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ORIGINAL PAPER

Development of an amperometric sulfite biosensor based on a gold nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline-modified gold electrode

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Abstract A sulfite oxidase (SOx) purified from leaves of *Syzygium cumini* (Jamun) was immobilized covalently onto a gold nanoparticles (AuNPs)/chitosan (CHIT)/carboxylated multiwalled carbon nanotubes (cMWCNTs)/polyaniline (PANI) composite that was electrodeposited onto the surface of a gold (Au) electrode. A novel and highly sensitive sulfite biosensor was developed that used this enzyme electrode (SOx/AuNPs/CHIT/cMWCNT/PANI/Au) as the working electrode, Ag/AgCl as the standard electrode, and Pt wire as the auxiliary electrode. The modified electrode was characterized by Fourier transform infrared (FTIR) spectroscopy, cyclic voltammetry (CV), scanning electron microscopy (SEM), and electrochemical impedance spectroscopy (EIS) before and after the immobilization of the SOx. The sensor produced its optimum response within 3 s when operated at 50 mVs^{-1} in 0.1 M phosphate buffer, pH 7.0, and at 35 °C. The linear range and detection limit of the sensor were 0.75–400 μM and 0.5 μM (S/N=3), respectively. The biosensor was employed to determine sulfite levels in fruit juices and alcoholic beverages. The enzyme electrode was used 300 times over a period of three months when stored at 4 °C.

Keywords Sulfite · Sulfite oxidase · Sulfite biosensor · CHIT · AuNPs · cMWCNT/PANI

Introduction

The determination of sulfite is important from biological and industrial perspectives [1]. Sulfite has been used extensively as a preservative because it is an antioxidant and an inhibitor of enzymatic or microbial activity in some foods, beverages and pharmaceutical products. The US Food and Drug Administration (FDA) requires that any food containing more than 10 mg/kg or beverage containing more than 10 mg/L of sulfite should have warning labels to this effect [2]. Sulfite oxidase (SOx) is a complex metalloprotein containing the molybdenum cofactor (Moco) and a cytochrome b5-type heme in two different domains of the protein, which are connected by a flexible loop region that is known to catalyze the oxidation of sulfite to sulfate. Sulfite oxidase also catalyzes the terminal reaction in the catabolism of sulfur-containing amino acids such as cysteine and methionine in mammals. A lack of functional SOx causes sulfite deficiency, which leads to neurological disorders, mental retardation, physical deformities, dislocated lenses, brain degradation, and early death. Thus, the sulfite level in the body must be tightly regulated. Various techniques for the quantitative determination of sulfite in different types of samples are available, such as iodimetry, spectrophotometry, anodic stripping voltammetry, reciprocal oscillographic chronopotentiometry, ion chromatography, enthalpimetry, chemiluminescence, gravimetry, ion selective electrode, gas chromatography, fluorometry, and HPLC [3–5]. However, these methods are labor-intensive, involve time-consuming sample preparation, and lack precision. Therefore, the development of an enzyme biosensor for the detection of sulfite is of considerable interest, since it would allow the fast, selective, and accurate determination of sulfite without the need for significant sample preparation. A number of sulfite biosensors have been reported, such as those based on a glassy carbon electrode coated with thin mercury film [2], a polytyramine-platinized glassy carbon electrode [6], a polyaniline-sulfonic acid layer [7], polypyrrole films [8], conducting polyaniline film [9], and a

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self-assembled monolayer of 11-mercaptoundecanol cast onto a Au electrode [10]. The disadvantages of these biosensors include a limited detection limit due to poor electron flow and poor storage stability of the enzyme electrode.

Recently, the development of biocompatible nanomaterials has opened up a new direction in the development of third-generation biosensors based on direct electron transfer between the enzyme and the electrode [11]. Multiwalled carbon nanotubes (MWCNTs) possess many useful properties, such as good electrical conductivity, strong adsorptive ability, and excellent biocompatibility [12], and thus have the ability to promote the electron transfer of hydrogen peroxide [13]. Chitosan, the *N*-deacetylated derivative of chitin, is a natural polymer product consisting of a repeating hexosamide residue with one amino group and two hydroxyl groups [14]. Its excellent film-forming ability, good adhesion, and high mechanical strength have led to growing interest in using it to immobilize biomolecules (especially enzymes) in recent years [15], and to construct amperometric biosensors [16]. Recently, metallic alloy NPs have gained considerable interest in the field of catalysis and sensors, as these NPs exhibit better catalytic properties than their nonmetallic counterparts [17]. Gold nanoparticles (AuNPs), a familiar and good biocompatible material, have been used to construct modified electrodes due to their high conductivity and high surface-to-volume ratio, which facilities the binding of biomolecules as well as direct, fast electron transfer in electrochemical reactions [18]. AuNPs can also be used for the electrostatic adsorption of a negatively charged material via the layer by layer (LBL) technique [19]. Polyaniline (PANI) is another attractive conducting polymer, as it exhibits two redox couples and facilitates efficient enzyme–polymer charge transfer [20]. The present report describes, for the first time, the use of the combination of AuNPs and carboxylated multiwalled carbon nanotubes (cMWCNTs)/polyaniline (PANI) in a matrix of the biopolymer chitosan (CHIT) to construct a new sulfite biosensor. Further, the covalent immobilization of enzyme onto a hybrid electrode is likely to overcome the problem of enzyme leakage and thus increase its storage stability. The present sulfite biosensor was also employed to measure sulfite levels in fruit juices and alcoholic beverages.

Experimental

Chemicals and real samples

Sephadex G-100 and DEAE-Sephacel from Sigma (Aldrich), sodium sulfite, chitosan and aniline (MW ~ 1×10^6; 75–85% deacetylation) from SRL (Mumbai, India), and carboxylated multiwalled carbon nanotubes (c-MWCNTs) (functionalized MWCNTs) from Intelligent Materials Pvt. Ltd. (Panchkula, India) were used. All other chemicals used were of analytical reagent (AR) grade. The mature leaves of *Syzygium cumini* (Jamun) plants that grow at the roadside of the new campus of MD University, Rohtak, India, were collected, brought to the laboratory immediately in a ice bath, washed thoroughly with distilled water, dried between folds of filter paper, and stored at −20 °C until use. Fruit juices and alcoholic beverages of various commercial brands were purchased from the local market.

Apparatus

All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (Eco Chemie BV, Utrecht, The Netherlands) with GPES 4.9 software. A modified Au electrode 1.0 mm in diameter, a KCl-saturated Ag/AgCl electrode, and a Pt electrode served as the working, reference, and counter electrodes, respectively, in all electrochemical experiments. All potentials were measured with respect to the reference electrode. All experiments were carried out at room temperature. Scanning electron microscopic (SEM) images were taken at the Department. of Chemistry at MD University, Rohtak, India.

Extraction and purification of sulfite oxidase from leaves of *Syzygium cumini*

Sulfite oxidase from frozen jamun leaves was extracted largely as described in [21], although the leaves were homogenized with cold 0.1 M Tris-HCl (pH 8) in a 3:1 ratio (w/v) in a chilled pestle and mortar. The extract was filtered through a muslin cloth, and the filtrate was centrifuged at 10,000×*g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and treated as crude enzyme. It was tested for sulfite oxidase activity and protein concentration by the Lowry method. The enzyme was purified as previously described [22], using a combination of 0–80% ammonium sulfate precipitation, gel filtration on Sephadex G-100, and ion-exchange chromatography on DEAE Sephacel using a linear gradient of KCl (0.1–0.6 M). The enzyme was purified 57-fold with a yield of 19.5% after the final step. The purified enzyme had a specific activity of 3638.6 unit/mg.

SOx assay

The SOx assay was carried out largely (i.e., with some modifications) as previously described [22], and was

based on the quantification of H_2O_2 generated from the sulfite using a color reaction consisting of 4-aminophenazone, phenol, and peroxidase as the chromogenic system. The reaction mixture, which contained 1.8 mL of 0.1 M Tris-HCl buffer, pH 8.5, and 0.1 mL of the enzyme preparation, was preincubated at 35 °C for 5 min. The reaction was started by adding 0.1 mL of 0.4 mM sodium sulfite. After incubating the mixture at 35 °C for 10 min in the dark, 1.0 mL of color reagent was added, and it was kept at room temperature for 15 min to develop the color. A_{520} was read and the H_2O_2 content generated during the reaction was interpolated from the calculated standard curve between $[H_2O_2]$ and A_{520} in 0.1 M Tris-HCl buffer, pH 8.5.

One unit of enzyme was defined as the amount of enzyme required to catalyze the formation of 1.0 nmol of H_2O_2 due to the oxidation of sulfite per min under standard assay conditions.

Preparation of the color reagent

The color reagent was prepared according to the method of Bais et al. [23], which utilized 50 mg of 4-aminophenazone, 100 mg of solid phenol and 1 mg of horseradish peroxidase per 100 mL of 0.4 M sodium phosphate buffer, pH 7.0. This was stored in an amber-colored bottle at 4 °C and discarded after one week.

Preparation of the chitosan (CHIT) solution

A chitosan solution (0.5 wt %) was prepared by dissolving 0.5 g of CHIT into 100 mL of 2.0 % acetic acid solution and stirring for 3 h at room temperature until complete dissolution was achieved.

Preparation of gold nanoparticles (AuNPs)

Gold nanoparticles were prepared according to the method of McFarland et al. [24] with a minor modification. Twenty milliliters of $HAuCl_4$ solution (1 mg/5 mL) were added to a 50 mL flask on a stirring hot plate. A magnetic stir bar was added and the solution was brought to boiling. To this boiling solution, 2 mL of 1% trisodium citrate dehydrate were added dropwise. A gold sol gradually formed as the citrate reduced the gold. A change in the color of the solution from blackish to wine red confirmed the aggregation of nanoparticles. The colloidal gold solution was then stored in a dark bottle at 4 °C after cooling.

Morphological characterization of the gold nanoparticles was carried out by a transmission electron microscope (TEM) at the Department of Anatomy, AIIMS, New Delhi.

Preparation of the AuNPs/CHIT/cMWCNTs/PANI/Au electrode

Prior to electrodeposition, the Au electrode (1.5×0.05 cm) was polished with alumina slurry. One gram of cMWCNTs was suspended in 1 mL of a 3:1 mixture of concentrated H_2SO_4 and HNO_3 and ultrasonicated for 24 h to get a finely dispersed black-colored solution of cMWCNTs. Aniline (50 μl) was added to 10.0 mL of 1 N HCl and mixed with 1.0 mL of the finely dispersed solution of cMWCNTs in a glass cell. cMWCNTs and aniline were electrodeposited onto the Au electrode through cyclic voltammetry (CV), by immersing the electrode into a solution (20 mL) containing 15 mL of electrolyte (0.1 N KCl) and 5 mL of Tris-HCl buffer (0.1 M, pH 8.5) and then applying 15 polymerization cycles at 0.0–1.5 V, as described previously [25]. The resulting cMWCNTs/PANI/Au modified electrode was washed thoroughly with distilled water to remove unbound matter and kept in a dry petri plate at 4 °C. Two milliliters of AuNPs were then dissolved into 2 mL of 0.5% chitosan solution (2% acetic acid solution). Next, the AuNPs/CHIT was adsorbed onto this electrode by dipping it in this solution for 3 h. Finally, the modified electrode (AuNPs/CHIT/cMWCNTs/PANI/Au electrode) was removed from this solution and dried in air.

Preparation of the enzyme electrode (SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode)

Purified SOx was immobilized covalently onto the AuNPs/CHIT/cMWCNTs/PANI-modified Au electrode surface by placing 100 μl of enzyme solution on it and keeping it at 4 °C overnight to facilitate the covalent immobilization of enzyme. The resulting enzyme electrode was washed in distilled water followed by reaction buffer, dried, and then stored in a refrigerator at 4 °C.

Measuring the response of the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode

All electrochemical characterizations and measurements were carried out using a conventional three-electrode system consisting of a SOx/AuNPs/CHIT/cMWCNTs/PANI-modified Au electrode as the working electrode, a Pt wire as the auxiliary electrode, and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode. Cyclic voltammetric measurements were carried out in a three-electrode cell containing 0.1 mL of sulfite (0.4 mM) and 15 mL Tris-HCl buffer (0.1 M, pH 8.5). Current was measured using cyclic voltammetry (CV) in the potential range between 0 and+0.8 V. To record cyclic voltammograms, the following instrumental parameters were used: step potential +6 mV and scan

rate 50 mV s^{-1}. All electroanalytical measurements were performed at room temperature.

The following electrochemical reactions occurred during the current response measurements of the sulfite biosensor:

$$\text{Sulfite} + O_2 + H_2O \xrightarrow{\text{SOx}} \text{Sulfate} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.47 \quad v} 2H^+ + O_2 + 2e^-$$

$$2e^- \xrightarrow{\text{AuNPs/CHIT/cMWCNTs/PANI}} \text{Au electrode.}$$

Optimization of the SOx/AuNPs/CHIT/cMWCNTs/PANI electrode/Au electrode

To determine the optimum working conditions of the enzyme electrode, the pH of the reaction buffer (0.1 M) was varied from pH 5.0 to 9.5 in steps of pH 0.5 using sodium phosphate buffer between pH 5.0 and 7.0 and Tris-HCl buffer between pH 7.5 and 9.5. Similarly, to determine the optimum temperature, the reaction mixture was incubated at 15–55 °C in steps of 5 °C. The optimum response time was studied by measuring the current at different timescales ranging from 1 s to 12 s. To study the effect of the substrate concentration, different sulfite concentrations in the range 0.75–400 μM were used. K_m and I_{max} for sulfite were calculated from a Lineweaver–Burk plot based on the following equation:

$$\frac{1}{I} = \frac{K_m}{I_{max}} \frac{1}{[S]} + \frac{1}{I_{max}},$$

where K_m is the Michaelis–Menten constant, I_{max} is the maximum current, and S is the sulfite concentration.

The amperometric response was also measured in the presence of potential interfering compounds found in real samples, such as glucose, fructose, ascorbic acid, cysteine, acetic acid, ethanol, and citric acid.

Evaluation

The following criteria were studied to evaluate the performance of this biosensor: linearity, detection limit, analytical recovery, precision, and correlation with the standard method. LOD was determined as the minimum concentration of the substrate (sulfite) required give a signal that can be distinguished by the biosensor from background noise. The signal-to-noise ratio was measured as follows: if the incoming signal strength in microvolts is *Vs*, and the noise level, also in microvolts, is *Vn*, then the signal-to-noise ratio, S/N, in decibels, is given by the formula S/N = 20 $\log_{10}(Vs/Vn)$. A recovery study was performed using alcoholic beverages with two different standard concentrations (20 and 40 μM) of sulfite, and the recovered concentrations measured by the present biosensor were compared with the concentrations added. To test the reproducibility and reliability of the present biosensor, the sulfite levels in six alcoholic beverages were determined five times during a single day (within-batch results), and five times again after storage at −20 °C for one week (between-batch results).

Amperometric measurement of sulfite in fruit juices and alcoholic beverages

Sulfite levels in fruit juices and alcoholic beverages were determined by the present biosensor in the same manner as described above for response measurement under optimal working conditions, except that sulfite was replaced with the sample. The current (μA) was recorded and the amount of sulfite was interpolated from the standard curve between sulfite concentration and current (μA) obtained under the optimal working conditions (Fig. 1).

Results and discussion

Electrode surface characterization by FTIR and SEM

The morphologies of the bare Au electrode, the cMWCNTs/PANI/Au electrode, the AuNPs/CHIT/cMWCNTs/PANI/Au electrode, and SOx immobilized onto the AuNPs/CHIT/cMWCNTs/PANI/Au electrode were studied by SEM. SEM of the bare Au electrode (Fig. 2a) showed a smooth and featureless morphology, whereas the nanocomposites (Fig. 2b and c) displayed stepwise immobilization of PANI, cMWCNTs, AuNPs and SOx onto the Au electrode. Figure 3a displays the FTIR spectra for cMWCNTs/PANI/Au (curve *a*) and SOx/AuNPs/CHIT/cMWCNTs/PANI/Au (curve *b*). The FTIR spectrum of the electrodeposited cMWCNT/PANI composite (curve *a*) shows benzenoid and quinoid ring stretching bands (C=C) at 1447.6 and 1594.2 cm^{-1}. The presence of peaks at 1129 and 3402 cm^{-1} is attributed to B–N(+)=Q stretching and –N–H stretching

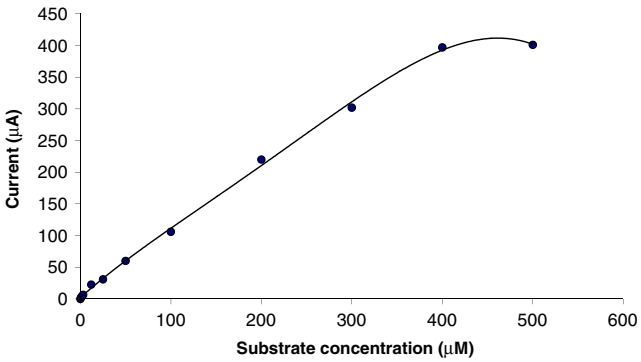


Fig. 1 The calibration curve for the sulfite biosensor

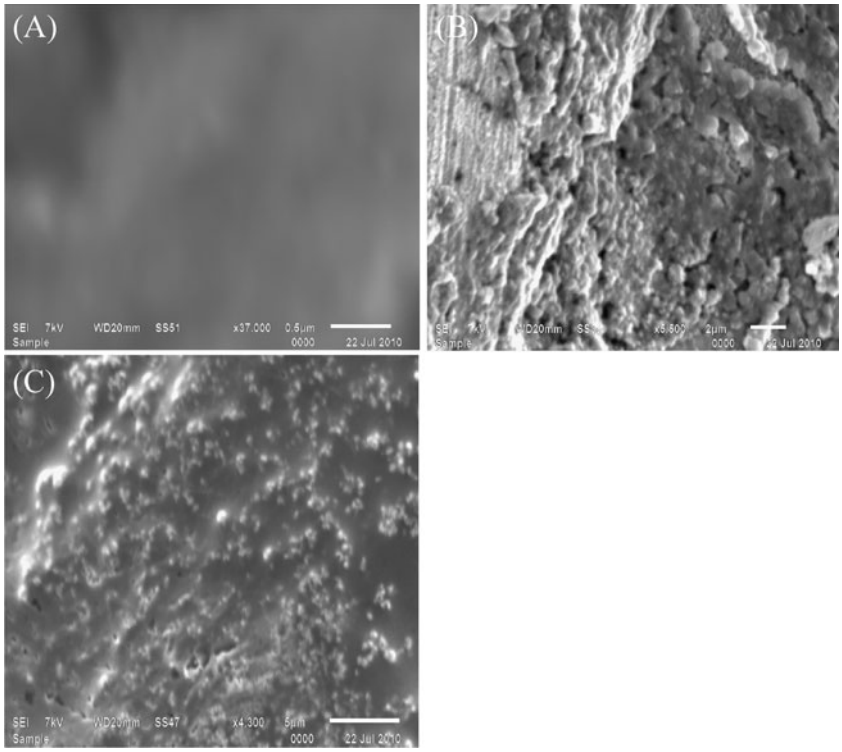


Fig. 2 **A**–**B** Scanning electron micrographs of **A** a bare gold electrode, **B** the cMWCNTs/PANI/Au electrode, and **c** the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode

vibrations of PANI in the composite. The peak at 1704 cm^{-1} is attributed to –C=O stretching vibrations and indicates the presence of a carboxyl group (−COOH). The presence of these peaks reveals the existence of both PANI and cMWCNTs on the Au electrode. The SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode (curve *b*) showed characteristic peaks at 1550 and 1600 cm^{-1} that are attributed to the primary and secondary amide linkages of enzyme molecules. The characteristic absorption band of the amino saccharides in chitosan occurred at 3415 cm^{-1}, which is due to the overlap of –OH and $–NH_2$ stretching bands.

The fabrication of the sulfite biosensor based on AuNPs/chitosan onto the cMWCNT/PANI-modified Au electrode is summarized in Scheme 1. First, PANI was electrodeposited onto the Au electrode, and the $–NH_2$ groups of PANI were linked to the –COOH groups of the cMWCNTs through amide bonds (−CO–NH). The electrodeposition method was used to produce the PANI/cMWCNTs layer on the electrode surface because this method is easy to perform and the layer thickness can be controlled. A positively charged AuNPs/chitosan solution was then adsorbed onto the negatively charged surfaces of the cMWCNTs. Experimental results showed that the AuNPs and cMWCNTs had a significant synergistic effect on the reduction of sulfite. When enzyme is applied onto the AuNPs/CHIT/cMWCNTs/PANI/Au electrode, amide bonds (−CO–NH–) are formed between NH_2 groups of the enzyme and free –COOH groups of the cMWCNTs. The formation of –CO–NH– bonds was observed using FTIR; see the peaks for amide linkages that occur at 1500 and 1600 cm^{-1} in curve *b* of Fig. 3a.

Figure 3b shows cyclic voltammograms (CVs) of Au electrodes in a solution containing 0.1 M KCl solution. The bare Au electrode (curve *a*) shows no redox peak, while the cMWCNTs/PANI/Au electrode (curve *b*) shows enhanced peak current and typical catalytic current. CV of the cMWCNTs/PANI/Au electrode revealed two redox peaks for the oxidation of aniline to a radical cation and then to a radical dication. The deposition of AuNPs/CHIT film onto the cMWCNTs/PANI-modified Au electrode surface leads to increased current intensity due to the increased electroactive area.

Response to sulfite at the AuNPs/CHIT/cMWCNTs/PANI/Au electrode

To evaluate the catalytic activity of sulfite oxidase at the AuNPs/CHIT/cMWCNTs/PANI/Au electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of sulfite in the potential range from 0 to +0.6 V. Figure 3c shows cyclic voltammograms of the AuNPs/CHIT/cMWCNTs/PANI/Au electrode in an unstirred 0.1 M KCl solution (20 mL) and 0.1 M Tris-HCl buffer, pH 8.5 (5 mL), with (curve *a*) and without (curve *b*) 0.4 mM sulfite solution at a scan rate of 50 mVs^{-1}. When 0.4 mM sodium sulfite was added, well-defined oxidation (0.47 V) and reduction

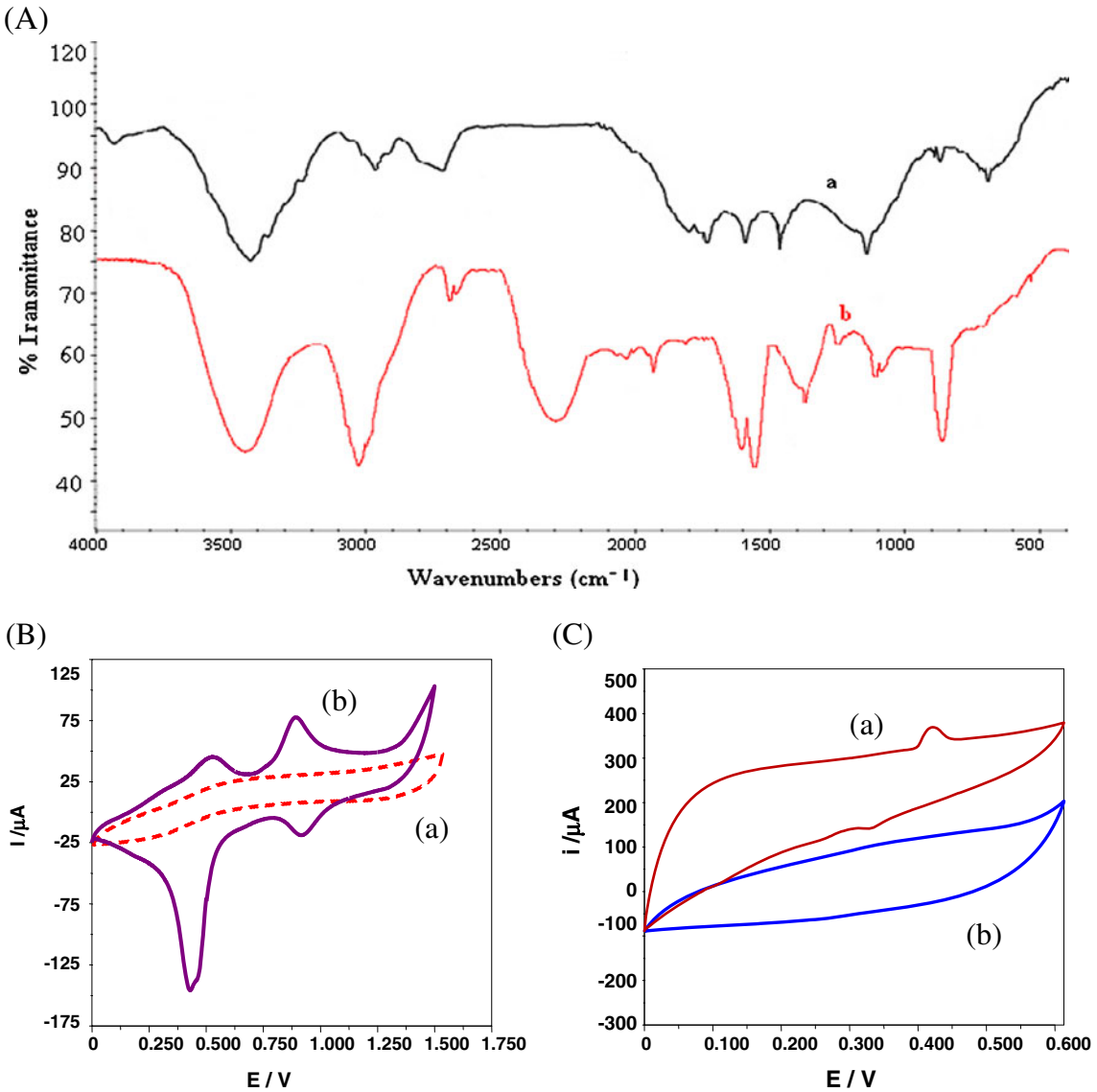
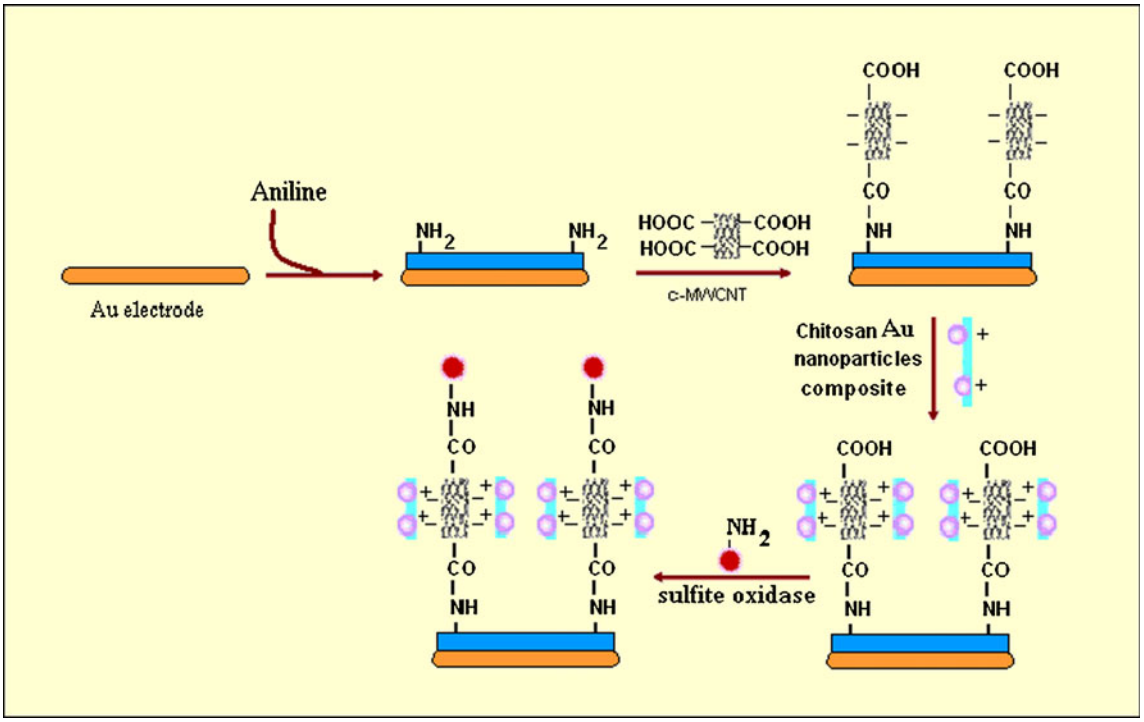


Fig. 3 **A** FTIR spectra of the cMWCNTs/PANI/Au electrode (*a*) and the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode (*b*). **B** Cyclic voltammograms of the bare gold electrode (*a*) and the electrochemical deposition of PANI/cMWCNTs onto the Au electrode (*b*). **C** Cyclic voltammetric (CV) curves of the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode in 0.1 M Tris-HCl buffer (pH 8.5) with (*a*) and without (*b*) 0.4 mM sodium sulfite solution. Supporting electrolyte: 0.1 M KCl solution; scan rate: 50 mVs^{-1}

peaks (0.35 V) (curve *a*) were observed, which clearly indicate the catalytic properties of the modified electrode.

Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) provides useful information on the impedance changes at the electrode surface during the fabrication process. The semicircular portion at higher frequencies corresponds to the electron-transfer-limited process, and its diameter is equal to the electron transfer resistance, which controls the electron transfer kinetics of the redox probe at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process [26]. Figure 4 displays the Nyquist plots of the EIS of the cMWCNTs/PANI/Au electrode (a) and the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode (b) in 0.1 M KCl. The Nyquist diameter (RCT) of the cMWCNTs/PANI/Au [curve (*a*)] was 1060 Ω, and this increased to 1580 Ω for the SOx/AuNPs/CHIT/cMWCNT/sPANI/Au electrode [curve (*b*)]. This increase in the RCT is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (<10 kHz), and thus hinder the electron transfer. These results indicate the binding of SOx onto the AuNPs/CHIT/cMWCNTs/PANI/Au composite.



Scheme 1 Scheme for the electropolymerization of AuNPs/CHIT/cMWCNTs/PANI onto an Au electrode, and the chemical reaction for the immobilization of enzyme (sulfite oxidase) on the modified electrode

Optimization of the experimental conditions for the biosensor

To determine the optimum response conditions for the biosensor (SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode), the working potential, pH, temperature, and scan rate were studied. The working potential was stepped up from 0 to 0.8 V. The steady-state current response increased with the working potential from 0 to 0.6 V, and then reached a plateau from 0.6 to 0.8 V. Therefore, 0.6 V was chosen as the working potential for the amperometric determination of the sulfite concentration. The effect of the pH of the reaction buffer on the biosensor response was studied in the pH range 5.0–7.0 for a 0.4 mM sodium sulfite solution using 0.1 M sodium phosphate buffer, and the pH range 7.5–9.5 using 0.1 M Tris buffer. The current response resulting from the enzyme-catalyzed reaction was maximized at pH 7.0, which is lower than that for SOx bound to polytyramine on a glassy carbon electrode (pH 7.5–8.5) [6], polyaniline on an aluminum surface (8.5) [27], a screen-printed strip-modified carbon electrode (pH 7.5) [28], and is comparable to that of SOx bound to a glassy carbon-coated thin mercury film (pH 7.0) [2], and polypyrrole film (pH 7.0) [8]. Therefore, a pH of 7.0 was used in subsequent

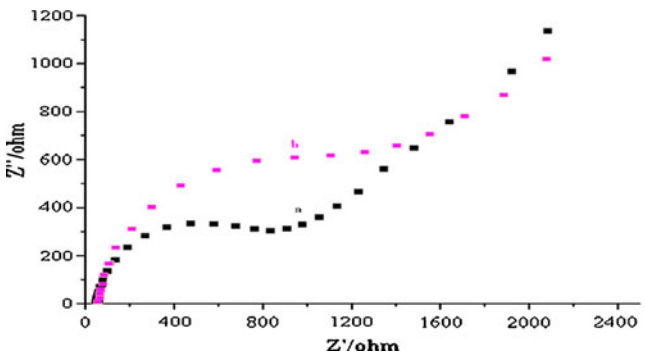


Fig. 4 Nyquist plots comparing the EIS of the cMWCNTs/PANI/Au electrode (*a*) and the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode (*b*) in 0.1 M KCl

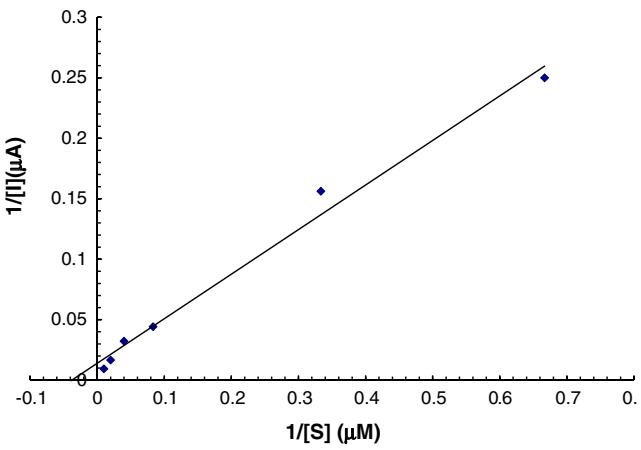


Fig. 5 Lineweaver–Burk plot for the effect of sulfite concentration on the response of the biosensor based on the AuNPs/CHIT/cMWCNTs/PANI/Au electrode

Table 1 A comparison of the analytical characteristics of the present sulfite biosensor with those of previously reported amperometric sulfite biosensors

Properties	Dinckaya et al. [2]	Ameer and Adelozu [8]	Spricigo et al. [7]	Bahmani et al. [9]	Kalimuthu et al. [10]	Present work
Support for immobilization	GC coated with thin Hg film	PPy	PASA-modified Au electrode	Conducting PANI film	SAM of 11-mercaptoundecanol cast onto an Au electrode	AuNPs/CHIT/ cMWCNTs/ PANI/Au electrode
Method of immobilization	Crosslinking	Entrapment	Adsorption	Entrapment	Adsorption	Covalent
Optimum pH	7	7	8.5	8.5	8	7
Optimum temperature	40 °C	ND	ND	35 °C	ND	35 °C
Response time	3 min	120 s	2 min	ND	3 s	3 s
Linearity (μM)	200–2800	0.9–400	I–60	6–500	1–6	0.75–400
Detection limit (μM)	317	0.9	1	2	0.000044	0.5
Michaelis constant (μM)	ND	ND	77	365	43	25.6
Stability (days)	13	30	60	ND	14	90

PPy polypyrrole, *PASA* polyaniline sulfonic acid, *SAM* self-assembled monolayer
PANI polyaniline, *c-MWCNT* carboxylated multiwalled carbon nanotubes, *ND* not detected

experiments. The optimum temperature of the biosensor was 35 °C, which is lower than that for a glassy carbon-coated thin mercury film (40 °C) [2]. As polyaniline is a good thermal insulator for enzymes, it may have provided a suitably protective microenvironment that insulated the enzyme from environmental and thermal variations, thus favoring the repeated use of the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode [12]. The optimum response of the present electrode was observed within 3 s. The effect of varying the scan rate from 10 to 100 mVs^{-1} on the biosensor response using 0.4 mM sodium sulfite in a 0.1 M Tris buffer solution (pH 8.5) was also investigated. A potential scan rate of 50 mVs^{-1} was selected, since the highest analytical signal and very good cyclic voltammogram profiles were obtained using this scan rate.

An amperometric method for determining sulfite in biological materials was developed using the present biosensor. The method has advantages over the standard colorimetric reference method in terms of its simplicity, rapidity, better sensitivity and specificity.

Table 2 Sulfite levels in different brands of fruit juices and alcoholic beverages as measured by the SOx/AuNPs/CHIT/cMWCNTs/PANI/Au electrode

Samples	Sulfite level (μM) Mean±SD* (n=4)
1. Fruit juices	
a) Pineapple	21.4±0.3
b) Orange	19.2±0.3
c) Guava	17.8±0.2
d) Mango	19.8±0.3
e) Lime	24.1±0.2
f) Lychee	18.5±0.3
2. Alcoholic beverages	
a) Officer's Choice	80.4±0.3
b) Royal Challenge	73.5±0.4
c) Aristocrat	78.2±0.2
d) Bagpiper	83.7±0.5
e) Bonnie	77.2±0.5
f) Vodka	68.6±0.4

* *SD* standard deviation

Evaluation of biosensor

Linearity

There was a linear relationship between the current (in μA) and the sulfite concentration in the range 0.75–400 μM (Fig. 1), which is a better linear range than those of earlier biosensors based on SOx immobilized onto a polytyramine-platinized glassy carbon electrode (2–300 μM) [6] or onto polyaniline-sulfonic acid (1–60 μM) [7], but this linear range is comparable to that seen for SOx immobilized onto polypyrrole films (0.9–400 μM) [8]. K_m for sulfite was 25.6 μM and I_{max} was 72.7 μA (Fig. 5).

Detection limit

The detection limit of the present sensor was 0.5 μM (S/N= 3), which is lower than that for enzyme immobilized onto a polytyramine-platinized glassy carbon electrode (2 μM) [6], polyaniline-sulfonic acid (1 μM) [7], and polypyrrole films (0.9 μM) [8]. The normal serum sulfite concentration has been found to lie between 0 and 10 μM by an HPLC

method [29]. This sulfite concentration rises rapidly five- to tenfold on the consumption of sulfite-containing beverages (e.g., wine). As the detection limit of the present sensor is 0.5 μM, it can be used to detect the consumption of wine.

Analytical recovery

The average recoveries of sulfite added to wine (at levels of 20 and 40 μM) varied from 95.40 to 97.56%, demonstrating the satisfactory accuracy of the present biosensor.

Precision

Within-sample and between-sample coefficients of variation (CVs) for the determination of sulfite in wine samples on the same day and after one week of storage were 2.2 and 4.3%, respectively. These high precisions highlight the good reproducibility and consistency of the present method, which can be attributed to the excellent immobilization of SOx onto the AuNPs/CHIT/cMWCNTs/PANI/Au electrode.

Correlation

The sulfite levels in 15 beverage samples, as measured by the present method (y), closely matched those obtained by the standard 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) method (x), as indicated by the good correlation (r=0.96) of the regression equation $y=0.9635x - 3.828$. These results indicate that the present biosensor gives highly accurate results for real samples.

A comparison of the analytical characteristics of the present sulfite biosensor with those of previously reported amperometric biosensors is summarized in Table 1.

Interference

The effect of the addition of possible interferents—ascorbic acid (4.8 μM), cysteine (0.24 μM), fructose (60 μM), glucose (22.2 μM), citric acid (100 μM), ethanol (70 μM), and glutamine (85 μM)—on the current response of the present biosensor was studied. Negligible interference was observed from these compounds (a decrease of 6.13% for glutamine, 5.76% for glucose, 5.26% for citrate, 4.21% for fructose, 3.76% for cysteine, and 2.35% for ascorbic acid). The decrease in the current in the presence of interferents may be due to the ionization of the interferent at high voltage, which might cause interference.

Stability and reusability of the biosensor

To examine the long-term storage stability of the biosensor, the change in the activity of the enzyme electrode over time was monitored. After each experiment, the electrode was washed with buffer solution. The electrode lost 30% of its initial activity after 300 uses over the course of three months when stored at 4 °C, which is much better than previously reported biosensors [7, 28], due to the covalent coupling of the enzyme.

Determination of sulfite in fruit juices and alcoholic beverages

Sulfite levels as measured by the present biosensor ranged from 17.8 to 24.1 μM in fruit juices and from 68.6 to 82.7 μM in alcoholic beverages (Table 2). These values are comparable to those stated in earlier reports [30].

Conclusion

The use of an AuNPs/CHIT/cMWCNTs/PANI-modified Au electrode resulted in a sulfite biosensor with improved analytical performance in terms of its low response time (3 s), high storage stability (90 days), and lack of interference from various potential interferents. However, the detection limit of the present sensor is no better than a previously reported biosensor [10], although it could be improved by modifying the matrix. These advantages indicate that this biosensor based on sulfite oxidase could be applied successfully to the determination of sulfite in fruit juices and alcoholic beverages.

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