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Preparation and characterization of magnetic poly(styrene–glycidyl methacrylate) microspheres for highly efficient protein adsorption by two-stage dispersion polymerization

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ABSTRACT: Micrometer-sized superparamagnetic poly(styrene–glycidyl methacrylate)/Fe_3O_4 spheres were synthesized by two-stage dispersion polymerization with modified hydrophobic Fe_3O_4 nanoparticles, styrene (St), and glycidyl methacrylate (GMA). The morphology and properties of the magnetic Fe_3O_4–P (St-GMA) microspheres were examined by scanning electron microscopy, transmission electron microscopy, vibrating sample magnetometry, thermogravimetric analysis, and attenuated total reflectance. The average size of the obtained magnetic microspheres was 1.50 μm in diameter with a narrow size distribution, and the saturation magnetization of the magnetic microspheres was 8.23 emu/g. The magnetic Fe_3O_4–P (St-GMA) microspheres with immobilized iminodiacetic acid–Cu^{2+} groups were used to investigate the adsorption capacity and selectivity of the model proteins, bovine hemoglobin (BHb) and bovine serum albumin (BSA). We found that the adsorption capacity of BHb was as high as 190.66 mg/g of microspheres, which was 3.20 times greater than that of BSA, which was only 59.64 mg/g of microspheres as determined by high-performance liquid chromatography. With a rather low nonspecific adsorption, these microspheres have great potential for protein separation and purification applications. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43005.

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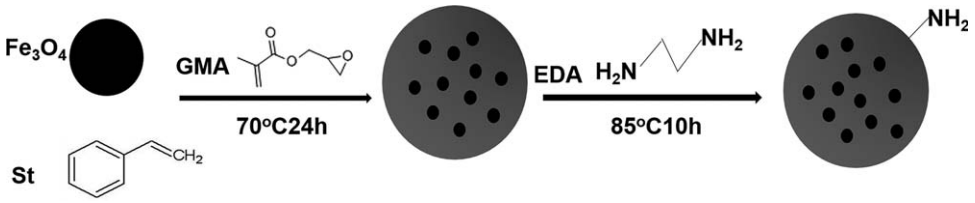
INTRODUCTION

Since the rapid development of biotechnology and biomedicine, magnetic separation as a reliable and efficient separation technology for the isolation and purification of biomolecules has been applied to various applications, such as cell isolation,[1,2] enzyme immobilization,[3,4] protein purification,[5,6] and immunoassay.[7] Microsized magnetic polymer spheres as an effective tool can be prepared via monomer polymerization, including dispersion polymerization,[8,9] suspension polymerization,[10] emulsion polymerization,[11,12] and the activated swelling method, which was developed by Ugelstad *et al.*,[13] and commercialized products (Dynabeads) are already used widely.

Among these methods, dispersion polymerization has a unique merit for producing monodisperse 1–10-μm microspheres[14,15]; these have a controllable and uniform particle size and evenly distributed magnetic particles. Moreover, the reaction is easy to carry out and scale up compared with the time-consuming successive seeded emulsion polymerization method[16] or multistage activated welling suspension polymerization method.[17,18] Zhang *et al.*[19] prepared magnetic poly(styrene–glycidyl methacrylate) [P(St–GMA)] microspheres with a high magnetite content but a broad size distribution and irregular shape by one-stage dispersion polymerization. Horák *et al.*[15] synthesized magnetic poly (hydroxyethyl methacrylate-glycidyl methacrylate) microspheres with a relatively narrow size distribution by one-stage dispersion polymerization, but the polydispersity index was still 1.04. When the comonomers were added at the beginning of dispersion polymerization, poor results were obtained,[20] and a large amount of functional groups were buried in the polymer with only a small part left on the surface.[21] The low functional group content and the low adsorption capacity of those magnetic microspheres synthesized by one-stage dispersion polymerization limits their application in separation and immunoassay.

Song *et al.*[22] prepared nonmagnetic monodisperse carboxylated micrometer-sized polystyrene (PSt) particles with a very narrow size distribution by two-stage dispersion polymerization. Cao *et al.*[23] prepared nonmagnetic crosslinked monodisperse polymer microspheres with spherical shapes and smooth surfaces by



Scheme 1. Schematic illustration for the synthesis of the magnetic Fe_3O_4–P (St-GMA) microspheres.

two-stage dispersion polymerization. The two-stage dispersion polymerization process can be separated into a nucleation stage (first stage) and a particle-growing stage (second stage).[24] The nucleation stage is short but more complex and sensitive, whereas the particle growing stage is relatively long, simple, and robust. However, the preparation of magnetic microspheres has rarely been reported in the literature.

The methods of protein purification include ammonium sulfate precipitation, column chromatography, electrophoresis, and magnetic separation. With magnetic microspheres as a solid medium, the magnetic separation method has already been used widely.[25] With magnetic separation, several stages of sample pretreatment (viz., centrifugation, filtration, and membrane separation) that are normally necessary to condition an extract before its application on packed-bed chromatography columns, can be eliminated.[26] With its magnetically responsive nature, magnetic separation can separate selected target species directly out of a crude biological solution by the application of a magnetic field. Compared with chromatography separation methods of protein, magnetic separation technology has the advantages of quickness, high purity, and high yield.

In this study, we prepared micrometer-sized superparamagnetic spheres by two-stage dispersion polymerization. The magnetic P(St–GMA)/Fe_3O_4 microspheres made by this method had the advantages of a narrow size distribution, superparamagnetic properties, and extensive amino groups available on the surface for functionalization. The morphology and properties of the magnetic P(St–GMA) microspheres were examined with scanning electron microscopy (SEM), transmission electron microscopy (TEM), vibrating sample magnetometry (VSM), thermogravimetric analysis (TGA), and attenuated total reflectance (ATR). Furthermore, to prove that the amine-modified magnetic microspheres could be applied in protein separation, bovine hemoglobin (BHb), and bovine serum albumin (BSA) were adopted as model proteins to investigate their affinity adsorption capacity.

EXPERIMENTAL

Materials

Original Fe_3O_4 nanoparticles (NPs; hydrophobic Fe_3O_4 NPs; size = 15 nm) were obtained from Beijing GiGNano Biointerface Co., and sodium chloroacetate ($CH_2ClCOONa$) was supplied by Shanghai Macklin Biochemical Co., Ltd. (Shanghai). Glycidyl methacrylate (GMA), styrene (St), poly(vinyl pyrrolidone) (PVP K30), ethylenediamine (EDA), cupric sulfate ($CuSO_4{\cdot}5H_2O$), sodium hydroxide (NaOH), glacial acetic acid, and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing). Disodium ethylenediamine tetraacetate (EDTA) and tris-(hydroxymethyl)aminomethane were acquired from Amresco. BSA and BHb were obtained from Roche (Switzerland). Deionized water was used throughout the study. All of the chemicals and solvents were used without any further purification.

Preparation of the Magnetic Fe_3O_4–P (St-GMA) Microspheres

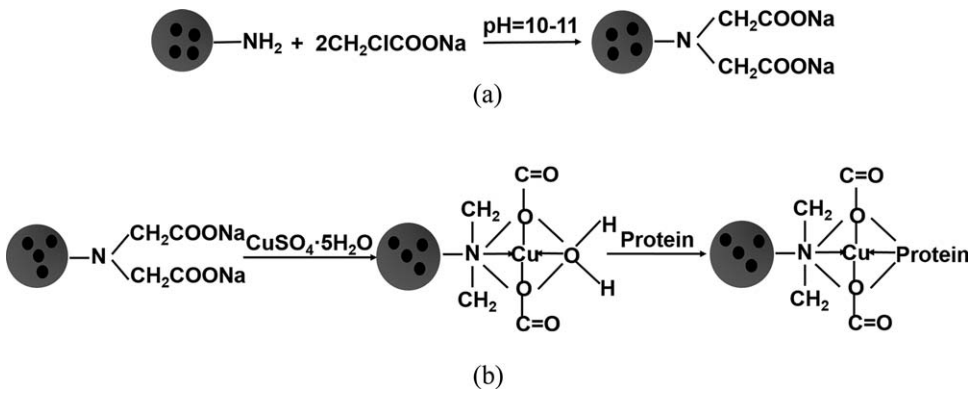
The magnetic Fe_3O_4–P (St-GMA) microspheres were prepared by a modified two-stage dispersion polymerization. The reaction is shown in Scheme 1. A given amount of hydrophobic Fe_3O_4 NPs was dispersed in 6.60 mL of St. After 5 min of ultrasonic dispersion, 0.80 g of AIBN was added. Then, a mixed solution of 41.20 mL of alcohol and 2.20 mL of water (volume ratio = 95:5) with 1 g of PVP K30 was added. After that, the solution was transferred to a 100-mL three-necked reaction flask equipped with a condenser, heated to 70°C, and stirred mechanically at 150 rpm. After the polymerization reaction had run for 2 h, 2 mL of GMA solution in an additional 8 mL of solvent was added to the flask by a micropump at a velocity of 2 mL/h. The reaction was continued for 24 h. Finally, the magnetic microspheres were thoroughly washed alternately with ethanol and deionized water three times and then dispersed in deionized water.

Modification of the Magnetic Fe_3O_4–P (St-GMA) Microspheres with EDA

To prepare the EDA-modified magnetic Fe_3O_4–P (St-GMA) microspheres, the following procedure was applied. A certain amount of magnetic Fe_3O_4–P (St-GMA) microspheres was put into a solution of 80 mL of water and 20 mL of EDA with 1 g of NaOH. The reaction continued at 85°C for 10 h under vigorous stirring, as described previously. After the particles were washed alternately with water and ethanol three times through a magnetic separation method, we successfully obtained amino groups instead of epoxy groups on the magnetic Fe_3O_4–P (St-GMA) microspheres.

Immobilization of Iminodiacetic Acid (IDA)–Cu^{2+} on the Surface of the Fe_3O_4–P (St-GMA) Microspheres

The scheme of immobilizing IDA–Cu^{2+} on the surface of the Fe_3O_4–P (St-GMA) microspheres is shown in Scheme 2. $CH_2ClCOONa$ (2 g) was dissolved in 50 mL of deionized water, and then, a certain amount of EDA-modified magnetic Fe_3O_4–P (St-GMA) microspheres was added to the solution. The mixture reacted at 80°C for 8 h in a constant-temperature oscillator at 200 rpm. NaOH solution was used to adjust the pH value to 10–11 throughout the whole process. The product, Fe_3O_4–P (St-GMA)–IDA microspheres, was washed with deionized water. Then, these microspheres were mixed with 50 mL of $CuSO_4{\cdot}5H_2O$ (0.40 mol/L) under continuous shaking at room



Scheme 2. Schematic illustrations of (a) the immobilization of IDA–Cu^{2+} on the surface of the Fe_3O_4–P (St-GMA) microspheres and (b) their use for protein adsorption.

temperature for 2 h. In the end, the Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres were washed with deionized water three times to remove the excess unbound Cu^{2+}. After that, a known amount of the Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres was treated with 0.10*M* EDTA to release the chelated Cu^{2+}, and the immobilized Cu^{2+} capacity was equal to the released amount.[27] The concentration of Cu^{2+} that was released in the EDTA solution was detected with an atomic absorption spectrophotometer (Shanghai Spectrum Instrument Co., Ltd.).

Characterization of the Magnetic Fe_3O_4–P (St-GMA) Microspheres

The morphology and structure of the magnetic Fe_3O_4–P (St-GMA) microspheres were observed by SEM and TEM. The magnetization curves of the samples were measured with VSM. ATR spectra of the magnetic Fe_3O_4–PSt microspheres, magnetic Fe_3O_4–P (St-GMA) microspheres, and EDA-modified magnetic Fe_3O_4–P (St-GMA) microspheres were obtained with ATR/Fourier transform infrared spectroscopy (Bruker, TENSOR-II). The particle size analysis of the Fe_3O_4–P (St-GMA) microspheres was done with a particle analyzer (Delsa Nano C, Beckman Coulter Ireland, Inc.). Quantitative analysis of the magnetic Fe_3O_4–P (St-GMA) microspheres and original Fe_3O_4 NPs was performed with a TGA instrument (Beijing Scientific Instrument Co., Ltd.) and was recorded over the range 25–800°C at a rate of 10°C/min.

Protein Adsorption of the Magnetic Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} Microspheres

BSA and BHb were selected as model proteins to explore the capacity and selectivity of magnetic Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres for protein adsorption. The experiments were carried out as follows. An amount 10 mg of the magnetic Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres was reacted with a binary mixture containing 2 mg/mL BHb and 2 mg/mL BSA in 10 m*M* tris(hydroxymethyl)aminomethane/glacial acetic acid buffer solution (pH = 8.28), and they were conducted in an oscillator at room temperature for 30 min. After the reaction, the microspheres were magnetically separated, and the supernatants were collected by high-speed centrifugation at a speed of 8000 rpm for 10 min. The peak areas of the initial and final concentrations for the binary mixture were measured by high-performance liquid chromatography (HPLC; LC-20A, Shimadzu, Japan) with a Shim-Pack WAX-2 column (Shimadzu, Japan).

RESULTS AND DISCUSSION

Preparation and Characterization of the Magnetic Fe_3O_4–P (St-GMA) Microspheres

Micrometer-sized magnetic Fe_3O_4–P (St-GMA) microspheres were prepared by a modified two-stage dispersion polymerization. Scheme 1 shows the preparation process of the amine-functioned magnetic Fe_3O_4–P (St-GMA) microspheres. With the existence of the magnetic particles, monomer (St), stabilizer (PVP K30), and initiator (AIBN), the functional comonomer (GMA) was added after the nucleation stage to form epoxy groups on the surface of the microspheres. Then, the additional EDA reacted with the microspheres and converted the epoxy groups into the amino groups.

The morphology and structure of the magnetic Fe_3O_4–P (St-GMA) microspheres were observed with SEM, as shown in Figure 1(a), and TEM, as shown in Figure 1(b). We defined the number-average diameter (D_n) of the particle diameter with the following equation by counting at least 100 individual particles from SEM microphotographs[28]:

$$D_n = \left(\sum {}^{n_i d_i} / {}_{n_i} \right) \quad (1)$$

where n_i is the number of particles with a diameter of d_i.

We calculated that the size of the microspheres was 1.50 μm. The particle size distribution of the microspheres was also measured by a laser particle size analyzer, as shown in Figure 2. The average size was 1.55 ± 0.17 μm. Figure 1(b) shows the TEM photograph of the magnetic Fe_3O_4–P (St-GMA) microspheres with magnetite dispersed in the network of polymers to form the composite structure.

The typical magnetization curves for the magnetic Fe_3O_4–P (St-GMA) microspheres was measured with VSM at 25°C, as shown in Figure 3(b). There was no hysteresis in the magnetization with both the remanence and coercivity being zero; this means that such magnetic microspheres were superparamagnetic. The saturation magnetization was found to be 8.23 emu/g. The saturation magnetization of the original Fe_3O_4 NPs was 60.80 emu/g, as shown in Figure 3(a). If we assumed that all of the

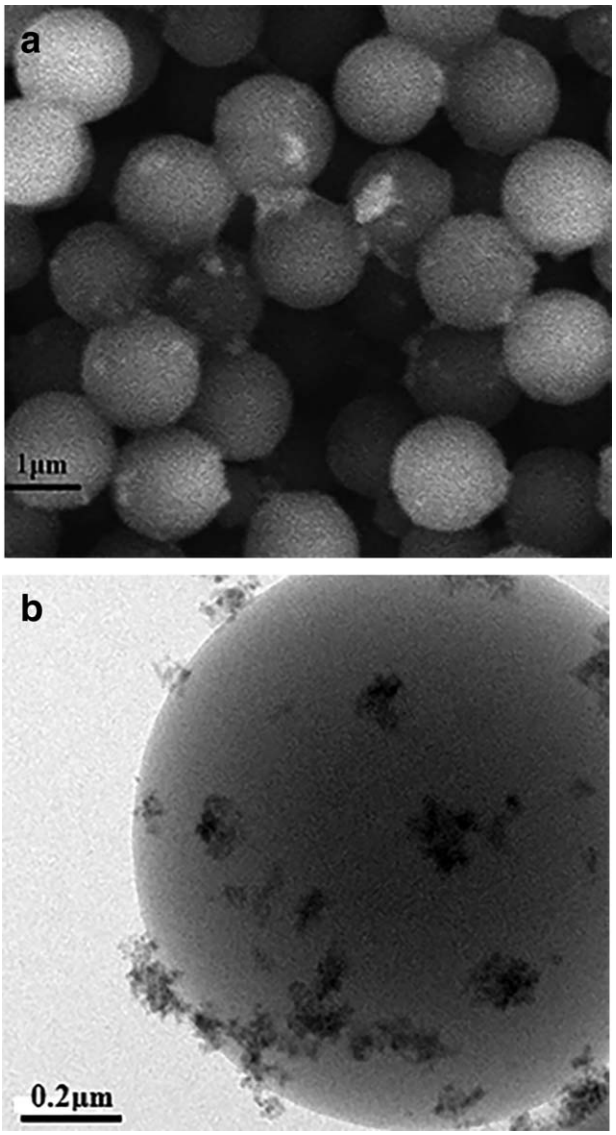


Figure 1. (a) SEM and (b) TEM micrographs of the magnetic Fe_3O_4–P (St-GMA) microspheres.

hydrophobic Fe_3O_4 NPs were inside of the microspheres, a 13.50 wt % magnetic particle content could be calculated. Microspheres with superparamagnetic properties could be redispersed rapidly when the magnetic field was removed; these properties play a crucial role in biomedical applications. Moreover, the relatively high saturated magnetization makes microspheres susceptible to magnetic fields, and they can be separated from liquid phases with an ordinary permanent magnet.

Figure 4(a–c) shows the ATR spectra of the Fe_3O_4–P (St-GMA), Fe_3O_4–P (St-GMA)–EDA, and Fe_3O_4–P (St-GMA)–IDA microspheres. The peaks appearing at 680 cm^{-1} in Figure 4(a–c) corresponded to the Fe—O vibrations of Fe_3O_4. The characteristic absorption peaks of the monomer St appeared at 756 cm^{-1} (C—H bending vibrations), 3000–3100 cm^{-1} (C—H stretching vibrations), 1450 cm^{-1}, and 1600 cm^{-1}. Peaks for the comonomer GMA appeared at 1729 cm^{-1} (C═O stretching vibrations), as shown in Figure 4(a–c). The absorbent peak at 3400 cm^{-1} in Figure 4(b) corresponded to the N—H stretching vibrations and provided evidence that amino groups were successfully immobilized on the surface of the Fe_3O_4–P (St-GMA) microspheres. The peaks appearing at 1550–1650 and 1400 cm^{-1} in Figure 4(c) corresponded to the C═O symmetric stretching vibrations and C—O antisymmetric stretching vibrations of carboxylate, respectively; these proved the existence of IDA on the surface of the Fe_3O_4–P (St-GMA) microspheres.

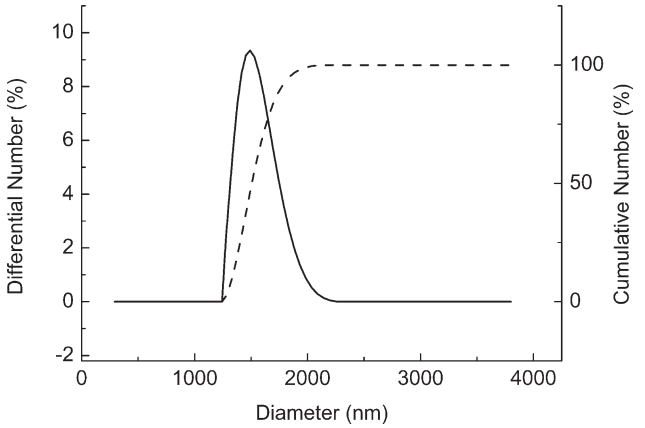


Figure 2. Particle size distribution of the magnetic Fe_3O_4–P (St-GMA) microspheres measured with a laser particle size analyzer.

TGA of the magnetic Fe_3O_4–P (St-GMA) microspheres is shown in Figure 5. The original hydrophobic Fe_3O_4 NPs (curve a) showed a 2.50 wt % loss because of the decomposition of hydrophobic groups on the surface of the NPs. The pure polymer microspheres (curve c) were prepared in a dispersion polymerization as in the synthesis of the magnetic polymer microspheres. A 73.00 wt % loss was observed in the thermogravimetry (TG) curves of the magnetic Fe_3O_4–P (St-GMA) microspheres (curve b) in the range 300–450°C; this resulted from the decomposition of organic compounds and hydrophobic groups of the original hydrophobic Fe_3O_4 NPs. Then, we worked out that the organic compounds of the microspheres amounted to 70.50 wt %, and the residual ash with magnetic

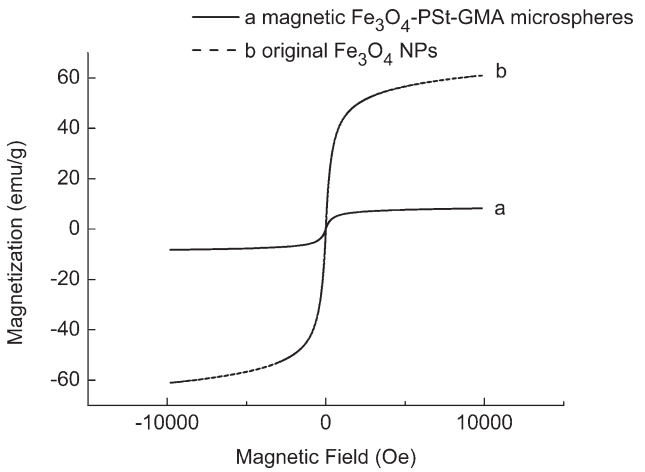


Figure 3. Magnetic hysteresis loops of the (a) magnetic Fe_3O_4–P (St-GMA) microspheres and (b) original Fe_3O_4 NPs.

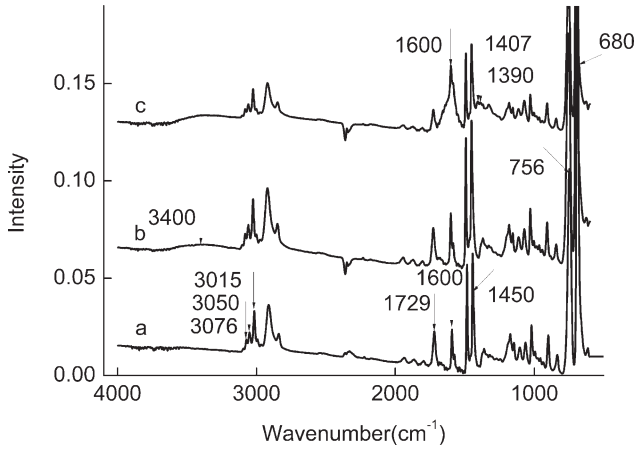


Figure 4. ATR spectra of the (a) Fe_3O_4–P (St-GMA), (b) Fe_3O_4–P (St-GMA), and (c) the Fe_3O_4–PSt–GMA–IDA microspheres.

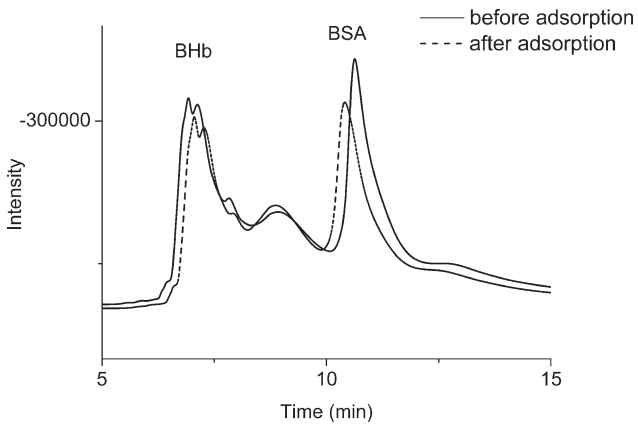


Figure 6. HPLC chromatograms of the binary mixture (2 mg/mL BHb and 2 mg/mL BSA) before and after protein adsorption by the Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres.

hydrophobic Fe_3O_4 NPs was 29.50 wt %. For the pure polymer microspheres (curve c), a residual ash content of 16.00 wt % was found, and a magnetic particle content of 13.50 wt % was deduced for the magnetic Fe_3O_4–P (St-GMA) microspheres. When we assumed that the magnetic content was 100%, saturation magnetization of the magnetic Fe_3O_4–P (St-GMA) microspheres was found to be 60.08 emu/g. Here, a 13.50% magnetic content was equal to the saturation magnetization of 8.23 emu/g; this corresponded well with that obtained by VSM.

Protein Absorption of the Magnetic Fe_3O_4–P (St-GMA) Microspheres with Immobilized IDA–Cu^{2+}

Scheme 2 illustrates the preparation process of immobilizing IDA–Cu^{2+} on the surface of the Fe_3O_4–P (St-GMA) microspheres and their use for the adsorption of proteins. The IDA group applied as a chelating ligand for Cu^{2+} had two carboxyls per molecule; this strengthened the binding force between Cu^{2+} and the magnetic Fe_3O_4–P (St-GMA) microspheres. The Cu^{2+} concentration released in the EDTA solution was detected with an atomic absorption spectrophotometer. According to the results of atomic absorption spectrophotometry, the concentration of Cu^{2+} chelated on the surface of the magnetic Fe_3O_4–P (St-GMA) microspheres was 0.029 mmol/g.

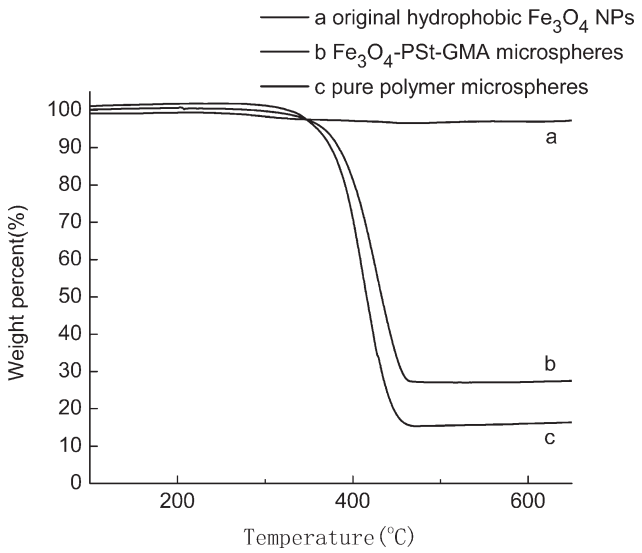


Figure 5. Thermogravimetric curves of the (a) original hydrophobic Fe_3O_4 NPs, (b) magnetic Fe_3O_4–P (St-GMA) microspheres, and (c) pure polymer microspheres.

The selectivity of protein absorption was confirmed by the change in the peak area belonging to BHb and BSA in HPLC chromatograms before and after the adsorption of proteins, as shown in Figure 6. The peak area of BHb decreased much more than that of BSA after it was absorbed by Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres. The protein adsorption capacity was determined by the following equation:

$$q = \frac{(c_0 - c)V}{m} \quad (2)$$

where q is the density of adsorbed protein at equilibrium (mg/g), c_0 is the initial concentration of protein (mg/mL), c is the equilibrium protein concentration (mg/mL), V is the volume of protein solution (mL), and m is the weight of Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres (g).

According to the standard curve of protein concentration and the corresponding characteristic peak area in the chromatogram of BSA and BHb, respectively, the two kinds of protein concentrations in supernatant after adsorption could be calculated.[29] Then, with eq. (2), the amounts of BHb and BSA adsorbed from the binary mixture by the microspheres were found to be 190.66 mg/g (0.0030 mmol/g) and 59.64 mg/g (0.0009 mmol/g), respectively. The result shows that the absorption capacity of BHb was 3.2 times greater than that of BSA; this was much higher than those of similar works, such as that of Xia *et al.*,[29] who prepared magnetic NPs with amine groups on the surface, whose selective adsorption capacity of BHb was only 2.5 times. Overall, the Fe_3O_4–P (St-GMA)–IDA–Cu^{2+} microspheres showed a higher selective adsorption capacity for BHb than for BSA in the binary mixture.

CONCLUSIONS

Magnetic Fe_3O_4–P (St-GMA) microspheres with immobilized Cu^{2+} for highly efficient protein adsorption were synthesized by

two-stage dispersion polymerization. The average size of the magnetic microspheres obtained was 1.50 μm in diameter with a narrow size distribution, and the saturation magnetization of magnetic microspheres was 8.23 emu/g. The adsorption capacity of BHb was as high as 190.66 mg/g of microspheres; this was 3.20 times greater than that of BSA, which was only 59.64 mg/g of microspheres with a rather low nonspecific adsorption. With the advantage of a narrow size distribution, superparamagnetic properties, rapid separation, high selective adsorption capacity, low nonspecific adsorption of proteins, and easy scalability, these microspheres have the opportunity to be used in the large-scale separation or purification of biomolecules. We believe that the combination of the two-stage method with the dispersion polymerization of magnetically prepared microspheres has a good future. A subsequent optimization experiment is in progress.

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