A comparative study on NiO nanocrystal modified graphite and Au electrode matrices as immobilization supports for laccase enzyme in amperometric biosensing for catechol detection

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Abstract

A comparative study of the electrochemical behaviours of two different electrode matrices used in the construction of amperometric laccase biosensors is reported here for catechol detection in water. The matrices considered are NiO nanocrystal (NC) modified graphite electrode (MCPE-NiO) and Au electrode of Clark type DO sensor. The laccase enzyme from *Trametes versicolour* was immobilized on electrode surfaces by co-crosslinking method using bovine serum albumin, a protein-based stabilizer, along with glutaraldehyde as the crosslinking agent. A comparison of the stability parameters of the electrode designs was carried out including sensitivity, calibration plots, analytical data and storage stability, and the biosensor performance was shown to be superior for MCPE-NiO-Lac compared to Au-Lac electrode. The NC modified system reached steady state within 6 seconds after the analyte contact and displayed a lower detection limit of 0.95 μM, while the Au electrode took 3 minutes to reach the same and had lower detection limit of 4 μM. Better reproducibility and longer linear response was also observed for MCPE-NiO system compared to the latter, all of which could be attributed to the microstructure of the electrode and the surface lattice arrangement in the embedded nanocrystals.

Keywords: Amperometric biosensor, laccase enzyme, NiO nanocrystals, clark type DO sensor, au electrode, carbon paste electrode, catechol detection.

Introduction

Phenol and its numerous derivatives are used extensively in industries catering to polymer materials, wood technology, petrochemicals, pharmaceutical and manufacture of abrasives, plasticizers and detergents **[1]**. Phenolic compounds can enter into food chain from waste water when discharged carelessly, causing dangerous and toxic effect on human and aquatic life. Toxicity of phenolic compounds provoke mutagenesis and carcinogenesis and also act as endocrine disrupters causing health hazards for human and other living organisms. Some of the principal methods for quantitative phenolic compound estimation are high performance liquid chromatography, capillary electrophoresis and gas chromatography **[2, 3]**. Although these methods are analytically capable, they are all time consuming detection processes involving complex pre-treatment steps and generally require highly trained operators. Alternately, enzyme based amperometric biosensors have been developed by researchers for estimating phenolic compounds due to their advantages such as good selectivity, rapid response with high accuracy, relatively easy operation, working possibility in aqueous medium, low cost and the potential for miniaturization and automation **[4]**. These biosensors developed for detecting phenol and their derivatives usually have working electrodes containing polyphenol oxidase or horseradish peroxidase enzymes **[5]** and have reversible oxidation of phenol derivative during the detection process.

Laccase is a polyphenol oxidase enzyme produced by plants and microorganisms, having multiple copper containing oxido-reductase (benzenediol:oxygen oxidoreductase, E.C.1.10.3.2). It reduces oxygen from atmosphere directly into water in a four-electron step transfer without intermediate formation of soluble

hydrogen peroxide **[6]** and at the expense of one-electron oxidation of the substrates (analytes). In third generation biosensors, immobilized redox enzymes act as electrocatalysts facilitating direct electron transfer between the electrodes and the substrate molecules and involving no mediator **[7]**. Mediators are reversibly electroactive systems that work as electron transferring agent by acting as bridge between the analyte and the electrode. These biosensors usually offer better sensitivity and selectivity, as they provide high integration between the biomolecule and the electrode surface and operate in a potential range closer to the redox potential of the enzyme and are thus exposed less to the interfering reactions.

The electron transfer rate depends strongly on the immobilization procedures and the type of support materials used. The support should be chemically stable in the analytical environment present and inert to the enzyme and detected analytes. They should also provide large surface area and least diffusion limitation for the transport of analytes and the products formed from the enzymatic reactions **[8]**. Laccase assembly for electrochemical sensing has been studied by several researchers on different solid supports such as glassy carbon, carbon paste, Pt and Au by immobilization methods such as direct adsorption, covalent binding, entrapment in polymeric membranes or gels and cross-linking procedures. Among them, graphite or carbon paste electrodes are attractive for electrochemical studies and various analytical applications due to their low background current, wider anodic potential range and chemical stability **[9]**. Recently laccase immobilized graphene-cellulose microfibers composite modified screen printed carbon electrode has also been used as catechol biosensor **[10]**. The high conductivity of graphene and good biocompatibility of cellulose microfibers helps in firm attachment of laccase on the composite modified screen printed electrodes exhibiting high sensitivity for the detection of catechol. The modification of electrode surfaces by nanomaterials is also studied due to their high conductivity, surface to volume ratio and interesting surface charge properties leading to rapid detection and specificity for molecular analytes **[11]**. Besides, they function as chemical modifiers to decrease any over-potential of the electrode through electron transfer reactions with reversible regeneration. They also provide excellent platform for interfacing bio-recognition elements for signal amplification **[12]**. Au surfaces also provide similar advantages as enhanced electrode conductivity and electron transfer leading to improved detection limit for molecules **[13]**. They have fascinating electronic and optical properties and serve as good candidate for the immobilization of enzymes for biosensing. The range of electrochemical methods available with carbon and Au electrodes display the high design rate in the development of these sensors.

The work presented here deals with the development of *Trametes versicolor* laccase based catechol biosensor on NiO NC modified carbon paste electrode as well as Au electrode surfaces, using co-crosslinking method of enzyme immobilization with a protein based stabilizing agent, bovine serum albumin (BSA). Enzyme modified electrodes show good sensitivity to selected compound detection depending on the enzyme being immobilized and the corresponding biochemical and electrochemical reactions **[14]**. The electrode configurations in each case is optimally designed to ensure that the electron transfer distance between the immobilized redox biomolecule and the electrode surface is short in order to provide fast electron transfer. The electrode design also focuses on achieving high efficiency for catechol biosensing. The sensitivity, calibration curves and the stability of the biosensors are investigated and compared.

Experimental

Reagents and apparatus

Laccase from *Trametes versicolor* having specific activity of 10 IU $mg^{-1}$and NiO NCs with diameter ~10 nm were procured from Sigma USA. Catechol, graphite powder, and silicon oil were obtained from SD Fine Chem Ltd. India. Glutaraldeyde, bovine serum albumin (BSA), disodium monohydrogen phosphate heptahydrate, and potassium dihydrogen phosphate were acquired from Hi media, India. All solutions were prepared with doubly distilled water.

Preparation and electrochemical measurements

(i) Bare and NiO NCs modified carbon paste electrodes

The unmodified or bare carbon paste towards electrode fabrication (BCPE) was prepared by grinding graphite powder (70.0% w/w) and silicon oil (30.0% w/w) together until a homogeneous paste was obtained. NiO NC modified carbon paste (MCPE-NiO) was prepared by mixing NiO NCs with graphite powder in different weight ratios of 1:2, 1:3, 1:4 and 1:5 (w/w) followed by the mixing of silicon oil at regular intervals until a uniform paste was obtained. For laccase immobilization on NiO NC modified carbon-paste electrode (MCPE-NiO-Lac), the enzyme solution containing 5 IU laccase, 20 µl of glutaraldehyde (5% (v/v) and 2.5 mg BSA were added into the graphite powder-NiO NC mixture. Finally, carbon paste was obtained by mixing with 10 µl silicon oil for 20 min. The different pastes were firmly placed in cavities of Teflon tube with 2 mm internal diameter and containing copper rod on one end. The surface of the nanocomposite electrode at the other end was smoothened by a wax paper before starting the electrochemical experiments. The obtained electrodes were stored in the refrigerator at 4°C when not in use.

The electrochemical experiments were performed at room temperature using an Autolab PGSTAT 3.0 Potentiostat/Galvanostat model in a three-electrode configuration. A Pt wire was used as the auxiliary electrode, the carbon paste electrode was the working electrode and a Calomel electrode was used as the reference electrode. The pH values of the buffer solutions were measured with a systronics model pH meter equipped with a glass electrode. The electrochemical

behavior of MCPE-NiO electrodes were examined by cyclic voltammetry (CV) in 0.1 M KCl solution containing 10 mM $K_3[Fe(CN)_6]$ and compared to the behaviour of BCPE. The electrocatalytic activity of MCPE-NiO and MCPE-NiO-Lac electrodes were investigated in the presence and absence of 1 mM catechol in 0.2 M phosphate buffer saline (PBS, pH 7.0). The cyclic voltagrams were recorded in the potential range between -0.2 and 0.8 V at a scan rate of 50 mV/s. The effect of scan rate on CV of MCPE-NiO-Lac electrode was also studied by varying the same between 10-200 mV/s. Electrochemical impedance measurements were carried out in 10 mM $Fe(CN)_6^{3-/4-}$ solution to analyze the charge transfer properties of bare and NC modified carbon paste electrodes at room temperature using a CHI604E electrochemical work station. A platinum foil and Ag/AgCl electrodes were used as the counter and reference electrodes respectively. The steady-state amperometric responses of the MCPE-NiO-Lac to different concentrations of catechol were determined by the successive addition of different volumes of 2 and 200 mM catechol into 20 ml of pH 7 PBS with stirring at 0.3 V against standard calomel electrode. First, the enzyme electrode was equilibrated in PBS at 0.3 V until a constant current (i_1) called as background current was obtained. Then, aliquots of catechol solution were added to the electrochemical cell. The steady-state current response (i_2) to the addition of catechol was recorded and the current difference ($\Delta i = i_2$-i_1) was determined with the change between the steady-state current and the background current. A calibration curve of Δi–catechol concentration was plotted.

(ii) Enzyme immobilized gold electrode of Clark type DO sensor

The two terminal Clark type DO (dissolved oxygen) electrode was purchased from M/S Century Instruments, Chandigarh, India and consisted of Au cathode and Ag/AgCl reference electrodes. The laccase enzyme was immobilized on Au surface (Au-Lac) for amperometric estimation of catechol in water. For this, 5IU of laccase was crosslinked with 20 µl glutaraldehyde (5%) on Au electrode surface using 2.5mg of BSA. After incubating at room temperature for 1hr, the electrode was washed with 0.2 M PBS solution (pH 7.0) to remove excess glutaraldehyde. The electrode was further immersed in a sample cell containing 5ml of the same PBS solution. The interconnectivity between the working electrode and the reference electrode was established through the electrolyte solution, which was phosphate buffer saline. The area of the working electrode was 1.2mm^2. The sample estimation by the prepared enzyme electrode was done using a detector system developed indigenously based on amperometric principles, which monitored the signals and amplified the same. The dependency of the amperometric response on the pH and working potential for Au electrode was carried out prior to this in 0.2 M PBS solution for optimum values using the same detector system and for catechol increments from 200-600 µM.

The dependency of the amperometric response on the pH and working potential for Au electrode was carried out in 0.2 M PBS solution for optimum values and for catechol increments from 200-600 µM. Further, the steady-state amperometric responses of the electrode to different catechol concentrations were determined by adding 50 µL of standard catechol solutions of varying strength in presence of the buffer. A similar procedure of estimation as adopted for NiO NC based electrode was used here. We tested the electrode-to-electrode reproducibility by preparing multiple biosensors independently under same conditions in both cases and subjected to electrochemical biosensing. The long-term stability of the electrodes was determined by performing activity assays within 20 days. The practical application of the biosensors prepared was demonstrated by investigating their responses in real water samples such as tap water and industrial effluents using 100µM catechol at optimum potentials. The experiments were repeated five times for better statistics.

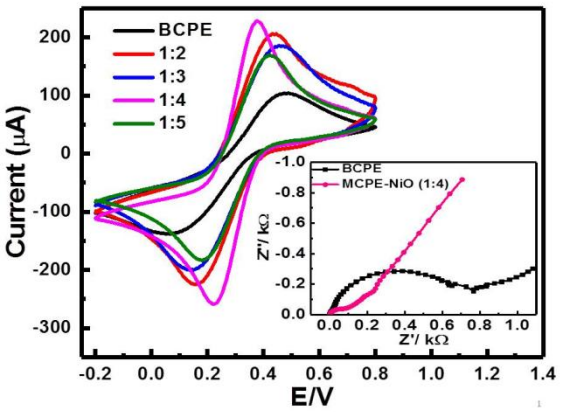

Fig. 1. CVs of 10 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ on various MCPE-NiO electrodes prepared with the mass ratio of NiO NCs to graphite powder as 1:2, 1:3, 1:4, and 1:5 at a scan rate of 50 mVs^{-1}. Inset picture gives EIS spectra of BCPE and MCPE-NiO (1:4) performed in 0.1M KCl and 10 mM $Fe(CN)_6^{3-/4-}$ solution.

Results and discussion

Electrochemical studies of NiO NC modified graphite electrodes and Au electrode of Clark type DO sensor

Highly crystalline NiO particles of diameter ~10 nm were commercially procured and were found to have a face centered cubic growth phase as understood from their XRD studies (pattern not shown here). MCPE-NiO electrodes were prepared at various mass ratios of NiO NCs /graphite (ie. 1:2, 1:3, 1:4, and 1:5) and their electron transfer features examined using $K_3[Fe(CN)_6]$ as redox probe and compared with those of BCPE. **Fig. 1** shows the CVs of different MCPE-NiO and BCPE electrodes prepared in $Fe(CN)_6^{3-/4-}$ redox couple solution exhibiting a pair of redox waves between -0.2 V and 0.8 V of the potential. The modified electrodes exhibited increased current flow and reduced peak-to-peak potential separation (ΔEp) between the cathodic and anodic waves compared to BCPE. ΔEp was found lowest for NC/graphite ratio of 1:4, while the anodic and cathodic

peak currents were the highest for the same electrode. These results denoted that the NiO NCs provide increase in electron transfer at the solution/electrode interface on incorporation into carbon paste as compared to BCPE, due to their enhanced electrocatalytic properties and extended electroactive surface area **[15, 16]**.

The electrochemical impedance spectra (Nyquist curves) of BCPE and MCPE-NiO (1:4) electrodes are shown in the inset of the figure. EIS is an effective technique to investigate the interface properties of electrodes **[17]**. The Nyquist curve for BCPE included a semicircle portion at high frequencies and a linear portion at low frequencies corresponding to an electron transfer limited process and a diffusion process, respectively. The semicircle diameter is equal to the electron transfer resistance (Ret) value of the system. The BCPE has a large semicircle, whereas the MCPE-NiO electrode exhibits almost a straight line, indicating that NiO NCs enabled fast electron transfer kinetics for the $Fe(CN)_6^{3-/4-}$ redox system. The good electron transfer ability of NiO NCs can further facilitate easy electrochemical oxidation of catechol on the electrode surface **[18]** during the electrocatalytic activity.

Fig. 2 depicts the cyclic voltammograms of different electrodes in pH 7 PBS solution containing 1 mM catechol at a scan rate of 50 mVs^{-1}. As seen in the figure, a small background current was obtained for BCPE in the potential range from -0.2 to 0.8 V, while a significant increase of current response was obtained for the MCPE-NiO electrodes (pink curve) for catechol detection. This could be attributed as mentioned above to the enhanced electrocatalytic activity by NiO NCs in the carbon paste **[19]**. However, the voltammogram of laccase enzyme immobilized MCPE-NiO (ie. MCPE-NiO-Lac) displayed much higher redox peak currents compared to other two electrodes as related to highly efficient catalysis of the enzyme towards the catechol oxidation. Detailed analyses of CVs obtained and the different redox processes involved during the electrochemical oxidation-reduction reactions and based on adsorption property of the enzyme on the NiO modified electrode is being carried out and prepared as a separate manuscript **[20]**.

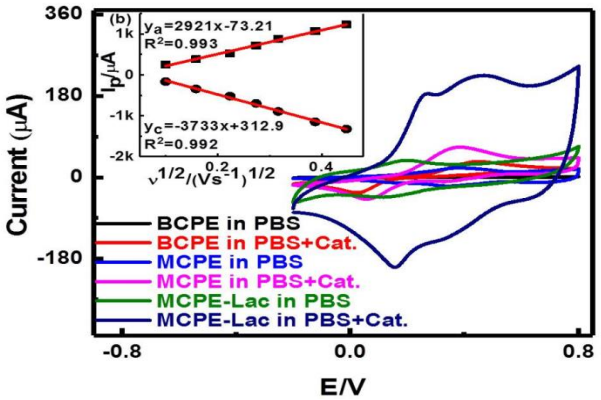

Fig. 2. Cyclic voltammograms of BCPE, MCPE-NiO, MCPE-NiO-Lac in 0.2 M pH 7 PBS buffer solution containing 1mM catechol at a scan rate of 50 mV s^{-1}. Inset picture shows the linear variation of the peak current as a function of the square root of the scan rate for MCPE-NiO electrodes.

The figure also displays the cyclic voltagrams of each of the three electrodes in presence of only phosphate buffer solution where in a similar trend of activity could be observed with much reduced overall current flow, compared to catechol oxidation-reduction reactions. The effect of scan rate on the voltammetric response of MCPE-NiO electrode was separately studied and the peak current was found to increase consistently as the scan rate increased. The variations of anodic and cathodic peak currents are shown in the inset of the figure. They exhibited a linear dependence on the square root of the scan rate indicating that the mass transfer phenomenon at the electrochemical probe and the electrode interface is mainly a diffusion-controlled process **[21]**.

The laccase enzyme was immobilized on NiO NC modified graphite electrode along with stabilizing and cross-linking agents such as BSA and glutaraldehyde in order to achieve maximum sensitivity. The biocomposite is then packed in Teflon tubes to fabricate amperometric biosensors. The incorporation of BSA along with glutaraldehyde during the process of enzyme immobilization contributed to the long term operational stability of the biosensor. This was investigated in our previous studies on laccase based sensors for analysis of substituted phenols **[12, 22]**. The concentrations of laccase, BSA and glutaraldehyde combination for maximum biosensor response with minimum reagent levels were optimized using Box-Behnken design of experiment **[23]**. The enzyme immobilized electrode was further analyzed using FT-IR spectroscopy for investigating the binding sequences and for nano-biocomposite formation.

Fig. 3 shows the FTIR spectra of MCPE-NiO-lac electrode material and that of laccase solution, which comprises of laccase enzyme, glutaraldehyde and BSA solutions in required proportions as discussed in the experimental section. The spectra for MCPE-NiO-Lac electrode material (**Fig. 3a**) showed multiple bands in the region between 350-700 cm^{-1} corresponding to Ni-O stretching and bending vibration modes. The peaks in the region 650–800 cm^{-1} were arising due to out of the plane N–H wagging modes, and the multiple peaks observed in the region 700-1100 cm^{-1} were due to M-OH and M-OH_2 stretching in the composite **[24]**. These peaks were absent in laccase solution spectra in **Fig. 3b**, which showed broad IR peak around 3400 cm^{-1} corresponding to the OH- and NH- stretching vibrations in the enzyme **[25]**. The peaks due to the amide linkages from the enzyme and the cross-linking agent were seen at 1420, 1450 and 1640 cm^{-1} in the laccase solution spectra. The intensity of the typical laccase solution spectra peaks were in general reduced in the nanocomposite due to their decreased weight proportion in the carbon paste matrix compared to the pure solution. IR absorption bands at 1024, 1075 cm^{-1} in **Fig. 3a** was due to C–N stretching, which was significant for the nanocomposite. All spectra showed peaks close to 2950 cm^{-1} attributed to symmetric and asymmetric stretching of NH_3 groups, while those at 1262 cm^{-1} could be due to C-O stretching vibrations in the nanocomposite. Unlike laccase solution pattern,

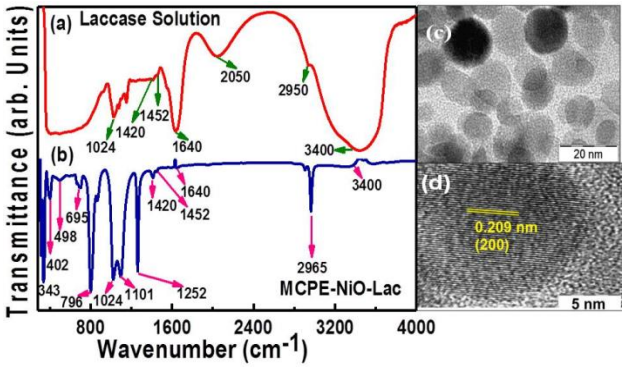

Fig. 3. FTIR spectra of (a) laccase solution (b) laccase immobilized NiO NCs-carbon paste. **(c)** TEM image of NiO particles in carbon paste and (d) HRTEM of the NiO nanocrystal imaged along (200) crystal plane.

Fig. 3a in general showed strong signatures of M-O, M-OH, M-OH_2, C-N and C-O stretching vibrations pointing to the formation of a nano-bio-composite. The **Fig. 3(c)** and **(d)** shows TEM images of NiO NCs neatly dispersed in the carbon paste matrix and the corresponding HRTEM image displaying (200) growth phase with 0.29 nm lattice plane separation. The value agreed well with that of the d value obtained from NiO NC XRD pattern recorded. We have previously reported the formation of nano-biocomposite in α-Fe_2O_3 NC based enzyme biosensor facilitating catechol detection from aqueous solutions **[12]**. Similar pattern is being observed here with much enhanced sensitivity for organic molecule detection on incorporation of NiO NCs in carbon paste matrix. As far as reaction mechanism goes, first, the catechol on contact with the enzyme was oxidized to 1,2-benzoquinone in the presence of molecular oxygen. Subsequently, the 1,2-benzoquinone was reduced electrochemically on the surface of the electrode. The obtained current in the process of electrochemical reduction of the 1,2-benzoquinone to catechol was proportional to the concentration of catechol present in the aqueous medium.

Amperometric biosensing of catechol by electrodes

To acquire optimal amperometric response for catechol biosensing, we investigated first the effects of solution pH and applied potential on the current values of laccase enzyme immobilized MCPE-NiO and Au electrode of Clark type DO sensor. As shown in **Fig. 4a**, the current value for laccase immobilized MCPE-NiO electrode slowly increased and reached maximum value at pH7, followed by a sudden decrease. The peak current value was obtained at pH 7 for Au electrode of Clark type DO sensor also, though peak rise was less sharp. **Fig. 4b** presents the influence of applied potentials on the responses of the electrodes. It can be seen from the figure that the maximum current was obtained at 0.3 V and 0.2 V respectively for MCPE-NiO and Au electrodes after enzyme immobilization. So, 0.2 M PBS solution with pH 7 was used and the applied potential was set at 0.3 V and 0.2 V respectively for NiO NC based and Au electrodes in the following experiments.

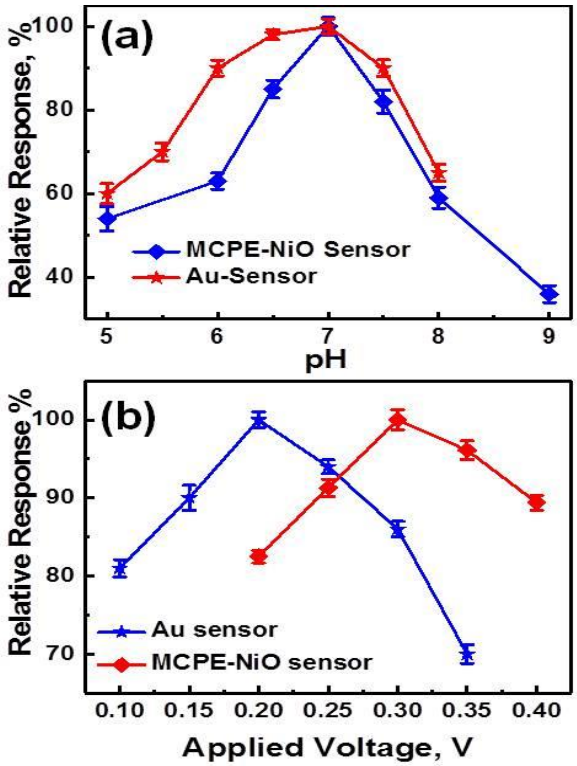

Fig. 4. Effect of solution pH (a) and applied potential (b) on the steady-state current response of MCPE-NiO-Lac and Au-Lac electrode of Clark type DO sensor in 0.2 M PBS solution containing 1mM catechol.

Further, the steady-state amperometric response of MCPE-NiO-Lac electrode to different concentrations of catechol was determined by successive addition of different volumes of 2 and 200 mM catechol into 20 ml of pH 7 PBS solution with stirring under the optimum conditions, ie at pH 7 & applied potential of 0.3 V. It can be seen from **Fig. 5a** that with the successive addition of catechol the steady-state current values increased gradually and the first current step happened on adding 2 μM catechol into the PBS solution. The corresponding calibration curves are shown in the inset **(b)** of the figure. The MCPE-NiO-Lac electrode showed a linear response range of 2-160 μM with linearity equation Y = 0.126 x+ 0.069 (R^2 = 0.997) and a limit of detection (LOD) of 0.95 μM for S/N = 3.

The steady-state amperometric response of Au-Lac electrode of Clark type DO sensor was also determined for different concentrations of catechol by similar successive addition as described above and for optimum conditions of pH 7 and applied potential of 0.2 V. The calibration curve of the response is shown in the **Fig. 5c**, which displayed more gradual increase of the current values with catechol addition and the limited response of the biosenor. The first current step happened for Au electrode when 10 μM catechol was added to the PBS solution. The linear range of 10-100 μM was displayed by the Au-Lac electrode with a LOD of 4 μM for the same signal to noise ratio. **Table 1** compares biosensing performance of several laccase modified electrodes toward catechol. Our NiO NC based biosensor showed a very low detection limit and one of the most wide linear

ranges with good sensitivity, among the different sensor electrodes known.

Laccase immobilized electrodes were further tested for repeatability, reproducibility and stability parameters in our study. The relative standard deviation (RSD) of the MCPE-NiO-Lac biosensor response to catechol was within 4.0% for 10 successive measurements indicative of the good repeatability of the electrode.

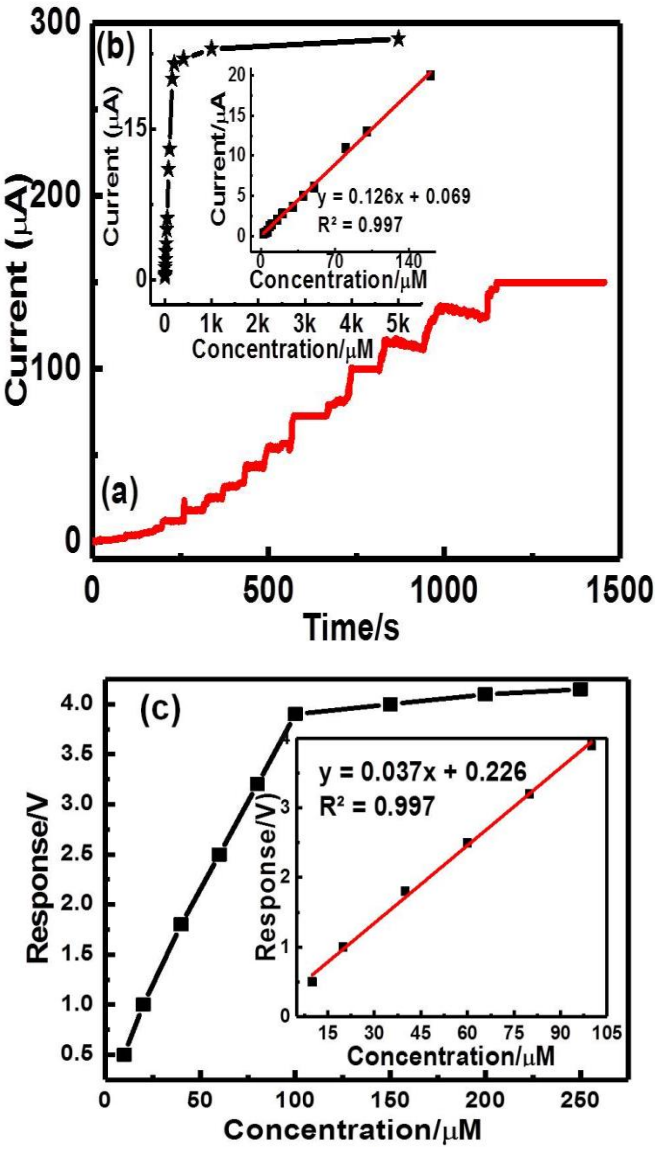

Fig. 5. (a) Chronoamperometric response of MCPE-NiO-Lac on successive addition of different concentration and volume of catechol solutions into pH=7, 0.2 M PBS solution at an applied potential of 0.3V; **(b)** Calibration curve with fitting for MCPE-NiO-Lac electrode. Inset: Calibration curve showing linear fitting in the range 0-150 μM; **(c)** Calibration curve with fitting for Au-Lac electrode. Inset: Calibration curve with linear fitting in the range 0-100 μM.

Five biosensors were prepared independently in each case under the same conditions to study the electrode-to-electrode reproducibility and the RSD of the five modified electrodes was 3.9 % and 4.9 % for MCPE-NiO-Lac and Au-Lac electrodes respectively, indicating of their good reproducibility. The long-term stability is a feature required for the satisfactory application of the biosensor and that was determined by performing activity assays for 20 days. The electrodes were stored in dry atmosphere at 4°C when not in use. An activity loss of 9% was observed on the 20th day for MCPE-NiO-Lac electrode, while the same was 15% in the case of Au-Lac electrode of Clark DO sensor type. Good long-term stability could be attributed to the mild immobilization procedure adopted for the assembly of laccase enzyme particularly on NiO modified carbon matrix and the beneficial nano-biocompatible environment for preventing any enzyme leakage. MCPE-NiO-Lac electrode achieved 95% of the steady-state current within 6s which is quite satisfactory for biosensor response. Such a fast response may reflect the increased electron transfer in the NC modified carbon paste. Au-Lac electrode surface on the other hand took about 3 minutes to reach the same state of 95% of the steady-state current.

Table 1. A comparison of sensing features of various laccase based catechol biosensors.

Electrode Description	Analyte	LOD (μM)	Linearity range (μM)	Ref.
Lac/carbon fibres	Catechol	NR	1 -90	Freire et al., 2001
Lac/AP-rGOs/Chit/GCE	Catechol	7.0	15-700	Zhou et al., 2013
MB-MCM-41/PVA/Lac	Catechol	0.33	4-87.9	Xu et al., 2009
Lac/CNTs−CS/GCE	Catechol	0.66	1.2-30	Liu et al., 2006
PDA-Lac-NiCNFs/MGCE	Catechol	0.69	1-9100	Dawei et al., 2014
Au-Lac	Catechol	4.0	10-100	This work
MCPE-NiO-Lac	Catechol	0.95	2-160	This work

The remarkable electrochemical performance of NiO NCs incorporated graphite electrode during the studies undertaken here and also when compared to Au electrode surface, on immobilization of laccase enzyme for catechol detection, deserves attention with regard to the details. While Au electrode used was a thin film with area of 1.2 mm^2 and thickness in micron range, NiO NCs were particles of diameter ~10 nm finely dispersed in a graphite matrix. The difference in the morphology would contribute differently to the effective surface area and roughness of the electrodes as seen by enzyme molecules

on immobilization. Laccase enzyme immobilized on electrode surface oxidizes the analyte catechol by reducing oxygen from ambience through a four electron step transfer and at the expense of one-electron oxidation of the analyte. Clark type DO sensor often requires continuous supply of oxygen for this as externally provided, during the catalytic oxidation of catechol. On the other hand, NiO NCs on electrode surfaces with (100) planar geometry as seen from HRTEM, has surface Ni atoms connected by bridging O atoms lying in the plane. The geometry enables adsorption of aminoacid sequence from enzyme favorably on surface oxygen atoms through electrostatic interactions (as understood by our detailed calculations **[20]**) and thereby facilitates faster catechol oxidation. Also amino acid sequences have comparable adsorption features on NiO with those of water molecules from the medium and would interact with each other as well as the oxide surface atoms. This circumstance as prevailed in the nano-biocomposite surface could do away with the requirement of external oxygen supply for catalytic oxidation of catechol. Further, NiO surface is well studied in literature for efficient water splitting reactions where the role of NiO toward water oxidation is clearly established **[26]**.

Real water sample application

The water samples used were filtered with a 0.2 μM membrane and mixed with 0.2 M PBS solution of pH 7 with 2-fold dilution before the amperometric measurements. On attaining constant current, 100 μM catechol was introduced through stirring at constant potentials determined previously (0.3 V for MCPE-NiO-Lac and 0.2 V for Au-lac). **Table 2** shows satisfactory recovery obtained in each case and confirmed the potential application of the biosensors in detecting phenols from real water samples and industrial effluents.

Table 2. Determination of catechol in real water samples.

Sample	C_{added} (μM)	MCPE-NiO-Lac		Au-Lac	
		C_{found} (μM)	Recovery (%)	C_{found} (μM)	Recovery (%)
Tap water	100	102.3	102.3	98	98
TIE-1	100	97.5	97.5	97	97
TIE-2	100	101	101	99	99

*****TIE= Textile industry effluent

Conclusion

One of the important challenges in the development of enzyme based amperometric electrodes is the establishment of satisfactory electrical communication between the active sites of the biomolecules and the electrode surfaces. The redox centers of most oxido-reductases are electrically insulated by protein shells. As a result enzymes cannot be oxidized or reduced at the electrode surface easily at any potential. The possibility of direct electron transfer between enzymes and the electrode surface could pave the way for the development of superior reagent-less biosensing devices, as it may obviate the need for co-substrates or mediators, allowing efficient transduction of biorecognition events. The judicious application of metal oxide nanocrystals is seen here to enable the fabrication of novel biosensing devices with efficient electrical communication with redox biomolecules/enzymes that may address several diagnostic requirements. The immobilized biomolecule also must have an appropriate orientation, which should facilitate communication between the active centre of the biomolecule and the electrode surface. Metal oxide NC modified graphite electrodes provided a biocompatible electroactive surface for enzyme immobilization with improved conformation, orientation and biological activity resulting in enhanced electron transfer and improved biosensing characteristics. In constructing enzyme-based biosensors, NiO NCs have been favorable because of their high isoelectric point for physical adsorption of enzyme molecules by electrostatic interactions **[27]**. From the comparison of the behaviour of two laccase immobilized electrode matrices studied here (ie. MCPE-NiO and Au), it may be concluded that both of them are useful for monitoring of catechol used as substrate. They are reusable and show good stability and robustness when compared with other laccase based biosensor designs found in the literature. Among the biosensors tested in this work, the MCPE-NiO-Lac electrode showed better biosensing performance, longer lifetime and sensitivity towards the amperometric measurements. MCPE-NiO-Lac also showed a broad linearity range for catechol detection and small limit of detection. Both the biosensors can be used in the monitorization of phenolic compounds in industrial waste waters.

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Author's contributions

ICL, BNM and KR have conceived the plan, while experiemental work and associated discussions have been undertaken by CS, MSS, ST and NK; Data analyses were done by CS and LD. And manuscript was writen by CS, LD and ICL. Authors have no competing financial interests.

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