Preparation and Characterization of Micron-Sized Non-Porous Magnetic Polymer Microspheres with Immobilized Metal Affinity Ligands by Modified Suspension Polymerization

Zhi-Ya Ma, Yue-Ping Guan, Xian-Qiao Liu, Hui-Zhou Liu

Laboratory of Separation Science and Engineering, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, P.O. Box 353, Beijing 100080, People's Republic of China

Received 4 June 2004; accepted 13 September 2004.
DOI 10.1002/app.21688
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A novel preparation method of micron-sized non-porous magnetic polymer microspheres with immobilized metal affinity ligands was developed. A modified suspension polymerization of methacrylate (MA) and divinylbenzene (DVB) was performed in the presence of oleic acid-coated magnetic Fe_3O_4 nanoparticles to obtain magnetic poly (methacrylate-divinylbenzene) (mPMA-DVB) microspheres. Through ammonolysis using ethylenediamine (EDA) and subsequent carboxymethylation with chloroacetic acid, magnetic polymer microspheres with chelate ligands of iminodiacetic acid (IDA) were obtained. Charging with copper ions resulted in magnetic polymer microspheres capable of binding proteins that display metal affinity. The morphology, magnetic properties, and composition of magnetic polymer microspheres were characterized with scanning electron microscopy (SEM), vibrating sample magnetometer (VSM), and Fourier transform infrared spectroscopy (FTIR), respectively. Bovine hemoglobin (BHb) was adopted as a model protein to investigate their affinity adsorption capacity. It was found that the adsorption capacity was as high as 168.2 mg/g microspheres and with rather low non-specific adsorption. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 2174–2180, 2005

Key words: magnetic polymers; copolymerization; functionalization of polymers; modified suspension polymerization; immobilized metal affinity adsorption

INTRODUCTION

Magnetic support-based separations are fast, gentle, and compatible with complex biological suspensions such as blood, milk, and cell disruptates, and have consequently become increasingly popular in biotechnology and bioseparation. However, the majority of published work on magnetic separation concerns their use with immobilized enzymes and in bench scale separation, such as cell sorting and molecular biology.[1,2] Comparatively, seldom is there published work on the application of magnetic affinity adsorbents in protein purification, and most of these have involved porous support structures.

A potential constraint of any porous adsorbent is its propensity to become "plugged" with biological foulants and/or suspended solids. Once fouled, in addition to being difficult of access of the target protein to a large fraction of potential binding sites, the difficulties in removing such substances can also be acute. In contrast, non-porous support particles are less prone to fouling and are easier to clean than their porous counterparts and, therefore, are potentially more useful in purification of substances in dirty fouling feedstreams.[3,4] However, in order to obtain a comparable surface area of a typical macro-porous support, the dimensions for a non-porous particle have to be in the order of micron-size.[5] In addition, for the sake of easy separation, the magnetic particles should possess a strong magnetic responsiveness and high stability.

Another aspect hindering the magnetic affinity separation in protein purification is the lack of suitable generic ligands; classical proteinaceous ligands (e.g., antibodies) are not an attractive choice because of their high cost, low binding capacity on immobilization, instability, and leakage. The application of pseudo affinity ligands, such as immobilized metal affinity separation, has been developed rapidly since its first introduction by Porath and co-workers in 1975.[6] It takes advantage of the selective interaction between immobilized metal ions and the functional groups of amino acids located at the surface of biomolecules, such as the imidazole group of histidine, thiol group of cysteine, and indolyl group of tryptophan.[6,7] Compared with classical biological affinity ligands, they are small, inexpensive, and chemically and physically

Correspondence to: H.-Z. Liu (hzliu@home.ipe.ac.cn).

stable, and can be easily coupled to matrices at high density, resulting in high capacity adsorbents.

Magnetic supports can be produced using inorganic materials or a number of synthetic and natural polymers. The main disadvantage of inorganic supports is their limited functional groups for selective binding. For a highly selective process, magnetic supports are more commonly made from polymers since they have a variety of surface functional groups that can be tailed for specific applications. Among the common preparation methods of magnetic polymer supports, monomer polymerization is more widely used, in which suspension polymerization is relatively easy to perform and results in high magnetite contents support with bead shape. However, the magnetic polymer microspheres obtained from conventional suspension polymerization methods are in the size of several hundred micrometers, with very broad size distribution.[8–10] It is scarcely reported in literature that micron-sized (several microns) magnetic spheres could be prepared with suspension polymerization.

In this work, we developed a novel modified suspension polymerization method for the production of non-porous magnetic polymer microspheres with micron size, which have narrow size distribution and high magnetite contents. Oleic acid-coated magnetic Fe_3O_4 nanoparticles were first prepared, and then the modified suspension polymerization of methacrylate (MA) and divinylbenzene (DVB) was performed in the presence of magnetic nanoparticles to obtain magnetic poly(methacrylate-divinylbenzene) (mPMA-DVB) microspheres. Through ammonolysis using ethylenediamine (EDA) and subsequent carboxymethylation with chloroacetic acid, magnetic polymer microspheres with chelate ligands of iminodiacetic acid (IDA) were obtained. The morphology, magnetic properties, and composition of magnetic polymer microspheres were characterized with scanning electron microscopy (SEM), vibrating sample magnetometer (VSM), and Fourier transform infrared spectroscopy (FTIR), respectively. After being coupled with copper ions, bovine hemoglobin (BHb) as a model protein was used to investigate their affinity adsorption capacity.

EXPERIMENTAL

Materials

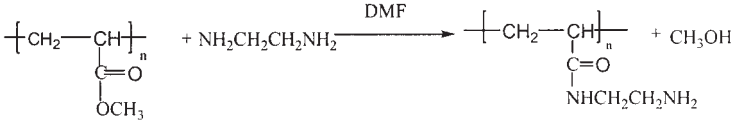
All chemicals used were purchased from Beijing Chemical Reagents Company (Beijing, China) unless otherwise stated. Methacrylate (MA) and divinylbenzene (DVB) were distilled under reduced pressure to remove the inhibitor before use. Benzoyl peroxide (BPO) was used as initiator. Poly (vinyl alcohol) (PVA-1788, degree of polymerization 1700, degree of hydrolysis 88%) was used as a stabilizer. Bovine hemoglobin (BHb) was obtained from Sigma. All other materials were analytical grade and used without further purification, including ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ammonium hydroxide (25% [w/w]), oleic acid, ethylenediamine (EDA), chloroacetic acid, *N, N′*-dimethylformamide (DMF), and copper sulfate ($CuSO_4 \cdot 5H_2O$).

Preparation of oleic acid-coated magnetic Fe_3O_4 nanoparticles

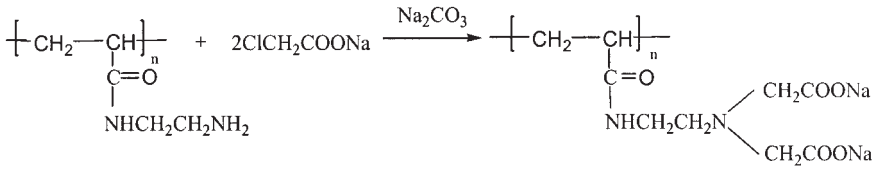
The preparation of oleic acid-coated magnetic Fe_3O_4 nanoparticles was by a co-precipitation method[11] with some modifications. Twenty three and a half grams of $FeCl_3$ $6H_2O$ and 8.6g of $FeCl_2 \cdot 4H_2O$ were dissolved in 800ml deionized water under nitrogen. When the solution was heated to 90°C, 30 ml $NH_3 \cdot H_2O$ were added rapidly. Then, 20 ml oleic acid was added dropwise within 20 min. The reaction was kept at 90°C for 1 h. The black lump-like Fe_3O_4 gel was cooled to room temperature and washed several times with deionized water.

Preparation of mPMA-DVB microspheres

The mPMA-DVB microspheres were prepared by a modified suspension polymerization method. In a typical experiment, 30g of the magnetic Fe_3O_4 gel and 3g of BPO were dispersed in a mixture of 95ml MA and 5ml DVB (crosslinker) to form the organic phase, and agitated until Fe_3O_4 was dissolved completely. The mixture was then transferred into a 2-liter beaker containing 25g PVA and 30g NaCl dissolved in 1000 ml H_2O, which was equipped with four vertical stainless steel baffleplates, a nitrogen inlet, and a 4-paddle mechanical stirrer. With agitation at 1000rpm, the mixture temperature was increased evenly from 45°C to 60°C within 1 h, then the temperature was maintained at 60°C for 2 h until finally the temperature was increased to 70°C and kept for 2 h. The reaction mixture was cooled to room temperature, and the resulting magnetic microspheres were thoroughly washed with deionized water and ethanol to remove the excess



Scheme 1



Scheme 2

amount of stabilizer and other impurities. The microspheres were stored in ethanol before use.

Modification of mPMA-DVB microspheres with EDA

In order to prepare the EDA-modified mPMA-DVB microspheres, the following procedure was applied. A 3g sample of mPMA-DVB microspheres was washed with DMF two times and put in a solution of 100ml DMF plus 100ml EDA. The mixture was agitated gently at 110°C for 12 h. After being cooled to room temperature, the mPMA-DVB microspheres were separated by magnetic decantation and washed with water and ethanol to remove the residual DMF. After modification, ester groups on the mPMA-DVB were converted into amino groups. The resulting microspheres were denoted as mPMA-DVB-NH_2. The reaction was as follows:

Carboxymethylation of mPMA-DVB-NH_2 microspheres with chloroacetic acid

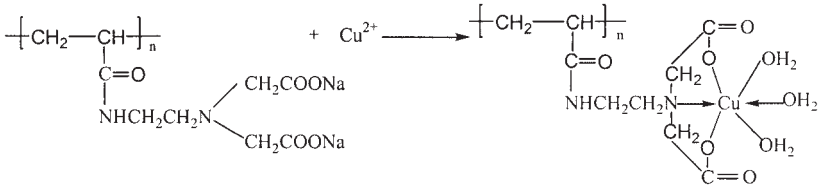
Carboxymethylation of the EDA-modified mPMA-DVB microspheres was carried out by the reaction of the microspheres with sodium salt of monochloroacetic acid solution, while 18.9g (0.2mol) of chloroacetic acid was dissolved in 30ml H_2O. Then 8g (0.2mol) NaOH in 30ml H_2O was added dropwise to the ice cooled solution of the chloroacetic acid with stirring. The stirring was continued until no foaming occurred. The sample of the above EDA-modified mPMA-DVB microspheres and 2g of Na_2CO_3 were placed in this solution, and the mixture was agitated at 70°C for 12 h. After carboxymethylation, amino groups in mPMA-DVB-NH_2 were transformed to iminodiacetic acid groups, which were used as chelating ligands for immobilization of metal ions. The produced microspheres were denoted as mPMA-DVB-IDA, and the reaction is shown below:

Charging of mPMA-DVB-IDA microspheres with copper ions

To charge the mPMA-DVB-IDA microspheres with copper ions, the microspheres were mixed with $CuSO_4 \cdot 5H_2O$ solution (50mg/ml) under continuous shaking at room temperature for 2 h. Then the mPMA-DVB-IDA-Cu^{2+} microspheres were washed several times with water and 20mM sodium phosphate buffer (pH 8.0) to remove the excess unbound Cu^{2+}. The microspheres were stored in 20mM sodium phosphate buffer (pH 8.0), and the coupling process can be shown as follows:

Characterization of magnetic microspheres

The morphology and the size of magnetic Fe_3O_4 nanoparticles were determined by transmission electron microscopy (TEM, Hitachi 8100). The morphology of the mPMA-DVB microspheres was observed by scanning electron microscopy (SEM, JEOL, JSM-6700F, Tokyo, Japan). The magnetization curves of the dried microspheres were recorded with a vibrating sample magnetometer (VSM) (model-155, Digital Measurement System, Inc.). Fourier transform infrared spectroscopy (FTIR) spectra of both the unmodified and modified magnetic microspheres were obtained with an FTIR spectrophotometer (FTIR, Bruker, Vector 22). All the microspheres were thoroughly washed with water and dried under vacuum. The dried samples were milled with potassium bromide (KBr) powder, and pressed into a disk. Then the spectra were recorded.



Scheme 3

Determination of immobilized Cu^{2+} capacity

A known amount of microspheres was suspended in 0.5M EDTA to release the bound Cu^{2+}. The Cu^{2+} concentration released in EDTA solution was detected with atomic absorption spectrophotometry (AAS, Analyst 100, Perkin–Elmer). The immobilized Cu^{2+} capacity is equal to the amount released.

Protein adsorption on magnetic microspheres

The adsorption of BHb on microspheres of mPMA-DVB, mPMA-DVB-NH_2, mPMA-DVB-IDA, and mPMA-DVB-IDA-Cu^{2+} was carried out by the batch adsorption method. The initial concentration of BHb was 1.0 mg/ml. BHb was incubated with approximately 1 mg of the microsphere suspension in 1.5ml binding buffer (20mM sodium phosphate buffer containing 1M NaCl, pH 8.0). After being shaken at room temperature for 2 h, which proved to be a sufficient period to reach equilibrium, the microspheres were separated by magnetic sedimentation and washed twice with binding buffer. The supernatant was assayed for residual protein concentration by UV-Vis spectrophotometer at 406nm.[12] The adsorption capacity of BHb was calculated by mass balance.

RESULTS AND DISCUSSION

Preparation and characterization of oleic acid-coated magnetic Fe_3O_4 nanoparticles

Magnetic Fe_3O_4 nanoparticles were obtained in a coprecipitation process by quickly adding a concentrated ammonium solution to a solution of Fe^{2+} and Fe^{3+}. By adding oleic acid as a surfactant, oleic acid was coated (adsorbed on) the surface of magnetic Fe_3O_4 nanoparticles because of the reaction of carboxylic groups with hydroxyl groups on magnetic Fe_3O_4 nanoparticles. TEM images showed the average diameter of oleic acid-coated magnetic nanoparticles was about 8nm (Fig. 1). It has been clear that, for ultrafine magnetically ordered particles, there exists a critical size below which the particles will exhibit superparamagnetism. The critical size was estimated to be 25nm.[13] This suggested that the magnetic particles prepared in this work were superparamagnetic.

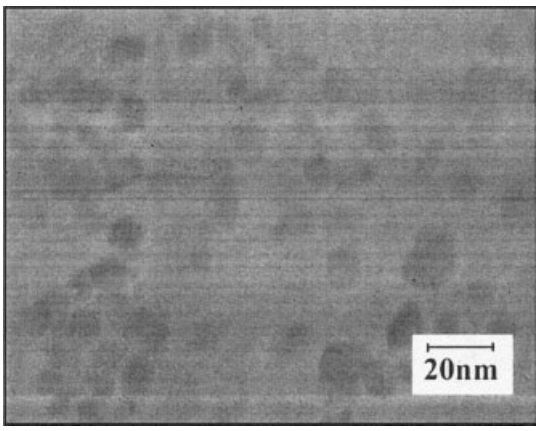


Figure 1 TEM of oleic acid-coated magnetic Fe_3O_4 nanoparticles.

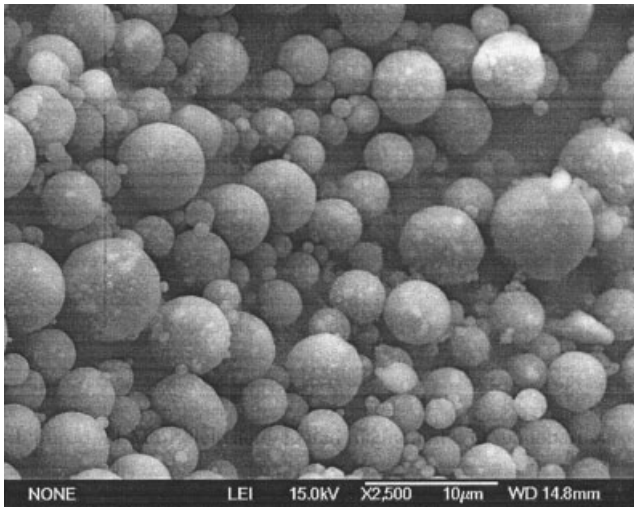


Figure 2 SEM micrograph of mPMA-DVB microspheres.

The good dispersibility of oleic acid-coated magnetic nanoparticles in monomer resulted from their hydrophobic property. The long alkyls of oleic acid not only stabilized the Fe_3O_4 nanoparticles, preventing their coagulation, but also enhanced the affinity of the Fe_3O_4 nanoparticles to the monomer in the polymerization step.

Preparation and characterization of magnetic microspheres

Figure 2 shows the morphology of mPMA-DVB microspheres observed by SEM. It can be seen that the size of microspheres ranged from 1 to 8μm, with a mean diameter of about 5μm.

The magnetic properties of mPMA-DVB and mPMA-DVB-NH_2 microspheres were recorded by VSM at room temperature. Figure 3 shows their magnetization curves. There was no hysteresis loop observed at this temperature, suggesting that the magnetic microspheres were superparamagnetic, which indicated that there would no magnetic interactions among magnetic microspheres in a zero magnetic field environment. This feature would result in the easy dispersion of the magnetic microspheres. The saturation magnetization of mPMA-DVB microspheres was 13.8emu/g, which was higher than those reported by other similar works.[8,10] With such high saturation magnetization, they could be easily and quickly sep-

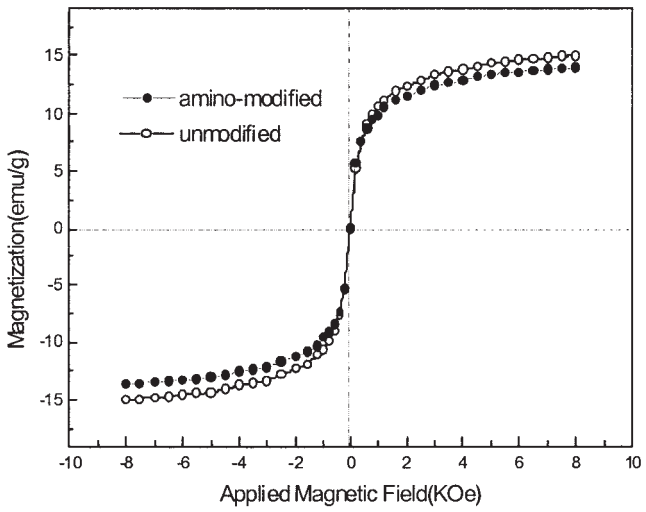


Figure 3 Magnetization curves of the amino-modified and unmodified mPMA-DVB microspheres.

arated from a suspension. This is very favorable to the magnetic separation of proteins on a large scale.

Micron-sized mPMA-DVB microspheres were prepared via a modified suspension polymerization method in this study. In suspension polymerization, the monomer phase containing an oil soluble initiator was dispersed by mechanical agitation in dispersion media to form droplets. The combination of continued agitation and the addition of a suitable stabilizer (often a surface active polymer) have a stabilizing effect, hindering both the coalescence and further breakage of monomer droplets. The size of the initial monomer droplets formed is dependent upon the balance between the droplet breakage and their coalescence. The average size of the polymer beads formed is expected to be approximately the same as that of the initial monomer droplets. However, in conventional suspension polymerization, because of the uneven distribution of monomer droplets, the resultant polymer beads have very broad size distribution. Moreover, the polymer beads are mostly in the size range of several hundred micrometers.

To overcome the drawbacks of conventional suspension polymerization, four modification measures were adopted in our study. First, the polymerization was conducted in a 2-liter beaker equipped with four vertical stainless steel baffleplates and a 4-paddle stirrer, so that the strength of mechanical agitation was enhanced and the monomer droplets could be dispersed more uniformly. Second, the reaction temperature was increased at a controlled rate. It is important to control the process of temperature to ensure the period of droplet dispersion and the formation of small droplets. A relatively long residence time in the temperature of 45°C to 60°C is advantageous to forming micron-sized uniform monomer droplets with high magnetite contents. During this period, the droplets' size would gradually change to smaller with the diffusion of monomer into the aqueous phase, and consequently the magnetite contents will become higher in each monomer droplet. In conventional suspension polymerization, on the other hand, the reaction is carried out directly at high temperature (70 or 80°C), without the long residence time in low temperatures. Therefore, the droplets' size was mainly dependent on the strength of mechanical agitation. Third, a large amount of PVA (more than 20 wt % of monomer) was added in the aqueous phase, which is much higher than that in conventional suspension polymerization (only 0.1–1 wt % of monomer). It has been proven in this study with the addition of a large quantity of PVA, the size distribution of magnetic microspheres would become narrower. Because more PVA would adsorb on the droplet surface, the extent of the droplets' aggregation decreased and the stability of droplets was enhanced. Fourth, compared with conventional suspension polymerization, in which an initiator of only 0.1–1 wt % of monomer was used, in this study, a high quantity of initiator BPO (3–5 wt % of monomer) was added for the sake of magnetite as a strong inhibitor to the polymerization process. On the other hand, a large quantity of initiator is favorable to shortening the nucleation period, which is helpful to the formation of magnetic microspheres with narrow size distribution.

Modification of mPMA-DVB microspheres with EDA

In order to introduce metal chelate ligand to the mPMA-DVB microspheres, the microspheres were first modified with EDA by ammonolysis. The result was proven by the comparison of FTIR spectra of mPMA-DVB (A) and mPMA-DVB-NH_2 (B), as shown in Figure 4. The FTIR spectra of EDA-modified mPMA-DVB microspheres give some absorption bands different from those of unmodified mPMA-DVB microspheres. The most important absorption bands at 1650 cm^{-1} and 1545 cm^{-1} represent amide I (mainly due to C═O stretching) and amide II (mainly due to N—H bending), respectively, due to EDA bonded to mPMA microspheres.

Carboxymethylation of mPMA-DVB-NH_2 microspheres with chloroacetic acid

The amino groups of the mPMA-DVB-NH_2 microspheres could be carboxymethylated by sodium salt of chloroacetic acid. FTIR spectra of chloroacetic acid modified magnetic microspheres are shown in Figure 4c. The —COO^- stretch band at 1394 cm^{-1} and the vibration band at 1606 cm^{-1} (antisymmetric stretch) appeared after the chloroacetic acid modification,

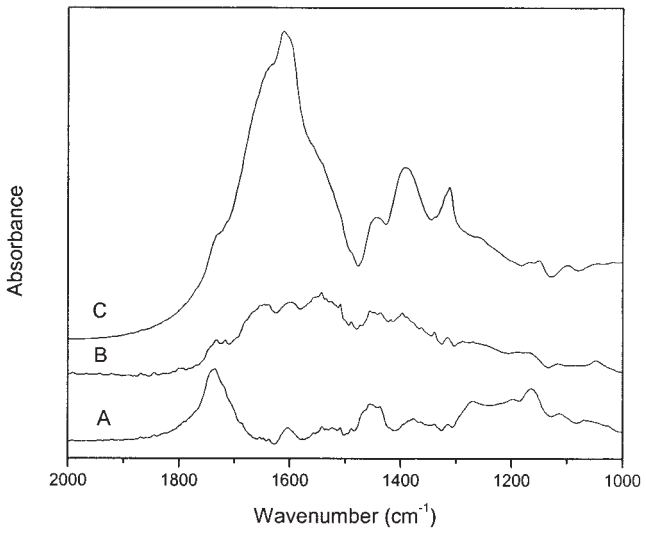


Figure 4 FTIR spectra of (A) unmodified mPMA-DVB, (B) EDA modified, and (C) chloroacetic acid modified.

which suggested the reaction of carboxymethylation occurred.

To obtain high conversions, it is critical to use sodium salt of chloroacetic acid. It has been proven that the direct reaction with chloroacetic acid yielded always a low degree of substitution. Moreover, due to the hydrolysis of chloroacetic acid, it is necessary to add more sodium salt of chloroacetic acid solution to the reaction system during reaction. The reaction resulted in the transformation of amino groups to iminodiacetic acid groups, which is a tridentate chelator and can coordinate with many metal ions.

Charging of mPMA-DVB-IDA microspheres with copper ions

Many metal ions, such as Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}, could coordinate with IDA groups on the magnetic microspheres. Cu^{2+} was selected in this study for its high coordinate capacity with IDA groups and high binding capacity with proteins having exposed histidines. The coordinate capacity of Cu^{2+} was 0.30mmol/g determined by AAS.

Cu^{2+} coordinating with IDA groups could be qualitatively proven. When the Cu^{2+} charged magnetic microspheres were treated with 0.5M EDTA, the EDTA solution turned light blue, indicating that Cu^{2+} was readily chelated, and the bound Cu^{2+} could be stripped easily from the microspheres by using EDTA.

Affinity adsorption of BHb

BHb, which has an estimated 24 exposed histidines,[14] was selected as a model protein to investigate the affinity adsorption capacity of mPMA-DVB-IDA-Cu^{2+} microspheres.

Adsorption of a protein to an immobilized metal affinity support has to be performed at a pH at which an electron donor group on the protein surface is at least partially unprotonated. In fact, the optimal pH for adsorption largely depends upon the nature of the metal ion, the chelating ligand, and the structure of the protein. For immobilized Cu^{2+}, which is directed chiefly toward accessible histidine residues on proteins, the optimal adsorption occurs in the pH range of 6–8.[15,16] So in this study, the adsorption of BHb was performed at pH 8.0.

With the non-porous structure, the magnetic microspheres were not prone to fouling and the mass transfer efficiency was high, for there was no inner diffusion but only outer diffusion and surface diffusion. Although equilibrium binding to Cu^{2+}-charged magnetic microspheres was achieved rapidly for BHb, 2 h contact was routinely employed to ensure the equilibrium was attained for all tested proteins and microspheres under investigation.

Adsorption of protein onto an immobilized metal ion is caused not only by the formation of a coordinated compound of protein with the immobilized metal ion (specific interaction), but also by non-specific interaction, for example, electrostatic and hydrophobic interactions.[17,18]

To evaluate the non-specific binding of protein to magnetic microspheres, mPMA-DVB, mPMA-DVB-NH_2, mPMA-DVB-IDA, and mPMA-DVB-IDA-Cu^{2+} were used in batch adsorption. The results are shown in Figure 5. The former three microspheres showed very little binding of BHb, while the binding capacity of Cu^{2+} chelated microspheres was significantly increased. This could be explained as follows: BHb adsorption onto mPMA-DVB is probably due to hydrophobic interactions. After modification with EDA, the surface became more hydrophilic, which resulted in the decrease of adsorption capacity. At pH 8.0, since the residual charged groups on the mPMA-DVB-IDA

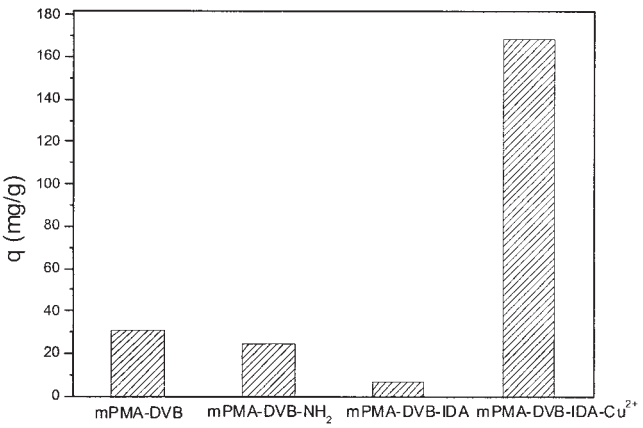


Figure 5 BHb adsorption on different magnetic polymer microspheres.

were anionic, BHb was expelled due to its net negative charge (the isoelectric point of BHb is 7.1[19]). The highest adsorption capacity on mPMA-DVB-IDA-Cu^{2+} indicated that the specific interaction is dominant in this case.

Regeneration of magnetic microspheres is important for their application in affinity separation. Both the bound protein and chelated Cu^{2+} could be stripped from the magnetic microspheres by the addition of EDTA or other chelating agents. After being reloaded with Cu^{2+}, the microspheres could be reused to affinity adsorb proteins. To investigate the reusability of the magnetic microspheres, the strip-reload cycles were repeated 10 times using the same microspheres. There is no significant protein adsorption capacity loss (data not shown) observed, indicating such magnetic microspheres could be repeatedly utilized for the affinity separation of proteins.

CONCLUSION

Micron-sized non-porous magnetic polymer microspheres were prepared via a novel modified suspension polymerization of MA and DVB in the presence of oleic acid-coated magnetic nanoparticles. The magnetic microspheres have narrow size distribution with high magnetic contents. After ammonolysis and carboxymethylation, IDA chelate ligands were introduced on the microspheres. When charged with metal ions (e.g., Cu^{2+}), the magnetic microspheres efficiently bound a model protein (BHb) with high adsorption capacity. With the advantages of not being prone to fouling, high mass transfer efficiency, high magnetic contents, and high adsorption capacity, these magnetic microspheres are a good candidate that could be used in large scale protein affinity separation or purification.

This work was financially supported by the National High Technology Research and Development Program of China (No.2002AA302211), the National Natural Science Foundation of China (No. 20206032), and the Outstanding Young Scientist Foundation of China (No. 29925617).

References

1. Šafarik, I.; Šafariková, M. J Chromatogr B 1999, 722, 33.
2. Levison, P. R.; Badger, S. E.; Dennis, J.; Hathi, P.; Davis, M. J.; Brue, I. J.; Schimkat, D. J Chromatogr A 1998, 816, 107.
3. Halling, P. J.; Dunnill, P. Biotechnol Bioeng 1979, 21, 396.
4. Halling, P. J.; Dunnill, P. Enzyme Microb Technol 1980, 2, 2.
5. O'Brien, S. M.; Thomas, O. R. T.; Dunnill, P. J Biotechnol 1996, 50, 13.
6. Porath, J.; Carlson, J.; Olsson, I.; Belfrage, G. Nature 1975, 258, 598.
7. Lönnerdal, B. O.; Keen, C. L. J Appl Biochem 1982, 4, 203.
8. Xue, B.; Sun, Y. J Chromatogr A 2002, 947, 185.
9. Cocker, T. M.; Fee, C. J.; Evans, R. A. Biotechnol Bioeng 1997, 53, 79.
10. Lee, Y.; Rho, J.; Jung, B. J Appl Polym Sci 2003, 89, 2058.
11. Guan, Y. P.; Liu, H. Z.; An, Z. T.; Ke, J. J.; Chen, J. Y. CN Pat. 98124516.1 (1998).
12. Avramescu, M. E.; Borneman, Z.; Wessling, M. J Chromatogr A 2003, 1006, 171.
13. Lee, J.; Isobe, T.; Senna, M. J Colloid Interface Sci 1996, 177, 490.
14. Wuenschell, G. E.; Naranjo, E.; Arnold F. H. Bioprocess Eng 1990, 5, 199.
15. Todd, R. J.; Johnson, R. D.; Arnold, F. H. J Chromatogr A 1994, 662, 13.
16. Kwon Y. J.; Kim J. H.; Lee H. Food Sci Biotechnol 1999, 8, 367.
17. Chen, W. Y.; Wu, C. F.; Liu, C. C. J Colloid Interface Sci 1996, 80, 135.
18. Wu, C. Y.; Suen, S. Y.; Chen, S. C.; Tzeng, J. H. J Chromatogr A 2003, 996, 53.
19. Causserand, C.; Kara, Y.; Aimar, P. J Membr Sci 2001, 186, 165.