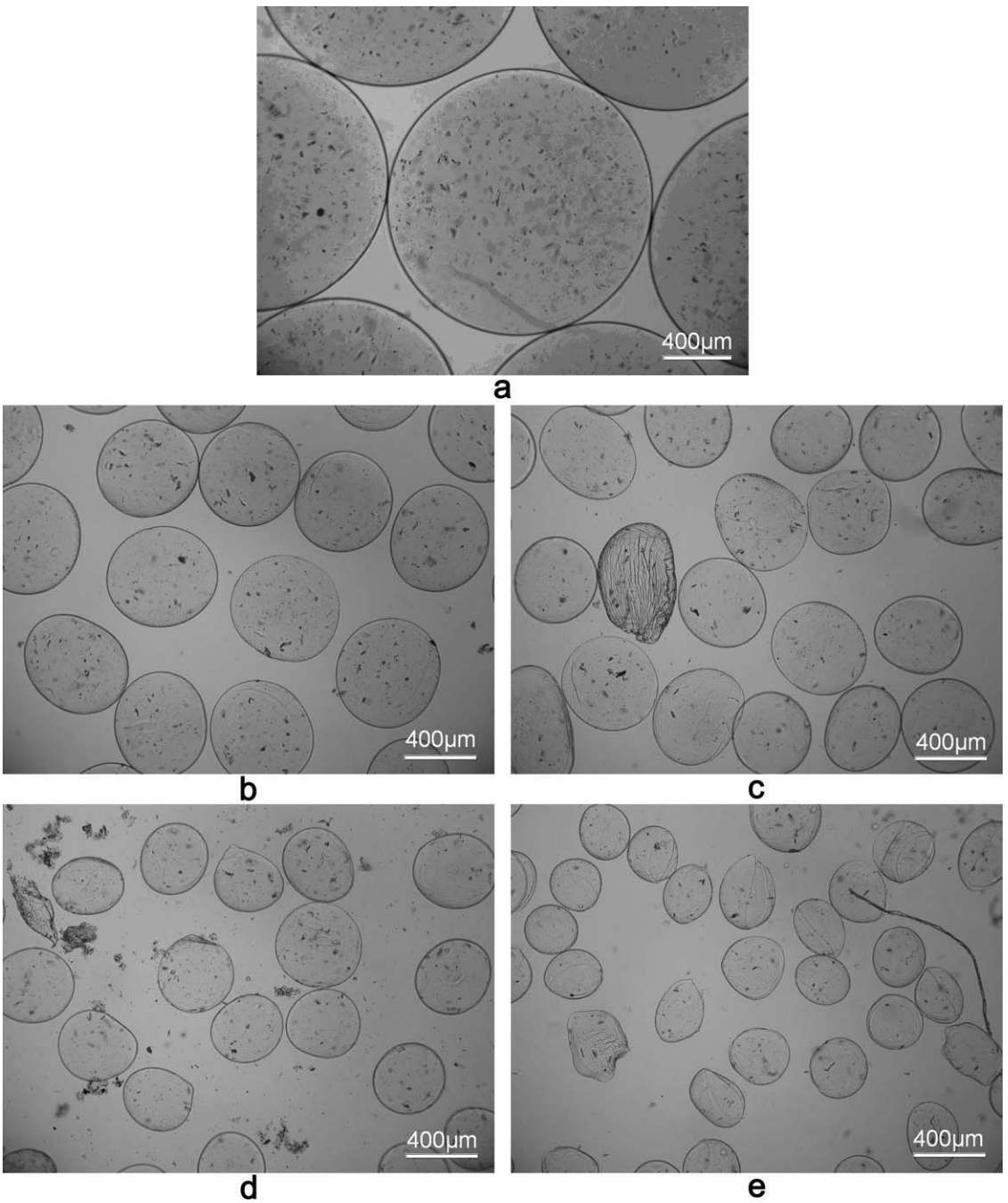

Figure 4 Microspheres prepared under nitrogen streams of different pressure The pressures of nitrogen are: (a) 0 kPa; (b) 2.0265 kPa; (c) 3.0398 kPa; (d) 4.053 kPa; (e) 5.066 kPa.

TABLE I
Nitrogen Stream Pressure and the Corresponding Diameter Statistics

Sample ID	Nitrogen pressure (kPa)	Mean *d* (μm)	SD (μm) *d* Er ±	Median (μm)	PDI
a	2.0265	587.64	16.58	588.45	0.0282
b	3.0398	488.58	19.96	486.33	0.0409
c	4.053	394.90	15.80	393.40	0.0400
d	5.066	300.82	16.51	298.05	0.0549

PDI is calculated as PDI = δ/d, wherein δ is the SD(standard deviation) and d is the mean diameter. Microspheres are prepared at an agitating speed of around 500 rpm. "Mean *d*" is the mean diameter of samples.

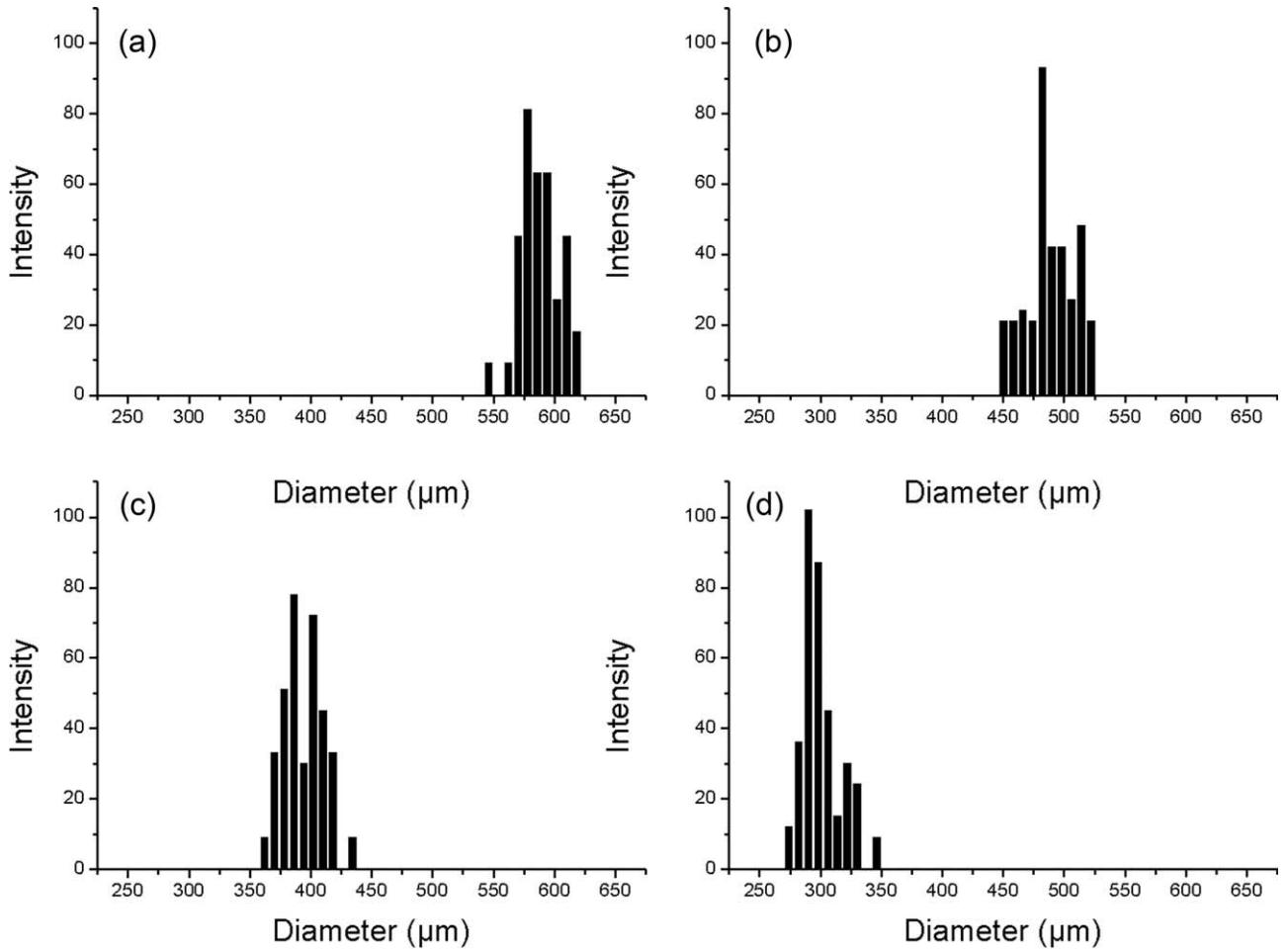

Figure 5 The histogram of microspheres' diameter statistic prepared under nitrogen pressure of: (a) 2.0265 kPa (20 cm H_2O); (b) 3.0398 kPa (30 cm H_2O); (c) 4.053 kPa (40 cm H_2O); (d) 5.066 kPa (50 cm H_2O).

equilibrium. Rebinding speed of larger beads (395 and 588 μm) is lower at first and takes more time (more than 120 s) to reach constant. The reason for this is probably the analyte transferring and beads specific surface area in connection with the particle diameters. Smaller diameter provides the microspheres with larger specific surface area and shorter radial path. The surface area rebinding is considered

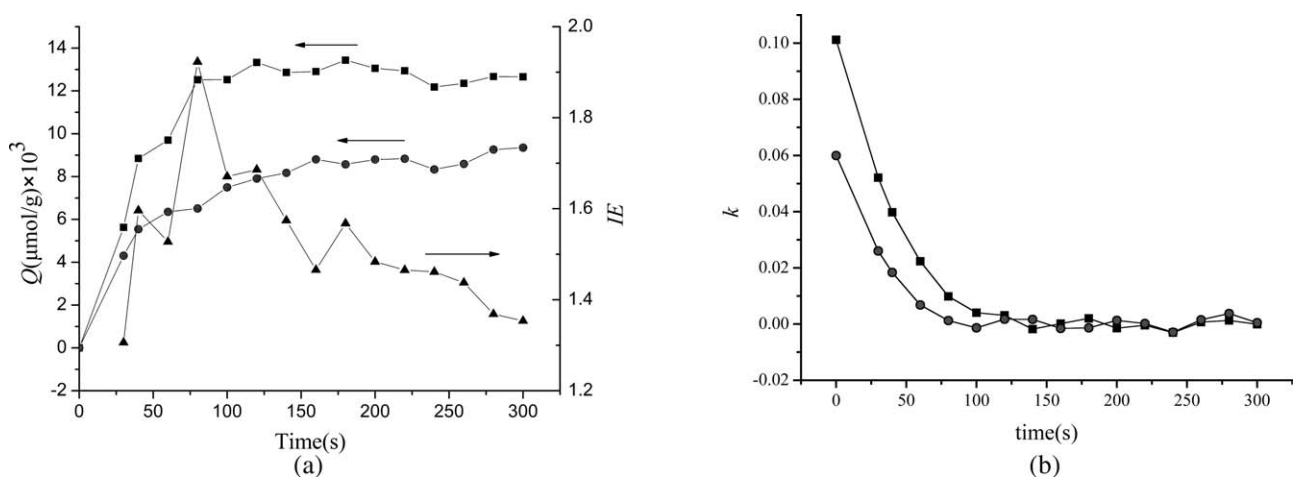

Figure 6 Rebinding on BSA-imprinted and nonimprinted microspheres as the function of time (a) ■: rebinding quantity of BSA-imprinted microspheres; ●: rebinding quantity of nonimprinted microspheres; ▲: the imprinting efficiency. (b) The rebinding primary kinetics of ■: BSA-imprinted microspheres and ●: nonimprinted microspheres. $k = d\ (\ln Q)/dt$.

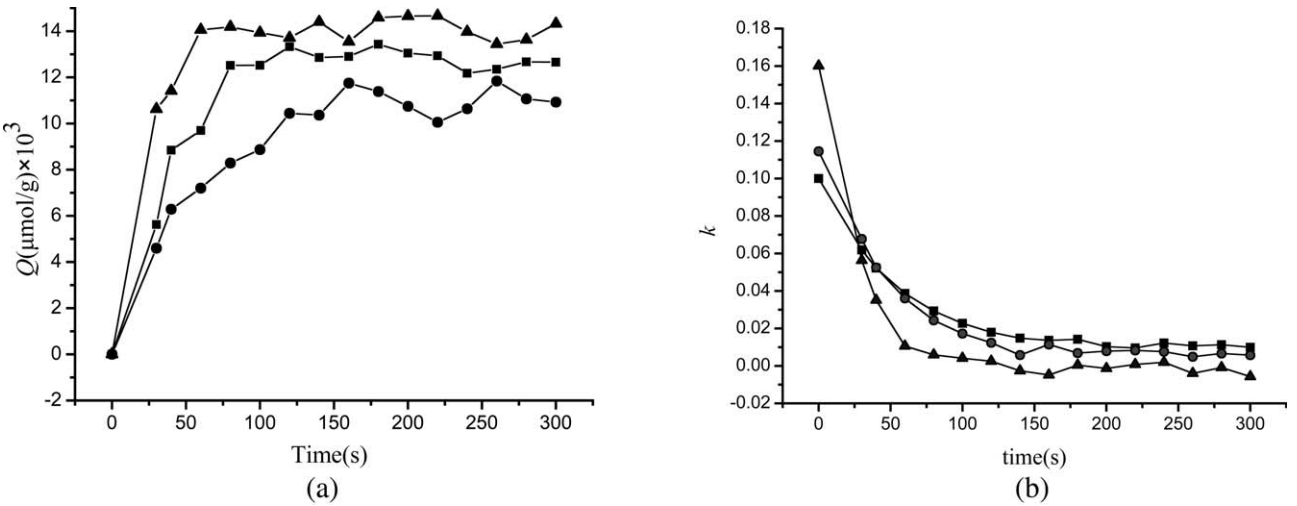

Figure 7 Rebinding on BSA-imprinted microspheres with different diameters. (a) ▲: 300 μm; ■: 395 μm; ●: 588 μm. (b). The rebinding primary kinetics of microspheres with mean diameter of ▲: 300 μm; ●: 395 μm; ■: 588 μm. $k = d\ (\ln Q)/dt$.

contributing a lot in the rebinding speed and quantity, especially in the macromolecular imprinted systems. Macromolecular templates, especially proteins, are remarkably hindered when transferring and entering the inner of the microspheres because of their giant volume.[22] A shorter way into the spherical center ensures effective utilization of inner rebinding sites, hence the higher imprint rebinding quantity and kinetics coefficient k.

The IE are calculated with formula 2 and are plotted against time as shown in Figure 8. Higher IE (approx. 2.16) is found in smaller sample at the beginning of rebinding but it decreases as the rebinding proceeds. The IE of larger microspheres is found much lower (about 1.3) but is maintained for a longer time. This could be explained by the size and swelling properties of the microspheres. Microspheres with shorter radial length swell at a higher rate and allow the protein to permeate through the globule much faster. Imprinting sites deep inside the microspheres are reached and bound with priority over those on the nonimprinted matrix. As a result the IE of the smaller microspheres is higher than that of the larger ones. Oppositely the larger ones with longer radial length and lower swelling rate will hinder the target molecules from permeating deep into the matrix and most of the protein is bound on surface and superficial depth of the microspheres.

The imprinting efficiencies decrease to about 1.62 and 1.39 in the small and medium-scaled diameters, which is probably caused by the swelling property of the hydrogel. In the hydrogel matrix, the imprinting effect is constructed by the hydrogel polymer chains in the form of specific sites orientation and segments arrange. When the microspheres swell, the specificity of the imprints deteriorates at the chain movement. The specific rebinding effect is gradually replaced by nonspecific rebinding, resulting in the IE reduction. Microspheres with relatively larger diameter are not easily swollen and the inside imprints can be preserved for a longer time to rebind successive target molecules. As a result, although IE is lower, it is maintained at a certain level when the microspheres are swelling through the test.

The rebinding test is also carried on in a series protein solution with different concentration and the rebinding isotherm is shown in Figure 9. The imprinting efficiency (IE, Fig. 10) is growing at a high speed when rebinding in lower BSA concentration. An apex IE is found in each sample when BSA concentration increases. It is indicated that the rebinding of protein imprinting requires a specific interaction between protein and the imprinted matrix. Once the layer of protein is formed on the matrix and the imprinted cavities are occupied, the rebinding quantity will increase in a different

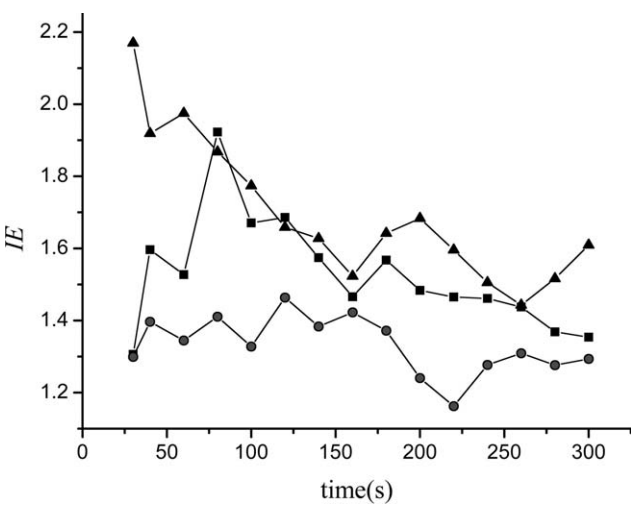

Figure 8 Imprinting efficiency of microspheres with a mean diameter of ▲: 300 μm; ■: 395 μm; ●: 588 μm.

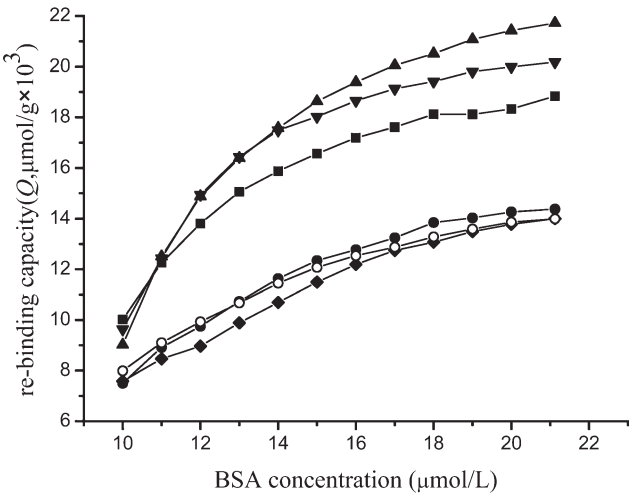

Figure 9 The rebinding isotherm of BSA-imprinted and nonimprinted microspheres ▲, ▼, and ■ are BSA-imprinted microspheres with an average diameter of: 300 μm, 395 μm, and 588 μm; ●, ○, and ◆ are nonimprinted microspheres with an average diameter of: 300 μm, 395 μm, and 588 μm.

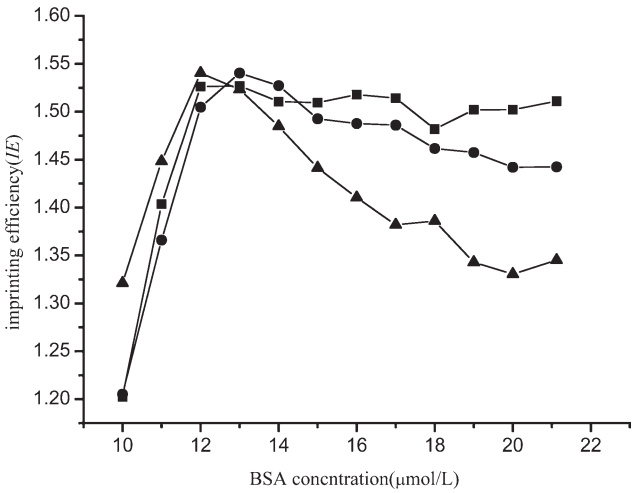

Figure 10 The imprinting efficiency of BSA-imprinted microspheres ■, ●, and ▲ are BSA-imprinted microspheres with an average diameter of: 300 μm, 395 μm, and 588 μm.

manner that is only affected by the concentration gradient. As the protein concentration increases, multilayer is formed on both of the imprinted and nonimprinted microspheres. Therefore the rebinding quantities become similar in higher BSA concentration and result in lower IE.

The imprinting efficiency of smaller microspheres is maintained at higher level as BSA concentration increases. This is because of the larger surface area and better diffusing property of smaller beads. As BSA concentration increases, it is much easier for protein to enter the micropsheres with larger surface. Moreover, BSA can transfer to the bead center quickly because of the short radius path of small beads, making full use of the inner rebinding sites. Therefore smaller beads are more likely to perform specific rebinding when BSA concentration keeps increasing, leading to a maintained IE.

Recognition specificity of BSA-imprinted microspheres

The recognition selectivity of molecular imprinted microspheres can be evaluated by the static distribution coefficient K_D and the separation factor α:[22,23]

$$K_D = C_P/C_S \quad (5)$$

Wherein C_p (μmol/g) is the concentration of BSA on microspheres, and C_S (μmol/mL) is the concentration in the solution.

$$\alpha = K_{D1}/K_{D2} \quad (6)$$

Wherein K_{D1} and K_{D2} are the static distribution coefficients of the template and the other molecules, respectively. The selectivity testing of BSA-imprinted microspheres was carried out under equilibrium binding conditions using OVA as contrastive molecules. Table II shows K_D and α of BSA-imprinted and nonimprinted microspheres with respect to OVA. It was found that the K_D of BSA onto BSA-imprinted microspheres was higher than that of OVA. The greatest separation factor of BSA-imprinted beads was over 1.8, indicating that BSA-imprinted microspheres exhibited good recognition selectivity for the template protein. In contrast, nonimprinted microspheres showed relatively lower K_D and α. The affinity for BSA is attributed to the imprints with high affinity generated by templates. The specific sites and complementary cavities are formed in the matrix during the gelation. The imprinted microspheres favor BSA more than other OVA, leading to a high K_D. The insufficient match between OVA and the imprinted matrix templated by BSA is reflected in the lower K_D and α.

TABLE II
K_D and α Values of BSA-imprinted Alginate Microspheres with Different Mean Diameter

Rebinding protein	Mean d = 300 μm		Mean d = 395 μm		Mean d = 588 μm	
	K_D	α	K_D	α	K_D	α
BSA	1.834	1	1.680	1	1.481	1
OVA	1.729	1.061	1.353	1.242	0.972	1.523

It is indicated that larger beads perform with better selectivity according to the higher α value. As a soft-wet hydrogel matrix, alginate microspheres are easily swollen in aqueous solution. Moreover, smaller beads swell in a higher rate than the larger ones because of their larger specific surface area. The specificity of the cavities and sites are partially destroyed by the hydrogel chain movements when swelling, resulting in a nonspecific rebinding tendency and lower α in smaller samples.

CONCLUSIONS

BSA-imprinted alginate polymer microspheres were prepared by a new nitrogen GJT method. This method possesses the advantages of continuous production in large quantity without using any organic solvent or surfactant. The particle diameter was controllable by adjusting the nitrogen stream pressure. Microspheres with a mean dimension ranging from 300 to 588 μm were prepared under the pressure from 2.027 to 5.066 kPa. Higher injecting pressure resulted in smaller beads dimension but worse uniformity and sphericity. Rebinding properties were investigated with samples of different diameters. The best imprinting efficiency is from 1.46 to 2.17 according to their different diameters. The selective rebinding tests were also conducted in the presence of a competitive molecule (OVA). The separation factor α from 1.061 to 1.532 indicated a specific selectivity of the BSA-imprinted microspheres towards its template.

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