A Novel Approach to Prepare PA6/Fe_3O_4 Microspheres for Protein Immobilization

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ABSTRACT: A novel method for preparation of polymer-based magnetic microspheres was proposed by utilizing melt reactive blending, which was based on selective location of Fe_3O_4 nanoparticles in PA6 domain of polystyrene (PS)/polyamide 6 (PA6) immiscible blends. The results showed that most of Fe_3O_4 was located in the PA6 microspheres. Magnetization data revealed the magnetite content of PA6/Fe_3O_4 microsphere could be up to 54 wt % with strong magnetic responsibility and high saturation magnetization. Carboxyl functional group, bonded with PA6/Fe_3O_4 microsphere by copolymerization of acrylic acid with PA6 chain in different concentration ethanoic acid (HAc) solution, was used as a ligand for protein adsorption. The amount of adsorbed bovine serum albumin (BSA) was optimized by changing the medium pH and the initial concentrations of BSA. The results denoted that the adsorption capacity of BSA reaches 215 mg/g microspheres, showing potentials to promising applications in bioseparation and biomedical fields. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 2271–2277, 2011

Key words: composite magnetic microspheres; Fe_3O_4; reactive blending; protein immobilization; PA6

INTRODUCTION

Polymer-based magnetic composite microspheres are usually composed of magnetic cores and polymeric shells. Compared with inorganic materials, polymeric shells provide a variety of surface functional groups that can be tailored according to separation requirement and protect from particle aggregation. Besides multitudinous characteristics of the conventional polymer microspheres, magnetic polymer microspheres can be rapidly and easily separated from the mixtures under a magnetic field due to their magnetic property. These microspheres exhibit superior properties than normal particles and hence have received much attention in recent years for wide potential applications such as enzyme immobilization,[1] cell and protein separations,[2–5] and drug delivery processes.[6] However, there are several significant demands which should be satisfied for life-related applications. The requirements of the particle matrix, such as biocompatibility, biodegradability, and stability in defined media, must be combined with a uniform size distribution and an appropriate size range and shape. The physical properties of magnetism, the magnetic susceptibility, or the content of magnetic particles must also be considered. Most of studies, however, have been handicapped by the disadvantages of poor magnetization and low encapsulation efficiency.

Generally, the composite magnetic microspheres were prepared by either *in situ*[7,8] or *ex situ* method.[9–14] In the first method, inorganic particles are directly precipitated inside the polymer micelle or sphere, but this is restricted to the particles which only can be synthesized or formed by decomposed corresponding compounds, also the magnetic particles are always prone to leak out from polymer matrix and lead to low magnetic content. In the *ex situ* method, magnetic particles are prefabricated and then combined with the polymer almost by *in situ* polymerization. In the latter one, magnetic particles can be encapsulated by two different principles. When the polymerization site is at the monomer droplets, which is classified as suspension polymerization[9] and mini-emulsion polymerization,[10] magnetic particles are embedded in polymer micelles or spheres during the polymerization. Another category of polymerization is initiated in water phase or micelle, such as emulsion polymerization,[11] soap-free polymerization,[12] deposition polymerization,[13] and dispersion polymerization.[14] In this kind of polymerization the inorganic particles are dispersed in water phase and monomer, and the polymerization occurs around the particles. Although the *ex situ* method seems to be more feasible than the *in situ* method, this method still has many

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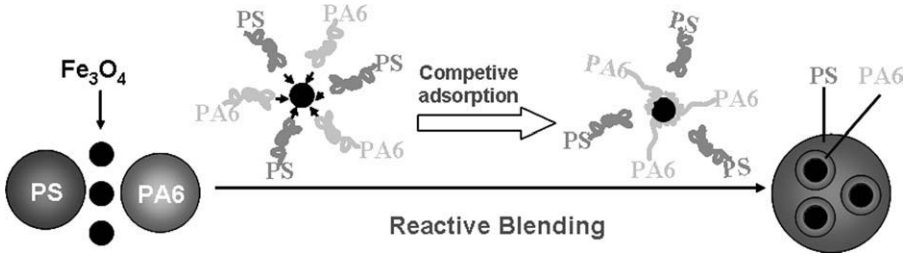

Scheme 1 Strategy of preparing composite magnetic microspheres by reactive blending.

disadvantages. First, the complexity of the particle nucleation mechanism and the difficulties in controlling the dispersion stability of inorganic particles result in the major obstacles in preparing magnetic polymer microspheres with a high encapsulation efficiency and high magnetic response. According to previous reports, magnetite contents of these microspheres are relatively low (<30 wt %); Secondly, the *ex situ* method is limited by the fact that many polymers can't be easily synthesized by radical polymerization; Finally, if the particles are too hydrophilic or too hydrophobic, they will segregate in the water or in the oil phase resulting in poor stability and it is difficult to design a suitable dispersant.

It is noticed that when nanoparticles are compounded with immiscible binary polymer blends, they always tend to selectively locate in one of the two phases.[15–18] Our strategy to fabricate composite magnetic microspheres in this article is by reactive blending Fe_3O_4 nanoparticles with two polymers, which are PA6 and PS. Due to selectively location of Fe_3O_4 in the more polar PA6 domains, the PA6-based composite magnetic microspheres could be obtained by selective extraction of PS. Moreover, the key to control the diameter of microspheres is the use of compatibilizer to tailor morphology. The sketch of the strategy was shown in Scheme 1. Carboxyl as surface functional groups are usually introduced onto PA6/Fe_3O_4 magnetic polymer microspheres by copolymerization with functional acrylic acid monomers.

EXPERIMENTAL

Raw materials

The polyamide 6 (PA6, M_n = 25,000, Toray Industries, Inc. Japan) and the polystyrene (PS, Mn = 100,000, Shanghai Petrochemical Co., China) were used in this study. The functionalized PS with a terminal maleic anhydride group (FPS, M_n = 71,000; maleic anhydride group = approximately 1.3 wt %) was used as a reactive compatibilizer for the PS/PA6 blends. Fe_3O_4 nanoparticles (200 nm) were provided by Tayca, Japan. Bovine serum albumin (BSA, >98%) was obtained from Bovogen Biologicals Co.; 3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC, Yanchang Biological Chemical Co., Shanghai) and phosphate buffer saline (PBS, Fuzhong Biological Chemical Co., Shanghai, China) were used for carboxyl activator and buffer solution, respectively. Acrylic acid (AA, Third Chemical Plant, Tianjin, China), benzoyl peroxide (BPO, Lingfeng Chenical Reagent Co., Shanghai, China), and ethanoic acid (HAc, Lingfeng Chenical Reagent Co., Shanghai, China) were of analytical grade and used without further purification.

Preparation of PA6/Fe_3O_4 microspheres

The blends were prepared in a Haake mixer (POLYLAB RC300P, Haake) at 240°C under a rotational speed of 100 rpm. Before processing, all the raw materials were dried overnight at 80°C in a vacuum oven. The mixing procedure was as follows: precompounding PA6, FPS, and PS for 2 min and then adding Fe_3O_4 for another 8 min. The effect of mixing protocol on the dispersed of Fe_3O_4 in PS/PA6 blend has been detailed in our previous report.[19] The mixtures were compressed into a 1mm-thick sheet at 260°C for 15 min. Then, the PA6/Fe_3O_4 composite magnetic microspheres were finally obtained by selective soxhlet extraction of the PS matrix in methylbenzene for 2 h.

Functionalize PA6/Fe_3O_4 microspheres

AA was used to copolymerize with PA6/Fe_3O_4 microspheres to introduce carboxyl groups on the particles. The dried PA6/Fe_3O_4 (40/50, weight ratio) microspheres, 0.5 g, was dispersed in different concentration (30 wt % or 35 wt %) ethanoic acid solution (HAc) and then the suspension was stirred under vigorous stirring at 120°C for 2 h in a three-necked flask equipped with oil bath. Subsequently, AA monomer was added dropwise at the same time 0.1 g BPO was introduced. Two concentration of AA in suspension, 10 vol % and 15 vol %, were investigated. The polymerization medium was kept at 80°C for 0.5 h with stirring speed of 600 rpm. The residuals (e.g., unconverted monomer, initiator, and other materials) were removed by an extensive cleaning procedure. The functionalized PA6/Fe_3O_4 microspheres were obtained by centrifugation.

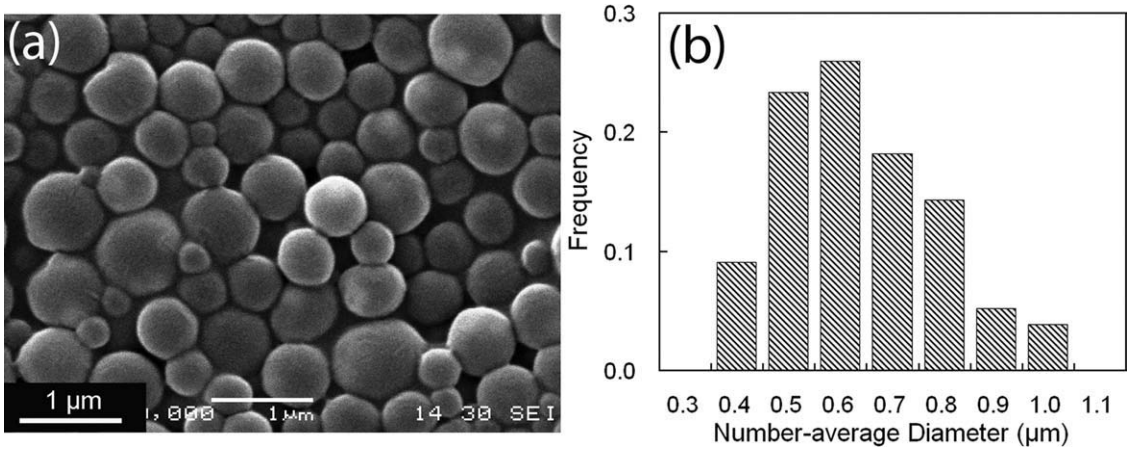

Figure 1 (a) The SEM image and (b) the size distribution of PA6/Fe_3O_4 microspheres extracted from the PS/PA6/Fe_3O_4 blends (60/40/50/6, weight ratio).

BSA adsorption and determination of immobilization capacity

BSA was selected as a model protein for adsorption on functionalized magnetic microspheres. BSA adsorption was performed in a batch adsorption system. An appropriate amount of BSA and 10 mg EDC were dissolved in 25 mL of buffer solution of specified pH. Three milligrams of functionalized microsphere was added to the mixture, and then sonicated for 30 min and shaken overnight at 37°C. The particles were collected under a strong external magnetic field. The BSA adsorbed onto microspheres (immobilization capacity, Q) was determined by measuring the initial and final concentrations of BSA in the medium, which can be calculated as follows [eq. (1)]:

$$Q = (C_0 - C)V/M(\text{mg/g}) \quad (1)$$

where, C_0 and C are the initial BSA concentration (mg/mL) and the residual BSA concentration in equilibrium, respectively. V is the BSA solution volume (mL) and M is the amount of the functionalized magnetic PA6/Fe_3O_4 microspheres added in the reaction.

Characterization

The morphology of composite magnetic microspheres was investigated by scanning electron microscopy (SEM, JSM-6360LV, JEOL). The number-average diameter (D_n) ($D_n = \Sigma N_i D_i / \Sigma N_i$, where N_i is the number of particles having the diameter of D_i) of microspheres was determined according to SEM micrographs.

The degree of magnetism of the composite magnetic microspheres was performed at room temperature in magnetic fields up to 150 KG using the vibrating-sample magnetometer (VSM 7407, America Lake Shore Co.), and the results were used to calculate the magnetic quality of the microspheres. Before these tests, the PA6-based microspheres were purified by a centrifugal separator to remove Fe_3O_4 particles without encapsulated by the PA6 domain and to remove PA6 spheres with a diameter less than 200 nm.

The surface functional carboxyl group of microspheres was qualitative analyzed by the FTIR (Nicolet 5700, Thermo Electron Scientific Instruments Co.) using a KBr compressed pellet method and quantitatively determined by conductometric titration.[20,21] Functionalized microspheres, 0.10 g, acidified with HCl, were titrated under nitrogen atmosphere with 0.01M NaOH solution at 30°C. Quantitative information was acquired from conductometric results using the standard extrapolation/intersection method to determine the titration endpoints.

The BSA concentration was measured on a Microplate spectrophotometer system (SPECTRA max PLUS[384], Molecular Devices Co.) at 562 nm via bicinchoninic acid (BCA).[22] A calibration curve, which is concerned with the relationship of absorbance and BSA concentration, can be constructed using dilutions of a stock 1 mg/mL solution of BSA. The BSA content of unknown samples can be determined spectrophotometrically by comparison with the calibration curve.

RESULTS AND DISCUSSION

Properties of PA6/Fe_3O_4 microspheres

Based on the previous study,[19] the result showed that PS/PA6 form sea-island structure where PA6 is the dispersed domain and most of Fe_3O_4 nanoparticles are selectively located in PA6 domain. Figure 1(a,b) are the SEM image and the size distribution of PA6/Fe_3O_4 microspheres extracted from the PS/PA6/Fe_3O_4/FPSblends (60/40/50/6, weight ratio).

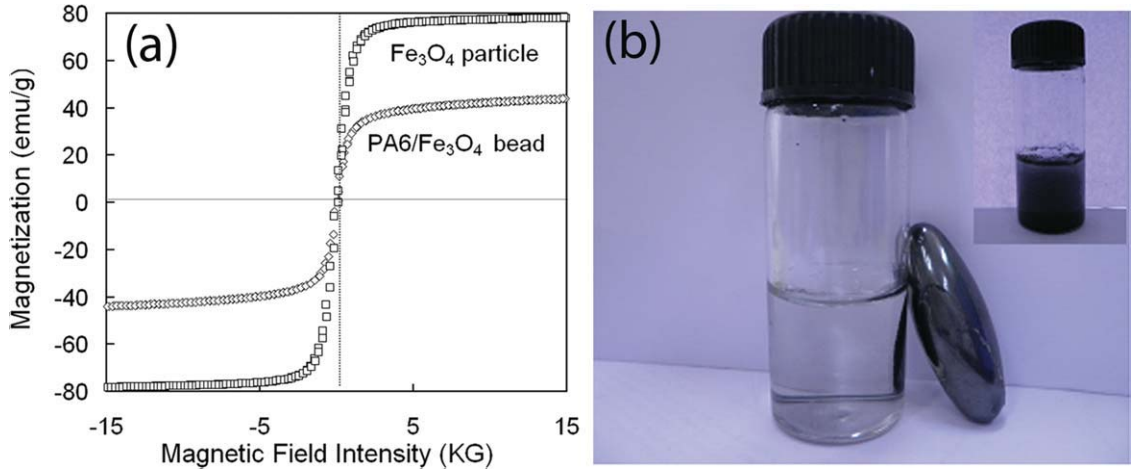

Figure 2 The magnetic properties of PA6/Fe_3O_4 (40/50, weight ratio) microspheres: (a) room temperature magnetization curve, and (b) separation from solution under an external magnetic field. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

As clearly seen here [Fig. 1(a)], the magnetic microspheres have a spherical form and few Fe_3O_4 particles were observed outside the PA6 domains, suggesting that most of the magnetic particles could be encapsulated within the micro PA6 domains. The size of the magnetic microspheres is quite uniform ranging from 0.4 to 1.0 μm [Fig. 1(b)].

The room temperature magnetization curve PA6/Fe_3O_4 microspheres [Fig. 2(a)] shows a little magnetic hysteresis loop, which depicts the strong magnetic response to a varying magnetic field. The saturation magnetization of Fe_3O_4 particles and PA6/Fe_3O_4 microspheres are about 78.1 emu/g and 42.3 emu/g, respectively. It demonstrated that the magnetic content of the microspheres is about 54 wt % which is higher than the polymer-based composite microspheres prepared by the emulsion polymerization approach (~ 30 wt %). The magnetic separability of such magnetic microspheres was tested in water by placing a magnet near the glass bottle. The black particles were attracted toward the magnet within 3 s [Fig. 2(b)], demonstrating directly that the core shell microspheres display an excellent magnetic response. This will provide an easy and efficient way to separate PA6/Fe_3O_4 microspheres from a sol or a suspension system and to immobilize protein under an external magnetic field.

Functional groups

The FTIR spectra of functionalized and as-prepared PA6/Fe_3O_4 microspheres are shown in Figure 3. In Figure 3(a), as-prepared PA6/Fe_3O_4 exhibits a number of characteristic spectral bands. The most characteristic of them are at 1634 cm^{-1} (amide I), 1528 cm^{-1} (amide II), and 1451 cm^{-1} (amide III), which belong to PA6 chains. The peak at 580 cm^{-1} is assigned to the stretching vibrations of Fe-O bond of Fe_3O_4 magnetic nanoparticles. Figure 3(b) is the spectra of the PA6/Fe_3O_4 microspheres copolymerized with 15 vol % AA in 35 wt % HAc. The PA6 characteristic absorption band of functionalized PA6/Fe_3O_4 microspheres as shown in Figure 3(b) is much weaker than that of the as-prepared ones in Figure 3(a), suggesting that some of PA6 chains thermo-oxidation degradation form radical which induced copolymerization with AA.[23] On the other hand, the wide peak at 3200 to 3500 cm^{-1} attributed to the stretching vibrations of O-H bond and the absorption band at 1740 cm^{-1} belonged to the —C=O group are much stronger compared with Figure 3(a). These thoroughly confirm the existence of carboxyl groups. In addition, the carboxyl concentration on the surface of functionalized microspheres was quantitative determined by titration of the microspheres dispersion with NaOH under a nitrogen atmosphere.

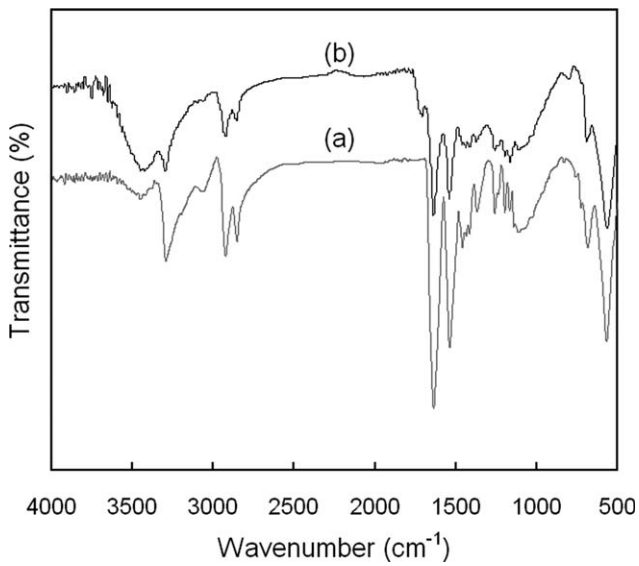

Figure 3 FTIR spectra of (a) as-prepared and (b) functionalized of PA6/Fe_3O_4 microspheres.

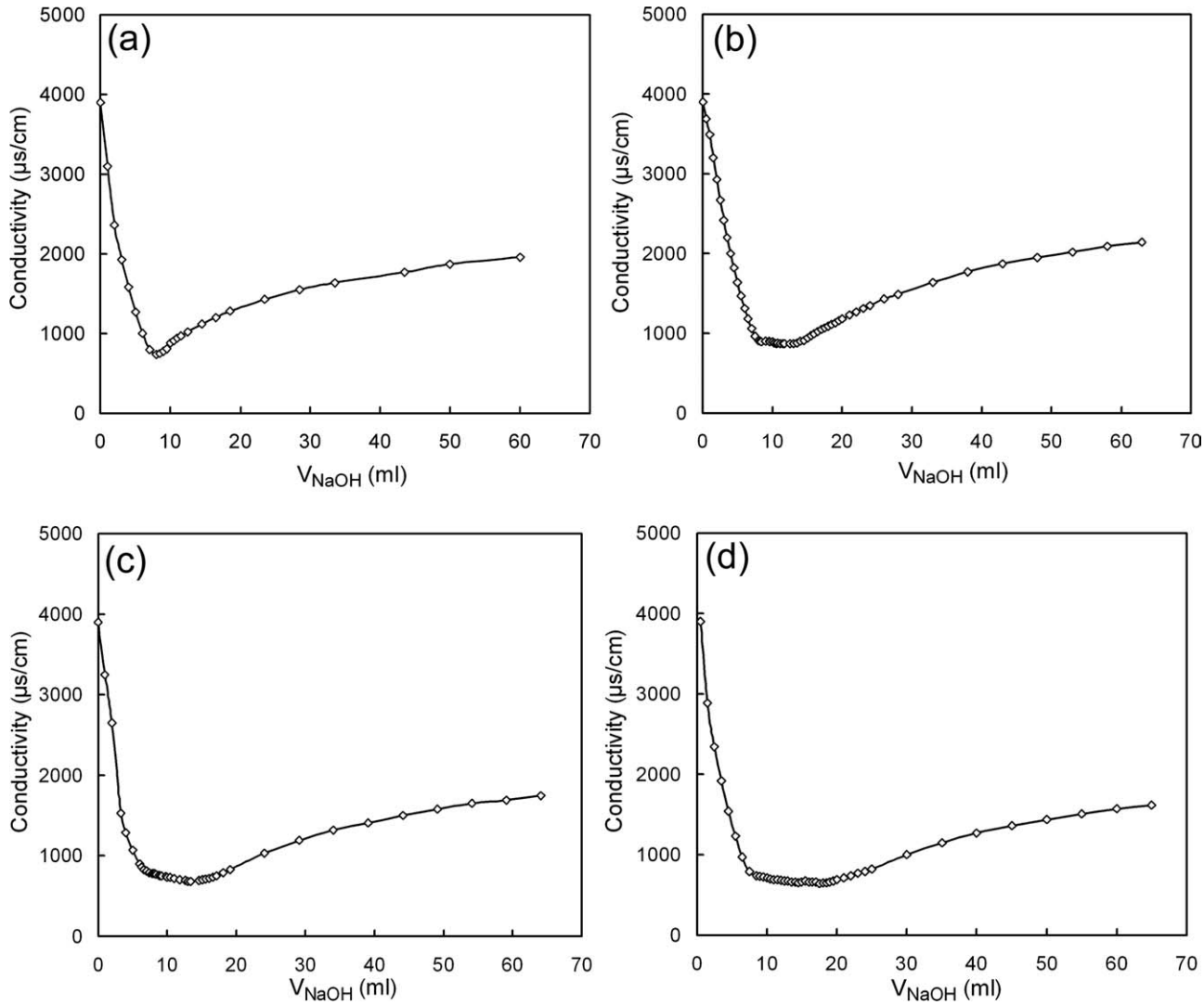

Figure 4 Conductometric titration curve of the PA6/Fe_3O_4 microspheres copolymerized with (a)10 vt % AA in 30 wt % HAc; (b) 10 vt % AA in 35 wt % HAc; (c) 15 vt % AA in 30 wt % HAc; (d) 15 vt % AA in 35 wt % HAc.

Figure 4 shows the electrical conductometric titration curve of the microspheres with different copolymerization condition. One can see that this is a typical conductometric titration curve of a strong acid group (HCl) and a weak acid group (—COOH) when neutralized with a strong base. There are three regions. First, the conductivity sharply decreases with increasing NaOH which due to the disappearance of free H^+ ions. Second, the conductivity value changes gradually with addition of NaOH, caused by the neutralization of —COOH. Third, the slope becomes a constant which is equal to the theoretical value for the conductivity of NaOH. Therefore, the surface concentration of carboxylic acid can be calculated as follows [eq. (2)]:

$$-\mathrm{COOH(mmol/g)} = M\Delta V/m \qquad (2)$$

where, M and ΔV are the mol concentration of NaOH solution and the consumed NaOH volume of —COOH, respectively. m is the weight of the functionalized magnetic PA6/Fe_3O_4 microspheres added in the titration. According to the Figure 4, the surface concentrations of carboxylic acid of microspheres in different copolymerization condition were listed in Table I. As demonstrated in Figure 4 and Table I, the surface concentration of carboxylic acid of microsphere was increased with the increasing concentration of AA and HAc. On the one hand, in general, the probability of successful copolymerization was increased with increasing AA concentration; on the other hand, the mobility of PA6 chains was improved with increasing the concentration of HAc, which must be in favor of the copolymerization. The surface concentration of carboxylic acid of the functionalized microsphere could be add up to 1.00 m mol/g when microsphere was copolymerized with 15 vol % AA in 35 wt % HAc.

TABLE I
Surface Concentrations of Carboxylic Acid of Microspheres Using Different Copolymerization Condition

Copolymerization condition	ΔV (mL)	M (mol/L)	m (g)	–COOH (m mol/g)
10 vt% AA, 30 wt% HAc	2.0	0.01	0.1	0.20
10 vt% AA, 35 wt% HAc	6.5	0.01	0.1	0.65
15 vt% AA, 30 wt% HAc	8.6	0.01	0.1	0.86
15 vt% AA, 35 wt% HAc	10.0	0.01	0.1	1.00

BSA adsorption

Effects of functionalization

Figure 5 presents the BSA immobilization capacity using the as-prepared and functionalized PA6/Fe_3O_4 microspheres with different copolymerization conditions. As can be seen, negligible amounts of BSA was adsorbed nonspecifically on the as-prepared PA6/Fe_3O_4 beads (6.3 mg/g), and the amount of the protein adsorbed on the functionalized PA6/Fe_3O_4 microspheres increased as the carboxylic acid was increased from 0.2 to 1.0 m mol/g. It is clear that this increase in adsorption capacity is due to specific interactions between the surface carboxylic acid groups and BSA molecules which promote the adsorption of albumin. Therefore, PA6/Fe_3O_4 microsphere copolymerized with 15 vol % AA in 35 wt % HAc is applied to immobilize BSA.

Effects of pH

Figure 6 reveals the effect of pH on the adsorption of BSA onto functionalized PA6/Fe_3O_4 microspheres. In all of the investigated cases, the maximum adsorption of BSA was observed at pH 6.0. At pH higher than 6, the BSA adsorption capacity decreased. This decrease can be attributed to electrostatic repulsion forces between the identically charged groups. At the isoelectric points of BSA (pH = 5),[24] proteins have no net charge and therefore the maximum adsorptions from solution are usually observed at these points. When neutrally charged, protein solubility in aqueous media decreases. On the other hand, basic medium caused the protein positively or negatively charged, increasing the solubility of protein in aqueous media. Consequently, higher pH values resulted in decreased adsorption capacities for BSA in aqueous media.

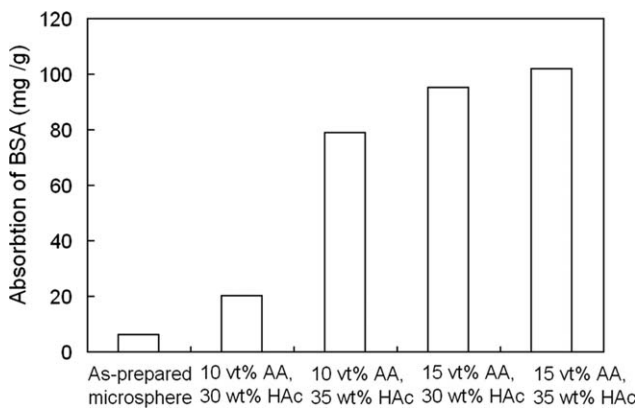

Figure 5 Effect of functionalization of microspheres on BSA adsorption: initial BSA concentration 0.35 mg/mL; pH, 7.4; T, 37°C.

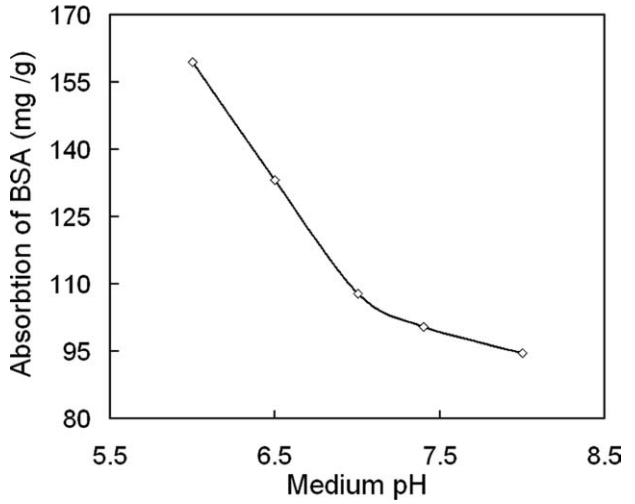

Figure 6 The variation of adsorbed BSA with medium pH at equilibrium (initial BSA concentration 0.35 mg/mL; T, 37°C).

Effects of BSA concentration

The amount of BSA adsorbed at pH 7.4 as a function of equilibrium BSA concentration was presented for magnetic microspheres in Figure 7. As seen in Figure 7, with increasing BSA concentration in solution, the adsorbed amount of BSA per unit mass of the beads increases until about 0.6 mg/mL and then

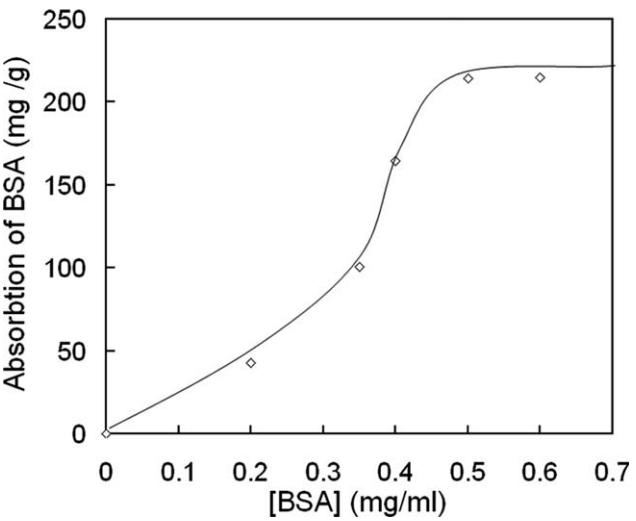

Figure 7 Effect of concentration of BSA on BSA adsorption: pH, 7.4; T, 37°C.

approaches saturation at 215 mg/g which was higher than the previous reported value of 85.37 mg/g.[25]

CONCLUSIONS

In this study, we proposed a novel way to prepare composite magnetic microspheres through blending Fe_3O_4 particles into immiscible PS/PA6 blends, where Fe_3O_4 selectively locate in the PA6 droplets. The obtained microspheres show quite uniform size ranging from 0.4 to 1.0 μm, and most of Fe_3O_4 was dispersed in the interior of the microspheres. The weightcontent of Fe_3O_4 in microspheres can reach 54% and the degree of magnetism of the magnetic composite microspheres is 42.3 emu/g. The PA6/Fe_3O_4 beads with copolymerization attached surface carboxylic acid groups were developed for affinity adsorption of BSA. The results show that adsorption of BSA under different pH values and BSA initial concentrations have revealed the high selectivity and affinity of carboxylic acid-attached PA6/Fe_3O_4 microspheres for BSA. In addition, the adsorption capacity of BSA reached 215 mg/g microspheres, showing potentials to promising applications in bio-separation and biomedical fields.

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