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An amperometric hydrogen peroxide biosensor based on immobilizing horseradish peroxidase to a nano-Au monolayer supported by sol–gel derived carbon ceramic electrode

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Abstract

A novel strategy for fabricating horseradish peroxidase (HRP)-based H_2O_2 sensor has been developed by combining the merits of carbon sol–gel supporting matrix and nano-scaled particulate gold (nano-Au) mediator. The thiol functional group-derived carbon ceramic electrode (CCE) was first constructed using (3-mercaptopropyl) trimethoxy silane as sol–gel monomer. Then, the stable nano-Au monolayer was obtained through covalent linkage between nano-Au and thiol group on the surface of CCE. The experimental results showed that nano-Au monolayer formed not only could steadily immobilize HRP but also efficiently retain its bioactivity. Hydrogen peroxide was detected with the aid of hydroquinone mediator to transfer electrons between the electrode and HRP. The process parameters for the fabrication of the enzyme electrode and various experimental variables such as the operating potential, mediator concentration and pH of background electrolyte were explored for optimum analytical performance of the enzyme electrode. The biosensor had a fast response of less than 8 s with linear range of 1.22×10^{-5} to 1.10×10^{-3} mol l^{-1} and a detection limit of 6.1×10^{-6} mol l^{-1}. The sensitivity of the sensor for H_2O_2 was 0.29 A l mol^{-1} cm^{-2}. The activation energy for enzyme reaction was calculated to be 10.1 kJ mol^{-1}. The enzyme electrode retained 75% of its initial activity after 5 weeks storage in phosphate buffer at pH 7.

Keywords: Amperometric biosensor; Horseradish peroxidase; Nano-Au monolayer; Hydrogen peroxide

1. Introduction

Enzyme-based biosensor is an active research field for their potential application to a wide range of analytical tasks, such as clinical diagnosis, food industry, environmental monitoring and bioassay [1–4]. In the design and fabrication of biosensors, the development of a simple and reliable procedure for immobilizing and stabilizing active enzymes on the sensor surface is a crucial step. Established avenues of enzymes immobilization include physical adsorption [5], embedding into electrode matrix [6], entrapment in electropolymerized polymer [7] or cast film [8] and covalent cross-linking [9]. But there are still several challenges concerning simplification of fabrication, efficient retention of enzyme activity and lifetime of biosensors. Therefore, investigation of new strategies for immobilizing enzymes for the purpose of improving the performance of sensor is still a prevailing subject in the design of sensor at present [10–15].

In recent years, there has been an increasing attention about the electrochemical behavior and application of metal nanomaterials [16,17]. Especially, nano-scaled particulate gold (hereafter abbreviated as nano-Au), a well-known nanomaterial, is now being applied in analytical chemistry for its size-dependent electronic and chemical characteristics [18–20]. It has been demonstrated that nano-Au can strongly adsorb some protein and is a potential mediator to immobilize biospecies to efficiently retain their biological activity [21–24].

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Since the pioneering work of Pankratov and Lev [25] and Tsionsky et al. [26], sol–gel technique has been widely used for the fabrication of amperometric biosensors. The sol–gel-derived carbon ceramic composite electrodes (CCE) possess a number of advantages, including tunability of physical characteristics, mechanical rigidity (surface renewal), chemical inertness, thermal stability, porosity and permeability in electroanalysis. These attractive features would be further enhanced by means of chemical modification of CCE or by the use of bifunctional sol–gel precursors, such as (3-mercaptopropyl) trimethoxy silane (MPTMOS) and (3-aminopropyl) trimethoxy silane [27,28].

In present work, we reported a novel methodology for the construction of horseradish peroxidase (HRP)-based H_2O_2 sensor by combining the merits of carbon sol–gel supporting matrix and active nano-Au mediator. MPTMOS, which contains a silane and a functional thiol group, was utilized as sol–gel precursor to form thiol group-containing CCE. The thiol group on the surface of CCE could be used to covalently attach to nano-Au. Thus, a stable nano-Au monolayer could be formed on the surface of thiol functional group-derived CCE. The nano-Au monolayer formed provided an ideal mediator for the immobilization of HRP. Consequently, a simple, efficient and steady immobilization of HRP was realized. The determination of H_2O_2 was carried out using hydroquinone as an electron mediator. The results obtained indicated that the HRP immobilized on nano-Au, which in turn covalently link with thiol group-containing CCE, demonstrated an improved catalytic ability to the reduction of H_2O_2. The main advantages of this approach include the simple operation for forming nano-Au monolayer, the relatively high stability of nano-Au monolayer resulting from covalent linkage between nano-Au and -SH, rapid response for H_2O_2 sensing and improved stability of the biosensor.

2. Experimental

2.1. Reagents and solutions

MPTMOS (98%) and HRP (E.C.1.11.1.7, RZ>3.0, A>250U/mg) were purchased from Sigma (USA) and used without purification. High purity graphite powder was the product of Shanghai Carbon Plant (Shanghai, China). Hydrogen peroxide (30%) and $HAuCl_4 \cdot 3H_2O$ were obtained from Shanghai Chemical Reagents (Shanghai, China). Other chemicals were of analytical grade and used as received. All solutions were prepared with the twice-distilled water.

Background electrolytes for electrochemical experiments were 0.067 mol l^{-1} phosphate buffer solutions containing 0.1 mol l^{-1} KCl (PBS), which were prepared by mixing standard stock solutions of K_2HPO_4 and NaH_2PO_4. The solutions were purged with highly pure N_2 for at least 10 min to remove O_2, and a N_2 atmosphere was kept over the solutions during measurements. The accurate concentrations of H_2O_2 solutions were determined by titration with $KMnO_4$.

2.2. Apparatus

The electrochemical measurements were performed at room temperature (25 °C except in experiments on temperature effects) in a conventional one-compartment cell with a three-electrode configuration using a PAR 283 potentiostat/galvanostat (EG & G Princeton Applied Research, Princeton, NJ, USA) linked to PC computer. The experimental operation was controlled with PAR M270 software. The working electrode was an enzyme modified CCE, the auxiliary electrode was a Pt foil and the saturated calomel electrode (SCE) served as the reference electrode. Cyclic voltammetric experiments were performed in unstirred solutions. A magnetic stirrer and a stirring bar provided convective transport during amperometric measurements. An appropriate amount of analyte (H_2O_2) was added to measurement solution after a steady-state background current had been first achieved and corresponding amperometric response was recorded. All potentials were measured and quoted *versus* SCE.

2.3. Preparation of colloidal gold sols

The colloidal gold sols were prepared according to the method reported elsewhere [21,22] with minor modifications. All glassware used in the preparation procedure were thoroughly cleaned in aqua regia (3 parts of HCl plus 1 part of HNO_3) and rinsed with twice-distilled water. Prior to use, $HAuCl_4$ and Na_3-citrate aqueous solutions were filtered through a 22-μm microporous membrane filter. In a 100-ml beaker, 1.0 ml of 1% Na_3-citrate was quickly added to a boiling 50 ml solution of 0.01% $HAuCl_4$ with vigorous stirring, giving a color change from blue to red-violet. The mixture was continually boiled for an additional 10 min and stirred for another 10 min after removal of the heater to produce about 15-nm-diameter colloidal Au particles. The prepared colloidal gold sols were stored in refrigerator.

2.4. Construction of thiol group-containing CCE

The CCE was constructed as follows: 1.0 ml of methanol, 0.5 ml of MPTMOS and 1.0 ml of 0.01 mol l^{-1} HCl were first mixed in a weighing vial at room temperature and then sonicated for nearly 20 min. The resultant homogenous sol was then sufficiently mixed with graphite powder (0.5 g) for nearly 20 min and the mixture was allowed for gelation in a desiccator for 4 days at ambient temperature. The thiol group-containing ceramic-graphite composite produced was tightly packed into a home-made Teflon tube (with 6 mm inner diameter and the length of composite material in the tube was about 0.5 cm) [29] with a copper wire for electric contact and dried in air.

2.5. Fabrication of the HRP electrode

The enzyme, HRP, was attached to the surface of above thiol group-containing CCE with the aid of nano-Au as an active mediator. The CCE was first polished on a piece of emery paper, smoothed again on weighing paper and rinsed with twice-distilled water to yield shiny surface. Subsequently, the cleaned CCE was placed in colloidal gold sol for 6 h, rinsed with twice-distilled water. Finally, the immobilization of HRP to active nano-Au mediator was realized by inserting the electrode in 5 mg ml^{-1} HRP PBS (pH 7) for 8 h to fabricate the H_2O_2 sensor. After thoroughly rinsing the sensor with PBS, it was stored in PBS (pH 7) in refrigerator when not in use.

3. Results and discussion

3.1. Construction of H_2O_2 sensor by immobilizing HRP to nano-Au mediator

The thiol group-containing CCE was used as supporting matrix for the formation of nano-Au monolayer and the immobilization of enzyme on its surface. It is well known that sol–gel preparation condition strongly influences the quality of the biosensors fabricated. In particular, the ratio of silicon alkoxide/water in the stock sol–gel solution for the acid-catalyzed hydrolysis has a profound effect on the porous nature of CCE and the ratio of graphite to sol solution greatly affects the conductivity of CCE. The resistance of CCE prepared according to Section 2.4 was below 20 Ω, which implies a nice conductivity. The cyclic voltammograms of $[Fe(CN)_6]^{4-}$ on the CCE showed that well-defined and reproducible shape (the figure not shown), which indicate the good quality of prepared electrode. The thiol groups on the surface of CCE could covalently attach to nano-Au. So a stable monolayer of nano-Au could be formed on the surface of CCE. The nano-Au monolayer formed provided active sites ideal for the immobilization of HRP. In such a way, the HRP was immobilized on the surface of CCE by means of nano-Au mediator. The association of HRP to nano-Au is possibly due to strongly electrostatic interaction between the citrate anion bound to the surface of the nano-Au particle and protonated amine residues of the lysine groups of HRP. It is reasonable to assume that the HRP immobilized on nano-Au could best retain its active configuration and sustain the rotational freedom, so maintaining the high biological activity.

3.2. Electrocatalytic behavior of nano-Au mediated HRP electrode to the reduction of H_2O_2

Cyclic voltammetry was utilized to test the electrocatalytic behavior of HRP modified electrode. Since the proposed HRP electrode did not show the direct electron communication between the heme group of HRP and the CCE, hydroquinone was used as the electron mediator. The cyclic voltammetric behavior of the proposed HRP electrode in an unstirred deoxygenated PBS (pH 7) containing 4.0 mmol l^{-1} hydroquinone was investigated at a scan rate of 100 mV s^{-1} (Fig. 1). In the absence of H_2O_2 (Fig. 1a), only one pair of oxidation/reduction peak, which represented the typical electrochemical behavior of hydroquinone was observed. But in the presence of 0.61 mmol l^{-1} H_2O_2 (Fig. 1b), the electrocatalytic behavior appeared with a obvious increase of the reduction current and a concomitant decrease of the oxidation current. The result showed the HRP immobilized on nano-Au possesses improved electrocatalytic ability for the H_2O_2 reduction and the hydroquinone could effectively shuttle electrons from the redox center of HRP immobilized on nano-Au and the CCE.

3.3. Optimization of measurement variables

The biosensor response to H_2O_2 is mainly influenced by the concentration of the mediator, the pH of background electrolyte and the working potential of chronoamperometry.

The effect of hydroquinone concentration on the HRP electrode response was studied in the presence of 0.22 mmol l^{-1} H_2O_2 (PBS pH 7, −0.17 v vs. SCE). The response of the HRP electrode increased sharply with the increase of the concentration of hydroquinone from 0.5 to 4.0 mmol l^{-1} and then leveled off. Such a behavior is typical of a mediator-based sensor [30]. At low mediator concentration, the current response will be limited by enzyme-mediator kinetics. When the mediator concentration is high, the current response will be limited by enzyme-substrate kinetics. However, a higher concentration of hydroquinone produced a higher background current. Thus, the concen-

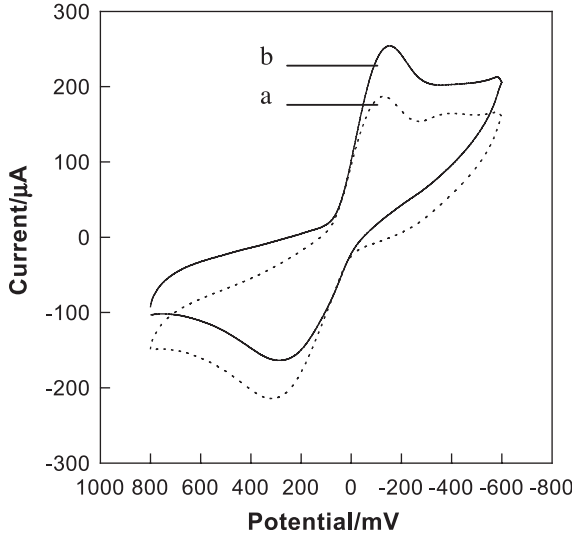

Fig. 1. Cyclic voltammograms recorded in 4.0 mmol l^{-1} hydroquinone solution in the absence (a) and presence of 0.61 mmol l^{-1} H_2O_2 at nano-Au mediated HRP electrode. Supporting electrolyte pH 7. PBS. Scan rate 100 mV s^{-1}.

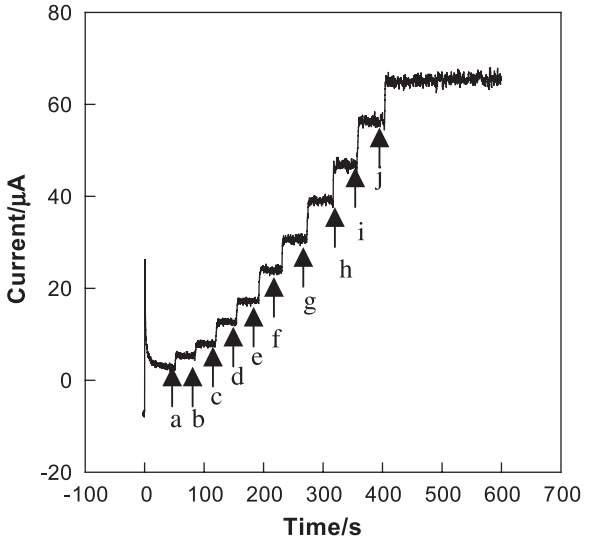

Fig. 2. The chronoamperometric response of the HRP electrode after successive additions of 20 (a), 20 (b), 40 (c), 40 (d), 60 (e), 60 (f), 80 (g), 80 (h), 100 (i) and 100 μl (j) of 36.5 mmol l^{-1} H_2O_2 into 30 ml of PBS (pH 7.0) containing 4.0 mmol l^{-1} hydroquinone at applied potential of −170 mV.

tration of hydroquinone was fixed at 4.0 mmol l^{-1} for all further experiments.

The pH dependence of the biosensor response was decided by two aspects: one was the activity of HRP, the other was the peak potential of hydroquinone. Taking account of the two factors, the effect of pH on H_2O_2 sensor response to 0.22 mmol l^{-1} H_2O_2 was investigated over the pH range 4–9 in PBS containing 4.0 mmol l^{-1} hydroquinone and an optimum pH 7 was adopted in following test, which was in agreement with that observed for soluble peroxidase [31].

The influence of operating potential on biosensor response was tested over the potential range 50 to −300 mV with 0.22 mmol l^{-1} H_2O_2 in PBS containing 4.0 mmol l^{-1} hydroquinone at pH 7. The sensitivity of the biosensor increased steadily with the operating potential decreasing from 50 to −170 mV since the enzymatically liberated benzoquinone species can be more easily reduced at more negative potential. But when the potential was further stepped negatively, the response currents actually leveled off. Therefore, a proper potential of −170 mV (vs. SCE) was selected for all subsequent experiments.

3.4. Electrode response characteristics and calibration of H_2O_2 sensor

The nano-Au monolayer formed on the surface of thiol-containing CCE by means of covalent bond provided a stable, active mediator for the immobilization of HRP as well as can efficiently retain its bioactivity. Fig. 2 displays a typical current-time response of the HRP biosensor for successive additions of varying volume H_2O_2 under optimized experimental conditions (4.0 mmol l^{-1} hydroquinone, pH 7, operating potential −170 mV vs. SCE). It is clear that a rapid and sensitive response to H_2O_2 was

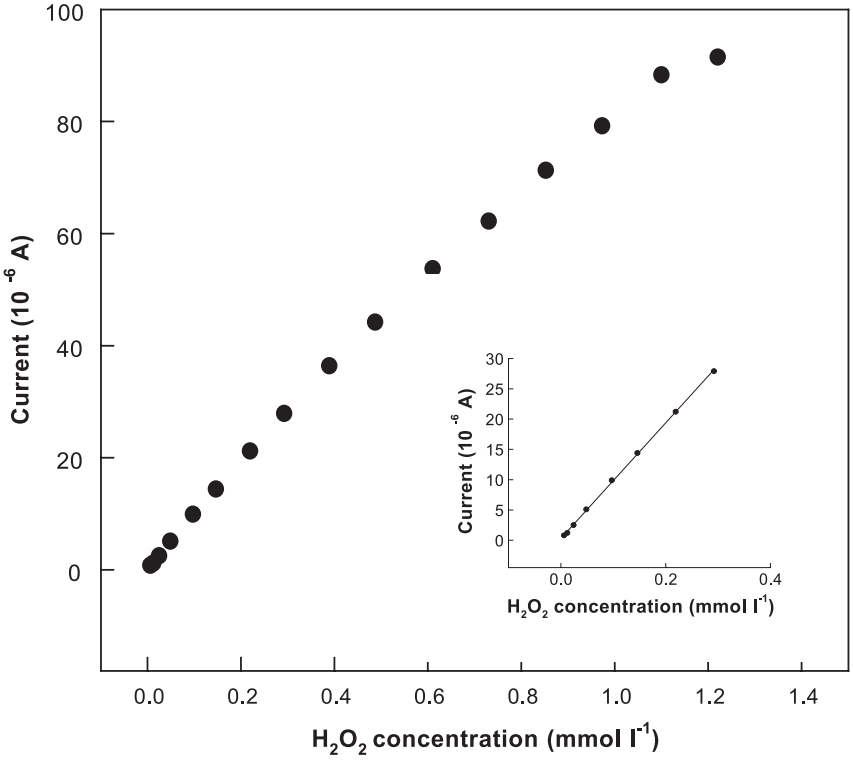

Fig. 3. Calibration curve of the HRP enzyme electrode to H_2O_2 concentrations in PBS at optimal experimental conditions.

achieved. The time to reach 95% of the steady-state current is within 8 s, which is attributed to the high bioactivity of HRP immobilized on nano-Au as well as the fast diffusion of the electron mediator to the prosthetic groups of HRP.

The proposed H_2O_2 sensor exhibited a linear calibration range from 1.22×10^{-5} to 1.10×10^{-3} mol l^{-1} with a detection limit of 6.1×10^{-6} mol l^{-1} at a signal-to-noise ratio of 3 under the optimized experimental conditions (Fig. 3). The regression equation is I (μA) = 2.26 + 80.92 C (mmol l^{-1}) with a correlation coefficient of 0.996 (n = 14). The response was saturated at the concentration of H_2O_2 higher than 1.10×10^{-3} mol l^{-1}, which can be attributed to the saturation of the enzyme-substrate or enzyme-mediator kinetics. In addition, the sensitivity of the biosensor to H_2O_2 can also be calculated to be 0.29 A l mol^{-1} cm^{-2}. This value is higher than that reported in the literatures [8,32,33], e.g., 0.19 A l mol^{-1} cm^{-2} based on immobilization of HRP in chitosan film [8], 0.06 A l mol^{-1} cm^{-2} at a pyrolytic graphitic electrode with HRP adsorption [32], 0.15 A l mol^{-1} cm^{-2} using silica sol–gel/chitosan film as immobilization of HRP [33]. The high sensitivity of the sensor reflects the high efficiency of the immobilization strategy in which the nature of immobilized HRP on nano-Au is essentially identical with that of native HRP.

3.5. Repeatability, reproducibility and stability of the HRP enzyme electrode

The measurement repeatability of the HRP enzyme electrode was examined at a H_2O_2 concentration of 0.22 mmol l^{-1} with the same enzyme electrode and the relative standard deviation (RSD) was 4.1% for eight successive assays. The fabrication reproducibility for four HRP electrodes gave RSD 4.9% for the amperometric determination at 0.22 mmol l^{-1} H_2O_2.

The long-term stability of the biosensor was investigated by measuring the current response of 0.22 mmol l^{-1} of

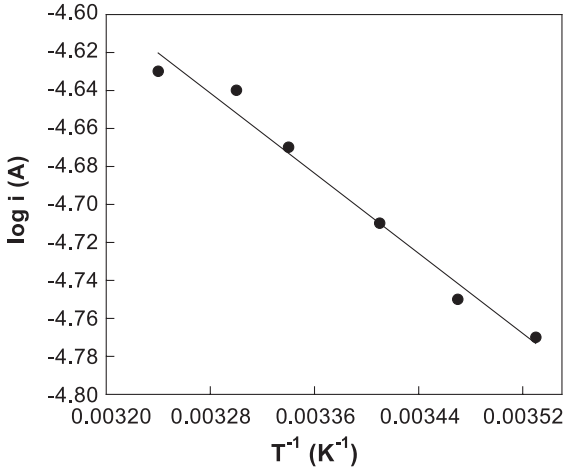

Fig. 4. log i vs. T^{-1} plot for the HRP electrode in PBS containing 0.22 mmol l^{-1} H_2O_2 at optimal experimental conditions.

Table 1
Measurement of H_2O_2 concentrations (g ml^{-1}) in disinfecting preparations

Sample	Proposed H_2O_2 biosensor[a,b]	$KMnO_4$ titration method[c]
1	0.12 ± 0.011	0.119
2	0.21 ± 0.015	0.209
3	0.26 ± 0.016	0.262

[a] Samples were diluted 2000 times.
[b] Mean ± SD of four measurements.
[c] Samples were diluted 200 times.

H_2O_2 every other day over 5 weeks. The results showed that the response decreased about 8% of its initial current after 2 weeks and retained about 75% of its original response after 5-week usage. This good long-term stability was attributed to covalent linkage of nano-Au with thiol group on the surface of CCE, strongly interaction between HRP and nano-Au as well as the favorable biologically microenvironment for HRP provided by nano-Au.

3.6. Effect of temperature and activation energy

The effect of temperature on HRP electrode response was studied in the temperature range of 283–318 K in the PBS containing 0.22 mmol l^{-1} H_2O_2 under optimum conditions. The response current, which correspond the activity of the immobilized HRP, increased with increasing temperature from 283 to 308K and then went down as the temperature going up further, which indicated that the enzyme began denaturation. The temperature effect can be described by the Arrhenius equation [34].

$$\log i = \log i_0 - \frac{E_a}{2.303RT}$$

where i_0 is the pre-exponential term, R is the gas constant, T is the temperature in Kelvin degrees and E_a is the activation energy. The activation energy for enzymatic reaction was determined by analysis of the slope in the appropriate region of temperature for the plot of the logarithm of the current versus the reciprocal of Kelvin temperature (Fig. 4). The E_a value obtained was 10.1 kJ mol^{-1}, which was small than that (13.8 kJ mol^{-1}) reported by Yang and Mu [35] for HRP immobilized in the polyaniline films. The lower activation

Table 2
Results of interfering experiment

Interferents	Current ratio[a]
Glocose	1.00
Ethanol	1.00
Acetic acid	1.00
Citric acid	1.01
Oxalic acid	1.02
Acetaminophenol	1.02
Ascorbic acid	0.76
S^{2-} (0.2 mmol l^{-1})	0.23

[a] Ratio of currents for mixtures of 1.0 mmol l^{-1} interferents and 0.22 mmol l^{-1} H_2O_2 compared to that for 0.22 mmol l^{-1} H_2O_2 alone.

energy indicates the immobilized HRP via nano-Au as interface possesses higher enzymatic activity. Though higher sensitivity could be obtained at higher temperatures, long-term stability of the sensors is compromised because of protein unfolding with temperature rising. Therefore, an appropriate temperature of 25 °C was adopted through the experimental work.

3.7. Preliminary application and selectivity against interferences

We evaluated the real analytical usefulness of the proposed biosensors by determining H_2O_2 concentration in disinfecting preparations. Results are shown in Table 1. The results obtained by proposed HRP biosensor were compared to those determined by the classical $KMnO_4$ titration method.

Eight potential interfering substances were examined to evaluate the selectivity of the proposed HRP sensor. The interference experiments were performed in PBS at optimal conditions by comparing the response current of 0.22 mmol l^{-1} H_2O_2 plus 1.0 mmol l^{-1} each interfering substance with the 0.22 mmol l^{-1} H_2O_2 alone. The results of the interference study are listed in Table 2. Glucose, ethanol, acetic acid, citric acid, oxalic acid and acetaminophenol do not cause any interference, within 2% under the determining conditions. Only sulfite and ascorbic acid interfered seriously with the H_2O_2 detection. The reasons of interference originate from that sulfide directly inhibit the activity of HRP [36] and ascorbic acid can reduce benzoquinone (the oxidation form of mediator), which destroyed the circulation of mediator.

4. Conclusions

A sol–gel technology was utilized to prepare a thiol group-containing carbon ceramic electrode using MPTMOS as a bifunctional sol–gel precursor. The stable nano-Au monolayer can be formed on the surface of CCE owing to the covalent linkage between nano-Au and the thiol group. The nano-Au monolayer formed on CCE provided a desirable mediator for the immobilization of HRP to fabricate a H_2O_2 sensor. The experimental results clearly exhibited that the immobilized HRP possesses excellent catalytic ability and well-retained activity. The developed biosensor showed high sensitivity, fast response and good stability.

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