

Sensitive fluorescent probes for determination of hydrogen peroxide and glucose based on enzyme-immobilized magnetite/silica nanoparticles

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Abstract Sensitive fluorescent probes for the determination of hydrogen peroxide and glucose were developed by immobilizing enzyme horseradish peroxidase (HRP) on $\text{Fe}_3\text{O}_4/\text{SiO}_2$ magnetic core-shell nanoparticles in the presence of glutaraldehyde. Besides its excellent catalytic activity, the immobilized enzyme could be easily and completely recovered by a magnetic separation, and the recovered HRP-immobilized $\text{Fe}_3\text{O}_4/\text{SiO}_2$ nanoparticles were able to be used repeatedly as catalysts without deactivation. The HRP-immobilized nanoparticles were able to activate hydrogen peroxide (H_2O_2), which oxidized non-fluorescent 3-(4-hydroxyphenyl)propionic acid to a fluorescent product with an emission maximum at 409 nm. Under optimized conditions, a linear calibration curve was obtained over the H_2O_2 concentrations ranging from 5.0×10^{-9} to 1.0×10^{-5} mol L^{-1} , with a detection limit of 2.1×10^{-9} mol L^{-1} . By simultaneously using glucose oxidase and HRP-immobilized $\text{Fe}_3\text{O}_4/\text{SiO}_2$ nanoparticles, a sensitive and selective analytical method for the glucose detection was established. The fluorescence intensity of the product responded well linearly to glucose concentration in the range from 5.0×10^{-8} to 5.0×10^{-5} mol L^{-1} with a detection limit of 1.8×10^{-8} mol L^{-1} . The proposed method was successfully applied for the determination of glucose in human serum sample.

Keywords $\text{Fe}_3\text{O}_4/\text{SiO}_2$ magnetic nanoparticles · Immobilized enzyme · Hydrogen peroxide · Glucose · Fluorometry · Catalysts · Clinical/biomedical analysis

Introduction

Glucose is the main source of energy for living bodies. Its level in the blood often reflects changes in glucose metabolism in vivo situation, indicating health conditions. Therefore, accurate determination of glucose is very important to clinical treatments. At the present time, the determination of glucose is generally based on the monitoring of hydrogen peroxide (H_2O_2) produced stoichiometrically during the oxidation of glucose by dissolved oxygen in the presence of glucose oxidase (GOx) [1]. Thus, the accurate determination of glucose is dependent the accurate determination of H_2O_2 . In fact, the determination of trace H_2O_2 itself is also very important and necessary in environmental and clinical analysis. There are many methods available for the quantification of H_2O_2 such as spectrofluorometric [2–5], spectrophotometric [6–8], electrochemical [9, 10], and chemiluminescent [11–13] methods, most of which are based on the enzyme catalytic reaction because of their unusual sensitivity and selectivity. Horseradish peroxidase (HRP) is the most commonly used catalyst in the enzymatic reaction [14, 15]. However, the application of the free HRP may be limited by its high cost due to the difficulty of its recovery and reuse. These deficiencies can be avoided by immobilizing the enzyme [16]. The immobilized enzymes are required to retain the same functionality and have merits of better storage stability [17], thermal stability, and operational facility in comparison with free enzymes in solution.

Fe_3O_4 magnetic nanoparticles are known to be biocompatible, displaying no hemolytic activity or genotoxicity with superparamagnetic properties [18], having potential applications in separation of biochemical products [19], magnetic resonance imaging [20], targeted drug delivery [21], biosensing [22], and so on. The magnetic nanoparticles may aggregate because of anisotropic dipolar

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attraction and undergo biodegradation when they are directly exposed to biological environment. This problem can be solved by encapsulating the nanoparticles in silica shell as the stabilizer, which prevents direct contact between the nanoparticles and modifies inherently their surface to link bioconjugators with interested biofunctionalities [23]. In addition, over the past few years, silica materials turn out to be a very good solid support for enzyme immobilization with good cost-effectiveness, biocompatibility, and stability in the biosystems. Yan and coworkers [24] synthesized SiO₂ particles as enzyme immobilization carriers to fabricate glucose biosensors. Yang [25] prepared silica-coated magnetite sol-gel nanomaterials by a reverse-micelle technique to immobilize gentamicin for biological analysis. Enzyme immobilization materials had better be magnetic, which allows them to be easily manipulated and recovered. Compared with the reverse microemulsion method, the Stöber method [26] is relatively simple and avoids the use of surfactants. Zhang [27] developed a hydroquinone amperometric biosensor based on the immobilization of laccase on the surface of modified magnetic core-shell (Fe₃O₄/SiO₂) nanoparticles, which synthesize by the Stöber method.

To our best knowledge, most of the researches on the quantification of H₂O₂ or glucose dealt with an electrochemical method for with high detection limit [9], and there are few reports that deal with the immobilization of HRP or GOx onto iron magnetic nanoparticles and their use as biosensing materials. The aim of the present work is to immobilize HRP on the surface of modified core-shell magnetic nanoparticles and to use the HRP-immobilized nanoparticles for fabricating H₂O₂ and glucose biosensing probes. In the probes, non-fluorescent 3-(4-hydroxyphenyl) propionic acid (HPPA) was selected as a classical substrate to develop a new method for determination of trace amounts of H₂O₂ in the presence of immobilized enzyme, which could activate H₂O₂ and then accelerate the oxidation of non-fluorescent HPPA to strongly fluorescent dimer. This probe exhibited sensitive and selective responses for H₂O₂ measurement in a wide range of H₂O₂ concentrations. This probe could be combined with the catalytic reaction of glucose via the use of GOx, which resulted in the development of a simple and sensitive fluorometric method for the determination of H₂O₂ in complex bio-samples.

Experimental

Reagents and apparatus

HRP and GOx were purchased from Tianyuan Biologic Engineering Corp. (China) and Sigma, respectively. All

other chemical reagents were analytical reagent grade. The water was double distilled water.

A stock solution of H₂O₂ (0.1 mol L⁻¹) was prepared from 30% H₂O₂ solution and standardized by titration with potassium permanganate. It was further diluted if necessary. HPPA solution of 5 × 10⁻³ mol L⁻¹ was obtained by dissolving HPPA (from Sigma) in distilled water and was protected from light in the dark before its use.

Fluorescence spectra were monitored on a FP-6200 spectrofluorometer (JASCO). The magnetic properties were evaluated on an ADE 4HF vibrating sample magnetometer at 300 K.

Synthesis of nanostructured silica-coated magnetite

Fe₃O₄ MNPs were prepared by the co-precipitation method with procedures similar to that reported previously [28]. The obtained Fe₃O₄ MNPs (1.0 g) were added into a mixture of ethanol (100.0 mL) and distilled water (20.0 mL), and the resulting dispersion was sonicated for 10 min. After adding ammonia water (2.5 mL), tetraethyl orthosilicate (TEOS, 3.0 mL) was added to the reaction solution. The resulting dispersion was mechanically stirred for 3 h at room temperature. Then (3-aminopropyl) triethoxysilane (1.0 mL) was added to the reaction solution. The reaction was allowed to proceed for 10 h under stirring. The core-shell magnetic nanoparticles being surface-modified with amine groups were collected by magnetic separation, washed with distilled water, and then dried.

Enzyme immobilization

The magnetic nanoparticles being surface-modified with amine groups (0.8 g) were treated with 1.6% glutaraldehyde in the PBS solution (pH 7.0) at 25 °C for 3 h. The nanoparticles were washed with PBS solution and distilled water. Then the nanoparticles were redispersed in water (20 mL) and stored at 4 °C for use, being referred to as solution I.

Solution I (5.0 mL) was mixed with 5.0 mL of 1.0 mg mL⁻¹ HRP solution and 10.0 mL PBS buffer solution (pH 8.0). The immobilization was carried out with shaking for 11 h at 25 °C. The nanoparticles were separated and washed with PBS buffer and water to remove the unbound enzyme. The enzyme-immobilized nanoparticles were then redispersed in water (20.0 mL) and stored at 4 °C, being referred to as solution II.

Assay procedure for H₂O₂

Reaction solution (2.00 mL) was obtained by mixing 0.20 mL PBS buffer (pH 7.0), 1.30 mL H₂O, 0.10 mL of 5 × 10⁻³ mol L⁻¹ HPPA, 0.20 mL of solution II, and

0.20 mL of H_2O_2 solution at a specified concentration (or the sample solution). The mixture was incubated in a water bath at 25°C for 30 min. At the end of reaction, the solution pH was adjusted to 10 by adding 0.30 mL of glycine–NaOH buffer solution. After the enzyme-immobilized particles were removed by magnetic separation, the supernatant solution was used for spectrofluorometric measurement at 409 nm with an excitation at 324 nm.

Assay procedure for glucose

Standard glucose solution (or diluted serum sample, 1.00 mL), GOx solution (5 U mL^{-1} , 0.10 mL), PBS buffer solution (pH 7.0, 0.40 mL), and H_2O (0.50 mL) were mixed and incubate at 30°C for 20 min. Into the incubated solution (1.00 mL), 0.70 mL of H_2O , 0.10 mL of $5 \times 10^{-3} \text{ mol L}^{-1}$ HPPA and 0.20 mL of solution II were then added. The further procedure was the same as that for H_2O_2 determination. All the procedures for the determination of H_2O_2 or glucose at each test were repeated three times, and the averaged values were reported throughout the present work. The standard deviations were generally less than 5%.

Influences of foreign species

The effects of foreign species as possible interferents were checked by adding the foreign species at various concentrations into the analyte in the presence of $1.0 \times 10^{-6} \text{ mol L}^{-1}$ H_2O_2 or $2.5 \times 10^{-6} \text{ mol L}^{-1}$ glucose. The influences of the interferents were evaluated by determining the concentration of H_2O_2 or glucose before and after the addition of the foreign species. If the relative error in the determination of the concentration of H_2O_2 or glucose is less than $|\pm 5.0\%|$, the existence of the foreign species at the added concentration was considered to be tolerable, and the maximal tolerable ratio was obtained as the molar ratio of the added foreign species and H_2O_2 or glucose when the error was equal to $|\pm 5.0\%|$.

Results and discussion

Characterization of Fe_3O_4 MNPs

The hysteresis loop of the dried Fe_3O_4 was recorded at room temperature (Fig. 1), which demonstrated that the Fe_3O_4 MNPs exhibited negligible coercivity (H_c) and remanence, being typical of superparamagnetic materials. The saturation moment per unit mass, M_s , for the Fe_3O_4 MNPs was measured to be 68.8 emu g^{-1} . The superparamagnetism of magnetic nanoparticles is attractive for a broad range of biomedical applications because the nano-

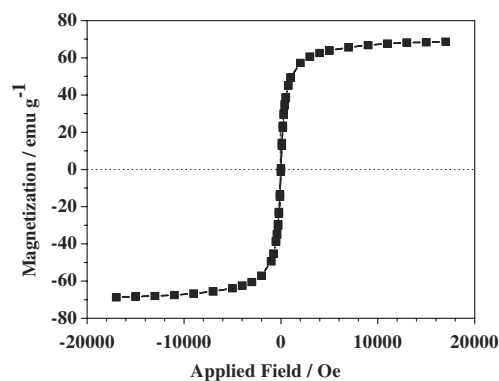


Fig. 1 Magnetization curves of Fe_3O_4 nanoparticles measured at 300 K

particles can be easily separated by external magnetic field in practice use and redispersed rapidly when the magnetic field is removed.

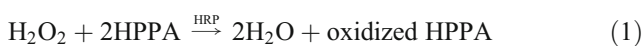
Immobilized peroxidase

HRP was immobilized on the magnetite/silica nanoparticles by using glutaraldehyde as a cross-linking agent. The effects of immobilization conditions (such as glutaraldehyde concentration, pH, temperature, and reaction time) on the activity of immobilized HRP were investigated. By examining the effect of glutaraldehyde concentrations ranging from 0.4 to 4 wt.% on the activity of the immobilized enzyme, it was found that the optimum glutaraldehyde concentration was 1.6 wt.%. When HRP was immobilized on the surface of the NH_2 -modified magnetic core–shell nanoparticles at different solution pH values from 6.0 to 9.5, the solution at pH 8.0 yielded the immobilized HRP showing the highest activity. In addition, we also investigated the influence of the temperature ranging from 15 to 35°C on the activity of the immobilized HRP. When the temperature was low, the cross-linking reaction was not sufficient, leading to low activity of the HRP-immobilized nanoparticles. The immobilized enzyme possessed a much higher activity when the reaction was carried out at 25°C . Higher temperatures over 25°C resulted in a decreased activity of the immobilized enzyme. Similarly, by varying the reaction from 2 to 20 h, it was observed that the optimum reaction time was 11 h. Therefore, the enzyme-immobilized nanoparticles were prepared by cross-linking with the addition of 1.6% glutaraldehyde at pH 7.0 at 25°C for 11 h.

Reaction system and its spectral characteristics

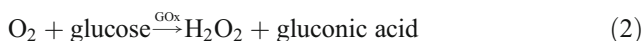
The determination of H_2O_2 is achieved by using its oxidizing ability. When HPPA is used as a non-fluorescent substrate, it can be oxidized by H_2O_2 to a

strongly fluorescent dimer, which needs the catalysis of peroxidase enzyme (Eq. 1),



The fluorescence intensity of oxidized HPPA is directly proportional to the concentration of H_2O_2 , allowing the establishment of a fluorescence probe for the determination of H_2O_2 concentration. In the present work, the immobilized HRP is used instead of free HRP.

It is known that glucose can be oxidized by O_2 in the presence of GOx via the following reaction,



When the oxidation of HPPA by H_2O_2 is coupled with the oxidation of glucose in the presence of GOx, the determination of glucose is easily achieved. Therefore, the reaction system for the determination of H_2O_2 is mainly composed of a substrate and hydrogen peroxidase, while the reaction system for the determination of glucose requires the further addition of GOx.

As discussed above, both the probes for the determination of H_2O_2 and glucose are based on the variation of fluorescence of the added substrate HPPA before and after the oxidation, which originates from the generation of the fluorescent product from the oxidation of HPPA. The product of the reaction of HPPA with H_2O_2 catalyzed by immobilized enzyme shows the excitation maximum and emission maximum wavelengths at 324 and 409 nm, respectively. Figure 2 compares the fluorescence spectra of the HPPA– H_2O_2 –enzyme system and the HPPA– H_2O_2 system. It was observed that H_2O_2 itself could slowly oxidize HPPA (curve 1 in Fig. 2), but the oxidation is promoted significantly by the immobilized enzyme (curve 2

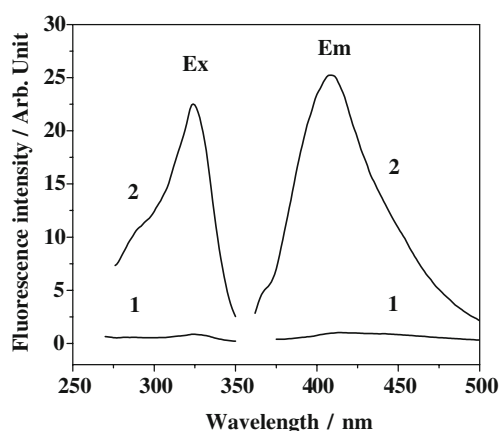


Fig. 2 Excitation and emission spectra of the solutions from the systems of HPPA– H_2O_2 (1) and HPPA– H_2O_2 –immobilized enzyme (2). Experimental conditions: H_2O_2 , $1.0 \times 10^{-6} \text{ mol L}^{-1}$; HPPA, $2.5 \times 10^{-4} \text{ mol L}^{-1}$; immobilized enzyme dispersion, 0.20 mL; pH of reaction solution, 7.0; reaction temperature, 25°C ; reaction time, 30 min, pH of solution for detection, 10.0

in Fig. 2). The excitation and emission spectra of the HPPA– H_2O_2 –enzyme system are similar in shape to that of HPPA– H_2O_2 system, but with much more increased intensity.

Effect of pH on the assays of H_2O_2 and glucose

Solution pH can markedly influence the assays of H_2O_2 and glucose from two aspects. At first, the catalytic activity of the immobilized enzyme is dependent on the pH of the reaction solution. Secondly, the fluorescence intensity of the oxidation product of HPPA is influenced by the solution pH during the fluorescence detection.

During the oxidation of HPPA by H_2O_2 being catalyzed by the immobilized HRP, the generation of the fluorescent product (the dimer of HPPA) is strongly influenced by the reaction solution pH. The solution acidity can change the dissociation status of the organic substrate HPPA, and the different dissociation forms of HPPA may have different resistances to the action of H_2O_2 . More importantly, the change of the solution acidity will vary the space conformation of the enzyme, influencing its ability of activating H_2O_2 . Therefore, the influence of solution pH on the immobilized enzyme-catalyzed reaction was studied in the pH range from 5.5 to 9.0 without changing the other experimental conditions. It was observed that the oxidation of HPPA by H_2O_2 in the absence of the immobilized enzyme was very slow, leading to only very small fluorescence intensity of the product solution in the whole tested range of pH. In contrast, the fluorescence intensity of the HPPA– H_2O_2 –enzyme system was greatly increased in the whole tested range of pH because the activation of H_2O_2 was catalyzed by the immobilized HRP. Moreover, the highest fluorescence intensity was observed at pH 7.0. Therefore, the reaction solution pH was optimized at pH 7.0 for the determination of H_2O_2 to guarantee a high catalytic activity of the enzyme.

Once the catalytic oxidation of HPPA was ended, the pH values of the resultant solution were adjusted to various values, and the effect of the detection solution pH on the fluorescence intensity of the generated product was examined from pH 7.0 to 12.0. The fluorescence intensity was increased initially with the increase of the detection solution pH and then kept almost constant over the pH range from 9.5 to 12.0. Thus, the detection solution pH was optimized at pH 10.0, which was controlled by adding a glycine–NaOH buffer solution in the present work. It should be noted that all the experimental conditions were optimized according to the fluorescence measurement in the final detection solution, which was different from the resultant solution after the oxidation reaction because of the addition of the buffer. However, for the simplicity of the description, we only use the expression of the

fluorescence intensity of the resultant solution hereafter, instead of the use of “the fluorescence intensity of the final detection solution.”

Effect of reaction time and temperature on the assays of H_2O_2 and glucose

Figure 3 gives the influence of reaction time on the fluorescence intensity of the resultant solution after the oxidation of HPPA. The oxidation of HPPA by H_2O_2 in the absence of immobilized enzyme is very slow as reaction time is increased, leading to a very low fluorescence intensity (curve 1 in Fig. 3). Because of the activation of H_2O_2 by the immobilized enzyme, the fluorescence intensity of the HPPA– H_2O_2 –enzyme system is initially increases rapidly and then reaches a platform beyond 25 min (curve 2 in Fig. 3). The saturation of the fluorescence intensity beyond 25 min indicates that all the added HPPA can be completely oxidized within 25 min under the experimental conditions. According to this observation, the reaction time was optimized at 30 min for the determination of H_2O_2 .

The effect of reaction temperature was investigated on the fluorescence intensity of the systems of HPPA– H_2O_2 and HPPA– H_2O_2 –enzyme. In the absence of the immobilized HRP, the fluorescence intensity was slightly increased with the increasing of reaction temperature. However, the fluorescence intensity in the HPPA– H_2O_2 –enzyme system is much greater than that in the of HPPA– H_2O_2 system. This is attributed to the accelerated oxidation of HPPA by H_2O_2 , which is catalyzed by the peroxidase-immobilized catalyst. From the dependence of the fluorescence intensity on the reaction temperature, a maximum intensity was observed at 25 °C. Therefore, the reaction temperature was optimized at 25 °C.

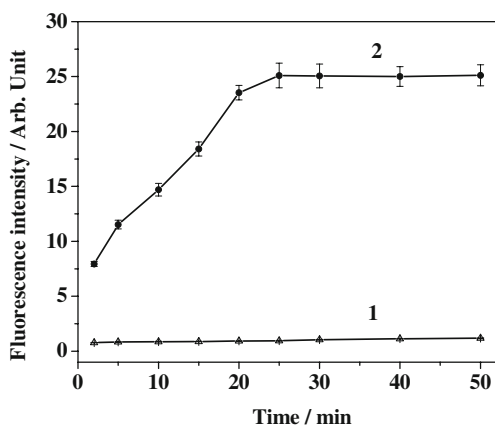


Fig. 3 Kinetic profiles for the oxidation of HPPA in the systems of (1) HPPA– H_2O_2 and (2) HPPA– H_2O_2 –immobilized HRP. Other experimental conditions were the same as in Fig. 2

Optimal concentration of substrate and Michaelis constants (K_m)

The effects of HPPA concentration on the fluorescence intensity of HPPA– H_2O_2 and HPPA– H_2O_2 –immobilized HRP systems are shown in Fig. 4. It can be seen that the fluorescence intensity of both systems is increased with the increase of the HPPA concentration in the range from 1.0×10^{-5} to 1.0×10^{-3} mol L^{-1} . Therefore, the fluorescence intensity of the former system is greatly enhanced due to the catalysis by the immobilized HRP. The fluorescence intensity of HPPA– H_2O_2 –immobilized HRP system is significantly promoted initially with the concentration of the HPPA up to 2.5×10^{-4} mol L^{-1} and then increased slowly beyond this concentration. Thus, the concentration of the HPPA was optimized at 2.5×10^{-4} mol L^{-1} in our method.

The enzyme kinetic constant K_m (Michaelis constant) is one of the most important parameters for the evaluation of the enzyme’s efficiency. For a enzyme catalytic process, the reaction rate (v) and the concentration of the substrate ($[c]$) are correlated via Michaelis–Menten equation, in which the basic parameters may be obtained by using Lineweaver–Burk double reciprocal plots according to Eq. 3,

$$\frac{1}{v} = \left(\frac{K_m}{v_{\max}} \right) \frac{1}{[c]} + \frac{1}{v_{\max}} \quad (3)$$

The Lineweaver–Burk double reciprocal plot (the plot of $1/(\Delta F/\Delta t)$ against $1/c_{\text{HPPA}}$) yielded a fitting equation $1/(\Delta F/\Delta t) \sim 1.112 + 0.271 \times 10^{-4}/c_{\text{HPPA}}$, where ΔF is the variation of the fluorescence intensity. Hence, the apparent K_m of the immobilized HRP enzyme with HPPA as the substrate was obtained as 2.43×10^{-5} mol L^{-1} at 25 °C. We also investigated the HPPA– H_2O_2 –free HRP system under the same conditions. The apparent K_m of free HRP was estimated as 1.28×10^{-5} mol L^{-1} , which was half of the

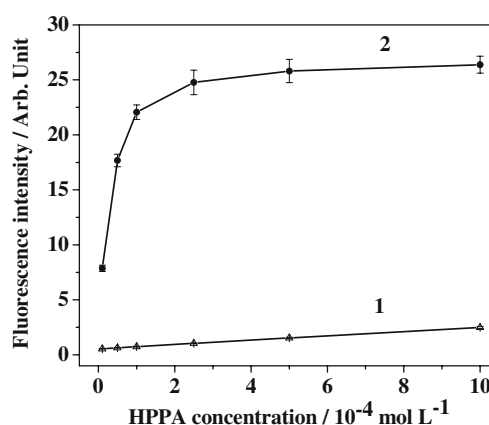


Fig. 4 Effects of HPPA concentration on the fluorescence intensity of (1) HPPA– H_2O_2 and (2) HPPA– H_2O_2 –immobilized enzyme systems. Other conditions were the same as in Fig. 2

immobilized HRP. The affinity of the immobilized enzyme to the substrate is weaker than that of the free enzyme to the substrate due to increased stereo-hindrance. However, such weakening is not so strong, and the affinity of the immobilized enzyme to the substrate is still comparable with that of the free HRP, which is attributed to the enzyme-like activity of Fe_3O_4 nanoparticles toward the activation of H_2O_2 [31].

Optimum addition of the immobilized enzyme

The effect of the addition amount of the immobilized enzyme (solution II) is shown in Fig. 5. The fluorescence intensity displays an obvious enhancement as the addition amount of solution II ranging from 0.01 to 0.5 mL. The catalytic ability of the immobilized enzyme is enhanced with the increase of enzyme load initially and then tends to reach a platform when the added volume is more than 0.1 mL. Therefore, the addition amount of the immobilized enzyme dispersion is optimized as 0.2 mL.

Analytical performance

Under the optimized reaction conditions and the detection conditions, a calibration curve was obtained by correlating the fluorescence intensity with H_2O_2 concentration. The calibration curve indicated that the fluorescence of the reaction system responds well linearly with H_2O_2 concentration (c_1 , $\mu\text{mol L}^{-1}$) in the range from 5×10^{-3} to $10.0 \mu\text{mol L}^{-1}$ under the optimal conditions, with a linear regression equation of $F \sim 0.9758 + 27.6270c_1$ and a correlation coefficient of 0.9993. The detection limit was $2.1 \times 10^{-3} \mu\text{mol L}^{-1}$ ($\text{DL} = 3 \sigma/k$, where σ is the standard deviation of the blank sample and k is the slope of the analytical calibration). The relative standard deviation was

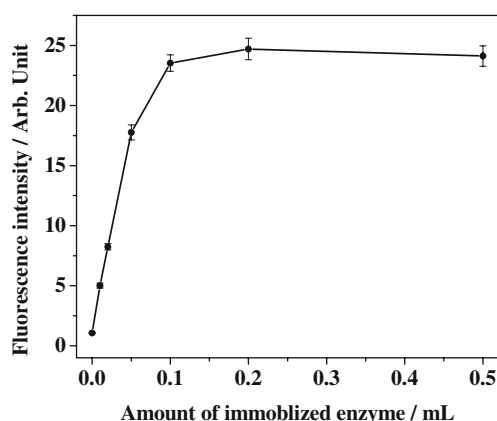


Fig. 5 Dependence of fluorescence intensity of the reaction system on the added amount of the immobilized enzyme solution (solution II). Other conditions were as same as in Fig. 2

2.3% for the determination of $1.0 \times 10^{-6} \text{mol L}^{-1} \text{H}_2\text{O}_2$ ($n=7$). The developed method exhibited sensitive and selective response toward H_2O_2 detection.

As discussed above, when GOx is added into the reaction system for the determination of H_2O_2 , the selective oxidation of glucose leads to the generation of H_2O_2 , which is coupled with the oxidation of HPPA by the in situ-generated H_2O_2 . This allows the successive determination of glucose. Based on the optimized experimental conditions for the determination of H_2O_2 , the reaction condition was further optimized by investigating the effects of the addition amount of GOx, which was optimized as 0.10 mL GOx solution (5U mL^{-1} , Fig. 6). Under the optimized conditions, a new method for glucose detection was also developed in the present work. From the dependence of the fluorescence intensity (F) on the concentration of glucose (c_2 , $10^{-6} \text{mol L}^{-1}$), a linear calibration curve with the expression of $F = -0.0588 + 3.3993c_2$ was obtained over glucose concentrations ranging from 5.0×10^{-8} to $5.0 \times 10^{-5} \text{mol L}^{-1}$, with a correlation coefficient of $r=0.9993$ and a detection limit of $1.8 \times 10^{-8} \text{mol L}^{-1}$. The relative standard deviation for the determination of $5 \times 10^{-6} \text{mol L}^{-1}$ glucose was 3.4% ($n=5$).

To compare the present method with other available methods, the H_2O_2 concentrations in three artificial samples were determined by using the present method and the reported *N,N*-diethyl-*p*-phenylenediamine sulfate (DPD)–HRP spectrophotometric method [32]. It is seen from Table 1 that for all the three samples, the present method yields analytical results being well consistent with that obtained by using the authorized DPD–HRP method. We have also carried out the F test to compare the two methods in Table 1. The results of the randomization F test shows that, in comparison with the tabulated critical value ($F_{(4,4)}=6.39$) at the level of $\alpha=0.05$, three samples are not significant since their F values are from 1.07 to 2.53, being considerably less than 6.39, which indicate that there are no significant differences between the two methods. By comparing with other methods for the determination of H_2O_2 and glucose as shown in Table 2, moreover, the method developed in the present work has merits of lower detection limit and wider linear range.

Interference

As described in the “Experimental” section, the possible interferences of common inorganic ions and organic compounds to the determination of H_2O_2 ($1.0 \times 10^{-6} \text{mol L}^{-1}$) were investigated when the proposed new method was used. Selection of the foreign ions was based on the possible use in real sample determinations. No interferences were found for K^+ , SO_4^{2-} , NO_3^- (5,000, maximal tolerance ratio in mol); NH_4^+ , CO_3^{2-} , PO_4^{3-} (2,000); Mg^{2+} , Zn^{2+} , Ca^{2+} (1,000);

Table 1 A comparison between the analytical results of the H₂O₂ determination by using the proposed systems and HRP–DPD system (*n*=5)

Sample	Immobilized HRP–HPPA		HRP–DPD		Relative difference (%)	<i>F</i> values in <i>F</i> test ^a
	Found (10 ⁻⁶ molL ⁻¹)	RSD (%)	Found (10 ⁻⁶ molL ⁻¹)	RSD (%)		
1	0.54	4.9	0.52	3.2	3.7	2.53
2	1.98	4.4	2.05	4.1	-3.5	1.07
3	9.76	1.3	10.02	0.9	-2.7	1.98

^a For comparison, $F_{(4,4)}=6.39$

Al³⁺, Ba²⁺ (900); L-phenylalanine (800); EDTA (80); Cu²⁺ (25); Fe³⁺ (3); and ascorbic acid (0.3). The interference effects of some foreign substances on the determination of 2.5 × 10⁻⁶ mol L⁻¹ glucose by the described procedure were also studied. No interferences were found for K⁺, SO₄²⁻, NO₃⁻ (5,000); NH₄⁺, CO₃²⁻, PO₄³⁻ (2,000); Mg²⁺, Zn²⁺, Ca²⁺, Al³⁺, Ba²⁺ (700); L-phenylalanine, arginine (800); tyrosine, cystine (200); EDTA (75); Cu²⁺ (18); Fe³⁺ (2); bilirubin (0.6); hemoglobin (0.3); and ascorbic acid (0.2). As shown above, the interferences from Cu²⁺ and Fe³⁺ ions are considerably marked. The presence of Cu²⁺ ions caused serious negative errors in the measurement, which is possibly because Cu²⁺ causes fluorescence quenching in the reaction. In contrast, Fe³⁺ ions yielded positive errors because Fe³⁺ ions might function as a catalyst like that in a Fenton process. However, the interferences from these metal ions may be eliminated by adding a small amount of EDTA as a masking agent. In addition, the serum sample requires to be heavily diluted for the determination of glucose in serum because the limit of detection limit of the present method is very low. This decreases markedly the levels of metal ions, bilirubin, hemoglobin, and ascorbic acid in the sample, and hence, the interferences from Cu²⁺ and Fe³⁺ ions, bilirubin, hemoglobin, and ascorbic acid can be effectively eliminated.

Determination of glucose in human serum

The applicability of this new method was evaluated by the determination of H₂O₂ and glucose in different practical samples. The two rainwater samples were collected on our

campus in Wuhan of China. After being filtered through a 0.22-μm pore size filter, the rainwater sample was analyzed immediately. As shown in Table 3, there were 4.84 and 5.92 μmol L⁻¹ of H₂O₂ in the two samples. Recovery experiments were also carried out by adding different amounts of H₂O₂ into the rainwater samples. The relative standard deviation was generally less than 3.6%, and the recovery ranged from 93.3% to 109.8%.

Serum samples were provided by Wuhan Centers for Disease Prevention and Control and used as testing samples. In order to eliminate the possible interference of proteins in human serum, the fresh serum samples were pretreated with 0.5 mol L⁻¹ trichloroacetic acid to precipitate the proteins. The samples were diluted 500 times and determined with the proposed method. It was found that there were 8.25 and 6.63 mmol L⁻¹ of glucose in two samples of human serum, as shown in Table 4. The recoveries in measurements ranged from 93.3% to 107.5%. The results demonstrate that the proposed method can be satisfactorily applied to analyze practical samples.

Stability and reusability of the immobilized enzyme

The chemical stability of the immobilized HRP was checked by two experiments. Firstly, both the immobilized HRP and free HRP solution were incubated at 50 °C for 3 h. After the heating treatment, it was observed that the immobilized HRP retained about 93% of its activity, while the relative activity of the free HRP dropped to 70% of its initial one. Secondly, both of the immobilized HRP and free

Table 2 Comparison of different methods for the determination of H₂O₂ and glucose

Method	Catalyst	Linear range (10 ⁻⁶ molL ⁻¹)	Detection limit (10 ⁻⁶ molL ⁻¹)	Reference
Fluorometry	HRP-immobilized nano-Fe ₃ O ₄ /SiO ₂	H ₂ O ₂ , 0.005–10.0 Glucose, 0.05–50.0	0.002 0.018	This work
FIA fluorometry	HRP	H ₂ O ₂ , 0.19–0.72	0.056	[4]
Fluorometry	FeTSPC	H ₂ O ₂ , 0.0–3.6	0.013	[29]
Chemiluminescence	Immobilized GOx and HRP on SiO ₂	Glucose, 10–1,000	5	[14]
Spectrophotometry	Hemin	H ₂ O ₂ , 0.3–32.0	–	[30]
Spectrophotometry	Fe ₃ O ₄ MNPs	H ₂ O ₂ , 0.5–150.0	0.25	[31]
Electrochemistry	Immobilized GOx on SiO ₂	Glucose, 20–10,000	1.5	[24]

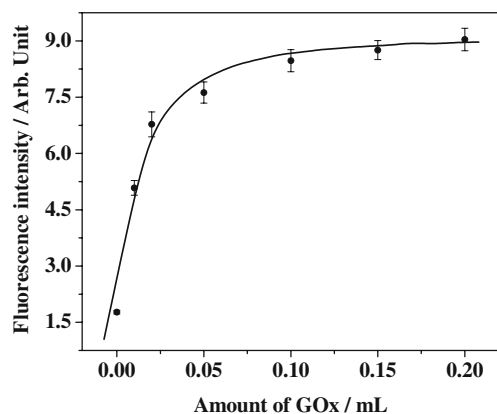


Fig. 6 Dependence of the fluorescence intensity of the reaction system on the added amount of GOx solution (5 U mL⁻¹). Experimental conditions: glucose, 2.5×10^{-6} mol L⁻¹; temperature, 30°C; reaction time, 20 min. The other procedures and conditions were the same as that for H₂O₂ determination

HRP were immersed in HCl solution (pH2) for 3 h. The acid immersion decreased the relative activity of free HRP to 80% of its initial one, but the immobilized HRP retained 97% of its initial activity after the treatment. The results suggested that the immobilized enzyme is more resistant to heat and acid than the free HRP.

The recovery of HRP-immobilizing magnetite/silica nanoparticles catalyst and its reusability were studied. For this purpose, the used catalyst was recovered by magnetic separation and washed with water to remove reaction solution. The recovered catalyst was reused in a subsequent reaction under the same experimental conditions as described in the “Experimental” section. Figure 7 gives the relative activity of the reused catalyst for the first four cycles, showing that the immobilized enzyme catalyst has high stability and can be almost completely recovered and reused because the decreasing in the activity is little.

Conclusions

Magnetic core-shell Fe₃O₄/SiO₂ nanoparticles were surface-modified and used as a substrate for the immobi-

Table 3 Determination results for H₂O₂ in practical rainwater samples ($n=5$)

Sample	Added (mmol L ⁻¹)	Found (mmol L ⁻¹)	RSD (%)	Recovery (%)
1	0	4.84	3.6	–
	4.00	9.23	2.9	109.8
	6.00	11.21	2.7	106.2
2	0	5.92	3.4	–
	4.00	9.65	2.8	93.3
	6.00	12.37	2.2	107.5

Table 4 Experimental data for the determination of glucose in serum samples ($n=5$)

Sample	Added (mmol L ⁻¹)	Found (mmol L ⁻¹)	RSD (%)	Recovery (%)
1	0	8.25	3.8	–
	4.00	12.14	3.2	97.3
	8.00	16.69	2.9	105.5
2	0	6.63	3.5	–
	4.00	10.30	4.1	91.8
	8.00	14.09	2.6	93.3

lization of hydrogen peroxidase HRP. The HRP-immobilized nanoparticles were used as an enzyme catalyst to activate H₂O₂, which was able to oxidize non-fluorescent organic substrate HPPA to its dimer, a strongly fluorescent product. The greatly increased fluorescence allowed the development of a fluorometric method for the determination of H₂O₂. Under the optimized conditions, this method could give linear responses to H₂O₂ in a range of 5.0×10^{-9} to 1.0×10^{-5} mol L⁻¹. When the HRP-immobilized magnetite/silica nanoparticles were used in couple with the addition of glucose oxidase, the above-mentioned method could be easily converted to a new method for the determination of glucose. By simultaneously using the immobilized HRP and free GOx, the new method yielded linear responses to the glucose concentrations ranging from 5.0×10^{-8} to 5.0×10^{-5} mol L⁻¹ with a detection limit of 1.8×10^{-8} mol L⁻¹. This method was successfully applied to determination of glucose in human serum samples. In comparison with the available methods for the determination of either H₂O₂ or glucose, the methods developed in the present work have marked merits of being highly sensitive with wide linear range and very low detection limit. Especially, the immobilized HRP on the surface of Fe₃O₄/SiO₂ nanoparticles is very stable. The HRP-immobilized nanoparticles can be easily recovered

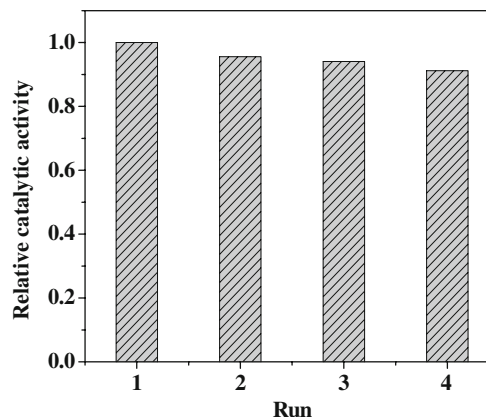


Fig. 7 The relative activity of the reused catalyst

with magnetic separation, and the recovery nanoparticles are able to be reused as enzyme catalyst.

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