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Detection of Catechol Using a Biosensor Based on Biosynthesized Silver Nanoparticles and Polyphenol Oxidase Enzymes

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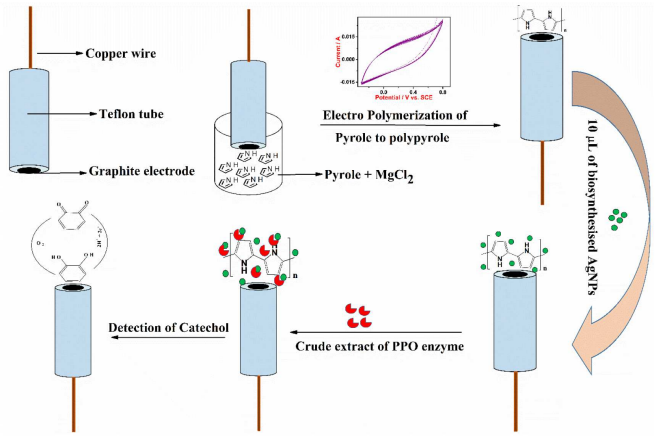

Abstract

In the present work, we report the development of a polyphenol oxidase (PPO) sensor for the selective and sensitive detection of catechol, using biosynthesized silver nanoparticles (AgNPs). For the sensor development, AgNPs biosynthesized using the leaf extract of *Convolvulus pluricaulis*, were successfully deposited onto a polypyrrole modified graphite electrode (Gr/PPy). The resulting Gr/PPy/AgNPs electrode was further used as a matrix for the immobilization of the PPO enzyme extracted from *Manilkara Zapota* (sapota). The morphological characteristics of the developed Gr/PPy/AgNPs/PPO sensor were studied using a scanning electron microscope (SEM). The sensor performance was evaluated and optimized using cyclic voltammetry (CV), differential pulse voltammetry (DPV), chrono amperometry (CA) and electrochemical impedance spectroscopy (EIS) methods. Under neutral pH conditions, the developed sensor showed excellent electro catalytic activity towards catechol detection. The sensor

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performed well in the concentration range of 0.001 to 0.015 mM, with the detection limit of 0.47 µM, and sensitivity of 13.66 $\mu M^{-1}cm^{-2}$. The biosensor response was found to be uninfluenced by some common interferents, and it also showed good storage stability and repeatability. The practical applicability of the PPO biosensor for catechol detection in real samples was assessed by examining the catechol content in a green tea sample. The sensor could detect catechol content in a green tea sample, to an accuracy of about 98%, thereby establishing its efficiency in real sample analysis.

Keywords: biosynthesized silver nanoparticles, polyphenol oxidase enzyme and catechol.

Introduction

Catechol (1,2-benzenediol/1,2-dihydroxybenzene) is an important dihydroxybenzene present in many natural sources such as tea, vegetables, fruits and other plants [1-2]. It is considered as one of the key fragments of tea catechins, and its concentration is high up to 18-36% weight of the fresh dry tea. It is believed to be the basis of many claims made regarding tea's health benefits [3]. Catechol quantification is important, because of its biological roles and environmental significance, in such topics as antioxidation, antivirus, toxicity and carcinogenicity [4-5]. Catechol can be determined using various analytical methods, such as spectrophotometry [6], gas chromatography [7], HPLC [8], etc. However, these techniques have some disadvantages, such as complicated operation, sample pre-treatment, poor selectivity and high cost. On the other hand, sensor technology is one of the most widely adopted in biological and environmental sample analysis, due to its several advantages, such as rapid response, shorter analysis time, no samples pre-treatment, low cost, high sensitivity and selectivity [9]. The sensor performance is, however, dependent of the electrode material and of its composition. PPO has received continuous attention from food chemists and processors, because it is involved in the enzymatic browning of many edible plant products, such as fruits and vegetables [10]. Catechol oxidase or PPO belongs to a group of oxidoreductases, and it is a copper containing an enzyme capable of catalyzing the oxidation of di or polyphenols to the corresponding quinones [11-12]. The positive attributes of high catalytic activity and the ability to utilize different phenolic compounds as substrates have led to a wide interest in its use for the construction of new polyphenol biosensors. The use of plant or fruit extract homogenates, to substitute the isolated enzymes as alternative biologic materials in the biosensors development, has received considerable attention in recent years, as these materials maintain the enzyme of interest in its natural environment, with better enzyme activity and higher stability [13].

The major challenge in the development of enzyme based electrochemical sensors is finding a suitable electrode matrix to immobilize the enzyme without denaturation or modification of its active sites [14].

Metal based nanoparticles such as gold, silver, platinum, copper, palladium, nickel, iron, etc., have attracted considerable attention as electrode materials for the development of ultrasensitive electrochemical sensors, due to their unique

magnetic, optical and electrocatalytic properties [15]. Biosynthesized metal nanoparticles are interesting materials, as they involve fast, simple and cost effective synthetic processes which do not require high temperatures or toxic chemicals [16]. In particular, biosynthesized AgNPs have gained more attention among the researchers in the field of biosensors, due to their high quantum electron transfer [17] and catalytic properties [18]. AgNPs have high conductivity and a high specific surface area that can adsorb biomolecules such as redox enzyme, proteins and cells, without the loss of activity, thus retaining their functional characteristics to a great extent [19]. Biosynthesized AgNPs can be excellent nontoxic biosensor materials.

Convolvulus pluricaulis is a medicinally important plant, because of its ability to treat a variety of medical conditions, namely, hypertension, neurodegenerative diseases, high blood pressure, epilepsy, vomiting and diabetes [20]. It also enhances memory and decreases cholesterol content in the body. The extract of *Convolvulus p.* plant is also a rich source of chemical constituents, such as alkaloids, phenolic, glycosides, tri-terpenoids and steroids, which reduce the metal salt to form the respective nanoparticles [21]. These reasons have motivated us to use the leaf extract of *Convolvulus p.* plant as a reducing agent for AgNPs biosynthesis.

In the present study, a catechol sensor was developed using a PPO enzyme extracted from *Manilkara Zapota* and biosynthesized AgNPs, by fabricating them onto a polypyrrole matrix. The developed sensor was successfully used for catechol detection and quantification in a tea sample.

Experimental

Chemicals and equipments

Catechol, pyrrole, magnesium chloride and acetonitrile used in the experiments were procured from Sigma Aldrich. For UV-visible spectral studies, Shimadzu – 1800 UV-visible spectrometer was used. The morphological aspects of the sensor surface were investigated using ZEISS EV040EP (Germany) SEM. CV and DPV were performed using SP-150 Biologic Science Instrument. CA and EIS were performed using Versastat 3 (Princeton Applied Research, USA). The EIS spectral data analysis was carried out using Zswimp win 3.2 simulation software.

AgNPs biosynthesis and characterization

AgNPs were prepared using *Convolvulus p.* leaves by a procedure earlier reported by our group [22]. 10 g of freshly collected leaves of this plant were thoroughly washed in deionized water and shade dried. They were crushed in 100 mL of distilled water, filtered through a sterile muslin cloth, and then through a Whatman-41 filter paper. 10 mL of this leaf extract were added to 50 mL of the 0.05 M $AgNO_3$ aqueous solution, and the mixture was incubated at room temperature, until the solution color changed from pale yellow to dark brown. After the incubation time, the reaction mixture was centrifuged at 10,000 rpm for 15 min, and purified by repeated washing with methanol. Finally, AgNPs were dried at room temperature by evaporating methanol.

Extraction of the PPO enzyme

The PPO enzyme used in this study was extracted from *Manilkara Z.* (sapota) fruit. 25 g of fruit pulp were homogenized with 25 mL of 0.05 M phosphate buffer (PBS), with a pH of 6.8, 1% polyvinyl pyrrolidone, 1% triton-X 100 and 1 mM phenyl methyl sulponyl fluoride, for 10 min. The homogenate was rapidly filtered and centrifuged at 10 000 rpm for 15 min, at 4 ºC. PPO activity was spectrophotometrically determined using 4-methyl catechol as substrate. The enzyme assay was carried out by taking 0.88 mL of PBS (pH 6.8, 50 mM) and 0.02 mL of the enzyme extract with 0.1 mL of catechol as substrate (0.1 M). The increase in absorbance at 420 nm was monitored for 3 min using a spectrophotometer, and the average change in absorbance per minute was calculated.

One unit of enzyme activity was defined as the increase in the enzyme amount that caused a change in absorbance of 0.1 min^{-1} at 420 nm for the o-quinone produced. The enzyme activity was found to be 620 enzyme units (EU). The extracted enzyme did not show any increase in absorbance when tyrosine was used as substrate. This indicates that there was no monophenolase activity and, therefore, the extracted enzyme is a catecholase. The resulting supernatant was stored at 4 ºC in a refrigerator, and used as a PPO source for further experiments.

Fabrication and characterization of the PPO biosensor

A graphite electrode was fabricated by inserting a cylindrical graphite rod (Gr) of 6 mm diameter into a teflon holder with the same internal diameter. A copper wire was used to establish electrical contact. The Gr electrode surface was polished using a PK-3 electrode polishing kit (0.05 µm aqueous polishing alumina and 1 µm polishing diamond), until a mirror shining surface was obtained; finally, it was several times sonicated to remove loosely bound particles, later rinsed with Milli-Q water, and dried with nitrogen gas. The pyrrole monomer was electro-polymerized on the Gr electrode to polypyrrole, by the previously described method [23], with a slight modification. Pyrrole electropolymerization on the Gr electrode was achieved by immersing the electrode in 20 mL of acetonitrile containing 0.134 g of pyrrole and 0.406 g of $MgCl_2$. Subsequently, the electrode was subjected to cyclic scanning in the range of -0.3 to 0.8 V, at a scan rate of 50 mVs^{-1} for 10 potential cycles, which resulted in PPy deposition on the Gr surface. The graphite surface was then rinsed with acetonitrile to remove an unreacted or loosely bound monomer, and then allowed to dry at room temperature. Further, a suspension of biosynthesized AgNPs in deionized water (w/v ratio of 10 mg of AgNPs in 1 mL) was prepared, and 10 µL were drop casted over the Gr/PPy electrode surface, and dried at room temperature, to get the Gr/PPy/AgNPs electrode matrix. Finally, PPO was immobilized by drop casting 10 µL of the enzyme (620 UmL^{-1}) onto the Gr/PPy/AgNPs electrode matrix, to get the modified Gr/PPy/AgNPs/PPO electrode. The fabricated electrode was dried in a refrigerator for 30 min, and later washed in deionized water, to remove loosely bound enzyme particles from

the electrode surface. The fabricated Gr/PPy/AgNPs/PPO sensor was stored at 4 ºC, to be used in further studies.

Electrochemical studies

All electrochemical experiments were performed using a three electrode system with modified Gr as working electrode, saturated calomel electrode (SCE) as reference electrode, and platinum wire as counter electrode. The electrochemical characterization of the bare and modified Gr electrodes was performed in a PBS buffer at pH 7.0, with 5 mM $[Fe(CN)_6]^{-3/-4}$ as electrochemical probe. The charge transfer process at the electrode/electrolyte interface of the modified electrode was studied by EIS technique, in the frequency range of 100 kHz to 0.1 Hz.

Sample preparation

The commercially available green tea sample of Lipton brand was purchased and used for experiments. The tea samples were first crushed to powder using a mortar, and then 0.1 g of dry powder was mixed to 100 mL of a 20 % (v/v) methanol solution. The mixture was shaken for 20 min at 80 ºC, and filtered. The filtered solution was diluted to 100 mL, and stored for further studies [24]. The catechol content in the tea sample extract was also quantified using standard HPLC method. The catechol content in 0.1 g of green tea powder extract was 0.279 mg/g.

Results and discussion

AgNPs biosynthesis

For the AgNPs synthesis, an aqueous solution of $AgNO_3$ was mixed with the leaf extract of *Convolvulus p.*, and incubated at room temperature. The reaction mixture initially showed a pale yellow color that gradually turned to dark brown, as the reaction proceeded, which suggested AgNPs formation through the reduction of Ag^+ ions to Ag^0.

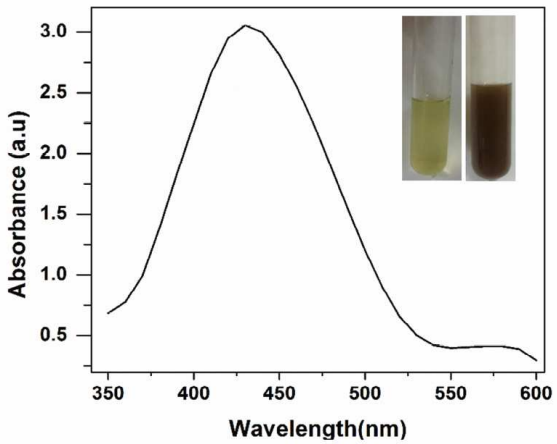


Figure 1. UV-vis spectra of biosynthesized AgNPs.

UV-visible spectroscopy was used as a tool to confirm AgNPs formation. The UV-visible spectral measurements of the reaction mixture at regular intervals

during the incubation period showed a gradual increase in absorbance. The absorbance reached a maximum, and almost stabilized after 10 hours of incubation, indicating the completion of the reduction of silver ions to AgNPs. Further, a strong and broad peak in the UV-visible spectrum, around 420 nm, clearly confirmed AgNPs formation (Fig. 1). This peak may be attributed to the surface plasmon resonance in AgNPs [25]. The UV-visible study clearly confirmed the completion of Ag^{+} reduction and AgNPs formation in nearly 10 hours.

PPO biosensor surface characterization using SEM

The PPO biosensor was fabricated through the Gr electrode surface modification in successive steps by PPy electropolymerization, followed by the drop-casting of the AgNPs and PPO enzyme. At each modification step, SEM was used as an effective tool to analyze the surface morphology of the modified electrodes.

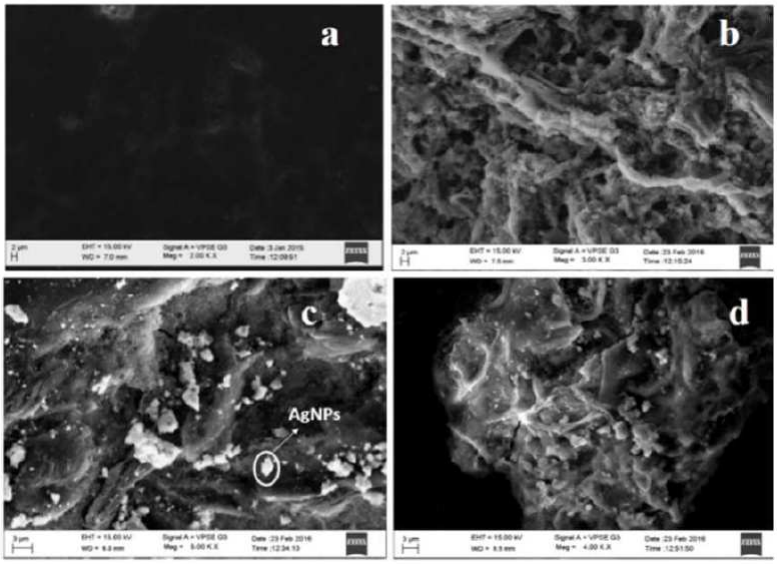


Figure 2. SEM images of **a)** Bare Gr, **b)** Gr/PPy, **c)** Gr/PPy/AgNPs and **d)** Gr/PPy/AgNPs/PPO electrodes surface.

Fig. 2 shows the SEM images of (a) bare graphite, (b) modified Gr/PPy, (c) Gr/PPy/AgNPs and (d) Gr/PPy/AgNPs/PPO electrodes.

PPO biosensor electrochemical characterization using CV and EIS

The redox behavior of the bare and modified Gr electrodes was studied using 5 mM $[Fe(CN)_6]^{-3/-4}$ as an electrochemical probe in PBS (pH 7.0), and compared after each modification step. From Fig. 3 A (a), it can be seen that the Gr electrode exhibits a pair of well-defined quasi reversible redox peaks, with a peak separation (ΔEp) of 133 mV. Comparatively, the CV of Gr/PPy [Fig 3A (b)] showed an increase in both oxidation and reduction currents, with a ΔEp of 125 mV, because of the polymer presence, which exhibits a conducting property. Further, when the electrode was modified with AgNPs (Gr/PPy/ AgNPs), it showed an enhanced oxidation and reduction current with a ΔEp value of 108 mV [Fig 3.A (c)]. The enhanced conductivity was attributable to the highly

conductive nature of AgNPs, which increased the electrode's surface area, and also facilitated the electron exchange between the electrode and the electrochemical probe. Finally, after the enzyme immobilization, the current at the Gr/PPy/ AgNPs/PPO electrode decreased with a ΔEp of 121 mV, indicating the enzyme's insulating property, which acted as a weak barrier to $[Fe(CN)_6]^{-3/-4}$ ion penetration [Fig 3.A (d)]. This confirmed the successful enzyme immobilization.

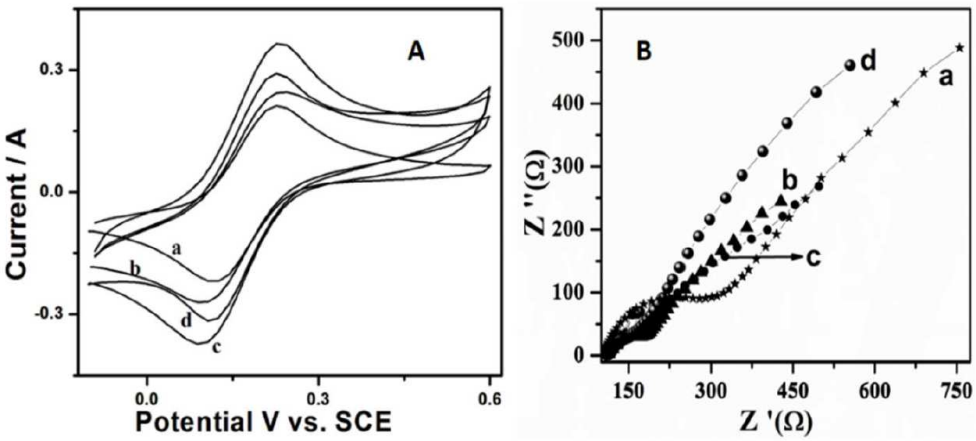


Figure 3. (A) CVs of a 5 mM $Fe(CN)_6^{-3/-4}$ solution obtained for a) bare Gr, b) Gr/PPy, c) Gr/PPy/AgNPs and d) Gr/PPy/AgNPs/PPO. **(B)** Impedance spectra of a) bare Gr, b) Gr/PPy, c) Gr/PPy/AgNPs and d) Gr/PPy/AgNPs/PPO.

EIS experiments were carried out to investigate the electrode surface features during each step of its modification. The charge transfer process occurring at the electrode/electrolyte interface was studied by carrying out EIS measurements in a frequency range of 100 KHz to 0.1 Hz, with an amplitude of 5 mV. Fig. 3 B shows the Nyquist plot of experimental data from EIS studies. The results of EIS studies indicate that the charge transfer resistance (R_{ct}) of the Gr electrode was much higher (200 Ω) than that of the Gr/PPy electrode. The reduced Gr/PPy (170 Ω) R_{ct} value may be attributed to the electrode's increased surface area. The modified Gr/PPy/AgNPs showed a further decrease in R_{ct} (47.89 Ω) value, indicating the enhanced surface area and conductivity, due to the AgNPs incorporation to the electrode surface. Further, when the electrode was modified with the PPO enzyme, this resulted in an increased R_{ct} (103.2 Ω), which was due to the insulating property of the PPO enzyme. These results are in clear agreement with the CV results above discussed.

Electrochemical detection of catechol using a developed Gr/PPy/AgNPs/PPO sensor

The electrocatalytic properties of the developed PPO biosensor were examined by measuring its cyclic voltammetric response for different catechol concentrations (0.001 – 0.009 mM), in an oxygen saturated PBS solution.

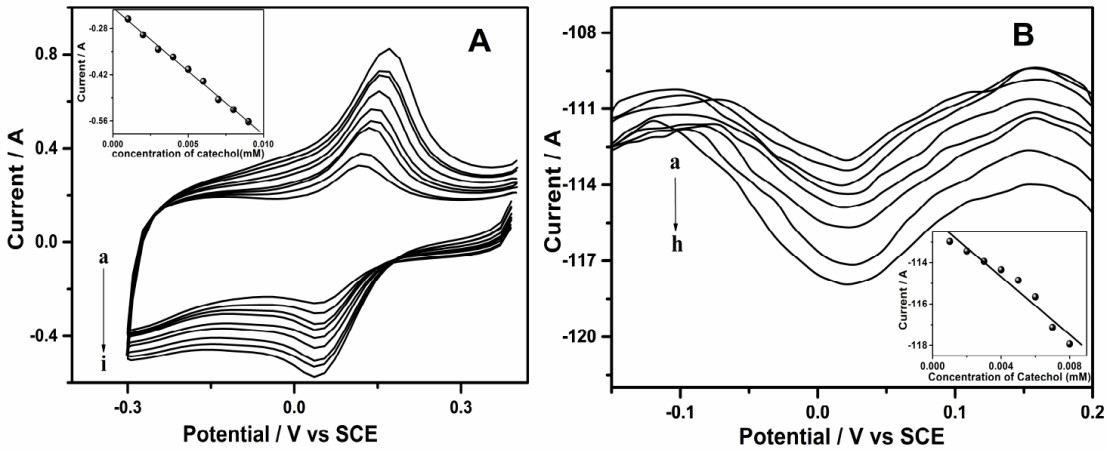
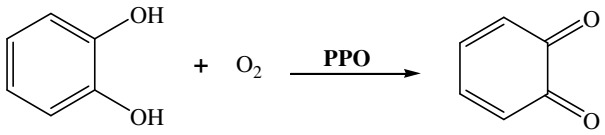
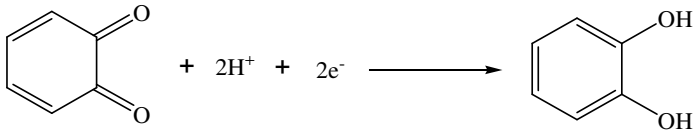


Figure 4. (A) Cyclic voltammograms of the Gr/PPy/AgNPs/PPO electrode with the successive addition of 0.001-0.009 mM catechol in 0.1 M PBS at pH 7. The inset shows the calibration plot of current vs. catechol concentration. **(B)** DPVs of the Gr/PPy/AgNPs/PPO electrode at varying catechol concentrations (0.001 – 0.008 mM) in a 0.1 M PBS solution. The inset plot shows the calibration plot of the peak current versus catechol concentration.

The CV results in Fig. 4(A) show an increase in the reduction peak, with an increase in catechol addition to the oxygen saturated buffer solution.
The PPO catalytic reaction is as follows:



O-quinone formation was detected by amperometric current measurements, during the reduction at the electrode:



The cathodic peak current for the reduction process linearly increased with an increase in catechol concentration, in the linear range of 0.001 to 0.009 mM (inset of Fig. 4 A), with a linear regression equation:

$$I_{pc}(A) = -0.218 - (-38.25)C_{catechol}(mM); R = -0.998$$

DPV is a pulse technique that shows higher sensitivity, while detecting the lower analyte concentration. DPV experiments were performed for the Gr/PPy/AgNPs/PPO electrode, in the potential range of +0.25 to -0.2 V. Fig. 4(B) shows a stable and well defined reduction peak, with an increase in catechol concentration. The calibration plot of I_{pc} vs. catechol concentration was plotted, and the peak current was in linear relationship with catechol concentration in the range of 0.001 to 0.008 mM (inset of Fig. 4 (B)), with a linear regression equation:

$$I_{pc}(A) = -111.8 - (-700.7)C_{catechol}(mM); R = -0.974$$

Scan rate effect

The effect of the scan rate on the cathodic peak current was studied, to investigate the nature of the electron transfer reaction occurring at the electrode surface. The study was carried out using 0.004 mM catechol in 0.1 M PBS (pH 7.0), for the different scan rates ranging from 10 to 100 mVs^{-1}, and the obtained results are shown in Fig. 5 (A). The results indicate that there was an increase in the oxidation and reduction peak current, with an increase in the scan rate from 10 to 100 mVs^{-1}. The linear regression equation was found to be:

$$I_{pc}(A) = -0.113 - (-1.004\times10^{-2})v(Vs^{-1}); R = -0.993$$

The inset plot of Fig. 5(b) also indicates that the cathodic peak current proportionally varies to the square root of the scan rate with a linear regression:

$$I_{pc}(A) = -0.303 - (-0.136)v^{1/2}; (Vs^{-1}); R = -0.999$$

These results suggest that the electrochemical reaction occurring at the Gr/PPy/AgNPs/PPO electrode surface is an adsorption controlled reaction.

pH effect

The pH is one of the major parameters that influence the biosensor amperometric response. The pH effect on the Gr/PPy/AgNPs/PPO biosensor performance was examined by measuring the current response of 0.004 mM catechol in the pH range of 2 to 10. The amperometric response of the developed biosensor at different pH values is shown in Fig 5(B). The biosensor showed maximum response current at pH 7.0, and the response decreased at high pH values, which could be attributed to the enzyme deactivation or leaching from the electrode surface [26].

Temperature effect

The temperature effect on the Gr/PPy/AgNPs/PPO modified electrode was examined over the temperature range of 10 ºC to 70 ºC, in 0.004 mM catechol. When the temperature was increased, the response current increased up to 40 ºC, and then it gradually decreased with an increased temperature, as shown in Fig. 5 (C). This may be due to the enzyme deactivation beyond optimum temperature. However, for practical reasons, all of our experiments were performed at room temperature.

Effect of interferences

To test the practical applicability of the developed biosensor, a selectivity study was obligatory. Despite high enzyme specificity, the enzyme based sensors may give erroneous results for real samples, due to the interference from electroactive species present in such samples. Some common interferents, such as glucose, ascorbic acid and gallic acid, present in natural samples, may often interfere in

catechol detection. The effect of these interferents in catechol detection was examined by measuring the reduction current on the subsequent addition of those interfering compounds. The sensor showed no significant changes due to the subsequent interferents' addition. This indicates that the developed Gr/PPy/AgNPs/PPO biosensor is highly selective towards catechol detection.

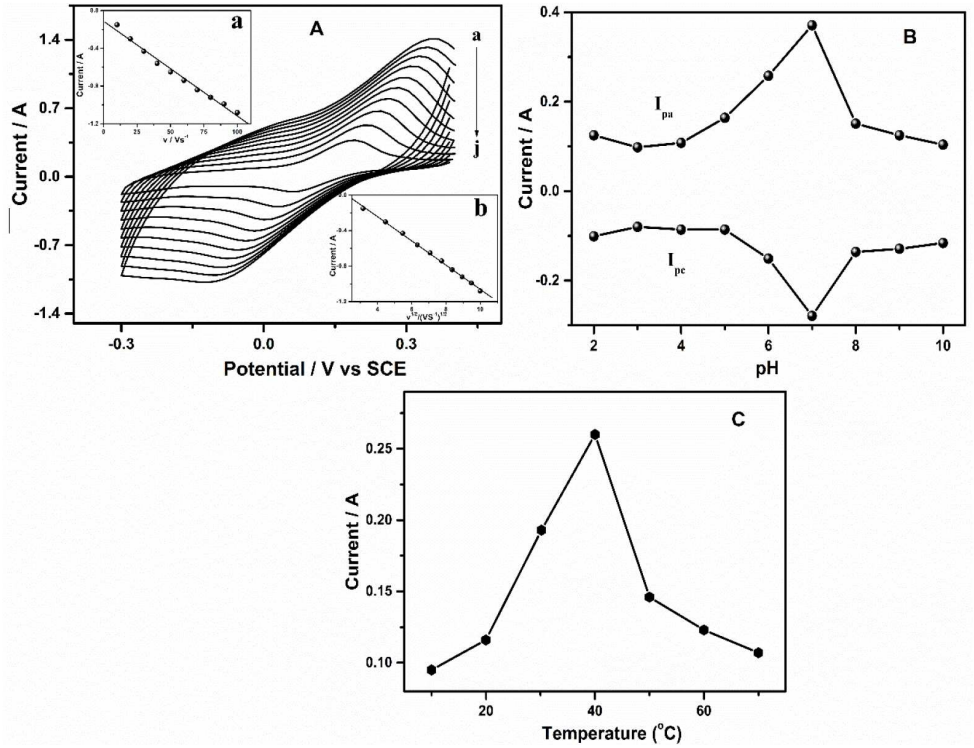


Figure 5. (A) Cyclic voltammograms of Gr/PPy/AgNPs/PPO in 0.1 M PBS at pH 7 containing 0.004 mM catechol at different scan rates (10-100 mVs^{-1}); inset plots a) peak current vs. scan rate, b) peak current vs. square root of scan rate. **(B)** Effect of varying pH (from 2 to 10) on the peak current. **(C)** Effect of varying temperature (from 10 to 70 ºC) on the peak current.

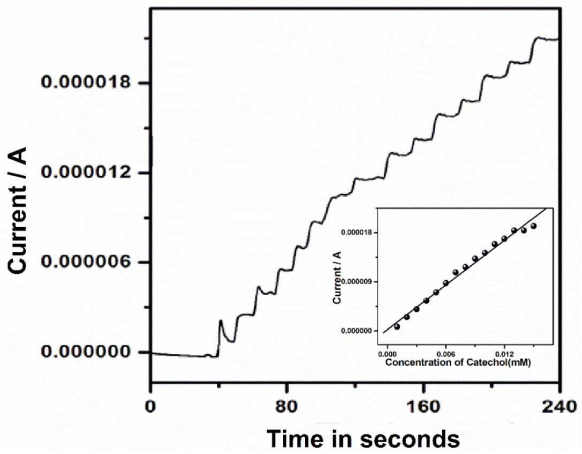


Figure 6. Chrono amperometric response of the Gr/PPy/AgNPs/PPO electrode in different catechol concentrations, at an applied potential of +0.025 V. The inset plot shows the calibration curve of the response current vs. catechol concentration.

Chrono amperometric response of the Gr/PPy/AgNPs/PPO biosensor

Chrono amperometry is a widely used technique to evaluate the change in current response as a function of time. Fig. 6 shows the amperometric response of the enzyme modified electrode on the successive catechol addition in varying concentrations, at an applied potential of +0.025 V. The enhanced current was recorded with incremental catechol addition. The inset of Fig. 6 shows the current calibration plot as a function of catechol concentration. The chrono amperometric results indicate that the sensor exhibited a linear detection range of 0.001 to 0.015 mM, with a regression coefficient of 0.993.

Sensitivity and detection limit

The detection limit of the developed sensor was determined according to:

$$\frac{3\sigma}{S}$$

where σ is the standard deviation of blank and S is the slope of the calibration plot [27]. The detection limit of the sensor was found to be 0.47 µM, with a sensitivity of 13.66 $\mu A m M^{-1} cm^{-2}$, which is better, compared to that of previously reported works (Table 1). The sensor developed by Ameer Q. et al., using a PPy matrix, showed a detection limit of 1 µM, while the sensor in the present work, developed using AgNPs in the PPy matrix, showed a detection limit of 0.47 µM. On the basis of this study, it may be concluded that the sensor developed using a combination of biosynthesized AgNPs and PPO enzymes from *Manilkara Zapota* (sapota) is more effective in catechol detection

Table 1. Comparison of the proposed PPO biosensor with other PPO immobilized electrodes towards catechol detection.

Electrode	Linear range (mM)	Detection limit (µM)	Reference
PPO entrapped in a garose-gaur gum	0.06-0.8	6.0	[28]
IDE/PEDOT/CNT/PPO/GAD/HFR	0.1-0.5	4.2	[29]
PPy/PPO	0.001 – 0.016	1.0	[30]
Gr/PPy/AgNPs/PPO	0.001 – 0.015	0.47	present work

Repeatability and storage stability of the developed biosensor

The stability and repeatability of the developed Gr/PPy/AgNPs/PPO sensor are the crucial factors for the sensor practical application. The repeatability of the developed sensor was investigated with a series of repetitive measurements using 0.004 mM of catechol. The results of successive CV measurements showed that the sensor could retain 96% of its initial current response. This indicates that the developed biosensor has good repeatability. Furthermore, the shelf life/stability of the biosensor was also studied for a period of four weeks. The current response of the sensor decreased by, approximately, 2% of its initial value, in one week, and by 18%, in four weeks. The decrease in response may be attributed to the partial enzyme denaturation.

Real sample analysis

The developed sensor was examined for its effectiveness in real samples analysis, by using a green tea sample. The green tea sample was first analyzed using HPLC method, and it was found to contain 0.279 mg/g of catechol. The standard catechol and green tea sample HPLC chromatograms are shown in Fig 7. The tea sample extract was further diluted to get a 2 µM catechol concentration, and later used for analysis using the developed sensor. The catechol amount detected by the Gr/PPy/AgNPs/PPO sensor was 1.96 µM, against 2 µM from the HPLC method, with an excellent recovery of 98%. This indicates that the developed sensor can be effectively used for catechol detection in real samples. The developed sensor can be very useful in the tea industry for quality assessment of the tea samples, as catechol is one key component in deciding the tea's taste and aroma.

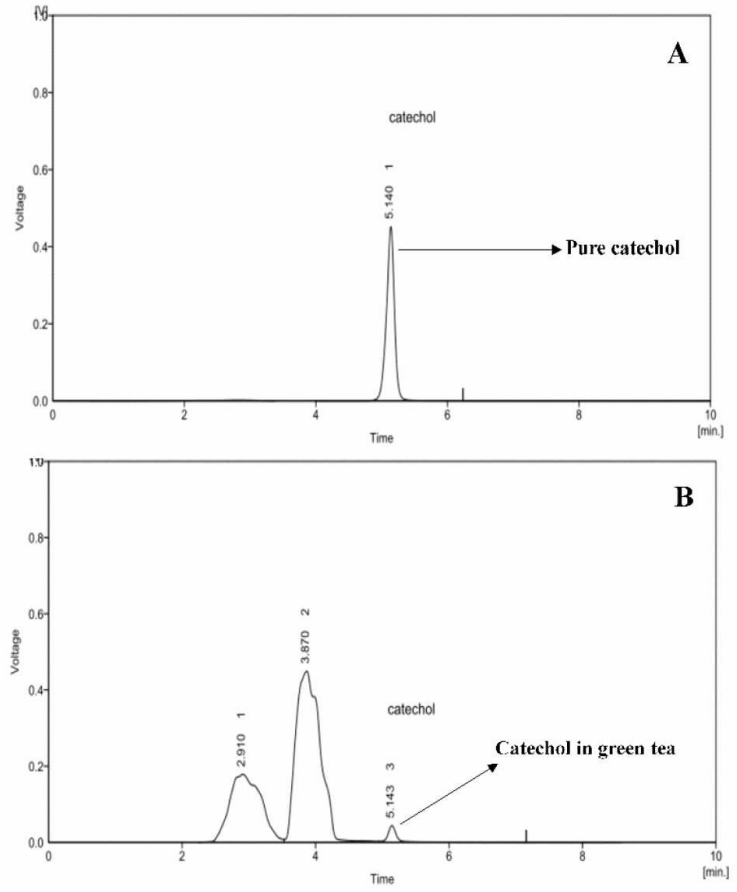


Figure 7. (A) HPLC chromatograms of standard catechol. **(B)** Green tea sample containing catechol. Catechol retention time: 5.14 min.

Conclusion

The proposed Gr/PPy/AgNPs/PPO sensor, employing biosynthesized AgNPs from *Convolvulus p.* and PPO extracted from *Manilkara Z.* (sapota), exhibited a better detection limit than that of some of the sensors reported in literature. The real sample analysis showed an excellent recovery of 98%, which indicates that

the development sensor is efficient enough to analyze real samples. Furthermore, the sensor performance does not require any sample pretreatment. Therefore, on the basis of the above results, it is concluded that the proposed sensor can be effectively employed in catechol analysis in samples.

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