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Stimuli-Responsive Recognition of BSA-Imprinted Poly Vinyl Acetate Grafted Calcium Alginate Core-Shell Hydrogel Microspheres

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ABSTRACT: Poly vinyl acetate grafted calcium alginate hydrogel microspheres were prepared with bovine serum albumin (BSA) as molecular template. The microspheres exhibited homogeneous and core-shell structure according to different preparation strategy. The rebinding and swelling property of microspheres showed responsiveness toward ionic strength, temperature, and pH. It was found the highest separation factor of 1.85 and imprinting efficiency of 1.75 when the Ca^{2+} ionic strength was 1.25 and 0.34 mol/kg, respectively. The separation factor was found decreased as temperature grew from 29 to 45°C while the imprinting efficiency reached a peak value at about 37°C. Separation factor of BSA imprinted microspheres at different pH was also recorded and two peaks were found, which were considered to be caused by the similar swollen state due to ionic and covalent crosslinking structure of modified microspheres. The recognition responsiveness is suggested to be influenced by environmental effects due to the changing in imprints' configuration at different swollen states. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

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INTRODUCTION

Protein-imprinted hydrogel is a kind of material prepared with protein templates presented during the hydrogel is assembling and gelating. The templates were then eluted to generate molecular imprints, which perform rebinding specificity toward templating molecules. Protein-imprinted hydrogels have found their wide application in many fields such as biological mimic sensor,[1–4] separation and purification,[5] clinical medicine,[6,7] high performance liquid chromatography,[8] macromolecular electrophoresis,[9] genetic engineering,[10] etc.

In most cases the detected object was opposed to complex biological and chemical stimuli. Much emphasis has been putting on the functionalization of protein-imprinted hydrogel because it was necessary to establish responsiveness in the recognition of water-rich system that was close to living systems.[11] Many stimulating factors like ionic strength, temperature, and acidity (pH) exert influences on releasing and rebinding properties.[12] Environmental sensitive hydrogel prepared with chitosan, alginate, polyacrylic,[13–18] etc. possess good penetrability, reversibility, and responsiveness that are critical for drug delivery and molecular imprinting. With the aim of realizing temperature-sensitiveness in protein-imprinted hydrogel, most efforts were focused on a series of *N*-substituted acrylamide derivatives (*N*-isopropylacrylamide) as thermal sensitive functional monomer. These monomers had lower critical solution temperature (LCST) at which the transmittance and swelling degree perform obvious changes. The sensitiveness of protein imprinted poly *N*-isopropylacrylamide could be regulated by functional monomer content and hydrogel penetrability.[19–21]

Protein separation and purification are two most important applications of protein-imprinted materials. Therefore, it is important for protein imprinted materials to adsorb and release efficiently, under relatively mild fluctuation in temperature, pH, etc. It is also favorable that the fragile template and imprints were well preserved during the preparation of imprinted materials. Although the results above achieved good responsiveness, they always suffer from low selectivity and bad repetitiveness. The main reason is probably the fragility of protein template. Protein is more active than hydrogel toward environmental effects in most cases and fails to be recognized (being adsorbed selectively) when the electrolyte concentration and temperature are changed. Moreover, most of the

preparations were conducted by initiating polymerization in the presence of template. The imprinting effect was weakened by irreversible binding and conformation damage to the templates due to radical oxidization and consumption of reactive functional groups.[22,23]

The work in this article provides a novel strategy other than conventional covalent grafting and ionic crosslinking, which are harmful to protein template or insufficient in preventing over swelling even erosion. BSA imprinted multisensitive calcium alginate (Ca-Alg) microspheres were prepared with poly vinyl acetate (PVAc)-grafted sodium alginate (Na-Alg). The material was covalently crosslinked without the present of protein and then ionically gelated by Ca^{2+}. Therefore, protein templates were protected from permanent entrapment and denaturation because the imprinting process was separated from radical grafting polymerization. The specific rebinding, separation, and swelling properties of BSA-imprinted microspheres were controllable through various environmental factors, i.e., ionic strength, temperature, and pH.

EXPERIMENTAL

Materials

Sodium alginate (Na-Alg, M_n = 35,000, M_w = 218,000) was purchased from Shanghai Chemical Reagents Corporation. VAc, potassium peroxydisulfate ($K_2S_2O_8$, C.P.), and sodium sulfite (Na_2SO_3, C.P.) were purchased from Shanghai Hengxin Chemical Reagent Co. Ltd (China). Bovine serum albumin (BSA, isoelectric point pH = 7.8, M = 66,000) and Ovalbumin (OVA, isoelectric point pH = 4.7, M = 41,000) were obtained from Fluka Chemie Gmbh. Tris-(hydroxymethyl) aminomethane (Tris, analytical grade) was from Institute of Biological Engineering Chinese Academy of Medical Science. $CaCl_2$ anhydrous, chloroform, hexane, Tween60, liquid paraffin, span 85, alcohol (100%), and ethyl ether (100%), chemical grade, were from Guangdong Guanghua Chemical Factory Co., Ltd. Span80 (chemical grade) was from Dazhong pharmaceutical factory, Shanghai. Alginate solution, monomer, and crosslinking agent were freshly prepared as needed in deionized water.

Preparation of Protein Imprinted PVAc-Grafted Calcium Alginate Microspheres

Two schemes have been used in preparation of PVAc-grafted calcium alginate (PVAc-Ca-Alg) microspheres, i.e., the prepolymerization-gelating (PPG) method and the gelating-*in situ*-grafting (GIG) method. In PPG method, the Na-Alg solution was first copolymerized with VAc at pregrafting stage and then crosslinked with Ca^{2+}($CaCl_2$, 2% w/w). The pregrafting was carried out under nitrogen atmosphere in a 250-mL three-necked flask equipped with a constant temperature bath in a magnetic stirring apparatus. Na-Alg was dissolved in distilled water at reaction temperature with constant stirring for 30 min. Then $K_2S_2O_8$ aqueous solution was added to reaction mixture and the solution was blended for 5 min. Freshly prepared Na_2SO_3 aqueous solution was slowly added to generate macromolecular radicals. After 15 min, VAc at required concentration was added slowly to the mixture and the total volume of the reaction mixture was made up to 50 mL with distilled water. A continuous supply of nitrogen was maintained throughout reaction period. The reaction was terminated after 3 h by allowing air into the mixture.

The product PVAc-Na-Alg was sampled three times for determination of VAc grafting and conversion percentage. Then the solution was used for preparing PVAc-Ca-Alg beads and determining swelling degree. Molecularly imprinted beads were produced by dissolving BSA in PVAc-Na-Alg solution at gentle stirring to a concentration of 15×10^{-3} mol/L. The beads were made by dropping BSA/PVAc-Na-Alg solution from a injector into $CaCl_2$ aqueous solution (2% w/w) and gelating for 24 h.

As for GIG method, Ca-Alg hydrogel microspheres were first formed by dropping Na-Alg solution (containing BSA template) into $CaCl_2$ aqueous solution and then immersed in VAc for 12 h. Redox initiator composed of $K_2S_2O_8$ and Na_2SO_3 was introduced into the mixture. After reaction for 3 h, the PVAc-Ca-Alg microspheres were separated from remained monomer and oligomer. Other reacting conditions were set similarly as described above. The imprinted microspheres were prepared at pH7.8 (the isoelectric point of BSA) so as to obtain the most stable configuration for molecular imprinting.

FTIR and NMR Tests

Fourier transformed infrared spectroscopy (FTIR) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) were used to confirm substitutions of vinyl acetate groups on hydroxyl sites of the modified alginate. The obtained PVAc-Ca-Alg used for the FTIR analysis was dried and ground into powder. The powder was then mixed with KBr and pressed into a disk. The sample was scanned on an FTIR spectrometer (Spectrum-2000 FTIR, Perkin-Elmer Corp. US.) from 400 to 4000 cm^{-1} under 25°C.

^{1}H-NMR studies were carried out with PVAc-Na-Alg and Na-Alg samples dissolved in deuterium oxide (J & K scienterium Ltd.). Analyses of the proton spectra were conducted on an NMR spectrometer (Avance III 500, Bruker Corp. Swiss). The substitution of vinyl acetate groups on hydroxyl sites of alginate were estimated by the emerging ^{1}H peaks on the methyl and methylene groups of vinyl acetate.

DSC Analysis of PVAc-Ca-Alg and Ca-Alg

DSC was used to determine the shift of alginate endothermic or exothermic peaks after modification and consequently detect interactions between PVAc and calcium alginate. Thermograms were obtained using a Shimadzu DSC-50 system (Shimadzu, Kyoto, Japan) in heating and cooling cycles to show glass transition temperature. Samples were obtained by lyophilization of hydrogel microspheres prepared with PVAc-Ca-Alg and Ca-Alg respectively. The materials were crimped in a standard aluminum pan and heated from 20 to 150°C at a heating rate of 10°C/min under constant purging of nitrogen at 20 mL/min.

Protein Removing and Stability Test

The removing percentage was estimated by testing eluant BSA concentrations with UV–vis Spectrophotometer (UV-2450,

Shimadzu) and the stability of protein template was estimated on fast protein liquid chromatography(FPLC, Pharmacia AKTA).

The templating and rebound protein (the protein taken in by the imprinted microspheres) were eluted[23,24] and freeze-dried as the samples of "eluted BSA template" and "rebound BSA molecule," respectively. Microspheres containing BSA were placed in 20.00 mL Tris-HCl buffer (pH = 7.8, 0.05 mol/L) at 25°C. The sample was agitated gently for 48 h and the buffer was refreshed every 12 h for four times. BSA in the Tris-HCl solution is applied directly in UV–vis adsorption determination. 1.36 mg of freeze-dried BSA from eluant was dissolved in 1 mL PBS(pH 7.5, 0.02 mol/L) and centrifuged at 10,000 r/min for 5 min. The supernatant was filtered with microfiltration membrane and injected into chromatographic column. The mobile phase was PBS (pH 7.5, 0.02 mol/L) at a flowing rate of 0.5 mL/min. The wavelength of detecting UV was set at 280 nm. The FPLC spectrograms of eluted BSA, rebound BSA, and intact BSA were compared.

Characterization of Grafted Calcium Alginate

The products of grafting reaction were precipitated in 95% ethyl alcohol for complete removal of homopolymers (PVAc) and remained initiator. The precipitant was separated by filtration and dried under vacuum at 50°C for 12 h. The grafting parameters were calculated as follows:

$$\text{Grafting percentage } (G\%) = [(W_2/W_1 \times W_{\text{all}} - W_{\text{NaAlg}})/W_{\text{NaAlg}}] \times 100\% \quad (1)$$

where W_1,W_2, W_{all}, and W_{NaAlg} were the weight of grafted copolymers, the dried precipitate, the total reactant, and the total NaAlg, respectively. For determination of VAc conversion percentage, grafting products were accurately weighed before and after vacuum drying at 50°C for 12 h. VAc conversion percentage was calculated as follows:

$$\text{Conversion percentage } (C\%) = [(W_2/W_1 - N) \times W_{\text{all}}/W_{\text{VAC}}] \times 100\% \quad (2)$$

where W_{VAc} was the weight of total VAC, N was the percentage of non-volatile and calculated as follows:

$$N = (W_{\text{all}} - W_{\text{water}} - W_{\text{VAC}})/W_{\text{all}} \quad (3)$$

where W_{water} was the total weight of distilled water. The calculation of grafting and conversion percentage in each experiment were worked out as the average of reactants sampled three times for accurate results.

The Ionic Strength Effects on Rebinding Property

In protein imprinting hydrogel, protein molecule is recognized (or to be adsorbed selectively) in the form of specific adsorption, i.e., protein is adsorbed at higher adsorption quantity (Q), greater imprinting efficiency (IE), and better separation factor (α). The recognition for protein involves two aspects including the shape complementary and the sites arrangement. When protein is recognized, the molecule matches the best with imprinted hydrogel, resulting in increased Q, IE, and α.

The rebinding experiments were all carried out in constant temperature oscillator (THZ-C-1, Taicang Experimental Equipment Factory, China). The imprinted microspheres were immersed in NaCl/$CaCl_2$-HCl and BSA solution (pH = 7.8). The concentration of BSA was detected by UV/vis spectrophotometer and the equilibrium rebinding quantity (Q) was calculated as follows:

$$Q = (C_0 - C_t)V/W \quad (4)$$

where C_0 and C_t were beginning and equilibrium concentrations of protein; V was the volume of protein solution, and W was the mass of microspheres. The IE was calculated as follows:

$$IE = Q/Q_N \quad (5)$$

where Q was the rebinding quantity of protein on imprinted microspheres, and Q_N was the adsorption quantity on equally weighted non-imprinted microspheres.

The swelling ratio (SR) of the microspheres was calculated as follows:

$$\text{SR} = (W_t - W_0)/W_0 \quad (6)$$

where W_t was the weight of swollen beads and W_0 was the dry weight before swelling.

The Temperature Effects on the Rebinding Property

The imprinted microspheres were immersed in NaCl-NaOH and BSA solution (pH = 7.8). The adsorption was allowed to perform under certain temperatures till equilibrium. Rebinding kinetic experiment was performed by automatic potential titrator (ZDJ-5) and conductivity meter (DDS-308A) (Shanghai Precision & Scientific Instrument Co, Ltd). The IE and SR were calculated with formula (5) and (6) and plotted as function of temperature.

The Acidity (pH) Effect on the Rebinding Property

A serious of buffer solution with pH ranged from 3 to 11 were prepared following two projects: (a) the basic solution (pH = 7–11) was prepared with NaCl-NaOH and the acidic (pH = 3–7) with HCl-NaCl. Ions contained in this solutions were Na^+, H^+, and OH^-; (b) the basic solution was prepared with $CaCl_2$-$Ca(OH)_2$ and the acidic with HCl-$CaCl_2$. Ions contained in these solutions were Ca^{2+}, H^+, and OH^-. The ionic strength in the above two projects was maintained at $I_{(Na+)} = 0.1$ mol/kg and $I_{(Ca2+)} = 0.05$ mol/kg.

BSA was dissolved in the above solutions to achieve a concentration of 20 μmol/L. Equal amount of imprinted microspheres were applied in adsorption experiments under 25°C till equilibrium. The rebinding quantity (Q) was determined by UV spectrometer and the IE was calculated by formula (5). The microspheres were also allowed to swell in the solutions till equilibrium and the SR was determined by formula (6). As last the IE and SR were plotted against pH.

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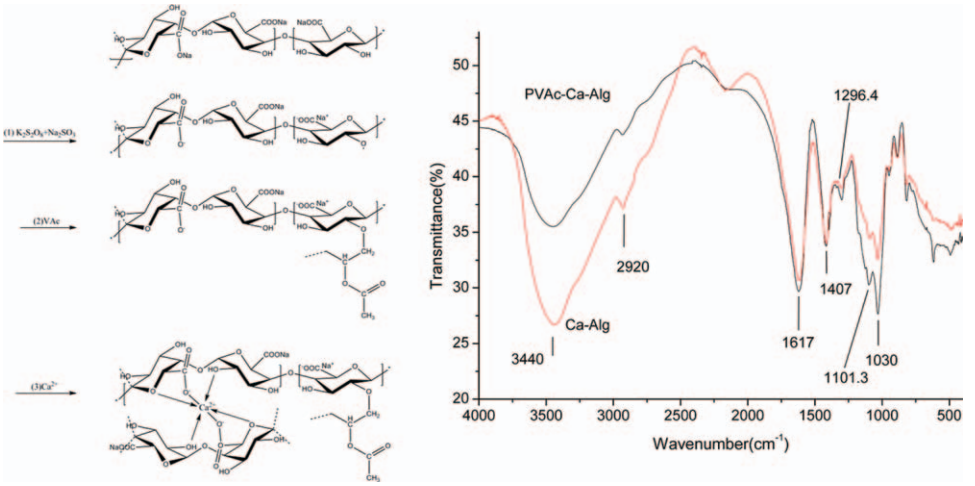

Figure 1. Pregrafting and ionic crosslinking reaction of calcium alginate and FTIR. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

Grafting Reaction of PVAc on Ca-Alg Hydrogel Microspheres

Alginate-based microspheres prepared following different steps (PPG and GIG) exhibited homogeneous and core-shell structure, respectively. In PPG method, pregrafting of VAc onto alginate was induced by generating macromolecular radical on hydroxyl groups (Figure 1). When it reached required reaction extent, the solution mixed thoroughly with BSA was dropped into $CaCl_2$ solution to form hydrogel microspheres. In GIG method, Ca-Alg microspheres templated with BSA were obtained by Ca^{2+} crosslinking and then soaking in VAc. When the beads were fully swollen, the *in situ* grafting reaction was induced by redox initiator and the ionic-covalently crosslinked microspheres were obtained.

The grafting of PVAc onto Ca-Alg was verified by FTIR as shown in Figure 1. Stretching vibration peak of O—H at 3446.5 cm^{-1} was weakened in PVAc-Ca-Alg, indicating a decrement in O—H amount after grafting. The asymmetric and symmetric stretching vibration of carboxylate group (—COO$^-$) at 1617 cm^{-1}and 1407 cm^{-1} was not change much at the modification, suggesting —COO$^-$ was well preserved during reaction. Peaks at 1101.3 cm^{-1} and 1030 cm^{-1} were originated from the stretching vibrations of group —C—O—. The strengthened of the two peaks was because of the increasing in ester groups of VAc segments. The result of FTIR analysis suggested that PVAc were grafted onto Ca-Alg backbone while maintaining the structure of carboxylate groups, which was crucial to further ionic crosslinking process.

^{1}H-NMR was also used to verify the grafting of PVAc onto sodium alginate (Figure 2). The chemical shift (δ) between 3.4 and 3.8 ppm (H_1, H_3, H_4, H_6) belonged to *H* of methylene on mannuronic and guluronic acid. Chemical shift located at 2.0 ppm (H_a) was due to *H* of methyl group. Peaks at 2.4 ppm (H_b) and 4.3 ppm (H_c) were from methylene groups of PVAc. The proton peak of methyl and methylene indicated grafting reaction between PVAc and sodium alginate.

The microspheres were observed by Zoom-stereo microscope (ZSA302, Chongqing Optical & electrical instrument Co., Ltd.). PPG samples possessed homogeneous matrix with PVAc grafted evenly on surface and center, as shown in Figure 3 (PPG samples). The histogram was also given as an overall illustration of the microspheres' diameters prepared following different procedures. Microspheres prepared by GIG had a unique core-shell appearance with white inner part surrounded with transparent hydrogel. Smaller SD and PDI indicated the diameter uniformity of grafted microspheres as shown in Table I. Core-shell structure could be attributed to the hydrophobic nature of VAc and gel effect of Ca-Alg. When microspheres were swollen in monomer, VAc was prohibited from diffusing out of hydrogel and accumulated at a higher concentration in the center than on surface. Moreover, radical life in the center was lengthened because of gel effect and also resulted in higher PVAc concentration.

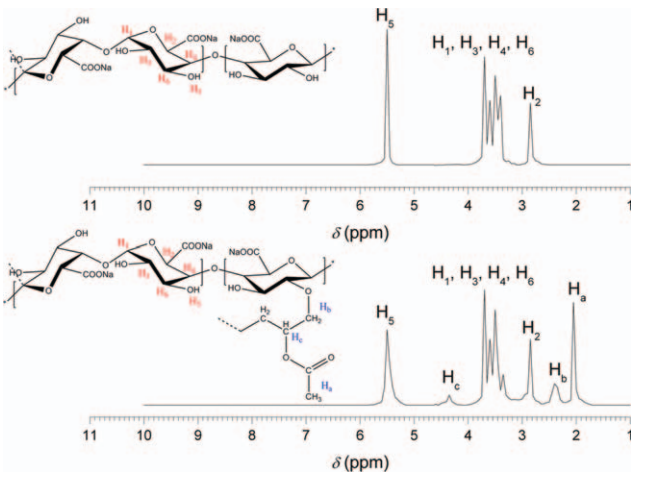

Figure 2. ^{1}H-NMR of Na-Alg and PVAc-Na-Alg. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

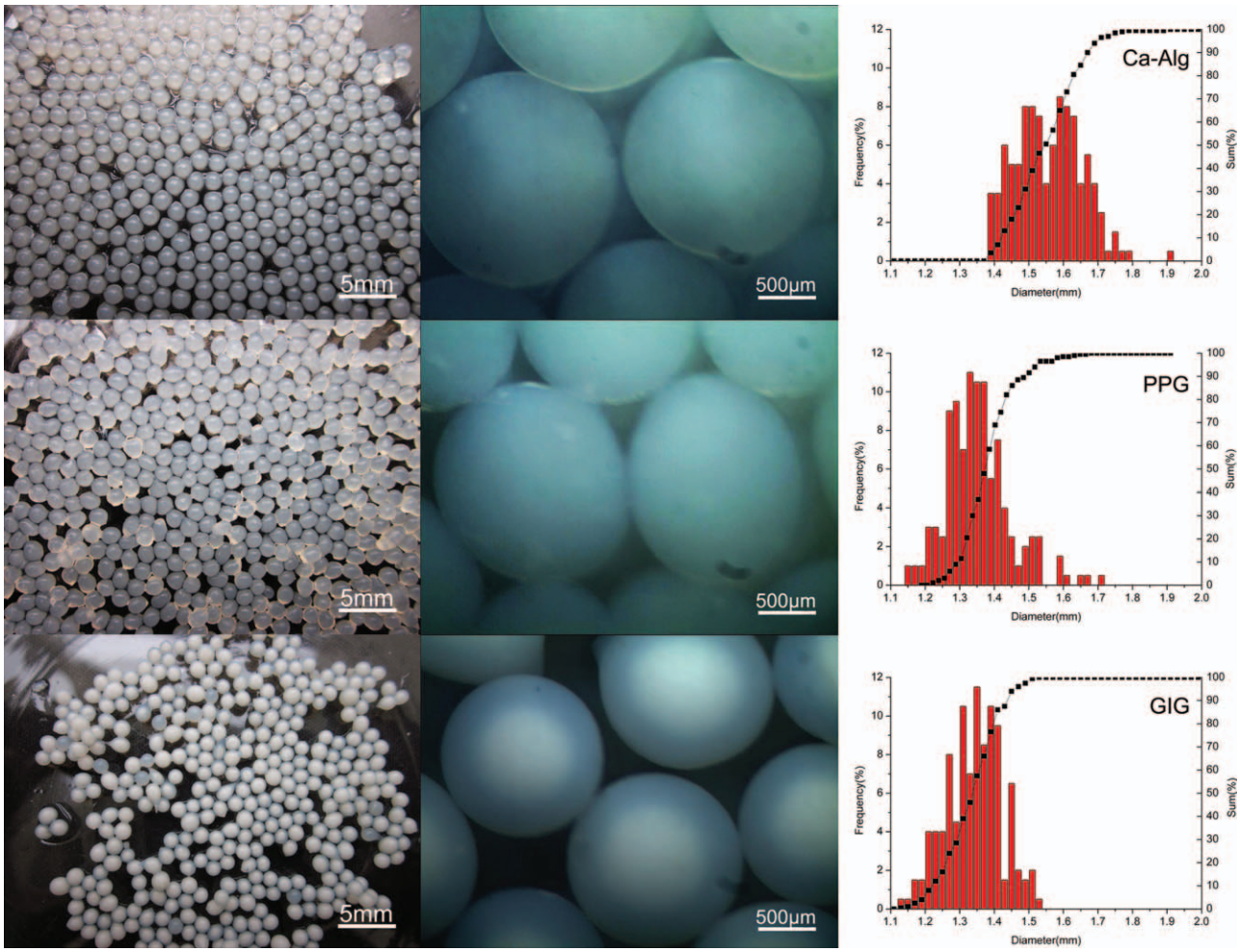

Figure 3. Photographs of Ca-Alg-based microspheres and the size distribution. Ca-Alg, sodium alginate hydrogel microspheres without modification; PPG, PVAc-Ca-Alg microspheres prepared with prepolymerization-gelating method; GIG, PVAc-Ca-Alg microspheres prepared with gelating-in-situ-grafting method. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Monomer concentration had a remarkable effect on VAc conversion percentage and grafting yield. VAc content varied from 2.0 $\times$ 10^{-3} to 9.0 $\times$ 10^{-3} mol/L while other conditions were kept constant. The results were listed in Table II. As VAc content increased, the diffusion of monomer into NaAlg matrix was promoted, resulting a higher conversion percentage and graft yield up to 83.9% and 78.0%.

For inspecting SR, microspheres were immersed in 0.9% NaCl. The lowest SR was found in samples with grafting percentage of 42.7% (PPG method) and 69.5% (GIG method). Appropriate grafting degree played an important role in reducing SR, which affected imprinting behavior remarkably. As grafting percentage was increased from 15.0% to 42.7%, SR was reduced to 115% because of the hydrophobic character of grafted copolymer. When the grafting percentage grew up to 78%, more hydroxyl groups were occupied by VAc and not capable of forming Ca^{2+} bridge, leading to a higher SR.

Similar works published by Wang and Wang[25] concerning alginate crosslinked with *N,N*-methylene-bis-acrylamide suggested an equilibrium water absorption in 0.9 wt % NaCl solution between 51 and 78 g/g, which is much higher than that of PVAc grafted alginate from 0.86 to 3.81 g/g. The main reason is the hydrophobic PVAc segment that prevents water from to permeating into the hydrogel. The grafting activity and swelling behavior were also studied as functions of reacting temperature. Experiment was carried out within a temperature range between 40.0°C and 50.2°C as shown in Table III. The total amount of VAc used in preparing was 0.005 g in order to reach an optimum grafting percentage.

According to Table III, conversion percentage increased quickly from 40 to 45°C then decreased when it approached 50°C. Similarly, grafting percentage increased very rapidly in the beginning and then decreased as temperature was further increased. The highest graft yield and conversion percentage obtained at 45.1°C were 82.2% and 85.4%. The enhancement in grafting with rising temperature could be ascribed to a higher rate of reaction between VAc and NaAlg. The increased temperature also enhanced the mobility of Na-Alg backbone, facilitating the diffusion of monomer and initiator into Na-Alg matrix. Nevertheless, the lowering of grafting parameters by the increase in

Table I. Statistics on the Diameters of Ca-Alg and PVAc-Ca-Alg Hydrogel Microspheres

Samples	Description	Mean diameter (mm)	Standard deviation (mm)	Median (mm)	PDI
a	Ca-Alg hydrogel microspheres	1.5523	0.09581	1.550	0.0617
b	Hydrogel microspheres prepared by PPG	1.34785	0.09498	1.340	0.0705
c	Hydrogel microspheres prepared by GIG	1.3361	0.08017	1.345	0.0600

PDI is calculated as PDI = δ/d, wherein δ is the standard deviation and d is the mean diameter.

temperature above 45°C was due to chain termination reaction, chain transfer reaction, and oligopolymer formation. The optimized reacting condition was suggested to be NaAlg(%) = 2.0, [VAC] = 3.25×10^{-2} mol/L, [$K_2S_2O_8$] = 1.1×10^{-3} mol/L and [Na_2SO_3] = 1.6×10^{-3} mol/L at 45.3°C.

Macromolecular-imprinted microspheres based on PVAc-Ca-Alg hydrogel could rebind target molecules by cavity shape complementary and functional sites specificity. Modified alginate hydrogel showed responsiveness to external stimuli, which results in the change of mesh density and permeability of imprinted polymer. In a microscale view within polymer segments, swelling of the imprinted material induced allosteric regulation of functional sites and cavities, which are fundamental elements for rebinding process. With the aim of investigating environmental factors on rebinding properties, IE and SR were tested as functions of external factors (ionic strength, temperature, and pH) and the rebinding dependence on swollen state was discussed.

Differential Scanning Calorimetery Analysis

As a ionic crosslinked hydrogel, PVAc-Ca-Alg and Ca-Alg's thermograms (second heating) consist of glass transition, exothermic and endothermic peaks at different temperature according to Figure 4. Endothermic peaks are correlated with melting while exothermic peaks are resulted from crystallization due to calcium and carboxyl groups.

It could be seen that the peaks of PVAc-Ca-Alg were shifted to higher temperature compared with those of Ca-Alg because grafted PVAc resulted in new chemical bonds. Crystallization exothermic peak of PVAc-Ca-Alg was more intensified compared with Ca-Alg which could be interpreted as an increasing tendency of forming ionic bridge between calcium and carboxyl groups. The grafted PVAc was more hydrophobic than polysaccharide and prohibited H_2O from forming hydrogen bond with carboxyl groups. Thus more regular "egg-box" structure is likely to be well constructed in dehydrated Ca-Alginate gel.[26] Both endothermic and exothermic peaks shifted to higher temperature and strengthened indicating more crystallizing crystal regions in modified hydrogel. Therefore, higher temperature and more energy were required to meltdown calcium carboxylic in alginate.

The glass transition temperature (T_g) was also increased at the modification of PVAc grafting. Alginate control sample exhibited T_g at 38.43°C while PVAc-grafted alginate showed T_g at 59.99°C, which is due to the hydrophobic effect of PVAc segments. The decrease in T_g has been attributed to the plasticization by water, which enhanced segmental movement.[27] As has been presented by Lim and coworkers,[27] water activity exerted a typical plasticization effect upon the hydrogel resulting the decrease in alginate T_g from 56.03°C to 35.12°C as water activity increased from 0.23 to 0.84. The grafting of PVAc achieves similar results as the reduction in water activity because of the hydrophilic acetoxyl groups that helps reducing the bound moisture around alginate backbone.

Removing Percentage and Stability Test of Templates

Stability of template was estimated by the sum of recovered BSA in all eluants and was calculated as proportion of eluted protein to total amount used as template. The concentration of BSA template in four batches of eluant (Table IV) was measured by UV–vis spectrophotometer at specific wavelength of 278 nm (Figure 5). Every batch was tested three times and taken the average. BSA was hardly detectable in the fourth sample and the templates were considered completely removed. According to

Table II. Conversion Percentage, Grafting Percentage of VAc, and Equilibrium Swelling Ratio of PVAc-Ca-Alg Microspheres Prepared with Different VAc Content

Sample ID	PPG1	PPG2	PPG3	GIG1	GIG2	GIG3
Na-alg (g)	1.0	1.0	1.0	1.0	1.0	1.0
VAc (g)	0.08	0.14	0.22	0.08	0.14	0.22
Swelling ratio (%)	381 ± 4.3	115 ± 1.9	214 ± 2.2	210 ± 3.1	86 ± 0.92	165 ± 1.4
Conversion percentage (%)	44.1 ± 0.57	69.0 ± 0.70	83.9 ± 1.1	62.1 ± 0.55	76.4 ± 1.2	91.6 ± 1.5
Grafting percentage (%)	15.0 ± 0.22	42.7 ± 0.50	78.0 ± 0.93	53.2 ± 0.46	69.5 ± 0.89	88.3 ± 1.3

Reaction condition: percentage of NaAlg (%) = 2.0; [$K_2S_2O_8$] = 1.1×10^{-3} mol/L; [Na_2SO_3] = 1.6×10^{-3} mol/L; H_2O = 50 g; Reaction time = 3 h. Reaction temperature: 30°C

Table III. The Conversion Percentage, Grafting Percentage of VAc, and Equilibrium Swelling Ratio of PVAc-Ca-Alg Microspheres Prepared Under Different Temperature

Sample ID	PPG4	PPG5	PPG6	GIG4	GIG5	GIG6
Na-alg (g)	1.0	1.0	1.0	1.0	1.0	1.0
Reaction temperature (°C)	40.0	45.1	50.2	40.2	45.3	51.9
Swelling ratio (%)	350 ± 4.6	550 ± 6.8	512 ± 4.3	195 ± 2.5	112 ± 2.1	352 ± 4.6
Conversion percentage (%)	60.3 ± 0.51	85.4 ± 0.94	45.5 ± 0.86	33.5 ± 0.45	79.1 ± 0.90	56.2 ± 0.93
Grafting percentage (%)	21.1 ± 0.30	82.2 ± 1.0	56.8 ± 0.78	32.3 ± 0.41	86.4 ± 1.55	68.2 ± 0.77

Reaction condition: percentage of NaAlg (%) = 2.0; [VAC] = 3.25×10^{-2} mol/L; [$K_2S_2O_8$] = 1.1×10^{-3} mol/L; [Na_2SO_3] = 1.6×10^{-3} mol/L; H_2O = 50 g; Reaction time = 3 h.

Table IV, 88.5% of the templates remained intact while eluting. Others (11.5%) were destroyed or trapped irreversibly in hydrogel.

FPLC results of eluted BSA (including template and adsorbance) and BSA solution were shown in Figure 6. It is indicated that eluted and rebound BSA exhibited absorption peaks with the intensity of 113.7 mAu and 110.5 mAu. Compared with BSA solution with an intensity of 128.4 mAu, about 88.6% of the protein was recovered from eluting process while 14% of the templated was denaturized or aggregated, as in the case of rebound protein the recovery is 86.1%. The eluted amount is indicative of an imprinting effect. Previous work published by Daniel M. Hawkins et al.[28] using SDS : AcOH as eluant indicated that only 50% of the template was removed. Moreover, the recovered protein (4.9–5.9 mg) during the washing steps after rebinding is less than the amount used for rebinding (7.25–7.5 mg), suggesting some irreversible rebinding (about 18.6%–34.7%) of the protein to the MIP. These data were probably due to protein entrapment and aggregation during imprinting and rebinding however was not addressed by the authors.

Rebinding in Different Ionic Strength

Molecular selectivity of imprinted microspheres was evaluated by static distribution coefficient K_D and separation factor α:

$$K_D = C_P/C_S \tag{7}$$

$$a = K_{D1}/K_{D2} \tag{8}$$

C_P (μmol/g) and C_S (μmol/ml) were equilibrium concentration of protein on microspheres and in solution. K_{D1} and K_{D2} were static distribution coefficients of template and competing molecules, respectively. The selectivity testing of BSA-imprinted microspheres was carried out under equilibrium rebinding conditions using BSA and OVA as contrastive molecules. The samples were tested in triplicate using UV/vis spectrophotometer and taken the average.

Figure 7 shows separation factor (α) of BSA-imprinted microspheres with respect to OVA. The separation factor increased with SR and reached peak value about α = 1.85. For OVA, it was between 1.05 and 1.45, indicating that BSA-imprinted microspheres exhibited good recognition selectivity for template

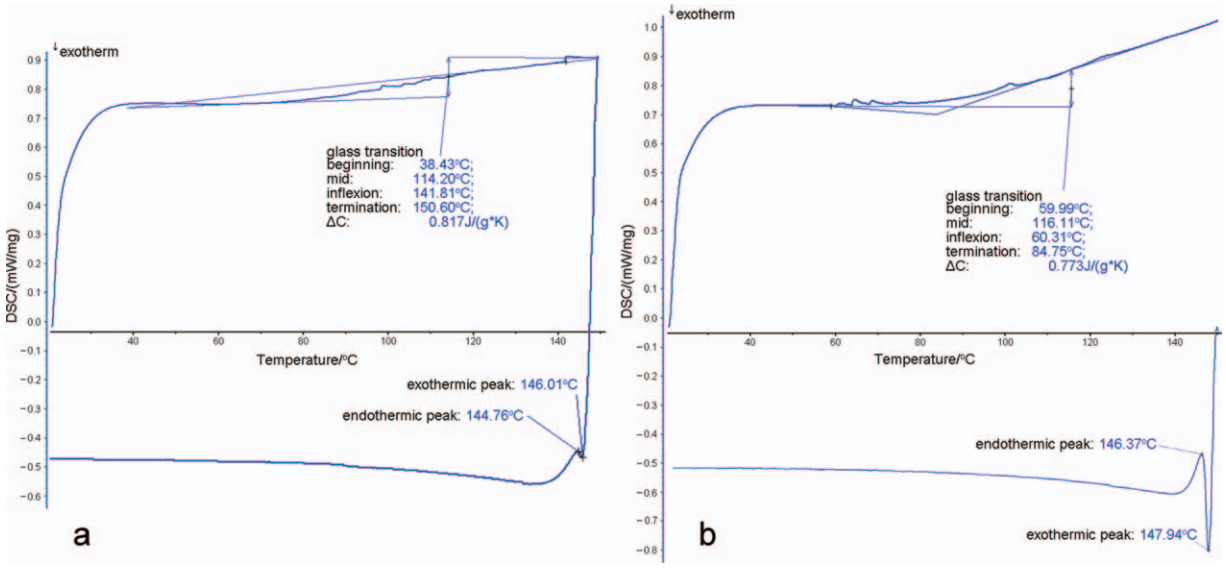

Figure 4. Thermograms of (a) Ca-Alg and (b) PVAc-Ca-Alg prepared by ionic crosslinking gelating. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table IV. BSA Concentrations in Four Batches of Eluant Measured by UV–Vis Measurement

	Eluting batches					
	I	II	III	IV	$\sum$ (= I+ II+ III+ IV)	Mass of total template (g)
BSA concentration in eluant (g/mL)	(8.13 ± 0.05) × 10^{-4}	(3.03 ± 0.02) × 10^{-4}	(1.49 ± 0.01) × 10^{-4}	(6.06 ± 0.05) × 10^{-5}		
Eluant volume (mL)	20.0	20.0	20.0	20.0		
Mass of eluted template (g)	(1.63 ± 0.01) × 10^{-2}	(6.06 ± 0.04) × 10^{-3}	(2.98 ± 0.02) × 10^{-3}	(1.21 ± 0.01) × 10^{-3}	(2.65 ± 0.02) × 10^{-2}	3.00 × 10^{-2}
Batch recovered percentage	(54.3 ± 0.33)%	(20.2 ± 0.13)%	(9.97 ± 0.17)%	(3.97 ± 0.03)%	(88.5 ± 0.66)% (Total recovered percentage)	

protein. Higher rebinding affinity for BSA was attributed to the rebinding sites and complementary cavities formed during the gelation reaction.[9] The imprinted microspheres created a microenvironment based on the combined interactions of complementary cavities and rebinding sites, leading to a high K_D. For OVA, relatively low K_D was due to poor match between protein and imprinted matrix produced by BSA. The separation of BSA and OVA with protein imprinted hydrogel has been previously performed in ionic crosslinked calcium alginate,[29] in which BSA imprinted alginate microspheres exhibited separation factor from 1.061 to 1.523 against OVA. The improvement in separation factor of the present work is attributed to grafted PVAc that provides stabilized meshwork and hydrophobic segment.

As inspection of swelling effect on specific rebinding behavior, IE were plotted against SR as the function of Na^+ or Ca^{2+} ionic strength (Figures 8 and 9). Swelling was facilitated as Na^+ ionic strength increased but restricted in Ca^{2+} solution due to different reactivity of Na^+ and Ca^{2+} with alginate carboxyl groups (—COO^-). IE increased from 1.15 to 1.45 with Na^+ ionic strength but reaches maximum (1.77) when Ca^{2+} ionic strength was I = 0.36 mol/kg, which was the same with crosslinking agent used for preparing the microspheres [$CaCl_2$ was 2.0% (w/w)]. This is because hydrogel in Na^+ kept protein molecules that were already adsorbed while swelling, however, samples in Ca^{2+} were not able to adsorb much unless the SR was high enough to accommodate protein. A similar cation environment with the preparing condition helped the beads regaining better imprinting conformation just as it was prepared, resulting higher IE. It is also noticeable that the extreme point (Figure 9) was located where Ca^{2+} ionic strength induced a SR of about 1.33 more than the expected value (SR = 1.00). The reason was speculated to be hydrogel shrinking as template was eluted, therefore, a little bit higher SR was needed to regain the best specific structure. However, the IE proved not as high as the work[30] performed in HEC (hydroxyl ethyl cellulose) interpenetrated alginate that had been published (2.02–2.81). Plenty of hydroxyl groups on alginate and HEC ensure hydrophilic environment and effective diffusion of hydrogel. The consumption of —OH groups at radical grafting as well as the accumulating of hydrophobic PVAc hinder protein from getting in the imprints and matching with specific sites, resulting relatively lower IE.

Rebinding Under Different Temperature

Rebinding temperature exerted influence on functional groups' electrification and bound moisture content inside hydrogel, affecting configuration of functional groups and cavities. Moreover, flexible protein molecular exhibited various configurations as temperature changed. The rebinding was performed from 26 to 48°C and both of IE and SR were found decreased.

As described in Figure 10, SR decreased significantly as temperature grew up because of bound moisture being squeezed out from hydrogel. The polymer matrix was contracted by electrovalent bond of calcium bridge[17] and covalent bond between hydroxyl groups of alginate and PVAc. The corresponding IE was found increased as temperature rose from 26 to 36.5°C

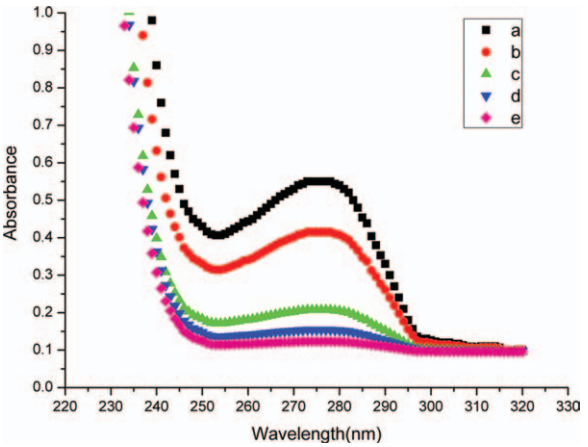

Figure 5. UV–vis spectrophotometer of BSA solution and four batches of eluant containing BSA template. (a) BSA 20 μmol/L; (b) UV of the first batch of eluant; (c) UV of the second batch of eluant; (d) UV of the third batch of eluant; (e) UV of the fourth batch of eluant. The curves showed the average taken in every tested three times. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

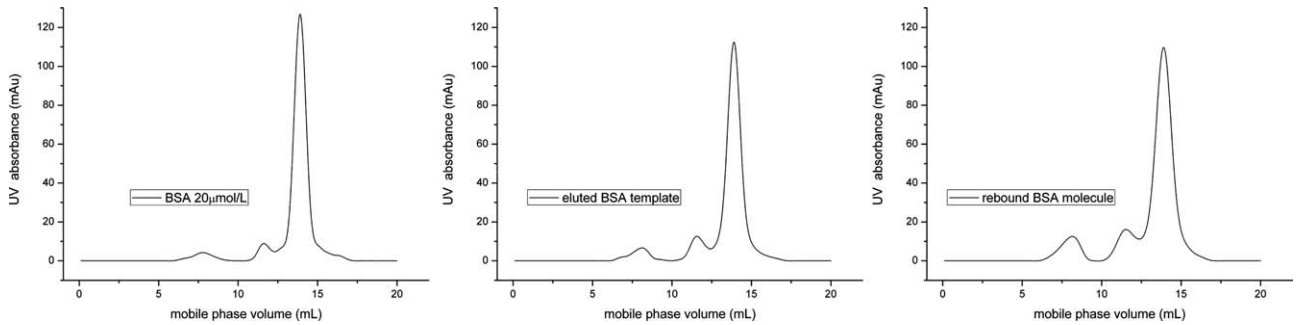

Figure 6. Fast protein liquid chromatography of 1.36 mg/gL BSA, eluted BSA and rebound BSA solutions.

because of compact meshwork and matching reorganization. It is noticeable that IE reached peak value when SR approached 1.65, suggesting an optimum swollen state required for the best performance of specific rebinding. With the aim of investigating selective properties, the separation factors were plotted according to SR and temperature (Figure 11). BSA-imprinted microspheres exhibited the best selectivity at around 33–34°C and slightly lower α at lower temperature (higher SR). This could be explained that microspheres took in BSA more than OVA at the beginning when SR was close to 1.65. As microspheres were further swollen, although the complementary was lost due to swelling, specific sites were preserved because of bound BSA. Moreover at lower temperature, adsorption was facilitated and the bound BSA did not easily break off from the matrix. Therefore, α was not as much decreased as it was higher than 34°C. The separation factor of non-imprinted microspheres was almost the same in all swollen states except for a slight increase due to the expanded porous surface of swollen polymer. Because of the lowered temperature, the in-diffusion of protein surpassed the out-diffusion and brought more diffused protein back to hydrogel. The non-imprinted microspheres rebind more competing protein through nonspecific adsorption therefore possess lower separation factors. The optimum α of imprinted microspheres was achieved at about SR = 1.65 and then approached equilibrium because that selective rebinding required the best matching between targets and matrix, which was gradually achieved as the material swelled and protein configurationally matched.

Rebinding in Different pH and Cation

In the studies of rebinding in solutions with different pH, the cations (Na^+ or Ca^{2+}) of basic buffers influenced differently on the swelling property of alginate-based hydrogel. The SR of microspheres was investigated as a function of pH (Figure 12). The reason for higher SR at alkaline may be explained by the crosslinking mechanism of alginate. Carboxyl groups of alginate existed in the form of carboxylic acid (—COOH) at lower pH and more hydrogen bonds were formed within their hydroxyl groups (—OH). Alginate hydrogel was therefore more densely crosslinked and was restricted from swelling. As in relatively neutral surrounding, the —COOH groups were partly transferred into carboxylate group ($—COO^-$). More electrostatic repulsion was established among polymer chains facilitating swelling. When the alkaline was further strengthen, excessive $—COO^-$ was crosslinked with Ca^{2+} and swelling was restricted again while the samples in Na^+ solution were further swollen till equilibrium.

The imprinted microspheres were allowed rebinding in BSA solutions of various pH with Na^+ and Ca^{2+} respectively and the

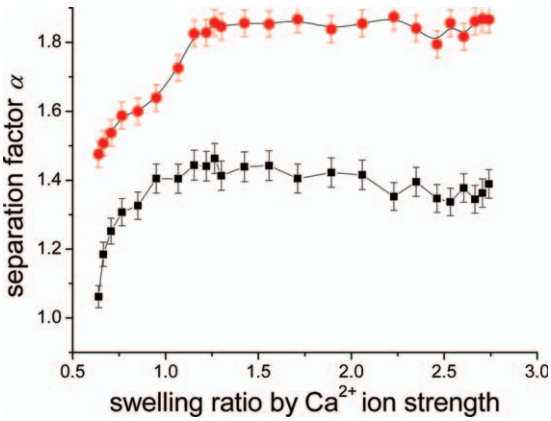

Figure 7. Separation factors of BSA imprinted and non-imprinted microspheres. ■, α of BSA-imprinted microspheres; ●, α of non-imprinted microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

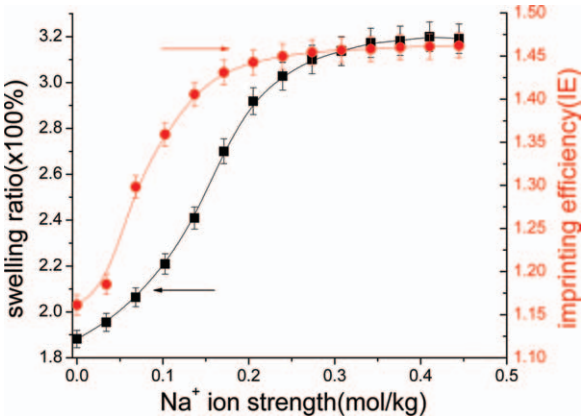

Figure 8. Imprinting efficiency and swelling ratio at different Na^+ ion strength. ■, swelling ratio; ●, imprinting efficiency. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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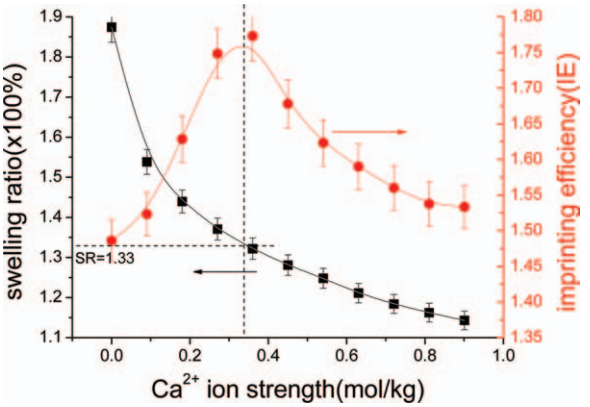

Figure 9. Imprinting efficiency and swelling ratio at different Ca^{2+} ion strength. ■, swelling ratio; ●, imprinting efficiency. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

IE was shown in Figure 13. Two peaks were found in IE curves of BSA solution with Ca^{2+} while only one peak was found in the case of Na^{+} solution. The pair of peaks in Ca^{2+} solution were located at pH 5.5 and pH 10.3, corresponding to SR = 1.67 according to Figure 12. This suggests an optimum swelling state required for the specific rebinding. Similarly, samples tested in BSA solutions with Na^{+} were also found rebinding the best when SR reached 1.67. However, only one peak was found in its IE curve because that SR in Na^{+} was monotonic increasing and never went back to the same lower level. This rebinding responsiveness to pH and cation was originated from the synergy of ionic and covalent crosslinking structure of PVAc-Ca-Alg hydrogel. When swelling, Ca^{2+} was almost substituted for Na^{+}, leaving covalent bond to prevent the mesh from swelling. Therefore, hydrogel matrix as well as the imprinting structure was maintained.

Considering the effect of pH on protein configuration, separation factors of BSA and OVA were plotted against pH (Figure 14) with Na^{+} and Ca^{2+} cation, respectively. Imprinted

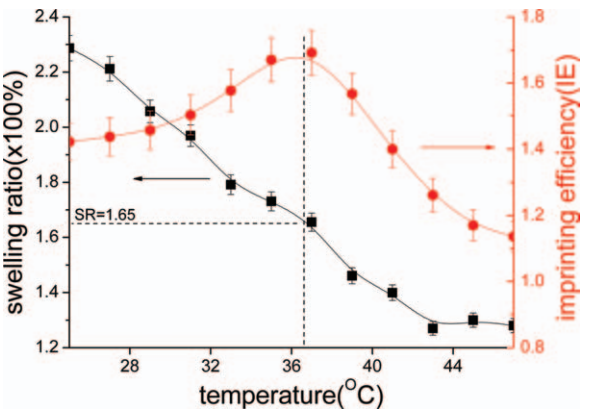

Figure 10. Swelling ratio and imprinting efficiency as the function of temperature. ■, swelling ratio as temperature ascending; ●, imprinting efficiency. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

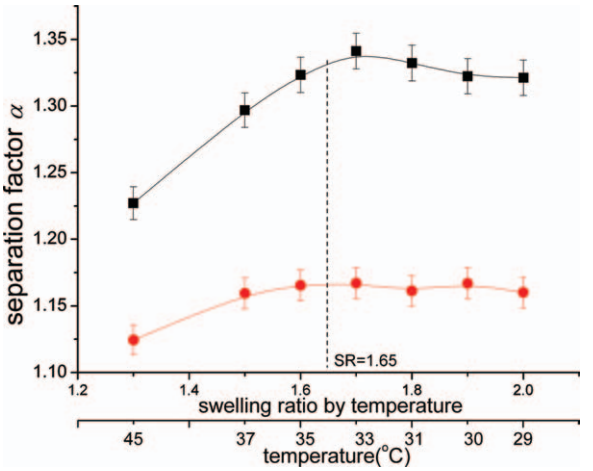

Figure 11. Comparison of separation factors of BSA and OVA under different temperature. ■, α of BSA-imprinted microspheres; ●, α of non-imprinted microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

microspheres possessed good selectivity toward templating molecule. When tested in Ca^{2+} surrounding, the separation factor seems to increase at lower pH, especially at about pH 4–5. The reason was that the competing molecule (OVA) was at its isoelectric point while BSA was positively charged. Therefore, recognition was facilitated by electrostatic attraction from —COO^{-}. Higher pH also resulted relatively higher α because of electrostatic repulsion established between —COO^{-} and negatively charged target molecules. Only those with the complementary structure (i.e., BSA) were allowed to bind on the sites. α curve in Na^{+} solution exhibited maximum value at around pH 4.4, at which hydrogel matrix was swollen to the most appropriate state (SR = 1.66). The curve decreased at higher pH because of deteriorate imprint structure at fully swollen state. With the aim of inspecting the swelling influence of rebinding, IE was plotted against SR, as shown in Figure 15. All of the three peaks of IE-pH curves were located at around SR = 1.66, indicating similar

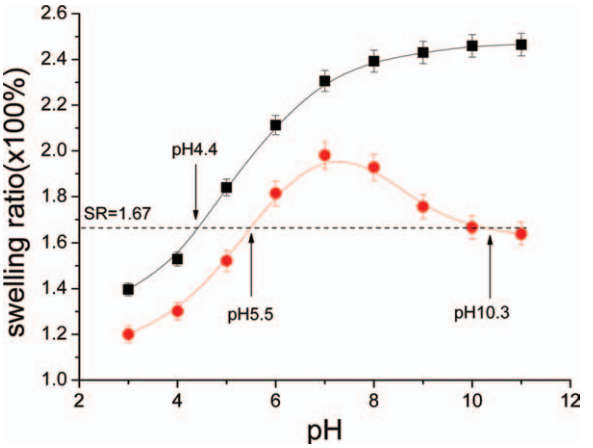

Figure 12. Microspheres swelling ratio as the function of pH. ■, buffer containing Na^{+}; ●, buffer containing Ca^{2+}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

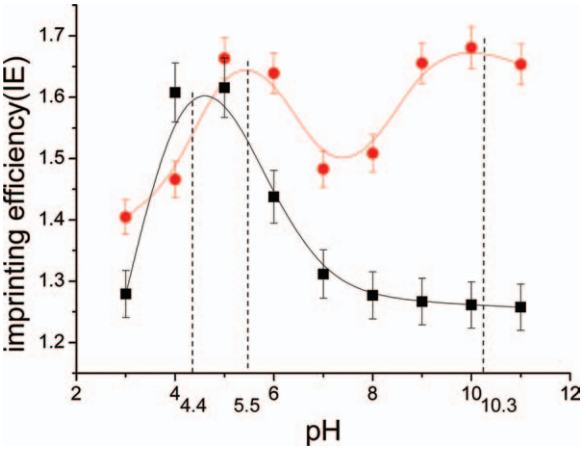

Figure 13. Imprinting efficiency of BSA as the function of pH. ■, buffer containing Na^+; ●, buffer containing Ca^{2+}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

swelling effects on the imprinting structure. The best matching of targets and matrix was accomplished as the hydrogel was swollen to required extent.

It has been published earlier[31] about the pH effect on molecular IE of calcium alginate with covalent crosslinked IPNs. The hydrogel microspheres with different crosslinkage showed varied IE from 1.35 to 1.00 as the function of pH, compared with the range between 1.40 and 1.65 in this work. The imprinting properties have been remarkably improved because of the well-preserved protein imprints due to PPG method and the hydrophobic segment induced by PVAc grafting.

It is interpreted as above the interaction between protein templates and hydrogel matrix under various conditions. The macromolecularly imprinted hydrogel was expected to dissolve, release, and finally rebind protein when imprinting, eluting, and

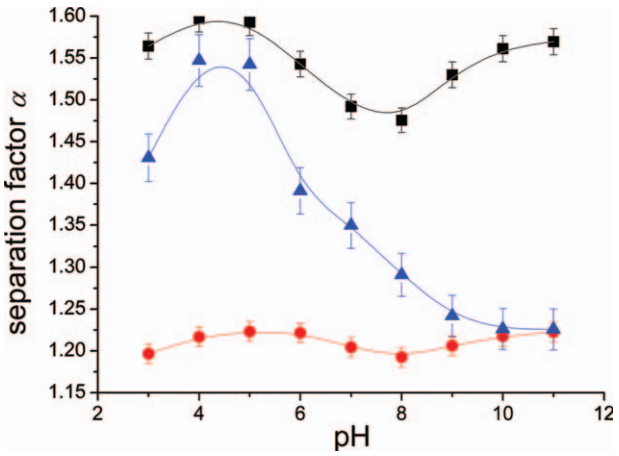

Figure 14. Separation factors of BSA and OVA in the BSA-imprinted microspheres under different pH in buffer containing Ca^{2+}. ■, α of BSA-imprinted microspheres in buffer containing Ca^{2+}; ▲, α of BSA-imprinted microspheres in buffer containing Na^+; ●, α of non-imprinted microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

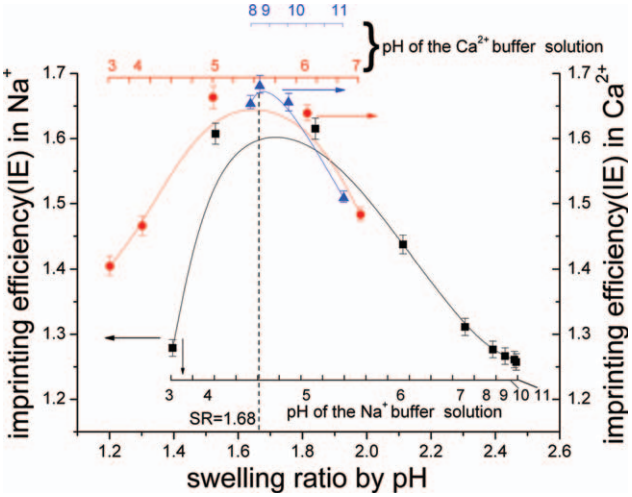

Figure 15. Imprinting efficiency as the function of swelling ratio. ■, buffer containing Na^+; ●, buffer from pH 3 to 7 containing Ca^{2+}; ▲, buffer from pH 8 to 11 containing Ca^{2+}.

rebinding of the target molecules, which required different interaction intensity within the interacting couples. The interactions were regulated by several environmental factors like ionic strength, temperature and pH. A relatively stable and intact protein configuration was required during these performances and therefore the protein solution was adjusted to isoelectric point. Proper concentration of Ca^{2+} was also used for the inhibition of hydrogel swelling during the hardening curing step.

The elution of templates required an expanded mesh structure. Besides, a weaker binding interaction was also favorable. Amino chelating reagent (TRIS) was used and the eluting solution was adjusted to weak alkalescency (pH = 7.6). As the polymer was swollen in basic solution, the templating macromolecules broke away more easily from imprints and binding sites, resulting in less destruction of the matrix. In addition, protein molecules were negatively charged in alkalescency and an electrostatic repulsion was appropriately established.

As for rebinding performance, hydrogel mesh dimension and functional sites configuration were expected to regain their structural features and binding force just as when the imprinted material was prepared. Therefore, calcium ion (Ca^{2+}) of the same ionic strength was applied in the adsorbate protein solution to provide a suitable matrix structure and good hydrogel stability. Furthermore, the adsorbate protein solution was adjusted around pH = 4 and pH = 5 at about 33°C so that the imprinted polymer exhibited the best selectivity.

CONCLUSION

Protein-imprinted PVAc-Alg-Ca hydrogel was prepared by PPG and GIG method without excessive destroy to the template and imprinting sites. Protein templates proved to be efficiently eluted at an average ratio of 88.5% and the rebinding protein exhibited good recovery up to 86%. The rebinding behavior showed responsiveness toward ionic strength, temperature and pH. The separation factor reaches maximum (α = 1.85) when

the Ca^{2+} ionic strength were 1.25. The highest imprinting efficiency (IE = 1.75) was achieved at $I_{(Ca2+)}$ = 0.34 mol/kg. The separation factor has been increased because of the hydrophobic effect and covalent cross-linking resulting from PVAc segments.

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