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Amperometric determination of total phenolic content in wine by laccase immobilized onto silver nanoparticles/zinc oxide nanoparticles modified gold electrode

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ABSTRACT

A method is described for construction of a highly sensitive amperometric biosensor for measurement of total phenolic compounds in wine by immobilizing laccase covalently onto nanocomposite of silver nanoparticles (AgNPs)/zinc oxide nanoparticles (ZnONPs) electrochemically deposited onto gold (Au) electrode. Scanning electron microscopy, X-ray diffraction, and electrochemical impedance spectroscopy were applied for characterization of the surface morphology of the modified electrode, and cyclic voltammetry was used to investigate the electrochemical properties of the proposed electrode toward the oxidation of guaiacol. The linearity between the oxidation current and the guaiacol concentration was obtained in a range of 0.1 to 500 μM with a detection limit of 0.05 μM (signal-to-noise ratio (S/N) = 3) and sensitivity of 0.71 μA μM^{-1} cm^{-2}. The electrode showed increased oxidation and reduced reduction current with the deposition of AgNPs/ZnONPs on it. R_{CT} values of ZnONPs/Au, AgNPs/ZnONPs/Au, and laccase/AgNPs/ZnONPs/Au electrode were 220, 175, and 380 Ω, respectively. The biosensor showed an optimal response within 8 s at pH 6.0 (0.1 M acetate buffer) and 35 °C when operated at 0.22 V against Ag/AgCl. Analytical recovery of added guaiacol was 98%. The method showed a good correlation ($r = 0.99$) with the standard spectrophotometric method, with the regression equation being $y = 1.0053x - 3.5541$. The biosensor lost 25% of its initial activity after 200 uses over 5 months.

Determination of total phenolic content, commonly called tannins in wine samples, is essential to measure their antioxidant properties, which definitely have a positive effect on human health. The phenolic content of wines depends on geographical and atmospheric factors as well as on different production systems. Normally, the content of phenolic compounds varies from 2 to 5 g/L on average in red wines and 0.1 g/L in white wines. Hence, it is of great importance to characterize a wine by its total phenolic content. A number of commonly used analytical methods are available for qualitative and quantitative determination of phenolic compounds such as spectrophotometry, gas chromatography, liquid chromatography, capillary electrophoresis, gas chromatography–mass spectroscopy (GC–MS),[1] and high-performance liquid chromatography. However, these methods require time-consuming sample preparation, expensive and unportable equipment, and a skilled person to operate [1]. Nevertheless, amperometric biosensors have advantages such as low-cost instrumentation, fast analysis, and improved sensitivity and selectivity. Therefore, there is an interest in developing a simple, sensitive, and rapid method such as biosensors for determination of phenolic compounds. A number of phenol biosensors have been developed in the past employing redox enzymes such as tyrosinase, peroxidase, and laccase (lac) [2]. Because laccase does not require hydrogen peroxide (H_2O_2) as a cosubstrate or any other cofactors for its catalytic action, the construction of laccase-based biosensors is much easier. A number of phenolic biosensors based on immobilized laccase have been reported [3,4], but most of them suffered from leakage of enzyme due to noncovalent immobilization of enzyme. Recently, analytic performance of biosensors has been improved using nanomaterials [5–7]. Nanostructure metal oxides are known to have a unique ability to promote faster electron transfer kinetics between electrode and the active site of the desired enzyme [8–10]. Among the metal oxide nanoparticles, zinc oxide nanoparticles (ZnONPs) have been exploited as a potential material for biosensing because of their unusual properties, namely high surface area for strong adsorption (high isoelectric point of 9.5), good biocompatibility, chemical stability, nontoxicity, and high electron communication. Nanoporous ZnONPs also enhance the surface area for strong adsorption of biomolecules [11,12]. Recent reports

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[1] *Abbreviations used:* GC–MS, gas chromatography–mass spectroscopy; lac, laccase; H_2O_2, hydrogen peroxide; ZnONP, zinc oxide nanoparticle; AgNP, silver nanoparticle; Au, gold; GA, glutaraldehyde; $Zn(NO_3)_2{\cdot}4H_2O$, zinc nitrate; DW, distilled water; CV, cyclic voltammetry; Pt, platinum; SEM, scanning electron microscopy; FTIR, Fourier transform infrared; XRD, X-ray diffraction; NaOH, sodium hydroxide; SAM, self-assembled monolayer; EIS, electrochemical impedance spectroscopy.

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indicate that silver nanoparticles (AgNPs) exhibit a catalytic activity for the reduction of H_2O_2 [13]. To the best of our knowledge, no attempts have been made toward the development of a phenolic biosensor based on this combination of nanocomposite (AgNPs/ZnONPs). Here we describe the construction of AgNPs/ZnONPs nanocomposite modified gold (Au) electrode and then covalent immobilization of laccase on this modified Au electrode for determination of total phenolic content in wine samples.

Materials and methods

Reagents

Sephadex G-100 and DEAE–Sephacel from Sigma–Aldrich (St. Louis, MO, USA); guaiacol, aniline, and cysteamine from SRL (Mumbai, India); glutaraldehyde (GA) from Merck India (Mumbai); and zinc nitrate ($Zn(NO_3)_2{\cdot}4H_2O$) and silver nitrate ($AgNO_3$) from Fluka (Mumbai) were used. All other chemicals used were of analytical reagent grade. Red wines of various commercial brands were purchased from local markets. Double distilled water (DW) was used throughout the study.

Apparatus

Cyclic voltammetry (CV) and amperometric measurements were carried out in a potentiostat/galvanostat (AUT83785, Autolab, Eco Chemie, Netherlands) with a conventional three-electrode system consisting of a modified Au electrode (diameter of 1 mm) as the working electrode, Ag/AgCl electrode as the reference electrode, and platinum (Pt) electrode as the counter electrode. All experiments were carried out at room temperature (30 °C). Scanning electron microscopy (SEM) images at different stages of construction of electrode were taken at Advanced Instrumentation Research Facility (Jawaharlal Nehru (J.N.) University, New Delhi, India) with a scanning electron microscope (Joel JSM-6510, Japan). Fourier transform infrared (FTIR) spectroscopy was performed with an FTIR spectrometer (iS10, Thermo Electron, USA). X-ray diffraction (XRD) studies of ZnONPs and AgNPs were carried out in the Department of Physics at Guru Jambheshwar (G.J.) University (Hisar, India) using an X-ray diffractometer (Rigaku Mini Flex II, Americas Corporation).

Purification of laccase

The cell-free extract of *Ganoderma* sp. Rckk02 grown in malt extract broth (MEB) [14,15] was used as crude laccase. The crude enzyme was purified using the method of Satyapal and Pundir [16] and consisted of 0 to 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) at 4 to 10 °C.

Assay of laccase

Assay of laccase was based on the oxidative polymerization of guaiacol [14].The reaction mixture contained 0.2 μmol of guaiacol, 230 μmol of acetate buffer (pH 5.0), and enzyme (4 μg protein) in a total volume of 2.5 ml. Change in absorbance of 0.01 min^{-1} ml^{-1} at 470 nm was studied. One enzyme unit was defined as

$$\text{Unit activity} = \frac{A_{470}/\text{min.} \times \text{total volume of reaction mixture}}{\text{extinction coefficient of guaiacol} \times \text{volume of enzyme}} \quad (1)$$

Protein content in various enzyme preparations was determined by the Lowry method using bovine serum albumin as standard protein.

Preparation of ZnONPs

ZnONPs were prepared according to the method described by Moghaddam and coworkers [17]. A 0.45-M aqueous solution of $Zn(NO_3)_2{\cdot}4H_2O$ and a 0.9-M aqueous solution of sodium hydroxide (NaOH) were prepared in DW. The beaker containing NaOH solution (100 ml) was heated at 55 °C. Then a previously prepared solution of 0.45 M $Zn(NO_3)_2{\cdot}4H_2O$ and 0.9 M NaOH in DW was added dropwise (slowly for 40 min) to the heated solution under high-speed stirring. The beaker was sealed at this condition for 2 h. The dried ZnONPs were cleaned with deionized water and ethanol and then air-dried at 60 °C.

Preparation of AgNPs

AgNPs were prepared as described previously [18]. When 10 ml of 1.0 mM silver nitrate was added dropwise to 30 ml of chilled 2.0 mM sodium borohydride solution under vigorous stirring, the solution turned light yellow initially, but then it turned a brighter yellow when all of the silver nitrate was added. The entire addition took approximately 3 min, after which stirring was stopped and the stir bar was removed.

Preparation of AgNPs/ZnONPs/Au electrode

Prior to the surface modification, Au electrode was cleaned using piranha solution (H_2SO_4/H_2O_2, 3:1) for 20 min and then rinsed thoroughly with DW. The Au electrode was then polished with alumina slurry. ZnONPs were electrochemically deposited onto Au electrode by CV by immersing it into a mixture of 23 ml of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and 2 ml of ZnONPs solution and then applying potential between –0.2 and +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 V s^{-1}. ZnONPs/Au electrode was rinsed with DW and then dried at room temperature. The electrodeposition of AgNPs onto the ZnONPs/Au electrode was carried out by immersing the modified Au electrode into a 23-ml solution of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and 2 ml of AgNPs solution and then applying potential between –0.2 and +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 V s^{-1}. The electrode was rinsed again with DW and dried in air. Amine-terminated self-assembled monolayer (SAM) of cysteamine was then formed on the surface of AgNPs/ZnONPs/Au electrode by immersing it in 1 mM ethanol solution of cysteamine for 10 h at room temperature. The sulfur atoms of the molecules bind to the metallic nanoparticles surface, whereas the amino groups can be employed for the attachment of other groups to the SAM. The resulting monolayer-modified electrode was rinsed thoroughly with DW to remove the physically adsorbed cysteamine and then dried in air.

Preparation of working electrode (lac/AgNPs/ZnONPs/Au)

The modified Au electrode was activated with GA by exposing its surface to 5% GA solution for 1 h. Then the GA-activated cysteamine monolayer of the electrode was rinsed with DW and dried in air. In the next step, an amide bond was formed between free $-NH_2$ groups on the surface of enzyme molecule and the free –CHO group of GA by dipping the electrode in a 2.5-ml solution of acetate buffer (0.1 M, pH 5.0) containing 50 μl of enzyme (protein, 40 mg ml^{-1}) for 24 h. After the immobilization of enzyme on cysteamine monolayer, the electrode was rinsed with a stream of a phosphate buffer (0.1 M, pH 7.0) to remove the residual monomer and unbound enzyme molecules. The protein concentration in washout solution was determined.

The principle of working of this biosensor was as follows. Guaiacol (standard phenolic substrate) was oxidized to its corresponding *o*-quinone by AgNPs/ZnONPs/Au electrode bound laccase and

then regenerated through electrochemical reduction of *o*-quinone, thereby forming a bioelectrocatalytic amplification cycle and generating electrons, which were passed to the working electrode from solution and are relayed to the potentiometer, which reads it as current. The electrons then reach Ag/AgCl electrode, where Ag^+ ions are reduced to Ag metal. The current (μAmp) was measured and was directly proportional to the guaiacol concentration.

The fabricated electrode was characterized by SEM and FTIR spectroscopy at different stages of its construction. The resulting enzyme electrode was stored in a refrigerator at 4 °C when not in use.

Electrochemical measurement

Electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the lac/AgNPs/ZnONPs/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. CV measurements were carried out in a three-electrode cell containing 20 ml of electrolyte (2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 1:1), 5 ml of acetate buffer (0.1 M, pH 5.0), and 0.1 ml of guaiacol (10 μM). Current measurements were performed applying CV in the potential range between −0.2 and +0.6 V. All electroanalytical measurements were carried out at room temperature.

Optimization of lac/AgNPs/ZnONPs/Au electrode

To determine the optimal working conditions of the enzyme electrode, the pH of reaction buffer (0.1 M) was varied from pH 3.0 to 7.0 at an interval of pH 0.5 using acetate buffer in an acidic pH range (pH 3.0–5.0) and phosphate buffer in a pH range of 6.0 to 7.0. Similarly, to determine the optimal temperature, the reaction mixture was incubated at 15 to 50 °C at an interval of 5 °C. To determine the optimal response time, the current response was measured from 2 to 12 s at an interval of 2 s. To study the effect of substrate concentration, different guaiacol concentrations ranging from 0.1 to 500 μM were used. Apparent K_m for guaiacol and I_{max} were calculated from a Lineweaver–Burk plot based on Eq. (1):

$$\frac{1}{I} = \frac{K_m}{I_{max}}\frac{1}{[S]} + \frac{1}{I_{max}} \quad (2)$$

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

Amperometric measurement of total phenolic content in wine

The biosensor was employed for determination of total phenolic content in five commercial brands of red wine. The samples were diluted by 10 times with acetate buffer (pH 6.0). Total phenolic content in red wine samples was determined by the biosensor in the same manner as described above for its response measurement under its optimal working conditions except that guaiacol was replaced by the wine sample. The current (μA) was recorded, and the amount of total phenolic content was interpolated from the standard curve between guaiacol concentrations and current (μA) prepared under optimal working conditions (Fig. 1).

Results and discussion

Electrode surface characterization by SEM, XRD, and FTIR

Scheme 1 shows the changes in surface morphology of Au electrode, as studied by SEM, after its modification by deposition of AgNPs/ZnONPs and then immobilization of laccase on it, in chronological order. The bare Au electrode shows a uniform surface (panel A), whereas the surface of ZnONPs modified Au electrode exhibits formation of an almost uniform layer of zinc oxide on the surface of Au electrode (panel B). SEM of lac/AgNPs/ZnONPs (panel C) clearly shows a regular globular structural morphology, indicating the successful immobilization of laccase on the surface of modified electrode.

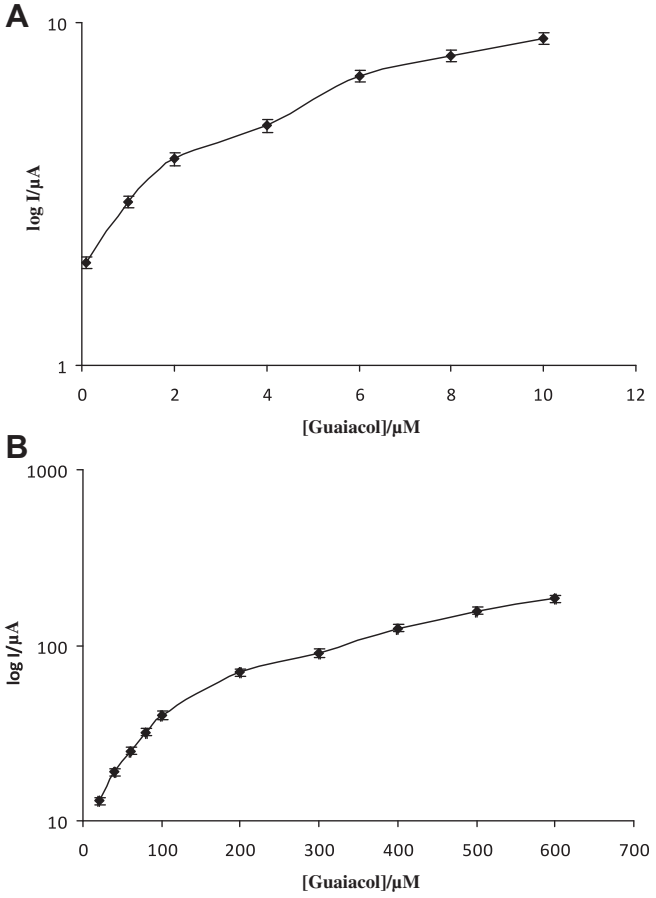
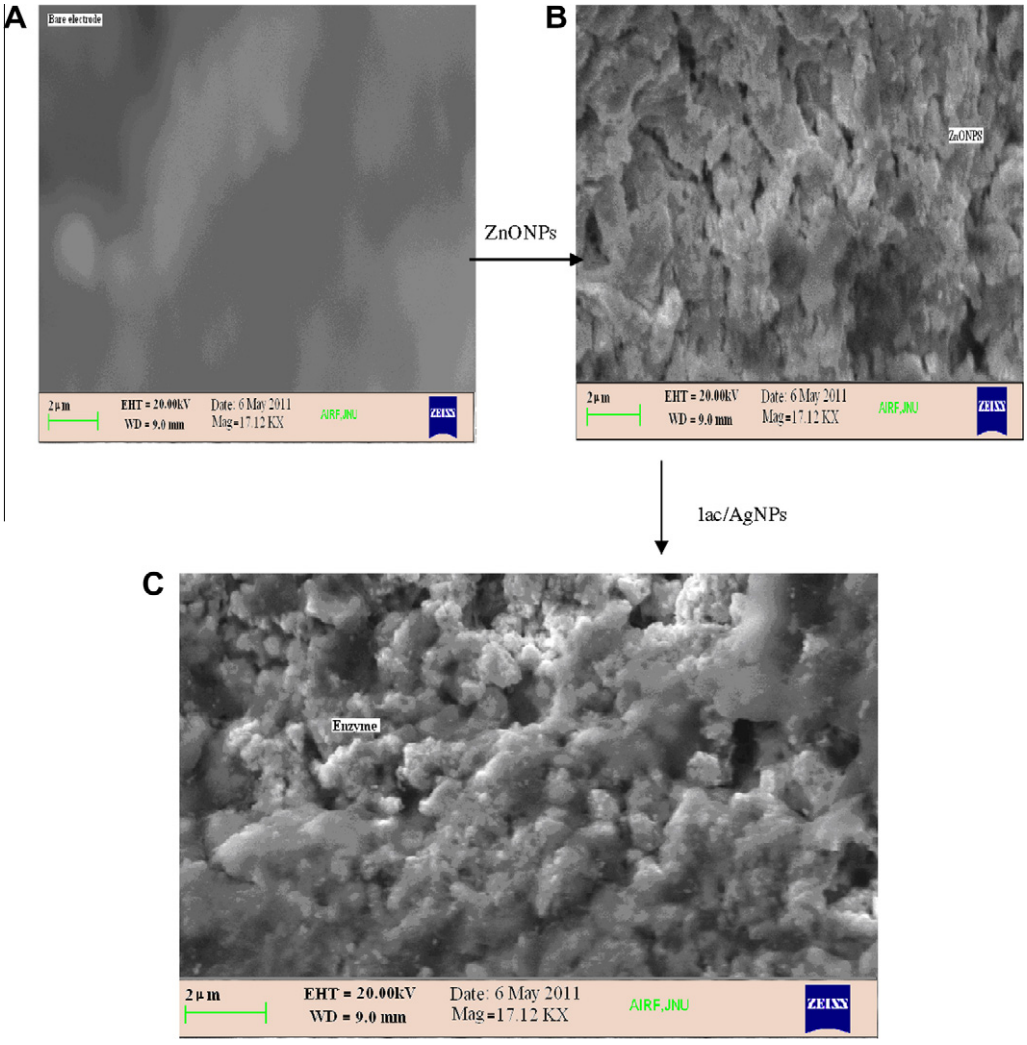


Fig.1. Effect of guaiacol concentration on the oxidation current response of AgNPs/ZnONPs/Au electrode at the lower concentration range (0.1–10 μM), with the linear equation being $y = 0.7047(\pm0.09)x + 2.2979(\pm0.19)$ (slope = 0.7047 and intercept = 2.2979) (A), and the higher concentration range (10–500 μM), with the linear equation being $y = 0.2935(\pm0.04)x + 8.0041(\pm0.35)$ (slope = 0.2935 and intercept = 8.0041) (B). Error bars represent the standard deviations for three independent measurements.

Fig. 2A shows the XRD pattern of ZnO composite films. All diffraction peaks can be indexed to hexagonal ZnO with lattice constants $a = 3.249$ Å and $c = 5.208$ Å. The characteristic peaks were higher in intensity and narrower in spectral width, indicating that the products were of good crystallinity. No peaks corresponding to impurities were detected, revealing that the final products consisted of purely ZnONPs. The characteristic peaks of ZnONPs at 31.80, 34.48, 36.28, and 47.50 corresponded to [100], [002], [101], and [102] diffraction peaks of the wurtzite form, indicating that the ZnONPs shell possessed a polycrystalline hexagonal crystal structure.

Fig. 2B shows the XRD pattern of AgNPs. All diffraction peaks corresponded to the characteristic face-centered cubic (FCC) silver lines. These diffraction lines, observed at 2θ angles 32.80, 38.20, 55.10, and 65.70°, have been indexed as [111], [200], [220], and [311], respectively.

The fabrication of lac/AgNPs/ZnONPs/Au electrode is summarized in Scheme 2. First, ZnONPs followed by AgNPs were electro-



Scheme 1. Scanning electron micrographs of bare gold electrode (A), ZnONPs/Au electrode (B), and lac/AgNPs/ZnONPs/Au electrode (C).

deposited on Au electrode. Amine-terminated SAM of cysteamine was then formed on the surface of AgNPs/ZnONPs/Au electrode. The enzyme was immobilized covalently onto this monolayer through GA coupling. In this coupling, one –CHO group of GA formed a –CH=N– bond with free the $–NH_2$ group of cysteamine layer, whereas its other –CHO group formed –CH=N– with the $–NH_2$ group on the surface of enzyme molecule.

CV study

To evaluate the charge transfer properties on the surface of the modified electrodes, CV was employed using potassium ferricyanide–potassium ferrocyanide solution as electrolyte. Cyclic voltammograms were recorded in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and 0.1 M acetate buffers (pH 5.5). The electrodeposition of AgNPs onto ZnONPs modified Au electrode surface led to an increase in current intensity as a result of an increase in electroactive area. Fig. 3A reveals the comparative cyclic voltammograms of the ZnONPs modified Au electrode (curve a) and AgNPs/ZnONPs/Au (curve b) in a solution of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and acetate buffer (0.1 M, pH 5.0) at 20 mV s^{-1}. The rise in current clearly indicates the role of AgNPs in the increase of the conductivity of ZnONPs modified electrode. The preservation of a quasi-reversible electrode process and the large increase in peak currents for the nanocomposite film modified electrode proved that AgNPs exerted an obvious improvement effect on ZnONPs/Au electrode properties.

Response toward guaiacol at lac/AgNPs/ZnONPs/Au electrode

To evaluate the catalytic activity of laccase at the AgNPs/ZnONPs/Au electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of guaiacol in the potential range between −0.2 and +0.6 V. Fig. 3B shows the cyclic voltammograms of the lac/AgNPs/ZnONPs/Au electrode in an unstirred 2.5-mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) solution and 0.1 M acetate buffer (pH 5.5) with (curve a) and without (curve b) guaiacol solution at a scan rate of 20 mV s^{-1}. It was observed that with the addition of 10 µM guaiacol, the oxidation current was increased and the reduction current was decreased, revealing the improved catalytic properties of modified electrode to the oxidation of guaiacol. A well-defined oxidation peak (0.22 V vs. Ag/AgCl) was observed, clearly indicating the catalytic properties of modified electrode. Thus, for further amperometric study of lac/AgNPs/ZnONPs/Au electrode, a potential of 0.22 V (vs. Ag/AgCl) was applied.

Electrochemical impedance measurements

Fig. 4 shows electrochemical impedance spectra of bare Au electrode (curve a), ZnONPs/Au electrode (curve b), AgNPs/ZnONPs/Au

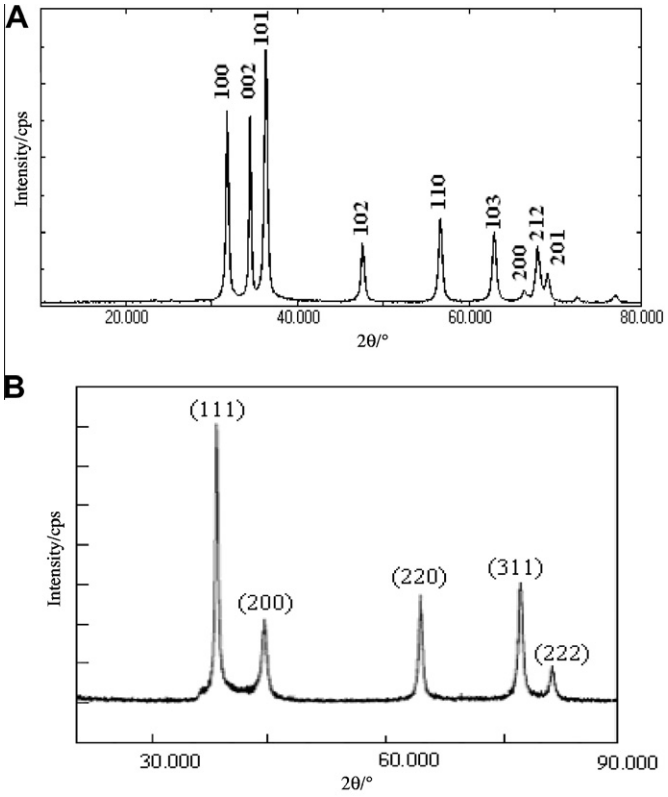


Fig.2. XRD patterns of ZnONPs (A) and AgNPs (B).

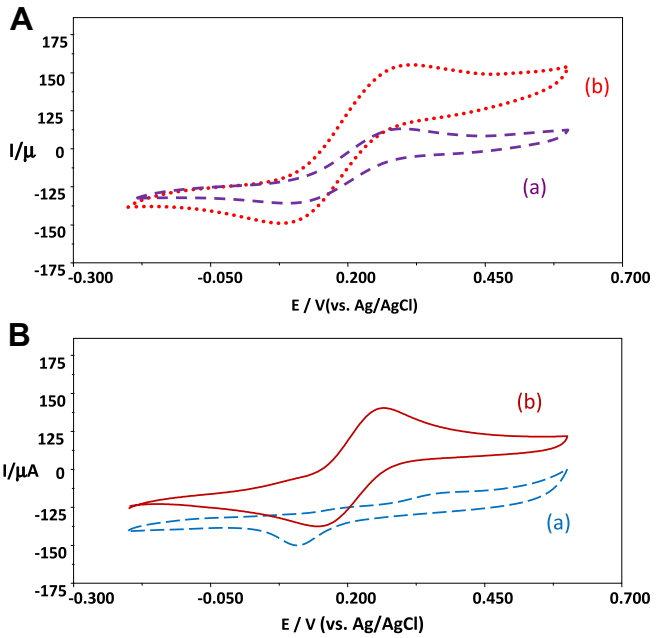
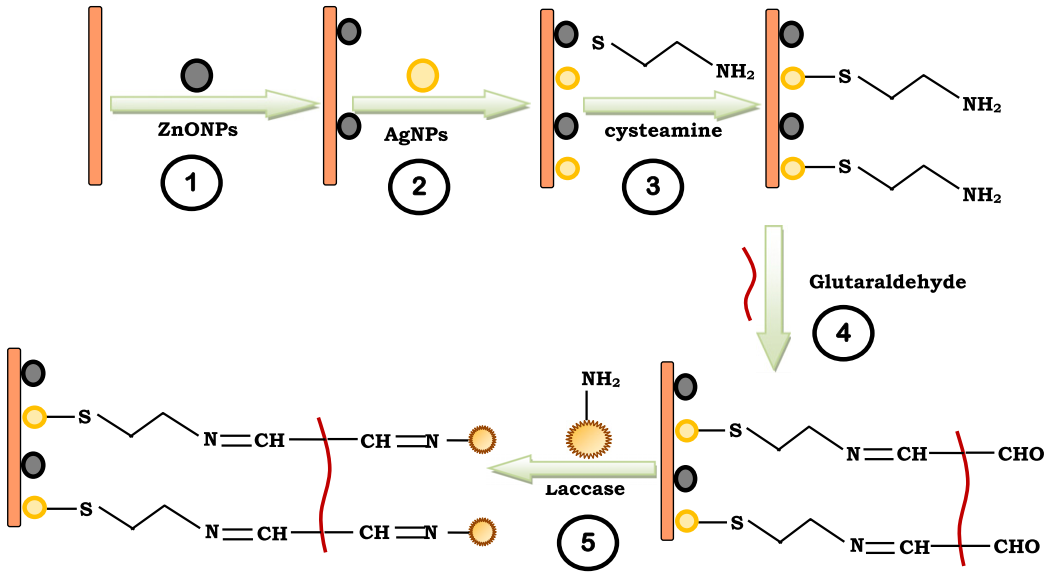


Fig.3. Comparative cyclic voltammograms: (A) ZnONPs/Au electrode (curve a) and AgNPs/ZnONPs/Au electrode (curve b); (B) lac/AgNPs/ZnONPs/Au electrode in 0.1 M acetate buffer (pH 6.0) with (curve a) and without (curve b) 10 μM guaiacol solution. Supporting electrolyte: 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1); scan rate: 20 mV s^{-1}.

electrode (curve c), and lac/AgNPs/ZnONPs/Au electrode (curve d) in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) at a polarization potential of 0.2 V in the frequency range of 0.1 to 10^4 Hz. Electrochemical impedance spectroscopy (EIS) analysis was employed to investigate the change in charge transfer after immobilization of laccase onto AgNPs/ZnONPs/Au electrode. The value of the charge transfer resistance (R_{CT}, semicircle diameter) depends on the dielectric and insulating features at the electrode/electrolyte interface. In EIS, the bare electrode exhibited an almost straight line (curve a), which is characteristic of a diffusional limiting step of the electrochemical process.

The Nyquist plot of ZnONPs/Au electrode (Fig. 4, curve b) gave an R_{CT} value of 220 Ω. AgNPs coated ZnONPs modified Au electrode (curve c) possessed a remarkably low R_{CT} of 175 Ω, implying that the AgNPs coated ZnONPs are acting as good electron conducting materials and there was an electron transfer between the redox probe and the electrode. When compared with the nanocomposite modified electrode, the semicircle diameter was increasing with the immobilization of laccase on AgNPs/ZnONPs modified Au electrode, showing an increased R_{CT} value of 380 Ω (curve d). This increase in R_{CT} value of lac/AgNPs/ZnONPs/Au electrode is



Scheme 2. Chemical sequence of electropolymerization of AgNPs/ZnONPs onto Au electrode and chemical reaction of immobilization of enzyme onto modified electrode.

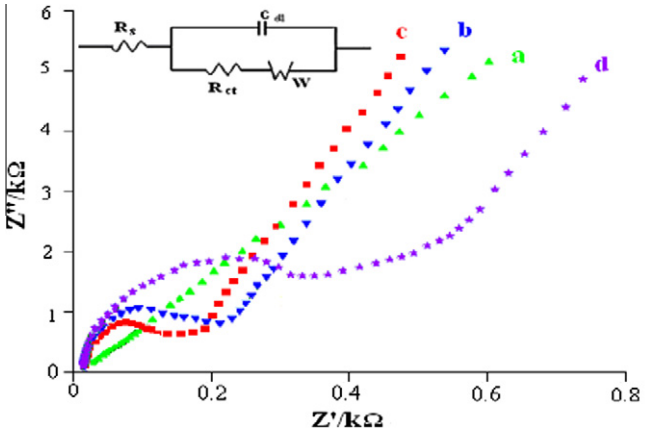


Fig.4. Electrochemical impedance spectra of bare Au electrode (curve a), ZnONPs/Au electrode (curve b), AgNPs/ZnONPs/Au electrode (curve c), and lac/AgNPs/ZnONPs/Au electrode (curve d) in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) at a polarization potential of 0.2 V in a frequency range of 0.1 to 10^4 Hz.

attributed to most biological molecules, including enzymes, which are poor electrical conductors at low frequencies and cause hindrance to the electron transfer. The above results confirm the successful immobilization of enzyme onto modified Au electrode.

Optimization of experimental conditions of biosensor

The optimal biosensor response was achieved at pH 6.0 (Fig. 5A). Therefore, a pH of 6.0 was used in further experiments. The optimal temperature of the biosensor was 35 °C (Fig. 5B). The biosensor showed a maximal response within 8 s (Fig. 5C). The effect of the scan rate from 25 to 200 mV s^{-1} on the biosensor response using 10 μM guaiacol in 0.1 M acetate buffer solution (pH 6.0) was also investigated. A potential scan rate of 20 mV s^{-1} was selected because with these experimental conditions the highest analytical signal and very good cyclic voltammogram profiles were obtained. The *Ganoderma* sp. laccase is known to have wide substrate specificity [19]. So, the current biosensor based on this enzyme was not selective.

Evaluation of biosensor

Linearity

There was a linear relationship between current (μA) and guaiacol concentration ranging from 0.1 to 10 μM (lower concentration range), with the linear equation being $y = 0.7047x + 2.2979$, and between 10 and 500 μM (higher concentration range), with the linear equation being $y = 0.2935x + 8.0041$ (Fig. 1), in the reaction mixture.

The detection limit of the current sensor was 0.05 μM (signal-to-noise ratio (S/N) = 3), which is lower/better than that of enzyme electrode based on carbon nanotubes–chitosan composite (0.66 and 0.275 μM) [20,6], copper-containing ordered mesoporous carbon/chitosan matrix (0.67 μM) [21], and copper nanoparticles/chitosan/carboxylated multiwalled carbon nanotubes/polyaniline/Au electrode (0.05 μM) [22].

Recovery

A recovery study was performed using wine samples by adding 0.1 mM guaiacol. The results showed that the average recovery of added guaiacol was 98.0%, demonstrating the high accuracy of the biosensor.

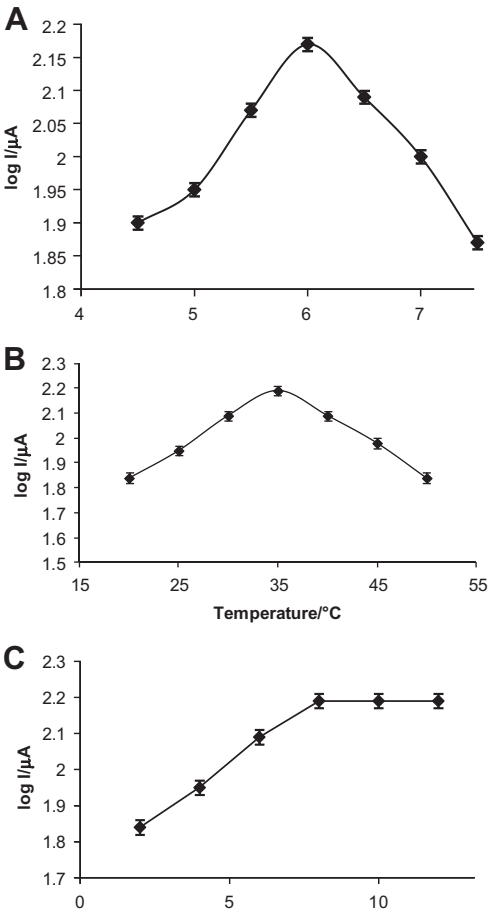


Fig.5. (A,B) Effect of pH value (A) and temperature (B) on the oxidation current response of 10 μM guaiacol at lac/AgNPs/ZnONPs/Au electrode. (C) Time course study of lac/AgNPs/ZnONPs/Au electrode with 10 μM guaiacol. Error bars represent the standard deviations for three independent measurements.

Precision

To test the reproducibility and reliability of the current biosensor, total phenolic content in six wine samples was determined on a single day (within batch) and five times again after storage at −20 °C for 1 week (between batch). The results showed that determinations were consistent and that within- and between-coefficients of variation were 1.7 and 2.9%, respectively, which is better than earlier reports [21,23]. The high precision indicated the reproducibility and consistency of the current method. The good stability, repeatability, and reproducibility observed for the proposed biosensor could be attributed to the excellent immobilization of laccase onto AgNPs/ZnONPs/Au electrode.

Correlation

Comparing the values of total phenolic content in 15 wine samples by the current method (y) with those obtained by the standard spectrophotometric method (x) [24] showed good correlation with $r = 0.99$ with a regression equation of $y = 1.0053x - 3.5541$ (Fig. 6). These results indicated a very good analytical performance of the current biosensor.

Determination of total phenolic content in wine samples

Total phenolic content in red wine ranged from 202 to 237 μM with the current sensor (Table 1).

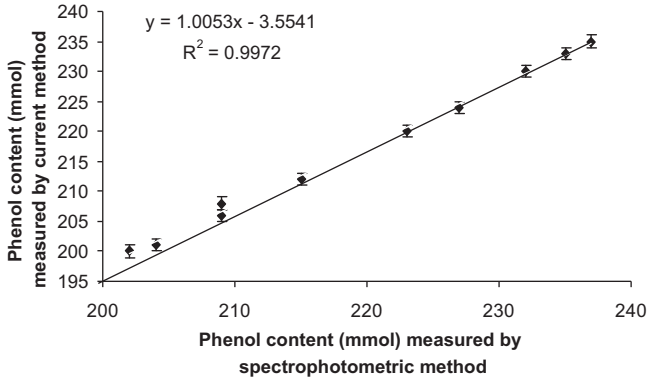


Fig.6. Correlation between total phenolic content values determined by the standard spectrophotometric method (x-axis) and the present biosensor employing the Lac/AgNPs/ZnONPs/Au electrode method (y-axis) with a regression equation of $y = 1.0053(\pm 0.33)x - 3.5541(\pm 0.21)$ with $r = 0.99$. Error bars represent the standard deviations for three independent measurements.

Table 1
Comparison of total phenolic content in different brands of wines as measured by lac/AgNPs/ZnONPs/Au electrode and standard spectrophotometric methods

Sample	Total phenolic content by current method (μM guaiacol, means ± SD, $n = 4$)	Total phenolic content by standard spectrophotometric method (μM guaiacol, means ± SD, $n = 4$)
Brand a	204 ± 0.012	200 ± 0.022
Brand b	235 ± 0.014	233 ± 0.025
Brand c	202 ± 0.016	199 ± 0.029
Brand d	237 ± 0.019	235 ± 0.031
Brand e	232 ± 0.014	230 ± 0.024
Brand f	209 ± 0.014	206 ± 0.022
Brand g	215 ± 0.016	212 ± 0.026
Brand h	223 ± 0.018	220 ± 0.026
Brand i	227 ± 0.016	224 ± 0.023
Brand j	209 ± 0.013	206 ± 0.020

Note: SD, standard deviations.

Interference study

The effect of the following possible interfering substances was tested on the biosensor response, each at a final concentration of 0.1 mM: ascorbic acid, acetic acid, fructose, glucose, citric acid, and cysteine. Fructose (26%), glucose (24%), acetic acid (20%), cysteine (15%), and ascorbic acid (15%) decreased the response (Table 2).

Stability and reusability of biosensor

The electrode lost 25% of its initial activity after its 200 uses during the span of 5 months when stored at 4 °C, which is much better than earlier biosensors [21,25].

Table 2
Effects of addition of compounds at a final concentration of 0.1 mM on the response measurement of lac/AgNPs/ZnONPs/Au electrode

Compound added	% Relative activity
None	100
Ascorbic acid	85
Acetic acid	80
Fructose	74
Glucose	76
Citric acid	90
Cysteine	85

Conclusions

The use of AgNPs/ZnONPs composite film in the construction of a biosensor for phenolic content has led to its improved analytical performance in terms of low working potential (0.22 V), short response time (8 s), sensitivity (0.71 $\mu A\ \mu M^{-1}\ cm^{-2}$), and high storage stability (5 months). Based on these observations, this composite could be employed for the improvement of other biosensors.

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