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Preparation and characterization of magnetic Fe_3O_4/CRGO nanocomposites for enzyme immobilization

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Abstract: A one-step hydrothermal procedure to form Fe_3O_4 nanospheres on chemically reduced graphene oxide (CRGO) surfaces was proposed, and these nanocomposites were used as substrates for enzyme immobilization. The as-prepared Fe_3O_4/CRGO nanocomposites were characterized using scanning electron microscopy (SEM), X-ray powder diffraction (XRD), FT-IR and vibrating sample magnetometer. Fe_3O_4 microspheres are randomly distributed on graphene sheets, and the average diameter of Fe_3O_4 microspheres is about 260 nm. Horseradish peroxidase (HRP) was used as a model enzyme to investigate the immobilization activity. The HRP loading was 23.3 mg/g supports and retained 70% of the first use after ten cycles. The catalyzed capability of immobilized HRP was investigated and the immobilized HRP exhibited broader pH stability and excellent reusability. The results show that the Fe_3O_4/CRGO nanocomposites are appropriate for the immobilization of enzyme, and could have potential use in practical.
Key words: chemically reduced graphene oxide (CRGO); Fe_3O_4; horseradish peroxidase (HRP); enzyme immobilization

1 Introduction

Enzymes can catalyze a large number of reactions with high specificity and speed. However, free enzymes are difficult to separate and reuse. To be used as practical biocatalysts, especially industrial biocatalysts, improvement of the activity, stability and recovery of enzymes are necessary [1]. Enzyme immobilization provides a preferable approach to achieve enzyme recovery [2], and establishes a better way to handle the enzyme and eliminate protein contamination of the product conveniently [3].

Plenty of nanoscaled materials, such as metal oxide nanoparticles [2,4,5], carbon nanotubes [6] and graphene oxide nanosheets, have been utilized as substrates for enzyme immobilization. Graphene oxide (GO) has received much attention in biological systems recently, because of its inertia and low toxicity under physiological conditions [7−9]. As for enzyme immobilization, chemically reduced graphene oxide (CRGO) can immobilize enzymes directly and simply through hydrophobic interaction as presented in Refs. [10,11]. The CRGO-enzyme conjugates exhibit high enzyme loading, better stability and higher activity than free enzymes. However, its separation is still a problem.

Fe_3O_4 labeled biological materials can be separated by an external magnetic field rapidly and efficiently, thus it has been widely used in separation and enrichment. Fe_3O_4 nanoparticles with various surface modifications have been used as support materials for the immobilization of enzymes, such as α-Chymotrypsin [12], pectinase [13] and lipase [14]. In many researches, Fe_3O_4 nanoparticles must go through a surface modification procedure before enzyme immobilization, which makes the material making process complex. CUI et al [15] used TEOS and APTES to modify the Fe_3O_4 nanoparticles, then lipase was immobilized by the coupling agent glutaraldehyde. REN et al [16] modified the surfaces of Fe_3O_4 nanoparticles with polydopamine before immobilizing lipase. They utilized the self-polymerization of dopamine to form MNPs with polydopamine coatings and enzyme was covalently linked to the modified surface.

Herein, a simple one-pot strategy was developed to obtain Fe_3O_4/CRGO nanocomposites directly from graphite oxide (GO) and ferric chloride ($FeCl_3·6H_2O$) in the presence of ethylene glycol. Through this in-situ approach, GO was partially reduced by ethylene glycol, at the same time the deposition of Fe_3O_4 nanospheres on

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CRGO sheets occurred. Thus, the superparamagnetism of Fe_3O_4 nanospheres was combined to separate rapidly in an external magnetic field, and the excellent enzyme immobilization ability of CRGO to build a Fe_3O_4/CRGO system to immobilize enzymes. Horseradish peroxidase (HRP) was used as a model enzyme here because it has been widely studied and used in many fields, such as phenol removal [11], biosensor [17], and organic syntheses [18]. This solvothermal method to prepare Fe_3O_4/CRGO nanocomposites is simple and environment friendly, which can be used to immobilize enzyme directly without any further modification. The enzyme loading and catalytic activity were evaluated using the Fe_3O_4/CRGO nanocomposites as substrate.

2 Experimental

2.1 Materials

Graphite oxide was prepared following the procedure in Refs. [19,20]. Ferric chloride hydrate ($FeCl_3·6H_2O$), citric acid monohydrate, urea, phenol (99%), H_2O_2 (30%), 4-aminoantipyrine(4-AAP), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4·12H_2O$) were purchased from Sinopharm Chemical Reagent Company, Shanghai, China. Ethylene glycol was obtained from Linfeng Chemical Reagent Company, Shanghai, China. Horseradish peroxidase (HRP) was purchased from Majorbio Biotech Company, USA. Deionized water was generated through a Millipore Water Purification System. All reagents were used as-received.

2.2 Synthesis of Fe_3O_4/CRGO nanocomposites

A convenient approach was developed to synthesize Fe_3O_4/CRGO nanocomposites by a one-pot solvothermal procedure.

In a typical experiment, exfoliation of graphite oxide was carried out by sonicating 160 mg graphite oxide in 40 mL ethylene glycol for 1 h, after which the suspension was vigorously stirred for 4 h to obtain a clear brown dispersion of graphene oxide. Then, 0.54 g $FeCl_3·6H_2O$, 1.2 g urea, 0.1 g citric acid monohydrate were added directly into the above graphene oxide suspension and stirred for 1 h to obtain a uniform suspension. Urea was introduced as a homogeneous precipitator [21] and citric acid monohydrate as dispersant. The suspension was transferred into a 50 mL Teflon reactor and heated at 200 °C for 8 h. After cooling down to room temperature (~15 °C), the solid product (Fe_3O_4/CRGO) was gained by magnetic separation, and was washed by deionized water and ethanol repeatedly until Cl^{-1} was no longer detected using a $AgNO_3$ solution, then dried in a vacuum at 40 °C for 8 h. The as-gained nanocomposites were stored at 4 °C before use.

2.3 Characterization of Fe_3O_4/CRGO nanocomposites

The morphology of the Fe_3O_4/CRGO nanocomposites was characterized by field emission scanning electron microscopy (SEM, Zeiss ultra 55, Germany) operated at an accelerating voltage of 5.0 kV, and the crystalline structure was recorded by X-ray powder diffraction (XRD, D8-Advance, Bruker, Germany) using Cu K_α radiation (λ=1.54178 Å), with a fixed power source (40.0 kV, 40.0 mA) and an aligned silicon detector. The surface functionalities of the nanocomposites were characterized by a Fourier transform IR spectrometer (FT-IR, EQUINOX 55 spectrometer, Bruker, Germany) in the range of 4000−400 cm^{-1}, and samples were run as KBr pellets. The magnetic properties of the samples were measured using vibrating sample magnetometer (VSM, Lakeshore 736, USA) at 300 K.

2.4 HRP immobilization

HRP was dissolved in phosphate buffer (0.1 mol/L) at pH 7.0. In a typical immobilization experiment, 200 mg Fe_3O_4/CRGO nanocomposites were incubated in 1 mL HRP solution with different concentrations, and the immobilization process was carried out at 4 °C in a shaking air bath for 3 h. The mixture was then separated using a magnet, and the sediments can be fully absorbed in 1 min. The sediments were collected and rinsed three times with phosphate buffer to remove non-specifically adsorbed enzymes. The supernatants were collected to determine the enzyme loading by measuring the amount of the residual enzyme. The sediments were stored at 4 °C for further measurements.

2.5 Enzyme loading determination

The enzyme loading amount onto Fe_3O_4/CRGO nanocomposites was determined by taking away the amount of unbound enzyme in the supernatants from the initial amount of enzyme added. The enzyme concentration in the supernatants was calculated by measuring the initial catalytic reaction rates with substrates through the 4-AAP method. In general, free or immobilized enzyme was added into 1 mL of 0.1 mol/L phosphate buffer (pH 7.0) which contained phenol (0.6 mol/L), H_2O_2 (1.58 mmol/L) as substrates and 4-AAP (14.38 mmol/L) as chromogen, and then reacted at room temperature for 10 s. Then, the catalytic rates were measured by a UV/Vis spectroscopy (Shimadzu UV−2550, Japan) at 495 nm. Free enzymes were used as control.

2.6 Enzyme activity assay

The catalytic activities of free and immobilized

enzyme were characterized using turnover number (K_{cat}) and enzyme efficiency (K_{cat}/K_m), by measuring the UV absorbance of the reaction mixture at 495 nm through 4-AAP method. K_m and K_{cat} values were obtained according to the Lineweaver-Burk equation.

2.7 pH stability and reusability of immobilized enzyme

The effect of pH on the activity of free and immobilized enzyme was investigated at room temperature (~15 °C). The hydrogen chloride and sodium hydroxide were used to adjust the pH of phosphate buffer (0.1 mol/L, pH 7.0) to a set pH. The immobilized HRP and free HRP were washed and immersed into different pH environments and their activities were measured respectively.

The reusability of immobilized enzyme was also measured by the 4-AAP method. After each run, the immobilized HRP was separated magnetically and washed with phosphate buffer (0.1 mol/L, pH 7.0) three times. The activities of enzyme were then measured and the recycled enzyme was repeatedly used in the next reaction. The residue activity of the recycled enzyme was compared with the enzyme activity of the first time (100%).

3 Results and discussion

3.1 Preparation of Fe_3O_4/CRGO nanocomposites

Figure 1 shows the typical morphologies of the nanocomposites. It shows that graphene sheets maintained their layer topography after reaction, and some nanospheres were randomly distributed on their surfaces, which have a uniform diameter distribution (Fig. 1(a)). The enlarged structure demonstrated that the average diameter of the nanospheres is about 260 nm, and they are nanocrystal clusters with spherical uniform size, as shown in Fig. 1(b).

Figure 2(a) shows the FT-IR spectrum of the gained nanocomposites. The peak around 608 cm^{-1} is owing to Fe—O vibration, which confirms the presence of magnetite nanoparticles [22]. The spectrum also shows that there are many oxygen-containing groups on the surface of the nanocomposites. For instance, there is a O—H absorption peak at 3422 cm^{-1} due to the physical adsorption of water, the peaks at 1576 cm^{-1} and 1387 cm^{-1} correspond to the symmetric and antisymmetric stretching of carboxylic groups respectively [21], which may belong to the remaining carboxylic groups on CRGO, or the citrate covalently bonded on the surface of the nanospheres. Peaks at 1174 cm^{-1} and 1095 cm^{-1} are of epoxy stretching vibration and alkoxy stretching, respectively. The intensity of these oxygen-containing groups is small, which indicates that graphene oxide was

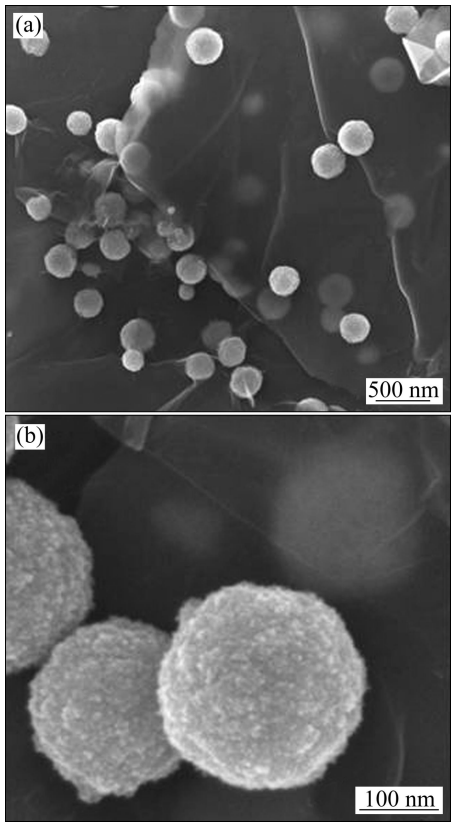

Fig. 1 SEM images (a) and (b) of Fe_3O_4/CRGO nanocomposites

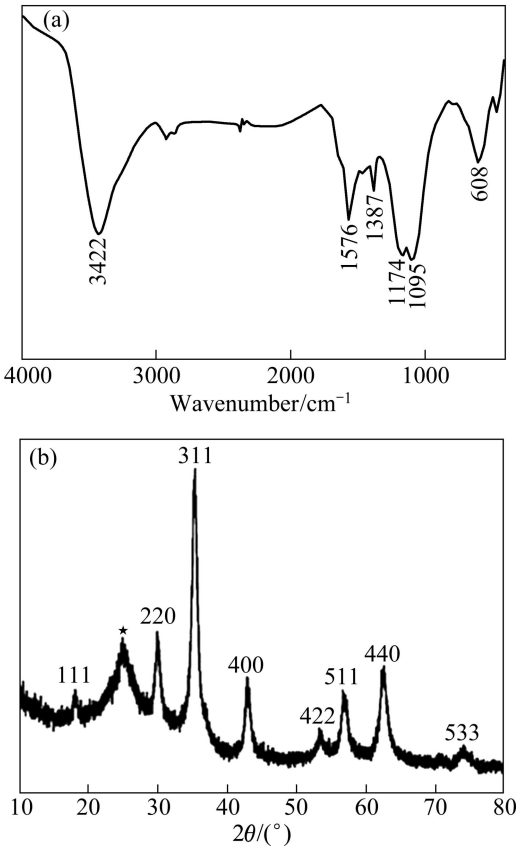

Fig. 2 FT-IR spectra (a) and XRD patterns of Fe_3O_4/CRGO nanocomposites (b)

partially reduced during solvothermal process.

The crystal structure of the gained sample was confirmed by XRD patterns. Figure 2(b) shows the XRD patterns of the nanocomposites. The diffraction peak centered at 2θ=25.1° is assigned to CRGO [23]. The broad peak indicates that graphene sheets are very poorly ordered along the stacking direction [24], which illustrates that the nanocomposites comprised free graphene sheets largely, because the formation of nanospheres prevented the restacking of graphene oxide during the solvothermal process. The remaining diffraction peaks in Fig. 2(b) all match well with crystal planes of Fe_3O_4 (JCPDS 65−3107). Therefore, the sample gained was Fe_3O_4/CRGO nanocomposites.

3.2 HRP immobilization and their activity

In Ref. [10], ZHANG et al [10] showed that horseradish peroxidase (HRP) can be immobilized directly on CRGO through hydrophobic interaction without any pretreatment. The results also indicated that CRGO was a good template to immobilize enzyme, but it could not be easily separated. So, in this work, we combined the magnetism of Fe_3O_4 and the ability of CRGO to directly immobilize enzyme to obtain a better enzyme immobilization system. Therefore, the enzyme loading and the catalytic activity of the loading system were investigated, as shown in Fig. 3.

Figure 3(a) gives the relationship between the amount of HRP loading and the initial addition of HRP when the addition of Fe_3O_4/CRGO nanocomposites is 200 mg/mL. It shows that the amount of HRP loading first linearly increases with the initial free HRP added. When the initial free HRP reaches around 25 mg/g substrates, the loading amount of HRP becomes stable, and gradually reaches maximum loading. Figure 3(b) shows the activity remains of the immobilized HRP as the loading amount increases. When the enzyme loading amount is low, the immobilized HRP shows a relatively high activity above 90%. However, as the loading amount increases, the relative activity decreases and finally reaches a balance state. It is considered that enzymes form an intermolecular steric hindrance as the loading amount gets higher, which restrains the diffusion between substrate and product. The phenomena have also been observed in previous reports [11]. Combining Figs. 3(a) and (b), the highest loading amount of HRP on the Fe_3O_4/CRGO nanocomposites is 23.3 mg/g, and at this time, the immobilized enzyme shows a remained activity at about 55.5%.

The catalytic activity of HRP immobilized on the nanocomposites was assayed using phenol (1−60 mmol/L) as a substrate at room temperature. Soluble HRP was characterized as a control. Their kinetic parameters are obtained from the Lineweaver-Burk equation, and the data were summarized in Table 1. Table 1 shows that when the enzyme loading amount is around 10 mg/g, K_m value of the immobilized HRP is lower than that of free HRP, suggesting that the immobilized HRP had a good affinity to the substrate. Though the K_{cat}/K_m valve values are slightly lower after immobilization, the catalyst activity of immobilized HRP still keeps at a relatively high level. This data show that the Fe_3O_4/CRGO nanocomposites are favorable for enzyme immobilization.

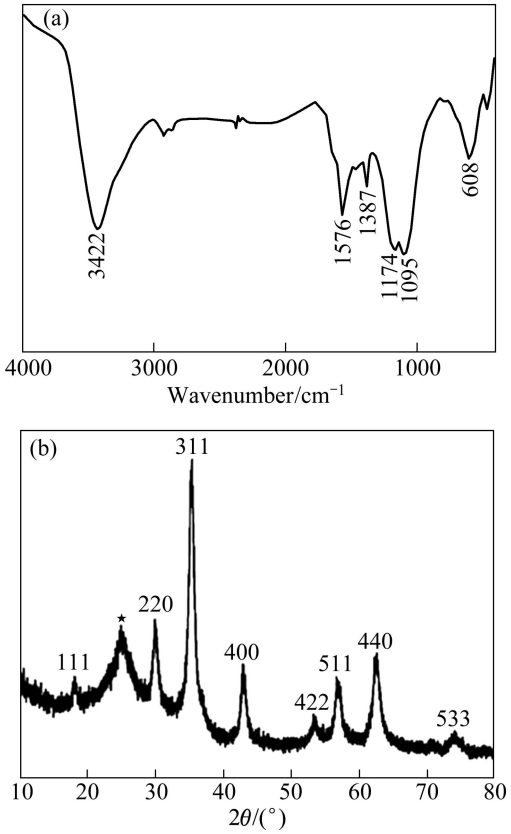

Fig. 3 Enzyme loading and catalytic activity of loading system: (a) HRP loading on Fe_3O_4/CRGO nanocomposites (The amount of Fe_3O_4/CRGO nanocomposites used is 200 mg/mL); (b) Activity remains of immobilized HRP with loading amount

Table 1 Kinetic properties of HRP immobilized on Fe_3O_4/CRGO nanocomposites

Type	Enzyme loading/ ($mg \cdot g^{-1}$)	K_m/ ($mmol \cdot L^{-1}$)	Kcat/s^{-1}	$\frac{K_{cat}}{K_m}$/ ($L \cdot mmol^{-1} \cdot s^{-1}$)
Free HRP	−	10.25±4.36	285.89±29.3	27.89±0.34
Fe_3O_4/CRGO immobilized HRP	10±0.2	8.81±1.34	189.35±3.00	21.49±1.56

3.3 Immobilization time

To better control the immobilization procedure, it is important to choose the optimum reaction time. Figure 4 illustrates the relative immobilizing efficiency of HRP at

different reaction time. The immobilized efficiency reaches 91% after 15 min, and arrives at maximum loading after proceeding for 140 min. So, the reaction time was set at 3 h to make sure fully immobilization, which is very short compared with some reported data. For instance, JUANG et al [25] immobilized enzyme on chemically modified chitosan beads for 18 h, and WEI et al [26] used Fe_3O_4/nano-Au to immobilize HRP overnight. Our work presented here shows short immobilization time and high immobilization efficiency, which is very convenient for practical use.

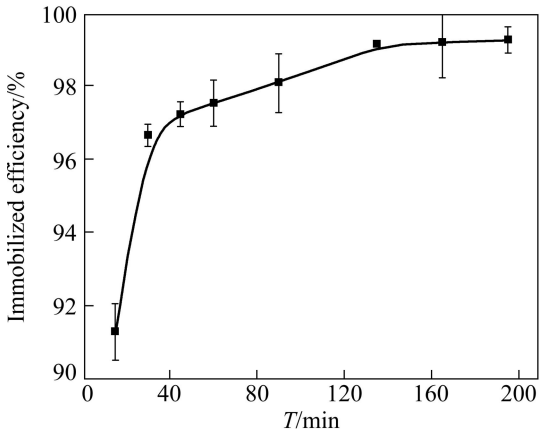

Fig. 4 Dependence of time on immobilization of HRP

3.4 Magnetic properties

The magnetic properties of Fe_3O_4/CRGO nanocomposites alone and after immobilized HRP were investigated using a vibrating sample magnetometer (VSM). For immobilized HRP, the sediments were separated by a magnet, washed several times with buffer, and dried in a vacuum at 40 °C for 4 h. Then, they were used to test the magnetic property. Figure 5 shows the magnetization hysteresis loops of Fe_3O_4/CRGO nanocomposites and Fe_3O_4/CRGO nanocomposites after immobilizing HRP at an applied magnetic field sweeping from −10 to 10 at 300 K. Both of the magnetic hysteresis loops are S-like curves, and no remanence was detected, indicating that they both have a superparamagnetic property, which is useful for separating the nanocomposites from the solution directly and rapidly using an external magnetic field. After immobilizing HRP, the saturation magnetization (M_s) of Fe_3O_4/CRGO nanocomposites decreased from 27.8 A·m^2/kg to 20.0 A·m^2/kg. This implied that HRP was successfully immobilized on the Fe_3O_4/CRGO nanocomposites.

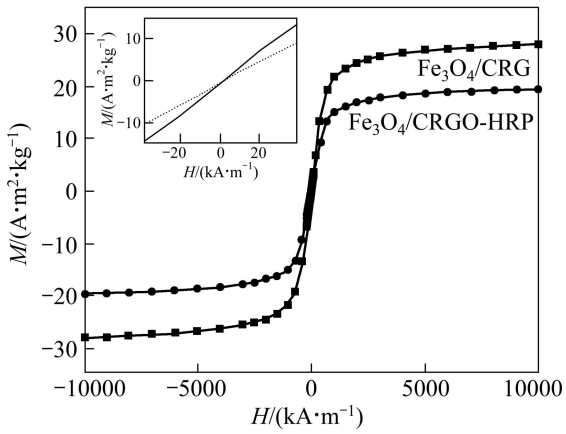

Fig. 5 Hysteresis loops of Fe_3O_4/CRGO nanocomposites before and after immobilize HRP

3.5 Dependence of pH on activity of free and immobilized enzymes

The effect of pH on the activity of free and immobilized enzymes was evaluated in the pH range of 4−9 at room temperature. Typical results are illustrated in Fig. 6. The optimal pH value is observed around pH 7 for both free and immobilized HRPs. Its catalytic performance is not generally affected too much by the immobilization. However, the immobilized HRP retained about 48% activity at pH 4, while for free HRP only 39% activity was left at the same pH. The immobilized HRP put up a better adaptability in a wider pH region.

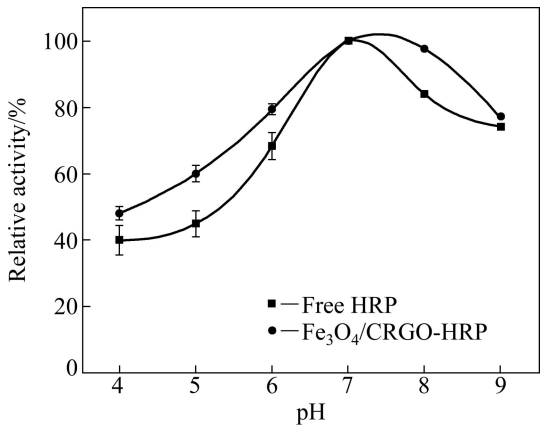

Fig. 6 Effect of pH on activity of free and immobilized HRP

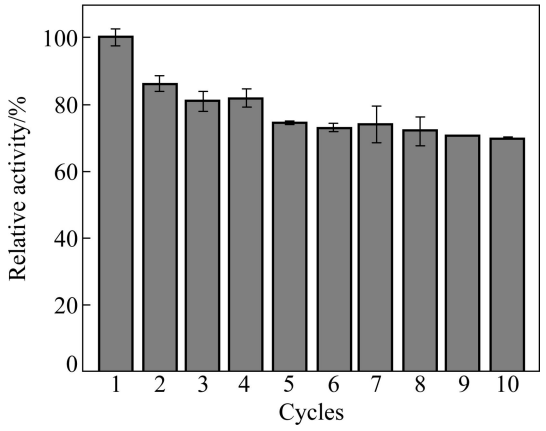

Fig. 7 Reusability of immobilized HRP (b)

3.6 Reusability assay

The reusability of the immobilized enzyme is a crucial parameter for potential practical applications, especially the reuse of costly enzymes. To evaluate the catalyst reusability, the immobilized enzyme was washed with 0.1 mol/L phosphate buffer (pH 7.0) three times after every run and reintroduced into a fresh reaction mixture to react at room temperature for 3 min. In our previous work, when HRP was immobilized on GO alone, the immobilized HRP only retained about 25% of its initial activity after sever cycles [11]. In this work, the reusability was improved greatly after the introduction of Fe_3O_4. As shown in Fig. 7, the immobilized HRP retained about 70% of the first use after ten cycles, which could be attributed to the strong separation ability of this system.

3.7 Immobilization of other enzymes

The Fe_3O_4/CRGO nanocomposites were also used to immobilize other enzymes. For instance, the maximum loading of lysozyme, glucose oxidase and BSA is 23.45, 60.0 and 57.5 mg/g, respectively, and they all show good activity and reusability. This implied the Fe_3O_4/CRGO nanocomposites prepared here are a simple, rapid and convenient template for enzymes immobilization.

4 Conclusions

1) Fe_3O_4/CRGO nanocomposites were obtained using a simple one-pot strategy from graphite oxide (GO) and ferric chloride ($FeCl_3·6H_2O$) in the presence of ethylene glycol.

2) Fe_3O_4 uniformly distributes on the surface of CRGO, and the nanocomposites inherit the strong separation ability of Fe_3O_4 and immobilization ability of CRGO.

3) The maximum HRP loading on the Fe_3O_4/CRGO nanocomposites is 23.3 mg/g and the immobilized HRP has a good affinity to the substrate. The catalyzed capability of immobilized HRP maintains high, and the immobilization time is very short.

4) The immobilized HRP exhibits better resistance to pH, and shows excellent reusability. These indicate the immobilization of enzyme directly onto Fe_3O_4/CRGO nanocomposites is facile and efficient, and would make the use of expensive enzymes economically viable.

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磁性 Fe_3O_4/CRGO 复合物的制备及其酶的固定化

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摘 要：四氧化三铁(Fe_3O_4)纳米颗粒可通过水热法一步复合在化学还原氧化石墨烯(CRGO)表面，得到的复合物 Fe_3O_4/CRGO 用于固载酶。通过扫描电子显微镜(SEM)、X 线衍射仪、红外光谱仪及振动样品磁强计对 Fe_3O_4/CRGO 的表观形貌及结构进行测试和表征。结果显示：Fe_3O_4 纳米颗粒随机分散在 CRGO 表面，Fe_3O_4 纳米颗粒的平均直径约 260 nm。利用辣根过氧化物酶(HRP)研究 Fe_3O_4/CRGO 复合物的酶固定化性能。HRP 的饱和固载量为 23.3 mg/g，固定化后的酶在重复利用 10 次后依然保留原始酶活性的 70%。固载后的 HRP 显示出更宽的 pH 耐受范围及很好的重复利用性，表示 Fe_3O_4/CRGO 复合物可作为固载酶的良好基底并具有实际应用价值。

关键词：化学还原氧化石墨烯；四氧化三铁纳米颗粒；辣根过氧化物酶；酶固载

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