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ORIGINAL PAPER

Preparation and characterization of protein imprinted agarose microspheres

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Abstract Agarose hydrogel microspheres imprinted with bovine serum albumin (BSA) were prepared by inverse suspension gelating method. The experimental conditions related with beads' shape and uniformity were studied, including temperature, surfactant content, and stirring speed. The size and distribution of the pores were found related with porogen and surfactant content. Rebinding properties were influenced by the diameter and porosity. The beads with the smallest diameter exhibited the greatest rebinding capacity, rebinding speed, and imprinting efficiency (IE). Smaller pores provided higher rebinding capacity and IE, but lower rebinding speed. Imprinted microspheres showed good specificity toward templating protein compared to others.

Keywords Imprinting · Protein · Agarose · Microspheres · Hydrogel

Instruction

Protein imprinting and rebinding techniques have received much attention in the fields such as separation, purification, biosensor, and diagnoses [1–4]. Unlike rigid templates, protein molecules are fragile and easily changed in configuration while imprinting and extracting, which is presumed to be responsible for the poor imprinting effects [5]. It has been verified in recent studies [6, 7] that numerous flexible regions of protein are of functional importance, allowing protein to interact with partners that are different in configurations.

As a kind of highly flexible matrix with loose mesh, hydrogels have received much attention as imprinting matrix since recently. The preparation and

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characterization of soft-wet hydrogel microspheres imprinted with BSA has been reported possessing higher rebinding efficiency toward the template [8, 9]. Other hydrogel polymers appropriate for protein imprinting are PHB [10] and PMAA [11], etc. Natural polysaccharides such as alginate [12] and agarose [13] are more popular as imprinted matrices because of their massive water content, moderate gelating condition, and good biocompatibility. As for the imprinting in hydrogel materials, it is believed the segments on polymer chains change shape to accommodate the conformation of the template protein [14]. Protein imprinted hydrogel materials are presumed to be essential in understanding the protein colloid interaction and mimicking natural molecular recognitions.

Agarose is a natural polysaccharide derivative from agar–agar. It is soluble in hot water (about 80–90 °C) and will not gelate until cooling down below 41 °C. Agarose has found wide application in DNA gel electrophoresis, isolation and identification of RNA [15–17] because of its good permeability, stability, and gelling property at room temperature. In the study of Melani et al. [18], agarose particles were prepared with an average diameter of 104 μm and packed in columns with a bed height between 5 and 6 cm. Mu et al. [19] studied the impact of compression load on the porous agarose beads prepared with different impeller speed. The deformation of particles manufactured at 500 and 1100 rpm are, respectively, 8% and 15%, suggesting good elasticity of the microsphere sample. The massive functional groups (like –OH and $-CH_2OSO_3^-$) and large mesh size facilitate protein delivering and accommodating. These make agarose an attracting candidate for protein imprinting matrix. The latest research on protein imprinted agarose membrane was reported by Lin et al. [13]. However, protein imprinted agarose microspheres (PIAMs) have seldom been reported.

In this study, PIAMs were prepared by inverse suspension gelating method. The emulsion templating method was applied for creating porous structure in the protein imprinted porous agarose microspheres (PIPAMs). Optical microscopy and laser particle size analyzer were used to investigate the bead diameter, pore size, and distribution in related with varying conditions such as bath temperature, porogen/surfactant content, and stirring speed. The rebinding property was then evaluated in the form of rebinding quantity, imprinting efficiency (IE), and recognition specificity.

Experimental procedure

Materials

Agarose was provided by Amersco[@], Solon, Ohio (gelling temperature: 34–38 °C, melting temperature: 87–89 °C, sulfate $\leq$0.15%). Soybean salad oil was used as suspending medium. Liquid paraffin and Sorbitan monooleate (Span80, $C_{24}H_{44}O_6$, $M = 428.61$) were from Damao chemistry reagent plant, Tianjin. Bovine serum albumin (BSA, fraction v, 66,000, isoelectric point p$I = 4.8$), bovine serum hemoglobin (Hb, Mw = 68,000, p$I = 6.7$), c-globulin (Glo, Mw = 160,000, p$I = 6.8$) were obtained from Institute of Hematology Chinese Academy of Medical

Science,electrophoretic grade. Ovalbumin (OVA, Mw = 45,000, and pI = 4.7) was from Sigma, electrophoretic grade.

Preparation of PIAMs and PIPAMs

Agarose hydrogel microspheres were prepared by means of inverse suspension gelating method. The preparation flow chart was shown in Fig. 1. For preparing PIAMs, 0.800 g agarose powder was dispersed thoroughly in 10 mL deionized water via magnetic stirring then heated in water bath (approx. 85 °C) to form a homogenous viscous solution. The solution was then kept in water bath at 43 °C, the temperature that was harmless to the templating protein and would not lead to the gelation of agarose. In preparing the agarose-BSA solution, exactly weighted 0.0264 g BSA powder was dissolved in 10 mL deionized water by slight stirring to form a 40 μmol/L BSA solution. The solution was then placed in the 43 °C water bath. Agarose and protein solution were thoroughly mixed and kept in 43 °C water bath for further use. The contents of agarose and protein in the mixture were, respectively, 4% (w/v) and 20 μmol/L (i.e., 0.132%(w/v)).

In our past researches on inverse suspending gelating method, chloroform and hexane were used as oil phase [12]. The microspheres prepared have good sphericity

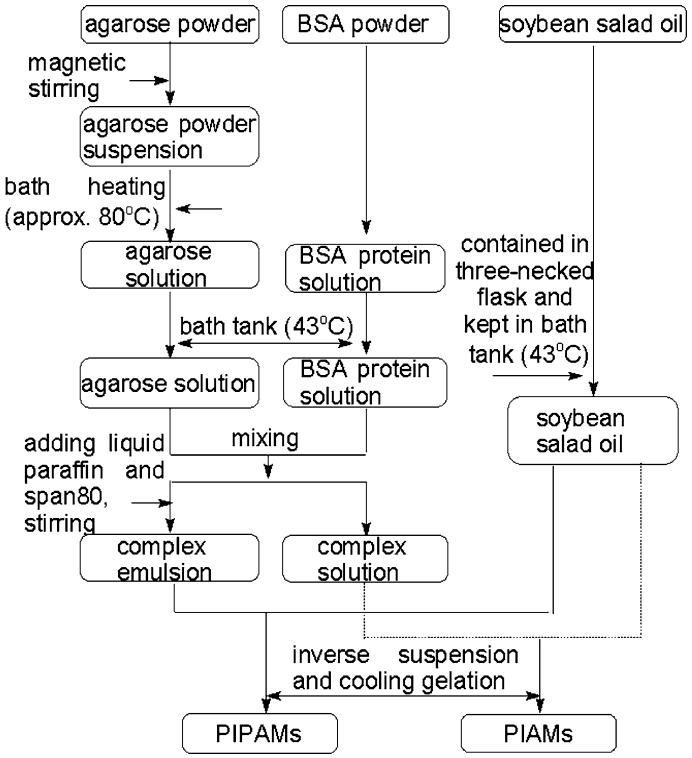

Fig. 1 The inverse suspension gelating method for preparing PIPAMs and PIAMs

and uniformity. However, we decided to replace these organic solvents with natural oils considering their toxicity. The options are readily available castor oil and soybean oil. The castor oil proves to be applicable as a suspending media though, the high viscosity [(E020 °C) > 14] is not conducive to forming microspheres with required sphericity [20]. Soybean oil has a much lower viscosity of [(E020 °C) ≈ 8.5] and is appropriate for suspending method.

As for preparing PIPAMs, liquid paraffin (0.5 g) and Span80 (0.02–0.1 g) were added in the agarose–protein solution and emulsified by magnetic stirring (approx. 750 rpm) for 1 h. The emulsion was also kept in 43 °C water bath to avoid any gelation.

The agarose–protein solution or the emulsion was dispersed in 40 mL soybean salad oil in 43 °C water bath by mechanical stirring for 30 min, as illustrated in Fig. 2. The rotating speed was set from 400 to 600 rpm. The reactor was then transferred into ice bath to gelate the agarose droplets into microspheres. The PIAMs (made from the agarose–protein mixture) or the PIPAMs (made from the emulsion) were separated from oil by filtering and washed to get rid of the residuals.

The liquid paraffin droplets in the PIPAMs were removed by treating with ethanol and ether [12]. Then, the microspheres were immersed in anhydrous ethyl ether for 24 h to elute the embedded paraffin droplets, and at last kept in deionized water at room temperature for further use. To remove protein templates, the

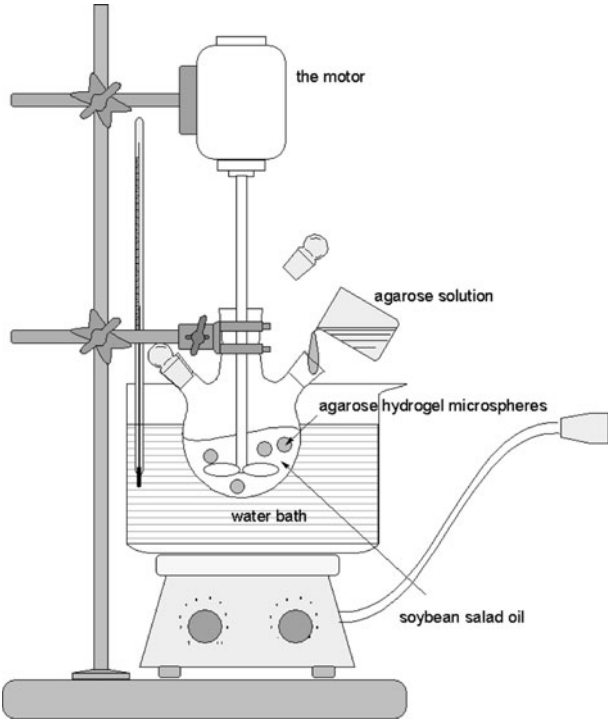

Fig. 2 The installation diagram of inverse suspension method for preparing agarose microspheres

microspheres were immersed in Tris–HCl buffer solution (pH = 7.5, 0.05 mol/L) and slightly shaken every 8 h until no protein was detected in the supernatant liquor.

As for the control samples, nonimprinted agarose microspheres (NIAMs) and porous agarose microspheres (NIPAMs) were also prepared following the above processes except no protein templates existed in the hydrogel.

Morphology and stability of microspheres

The preparing conditions such as water bath temperature and span85 content in the suspending medium were considered affecting the shape and dimension of the microspheres. Therefore, the beads prepared under different conditions were inspected by optical microscope for their morphological study. The microspheres sphericity and porosity were observed by means of inverted optical microscope (Axiovert 25, Carl Zeiss). The diameter distributions were analyzed by means of laser size analyzer. To confirm that the microspheres possess good mechanical stability, an agitation test was performed in culture table with a vibrating frequency of 120 rpm and amplitude of 1 cm at 25 °C. The agarose microspheres were placed in a beaker together with some glass beads (200 μm). The experiment continued for at lest 6 h.

Rebinding tests

In testing the rebinding property of the PIPAMs and PIAMs, exactly weighted microspheres (1.500 g) were immersed in 20 mL protein solution (20 μmol/L, pH = 4.8) and allowed to adsorb till equilibrium. The supernatant liquor was detected every 2 min by UV–vis spectrophotometer. Thus, the concentration of BSA in the adsorbate could be monitored and recorded timely. The microspheres' mass rebinding capacity was calculated as follows:

$$Q = (C_0 - C_t)V/W, \tag{1}$$

where Q is the rebound mass of protein on the microspheres; C_0 and C_t are the concentration of protein solution before and after rebinding; V is the volume of BSA solution; and W is the mass of microspheres. The IE could be calculated as follows:

$$\mathrm{IE} = Q/Q_N, \tag{2}$$

where IE is the imprinting efficiency and the Q_N is the adsorption mass on the nonimprinted microspheres.

Results and discussion

The samples prepared in different experimental conditions were observed with optical microscope. The sphericity and diameter distribution of PIAMs were influenced by temperature, surfactant content, and agitation frequency. The morphology of PIPAMs with and without paraffin droplets was also studied.

Morphology of PIAMs prepared under different temperature

The viscosity of the agarose solution slightly increases as temperature decreases till 38 °C [21]. When suspended in oil, agarose sol was agitated and scattered into oil medium forming liquid drops, being shaped in the most stable dimension until microspheres were gelated as the suspension cooling down. Therefore, the viscosity corresponding to temperature played an important role during the microspheres forming and resulted in different shapes. Microspheres prepared under different temperatures between 36 and 42 °C were observed by optical microscopy and were found much different in their appearance as shown in Fig. 3.

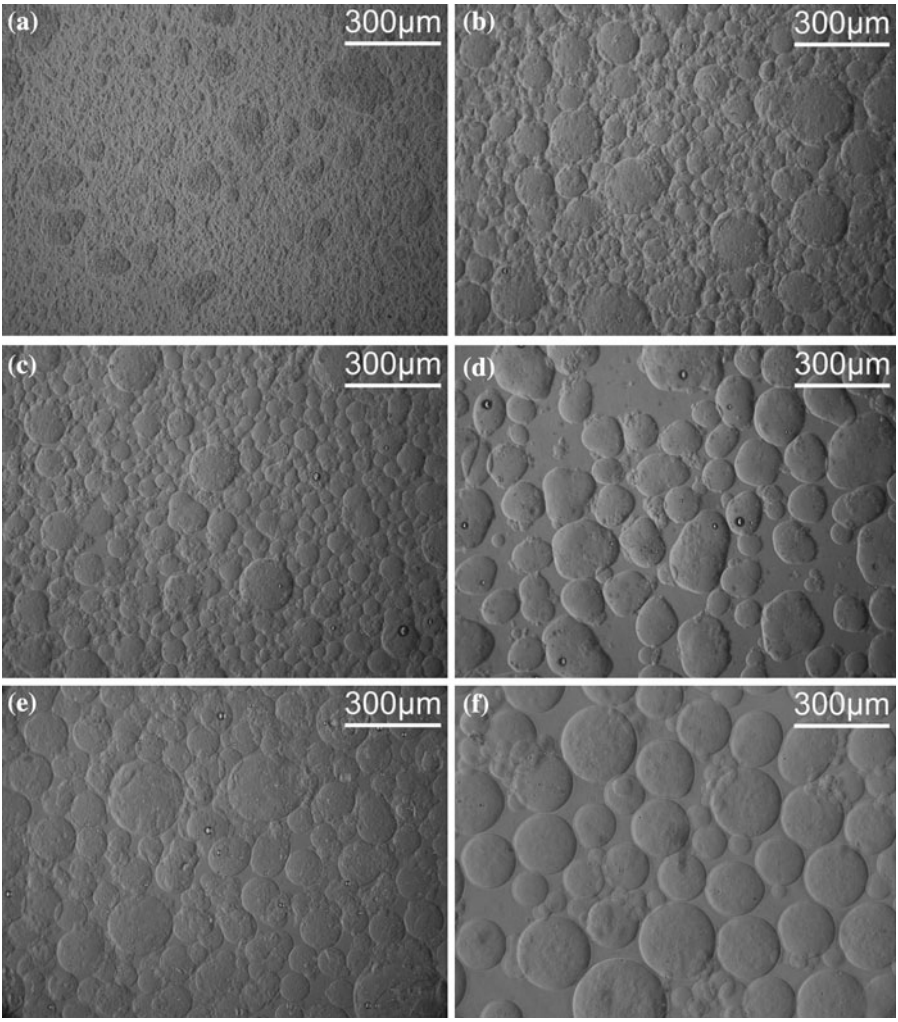

Fig. 3 PIAMs prepared by inverse suspension gelating method in water bath with temperature range from 36 to 42 °C. The microspheres were prepared in a water bath of **a** 36.0 °C; **b** 37.9 °C; **c** 38.4 °C; **d** 40.1 °C; **e** 40.9 °C; and **f** 42.2 °C

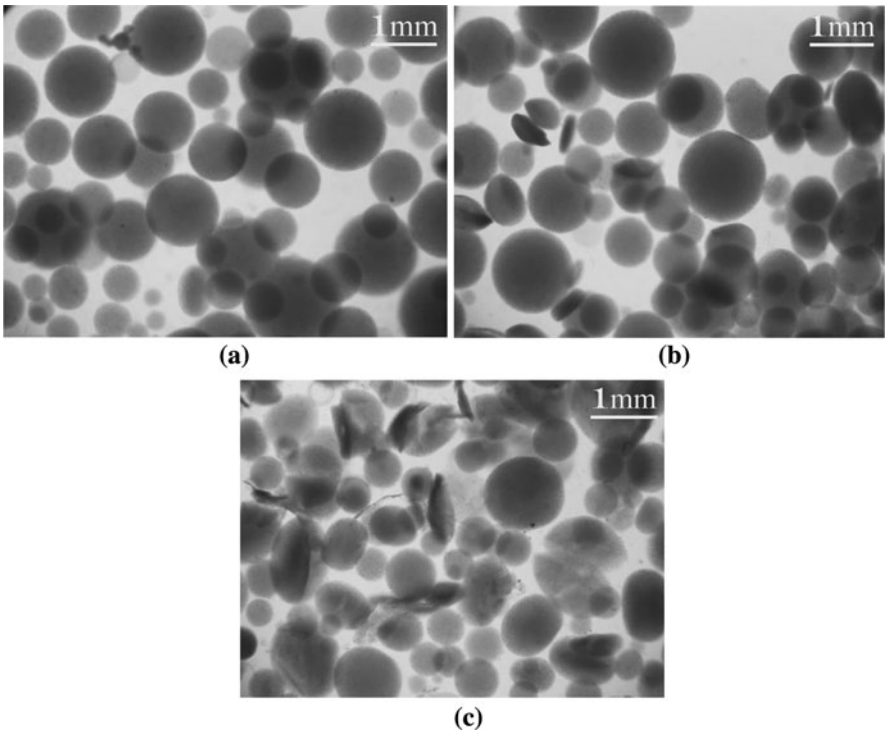

Fig. 4 Agarose microspheres tested by mechanical collision in culture table. **a** The original morphology; morphology after vibrating **b** 3 h, and **c** 4.5 h

Preferable microsphere samples with better sphericity, integrality, and more uniform diameters were those prepared at 42 °C (Fig. 3f). The sphericity became worse as the temperature reduced to 40 °C and irregularly shaped pieces were observed (Fig. 4d, e). It was considered that the increasing viscosity prevents the sol block from sufficient dispersion; as a result, agarose sol was hardly stabilized in the form of globe before gelling. As it went on cooling down till 36 °C, the pieces broke down and more fragments were found (Fig. 3a–c). Agarose sol was partially gelated below 38 °C and broken into pieces by stirrer, resulting in massive fragments. Temperature higher than 43 °C might provide beads even better in morphology, however would denaturize the protein templates and therefore impracticable. The microspheres were also tested against mechanical collision. No deformation or fragment was detected during the first 3 h (Fig. 4a, b). It is, therefore, suggested that the agarose microspheres exhibit the required mechanical stability.

The hydrogel microspheres are composed of crosslinked polymer chains and bound water up to 90% of the mass. They exhibit considerable stability after isolated from the suspension and is easily handling in further operations of rebinding and eluting. The SEM of dehydrated agarose microspheres were shown in

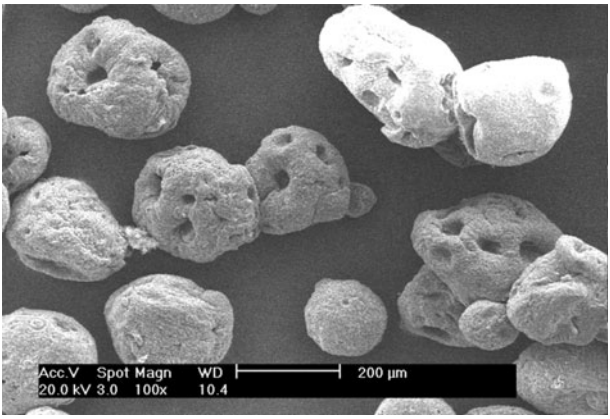

Fig. 5 Scanning electron microscopy of agarose microspheres treated by freeze-drying

Fig. 5. These samples were prepared by freeze-drying of hydrogel microspheres and are not applicable to rebinding experiments unless they are swollen again. Fortunately, all of the rebinding tests are performed in protein solution. The idle samples can be preserved in aqueous solution for as long as 3 months before next use.

PIAMs prepared with different surfactant content

The PIAMs were prepared by means of inverse suspension gelating method in 40 mL Soybean salad oil as suspending medium (Fig. 6). Span80 was added quantitative as surfactant to guarantee good sphericity and uniform dimension. It was found the diameters and distribution were affected remarkably by Span80 content (as presented in Table 1). Samples with relatively uniform diameters were obtained with 0.2 g of Span80 added in the suspension medium, as indicated by the smallest standard deviation (SD) value and polydispersity index (PDI) of sample b in Table 1.

The usage of Span80 reduced the surface tension of sol droplets and to guarantee dimensional uniformity. More large and small beads were observed in the samples prepared with less Span80 (0.1 g) as shown in Fig. 6a. It was probably because of the sol drops formed at stirring collision and incorporated into larger ones due to the insufficient dispersive capacity. The dimensional uniformity was improved at the increase of Span80 (up to about 0.2 g), as proved by the smallest SD and PDI in Table 1, indicating better dimensional uniformity. This sample was the most preferable in especially rebinding tests required by the performing consistency and data stabilization. In addition, smaller diameter provided larger specific surface area, which was helpful for protein contacting and diffusing. Excessive Span80 up to 0.5 g was not either suitable for preparation of good samples because much small beads were formed and hard to handle with.

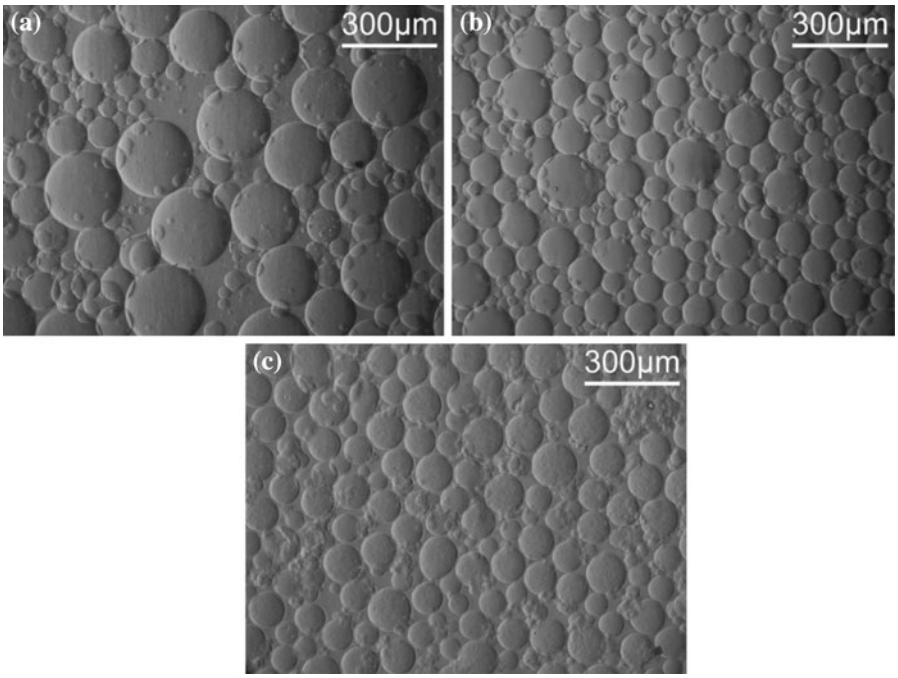

Fig. 6 PIAMs prepared with different content of Span80. The Span80 added in suspension medium was **a** 0.1 g; **b** 0.2 g; and **c** 0.5 g

Table 1 Span80 content in the oil medium and the corresponding diameter statistics

Sample ID	Span80 dosage (g)	Mean d (μm)	SD (μm) dEr±	Median (μm)	PDI
a	0.1	108.37	65.47	87.94	0.604
b	0.2	86.62	27.64	88.52	0.319
c	0.5	83.32	30.93	79.40	0.371

PDI is calculated as PDI = δ/d, where 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

The PIAMs prepared under different stirring speed

The agarose sol was dispersed and suspended in the oil media by mechanical stirring before gelating. The diameter of the microspheres was affected remarkably by the agitating speed. To achieve the best dispersity and sphericity, 0.2 g of Span80 was added in the suspension solution. PIAMs prepared at different stirring speeds were shown in Fig. 7. Microspheres with smaller diameter were prepared at higher agitating speed up to 610 rpm providing enough shearing force. Higher stirring speed leads to lower SD and PDI calculated according to photos (Table 2). In consideration of operation convenience in rebinding test, the agitating speed in later preparations was set at 500 rpm.

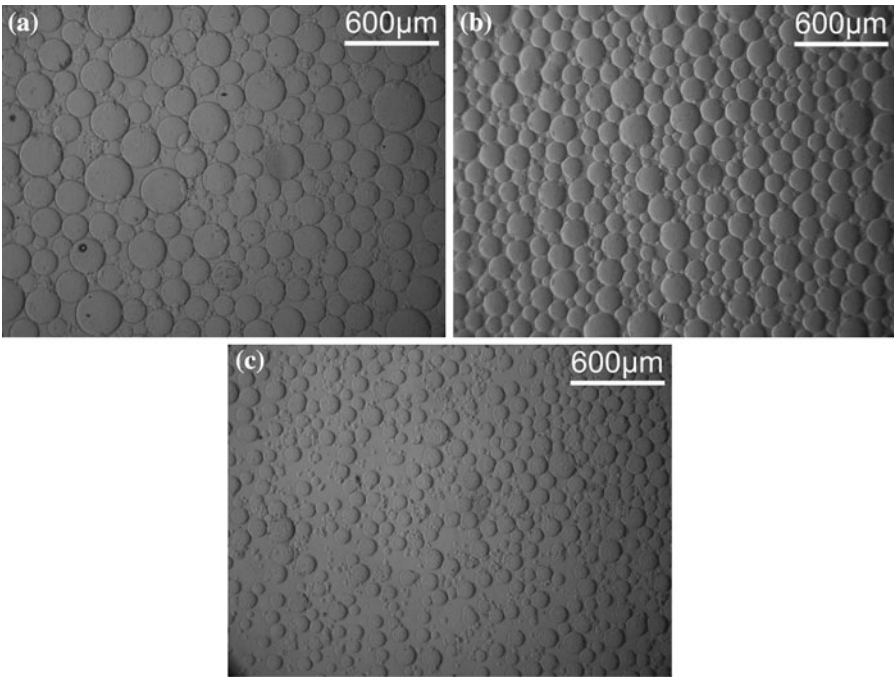

Fig. 7 PIAMs prepared at a stirring speed of **a** 429 rpm; **b** 504 rpm; and **c** 610 rpm

Table 2 Agitating speed and the corresponding diameter statistics

Sample ID	Agitating speed (rpm)	Mean *d* (μm)	SD (μm) *d*Er±	Median (μm)	PDI
a	429	163.07	51.63	156.8	0.317
b	504	117.50	36.78	111.06	0.313
c	610	100.74	18.90	99.3	0.188

Microspheres are prepared with 2.0 g Span80 as the surfactant

The PIPAMs prepared with different porosity

With the aim of increasing the contacting surface and permeability of the microspheres, porous structure was introduced in agarose hydrogel by means of emulsion templating method [12]. The samples were observed under optical microscope as shown in Fig. 8. The emulsion droplets dimension and distribution were conditioned by the content of liquid paraffin and Span80 (Table 3).

Microspheres with more uniform and smaller pores were prepared using 0.1 g of Span80 as the emulsifying agent, compared with the samples prepared with 0.02 and 0.06 g of Span80. Several large holes were found in Fig. 8a and b, which was considered caused by insufficient emulsification. The emulsifying capacity provided by adequate Span80 would separate and disperse any larger paraffin droplets into

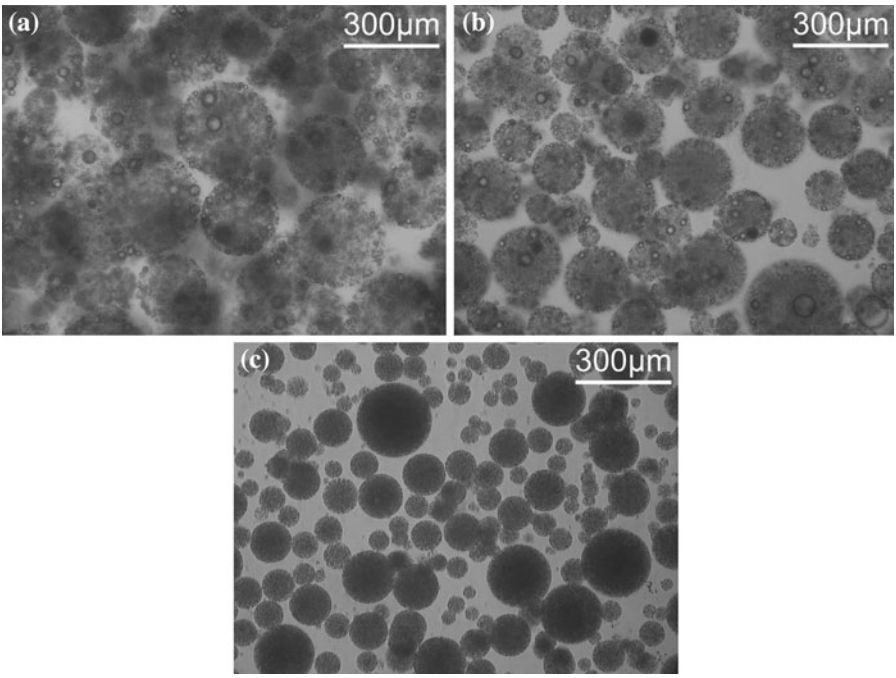

Fig. 8 The emulsion imprinted microspheres prepared with different Span80 dosage (g) before removal of paraffin. **a** 0.02 g; **b** 0.06 g; and **c** 0.1 g

Table 3 Statistics of porous microspheres prepared with liquid paraffin and different Span80 dosage

Sample ID	Paraffin (g)	Span80 (g)	Mean *d* (μm)	SD (μm) *d*Er±	PDI	Mean *d*p (μm)	SD (μm) *d*pEr±	PDI
a	0.5	0.02	118.32	66.28	0.560	25.13	6.01	0.239
b	0.5	0.06	104.24	48.16	0.462	16.04	3.16	0.197
c	0.5	0.1	98.17	53.99	0.550	5.95	0.98	0.165

The Span80 contents listed in the table refer to that used as the emulsifying agent. "Mean *d*" is the mean diameters of microspheres; "Mean *d*p" is the mean diameters of pores in microspheres

smaller ones. The PIPAMs could not be applicable in rebinding experiments until the emulsion droplets were removed. The liquid paraffin droplets could be eluted by soaking in gradient ethanol and ether solution and the microspheres take on transparent appearance as the paraffin completely removed (Fig. 9).

The main purpose of introducing pore structure is to investigate the swelling and diffusing influences on rebinding behavior. The rebinding quantity and IE are studied related with the relative differences among the pore size. However, the transparent appearance of the particles after removal of the emulsified paraffin makes it almost impossible to estimate the pore size by observing the photograph. Nevertheless, the description of pore size as exactly the emulsion droplet size is not

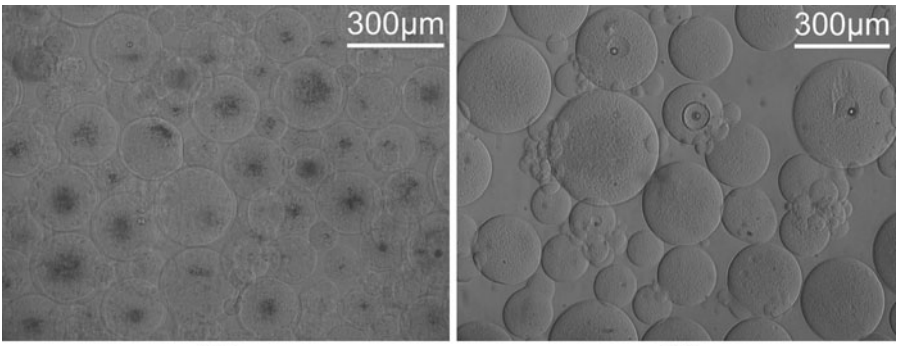

Fig. 9 PEIAMs eluted off the liquid paraffin. **a** Paraffin partly removed and **b** paraffin completely removed

quite accurate. We decided to develop a more appropriate method of pore size measuring in future works.

Rebinding test of PIAMs with different particle diameters

The elution percentage of the BSA template is estimated by the UV–vis measurement of BSA concentration in three batches of eluate (Table 4). The BSA is hardly detectable in the third eluate, and it is considered that the templates have been removed. The percentage of liberated imprints is calculated as the proportion of eluted protein to the total amount used as template. According to Table 4, 82.20% of the templates have been eluted; others are trapped in the hydrogel becoming part of the microspheres.

The rebinding experiments were performed by immersing exactly weighted microspheres (1.500 g) in 20 mL 20 μmol/L protein solution (pH = 8.2) and allowed to adsorb till equilibrium. Rebinding quantities (Q) and IE were plotted against time as shown in Fig. 10. The PIAMs possess higher rebinding quantity than the NIAMs approaching equilibrium. The IE increased most at certain period, during which the PIAMs were considered to perform the best.

Table 4 Calculation of the liberated imprints percentage of microspheres by UV–vis measurement

Mass of BSA template (g)	Eluting process				Percentage of liberated imprints (%)
		First	Second	Third	
	BSA concentration in eluting solution (g/mL)	7.93×10^{-4}	2.56×10^{-4}	2.04×10^{-5}	
	Eluting solution volume (mL)	20.21	20.53	19.30	
	Mass of eluted BSA (g)	0.0160	0.0053	0.0004	
0.0264		$\Sigma = 0.0217$			82.20

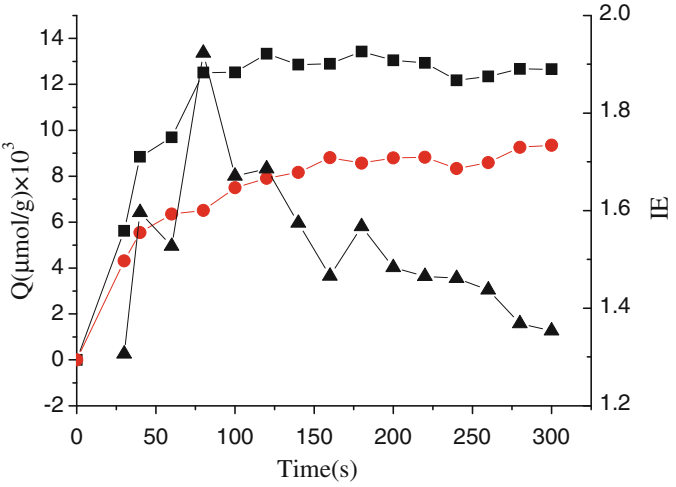

Fig. 10 Rebinding quantities and efficiency on protein imprinted and nonimprinted microspheres as the function of time. *Filled square*: re-binding quantity of protein imprinted agarose microspheres (PIAMs); *filled circle*: re-binding quantity of nonimprinted agarose microspheres (NIAMs); and *filled triangle*: IE of PIAMs

Macromolecular imprinting in hydrogel matrices usually involves multipoint rebinding among specifically arranged sites and segment or region interactions between the polysaccharide chains [9]. In a soft and wet material like agarose containing water more than 90%, the folding and assembling configuration of the chains are keeping changing as water and solute exchanges. Certain configuration provides the most appropriate arrangement of the specific sites and regions and therefore the IE is the greatest.

PIAMs with different particle diameters were found different in their rebinding properties. The smallest microspheres exhibited the highest rebind speed and rebinding quantity (Q). Oppositely the Q of larger ones increases much slower (Fig. 11). The reason is probably the transferring factors and specific surface area in connection with the particle diameters. Smaller microspheres have larger specific surface area and shorter radial path. The rebinding on surface is considered contributing a lot in rebinding speed and quantity, especially in the macromolecular imprinted systems where template volume plays a decisive role in transferring. A shorter way into the spherical center also ensures fully utilization of inner rebinding sites, leading to higher rebinding quantity.

The imprinting efficiencies were calculated by formula (2) and plotted as a function of time (Fig. 12). High IE was found in small sample at the beginning of rebinding (IE approx. 2.16) but decreased soon. The IE of larger microspheres was much lower (about 1.3) but was kept at certain level for a longer time. This could be explained by the size and swelling properties of the microspheres. Samples with shorter radial length swelled at higher rate and allowed the protein to permeate faster. Imprinting sites deep inside the microspheres were more easily reached and bound by protein molecules. As a result the IE of smaller microspheres was higher

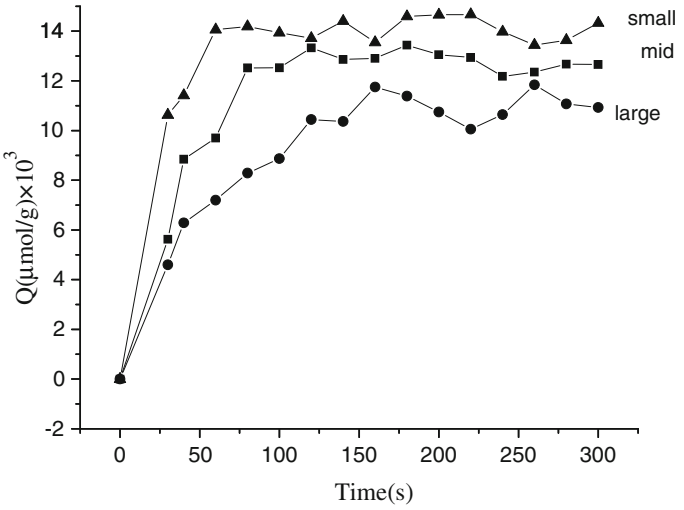

Fig. 11 Rebinding quantities of microspheres with a mean diameter of: *filled triangle*: small, 90 μm; *filled square*: mid, 120 μm; and *filled circle*: large, 150 μm

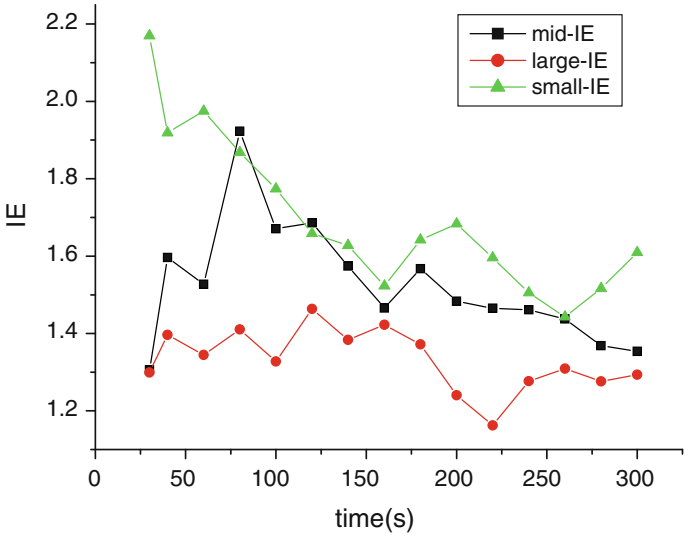

Fig. 12 Imprinting efficiency of microspheres with a mean diameter of: *filled triangle*: small, 90 μm; *filled square*: mid, 120 μm; and *filled circle*: large, 150 μm

than that of the larger ones. Oppositely, the larger microspheres with longer radial length and lower swelling rate will block the target molecules permeating deep into the matrix, and most of the protein was adsorbed on surface and superficial depth of the microspheres.

The IEs of small- and medium-scaled beads decreased to about 1.62 and 1.39, which was probably caused by the swelling property of the hydrogel. In the

hydrogel matrix, the imprints were constructed by the hydrogel polymer chains with specific sites orientation and segments arrangement. When the microspheres swelled, the specificity of the imprints deteriorated at the chain movement. The specific rebinding effect of the PIAMs was gradually replaced by nonspecific rebinding, resulting in the IE reduction. Microspheres with relatively larger diameter were not easily swollen and the inside imprints could be preserved for a longer time to rebind successive molecules. As a result, although IE was lower, it was kept at a certain level when the microspheres were swelling.

Rebinding test of PIPAMs with different pore size

Microspheres with different pore size were prepared by adjusting the content of liquid paraffin and Span80 in the emulsion. Samples with large pores had relatively higher rebinding speed than others; however, they approached equilibrium at the lowest level of Q (Fig. 13). Pore size was considered to affect swelling and diffusion properties of microspheres. Beads with larger pores swelled faster in protein solution and enhanced the inner diffusion and rebinding of protein. Larger pores take up more space from the hydrogel for imprinting sites and result in lower rebinding quantity.

The IE were calculated and plotted against time and showed in Fig. 14. The microspheres with smaller pores were provided with higher IE (about 2.11). As for the sample with larger pores, the configuration of the specific rebinding sites and segments will not be preserved for a long time because these microspheres were

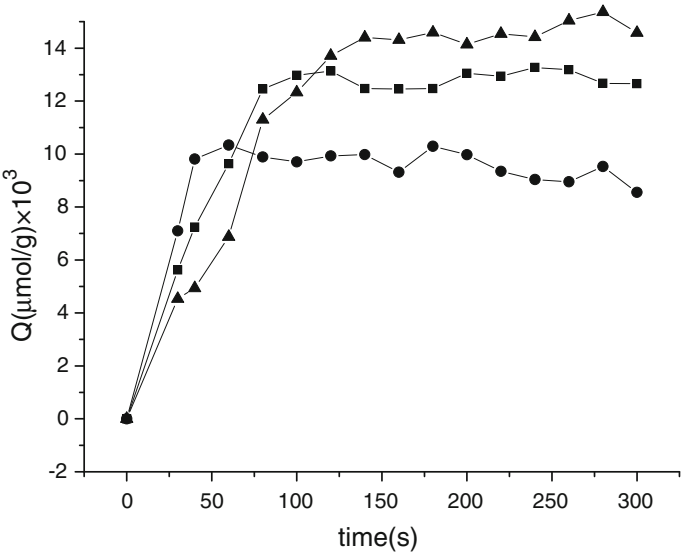

Fig. 13 Rebinding quantities of microspheres with a mean pore diameter of: *filled triangle*: small, 6 μm; *filled square*: mid, 16 μm; *filled circle*: large, 25 μm. The particle diameters of each sample are all 120 μm

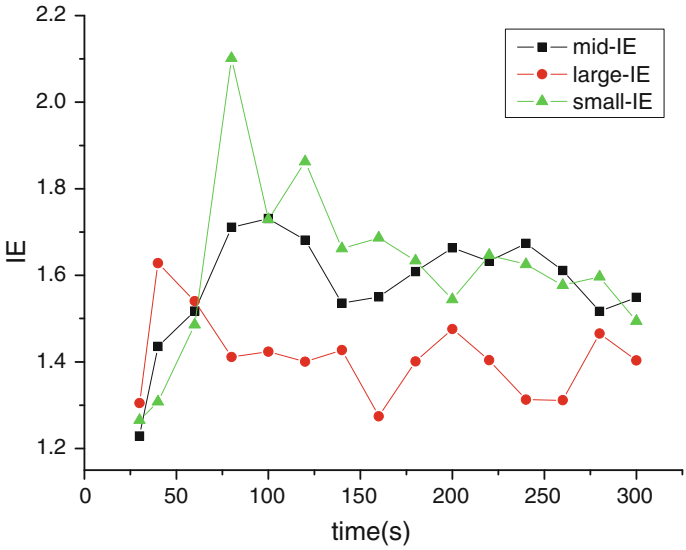

Fig. 14 Imprinting efficiencies of microspheres with a mean pore diameter of: *filled triangle*: small, 6 μm; *filled square*: mid, 16 μm; and *filled circle*: large, 25 μm. The particle diameters of each sample are all 120 μm

swelling faster. The specific rebinding sites were soon deteriorated after about 40 s and the specific rebinding was gradually replaced by nonspecific rebinding.

All of the IE curves of the microspheres were found with peak values, which could be defined as the molecular imprinting specific point (MISP). Unlike micromolecular imprinting materials, the macromolecular imprinted hydrogel material has a rebinding strategy consisting of multipoint rebinding and segment (region) interaction, both of which are connected with the chain movement of the hydrogel matrice. While rebinding protein targets, the imprints in hydrogel matrice are not always best matching with the targets until the hydrogel swells to certain level. The peak value of IE is pointing right to the time when the best matching is achieved. Study of MISP may be a feasible approach to better understanding macromolecular (especially protein) imprinted hydrogel systems.

The differences between PIAMS and PIPAMS lie in porous structure of PIPAMS. The pore structure is formed by emulsification of agarose solution with liquid paraffin droplets as hydrophobic phase. The main purpose of creating pores is to enlarge surface, construct channels, and increase swelling. As is known to all that a major drawback of protein imprinting is the large volume of protein used as template. The template is usually trapped in the polymer matrix in the eluting process. It is also difficult for protein to diffuse into polymer when rebinding. Pore structure provides the microspheres with channels and larger surface area, so that protein may perform more efficient internal diffusion and adequate contacting with polymer matrix. The rebinding sites deep inside become accessible and the IE is

Table 5 K_D and α values of PIAMs, PIPAMs, NIAMs, and NIPAMs

Target protein	PIAMs Mean d = 100.7 (μm)		PIPAMs Mean d = 98.2 (μm) Pore size = 6.0 (μm)		NIAMs Mean d = 99.8 (μm)		NIPAMs Mean d = 100.1 (μm) Pore size = 5.9 (μm)	
	K_D	α	K_D	α	K_D	α	K_D	α
BSA66000	1.25	1.00	2.71	1.00	0.72	1.00	1.18	1.00
OVA45000	0.81	1.54	1.25	2.17	0.80	0.90	1.21	0.98
Hb68000	0.72	1.74	0.96	2.82	0.71	1.01	1.16	1.02
Glo160000	0.78	1.60	0.92	2.94	0.63	1.14	1.00	1.18

The templating protein is BSA

therefore increased. Trapped templates in the polymer matrix are more easily eluted in the presence of pore structure.

Recognition specificity

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

$$K_D = C_P/C_S,$$

where C_P (μmol/g) is the concentration of the target molecule on microspheres (i.e., $C_P = Q_e$), and C_S (μmol/ml) is the concentration in the solution.

$$\alpha = K_{D1}/K_{D2},$$

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

The selectivity testing of BSA-imprinted agarose microspheres was carried out under equilibrium binding conditions using OVA, Hb, and Glo as contrastive molecules. Table 5 shows K_D and α values of BSA-imprinted and nonimprinted microspheres with respect to OVA, Hb, and Glo. It was found that the K_D of BSA onto BSA-imprinted beads was much higher than that of different proteins. The separation factor of BSA-imprinted microspheres increased with the increase of the proteins' Mw. For OVA, Hb, and Glo, the separation factors of PIAMs and PIPAMs were over 1.00 and 2.00, indicating that BSA-imprinted microspheres exhibited good recognition selectivity for the template protein. In contrast, nonimprinted microspheres showed lower K_D and α values.

The higher affinity of the imprinted microspheres for BSA is attributed to the generation of rebinding sites and the complementary cavities in the matrix during the gelation reaction [9]. The imprinted microspheres created a microenvironment based on the complementary cavities and combined interactions. In the rebinding process, imprinted microspheres favored BSA adsorbing, leading to a high K_D. For OVA, Hb, and Glo, the relatively low K_D was due to the poor match between proteins and the imprinted matrix produced by BSA.

Conclusion

Protein imprinted hydrogel microspheres were prepared through inverse suspension gelating method using agarose as a new matrix. The sphericity, integrality, diameter distribution, and pore dimension of the PIAMs and PIPAMs could be controlled by adjusting the gelating temperature, agitating rate, and the dosage of liquid paraffine and Span80. Sample with preferable size and shape were obtained under 42 °C at about 500 rpm with 0.2 g Span80 added in the 40 mL suspending medium. The smallest microspheres with a mean diameter of 90 μm exhibited the highest rebinding speed, rebinding quantity and IE at the very beginning of rebinding process. PEIAMs with the most uniform and smallest pores were prepared with 0.1 g of Span80 as the emulsifying agent and paraffine 0.5 g as oil phase. Smaller pores provided higher rebinding quantity and IE peak value. These facts were due to the swelling and permeating property of hydrogel. The imprinted agarose microspheres exhibit good recognition properties for the template protein. The peaks of the IE curves indicate an optimum swollen state and preferable state of the polymer chains in which the target molecules are best matching with the imprints in the hydrogel matrix. As a biocompatible natural polysaccharide, agarose is a promising material for protein imprinting and helpful in understanding interactions of protein templates and hydrogel matrix.

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