# α-Amylase Immobilized by $Fe_3O_4$/Poly(styrene-*co*-maleic anhydride) Magnetic Composite Microspheres: Preparation and Characterization

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**ABSTRACT:** $Fe_3O_4$/poly(styrene-*co*-maleic anhydride) core–shell composite microspheres, suitable for binding enzymes, were prepared using magnetite particles as seeds by copolymerization of styrene and maleic anhydride. The magnetite particles were encapsulated by polyethylene glycol, which improved the affinity between the magnetite particles and the monomers, thus showing that the size of the microspheres, the amount of the surface anhydrides, and the magnetite content in the composite are highly dependent on magnetite particles, comonomer ratio, and dispersion medium used in the polymerization. The composite microspheres, having 0.08–0.8 μm diameter and containing 100–800 μg magnetite/g microspheres and 0–18 mmol surface-anhydride groups/g microsphere, were obtained. Free α-amylase was immobilized on the microspheres containing reactive surface-anhydride groups by covalent binding. The effects of immobilization on the properties of the immobilized α-amylase [magnetic immobilized enzyme (MIE)] were studied. The activity of MIE and protein binding capacity reached 113,800 U and 544.3 mg/g dry microspheres, respectively. The activity recovery was 47.2%. The MIE had higher optimum temperature and pH compared with those of free α-amylase and showed excellent thermal, storage, pH, and operational stability. Furthermore, it can be easily separated in a magnetic field and reused repeatedly. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 95: 328–335, 2005

**Key words:** core–shell polymers; nanocomposites; enzymes; magnetic polymers; immobilization

## INTRODUCTION

Magnetic polymer microspheres are characterized by a potent magnetic susceptibility and reactive polymer surfaces with large specific surface area, on which functional molecules, such as enzyme, antibody, antigen, cell, and biotin, can be immobilized by different methods.[1,2] With a powerful magnetic field, they can be moved directly and, separated from dispersion medium, can be repeatedly reused.[3] The magnetic polymer microspheres generally have a magnetite core and polymer shell. The polymer can be one of the natural macromolecules or synthetic materials formed by polymerizing monomers in the presence of a magnetite core. Encapsulation of natural macromolecules often results in polydispersity in the microsphere diameter. In addition, controlling the diameter is also difficult for copolymerization.[4,5] Smaller size and higher reactive surface groups are favored for immobilizing an enzyme and can easily preserve the greater advantage of free enzyme and higher enzyme activity.

We copolymerized styrene and maleic anhydride in the presence of magnetite colloid particles. The products, poly(styrene-*co*-maleic anhydride) [poly(St-*co*-MA)] magnetic composite microspheres with nanosize magnetite particles, have monodispersed diameters and highly reactive surface groups and better magnetic susceptibility in a given magnetic field.

α-Amylase is a hydrolase of starch produced by a microorganism, extensively used in the food, textile, fermentation, and pharmaceutical industries, but it has poor stability and cannot be used repeatedly.[6,7] This article reports that α-amylase was covalently bound onto the magnetic poly(St-*co*-MA) microsphere by reacting with the surface anhydride groups. Immobilization of the enzyme improved its thermal and operational stability, but hardly changed its kinetics. The activity recovery during immobilization approached nearly 50% and the magnetic immobilized enzyme (MIE) could be conveniently reclaimed by a given magnetic field and used repeatedly. The MIE activity retention was above 80% after the tenth operation, which could potentially decrease production cost.

The MIE not only reserves the activity and selectivity of free enzymes, but also has excellent thermal, pH, and operational stability. In addition, the MIE could conveniently be reused repeatedly in a magnetic field.

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**TABLE I**
**Preparation of $Fe_3O_4$/Poly(St-*co*-MA) Composite Microspheres[a]**

| No. | MP (g) | KPS soln (mL) | St (g) | MA (g) | DVB (mL) | EtOH (mL) | $H_2O$ (mL) | Diameter ($\mu$m) |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.0 | 100 | 13.5 | 2.5 | 0.2 | 15.0 | 105 | 0.086 |
| 2 | 0.6 | 100 | 19.8 | 2.5 | 0.2 | 85.0 | 35 | 0.85 |
| 3 | 0.9 | 100 | 15.2 | 2.5 | 0.2 | 20.0 | 100 | 0.13 |
| 4 | 0.8 | 100 | 15.2 | 3.4 | 0.2 | 25.0 | 95 | 0.29 |
| 5 | 1.2 | 100 | 17.5 | 3.0 | 0.2 | 20.0 | 100 | 0.25 |

[a] 70°C, $N_2$, 10 h. MP, magnetic colloid particles; St, styrene; MA, maleic anhydride; DVB, divinylbenzene; EtOH, ethanol; KPS soln, aqueous solution of saturated potassium peroxydisulfate.

Thus, the MIE is adapted to the successional and automatic production and shows the potential of a wide range of industrial applications in many fields, such as food, textile, fermentation, pharmaceutical industries, wastewater treatment, and so on.

## EXPERIMENTAL

### Materials

Magnetite colloid particles (MPs, ∅: ~ 20 nm) were prepared following the method by Qiu et al.[8] Styrene (St), divinylbenzene (DVB), and maleic anhydride (MA) were purified by vacuum distillation. Potassium peroxosulfate (KPS) and ethanol were analytical-grade reagents. $\alpha$-Amylase (industrial activity: 6000 U/g) was purified with an anion-exchange resin. Soluble starch, bovine serum albumin, and amylomaltose were commercially available. Water was distilled twice before use.

### Magnetic composite microspheres

Ferrous ions were deposited by alkali in an aqueous solution of $H_2O_2$ and polyethylene glycol (PEG), with stirring and nitrogen purging. The resulting magnetite colloid particles (MPs) were encapsulated by PEG with an average particle size of 20 nm.[8] They were then dialyzed for 1 week and used as the polymerization seeds. MPs were first immersed in an aqueous solution saturated by KPS to absorb the initiator (KPS). They were then swelled by the ethanol solution with the monomers (St, MA, DVB) for 24 h. The mixture was poured into a 250-mL round-bottom, four-neck flask with an incubating dispersion medium of EtOH/water. MA monomer was added to the solution to copolymerize with St on the surface of MPs at 70°C. The reaction mixture was stirred at 400 rpm for 10 h with the reaction temperature at 70°C and constant $N_2$ purge. The magnetic latex microspheres were separated by a given magnetic field (0.5 Wb/m$^2$), and then washed repeatedly with distilled water and ethanol, and finally dialyzed for 1 week against water. The synthesis conditions are given in Table I.

The average diameter of the composite microspheres was estimated from the micrographs using a JEM-100CX electron microscope (TEM; JEOL, Tokyo, Japan). The dispersion parameter ($\hat{\delta}/\bar{\chi}$) was calculated with

$$\hat{\delta} = \left[ \sum_{i=1}^{n} (X_i - \bar{X})^2 / n - 1 \right]^{1/2}$$

where $\hat{\delta}$ is the mean-square deviation, $X_i$ is the diameter of a microsphere, and $\bar{X}$ is the average diameter of the microspheres.

The magnetite and surface maleic anhydride contents of the composite microspheres were determined from atomic absorption spectrophotometry and conductometric titration, respectively.[9]

### Coupling of $\alpha$-amylase onto composite microspheres

Immobilization of $\alpha$-amylase is based on the formation of a covalent bond between the enzyme and carrier microspheres. Covalent linkages are strong and do not release the enzyme into solution in the presence of a substrate or in high ionic strength solution.[10] The magnetic composite microspheres, used as carriers, were purified and dispersed supersonically in a phosphate-buffered solution (PBS: 0.1*M*, pH 7.5) for 5 h, then separated in a 0.5 Wb/m$^2$ magnetic field and added to 5 mL of 0.1*M* $\alpha$-amylase solution incubated with 1*M* $NaHCO_3$, in which the enzyme was purified with anion-exchange resin. The mixture was oscillated at 20–25°C for 24 h. The MIEs, covalently bounded on the magnetic microspheres, were separated from the mixture by a 0.5 Wb/m$^2$ magnetic field, and washed by 0.1*M* $NaHCO_3$, followed by 1*M* NaCl solution and distilled water. They were washed repeatedly until no protein was detected in the wash water by spectrophotometer. The bound enzyme, separated by the magnetic field, was freeze-dried under vacuum and stored at 4°C until later use. The composition and the properties of the products were investigated.

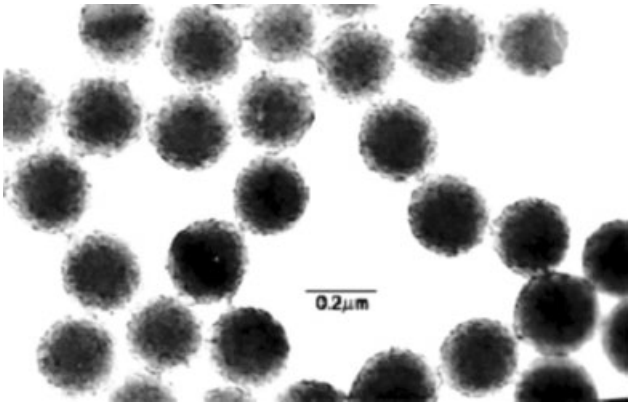


**Figure 1** TEM microphotograph of $Fe_3O_4$/poly(St-*co*-MA) core–shell composite microsphere.

### Kinetic measurement of the enzyme

The activities of the bound enzyme (MIE) and the free enzyme were determined by amylase-catalyzed reaction of 1% starch solution, following the method presented in Bergmeyer.[11] The substrate was soluble starch and the content of the maltose produced in the reaction was determined by salicylic acid colorimetry.[12] The activity unit of $\alpha$-amylase was defined as the amount of the enzyme needed in the enzyme-catalyzed hydrolysis reaction of starch for producing 1 mg maltose/h. The specific activity of MIE was defined as the enzyme activity per milligram protein. The activity retention was calculated as follows:

$$\text{Activity retention (\%)} = \frac{c}{a - b}$$

where $a$ and $b$ are the activity of free enzyme in the solution before and after immobilization, respectively; and $c$ is the activity of the bound enzyme (MIE).

The amount of enzyme protein was determined by the Lowry method.[13] Bovine serum containing 75.0 $\mu$g protein/mL was used as the standard. The protein binding capacity of MIE was defined as the amount of the protein bound on 1 g carrier microspheres during the immobilization of the enzyme, and calculated from

$$\text{Protein binding capacity (mg protein/g carrier)} = \frac{A - B}{C}$$

where $A$ and $B$ are the protein content of the free enzyme in solution before and after immobilization, respectively; and $C$ is the mass of the magnetic composite microspheres.

**TABLE II**
**Magnetic Susceptibility of the Microspheres versus the Magnetite Content in the Microspheres**

| Magnetite in the microspheres ($\mu$g/g)[a] | Setting distance in magnetic field (mm) | |
|---|---|---|
| | 0.5 Wb/m$^2$, 30 min | 0 Wb/m$^2$, 30 d |
| 103.1 | 18 | 0 |
| 239.1 | 24 | 0 |
| 386.9 | 48 | 0.5 |
| 478.3 | 51 | 0.5 |
| 725.6 | 72 | 1.0 |

[a] Diameter: 0.1–0.2 $\mu$m; dispersion medium: water; concentration of the microspheres: 20 wt %.

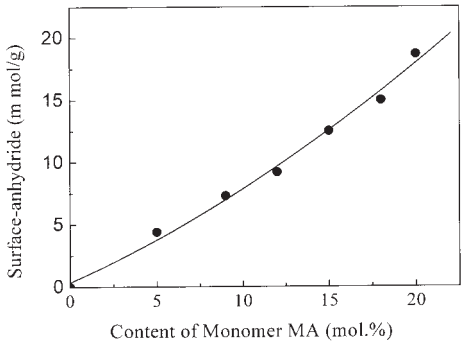


**Figure 2** Surface anhydride increase by copolymerization as functions of copolymerization monomer maleic anhydride.

## RESULTS AND DISCUSSION

### $Fe_3O_4$/P(St-*co*-MA) composite microspheres

Copolymerization of MA and St can be performed on the surface of MPs to generate $Fe_3O_4$/poly(St-*co*-MA) core–shell composite microspheres with reactive anhydride groups on the surface of the microspheres. Reaction conditions were controlled by the following

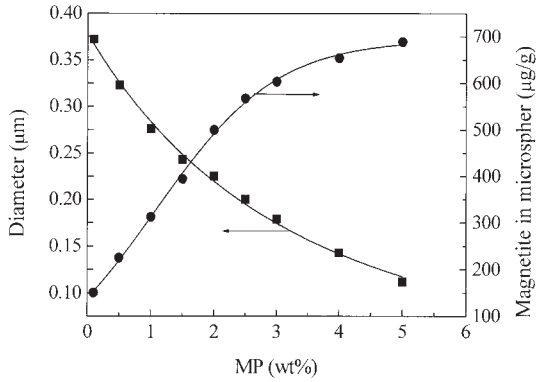


**Figure 3** Effects of MP on the diameter and magnetite content of the composite microspheres at a monomer concentration of 14 wt % (St/MA, 4/1) and ethanol concentration of 20 vol %.

**TABLE III**
**Effects of Ethanol on Polymerization Behavior[a]**

| Content of EtOH (vol %) | Average diameter ($\mu$m) | Dispersion parameter | Coagulum (g) | Conversion (%) |
|---|---|---|---|---|
| 18.0 | 0.085 | 0.032 | 3.0 | 99.1 |
| 20.0 | 0.106 | 0.038 | 0.9 | 98.5 |
| 25.0 | 0.154 | 0.076 | 0.1 | 97.8 |
| 30.0 | 0.213 | 0.098 | 0 | 96.4 |
| 40.0 | 0.328 | 0.178 | 0 | 94.7 |
| 50.0 | 0.400 | 0.246 | 0 | 91.4 |
| 60.0 | 0.562 | 0.381 | 0 | 78.6 |
| 70.0 | 0.728 | polydispersion | 0 | 66.2 |

[a] $N_2$, 70°C, reaction time: 10 h.

procedures: first, the magnetite particles were encapsulated with PEG to improve the affinity between MPs and initiators and monomers; then before polymerization, the MPs were soaked and swelled in an aqueous solution of KPS and alcohol solution of monomers, respectively, to enhance the adsorption of initiators and monomers; and, finally, an alcohol/water mixture was used as the dispersion medium for the polymerization reaction to control the interface tension of the composite microspheres and improve their dispersion stability. The $Fe_3O_4$/poly(St-*co*-MA) composite microspheres have a core–shell structure and monodispersity, as illustrated in Figure 1.

The infrared spectra of the composite microspheres indicated that the maleic anhydride had been copolymerized with styrene and introduced into the composite microspheres (1860, 1790 $cm^{-1}$, stretching of $-\overset{\overset{O}{\|}}{C}O\overset{\overset{O}{\|}}{C}-$ of maleic anhydride; 1250 $cm^{-1}$, stretching of –CO—O—OC– of maleic anhydride; 1600 $cm^{-1}$, loop vibration of benzene ring; 1065 and 698 $cm^{-1}$, distorting of ═C—H of benzene ring). The infrared absorption band, corresponding to $Fe_3O_4$ at 580 $cm^{-1}$, showed that the magnetite particles were entrapped in the composite polymer microspheres. The content of the magnetite in the composite microspheres was determined by atomic absorption spectrophotometry, and the magnetic susceptibility of the composite microspheres increased with the magnetite content (see Table II).

For the copolymerization of St ($M_1$) and MA ($M_2$), the monomer reactivity ratios were $r_1 = 0.01$ and $r_2 = 0$, respectively, and thus the copolymerization is a typical alternative reaction.[14] MA was instilled into the reaction flask during the late stage of the reaction to enhance anhydride incorporation. The amount of anhydride groups was determined by the titration method. An excess amount of 0.1*M* aqueous hydrochloride was added to the suspension and stirred at room temperature for 0.5 h to change surface anhydride groups into carboxyl groups, which were titrated by a conductometer with 0.01*N* NaOH aqueous solution used as titrant.[9] Assuming complete conversion, the amount of anhydride groups equals that of the carboxyl groups. Figure 2 indicates that the content of the anhydride groups on the surface of composite microspheres increases with increasing amount of MA.

In this polymerization, surfaces of MPs were the initiating centers, around which the polymerization of

**TABLE IV**
**Effect of Proportion of Enzyme to Microspheres on Immobilization[a]**

| | Immobilized enzyme properties | | | | |
|---|---|---|---|---|---|
| Enzyme (mg) | Total activity (U/g carrier) | Protein binding capacity (mg/g)[b] | Protein binding capacity (mg/mmol)[c] | Specific activity (U/mg protein) | Activity recovery (%) |
| 7.1 | 14,400 | 53.4 | 13.0 | 269.6 | 34.2 |
| 17.8 | 69,900 | 255.0 | 62.2 | 274.0 | 38.9 |
| 24.0 | 90,000 | 300.0 | 73.2 | 300.0 | 45.2 |
| 31.1 | 88,800 | 306.0 | 74.6 | 290.2 | 43.1 |
| 48.1 | 88,800 | 309.0 | 75.4 | 287.4 | 43.2 |

[a] Magnetic composite microspheres: dosage, 50 mg; diameter, 0.236 $\mu$m; surface anhydride, 4.1 mmol/g microsphere. 1 *M* $NaHCO_3$.
[b] Amount of the enzyme protein bound on 1 g of magnetic composite microspheres.
[c] Amount of the enzyme protein bound with 1 mmol anhydride on the surface of magnetic composite microspheres.

**TABLE V**
**Effect of the Properties of the Carrier Microspheres on Immobilization[a]**

| Microsphere properties | | Properties of MIE | | | |
|---|---|---|---|---|---|
| | | Protein binding capacity | | | |
| Diameter ($\mu$m) | Surface-anhydride (mmol/g) | (mg/g)[b] | (mg/mmol)[c] | Total activity (U/g carrier) | Activity recovery (%) |
| 0.092 | 4.8 | 386.7 | 80.6 | 106,000 | 46.8 |
| 0.225 | 4.6 | 348.2 | 75.7 | 98,500 | 46.0 |
| 0.436 | 4.7 | 285.9 | 60.8 | 70,800 | 40.2 |
| 0.660 | 4.8 | 250.2 | 52.1 | 60,600 | 37.8 |
| 0.219 | 3.0 | 221.4 | 73.8 | 58,600 | 36.5 |
| 0.246 | 5.5 | 401.5 | 73.0 | 113,800 | 47.2 |
| 0.207 | 7.2 | 544.3 | 75.6 | 102,400 | 42.8 |

[a] Enzyme, 25 mg; magnetic microspheres, 50 mg; 1 *M* $NaHCO_3$.
[b] Amount of the enzyme protein bound on 1 g of magnetic composite microspheres.
[c] Amount of the enzyme protein bound with 1 mmol anhydride on the surface of magnetic composite microspheres.

the monomers was carried out to form core–shell composite microspheres. The effects of MPs on the behavior of polymerization and property of the composite microspheres were significant. As the amount of MPs increased, the "incipient latex particles" increased and the concentration of the monomer in the latex particles decreased. As a result, the polymerization rate slowed down and the diameter of the composite microsphere decreased, as shown in Figure 3. It was shown that the content of magnetite in composite microspheres increased with increasing concentration of MPs. When the concentration of MPs exceeded 3 wt %, the weight fraction of magnetite began to level off, which could be a result of the coagulation of the excess MPs.

During copolymerization of St and MA, the monomers were mainly distributed in both latex particles and dispersion medium. The polarity and solubility parameter of the dispersion medium had substantial effects on the distribution of the monomers between the growing latex particles and dispersion medium, which affected the polymerization rate and the size of the composite microspheres. Table III indicates that the size of the composite microspheres noticeably increased with increasing ethanol vol %, until it reached 60 vol %, but the monodispersity and the monomer conversion decreased with increasing ethanol content. The composite microspheres could not be formed until the ethanol content exceeded 18 vol %.

### Covalent coupling of $\alpha$-amylase on magnetic composite microspheres

The covalent coupling of $\alpha$-amylase on the magnetic composite microspheres was mainly based on the formation of amido bonds between the amino groups of the enzyme and the anhydrides on the surface of the carrier microspheres. The reaction was performed under mild conditions. The effects of enzyme/microsphere ratio, microsphere properties, and pH of incubated solution on immobilization of enzyme were investigated. Table IV indicates that the protein-binding capacity of the magnetic composite microspheres, MIE activity, and activity recovery distinctly increased with increasing ratio of enzyme to microspheres until the amount of enzyme reached 480 mg per gram of carrier. When the amount of the enzyme exceeded 480 mg/g microspheres, the protein-binding capacity tended to reach saturation, whereas MIE activity and activity recovery began to decrease slightly. It is reasonable to conclude that the binding sites on the surface of the magnetic composite microspheres were limited and the enzyme molecules needed enough

**TABLE VI**
**Effect of pH of Reaction Medium on Immobilization[a]**

| | Properties of MIE | | | |
|---|---|---|---|---|
| pH | Total activity (U/g carrier) | Protein binding capacity (mg/g carrier) | Specific activity (U/g protein) | Activity recovery (%) |
| 5.5 | 66,600 | 248.7 | 267.8 | 19.2 |
| 6.8 | 86,280 | 287.7 | 299.8 | 28.8 |
| 7.6 | 99,840 | 300.0 | 332.8 | 37.1 |
| 8.2 | 100,600 | 306.8 | 327.9 | 36.6 |
| 9.1 | 88,100 | 291.2 | 302.5 | 34.6 |

[a] Enzyme, 25 mg; magnetic microspheres, 50 mg; 1 *M* $NaHCO_3$.

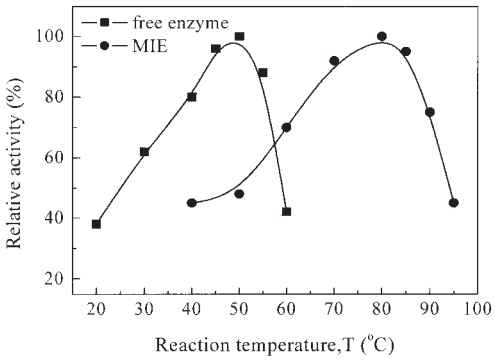


**Figure 4** Effect of temperature on the free enzyme and MIE measured at pH 8.0, 1% soluble starch.

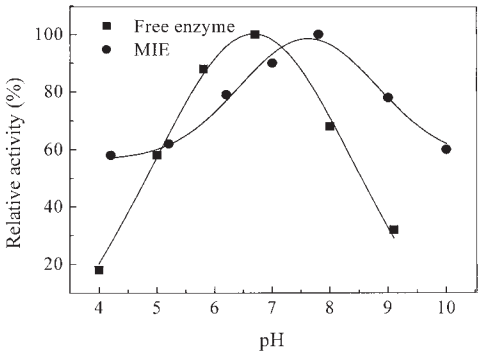


**Figure 5** Effects of pH on the free enzyme and MIE measured at 50°C, 1% soluble starch.

space for catalyzing the reaction of the substrate, and thus the overdenseness of the enzyme on the microspheres resulted in steric hindrance.

The specific area of the magnetic composite microspheres increased with the decrease in their size, so they could bind more enzyme and MIE activity and the percentage of recovery was higher. When the amount of enzyme bound on the surface of the carrier microspheres exceeded 98,500 U/g carrier, the activity recovery of MIE tended to level off, as demonstrated in Table V, showing that, as the amount of surface anhydride increased, the amount of protein per gram carrier microspheres substantially increased, whereas the amount of protein bound per mmol surface anhydride hardly changed. The MIE activity and recovery increased with increasing surface anhydrides until the protein binding capacity reached 73 mg/mmol surface anhydride, beyond which MIE activity and recovery began to decrease (Table V).

The immobilization of the enzyme on the magnetic microspheres was performed in the buffers with various pH values, the results of which are illustrated in Table VI. It was demonstrated that the protein-binding capacity of the microspheres and MIE activity and recovery reached the maximum values at about pH 8.0. This phenomenon is attributed to the alkalescent reaction medium, which is propitious for the formation of the covalent linkages between the anhydride groups of the microspheres and the amino groups of the enzyme.

### Kinetic characteristics of MIE

MIE and free $\alpha$-amylase were incubated in the PBS buffer solution (pH 8.0) with 1% soluble starch as substrate for 10 min at different temperatures, ranging from 20 to 100°C. Figure 4 indicates that the optimum temperatures of MIE and free enzyme are 80 and 50°C, respectively, and the optimum temperature of MIE is 30°C higher than that of free enzyme, which is highly significant for the application of MIE in industry.

MIE and free $\alpha$-amylase were mixed with 1% (w/v) soluble starch solution incubated in $Na_2HPO_4$–citric acid buffer solutions, with pH values ranging from 4.0 to 10.0. The mixture was oscillated at 50°C for 10 min, after which their activities were determined. Figure 5 indicates that the curve of the relationship of MIE activity versus pH was shifted to the right, and the optimum pH value of MIE was 8.0 and higher compared with that of free $\alpha$-amylase (pH 6.8). The reason for this difference is explained by the nonuniform distribution of hydrogen ions between the microenvironment of MIE and the bulk solution, which could be attributed to the excess carboxyl groups on the surface of the magnetic microspheres.

The rates of MIE and free enzyme reaction were determined at different substrate concentrations, and the double-reciprocal plots of velocity of enzyme reaction versus substrate concentration, according to Lineweaver–Burk method, are given in Figure 6.

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \cdot \frac{1}{S} + \frac{1}{V_{\max}}$$

where $V$ is the reaction rate; $S$ is the substrate concentration; $V_{\max}$ is the maximum reaction rate; and $K_m$ is the kinetic constant.

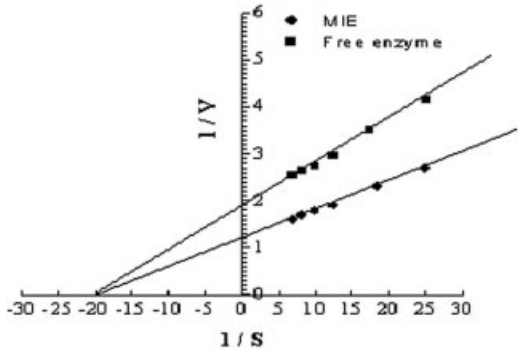


**Figure 6** Double-reciprocal plots of enzyme reaction rate versus substrate concentration measured at 50°C.

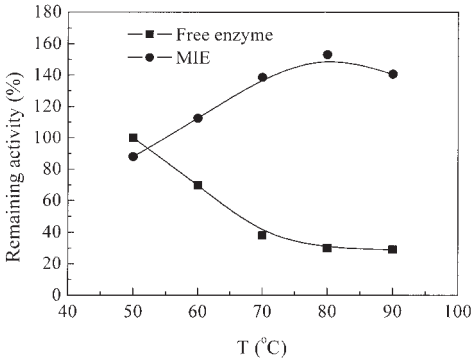


**Figure 7** Remaining activities of free α-amylase and MIE incubated at different temperatures for 2 h without the substrate.

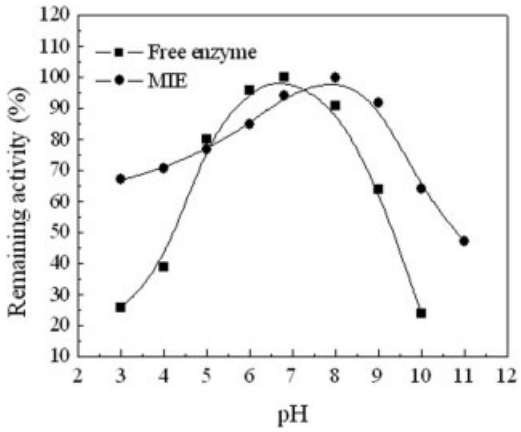


**Figure 8** Remaining activities of free α-amylase and MIE incubated at different pH values for 2 h without the substrate.

The kinetic constants of MIE and free α-amylase ($K_m$) were taken by extrapolation, which were 0.05% ($5 \times 10^{-4}$ g/mL) and 0.049% ($4.9 \times 10^{-4}$ g/mL), respectively. There was hardly any difference between the $K_m$ values of free enzyme and MIE, suggesting that the covalent binding between the α-amylase and magnetic microspheres did not take place at the active sites of the α-amylase. This means that the immobilization hardly affects the affinity between the α-amylase and substrates and changes the mode of action and specificity of α-amylase.[15]

Free α-amylase and MIE were incubated at different temperatures, ranging from 50 to 100°C for 2 h with sodium hydroxide solution (10 g/L) in the absence of a substrate. Both mixtures were then cooled to 25°C by cooling water. Finally, their remaining activities were determined. The curves of the enzyme activity versus temperature are shown in Figure 7, which indicates that the thermal stability of MIE was better than that of free α-amylase. It was evident that high temperature suitably activated the MIE and increased 53% of its activity. This was probably because the configuration of α-amylase was stabilized after α-amylase was immobilized by magnetic composite microspheres.

Free α-amylase and MIE were incubated in different pH buffer solutions in the absence of substrate and then placed in a refrigerator maintained at 4°C for 2 h. Their remaining activities were finally determined after pH values of solution were adjusted to 6.8 and 8.0, respectively. The curves of enzyme remaining activity versus pH are shown in Figure 8, which indicates that pH stability of α-amylase changed after the immobilization. The activity of MIE still maintained about 70% for pH 4.0–10.0, whereas free enzyme was stabilized in a smaller range of 5.0 to 8.0.

Remaining activity values of MIE, preserved in the absence of substrate at 4°C for 20 and 150 days, were 95 and 67%, respectively, whereas that of free enzyme stored at the same conditions for only 15 days was 22%. It was quite evident that MIE was more stable than free enzyme. Operational stability of MIE is very important for estimating application values of MIE. The situation of activity maintenance of MIE after repeated use is shown in Table VII. MIE was separated in a magnetic field (0.5 $Wb/m^2$) after each use.

## CONCLUSION

The nanosized core–shell composite spheres with magnetic inductivity and active anhydride surface groups were prepared. α-Amylase was covalently bound onto the surface of the spheres. The kinetic behaviors of the bound enzyme were investigated. The bound enzyme was separated from the reaction medium by a magnetic field, and then reused repeatedly. The bound enzyme had excellent operational

**TABLE VII**
**Operational Stability of the Magnetic Immobilization Enzyme**

| | MIE cycle number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Activity of MIE ($U/g \times 10^{-4}$) | 9.85 | 9.70 | 9.52 | 9.38 | 9.10 | 8.95 | 8.77 | 8.51 | 8.36 | 8.06 |
| Relative activity (%) | 100 | 98.5 | 96.6 | 95.2 | 92.4 | 90.9 | 89.0 | 86.4 | 84.9 | 81.8 |

stability, the activity of which remained above 80% of the initial state after the tenth use, whereas free enzyme can be used only once.

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