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Nanostructured nickel oxide film for application to fish freshness biosensor

Surendra K. Yadav,[1,2,a)] Jay Singh,[1,3,a)] Ved Varun Agrawal,[1] and B. D. Malhotra[4,b)]
[1]*Department of Science and Technology Centre on Biomolecular Electronics, Biomedical Instrumentation Section, National Physical Laboratory, Dr. K.S. Krishnan Marg, New Delhi 110012, India*
[2]*Department of Physics, Motilal Nehru National Institute of Technology, Allahabad, Allahabad 211004, India*
[3]*Department of BIN Fusion Technology and Department of Polymer-Nano Science and Technology, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea*
[4]*Department of Biotechnology, Delhi Technological University, Shahbad Daulatpur, Delhi 110042, India*

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Xanthine oxidase (XO_x) has been physisorbed onto nanostructured nickel oxide (n-NiO, ~41 nm), electrophoretically deposited onto indium-tin-oxide (ITO) glass substrate using poly-ethylene glycol via co-precipitation. The electrochemical response studies of the XO_x/n-NiO/ITO bioelectrode show linear response in the xanthine concentration range as 10–200 μM ($R^2 = 0.9989$), sensitivity as 0.6214 μA/μM cm^2, detection limit as 2.989 μM, standard deviation as 0.6192 μA, response time of 15 s with the stability of 100 days. The observed low value (0.0306 mM) Michaelis–Menten constant (K_m) indicates high affinity of XO_x towards xanthine. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4736578]

The past decade has seen considerable rise in the demand for fish as a nutritious and healthy commodity both in developing and the developed countries.[1] There is thus an urgent need for the availability of a device that can be used for evaluation of fish freshness. In this context, xanthine has been found to be a major metabolite in degradation of adenosine triphosphate (ATP) contained in the fish meat, whose concentration increases with storage.[2] Xanthine can thus be used as an indicator for estimation of fish freshness. Various analytical methods like high performance liquid chromatography (HPLC) and high performance capillary electrophoresis (HPCE) are being used for detection and quantification of xanthine.[3,4] However, these methods are time-consuming, expensive, and require expertise. Enzyme biosensor based on xanthine oxidase (XO_x) has recently been suggested for estimation of xanthine.[5] In this context, immobilization of XO_x onto a desired matrix including nanostructured metal oxide is considered very important.[6,7] Among the various nanostructured metal oxides, nano-structured nickel oxide has aroused much interest for fabrication of a desired biosensor. This has been attributed to its large surface area, high conductivity, efficient catalytic activity, wide band gap, high carrier mobility, good biocompatibility, chemical stability, and excellent electrochemical properties.[8] Moreover, the high isoelectric point (IEP) of NiO (~10.7) can be helpful to immobilize a given biomolecules with low IEP via electrostatic interactions. We report results of the studies relating to fabrication of a fish freshness biosensor based on nanostructured nickel oxide.

All chemicals of analytical grade have been purchased from Sigma Aldrich. Nanostructured n-NiO nanoparticles have been prepared by using $Ni(NO_3)_2$. $6H_2O$ of (0.5 M) and NaOH of (2 M) are separately dissolved in 100 ml distilled water and n-NiO has been prepared using co-precipitation method to adjust *p*H up to ~12 by utilizing 2–3 ml of polyethylene glycol (PEG) used as a surfactant. The observed green precipitate washed with deionized water and ethanol for 4–5 times is dried at 70 °C for about 12 h and is then calcined at 400 °C for about 8 h. The n-NiO (10 mg/ml) dispersed in double distilled water is ultrasonicated for about 30 min and is then electrophoretically deposited (EPD) at 35 V for 60 s to form a uniform film onto an indium-tin-oxide (ITO) (0.25 cm^2) glass surface. XO_x (0.2 unit/ml), in phosphate buffer, 50 mM, *p*H 7.0) is immobilized onto n-NiO/ITO electrode via physisorption and is kept overnight in a humid chamber for drying. The higher activity of XO_x/n-NiO/ITO bioelectrode toward the substrate (xanthine) is obtained at *p*H 7.0 (Fig. 2, inset (ii)).

The n-NiO particles have been characterized using X-ray diffraction (Rikagu) Fourier transform infrared spectra (FTIR, PerkinElmer, Spectrum BX II), scanning electron microscopy (SEM, LEO-440), transmission electron microscopy (TEM, Hitachi Model H-800) with a tungsten filament at an accelerating voltage of 200 kV the electrochemical analysis has been conducted by an Autolab Potentiostat/Galvanostat (Eco Chemie, The Netherlands) using a three-electrode system with XOx/n-NiO/ITO bioelectrode as the working electrode, a platinum (Pt) wire as the counter electrode, and saturated Ag/AgCl electrode as a reference electrode in phosphate buffer saline (PBS) (50 mM, *p*H 7.0, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. The contact angle (Dataphysics, Model OCA15EC) measurements have been done by Sessile drop method to delineate the hydrophilic/hydrophobic nature of the electrode.

The XRD pattern of n-NiO nanoparticles (Fig. 1) performed in the 2θ range exhibits characteristic peaks indexed at (111), (200), (220), (222), and (311) corresponding to nickel oxide (JCPDS file 78-0429) having fcc crystal structure. The average crystallite size has been obtained by Debye–Scherrer equation ($\lambda = 1.5406$ Å) and it turns out to be ~41 nm, lattice parameter (4.166 Å). The strain in the lattice of the n-NiO has been evaluated via Hall–Williamson relation[12] using Scherrer constant (k = 0.94), ($\lambda = 1.5406$ Å) and of linear fit of the data points gives ($\eta = -3.46 \times 10^{-3}$). The

[a)]S. K. Yadav and J. Singh contributed equally to this work.
[b)]Author to whom correspondence should be addressed. Electronic mail: bansi.malhotra@gmail.com.

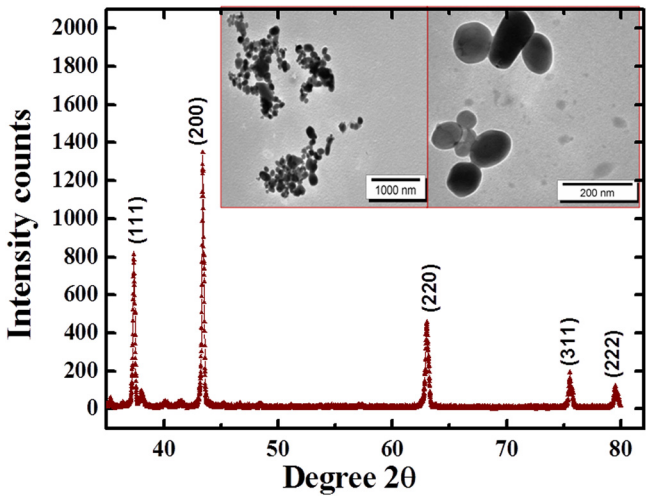

FIG. 1. X-ray diffraction pattern and inset transmission electron microscopy of n-NiO nanoparticles.

negative value of η for the n-NiO is due to presence of the vacancy defect that increases local charge density of states near the Fermi Energy due to its dangling bonds.[9] TEM image of n-NiO nanoparticle (Fig. 1, inset) shows that particle is spherical in shape with average diameter of the particle ~30–35 nm.

The FTIR spectra[12] of pure n-NiO nanoparticles (curve a) exhibits broad bands at 3462 cm^{-1} corresponding to O–H stretching arising due to physically adsorbed water on NiO surface, and the small peak seen at 1630 cm^{-1} is ascribed to adsorbed carbonate and chlorate moieties. The absorption band of n-NiO nanoparticles appears at 682 and 462 cm^{-1} belonging to the stretching vibration mode and torsional vibration mode of metal oxide (Ni-O) bonds with the tetrahedral and the octahedral sites. The FTIR spectral band of XO_x/n-NiO/ITO bioelectrode (curve b) shows sharp peaks at 3746 and 3090 cm^{-1} due to O-H stretching band and organic backbone (-CH_2-) of enzyme. Two amide bands found in the 1830–1700 cm^{-1} range pertain to C=O (amide I) stretching vibration of peptide linkage and amide II resulting due to combination of N-H in- plane bending and C-N stretching of peptide groups. The sharp peak seen at 1169 cm^{-1} is assigned to N–C stretching band and the bands observed at 780 and 682 cm^{-1} indicate presence of the metal oxide band. The 1377 cm^{-1} peak is due to the bonding in the protein indicating immobilization of XO_x onto n-NiO/ITO bioelectrode via electrostatic interactions.

The results of surface morphology studies conducted on n-NiO and XO_x modified n-NiO/ITO bioelectrodes are shown in the supplementary data.[12] The SEM image of n-NiO/ITO (images a and b)[12] film at different magnification shows homogeneous distribution of spherically shaped n-NiO particles (40 nm) with relatively smooth and crack free uniform surface. On immobilization of XO_x, the granular and spherical morphology of n-NiO/ITO film changes into homogeneous globular porous morphology due to ionic interactions between n-NiO and biomolecules, indicating immobilization of XO_x enzyme (images c and d are in different magnification).[12] The contact angle (CA) for the bare ITO, n-NiO/ITO, and XO_x/n-NiO/ITO bioelectrode have been found to be as 120°, 98°, and 68°, respectively.[12] The lower CA value obtained for XO_x/n-NiO/ITO electrode reveals the presence of hydrophilic group in the enzyme (XO_x).

The electrochemical impedance spectroscopy studies[12] reveal that the charge transfer resistance (Nyquist diameter) value determined using the semicircle of the n-NiO/ITO electrode ($R_{CT} = 2.57$ KΩ, curve a) is less than that of a bare ITO electrode (curve a, $R_{CT} = 4.40$ KΩ) but is greater than XO_x/n-NiO/ITO bioelectrode (curve c, $R_{CT} = 7.40$ KΩ). These results indicate immobilization XO_x onto n-NiO/ITO electrode facilitates easier electron transfer between the bioelectrode and substrate. The value of R_{CT} depends on the dielectric and insulating characteristics at the electrode/electrolyte interface.

The results of cyclic voltametery (CV) studies (Fig. 2) reveal that magnitude of the current (0.559 mA) of n-NiO/ITO electrode is enhanced (curve b) as compared to that of bare ITO electrode (0.499 mA) arising due to presence of the positively charged Ni^{2+} ion on the ITO surface. The redox potential of n-NiO/ITO electrode shifts towards the higher side, indicating increased electron mobility at the electrode surface resulting in enhanced electron transfer. However, after the immobilization of XO_x onto n-NiO/ITO electrode, magnitude of response current decreases (curve c) due to strong binding of XO_x with the n-NiO/ITO electrode that perhaps hinders transport of the charge carriers. It appears that insulating characteristics of XO_x perhaps perturb transfer of electrons between the medium and XO_x/n-NiO/ITO bioelectrode.[10]

The CV studies have been carried out on XO_x/n-NiO/ITO bioelectrode as a function of scan rate[12] varying from 10 to 100 mV/s. The magnitudes of both anodic (Epa) and cathodic (Epc) peak currents and the potential peak shift ($\Delta Ep = Epa - Epc$) increases linearly with square root of the sweep rate ($\nu^{1/2}$), revealing it as a diffusion-controlled electron-transfer process. The value of diffusion coefficient (D) of the redox species of XO_x/n-NiO/ITO bioelectrode has been estimated from slope of the Ip versus $\nu^{1/2}$ plot by using the Randles-Sevcik equation. The calculated value of D has been found to be as 1.33×10^{-8} $cm^2 s^{-1}$, with linear regression coefficient, $R^2 = 0.99$, indicating improved electrocatalytic behaviour.

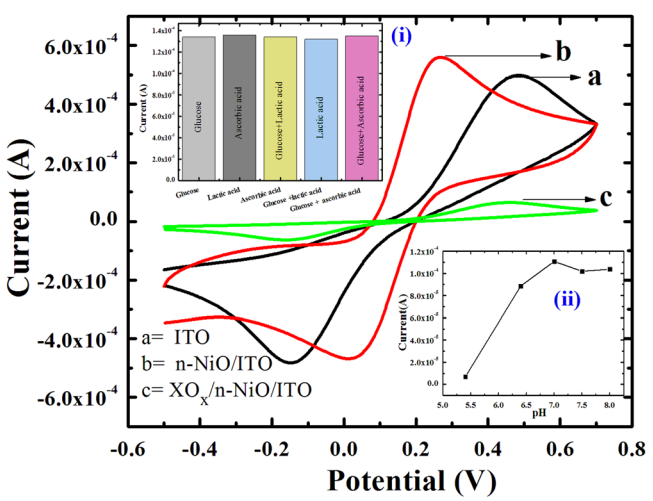

FIG. 2. CV of [inset (i) effect of interfering substances and inset (ii) effect of *p*H on bioelectrodes] ITO electrode (a), n-NiO/ITO electrode (b), and XOx/n-NiO/ITO bioelectrodes (c).

The surface concentration (SC) of the XO_x/n-NiO/ITO bioelectrode has been estimated using the Brown-Anson model (Eq. (1))

$$I_p = \frac{n^2F^2\Gamma AV}{4RT}, \quad (1)$$

where n is the number of electrons transferred (1), F is the Faraday constant (96 485 C/mol), Γ is the SC (mol cm^{-2}), A is surface area of the electrode (0.25 cm^2), V is scan rate (50 mV/s), R is gas constant (8.314 J $mol^{-1}K^{-1}$), and T is temperature (300 K). The SC of n-NiO/ITO (2.999×10^{-12} mol cm^{-2}) is higher than that of XO_x/n-NiO/ITO bioelectrode (1.527×10^{-12} molcm^{-2}).

The value of heterogeneous electron transfer rate constant (ks) of XO_x immobilized n-NiO/ITO electrode has been calculated using the Laviron model[11]

$$K_s = \frac{mnF\upsilon}{RT}, \quad (2)$$

where m is the peak-to-peak separation (0.59 V), F is Faraday constant (96 485 C/mol), υ is scan rate (50 mV/s), n is the number of transferred electrons (n = 2), and R is gas constant (8.314 Jmol^{-1}K^{-1}) (T = 300 K).The value of ks is obtained as 2.3 s^{-1}, indicating fast electron transfer between immobilized XO_x and electrode due to the presence of n-NiO in the matrix.

The results of electrochemical response (Fig. 3) studies conducted on the XO_x/n-NiO/ITO bioelectrode as a function of xanthine concentration (10–200 μm) reveal that magnitude of current response increases on successive addition of xanthine solution. This may attributed to well-aligned and cubic closed packed network of n-NiO nanoparticles that act as good accepter of electrons generated during reoxidation of XO_x. These electrons are transferred to the electrode via Ni(II)/Ni(III) redox couple resulting in increased electrochemical current response. Moreover, XO_x/n-NiO/ITO bioelectrode in PBS displays a pair of well-defined redox peaks at about Epc = −0.2 V, Epa = 0.42 V, and the ratio of anodic to cathodic peak currents is found to be as about one. This indicates that the bioelectrode shows a quasi-reversible redox process. The magnitude of the oxidation peak increases linearly with increase in the xanthine concentration (Fig. 3, inset (i)). The biochemical reaction between XO_x and xanthine follows Eqs. (3)–(5):

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{XO_x} \text{Uric acid} + H_2O_2, \quad (3)$$

$$H_2O_2 \xrightarrow{\text{Electrochemical Oxidation}} O_2 + 2H^+ + 2e^-, \quad (4)$$

$$2e^- \xrightarrow[Fe(CN)_6^{-3/-4}-3/-4]{\text{n-NiO}} \text{Electrode}. \quad (5)$$

The response time can be defined as the time required for a sensor to get response of sensor to its maximum output. It can be evaluated by measuring the current response of bioelectrode as a function of equilibrium time for electrochemical reaction. The short response time of the XO_x/n-NiO/ITO bioelectrode[12] found to be as 15 s is attributed to faster electron communication feature of n-NiO/ITO electrode. The

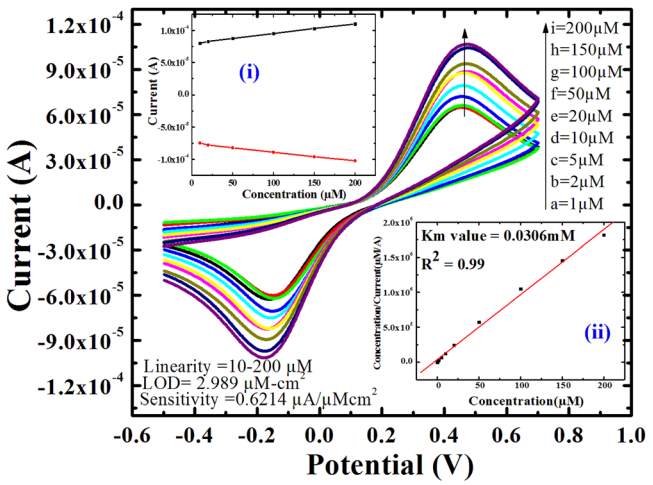

FIG. 3. Electrochemical response of XO_x/n-NiO/ITO bioelectrodes with respect to xanthine concentration (1–200 μM); inset (i) show calibration curve, variation in current as a function of xanthine concentration and inset (ii) is Hanes plot between the substrate concentration and substrate concentration/current.

XO_x/n-NiO/ITO bioelectrode exhibits broad linear range (10–200 μm), detection limit of 2.989 μM, and reproducibility of more than 15 times with linear regression coefficient ($R^2 = 0.9989$).

The sensitivity of the XO_x/n-NiO/ITO bioelectrode calculated from the slope of the curve is found to be as 0.6214 μA/μMcm^2. The value of standard deviation and correlation coefficient obtained from the linear regression analysis of the bioelectrode has been found to be as 6.45 μA and 0.9666, respectively.

The value of Michaelis-Menten constant (K_m) for the XO_x/n-NiO/ITO bioelectrode estimated by Hanes plot (Fig. 3, inset (ii)) has been found to be as 0.0306 mM. The observed K_m value indicates higher affinity of XO_x/n-NiO/ITO bioelectrode that is attributed to favourable microenvironment for XO_x resulting in higher loading onto n-NiO/ITO electrode surface.

The selectivity of XO_x/n-NiO/ITO based xanthine biosensor has been determined by measuring its electrochemical response by adding normal concentration of different interferents (Fig. 2, inset (i)), such as glucose (5 mM), lactic acid (0.5 mM), and ascorbic acid (0.05 mM) with physiological normal level of xanthine presence in fish meat, indicating maximum interference of 2%. No noticeable changes in the current response have been detected informed in presence of glucose, ascorbic acid, and lactic acid.

Stability is the ability of a sensor to provide reproducible results during a certain period of time. The stability of the XO_x/n-NiO/ITO bioelectrode[12] has been evaluated by measuring the amperometric response as a function of xanthine concentration of 100 μM over a period of 14 weeks. The XO_x/n-NiO/ITO bioelectrode response gradually decreases after about 5 weeks and to approximately 30% after 14 weeks. This suggests that XO_x/n-NiO/ITO bioelectrode has a good stability of enzyme electrode. The reason for the stability of XO_x/n-NiO/ITO bioelectrode is because the electrophoretically deposited n-NiO with high IEP (10.7) n-NiO binds with XO_x having low IEP (4.2) via electrostatic interactions. The results obtained in the current studies,

along with those reported in the literature, are summarized in Table I shown in the supplementary data.[12]

Xanthine in fish meat has been estimated by the following procedure. The fine paste of canned tuna fish is mixed with 5 ml of 0.5 M $HClO_4$ for precipitation of the proteins. The solution is diluted 10 times to maintain *p*H of the supernatant upto 7.0. The magnitude of current response is recorded upto 7 days. The result indicates that the xanthine level increases as the storage time increases as shown in supplementary file.[12]

The XO_x/n-NiO/ITO based xanthine biosensor shows improved detection range 10–200 μM, high sensitivity of 0.6214 μA/μM-cm^2, low detection limit of 2.989 μM, linear regression at 0.998, response time of 15 s, and a shelf-life of 14 weeks. The low K_m value of (0.0306 mM) indicates high affinity of XO_x/n-NiO/ITO bioelectrode to xanthine. This nanostructured metal oxide bioelectrode has implications towards the estimation of freshness of other foods including rice, sweet corn, and oats.

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[12]See supplementary material at http://dx.doi.org/10.1063/1.4736578 for Scheme, Hall–Williamson plot, and comparison table, and the results of FTIR, SEM, contact angle measurement, EIS, scan rate, response time, stability curve, and real sample analysis.