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ORIGINAL PAPER

Amperometric biosensor based on hemoglobin immobilized on Cu_2S nanorods/nafion nanocomposite film for the determination of polyphenols

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Abstract A novel biosensor was fabricated based on hemoglobin (Hb) immobilized onto cuprous sulfide (Cu_2S) nanorods/nafion nanocomposite film for the detection of polyphenols in the presence of hydrogen peroxide (H_2O_2). The nanostructured inorganic–organic hybrid material formed by Cu_2S nanorods and nafion provided a biocompatible microenvironment for Hb and increased the sensitivity for polyphenols detection. The modified electrodes were characterized by electrochemical impedance spectroscopy and linear sweep voltammetry. Parameters such as pH, H_2O_2 concentration, and the applied potential were optimized. Under optimum conditions, the biosensor gave linear response ranges of 7.0–110, 0.6–10, and 8–100 μM for catechol, hydroquinone, and resorcin, with the detection limits of 0.5, 0.03, and 0.6 μM (*S*/*N*=3), respectively. The developed biosensor exhibited a short response time within only 8 s with good stability and reproducibility. Such a novel biosensor showed great promise for rapid, simple analysis of polyphenols contents in real samples.

Keywords Hemoglobin · Cuprous sulfide nanorods · Nafion · Polyphenols · Biosensor

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Introduction

As a kind of the important natural product, plant polyphenols widely exist in the plant kingdom [1, 2], which mainly include flavonoids, tannins, curcuminoids, gallocatechins, stilbenes, and anthocyanidins [3, 4]. They play an important role in food and medicine [5–9] and are also commonly used in the manufacture of resins, polymers, paints, and herbicides products [10, 11]. Being one kind of rich reserves and renewable green resources, plant polyphenols will certainly become capital stocks for humanity use. Therefore, the determination of polyphenols is very important. The general methods, which are used to examine plant polyphenols, are high-performance liquid chromatography, thin-layer chromatography, capillary electrophoresis, and nuclear magnetic resonance [12–15]. However, these methods are quite expensive and time consuming. Moreover, they also need skilled operators and complicated samples pretreatment process. Therefore, new methods are required which are simple and rapid for effective and sensitive determination of plant polyphenols.

Amperometric biosensors-based enzymes have been considered to be an alternative method for the determination of polyphenols. At present, many enzymes, such as tyrosinase [16–18], laccase [19, 20], and peroxidase [21, 22] have been widely applied to fabricate biosensors for polyphenols determination. Enzyme molecules can be rereduced by phenolic compounds after they are oxidized by oxygen (for tyrosinase and laccase) or hydrogen peroxide (for peroxidase) on the surface of the electrode [23, 24]. The tyrosinase biosensors are effective to detect phenolic compounds with

at least one free orthoposition [18]. The laccase biosensors can catalyze the *ortho*- and *para*-diphenols, aminophenols, and polyphenols [25]. And the peroxidase biosensors are proposed to be sensitive to a wider range of phenolic compounds, since they can work as electron donors to peroxidase [21]. But these kinds of enzyme biosensors have the weakness of high cost and low stability, which reduce their practical application in experiments. To solve this problem, many researchers have devoted their attention to new enzymes for building more satisfactory biosensors, especially hemoglobin (Hb). As heme protein, Hb can store and transport oxygen in the red blood cells. It is reported that Hb can be used as a substitute of horseradish peroxidase to catalyze the reduction of hydrogen peroxide (H_2O_2). For polyphenols detection, a double displacement mechanism of Hb-modified biosensors is expressed as follow: Hb molecules are oxidized by H_2O_2, followed by its reduction by the phenolic compounds at the electrode surface. The latter reaction converts phenolic compounds into free radical products, which are electroactive and can be electrochemically reduced on the electrode surface [26–28]. Compared with other type of peroxidase, Hb has many advantages such as commercial availability, low cost, and its known and documented structure besides its intrinsic peroxidase activity. Therefore, the employment of Hb for the construction of phenolic measured biosensors is possible [29].

Recently, a considerable amount of interest has been focused on semiconductor transition metal oxides and chalcogenides owing to their excellent properties and wide-range potential applications. These semiconductors could retain the activity of enzyme well due to an increase in the stability and sensitivity of enzymatic sensors. So they can be used for immobilization of proteins and enzymes. Cuprous sulfide (Cu_2S) is an interesting material for its semiconduction and photovoltaic capabilities. Because of its stoichiometric composition, valence states, nanocrystal morphologies, complex structures, and different unique properties, it has been widely applied in numerous fields, such as solar cells, cold cathodes, electrochemical sensor, and nanoscale switches [30–33]. Nafion is a polyanionic perfluorosulfonate ionomer, which structure contains hydrophobic chain and hydrophilic group. It has unique properties with respect to thermal stability, solubility, and ionic conductivity and excellent film-forming ability, which make them suitable for a variety of applications, especially as immobilization material used in electrochemical biosensors [34–37].

In the present paper, we carried out a new solventless synthesis of size- and shape-monodisperse Cu_2S nanorods (Cu_2S NRs) by copper thiolate thermolysis according to previous report. A nanostructured inorganic–organic hybrid material was formed by combining Cu_2S nanorods and nafion, which was explored to build electrochemical biosensor by immobilizing Hb firmly on the surface of electrode. The inorganic–organic hybrid material offered a biocompatible microenvironment for Hb, enhanced the amperometric detection for polyphenols, and made the biosensor with high sensitivity and stability. The performance of this fabricated biosensor was investigated and its application in determining the total polyphenols contents of plant extracts was also studied.

Experiments

Reagents and apparatus

Hemoglobin was purchased from Beijing Biodee Biotechnology Co., Ltd. (China) and used as received. Catechol was purchased from Shanghai Zhanyun Chemical Co., Ltd. (China). Hydroquinone and resorcin were acquired from Beijing Chemical Reagent Institute (China). H_2O_2 and $Cu(NO_3)_2{\cdot}5H_2O$ were purchased from Tianjin Basf Chemical Co., Ltd. (China). Sodium octanoate and dodecanethiol were purchased from Sigma-Aldrich. Phosphate buffer solution (PBS) was prepared by mixing a stock solution of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusting the pH either with 0.1 M H_3PO_4 or 0.1 M NaOH. All of the chemicals were of analytical reagent grade, and all of the solutions were prepared with redistilled deionized water.

Electrochemical experiments were performed with CHI660C electrochemical workstation (Shanghai Chenhua Co., China) with a conventional three-electrode cell. A bare glassy carbon electrode (d=3 mm) or a modified glassy carbon electrode was used as the working electrode. A Pt wire and a saturated calomel electrode (SCE) were used as the counter and the reference electrodes, respectively. X-ray diffraction (XRD) pattern of the synthesized Cu_2S nanorods was obtained by D8 Advance X-ray diffractometer (Bruker). Scanning electron microscopy (SEM) image was gained using the JEOL JSM-7600F (Japan). A PHS-3 C Exact Digital pH meter (Shanghai KangYi Co. Ltd., China) was used for adjusting the pH of the buffer solution.

Preparation of Cu_2S nanorods

Cu_2S NRs were prepared similarly as in a previous report [38]. Briefly, a copper precursor is made by combining 24.5 mL of chloroform with a 36-mL solution containing 0.21 g $Cu(NO_3)_2$. Then, the sodium octanoate (0.18 g, Aldrich, 98%) was added as a phase transfer catalyst to solubilize the copper cations in the organic phase. The aqueous phase is discarded, when the blue copper octanoate complex transfers into the organic phase. After the

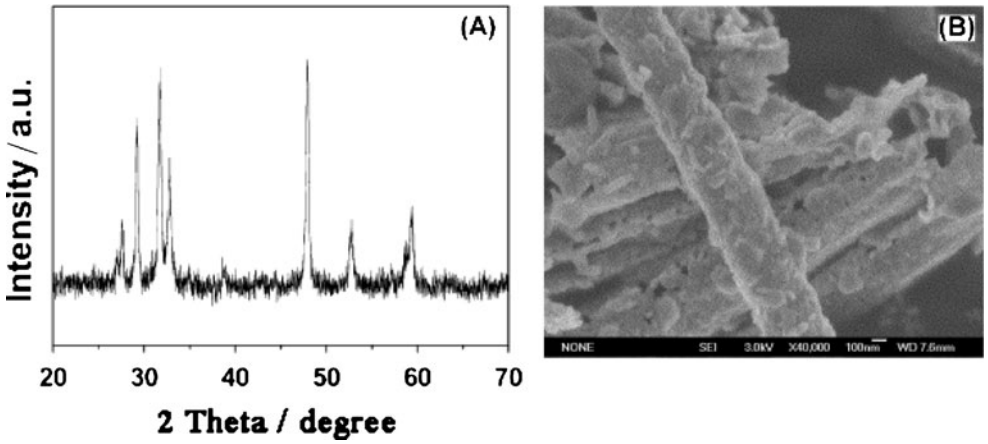


Fig. 1 XRD (**a**) and SEM (**b**) images of the synthesized Cu_2S nanorods

dodecanethiol (240 μL, Aldrich, 98%) is added to the organic solution, the color changes from blue to green. The organic solvent was evaporated to leave a waxy residue, which consists of the copper precursor species. The resulting residue is heated to 148 °C for 140 min to produce a brown solid material. Then this material redisperses in chloroform to remove unreacted surfactant and byproducts, and the resultant precipitates were attained with ethanol.

Preparation of Hb/Cu_2S NRs/nafion-modified glassy carbon electrode

Before the surface modification, the bare glassy carbon electrode (GCE) was successively polished with 0.3 and 0.05 μm alumina slurry and then rinsed with water, ultrasonicated in ethanol and redistilled deionized water, respectively. Finally, it was dried in air for further use. The preparation of the enzyme electrode was as follow: a 5-mg/mL Hb solution was first obtained with 0.1 M PBS (pH 7.0). Then, a mixture, which finally contained 5 mg/mL Hb, 2 mg/mL Cu_2S nanorods, and 0.25% nafion, was prepared by mixing appropriate amount of Cu_2S nanorods (2 mg) and nafion solution (5%, 50 μL) to 1 mL prepared Hb solution with the agitation for 5 h. Finally, 5 μL of the mixture solution was cast onto the surface of a freshly polished GCE to prepare the Hb/Cu_2S NRs/nafion/GCE. When not in use, the dried Hb/Cu_2S NRs/nafion/GCE was stored at 4°C in a refrigerator.

Extraction of polyphenols

In brief, 1 g of dried sample leaves powder was added to 50 mL ethanol/water (1:1), then centrifuged for 10 min at 4 °C. The supernatants were filtered using Whatman No. I filter paper, followed by 0.045-μm microbial filter according to a published procedure [39]. The filtrates were condensed with a rotary evaporator under reduced pressure below 40 °C and freeze-dried. The ethanol extract of the plant leaves was concentrated separately under vacuum using the rotary evaporator.

Results and discussion

Structural and morphology characterization of Cu_2S nanorods

The crystal structure and phase composition of the obtained Cu_2S nanorods were firstly analyzed by XRD. As can be seen from Fig. 1a, the diffraction peaks of 27.61, 31.75, 47.96, and 52.79 at 2θ angles with the crystal plane of (111), (200), (220), and (311) were corresponded with the cubic phase Cu_2S (JCPDS card, no. 53–0522). The SEM image in Fig. 1b indicated that the as-prepared Cu_2S owned the

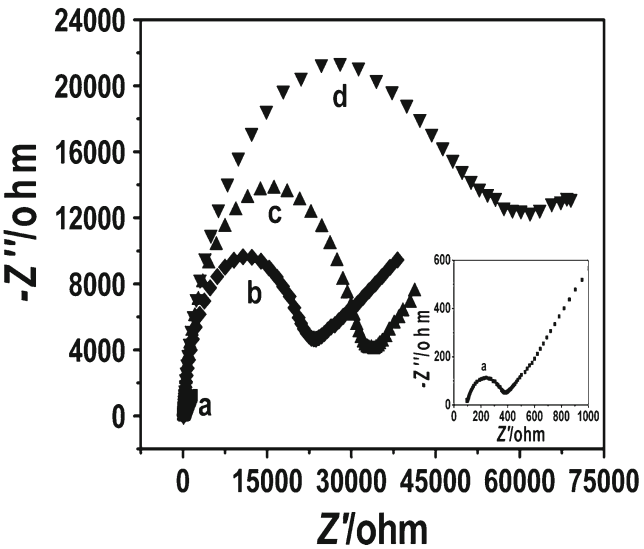


Fig. 2 Electrochemical impedance spectra of bare GCE (**a**), Cu_2S NRs/GCE (**b**), Hb/Cu_2S NRs/GCE (**c**), and Hb/Cu_2S NRs/nafion/GCE (**d**) in 5.0 mM $Fe(CN)_6^{3-/4-}$ (1:1) containing 0.1 M KCl with the frequencies swept from 10^5 to 10^{-1} Hz. *Inset* electrochemical impedance spectra of bare GCE

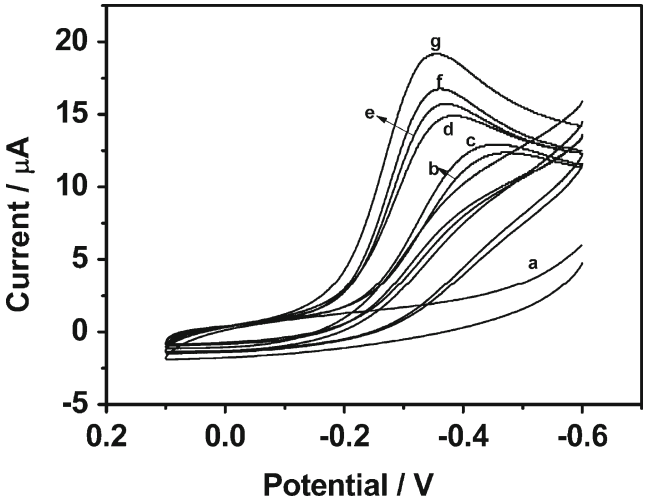


Fig. 3 Cyclic voltammograms of bare GCE (**a**), Hb/GCE (**b**), Hb/nafion/GCE (**c**), Hb/Cu_2S NRs/nafion/GCE (**d**), and Hb/Cu_2S NRs/nafion/GCE (*e–g*) in absence and presence of 50 μM catechol (**e**), 20 μM H_2O_2 (*f*), 50 μM catechol, and 20 μM H_2O_2 (**g**) in 0.1 M PBS (pH 7.0)

structure of nanorods with the average diameter of around 400 nm and length of about 7 μm.

Characterization of the modified electrodes

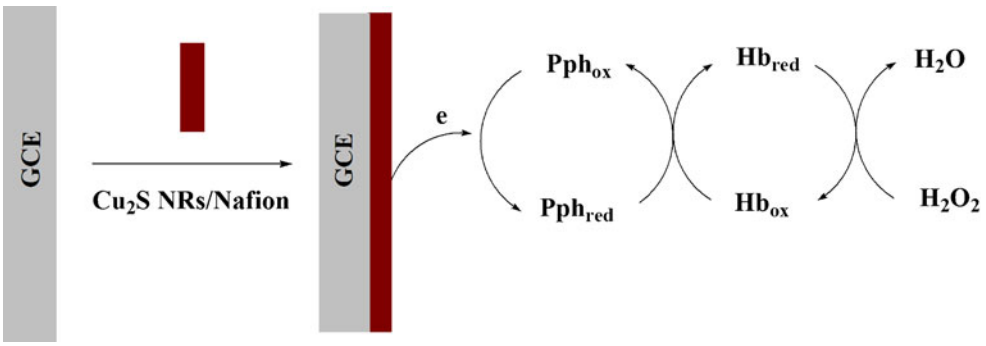
Electrochemical impedance spectroscopy was carried out to characterize the process of surface-modified electrodes using $Fe(CN)_6^{3-/4-}$ redox couples as the electrochemical probes. Figure 2 showed the typical Nyquist plot of bare GCE (a), Cu_2S NRs/GCE (b), Hb/Cu_2S NRs/GCE (c), and Hb/Cu_2S NRs/nafion/GCE (d). A higher R_{et} of Cu_2S NRs/GCE (b) was observed compared with the bare GCE (a), indicating that the Cu_2S NRs were successfully modified on the surface of the electrode. When the electrode was coated with Hb/Cu_2S NRs (c), the positively charged Hb attracted $Fe(CN)_6^{3-/4-}$. But this effect was canceled by the bulky volume of the Hb molecules, which blocked further the access of electrode surface to the redox pair $Fe(CN)_6^{3-/4-}$, leading to a higher R_{et} of Hb/Cu_2S NRs/GCE. After Hb/Cu_2S NRs/nafion film (curve d) was modified on the bare GCE, an obvious increase in R_{et} was attained, which indicated that the presence of nafion could limit the electron transfer. All these results illustrated the successful immobilization of Hb/Cu_2S NRs/nafion on the surface of the electrode.

Characterization of electrochemical behavior of electrodes

The linear sweep voltammograms of the modified electrodes in 0.1 M PBS (pH 7.0) in the presence of different solutions were shown in Fig. 3. As can be seen, there was a small reduction peak at Hb/GCE (b) and Hb/nafion/GCE (c), and an obvious reduction peak at −355 mV (vs. SCE) was observed when immobilizing Hb/Cu_2S NRs/nafion on the surface of the bare GCE (d), while no peak appeared in the selected potential range in 0.1 M PBS (pH 7.0) at bare GCE (a). This phenomenon showed that Hb maintained high activity on Cu_2S/nafion/GCE. The reason may be that Cu_2S/nafion composite film provided a biocompatible microenvironment for Hb. The results of UV–Vis analysis (Fig.S1, Support information) further confirmed this point. When only catechol was added to the solution (e), no obvious change in reduction current appeared for this Hb-modified electrode. After H_2O_2 was added to the solution (f), an increase in reduction current was gained, which was attributed to the catalytic reduction of H_2O_2 on the electrode. There appeared a larger reduction current (g) than (e, f) in the presence of both catechol and H_2O_2, which was due to that the heme group of Hb was oxidized by H_2O_2, then followed of its reduction by the phenolic compounds. And in this process, phenolic compounds were mainly converted into free radical products and could be electrochemically reduced on the electrode surface. In a word, the presence of H_2O_2 enhanced the sensitivity for polyphenols detection in this kind of biosensor.

The principle of the Hb-modified electrodes for polyphenols detection

The Hb biosensors show a response mechanism to polyphenols called double displacement mechanism in which two substrates, H_2O_2 and phenolic compounds, are involved [28]. The mechanism can be expressed as Scheme 1. At the electrode surface, Hb molecules are oxidized by H_2O_2, followed by its reduction by the phenolic compounds. The latter reaction



Scheme 1 Schematic representation of phenol detection using hemoglobin. Pph_{ox} and Pph_{red} are the oxidized and reduced forms of the polyphenolic compounds, respectively

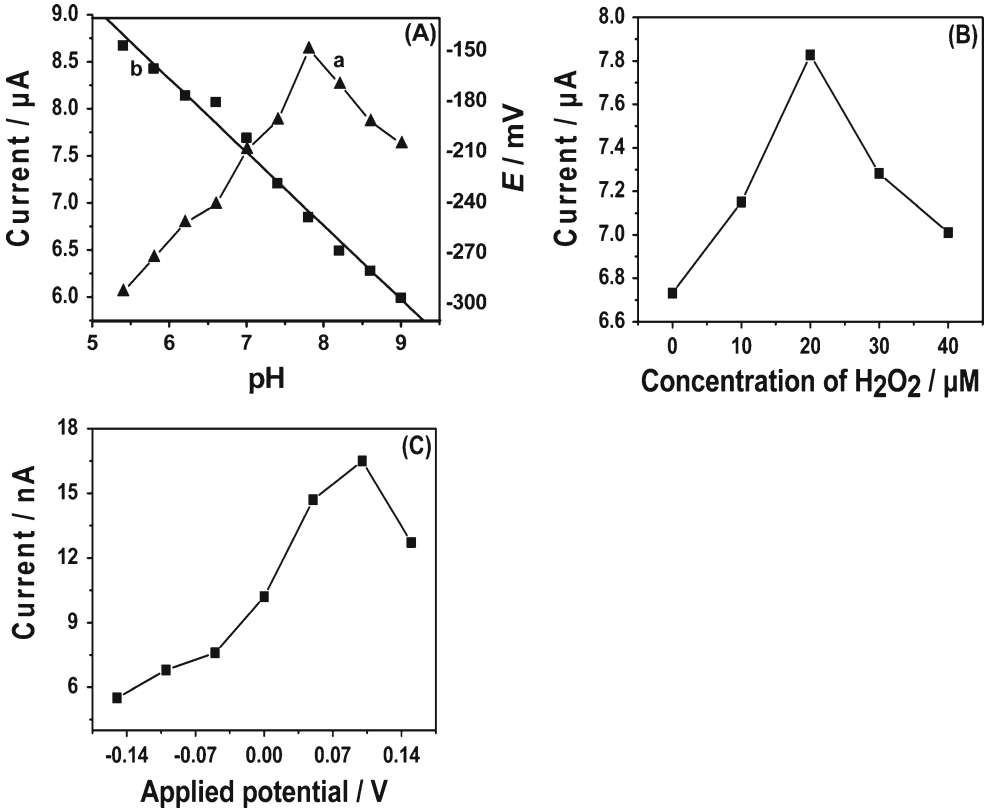


Fig. 4 Effect of pH on the reduction peak current (*a*) and reduction peak potential (*b*) in 0.1 M PBS containing 50 μM catechol and 20 μM H_2O_2 (**a**), H_2O_2 concentration on the biosensor response for polyphenols in PBS containing 50 μM catechol (**b**), applied potential on the biosensor response in the presence of 50 μM catechol and 20 μM H_2O_2 in 0.1 M PBS (**c**)

converts phenolic compounds into free radical products, which are electroactive and can be electrochemically reduced on the electrode surface. The reduction current is proportional to the phenolic compounds concentration in the solution.

Optimization of experimental parameters

The effect of the Hb/Cu_2S NRs/nafion biosensor depending on the solution pH ranging from 5.4 to 9.0 was studied in 0.1 M PBS containing 50 μM catechol and 20 μM H_2O_2. As can be seen from Fig. 4a, the reduction peak current changed gradually with an increasing pH and reached the maximum response at pH 7.8. This result also proved that the Cu_2S NRs/nafion matrix did not destroy the structure of Hb. In order to obtain maximum sensitivity, pH 7.8 was chosen in subsequent experiments. With the increase of pH, the reduction peak potential (E_p) shifted negatively, and a linear equation could be expressed as

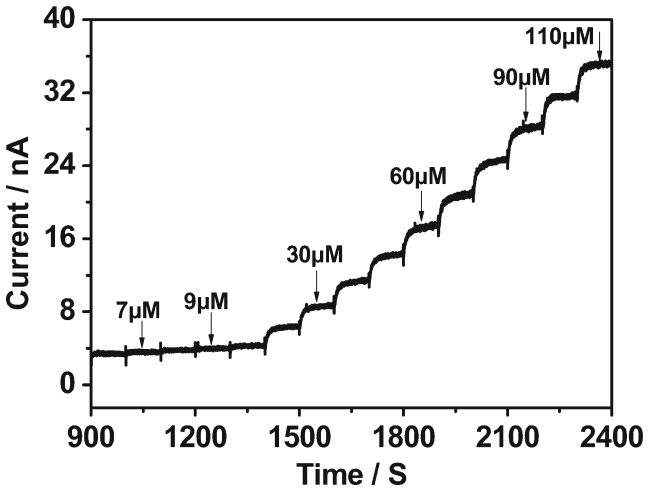


Fig. 5 Current–time curve obtained at the Hb/Cu_2S NRs/nafion/GCE measured for different concentrations of catechol with each step added with 10 μM and H_2O_2 in 0.1 M PBS (pH 7.8) at an applied potential = 100 mV vs. SCE

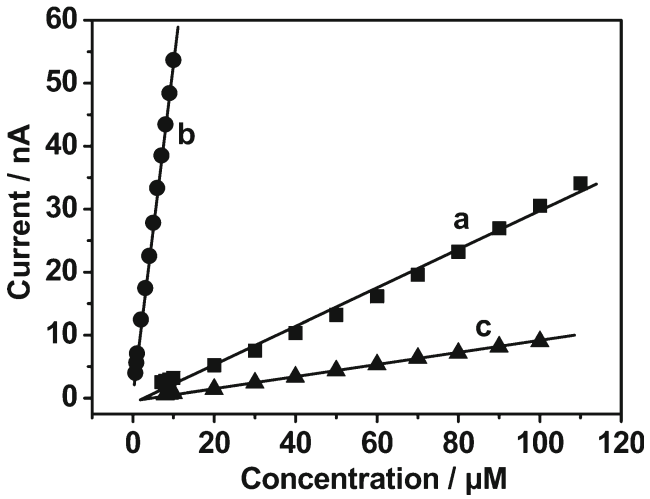


Fig. 6 Calibration curve of Hb/Cu_2S NRs/nafion/GCE for catechol (**a**), hydroquinone (**b**), and resorcin (**c**) in 0.1 M PBS (pH 7.8). Applied potential = 100 mV vs. SCE

Table 1 Estimation of total polyphenols contents in extracts of three different plants by Hb/Cu_2S NRs/nafion/GCE biosensor

Sample	Contained (μmol/L)	Added (μmol/L)	Found (μmol/L)	Recovery (%)	R.S.D. (%)[a]
Mulberry leaves	5.8	15	21.2±1.3	101.9±6.3	2.9
Cherry leaves	4.6	15	18.6±0.8	94.9±4.1	3.6
Jujube tree leaves	3.0	15	17.3±0.2	96.1±1.1	5.1

[a]R.S.D. (%) calculated from five separate measurements

E_p=95.56−43.60 pH (R=−0.9936, E_p in millivolts). A slope of −43.60 mV/pH with correlation coefficient of −0.9936 was obtained, which was smaller than the theoretical value of −59.16 mV/pH at 18 °C for a single proton transfer coupled to reversible single electron transfer [40]. It may attributed to the influence of the protonation states of trans ligands to the heme iron and amino acids around the heme, or the protonation of the water molecules coordinated to the central iron [41].

The H_2O_2 concentration is very important in Hb reactions to produce excellent sensitivity and it is necessary to avoid the inactivation of the enzyme due to high concentration of H_2O_2 [28]. The dependence of H_2O_2 concentration to the biosensor response for a fixed concentration of catechol was discussed and shown in Fig. 4b. As could be seen, the electrode response increased with the increase of H_2O_2 concentration and reached the maximum at 20 μM and then decreased slightly as the concentration of H_2O_2 increased more. Thus, the minimum ratio of [catechol]/[H_2O_2] was fixed as 5:2 to warrant an enough substrate concentration without forming the inactive enzyme throughout the study.

The applied potential dependence of the enzyme electrode for current response was studied and shown in Fig. 4c. The maximum current was obtained at a potential of 0.1 V versus SCE. As can be seen, sensitivity current response decreased as applied potential decreased, which can be attributed to the formation of a high formal oxidation state of Hb, and the Hb molecules can be inactivated when at more negative applied potential. Taking high sensitivity of the enzyme biosensor into consideration, 0.1 V was selected as the working potential in further experiment.

Amperometric response of the biosensor

Figure 5 illustrated the typical current–time recording of the modified biosensor obtained for successive additions of catechol and H_2O_2 at 0.1 V under continuous stirring. With the increase of catechol and H_2O_2 concentration, the enzyme biosensor exhibited a rapid change in reduction current response and reached 95% of steady-state current within 8 s. Figure 6 displayed the calibration curve of Hb/Cu_2S NRs/nafion/GCE for catechol (a), hydroquinone (b), and resorcin (c) under optimum conditions. The biosensor showed sensitivity of 0.23, 5.23, and 0.10 nA/μM for the substrates in wide linear ranges of 7.0–110, 0.6–10, and 8–100 μM, with detection limits of 0.5, 0.03, and 0.6 μM with a signal-to-noise ratio of 3 and good correlation coefficients of 0.996, 0.999, and 0.999 for catechol, hydroquinone, and resorcin, respectively.

Stability and reproducibility of the enzyme biosensor

The long-term stability of the Hb/Cu_2S NRs/nafion-modified biosensor was monitored by measuring the biosensor response to 50 μM catechol over a month. When not in use, the electrode was stored at 4 °C in a refrigerator. Two weeks later, the biosensor response lost 7% of its initial value. The biosensor still retained 85% of its original response after 30 days, which indicated the good stability of the biosensor.

To study the fabrication reproducibility, five electrodes made independently by the same procedure were also examined and a satisfactory relative standard deviation (R.S.D.) of 2.6% for 50 μM catechol was obtained. The reproducibility of

Table 2 Estimation of total polyphenols contents in plants extracts determined by the proposed and the Folin–Ciocalteau (FC) methods using catechol as the standard

Samples	Biosensor method			FC method		
	Total content (μmol/L)	Recovery (%)	R.S.D. (%)[a]	Total content (μmol/L)	Recovery (%)	R.S.D. (%)[a]
Mulberry leaves	5.8	101.9±6.3	2.9	6.1	102.4±3.7	2.4
Cherry leaves	4.6	94.9±4.1	3.6	4.5	95.2±2.5	4.2
Jujube tree leaves	3.0	96.1±1.1	5.1	2.7	98.5±2.4	3.8

[a] R.S.D. (%) calculated from five separate measurements

the same biosensor was also evaluated. The R.S.D. of the biosensor response to 50 μM catechol for eight successive measurements was 2.6%, indicating a good reproducibility.

Application to real samples

The potential applicability of the Hb/Cu_2S NRs/nafion-modified biosensor for the analysis of real samples was studied by estimating the contents of polyphenols in the extracts of three different plants leaves. The samples gathered in the field were determined after a simple pretreatment process to extract the plant polyphenols, then analyzed using catechol as standard. The biosensor showed sensitivity for the real samples and the total polyphenols contents of three different plant leaves were following the order of mulberry leaves > cherry leaves > jujube tree leaves. The results obtained from the present system were shown in Table 1. From the results, it could be seen that the sensor method in our work has good sensitivity and selectivity, and can determine contents of polyphenols in real samples.

Accuracy

To determine the accuracy of the method, the samples were determined by standard Folin–Ciocalteu colorimetry [42]. Total phenolic content in the sample was extrapolated from the standard curve between catechol concentration and absorbance. In the determinations of polyphenols, the catechol was used as standard compound in both methods. Table 2 reports the polyphenols content in mulberry leaves, cherry leaves, and jujube tree leaves samples measured with Folin–Ciocalteau spectrophotometric method and proposed biosensor. These evaluation studies showed that the method was fairly reliable with high recovery and in agreement with the standard method.

Conclusions

In this work, a novel amperometric biosensor was constructed by immobilizing Hb on the surface of Cu_2S NRs and nafion-modified electrode for determining ploypheno ls in the presence of H_2O_2. The suggested nanostructured inorganic–organic hybrid material formed by Cu_2S NRs and nafion could not only immobilize Hb firmly on the surface of the electrode and keep the biological activity of Hb but also enhance the amperometric detection for polyphenols. The proposed biosensor exhibited good sensitivity, wide linear ranges, excellent stability, high reproducibility, and short response time. Furthermore, the biosensor was also appropriate for the quantitative determination of polyphenols in natural extracts after a simple pretreatment of the samples. The developed Hb/Cu_2S NRs/nafion/GCE biosensor will be a new kind of amperometric biosensor for ploypheno ls detection.

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