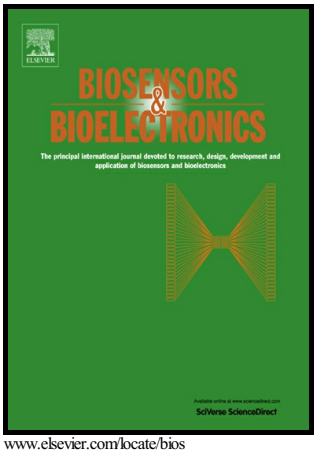

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A highly sensitive electrochemical biosensor for catechol using conducting polymer reduced graphene oxide-metal oxide enzyme modified electrode

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Abstract

The fabrication, characterization and analytical performances were investigated for a catechol biosensor, based on the PEDOT-rGO-Fe_2O_3-PPO composite modified glassy carbon (GC) electrode. The graphene oxide (GO) doped conducting polymer poly (3,4-ethylenedioxythiophene) (PEDOT) was prepared through electrochemical polymerization by potential cycling. Reduction of PEDOT-GO was carried out by amperometric method. Fe_2O_3 nanoparticles were synthesized in ethanol by hydrothermal method. The mixture of Fe_2O_3, PPO and glutaraldehyde was casted on the PEDOT-rGO electrode. The surface morphology of the modified electrodes was studied by FE-SEM and AFM. Cyclic voltammetric studies of catechol on the enzyme modified electrode revealed higher reduction peak current. Determination of catechol was carried out successfully by Differential Pulse Voltammetry (DPV) technique. The fabricated biosensor investigated shows a maximum current response at pH 6.5. The catechol biosensor exhibited wide sensing linear range from 4×10^{-8} to 6.20×10^{-5} M, lower detection limit of 7×10^{-9} M, current maxima (I_{max}) of 92.55 µA and Michaelis - Menten (K*m*) constant of 30.48 µM. The activation energy (Ea) of enzyme electrode is 35.93 $KJmol^{-1}$ at 50 $^{\circ}$C. There is no interference from D-glucose and L-glutamic acid, ascorbic acid and o-nitrophenol. The PEDOT-rGO-Fe_2O_3-PPO biosensor was stable for at least 75 days when stored in a buffer at about 4 °C.

Keywords: Catechol, Tyrosinase, Hydrothermal, Electrochemical, Biosensor.

1. Introduction

Nowadays, composites are attracting a great deal in an integrated miniaturized device using biological element (Pratima et al., 2011). Phenols are present in vegetables and fruits and they can be divided into various substances like flavones, flavanols, catechins etc. Since they play a significant role in human diet and health, highly sensitive detection of phenols has attracted increasing attention (Zhimin et al., 2005; Anna et al., 2008; Djeridane et al., 2006; Yi-Zhong et al., 2006; Alan Crozier et al., 2010). The phenolic compounds are detected by various methods such as HPLC, Flow-injection, Electrochemical, etc., (Proestos et al., 2006; Campuzano et al., 2003; Lidong et al., 2012) The HPLC and other methods have many disadvantages such as more laboratory setting, lack of rapid analysis, shorter limit of detection, higher cost and inability to field analysis. Comparatively, the electrochemical methods are cost effective, simple, and capable of carrying out field analysis and result in broader limit of detection and higher reproducibility (Jianping et al., 2012). The bulky nanostructure carbon materials such as fullerene, carbon nanotubes and graphene are known for their electrochemical applications (Xuan et al., 2010; Changsheng et al., 2010). Graphene is widely used due to their low residual current, high potential range and very good chemical stability in various electrolytes (Lu et al., 2012). In recent years, graphene has attracted much attention for construction of electrochemical modified electrodes for sensors. The graphene oxide (GO), which has poor electrical conductivity (Tapas et al., 2010; Yao et al., 2012), was reduced to obtain reduced graphene oxide (rGO) by chemical, electrochemical and thermal methods. In the above methods, electrochemical reduction was more suitable for sensor application due to the uniform deposition (Jianfeng et al., 2011; Si et al., 2012). In conducting polymers, the poly(3,4-ethylenedioxythiophene) (PEDOT) was used as one of modifier for electrode for biosensor applications as it has stable, eco-friendly, fast electron transfer and easy formation of strong-willed films (Jun et al., 2010). Moreover, the PEDOT-rGO modified electrode has electrocatalytic effect which could be used for different sensor applications

(Yajie et al., 2014; Xiaoliang et al., 2014; Wang et al., 2014). It can be used as the base for metal-oxide enzyme, incorporating on the modified electrode. Among the various immobilizing metal oxide matrix developed in recent years for excellent prospects of interfacial biological recognition (Zou et al., 2010; Qu et al., 2007; Yu et al., 2006). Hematite, α-Fe_2O_3 was used because of its environmental stability and catalytic effect (Xin et al., 2013; Ming et al., 2014). Less preparation cost, good adsorbent for enzymes and non-toxic nature with an n-type semiconductor in band gap of E_g= 2.2eV at ambient conditions are the advantages for hematite. More approaches were used to prepare α-Fe_2O_3 like microwave irradiation, pyrolysis, thermal annealing, sonochemical and hydrothermal methods (Mohammadi et al., 2012; Mammah et al., 2012; Qingtao et al., 2009; Dong et al., 2012). Though, microwave irradiation has advantages such as rapid heating and selective formation of one phase over another (Zhu et al., 2014), it has disadvantages of scaling-up and poor particle dispersion (Bilecka et al., 2011). Absence of side effect and formation of uniform film surface are advantageous for pyrolysis process but bigger particle size, low crystallinity (Patil, 1999) and surface compositions are the disadvantages (Miller et al., 2004). Thermal annealing results in improved targeting through heating (Wang et al., 2012) but saturation flux density, poor mechanability and inability to withstand thermal shocks are drawbacks (Lee et al., 2008). Sonochemical method leads to smaller size particles and inexpensive (Gedanken, 2004). But this method has disadvantages of acoustic cavitations, high energy loss and production of heat (Zhu et al., 2013). For the synthesis of metal oxides, hydrothermal method has more advantages over other methods. Soft chemical routes, structure directing method and crystal growth and defined crystal structures are the advantages (Ming et al., 2014). Hydrothermal method has already been used for the preparation of metal oxides by many researchers and in this paper hydrothermal method was used to prepare the α-Fe_2O_3 (Ming et al., 2014; Radhakrishnan et al., 2014; Wheeler et al., 2012; Chaudhari et al., 2011). The enzyme tyrosinase, polyphenol oxidase (PPO) based

biosensor for polyphenols, provides a faster, highly selective, excellent reproducibility, and higher sensitivity (Andreescu et al., 2004). Tyrosinase has two copper atoms within the active sites to oxidise the mono and diphenol compounds into quinone. Here, catechol is used as a model compound for polyphenol. The PPO enzyme mediator was used for $2e^-$ transfer process of the electrochemical biosensor (Sethuraman et al., 2013). In this context, we fabricated an electrochemical biosensor for catechol using enzyme modified electrode containing PEDOT-rGO-Fe_2O_3-PPO composite. The formation of Fe_2O_3 from $FeCl_2$ was confirmed by XRD and Micro Raman and the particle size of the iron oxide was evaluated using the same characterization. The presences of various functional groups of the modified electrodes were studied by FT-IR spectroscopy. The electrochemical performance was evaluated for the bioelectrode and catechol detection and it was carried out by Differential Pulse Voltammetry (DPV). Real sample analysis was carried out by using green tea.

2. Experimental

2.1. Materials

3,4-Ethylenedioxythiophene (EDOT), ferrous chloride, tyrosinase other name polyphenol oxidase (PPO) from mushroom species is agaricus bisporus, catechol and glutaraldehye were purchased from Sigma-Aldrich (India). Other chemicals such as sodium hydroxide, potassium nitrate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Merck (India). All the chemicals purchased were of AR grade and used without any further purification. All solutions were prepared by using MilliQ TKA-Lab pure water. GO was prepared from graphite powder by modified Hummer's method (Sasha et al., 2007).

2.2. Instruments

The morphology of the prepared electrode was studied with the help of FEG Quanta 250, Czech Republic FE-SEM. X-ray diffraction (XRD) analysis for Fe_2O_3 powder was recorded using Cu kα radiation (λ=1.54060 Å) using X-Pert Prop analytical X-ray

diffractometer system. Micro-Raman analysis was carried out using the instrument of Princeton Acton SP2500, CS spectrometer, 0.5 focal length, triple grating monochromator excitation source Ar^+ laser, 514.5 nm wavelength. FT-IR spectra were recorded using Nicolet 6700, Japan. Voltammetric experiments were carried out using CHI760 electrochemical workstation (CH Instruments, USA) with an accuracy of 50 pico Å was used to determine the response current at ambient temperatures. For voltammetric studies, a 3 mm dia glassy carbon electrode was used as a working electrode, auxiliary electrode was platinum wire and Ag/AgCl was used as reference electrode. Nitrogen gas was purged to the sample container for 20 minutes to displace dissolved oxygen and then electrochemical experiments were performed for the detection catechol using fabricated electrode. Electrochemical studies are carried out in the presence of $[Fe(CN)_6]^{3-/4-}$ in 0.1 M of KCl redox couple. Cyclic voltammograms were between the applied potentials -0.5 V to 0.5 V. Electrochemical Impedance Spectrometric (EIS) measurements were carried out in the presence of $[Fe(CN)_6]^{3-/4-}$ with the frequency range from 1-1,00,000 Hz and the applied voltage from 25 -200 mV. DPV was used for sensor studies of catechol. Parameters such as quiet time, pulse period, sampling width, amplitude, pulse width, scan increment and sensitivity of DPV technique were fixed as are 2 S, 2 mS, 16.7 mS, 50 mV, 50 mS, 8 mV and 1 x 10^{-4} A, respectively. The general reaction of catechol formation from o-quinone is used to detect the current response by tyrosinase enzyme, using a redox enzyme catalyst. The experiments were repeated four-times and the relative error were found to be less than 3.0%.

2.3. Fabrication of PEDOT-rGO-Fe_2O_3 – PPO

GC electrode was polished first with 0.3-0.05 μm alumina slurry. After thoroughly rinsing with double distilled water, the electrodes were sonicated in absolute ethanol and double distilled water for about 1 min. Before the experiment, the glassy carbon electrodes were scanned within the potential range of -0.1 to 1.5 V *vs* Ag/AgCl in 1 M freshly prepared H_2SO_4 solution until stable curves of a cyclic voltammogram were obtained. GO doped

PEDOT film was deposited by electropolymerisation method on the GC electrode by potential cycling from -0.2 to 1.2 V *vs* Ag/AgCl at scan rate of 50 mV/s and the CV is presented in the Fig S1. Solution containing 10 mM of EDOT and 1mg ml^{-1} of GO was used. The electrochemical reduction of PEDOT-GO was carried by amperometric method by applying -0.9 V for 600 s in the presence of pH 7.4 (Wang et al., 2014). Fe_2O_3 was synthesized by hydrothermal method. In brief, 0.1g of $FeCl_2$, 0.05 g of sodium fluoride and 0.01g of ammonium hydroxide were mixed and stirred the solution for 15 min and the mixture was transferred into the 30 mL telfon sealed autoclave (Dong et al., 2012; Ming et al., 2014). The mixture was heated at 170 °C for 12 h and then cooled at room temperature. The formed precipitate was washed with ethanol and deionised (DI) water 3:1 subsequently. Finally, α-Fe_2O_3 was dried under 80°C for a day. 1 mg of α-Fe_2O_3, 2 $mgmL^{-1}$ of tyrosinase enzyme in phosphate buffer (pH 7.0) and 2.5% wt of glutaraldehyde as cross linking agent were prepared about 10 mL and shaked well 15 min using shaker applying 60 rpm at 0 °C for entrapment of enzyme. 10 µL of the mixture was drop casted over the PEDOT-rGO electrode and dried at 4°C. Finally, the PEDOT-rGO- Fe_2O_3-PPO enzyme electrode was washed with the same buffer to remove loosely bounded cross linked enzyme on the surface.

The DPV current generated on the biosensor was measured, when the steady state was reached. The general reaction of catechol formation from o-quinone is used to detect the current response by enzyme, in which the redox enzyme was used as catalyst and schematic diagram represented in the Fig.1. Initially, the background current (*Io*) of PEDOT-rGO-Fe_2O_3-PPO biosensor in buffer without substrate was measured. Similar procedure was followed and the catechol response current (*Is*) was measured. The actual response current was calculated by subtracting *Io* from *Is* (*I = Is- Io*) (Sethuraman et al., 2013; Wang et al., 2009). Lipton Green tea was purchased from a shop in Karaikudi produced in Kerala and was powdered using a pestle and mortar. Catechol was extracted from 0.2 g of dry tea powder with 100 mL of 30% (v/v) methanol solution, with continuous shaking at 80 °C for 15 min,

and then sonicated for 20 min. The mixture was filtered by 0.45 µm filter paper and the filtrate was made up to 100 mL for further measurement (Wang et al., 2012).

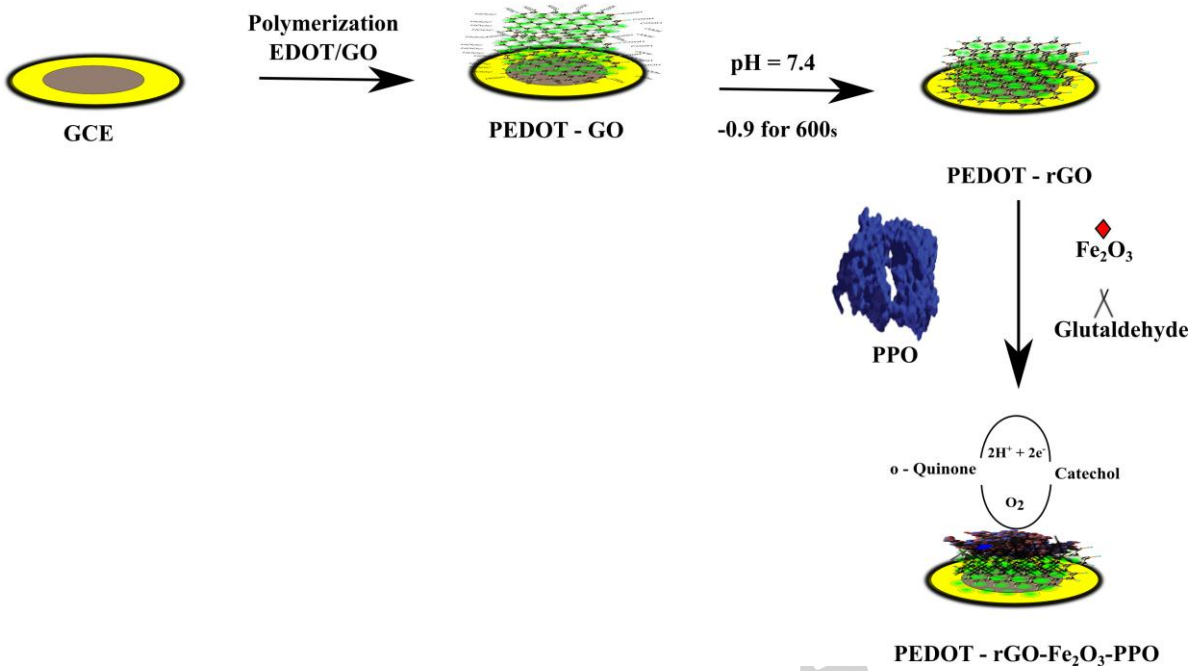

Fig.1. Schematic diagram of PFDOT-rGO- Fe_2O_3-PPO electrode for catechol.

3. Results and Discussions

3.1. Characterization of PEDOT-rGO-Fe_2O_3-PPO

The surface morphology of PEDOT-GO, PEDOT-rGO, PEDOT-rGO-Fe_2O_3-PPO and Fe_2O_3 were studied by FE-SEM. The SEM image of PEDOT-GO modified electrode shows high roughness, loose structure and resembled as wrinkled paper like sheets. Undoubtedly, such an open structure leads to large surface area as shown in Fig 2a. Fig 2b represents the crumpled morphology for PEDOT-rGO which resulted in higher surface area. The change of morphology (Fig 2d) Fe_2O_3-PPO indicates that incorporated onto the PEDOT-rGO modified electrode. As per the report (Kiran et al. 2012) α-Fe_2O_3 was biocompatible with much lesser toxicity and this facilitates their direct use for biological and biochemical application. Based on the explanation given, it can be concluded that the nano Fe_2O_3 can hold more enzyme molecules and stabilise them. The Fe_2O_3 was demonstrated with a hexagonal bipyramidal shape. The particle size of the prepared α-Fe_2O_3 particles found out from the surface

morphology (Fig 2c) was 200-450 nm (Ming et al. 2014, Huang et al., 2015). Since, α-Fe_2O_3 has homogeneous surface it can hold more enzyme on the surface which will increase the sensitivity of the biosensor. 3D topology was studied using AFM analysis. The surface roughness parameters, indicated as mean roughness (Ra), root mean square of Z data (Rq) and mean difference in the five highest peaks and five lowest valleys (Rz) were presented in Table ST1. The surface roughness of PEDOT-rGO (Fig 2f) is more than that of PEDOT-GO (Fig 2e) because reduced graphene oxide has lesser functionality. The GO was electrochemically reduced and surface exhibited higher roughness as shown in AFM. The pristine Fe_2O_3 exhibited uniform denser structure and the average particle size was 121 nm (Fig 2g). Cloudy morphology was observed for PEDOT-rGO-Fe_2O_3-PPO electrode which confirms incorporation of more enzymes in the electrode (Fig 2h). The powder X-ray diffractograms analysis of synthesized Fe_2O_3 particles is shown in Fig S2. The XRD peaks located at (2θ) 24.21, 33.25, 35.72, 40.97, 49.60, 54.23, 56.31 and 57.60° corresponds to (012), (104), (110), (113), (024), (116) (211) and (122) planes of the Fe_2O_3. Rhombohedral cantered with hexagonal bipyramidal structure of α-Fe_2O_3 was observed and it matched with the standard data (JCPDS File No.89-1166). From the XRD data the average crystallite size was estimated using Scherrer equation (Wang et al., 2014) and the estimated average crystallite size of the pure Fe_2O_3 particle was 145 nm. The Raman analysis was performed for α-Fe_2O_3 sample. The peaks at 221 and 491 cm^{-1} are conferring to A1g modes. The peaks at 244, 292, 406 and 611 are attributed to Eg modes (Fig S3) (Xin et al., 2013). The particle size was measured for α-Fe_2O_3 using Micro-Raman and it shows 127 nm which is in conformity with that calculated from AFM. The functional group of prepared electrodes were characterised by FT-IR spectra explained and presented in Fig S4a-d. The FT-IR studies reveal that the enzyme in the composite electrode was entrapped on the modified electrode.

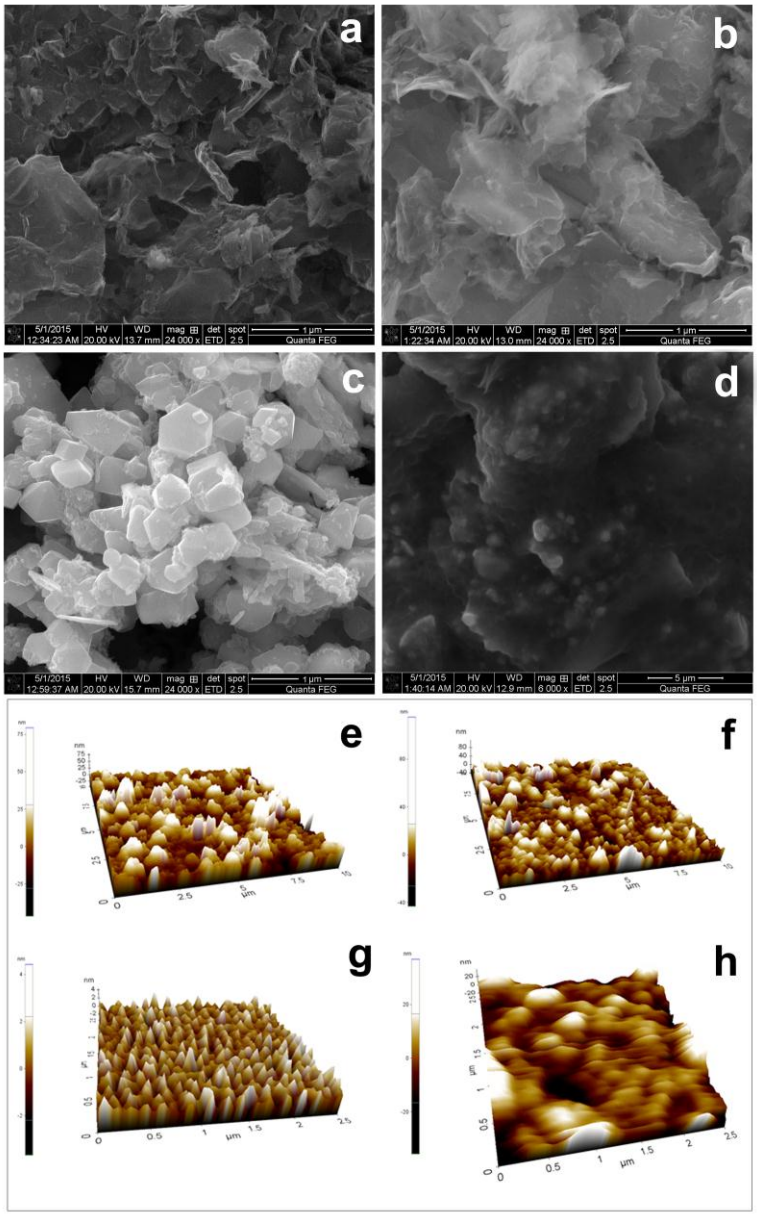

Fig.2. FE- SEM images of (a) PEDOT-GO, (b) PEDOT-rGO, (c) Fe_2O_3 (d) PEDOT-rGO-Fe_2O_3-PPO modified electrodes. AFM images of (e) PEDOT-GO, (f) PEDOT-rGO, (g) Fe_2O_3 (h) PEDOT-rGO-Fe_2O_3-PPO modified electrodes.

3.2. Electrochemical Studies

The cyclic voltammograms (CV) of 1mM $[Fe(CN)_6]^{3-/4-}$ on the bare GC electrode (curve a), PEDOT-GO (curve b), PEDOT-rGO (curve c) and PEDOT-rGO-Fe_2O_3-PPO (curve d) modified electrodes were recorded in 0.1M of KCl at a scan rate of 50 mVs^{-1} (Fig 3A). Modification of the PEDOT-GO (curve b), PEDOT-rGO (curve c), onto the GC

electrode surface by electrochemical method leads to an increase in the peak current of $[Fe(CN)_6]^{3-/4-}$ (i_{pa} : 28.15 µA) and (i_{pa} : 33.20 µA) respectively, compared to the bare GC electrode (i_{pa} : 19.75 µA). This could be attributed to high surface coverage and electrocatalysis. Now, the modification of PEDOT-rGO-Fe_2O_3-PPO onto the GC electrode significantly deteriorated the redox peak current of $[Fe(CN)_6]^{3-/4-}$ (i_{pa} : 7.45 µA) compared to the other modified electrodes. This is due to the non-conducting surface of enzyme on the electrode.

Electrochemical Impedance Spectroscopy (EIS) provides helpful information on the modification on the electrode surface during the fabrication process. The charge transfer is known by semicircle portion corresponds to the electron- transfer limiting process. The charge transfer process of the PEDOT-rGO-Fe_2O_3-PPO modified GC electrode was recorded by monitoring charge transfer resistance (R_{ct}) at the electrode/electrolyte interface. The Randels equivalent circuit for prepared electrode is R(QR)(QR). Fig 3B displays of the EIS of bare GC (curve a), PEDOT-GO (curve b), PEDOT-rGO (curve c) and PEDOT-rGO-Fe_2O_3-PPO (curve d) modified electrodes at the polarization potentials 25 mV and frequency of 1-1,00,000 Hz. The Nyquist diagrams for bare, PEDOT/GO, PEDOT-rGO, and PEDOT-rGO-Fe_2O_3-PPO modified electrodes were recorded and the R_{ct} values are 395, 143, 105 and 3879 Ω respectively. The R_{ct} value of PEDOT-rGO-Fe_2O_3-PPO is higher than that of other three electrodes. These results demonstrate that the non-conducting enzymes are on the surface in large quantity in PEDOT-rGO-Fe_2O_3-PPO. This can lead to good enzymatic reaction pathways between the electrode/electrolyte interactions and provides a good platform for sensing application. The influence of pH on the electroactivity of catechol was investigated by carried out DPV measurements in the presence 0.1 mM of catechol at various pH in the range 4-8 where the enzyme is having stability. Fig 3C shows DPV response of catechol at pH are 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 DPV responses studied. Maximum peak current response is observed at pH 6.5.

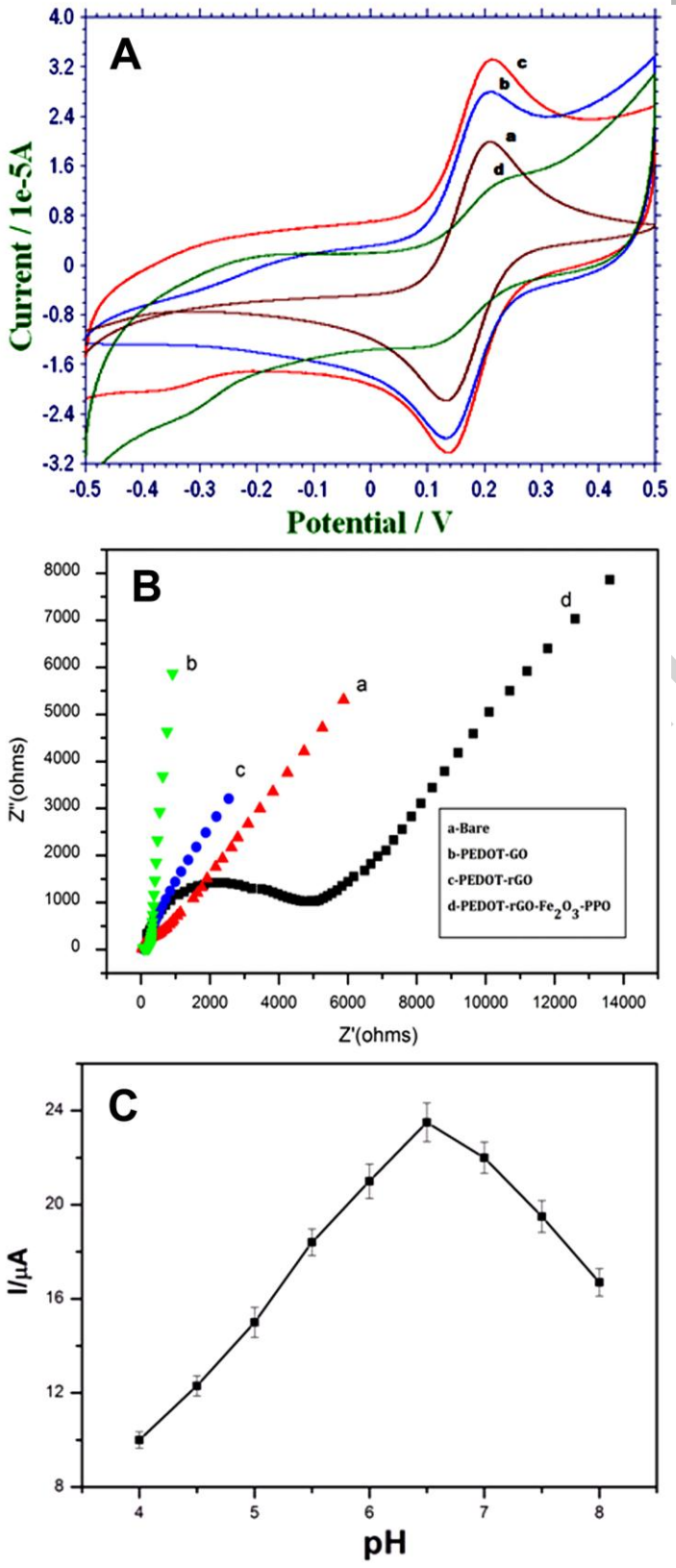

Fig.3A. A CV behavior of the modified GC electrodes in presence of 1 mM $[Fe(CN)6]^{3-/4-}$ in 0.1 M KCl at a scan rate of 50 mVs^{-1}. (B) EIS behavior of the modified GC electrodes measured in presence of 1 mM $[Fe(CN)6]^{3-/4-}$ in 0.1 M KCl. Bare GC electrode (a), PEDOT-

GO (b), PEDOT-rGO (c) and PEDOT-rGO-Fe_2O_3-PPO (d) modified electrodes. (C) Correlation between response current and pH of 50 µM catechol at 25.0°C.

The enzyme PPO's activity in different pH has influenced the response. Similar results have been already reported for similar systems such as PPO entrapped into PPO immobilized onto PANI film (Wang et al., 2009), PPO entrapped into PANI film (Tan et al., 2010). Hence pH 6.5 is chosen for the development of biosensor using PEDOT-rGO-Fe_2O_3-PPO.

3.3. Electrochemical determination of catechol

Since, PEDOT-rGO-Fe_2O_3-PPO modified electrode has good surface coverage of enzyme; it was used to elucidate the redox behaviour of catechol by following CV experiments. The cyclic voltammograms of bare GC electrode (curve a), PEDOT-GO (curve b), PEDOT-rGO (curve c) and PEDOT-rGO-Fe_2O_3-PPO (curve d) modified electrodes were recorded in the presence of 0.01mM of catechol in pH 6.5 at scan rate of 50 mVs^{-1}. Fig 4A present CVs and the anodic peak current observes for catechol are bare (i_{pa} : 14.02 µA), PEDOT-GO (i_{pa} : 16.75 µA), PEDOT-rGO (i_{pa} : 21.12 µA), and PEDOT-rGO-Fe_2O_3-PPO (i_{pa} : 15.45 µA). The formation o-quinone to catechol by applying current is also studied in a same graph. The cathodic current for catechol on electrodes are bare (i_{pc} : 11.52 µA), PEDOT-GO (i_{pc} : 10.52 µA), PEDOT-rGO (i_{pc} : 14.52 µA), and PEDOT-rGO-Fe_2O_3-PPO (i_{pc} : 18.52 µA). The increase in the reduction peak current of enzyme electrode is due to reduction of more o-quinone convert to catechol by $2e^-$ transfer process. Thus the modification of the electrode resulted in higher enzyme loading which in turn increase the sensitivity of determination of catechol. PEDOT-rGO-Fe_2O_3-PPO can give good enzymatic electron pathways between electrode and electrolyte and could be good platform for sensing applications. This bioelectrode is then used to determine catechol by following DPV experiments to increase the sensitivity further.

Differential pulse voltammetry technique gives improved sensitivity by avoiding most of the charging current by sampling the total current as late as possible after the application of each potential pulse. The DPV has conventional sweep technique when detecting very low level of analyte in the solution. Fig 4B shows the DPV response currents for different concentrations of catechol in pH 6.5 at 25 ºC under optimized experimental conditions. The DPV showing respective responses are the sensor upon the successive addition of different concentration of catechol illustrated. Under this optimized condition it is used to estimate the detection limit of catechol.

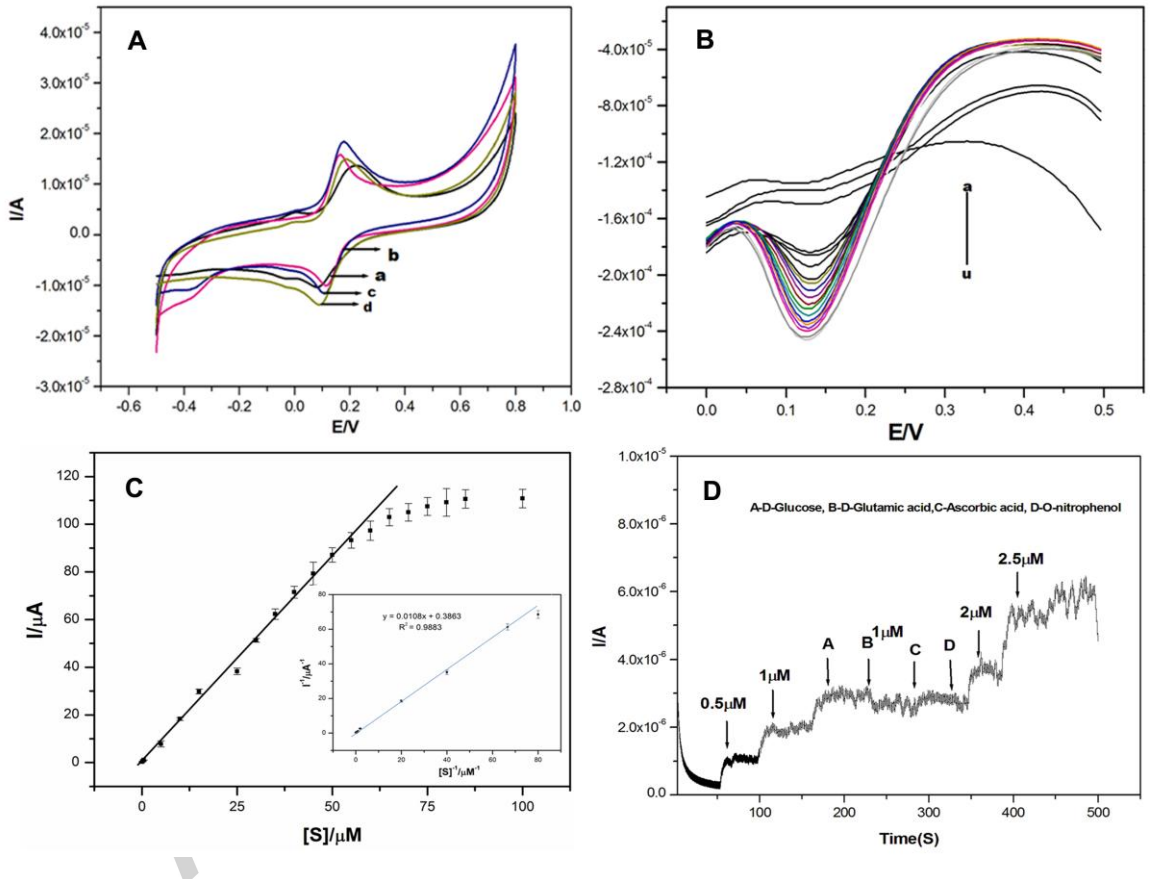

Fig.4A. A CVs obtained for nitrite 0.01mM at the (a) bare GC, (b) PEDOT-GO, (c) PEDOT-rGO, and PEDOT-rGO-Fe_2O_3-PPO (d) modified electrode recorded in PB solution (pH 6.5) at a scan rate of 50 mVs^{-1} at a potential between -0.5 and 0.8 V. (A) DPVs of PEDOT-rGO-Fe_2O_3-PPO film catechol concentrations buffer, 0.025, 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 µM. a-u (B) Correlation between response current and

catechol concentration at pH 6.5 and 25.0°C (C). Effect of interference on the response of the catechol biosensor (D).

The enzymatic oxidation of catechol generated on the modified electrode is directly proportional to the catechol concentration in the solution. The response current gives the catechol concentration a linear range from 4×10^{-8} to 6.20×10^{-5} M and the lower limit of detection is 7×10^{-9} M which was comparable to other electrodes (Fig 4C). The lower limit of detection of catechol observed is due to higher concentration of enzyme incorporated on the surface which in turn catalytically oxidised the catechol. The enzyme catalytic reaction follows first-order kinetics, since the response current increases slowly as the concentration increases and attains at a steady state and it becomes zero-order kinetics. A plot of inverse current and substrate concentration was constructed to find out maximum current (I*max*) and Michaelis-Menten (K*m*) (inset in Fig4C). The I*max* is 92.55 µA and K*m* is 30.48 µM. The lower Michaelis-Menten constant confirms the utilization of this biosensor for the detection of both low and high concentrations of catechol (Tang et al., 2008, Wang et al., 2008; Zhang et al., 2009; Perez Lopez, 2011; Wang et al., 2009; Tan et al., 2010; Mu, 2006) presented in Table 1.

Table. 1. Comparison of efficiency of PEDOT-rGO-Fe_2O_3-PPO based biosensor for catechol with reported results.

Different matrix	***K'm*** **(µM)**	**Linear range[M]**	**LOD [M]**	**pH**	**Temp (°C)**	**E*a*** **(KJ mol^{-1})**	**Ref.**
HRP immobilized onto poly(aniline-co-*o*-aminophenol)	--	5×10^{-6} to 8×10^{-5}	1×10^{-6}	5.0	35	23.6	Mu et al., 2006
PPO entrapped using agarose-guar gum	22	6×10^{-5} to 8×10^{-4}	6×10^{-6}	6.0	--	--	Sanket et al., 2007
PPO immobilized into PANI –ionic liquid film	1.44	4×10^{-10} to 2.1×10^{-6}	1×10^{-10}	7.0	35	38.8	Zhang et al., 2009
PPO immobilized onto PANI film	117	$2\times10^{-7}$to 8×10^{-5}	1×10^{-7}	6.0	40	30.23	Wang et al., 2009
PPO entrapped into PANI film	146	1.25×10^{-6} to 1.5×10^{-4}	--	6.5	40	31.1	Tan et al., 2010
Tyr-NGP-Chi/GC	--	$1x10^{-5}$to $2x10^{-6}$	3.3×10^{-7}	7.0	35	--	Lidong, et al., 2012
PPO entrapped into PANI film in presence of surfactant	85.44	$5\times10^{-7}$to 1.01×10^{-4}	1.5×10^{-7}	6.0	45	41.74	Sethuraman et al., 2013
PEDOT/RGO/GCE	--	0.1to175 µM	--	7.4		--	Wang et al., 2014
PEDOT-rGO-Fe_2O_3-PPO	30.48	4×10^{-8} to 6.20×10^{-5}	7×10^{-9}	6.5	50	35.93	This work

The amperometric responses of catechol in presence of potential interferences such as D-glucose, L-glutamic acid, ascorbic acid and o-nitrophenol in the phosphate buffer pH 6.5 are presented in Fig. 4D. The interfering substances were added sequentially in the same solution and the amperometric measurements were carried out. Since there is no significant change in the current response, the selectivity of the biosensor is good.

3.4. Effect of Temperature

DPV current response for 0.1 mM of catechol at the biosensor was studied at pH 6.5 by varying the temperature of the cell solution from 0 to 60 °C using controlled thermostatic

bath. The current response increases with temperature increased upto 50 °C and then decreases. Maximum current response was observed of the PEDOT-rGO-Fe_2O_3-PPO electrode due to higher stability of metal oxide and enzyme for the electrode upto 50 °C (Fig 5a) and showed a deceasing response current above 50 °C due to unfolding of proteins i.e., the denaturation of PPO is possible at high temperature. The PEDOT-rGO-Fe_2O_3-PPO electrode has environment stability to protect the PPO and improves heat stability of the PPO upto 50 °C.

According to the Arrhenius equation:

$$\ln k = \ln A - \frac{Ea}{RT}$$

Where, *k* is the rate constant and *Ea* is the apparent activation energy. The electrode surface area and the response current are directly proportional to rate constant *k* at fixed concentration, pH and quantity of enzyme. Therefore, the ln*k* was replaced as ln*I* in the Arrhenius formula. From the slope of the linear relationship of ln*I* versus 1/T, the activation energy was calculated (Fig 5b). The activation energy for PPO tyrosinase and peroxidase reactions also changed during decomposition. The value of *Ea* for PEDOT-rGO-Fe_2O_3-PPO in pH 6.5 is found to be 35.93 KJmol^{-1} which is comparable to the other biosensors reported (Wang et al., 2009; Tan et al., 2010; Zhang et al., 2009; Mu et al., 2006; Sethuraman et al., 2013).

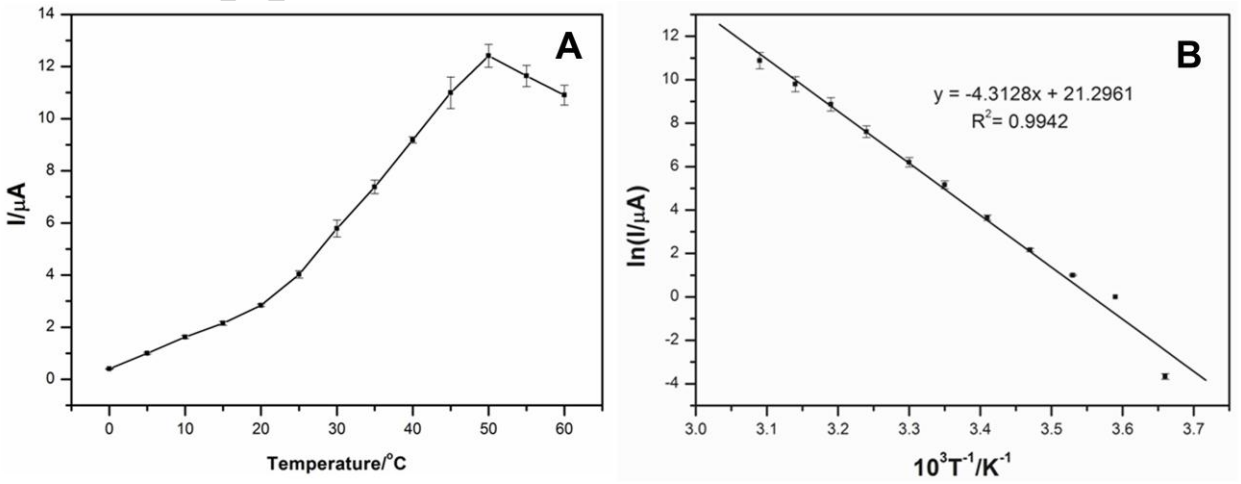

Fig.5a. The relationship between response current and temperature of 50µM catechol pH 7.0 PEDOT-rGO-Fe_2O_3-PPO modified electrodes. Fig. 5b Plots of *lnI* versus *1/T* according to the data in Fig.5a.

3.5. Interference, Recovery, Stability and Reproducibility

The response current was measured for 2µM catechol by employing the fabricated electrode. The electrode was washed with DI water and kept for one week at 4 ^{o}C. The response current was measured again and only 3-4% R.S.D in the response current was observed. This shows the stability of the electrode.

The recovery results obtained suggested an absence of matrix effect on those determinations. Recoveries from 96 to 103.5% of green tea from three commercial products were obtained using this procedure. The reproducibility of the biosensors were showed only 3% variation. The prepared biosensor was studied for the reproducibility using six electrodes, and the results showed a satisfactory reproducibility with a R.S.D of 3.2% for the current determined at 0.01 mM catechol concentration. The stability of the biosensor was investigated for a period 75 days measurements were made every 5 days. The stability determined for the biosensor was 79% confirming, as accepted, the higher stability of catechol with RSD of 3.5% (Fig S5). The proposed biosensor has been successfully applied for the accurate determination of catechol by carrying out 50 successive experiments and the results indicate good repeatability for catechol Fig S6.

The performance of the fabricated electrochemical biosensor using PEDOT-rGO-Fe_2O_3-PPO was compared with that of HPLC. HPLC was performed for the determination of catechol in the same green tea sample by external standard method. Quantification of catechol present in green tea extract was done by addition of three different concentrations of catechol using the developed biosensor. Fig S7 illustrates the chromatogram of the standard of catechol and one tea sample. Quantification of catechol and recovery percentages were found out from the obtained parameters and the results were presented in Table ST2. The

results obtained from the present method and HPLC are comparable and hence the developed biosensor can very well be used for the determination of catechol in tea samples.

4. Conclusion

The fabricated PEDOT-rGO-Fe_2O_3-PPO electrode shows higher loading capacity of enzyme and hence results in better electron-transfer efficiency. Current maxima (I_{max}) of 99.25µA and lower Michealis - Menten (K*m*) constant of 30.48µM were observed. The lower K*m* indicates the possibility of detection of both low and high concentration of catechol. The important interference compounds have no influence in the biosensor response. The PEDOT-rGO-Fe_2O_3-PPO biosensor was stable for at least 75 days when stored in a buffer at about 4 °C. We believe that the electrode could be used in real life application. Biosensor efficiency and incorporation in reduced graphene oxide with different polymer with metal oxide based material are currently in evolution.

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Highlights

- PEDOT-GO was prepared by potential cycling and GO was reduced by amperometric method.
- Fe_2O_3 nanoparticles were synthesized in hydrothermal method.
- Fe_2O_3 and Polyphenol oxidase (PPO) was immobilized over the prepared electrode.
- PEDOT-rGO- Fe_2O_3-PPO biosensor exhibit excellent sensing capability with selectivity for catechol.