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Enhanced Stability of Glucose Dehydrogenase by Immobilization onto Graphene Oxide Magnetic Nanoparticles

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Published Online: Nov 04, 2020

eBook: Importance & Applications of Nanotechnology

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Glucose dehydrogenase; Immobilization; Graphene oxide; Magnetic nanoparticles.

Abstract

Pesticides are chemical compounds that are employed to eliminate, repel, or control certain forms of plant or animal life that are deemed as pests. Usage of pesticides enhances the crop yield, but they are potentially hazardous to humans, animals, and the environment. Toxicity of pesticides including insecticides, fungicides can result in diseases such as nausea, dizziness, vomiting, abdominal muscle cramps, muscle twitching, tremors, weakness or loss of coordination; making their detection the need of the hour. Conventional techniques including mass spectrometry and gas chromatography suffer from limitations such as operational complexities, requirement of sophisticated instruments and issues related to portability. High sensitivity and stability of nanomaterials based biosensors makes them suitable candidates for on-site detection of pesticides. This report reviews different biosensors that have been employed for detection of pesticides, laying down their specific limitations. It also discusses the need to develop alternate nanoparticle based sensors with high specificity, sensitivity and capability of on-site analysis.

Introduction

Glucose dehydrogenase (GDH, E.C. 1.1.1.47) belongs to the family of short-chain dehydrogenases, which can specifically convert glucose into gluconate-δ-lactone [1]. In industry, it is a rate-limitation enzyme employed for production of 2-keto-D-gluconic acid from glucose [2]. Because of the important nature, it has been widely used in glucose measuring, especially in blood glucose detection. This application further makes it as an important catalyst for fabrication of biosensor in clinical diagnosis. Exception of this, the enzyme can be used for recycle nicotinamide cofactor in the enzymatic biotransformation catalyzed by oxidoreductase which account for around 30% of the all known enzymes [3]. The crucial role of oxidoreductase in the preparation of enantiomers, fine chemicals and pharmaceuticals attracts the increasing attention of GDH because it could significantly reduce the cost due to the cofactor regeneration. Thus, GDH has great potential applications in chemical and pharmaceutical industry, clinic therapy, and medicinal diagnosis.

Large scale applications of free enzymes will result high cost because of the low stability and difficulty in recycle or recovery. To overcome these drawbacks, the enzyme has to be limited in fixed space with physical or chemical methods for recycle using. These methods named immobilization include adsorption, entrapment and covalent attachment [4]. Adsorption is the simplest method [5] in which the enzyme and carrier could be combined together through ion bonds, hydrophobic interaction or Van der Waals' force, but it is not stable because of the weak interactions. Another immobilization method called entrapment also has the drawback like transfer limitations due to the entrapment affect the entry and release of the substrate and product [6]. Compared with these two methods, covalent binding is more interesting for the strong covalent bonds formed and highly stable immobilized enzyme could be obtained [7]. To increase the stability and recyclable use of GDH, many researches have been performed. Karagoz et al [2] used a hierar-

chically porous silica support (MM-SBA-15) to immobilize GDH, but there was only ~10% activity remained after 10 cycles. Five agaroses with different functional groups were selected to immobilize GDH and the immobilized enzyme could be coupled with the immobilized cytochrome P450 for cofactor regeneration [8]. In another report, mesoporous SBA 15 silica was used to immobilize GDH and xylose dehydrogenase to produce gluconic acid and xylonic acid [9]. For efficient immobilization of proteins or enzymes, carrier with abundant functional groups and large surface area is a good option.

Graphene Oxide (GO), has strong mechanical rigidity and good biocompatibility [10]. GO is an ideal carrier for immobilization of proteins or enzymes because of its rich carboxyl functional groups, high water solubility and very large special surface area [11]. In recent years, due to its unique physical and electronic properties, graphene has been applied in industry [3,12-15]. Magnetic nanoparticles with core-shell structure have been widely used in the fields of biological separation, protein adsorption, enzyme immobilization and cell immobilization. From this point of view, magnetic nanocomposites are easy to recover and are ideal carriers for immobilized enzymes.

In the present work, Fe_3O_4@SiO_2-GO composites were prepared for immobilization of GDH. The immobilization conditions were optimized to get high yield and activity recovery. The immobilized enzyme was characterized and compared with the free one to check the enhanced thermal stability.

Materials and methods

Materials

Glucose, tris (hydroxymethyl) aminomethane (Tris), sodium phosphate dibasic dodecahydrate, sodium phosphate monobasic dihydrate, NAD^+, carbazole, glycine, citric acid, sodium citrate were purchased from Aladdin (Shanghai, China). Isopropyl-β-D-thiogalactoside (IPTG) and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all reagents and chemicals were analytical or biological grade.

Enzyme assay

The activity of GDH was measured based on the amount of glucose consumed in the enzymatic reactions at specific conditions [16]. Typically, for the free enzyme, the total reaction volume of 1 mL including a final concentration of 10 mM substrate, 10 mM NAD and 0.2 ml enzyme was incubated for 5 minutes at 50°C. The reaction was terminated by placing the reaction tube in a boiling water bath for 5 minutes. Withdraw 0.2 mL of the reaction solution, added 1 mL of sulfuric acid and 0.05 mL of 0.1% carbazole to react in a boiling water bath for 20 min. Then the absorbance at OD_{530} was measured to calculate the remaining amount of glucose and the enzyme activity. For the immobilized enzyme, the same conditions were used exception of using 10 mg the immobilized GDH to replace the free enzyme. After centrifugation, the glucose concentration of the supernatant was measured to calculate the activity.

Preparation of GDH

The recombinant *Escherichia coli* harboring GDH was obtained as previous report [17]. The cells were cultured with induction using 0.1 mM of isopropyl-β-D- thiogalactopyranoside (IPTG) and harvested by centrifugation at 10,000×g for 15 min at 4°C, rinsed using phosphate buffered saline. To get the purified GDH, cell pellets were resuspended in 100 mM Tris–HCl buffer (pH 8). The cell disruption was carried out by sonication for 5 min at 4°C, and the lysate was centrifuged at 10,000×g for 15 min at 4°C to remove the cell debris. The resulting crude extracts were retained for purification. The purification using Ni-NTA column was performed according to the manufacturer's protocol (Transgene, China).

Preparation of the carrier for the immobilization

The preparation of the carrier was performed according to the previous work [11]. Fe_3O_4 nanoparticles were synthesized with coprecipitation method. Briefly, 60 mmol $FeCl_3 \bullet 6H_2O$ and 30 mmol $FeCl_2 \bullet 4H_2O$ were dissolved in 75 ml 0.5 M HCl, then added them dropwise to a flask containing 750 ml 1.5 M NaOH solution with vigorous stirring under a nitrogen flow. The Fe_3O_4 nanoparticles were obtained and washed several times with deionized water and dried 10 h at 50°C under vacuum. Silica-coating on the Fe_3O_4 nanoparticles were performed. Suspended 3 g of Fe_3O_4 nanoparticles in 650 ml deionized water, and then added 65 ml of 2.0 M sodium silicate dropwise to flask with stirring in a water bath 80°C under the protection of nitrogen flow. Adjusted the pH of suspension to 6.0 with 2 M HCl. Continous 3 h stirring, collected the product by magnetic separation and washed several times with deionized water, then drying 12 h at 50°C under vacuum. Then suspended 1 g of Fe_3O_4@SiO_2 in 35 ml of anhydrous ethanol, added 15 ml of ammonium hydroxide and 3 ml of APTMS in the suspension. Under a nitrogen flow, the reaction was continued 5 h at 25°C with vigorous stirring. The Fe_3O_4@SiO_2-NH_2 nanoparticles were obtained by magnetic separation and washed with anhydrous ethanol and distilled water several times. To prepare Fe_3O_4@SiO_2-GO composites, dispersed GO (100 mg) in 300 ml water and sonicated 3 h, added 50 mg EDC and 40 mg NHS into the solution. The mixture was stirred 2 h and sonicated another 1 h. Then 100 mg Fe_3O_4@SiO_2-NH_2 was added into the suspension and the mixture was sonicated for another 1 h. The reaction was kept 1 h at 80°C under stirring. The obtained solid materials were referred to Fe_3O_4@SiO_2-GO composites, and collected by magnetic separation and washed several times with distilled water.

Immobilization of GDH

10 mg Fe_3O_4@SiO_2-GO magnetic nanocomposite was dispersed in 1 mL PBS (0.1 M, pH 7.0), then 20 µL 25% (v/v) glutaraldehyde solution was added to activate the surface functional groups at room temperature for 10 h. The obtained nanocomposites were washed 3 times with PBS (0.1 M, pH 7.0) containing 0.1 M NaCl. Subsequently, 10 mg of the magnetic nanocomposites were added to 0.2 ml GDH solution and mixed at room temperature for 12 hours for the immobilization. The immobilized GDH was collected by centrifugation and washed with 0.1 M phosphate buffer (pH 7.0). The immobilization yield was calculated by measuring the protein concentration of the supernatant, and the activity recovery of the immobilized GDH was measured.

Immobilized yield = $[(P_0-P_1)/P_0]\times100\%$;

Activity recovery = $(A_1/A_0)\times100\%$;

Where A_1 is the total activity of the immobilized enzyme, A_0 is the total activity of the free enzyme; P_0 is the total protein content of added enzyme; P_1 is the total protein concentration of the supernatant.

Effect of the enzyme/support ratio, immobilization time, temperature and pH for immobilization of GDH

Suspended 10 mg of carrier in 1mL PBS, then added 100 uL

of 25% glutaraldehyde to the mixture for of the functionalization groups on the surface. Then wash the obtained functionalized carrier several times with PBS containing NaCl (0.1M). For the immobilization of the GDH, disperse the functionalized carrier in solution, add 0.2 mL of GDH into the mixture and gently mixed 12 hours in the pH range of 5.0-9.0 and temperature of 4-40°C. After the immobilization, the mixture was centrifuged at 8,000 rpm for 3 minutes. Wash the immobilized enzyme with PBS containing NaCl (0.1M) several times. The activity of the immobilized enzyme was measured with the enzyme assay. The protein concentration of the supernatant was also determined. Every experiment was performed in triplicate and the standard deviations were calculated.

Characterization of the immobilized GDH

The effects of temperature and pH on the free and immobilized GDH activity were evaluated in the temperature range from 30 to 80°C and pH range from 5.0 to 9.0. The activity of immobilized GDH was measured in 0.1 M citrate buffer (pH 5.0 and 6.0), phosphate buffer (pH 6.0, 7.0 and 8.0) and Tris-HCl buffer (pH 8.0 and 9.0). The thermostability of the enzyme was investigated by incubating the enzyme at 37 and 50°C for different times of 5 to 120 minutes. The reusability of the immobilized GDH was examined under the same condition as described in the activity measurement. The immobilized enzyme was reused 10 times at 50°C. The activity obtained each run was compared to the initial activity to calculate the relative activity.

Result and discussion

The carrier preparation and Characterization

All the FT-IR spectra of the carrier materials are presented in **Figure 1**. The peak around 582 cm^{-1} of Fig. 1a corresponds to the Fe-O vibration at magnetite phase [18]. And the peaks at 470, 800 and 1107 cm^{-1} of Fig. 1b are ascribed to the Si-O vibration because of the silica coating [12]. The weak peak at 3400 cm^{-1} in Fig. 1c was observed due to the -NH_2 vibration for the modification of APTMS which proved that the functional -NH_2 group on the surface of nanoparticles was successfully grafted [11]. Compared with spectra of GO in Fig. 4d, a new peak appears at 1607 cm^{-1} of Fe_3O_4@SiO_2-GO corresponding to C=O characteristic stretching band of the amide group. The peak at 470 and 1107 cm^{-1} proved that the GO was covalently bonded to Fe_3O_4@SiO_2 nanoparticles[10].

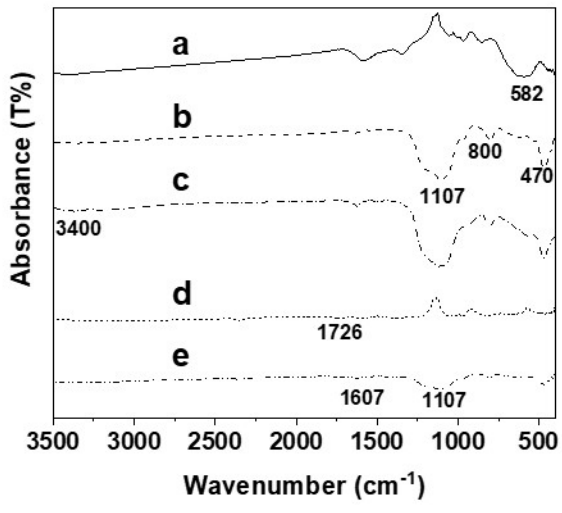

Figure 1: FT-IR spectra of (a) Fe_3O_4, (b) Fe_3O_4@SiO_2, (c) Fe_3O_4@SiO_2-NH_2, (d) GO, (e) Fe_3O_4@SiO_2-GO.

Effects of enzyme/support ratio, temperature, pH and immobilization time on the immobilization of GDH

In the immobilization process, the factors affecting the immobilization include the enzyme/support ratio, temperature, pH and immobilization time. To obtain high immobilization efficiency, these factors were investigated to check their influence on the immobilization.

As shown in **Figure 2**, the immobilization yield decreased from 98% to 75% when the enzyme/support ratio increased from 4 to 24 mg/g. With the enzyme concentration increased in the reaction mixture, the competition of enzyme molecules on the surface of support becomes more intense which leads to the stacking effect on the support [13]. Thus, the immobilization yield decreased with the increase of enzyme/support ratio. The highest activity recovery of 93% could be achieved when the enzyme/support ratio was 12 mg/g. When the ratio continued increasing, the activity recovery decreased to 70% when it was 24 mg/g. Apparently, the immobilized GDH couldn't present the highest activity because of the stacking effect or competition.

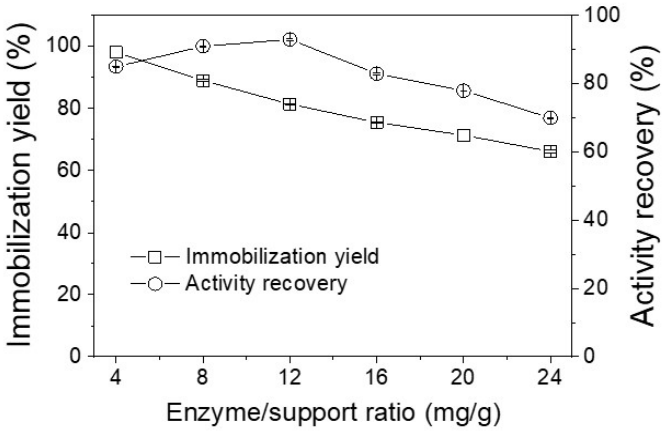

Figure 2: Effect of the enzyme/support ratio on the immobilization of GDH.

Effect of the immobilization time on the immobilization is shown in **Figure 3**. The time profile of the immobilization in the figures showed that the immobilization of GDH onto magnetic GO nanoparticles was quick. After 1-hour immobilization, around ~60% immobilization yield and 70% activity recovery were obtained. And both the immobilization yield and activity recovery reached the highest value (91% and 93%) after 12 hours immobilization. According to the previous research, the immobilization of cellulase onto GO carriers was very fast, and it finished in 10 minutes and the immobilization yield and activity recovery were higher than 90% [7]. In this work, the lysine residues on the enzyme surface would be the main active groups for the immobilization. The reaction between enzyme and support was finished in 12 hours.

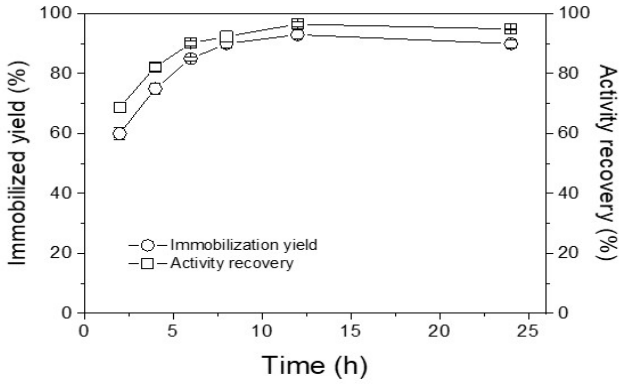

Figure 3: Time profile for the immobilization of GDH.

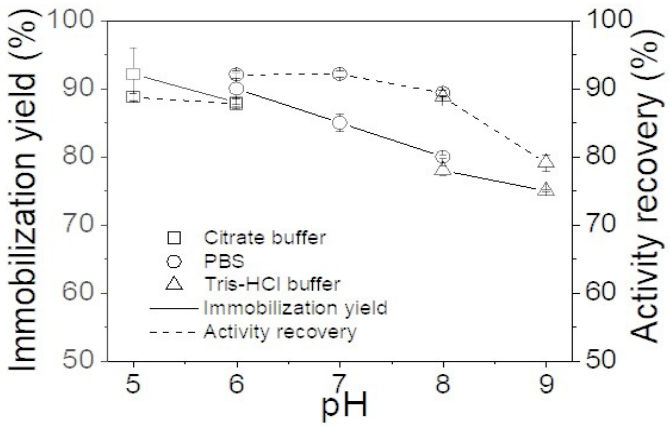

Figure 4: The effect of pH on the immobilization of GDH.

The effect of pH on the immobilization is shown in **Figure 4**. The immobilization yield of 93.8% was obtained at pH 7.0, and the highest activity recovery of 92% was also achieved at pH 7.0. With the pH increased from 5.0 to 9.0, both the immobilization yield and activity recovery decreased. While the immobilization yield decreased to 75% when pH was 9.0. Generally, pH is an important factor influence the steric structure of protein and the deprotonation of the amino acid residues of the enzyme. The GDH has an isoelectric point of 5.4 which means the enzyme has negative charges in the immobilization pH range. This explained that the highest yield and activity recovery were obtained at pH 5.0 which is close to the isoelectronic point because the enzyme has the lowest solubility around the isoelectronic point. The enzyme activity could be inhibited in citrate and Tris buffer, so there were 5-10% activity recovery lost than that in phosphate buffer. Consideration of the catalytic pH and the stability of the enzyme. Phosphate buffer (6.0) was chosen as the immobilization solution for the next experiments.

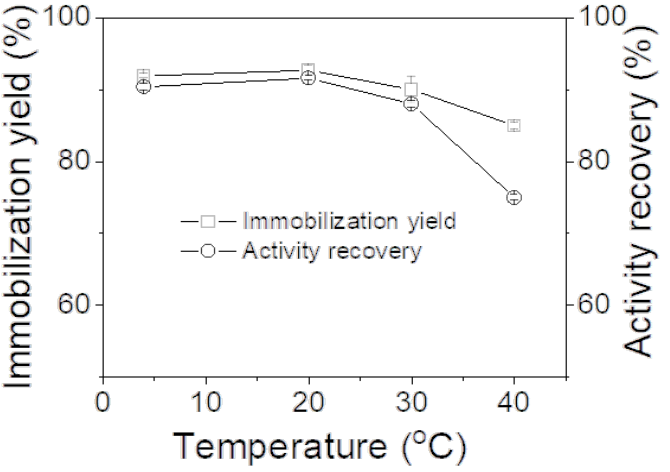

Figure 5: The effect of temperature on the immobilization of GDH.

Figure 5 shows the effect of temperature on the immobilization of GDH onto Fe_3O_4@SiO_2-GO nanoparticles. We checked the effect of temperature in a range from 4 to 20°C on the immobilization of GDH. It was found that with the increasing temperature, the immobilized yield gradually increased from 91.9% to 94.1%. And the activity recovery had the similar trend at this temperature range. At 20°C, the activity recovery and the immobilization yield were 91.8% and 92.8%, respectively. When the temperature was continuously increased to 40°C, both the immobilization yield and activity recovery decreased to 84.6% and 75.3%, respectively. The temperature is crucial for the enzyme immobilization because it affects the interactions between carriers and enzyme protein [19]. Higher temperature will accelerate the thermal motions of enzyme and provide enough energy for the immobilization [20]. But higher temperature also could induce the deactivation or denaturation of the protein [7]. This could explain that the immobilization yield was higher than activity recovery. By balancing these two sides, a proper temperature below 20°C will benefit the immobilization.

Characterization of the immobilized GDH

To compare the difference between the free and immobilized GDH, characteristics of pH and temperature profile was checked. The effect of temperature on the activity of GDH was measured from 4 to 70°C. As shown in **Figure 6**, both the free and immobilized GDH have the optimal temperature at 50°C. When the temperature was increased from 20 to 50°C, there was about 20% relative activity improved. After that, the relative activity of free and immobilized GDH decreased to 90% when the temperature was increased to 70°C. These results indicated that the free and immobilized enzyme can be used in a broad range temperature from 30 to 70°C. This could make it more practical in clinic or diagnosis.

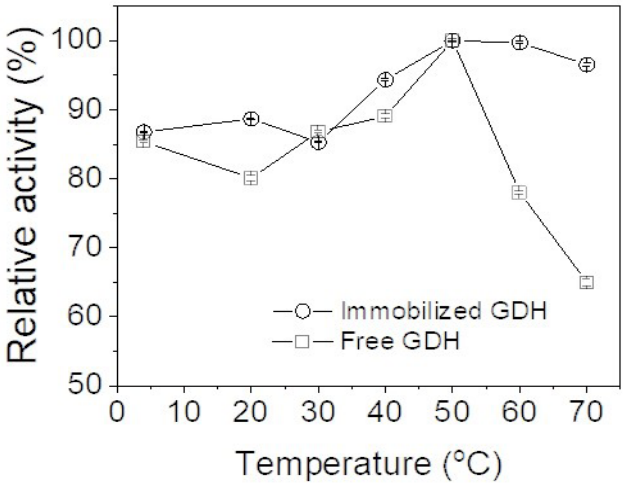

Figure 6: Effect of temperature on the activity of free and immobilized GDH.

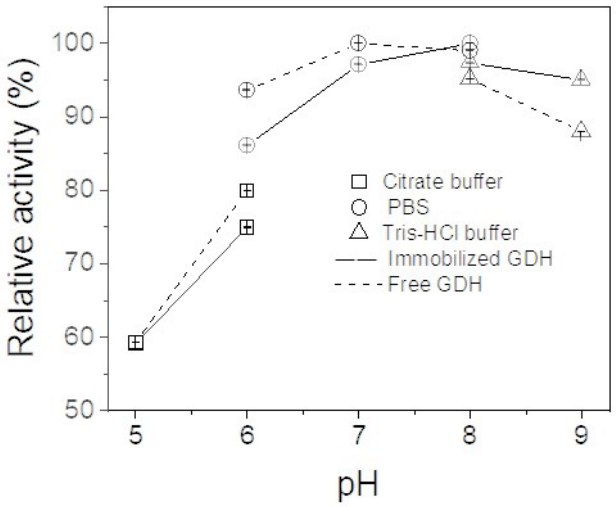

Figure 7: Effect of pH on the activity of the free and immobilized GDH.

Figure 7 shows the effect of pH on the free and immobilized GDH activity. The results showed that the free and immobilized GDH has relative low activity (~60-80%) in citrate buffer (pH 5-6). In phosphate buffer (pH 6-8), both enzymes had relative high activity (>85%), and has the highest activity at pH 8.0 which is the optimal pH for the immobilized GDH. Compared with the free enzyme, the optimal pH was shifted from 7.0 to 8.0. This will favor the enzyme because it could have high activity at pH 8.0. The optimal pH shift could be that the immobilization increased tolerance of the enzyme to changes in environmental pH [21].

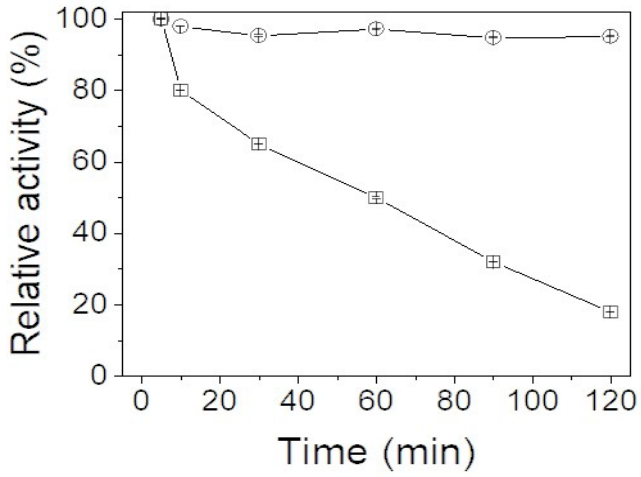

Figure 8: The thermal stability of the free and immobilized GDH.

The thermal stability of immobilized GDH was investigated by incubating them at 50°C. As shown in Figure 8, the immobilized GDH retained more than 95% of its original activity after heat treatment 3 h at 50°C, but the free enzyme only remained 18% of the initial activity. The immobilized GDH has a $t_{1/2}$ of 398 min, while that of the free enzyme was 58 min, and the thermal stability was improved 6.9-fold after the immobilization. The increase in thermal stability of the immobilized GDH can be attributed to the conformational stability of the enzyme by covalent immobilization between GDH and the support [22]. The abundant groups on the surface of carrier and the rigidity of GO nanoparticles might have contributions on the improved thermal stability.

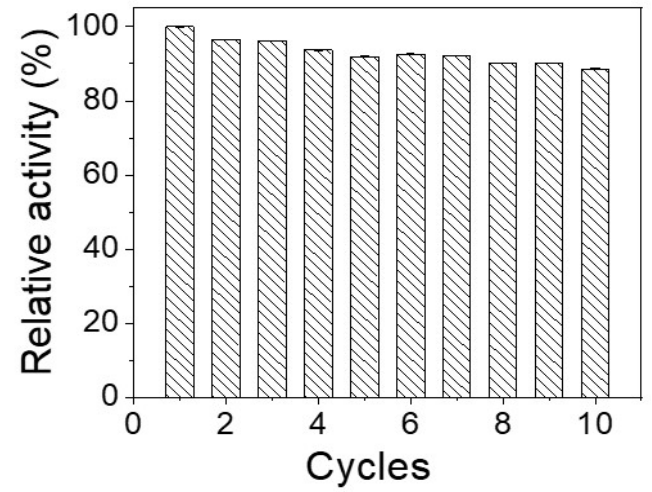

Figure 9: The reusability of the immobilized GDH.

The reusability of immobilized GDH was also checked through repeated cycles. According to **Figure 9**, the GDH showed a preferable stability by immobilization onto the surface of Fe_3O_4@SiO_2-GO. It reserved almost 88% of its initial activity after 10 cycles. The decrease in activity may be due to enzyme inactivation on the carrier and denaturation of the enzyme reuse. Although the immobilized GDH activity is slightly lowered, the immobilized GDH has good durability and can be easily recovered by magnetic separation. This indicates that the magnetic Fe_3O_4@SiO_2-GO nanoparticles can be used for the immobilization of the enzyme.

Conclusions

In summary, magnetic Fe_3O_4@SiO_2-GO nanoparticles were prepared and used for covalent immobilization of GDH. The highest immobilized yield and activity recovery were 92% and 91%, respectively. In addition, good reusability and thermal stability were obtained, which makes them more attractive for industrial applications. The immobilized GDH showed 6.9-folds higher stability than that of the free enzyme. These results indicate that the immobilized enzyme has good industrial applications.

Acknowledgement

This work was supported by Chinese college student's innovation training project (No. 201912026014).

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