Fabrication of Amperometric Bienzymatic Glucose Biosensor Based on MWCNT Tube and Polypyrrole Multilayered Nanocomposite

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ABSTRACT: A novel amperometric glucose biosensor based on bienzyme system such as glucose oxidase (GOx) and horseradish peroxidase (HRP) was developed based on the carboxy modified multiwalled carbon nanotubes (MWCNT) and polypyrrole (PPY) nanocomposite film, electrochemically deposited onto indium-tin-oxide (ITO) electrode using layer by layer deposition technique. The glucose oxidase (GOx) and horseradish peroxidase (HRP) have been coimmobilized onto the MWCNT/PPY/ITO nanocomposite electrode using gluteraldehyde as a crosslinking agent. The bienzymatic nanobioelectrodes GOx-HRP/MWCNT/PPY/ITO have exhibited 3 times higher sensitivity than mono enzymatic bioelectrode (GOx-HRP/MWCNT/PPY/ITO). Minimum interferences have been observed from ascorbic acid, uric acid, sodium pyruvate, and sodium ascorbate for both single and bienzyme systems. It is inferred that bienzyme-based nanobioelectrodes offer wider linearity, higher sensitivity, correlation coefficient, shelf life, and accuracy for testing of blood serum samples than mono enzyme system. Mechanism of the overall biochemical reaction has been proposed to illustrate the enhanced bio-sensing performance of the bienzyme system. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: E235–E246, 2012

Key words: polypyrrole; multiwalled carbon nanotube; glucose oxidase; horseradish peroxidase

INTRODUCTION

The carbon nanotubes (CNT) along with conducting polymers-based biosensor-matrices have attracted the attention of researchers worldwide due to their robust mechanical, electronic, and adsorptive properties as well as their good chemical stability.[1] Integration of CNT and conducting polymers with synergistic effect has shown particular promise in biosensing characteristics as they can play very interesting role such as (1) a biocompatible enzyme friendly platform, (2) fast electro catalytic oxidation or reduction of the product generated during biochemical recognition process at the CNT surface to reduce overvoltage and avoid interference from other coexisting electroactive species, and (3) an enhanced signal because of its fast electron transfer and large working surface area. Out of all conducting polymers, polypyrrole (PPY) is one of the promising matrix for biosensor applications due to its ease of synthesis at neutral pH, high mechanical and environmental stability, redox activity, ability to form nanowires with room temperature conductivity ranging from 10^{-4} to 10^{-2} S cm^{-1}, ion-exchange and ion discrimination capacities, electrochromic effect depending on electrochemical polymerization conditions and charge/discharge processes, strong absorptive properties towards gases, proteins, DNA, catalytic activity etc.[2] Recently, polypyrrole is found to be one of the major tools in nanobiotechnological applications due to its biocompatibility, capability to transduce energy arising from interaction of analyte, and analyte-recognizing-site into electrical signals that are easily monitored; capability to protect electrodes from interfering materials.[2] Wang et al. have fabricated CNT doped PPY/CNT nanocomposite electrode for glucoses sensing with improved electrochemical response properties.[3] They have shown that anionic CNT can act as dopant in the preparation of conducting-polymer enzymes electrodes and such dopant facilitates a highly sensitive bio sensing of glucose. The glucose oxidase (GOx) concentration within the PPY/MWCNT (multiwalled carbon nanotubes)/GOx nanobiocomposite and the film thickness have been found to be crucial to determine the efficiency of the glucose biosensor. The amperometric response of the optimized GOx/PPY/MWCNT glucose bioelectrode (1.5 mg/mL GOx, 141 mC/cm^2

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total charge) has displayed a sensitivity of 95 nA m*M*-1, a linear range up to 4 m*M*, and a response time of about 8 s.[4] Gao et al. have designed PPY/MWCNT-based platform for glucose sensing with a low detection potential and improved sensitivity.[5] A sensitive electrochemical biosensor for monitoring DNA hybridization, fabricated on MWCNT//PPY/GC (glassy carbon) electrode has been reported to exhibit a linearity in the range of 6.9×10^{-14} to 8.6×10^{-13} mol/L, and the detection limit of 2.3×10^{-14} mol/L of target oligonucleotide.[6]

The amperometric glucose biosensor is of special importance because of the advantages of good selectivity. Glucose biosensor has attracted great attention in clinical diagnosis because the accurate measuring of glucose can help in preventing euglycemia, which later causes diabetes. In diabetic patients, the consistently high glucose levels result in long-term complications, including retinopathy, nephropathy, and neuropathy which often leads to amputation of extremities. The basic research on glucose oxidase (GOx) based amperometric biosensor is still an active area due to its huge application in the field of blood glucose monitoring as well as the stability and inexpensive cost of the enzyme.[7] In amperometric biosensor, glucose quantification is usually performed by measurement of the current associated with the oxidation of hydrogen peroxide, a side product in the course of glucose oxidase enzyme-catalyzed oxidation of glucose at anodic potentials (>+0.6 V versus Ag/AgCl).[8,9] However, at this relatively high potential, there may be interferences from other oxidizable species such as ascorbic acid (AA), uric acid (UA), and acetaminophen (AAP). To avoid interferences, some improved biosensors have been used to detect hydrogen peroxide at low potential.[10,11] In such cases, the coupled enzyme reactions for analyte conversion provide a more favorable alternative. The primary product i.e., produced by the reaction of the analyte with the first enzyme is further converted by a second enzyme to produce products detectable by a transducer.[12] Coupled enzyme reactions are also employed to filter out chemical signals by eliminating the interference on the enzyme.[13,14] A reduction in the electrical signal can then be inversely correlated to the analyte concentration in the solution.[15,16] The immobilization of the GOx together with horseradish peroxidase (HRP) is thought to either help the protein to assume a favorable orientation or to make possible conducting channels between the prosthetic groups and the electrode surface. Both can reduce the effective electron transfer distance and thereby facilitates the charge transfer between the electrode and the enzyme.[17]

Yao et al. have fabricated bienzyme glucose biosensor based on the coimmobilization of HRP and GOx in Nafion and found high sensitivity at zero potential.[18] The bienzyme biosensor based on GOx and HRP, immobilized on polyamidoamine(PAMAM)/CNT has exhibited a linear response range for glucose from 4.0–1.2 m*M* ($R = 0.9971$, $N = 15$), with a detection limit of 2.5 μ*M*.[19] The glucose biosensor, coimmobilized with HRP and GOx, based on gold nanoparticles encapsulated mesoporous silica SBA-15 composite (AuNPs-SBA-15) has shown the detection limit of glucose of 0.5 μ*M* with a linear range from 1 to 48 μ*M*.[20] Li et al. have developed the bienzyme biosensor based on glycidoxypropyltrimethoxysiloxane (GPTMS)/chitosan (CS) which has shown a linear range of 1–351 μ*M* with a detection limit of 0.3 μ*M*.[21] Jeykumari et al. have coimmobilized HRP and GOx onto poly(toluidine blue O) and MWCNT-modified Nafion and achieved a linear calibrations upto 1.4×10^{-7} to 1.6×10^{-3} *M* for glucose detection.[22] The multienzyme system is a comparatively complicated format with respect to the enzyme kinetics and mechanism. From the above discussion, it has been inferred that the so far reported glucose biosensors have the limitations of reproducibility, reusability and stability which are the most essential criteria for commercial biosensor. In this context, there is indeed a need to develop a simple and reliable glucose biosensor using a multienzyme system as this format theoretically proves its efficiency.

In this manuscript, an attempt has been made to develop a novel glucose biosensor by taking the advantage of the merit of direct electron transfer property of HRP with carbon nanotubes-PPY nano composite electrode based on coentrapment of GOx and HRP into multilayer polypyrrole and MWCNT nano composite electrode. In this study, CNT, particularly MWCNT are used because they have well defined nanoscale geometry and cheaper and easier to be treated by acid than SWCNT without losing their electroconductivity. The analytical characteristics of the resulting bienzymatic glucose biosensor in relation to the determination of glucose has been investigated and compared with those of a monoenzyme glucose biosensor, constructed in a similar way. The novelty of this experiment lies on the ease and novelty of fabrication process, excellent and reliable electrochemistry of the developed bienzymatic biosensors, and testing on blood serum.

EXPERIMENTAL

Reagent

Pyrrole (PPY) (M.W:67.09) from spectrochem, India was doubled distilled prior to polymerization. Multiwalled carbon nanotubes (MWCNT, 96% 40–80 nm, aspect ratio of 1 : 2000), Glucose oxidase (GOx, E.C. 1.1.3.4, 151 U/mg, from *Aspergillus niger*) and Horseradish peroxidase (HRP, E.C1.11.1.7, ≥250 U/mg,

from Horseradish) were purchased from Sigma, USA. β-*d*-Glucose was provided by Glaxo, India. All other chemicals are of analytical grade and are used without further purification and the solutions are prepared in deionized water.

Preparation of PPY/ITO and MWCNT/PPY/ITO electrodes

The application of MWCNT is restricted due to its poor solubility in most solvents. The challenge of solubilizing MWCNT can be addressed through their covalent modification, such as carboxy modification. The MWCNT (2 mg) were functionalized with carboxylic acid groups by refluxing in a 3 : 1 sulfuric-acid/nitric-acid mixture (20 mL) for 8 h, in accordance to the procedure.[23] Subsequently, the pretreated MWCNT(carboxy modified) were washed with water, then with 0.1*M* NaOH (to reach the pH of 7.0), filtered, and dried for 1 h at 100°C. The 1 mg of carboxy modified MWCNT were dispersed in 1 mL methanol by sonication for 1–2 min. The PPY films were electrochemically deposited onto ITO coated glass plates in a three-electrode cell containing 0.1*M* pyrrole in 0.1*M* *p*-toluene sulfonic acid (PTS) solution in deionized water by chronopotentiometric technique with a constant current of 0.65×10^{-4} amp (A) for 30 s using a Potentiostat/Galvanostat and after that 15 μL of MWCNT-methanol dispersion was spread on PPY thin film by simple casting. The thin film was dried in vacuum oven for 5–8 min at 40°C. The MWCNT/PPY multilayered nano composite film was formed by repeating the whole process once again and multilayer film is denoted as $[PPY/MWCNT]_2$. There after the PPY film was again electrodeposited once again for another 30 s on $[[PPY/MWCNT]_2]$ film.[23] So the construction of multilayered nanocomposite electrode can be described as [PPY/MWCNT]2/PPY/ITO. But we are using the symbol MWCNT/PPY/ITO for describing multilayered electrode ([PPY/MWCNT]2/PPY/ITO) in our manuscript. The MWCNT/PPY/ITO electrode was washed with deionized water (milli- Q) followed by phosphate buffer, (50mM, pH 7.0) to maintain pH over the electrode and stored at 4°C. The PPY/ITO electrode was prepared using 0.1*M* pyrrole solution mixture containing 10 mL of 0.1*M* PTS solution by the chronopotentiometry technique with a constant current of 0.65×10^{-4} amp for 120 s using a Potentiostat/Galvanostat.

Preparation of GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nano bioelectrodes

GOx and HRP were covalently immobilized onto the MWCNT/PPY/ITO nano composite electrodes through the covalent bond formation between the -NH group of PPY-MWCNT nanocomposite and NH_2 of the enzymes by using glutaraldehyde as the crosslinking agent. Before enzyme immobilization, the MWCNT/PPY/ITO electrodes were treated with 10 μL of 1% glutaraldehyde solution and kept in a humid chamber for 3 h. Totally, 10 μL of freshly prepared solution of GOx (1 mg/mL) and GOx-HRP (1 mg/mL, 1 : 1 volume ratio) were uniformly spread onto glutaraldehyde treated MWCNT/PPY/ITO electrodes and were kept in a humid chamber for 12 h at 4°C to construct GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nano bioelectrodes. The nano bioelectrodes were washed with 7.0 pH buffer solution to wash out the unbound enzyme from the electrode surface. When not in use, the electrodes were stored at 4°C in a refrigerator.

Instrumentation

The surface chemistry of the pristine MWCNT, carboxy-modified MWCNT, PPY/ITO, MWCNT/PPY/ITO, GOx/MWCNT/PPY/ITO electrodes was studied by Fourier transform infrared spectroscopy (Perkin–Elmer Spectrophotometer BX-1 in the frequency range, 400–4000 cm^{-1}). The Thermo Gravimetric Analysis (TGA) was carried out on Mettler Toledo model TGA/SDTA 851^e up to 1000°C in air at a heating rate of 10°C/min. The surface morphologies of PPY/ITO, MWCNT/PPY/ITO, GOx/MWCNT/PPY/ITO, and GOx-HRP/MWCNT/PPY/ITO electrodes have been investigated by scanning electron microscope (LEO Model). Electrochemical measurements have been conducted in phosphate buffer (50 m*M*, pH 7.0, 0.9% NaCl) containing 5 m*M* $[Fe(CN)_6]^{3-/4-}$ in a three-electrodes cell consisting of Ag/AgCl as reference, platinum (Pt) as counter electrode and ITO as a working electrode (0.25 cm^2) using Potentiostat/Glavanostat [Model (PGSTAT 302*N*)].

RESULTS AND DISCUSSION

Characterization of carboxy-functionalized MWCNT

Figure 1 shows the FTIR spectra of the pristine MWCNT [Fig. 1(a)] as well as carboxy modified MWCNT [Fig. 1(b)]. In case of carboxy modified MWCNT, the broad peak at 3452 cm^{-1} is due to O—H stretching vibration of hydroxyl groups and the peak at 1749 cm^{-1} corresponds to the carboxylic (C=O) group attached to MWCNT. The peak at 1382 cm^{-1} corresponds to the C—O stretching vibration. Furthermore, the peaks at 1133 and 1084 cm^{-1} are attributed to C—C stretching vibration of main structure of MWCNT.[24,25] The formations of the functional groups are also confirmed by TGA

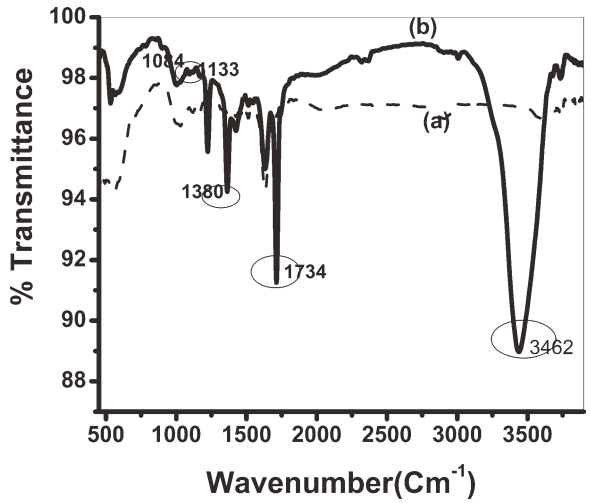


Figure 1 FTIR of (a) pristine MWCNT and (b) carboxy functionalized MWCNT.

studies as shown in Figure 2. As seen in the Figure 2, a sudden weight loss is observed at 550°C due to thermal oxidation of MWCNT. However, additional weight loss is observed at 350 and 270°C for carboxy-modified MWCNT respectively, due to decomposition of the functional groups formed on the MWCNT surface. In the temperature range of 270–350°C, about 10% weight loss is observed for carboxy-modified MWCNT which is due to the presence of functional groups and amorphous carbon.[24]

Characterization of PPY/ITO, MWCNT/PPY/ITO, GOx-HRP/MWCNT/PPY/ITO electrodes

Optimization of GOx to HRP ratio in the bioenzymatic nano bioelectrodes

It is known from the literature that the bienzymatic glucose biosensor based on immobilization of GOx and HRP in one layer (phase) functions better than that in two separated but closely contacted layers (phase).[26,27] In the presence of O_2, the glucose reacts with GOx to produce H_2O_2. For GOx and HRP in one layer (phase), most of H_2O_2 produced at a GOx site can easily diffuse to a nearby HRP site and exchange electron with it because GOx and HRP molecules are adjacent to each other. Moreover, the reaction rate of H_2O_2 in the single-layer configuration has been found to be higher than that of the same amount of both enzymes in solution.[27] Since GOx and HRP played different roles, the ratio of these two enzymes on electrode surface might influence the overall performance of the bienzyme biosensors. The largest current response has been observed for GOx/HRP ratio of 2/1 (data not shown) because HRP is approximately three times more catalytically active than GOx.[28] So we have used 2 : 1 ratio of GOx and HRP for our sensor development.

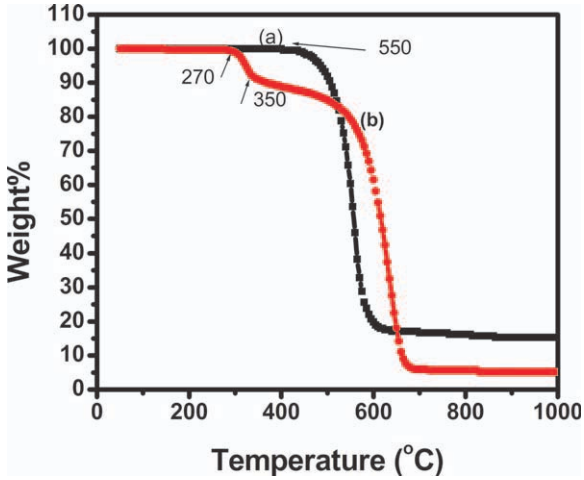


Figure 2 TGA curves of (a) pristine MWCNT and (b)carboxy functionalized MWCNT. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FTIR study

The Figure 3 represents the FTIR spectra of (a) PPY/ITO, (b) MWCNT/PPY/ITO, (c) GOx/MWCNT/PPY/ITO electrodes. The Figure 3(a) shows the characteristics absorption bands of polypyrrole. The peaks at 1580 and 1470 cm^{-1} corresponds to the fundamental vibration of the pyrrole ring.[29] The peaks at 1105,1055, and 1320 cm^{-1} can be assigned to the =C—H in plane vibration.[29] The Figure 3(b) depicts the characteristics absorption bands of polypyrrole as well as carboxy modified MWCNT. The peaks at 1670 cm^{-1} is due to C=0 of COOH and 3452 cm^{-1} corresponds to O—H of COOH.[24] The FTIR spectra of GOx/MWCNT/PPY/ITO is shown by Figure 3(c). When GOx is immobilized on to the MWCNT/PPY/ITO electrode, the typical absorptions bands attributed to amide I at 1665 cm^{-1} and amide to 1530 cm^{-1} are observed in Figure 3(c). Furthermore, the

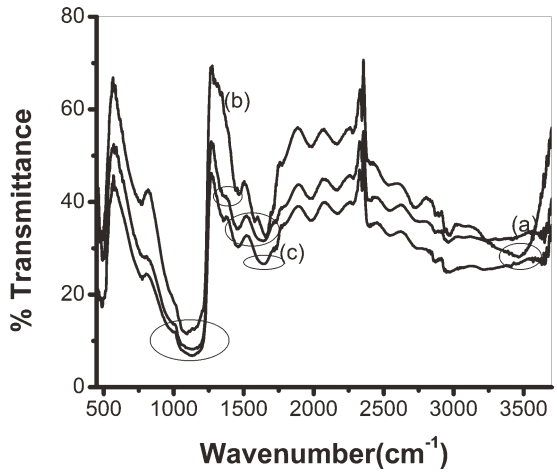


Figure 3 FTIR spectra of (a) PPY/ITO, (b) MWCNT/PPY/ITO (c) GOx/MWCNT/PPY/ITO

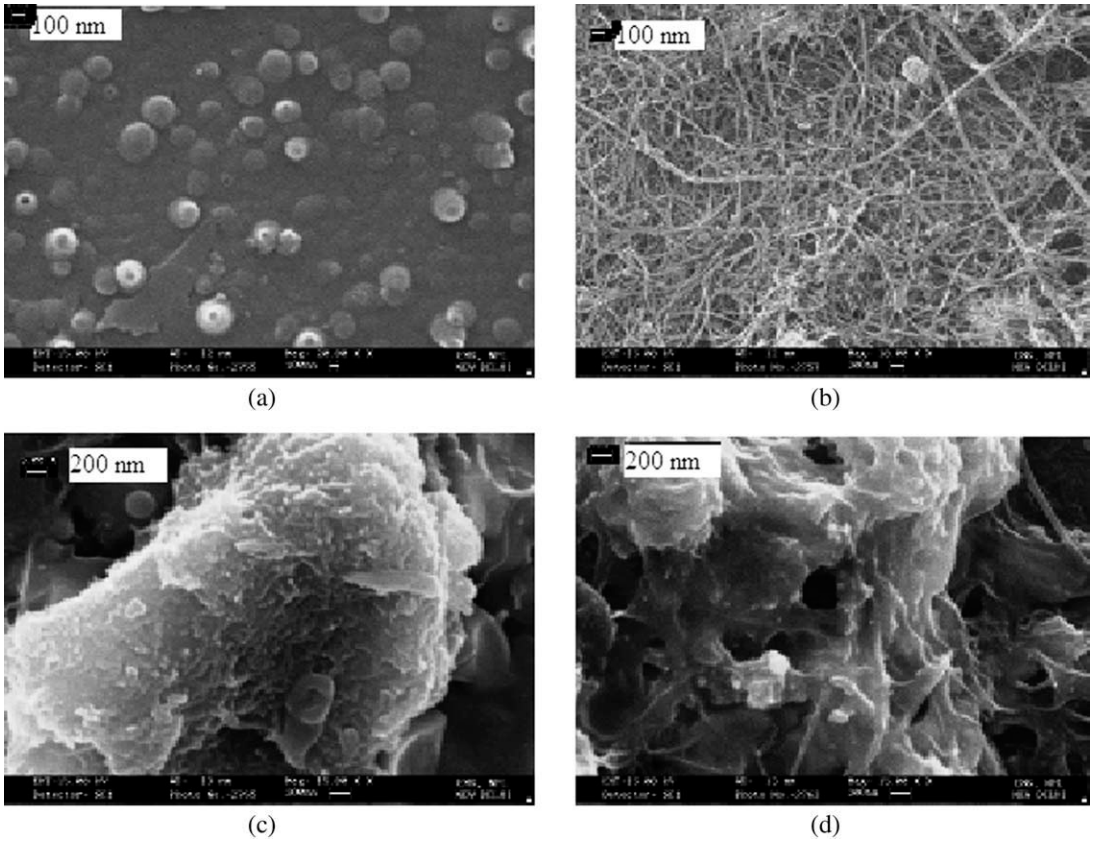


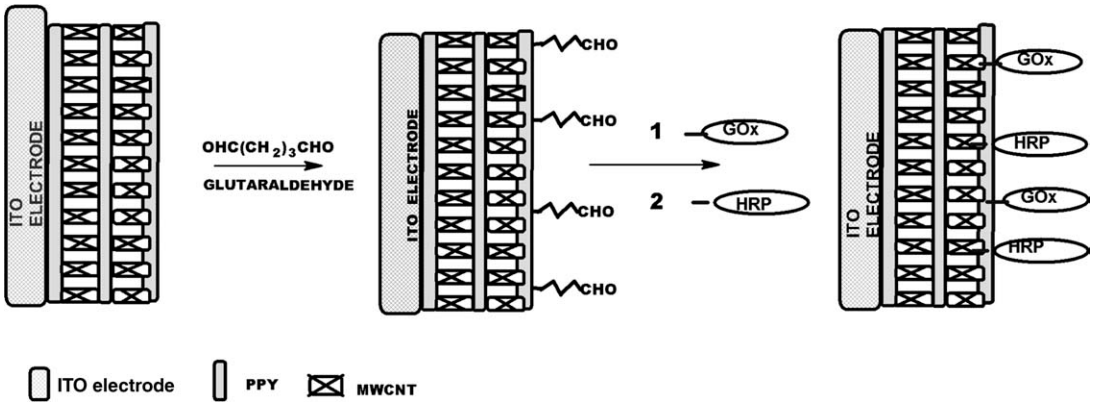
Figure 4 SEM images of the modified electrodes. (a) PPY/PTS, (b) MWCNT/PPY/ITO (c) GOx/MWCNT/PPY/ITO and (d) GOx-HRP/MWCNT/PPY/ITO

absorption peaks at 2950 cm^{-1} and 1347 cm^{-1} are also seen in the Figure 3(c) which is assigned to the typical absorption peaks of native GOx.[30] The peaks at 1200 cm^{-1} (—C—N) and at 3433 cm^{-1} (—OH of *N*—C—OH) in the spectrum of GOx/MWCNT/PPY/ITO can not be unambiguously assigned because it falls into highly mixed vibrational range, in which the contributions from different functional groups overlap extensively.[30,31]

SEM study

The surface morphology of the electrodes was studied using SEM images as shown in Figure 4 (a) PPY/ITO, (b) MWCNT/PPY/ITO (c) GOx/MWCNT/PPY/ITO, and (d) GOx-HRP/MWCNT/PPY/ITO. The SEM image of PPY/ITO electrode [Fig. 4(a)] shows microspheres that result in protruded globular appearance on the surface indicating porous nature of the surface. The incorporation of large dopant anion PTS during electrochemical polymerization results in increased porosity in the film.[32] The MWCNT/PPY/ITO electrode substrate, as shown in Figure 4(b), is mostly covered with homogeneous MWCNT net work. The carboxylation of MWCNT helps to provide a homogeneous MWCNT net work in polypyrrole matrix, which infact can enhance the electron transfer rate between the electrode and electroactive center. The SEM image has also revealed that the MWCNT, with a diameter ranging from 30 to 80 nm, are well distributed on the surface and that most of the MWCNT are in the form of small bundles or single tubes. It can be assumed that the layer-by-layer deposition of PPY and MWCNT has provided an interlinked and uniform network of MWCNT and PPY enabling the development of reliable and stable matrix. Such small bundles and single tubes, assembled homogeneously on the substrate, are believed to be very beneficial for the sensor performance because most of the well-dispersed MWCNT are electrochemically accessible. The porous MWCNT network [Fig. 4(b)] has large surface area which provides an ideal platform for the distribution of enzymes. From the Figure 4(c,d), it can be seen that the enzymes are uniformly distributed on the electrode surfaces. The MWCNT are almost hardly to be visualized on the surface [Fig. 4(c,d)] suggesting that they are totally wrapped by the huge protein molecules. Therefore, it is convenient for the MWCNT to be in close contact with the active center of HRP and act as electron conducting wires to establish direct electron communications.[33]

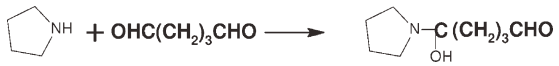
Scheme 1 shows the proposed pathway of immobilization of enzymes (GOx and GOx-HRP) onto



Scheme 1 Predicted composition of nanobioelectrode (GOx-HRP/MWCNT/PPY/ITO)

nanobiocomposite using glutaraldehyde chemistry. It appears that available NH_2 groups of enzyme get covalently attached with aldehyde unit of glutaraldehyde(GA) [eq. (1)] whereas *N*—H group of the carboxy functionalized MWCNT-PPY nanocomposite reacts with aldehyde group of GA [eq. (2)]. It appears that gluteraldehyde are linked by CH=NH group with enzyme and *N*—C—OH group with MWCNT/PPY nano composite.

$$\mathrm{OHC-(CH_2)_3CHO + NH_2-Enz \longrightarrow OHC-(CH_2)_3CH=NH-Enz} \quad (1)$$



(2)

Electrochemical studies

EIS study

Figure 5 shows Nyquist plots obtained for (a) PPY/ITO, (b) MWCNT/PPY/ITO (c) GOx-MWCNT/PPY/ITO, and (d) GOx-HRP/MWCNT/PPY/ITO electrodes, respectively, in the frequency range of 0.01–10^5 Hz in phosphate buffer (50 m*M*, pH 7.0, 0.9% NaCl) containing 5 m*M* $[Fe(CN)6]^{3-/4-}$ that yields information about electrical properties at desired interfaces. Nyquist diameter (real axis value at lower frequency intercept) indicates the value of charge transfer resistance (R_{CT}) i.e., hindrance provided by the electrode material to transfer charge from solution to the electrode that can be correlated with the modification of the surface. The values of electron-transfer resistance (R_{CT}) derived from the diameter of semicircle of impedance spectra are obtained as 14.86 Ω for PPY/ITO electrode (Curve a) and 12 Ω (Curve b) for MWCNT/PPY/ITO electrode. Compared with PPY/ITO electrode, charge transfer resistance (R_{CT}) value obtained for MWCNT/PPY/ITO electrode (Curve b) decreases resulting in enhanced electron transfer or conductive pathway towards electrode. This suggests that presence of MWCNT enhances ionic transport in MWCNT/PPY/ITO nano composite film. It can be seen that R_{CT} increases to 58.8 Ω and 20.015 Ω for after immobilization of GOx onto GOx/MWCNT/PPY/ITO nanobiocomposite (Curve c) and GOx-HRP onto GOx-HRP/MWCNT/PPY/ITO (Curve d), respectively, due to the hindrance provided by macromolecular configuration of GOx and GOx-HRP to electron transport between electrode and redox mediator indicating immobilization of single and bienzyme system on the MWCNT/PPY/ITO nano composite surface. Interestingly, the charge transfer resistance (R_{CT}) of GOx-HRP/MWCNT/PPY/ITO (Curve d) electrode is 2.98 times lower than that of GOx/MWCNT/PPY/ITO suggesting more conducting nature of the former bioelectrode. The increase in charge transfer resistance (R_{CT}) in the nano biocomposite electrodes (both mono and bienzymatic)

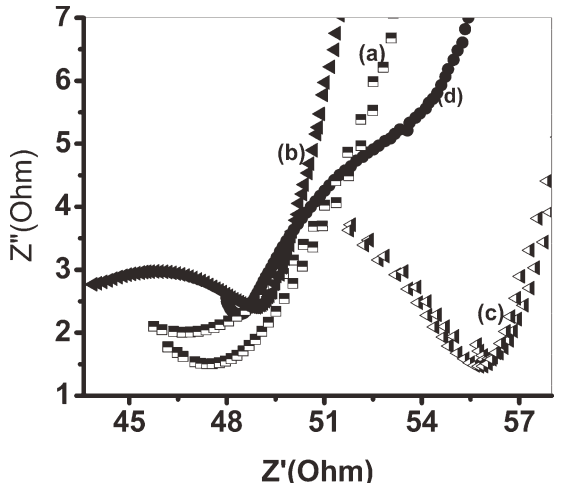


Figure 5 Electrochemical Impedance study of (a) PPY/ITO, (b) MWCNT PPY/ITO, (c) GOx/MWCNT/PPY/ITO and (d) GOx-HRP/MWCNT/PPY/ITO

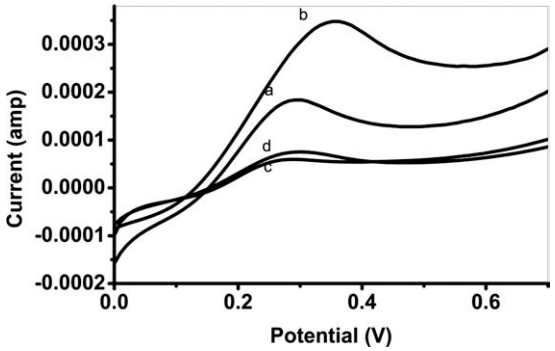


Figure 6 Differential pulse voltammetry (DPV) of (a) PPY/ITO and (b) MWCNT/PPY/ITO (c) GOx/MWCNT/PPY/ITO and (d) GOx-HRP/MWCNT/PPY/ITO.

reveals the immobilization of insulating enzyme molecules onto MWCNT/PPY/ITO nanocomposite electrodes.[34]

DPV study

DPV experiments have been conducted in phosphate buffer (50 m*M*, pH 7.0) containing 5 m*M* $[Fe(CN)_6]^{3-/4-}$ in the range 0.0–0.8 V (Fig. 6). The value of maximum response current obtained as 1.80×10^{-4} A for PPY/ITO (Curve a) increases to $(3.5 \times 10^{-4}$ A) on incorporation MWCNT into PPY. After addition of MWCNT in PPY, magnitude of current increases (Curve b) due to formation of complex between functionalized COOH group and NH group of PPY. In the case of PPY and MWCNT (Curve b), the value of oxidation potential is shifted towards the higher side as compared to that of PPY (Curve a). For nanobioelectrodes, the magnitude of peak current decrease to 6.19×10^{-5} A and 7.78×10^{-5} for GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO, respectively, indicating slow redox process at nanobiocomposite electrodes due to insulating characteristics of enzymes revealing immobilization of enzymes on nanobioelectrodes. The magnitude of peak current of GOx-HRP/MWCNT/PPY/ITO (Curve d) is found to be higher as compared with GOx/MWCNT/PPY/ITO (Curve c) and the peak potential is also shifted towards the higher side. The increase in peak current and the shift of peak potential towards higher value are the indication of increased number of electron transfer since E_p (peak potential)is inversely proportional to the number of transferred electrons ($E_p \propto 1/n$, n is the number of electrons).[34]

Cyclic voltammetry study

Figure 7 shows cyclic volammograms of nanobioelectrodes of (A) GOx/MWCNT/PPY/ITO and (B) GOx-HRP/MWCNT/PPY/ITO as a function of scan rate (20–160 mV/s). It can be seen that the peak potentials and magnitude of peak currents are dependent on the scan rate. As shown in Insets [Fig. 7(A): Inset (a) and Fig. 7B: Inset (a)], the peak current exhibits a linear relationship with the square root of

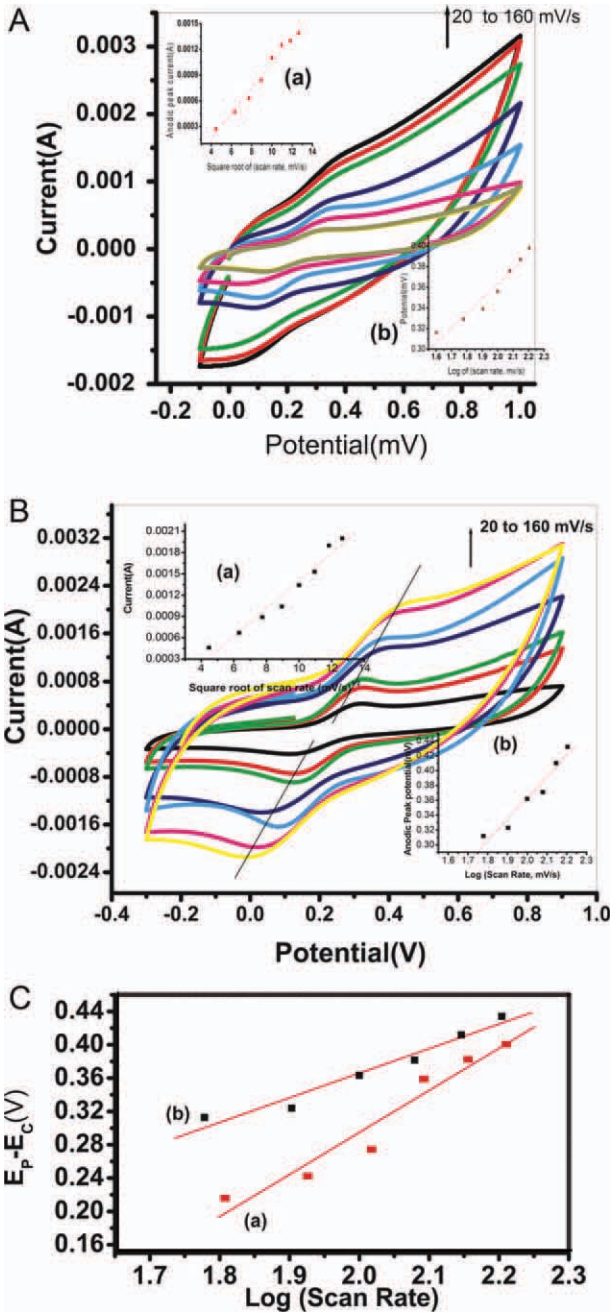


Figure 7 A Cyclic voltammogram of GOx/MWCNT/PPY/ITO nanobioelectrode as a function of scan rate (40–160 mV/s). Inset: a) variation of current and scan rate, b) variation of potential and scan rate. B: Cyclic voltammogram of GOx-HRP/MWCNT/PPY/ITO nanobioelectrode as a function of scan rate (20–160 mV/s). Inset: a) variation of current and scan rate, b) variation of potential and scan rate. C: Plot of difference of peak potential against scan rate (a) GOx/MWCNT/PPY/ITO and (b) GOx-HRP/MWCNT/PPY/ITO. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sweep rate suggesting that electrochemical reaction on both type of nano bioelctrodes is a diffusion-controlled process and follows the eqs. (3) and (4):

$$I(\text{mA}) = -0.43 + 0.14 \times \text{Square root of scan rate}(\sqrt{v}, (\text{mV/S})^{1/2}) \quad R = 0.99(\text{GOx/MWCNT/PPY/IYO}) \tag{3}$$

$$\text{I(mA)} = -0.55 + 0.19 \times \text{Square root of scan rate}(\sqrt{v}, (\text{mV/S})^{1/2}), \quad R = 0.99(\text{GOx} - \text{HRP/MWCNT/PPY/IYO}) \tag{4}$$

where, v = scan rate.

The peak potential increases linearly as a function of scan rate [Fig. 7(A): Inset (b) and Fig. 7(B): Inset (b)] indicating facile charge transfer kinetics in the 20–160 mV/s range of scan rates and follow eqs. (5) and (6):

$$\text{E(mV)} = 0.08 + 0.14 \times \log \text{scan rate} \quad R = 0.97(\text{GOx/MWCNT/PPY/ITO}) \tag{5}$$

$$\text{E(mV)} = 0.22 + 0.29 \times \log(\text{scan rate})\ (\log v) \quad R = 0.98(\text{GOx} - \text{HRP/MWCNT/PPY/ITO}) \tag{6}$$

where, v = scan rate.

The slopes as obtained from the plot of current versus scan rate and the potential versus scan rate of (GOx-HRP/MWCNT/PPY/ITO) nano bioelectrode are found to be higher than the (GOx/MWCNT/PPY/ITO) indicating that HRP enhances the electron transfer process. This can be explained by the fact that the direct electron communication between HRP and the conducting substrate is facilely bridged by the MWCNT.[34]

Figure 7(C) shows the plot of peak to peak separation potential against scan rate. The peak-to-peak separation potential ($\Delta E = E_a - E_c$) [Fig. 7(C)] increases as a function of scan rate, indicating facile charge transfer kinetics in the 60–160 mV/s range of scan rates according to equation:

$$\Delta E(\text{mV}) = -0.224 + 0.295 \log(\text{scan rate}) \times (\text{GOx/MWCNT/PPY/ITO}) \quad R = 0.98 \tag{7}$$

$$\Delta E(\text{mV}) = -0.803 + 0.539 \log(\text{scan rate}) \times (\text{GOx} - \text{HRP/MWCNT/PPY/ITO}) \quad R = 0.97 \tag{8}$$

The surface concentrations of GOx/MWCNT/PPY/ITO, GOx-HRP/MWCNT/PPY/ITO nanobioelectrodes have been estimated from the plot of current versus potential using equation: $I_p = n2F2I^*Av/4RT$ (Brown-Anson model) where n is the number of electrons transferred which is 1 in this case, F is the Faraday constant (96,584 C/mol), I^* is the surface concentration (mol/cm^2) obtained for the nano bioelectrode matrix, A is the surface area of the electrode (0.25 cm^2), v is the scan rate (60 mV/s), 250 R is the gas constant [8.314 J/(mol K)], and T is the absolute temperature (298 K). The values of surface concentrations for GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nano bioelectrodes have been found to be as 3.93×10^{-11} and 4.46×10^{-11} mol/cm^2, respectively. The bienzymatic electrode provides effective higher surface area.

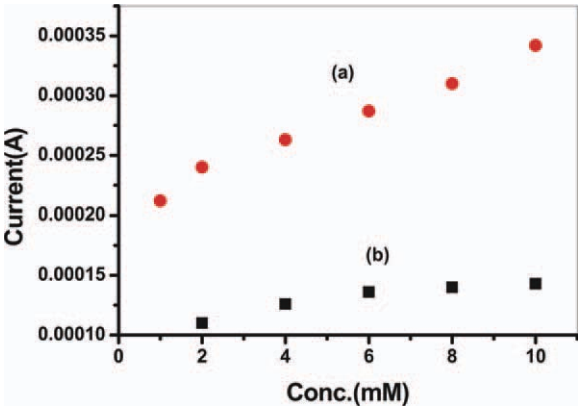
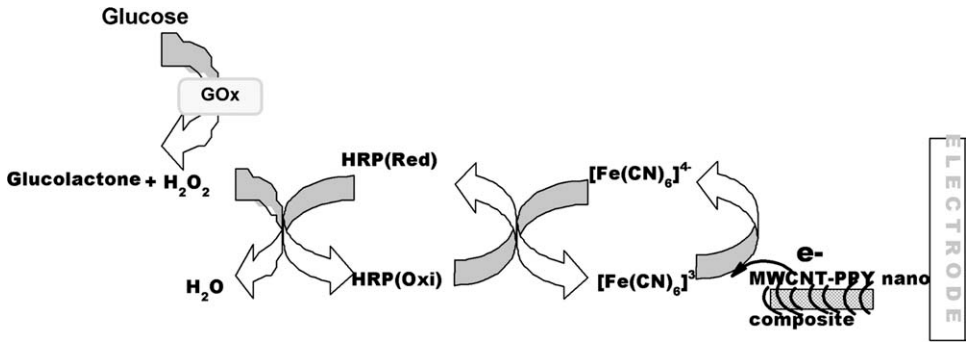


Figure 8 Calibration curves of (a) GOx-HRP/MWCNT/PPY/ITO and (b)GOx/MWCNT/PPY/ITO nano bioelectrodes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Response studies of GOx/MWCNT/PPY/ITO and GOx-HRP/Mwcnt/PPY/ITO nano bioelectrodes

Calibration curves for standard glucose solution are presented in Figure 8. A linear relationship between the glucose concentration and the response current of MWCNT/PPY/ITO for both the mono as well as bienzyme-based nano bioelectrodes is observed. The linear regression curve [Fig. 8(b)] of the GOx/MWCNT/PPY/ITO nano bioelectrode which is used to detect glucose in the range of 1–6 mM, follows the equation; I (current (mA) = 0.1 (mA) + 0.00441 (mA mM^{-1}) × glucose concentration (mM)] with 4 μA and 0.961 as standard deviation and correlation coefficient respectively. The sensitivity of the bioelectrode has been found to be 4.4 μA mM^{-1}. The linear equation of GOx-HRP/MWCNT/PPY/ITO in Figure 8(b) is represented by the equation I (current) (mA) = 0.2 (mA) + 0.013 (mA mM^{-1}) × glucose concentration (mM)] with 5 μA and 0.994 as standard deviation and correlation coefficient respectively. Furthermore, the GOx-HRP/MWCNT/PPY/ITO nanobioelectrodes exhibit a higher sensitivity of 13.48 μA mM^{-1}, three times higher than the single enzyme-based electrodes (GOx/MWCNT/PPY/ITO) and linear range of 1 to 10 mM/L which is also



Scheme 2 Proposed biochemical reaction on the GOx-HRP/MWCNT/PPY/ITO

much higher than the GOx/MWCNT/PPY/ITO. The response current and sensitivity are also much higher for bienezymatic sensor than the mono enzymatic nano biosensor suggesting effective reduction of H_2O_2 catalyzed by HRP. All of the experiments have been carried out in triplicate sets, and the results reveal reproducibility of the system within 3% error. The values of response time of the (GOx/MWCNT/PPY/ITO) and (GOx-HRP/MWCNT/PPY/ITO) are found to be as 13 and 9 s, respectively, which are measured by measuring the time taken to reach the steady state current after applying a steady voltage of 300 mV for 3×10^{-3} *M* of glucose solution in 7.0 pH PBS buffer containing 5 m*M* $[Fe(CN)_6]^{3-/4-}$. The values of apparent Michaelis-Menten constant (K_m) have been estimated using Lineweaver-Burke plot for GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO as 0.42 and 0.52 m*M*, respectively. The slightly higher value for the bienzymatic system which could be the relay type reaction mechanism happened in the bienzymatic nano biosensor. The working mechanism of the bienzymatic sensors for the detection of glucose can be depicted as follows: the substrate glucose, diffused from the bulk solution into the PPY film, reacts with the GOx in the presence of the O_2, to produce H_2O_2. The hydrogen peroxide then serves as substrate for HRP and the oxidized state of HRP is in turn recycled at the electrode surface which is stimulated by the sandwiched MWCNT. The overall biochemical reaction for GOx-HRP/MWCNT/PPY/ITO are shown by the equations (9–13) and Scheme 2.

Glucose oxidase catalyzes the oxidation of glucose to gluconic acid, in the presence of O_2, with production of H_2O_2 [eq. (9)].

$$\beta - \mathrm{D} - \text{glucose} + \mathrm{O_2} \longrightarrow \delta - \text{glucono} - \beta - \text{lactone} + \mathrm{H_2O_2} \quad (9)$$

HRP reduces H_2O_2 and in this process, is getting oxidized which is then reduced by the MWCNT, which in turn is reduced electrochemically. H_2O_2 formed by GOx undergoes a series of reactions (9–11).

$$\mathrm{HRP(Fe^{3+}) + H_2O_2 \longrightarrow HRP - I + H_2O} \quad (10)$$

In the first step (two electron step), H_2O_2 oxidizes ferriheme prosthetic group of HRP (Fe^{3+}), producing an unstable intermediate HRP-I consisting of a π cation radical of heme with Fe(IV) to which an oxygen atom is coordinated ([Fe(IV) O]):

The reduction of HRP-I to HRP (Fe^{3+}) takes place through two successive one-electron steps via the electron transfer through MWCNT.

$$\mathrm{HRP - I + MWCNT \longrightarrow HRP - II + MWCNT(+)} \quad (11)$$

$$\mathrm{HRP - II + MWCNT \longrightarrow HRP(Fe^{3+}) + MWCNT(+)} \quad (12)$$

where HRP-II, an intermediate state, possesses a heme with Fe(IV) to which OH is coordinated ([Fe(IV) OH]). Oxidized MWCNT is reduced at the electrode.

$$\mathrm{MWCNT(+) + \mathit{e} \longrightarrow MWCNT(at\ the\ electrode)} \quad (13)$$

The redox center of enzyme is electrically insulated by a glycoprotein shell. Because of this protein shell, electrical contact of redox enzymes with electrode surface is a challenge. MWCNT can help a direct electron transfer between electrode and the redox center of adsorbed enzyme.[35]

Effect of pH, Interferent and shelf life studies of GOx/MWCNT/PPY/ITO and GOx-HRP/Mwcnt/PPY/ITO nano bioelectrodes

The response current of the GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nano bioelectrodes studied in the pH range 5.0–8.0 (data not shown), suggests that both the bioelectrodes exhibit

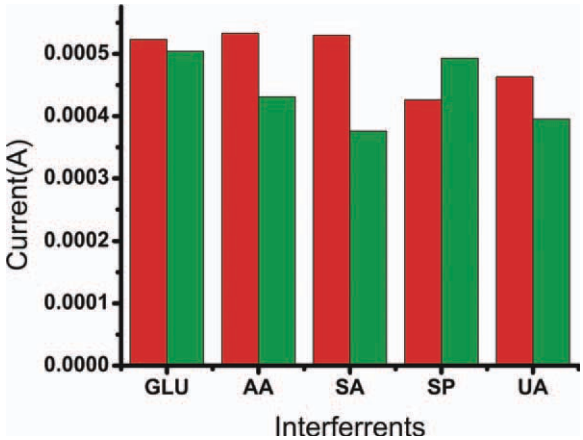


Figure 9 Interferent study of GOx/MWCNT/PPY/ITO and GOX-HRP/MWCNT/PPY/GOx bioelectrode. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

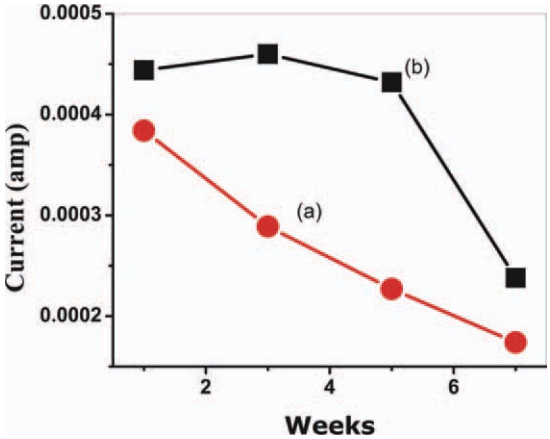


Figure 10 Results of shelf life of (a)GOX/MWCNT/PPY/ITO and (b)GOX-HRP/MWCNT/PPY/ITO nano bioelectrodes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

maximum activity at around pH 7.0. This could also be assigned to the fact the polypyrrole, unlike other conducting polymers like polyaniline and polythiophen, shows good conductivity and electroactivity even at the neutral pH.[2] The practical usefulness of an amperometric biosensor often rests upon the selectivity, i.e., the interference level from electroactive species. It is known that CNT showing extraordinary catalytic property for electrochemical oxidation of ascorbic acid (AA), uric acid (UA) and sodium ascorbate (AP), which are the common interferents in glucose determination.[12] The response characteristics of GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO in the presence of some possible interfering substances, like ascorbic acid(A A; 0.05 m*M*), sodium ascorbate (S A, 0.05 m*M*), sodium pyruvate (SP; 0.1 m*M*), and uric acid (UA;0.1 m*M*) is shown in Figure 9. The Figure 9 shows that the both the nanobiolectrodes experience negligible interference from the above interfering agents. Such observation can be correlated with the excellent permselectivity of polypyrrole which protects electrodes from fouling by proteins and another biological substances present in the real samples as blood serum and urine.[36] It is also revealed from the Figure 9 that GOx-HRP/MWCNT/PPY/ITO electrodes experience minimum interference as compared with GOx/MWCNT/PPY/ITO electrodes due to faster response and lower detection potential.[37,38]

The shelf life of GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO bioelectrodes have been determined by measuring the response current at regular interval of one week for about two months. Figure 10 demonstrates the shelf life of the GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nanobioelectrodes. The GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO bioelectrode are stored at 4°C when not in use. It has been found that GOx-HRP/MWCNT/PPY/ITO nano bioelectrode exhibit 100% activity after about five weeks after that response current drastically decreases while GOx/MWCNT/PPY/ITO nano bioelectrode shows a decrease in activity after 2.5 weeks (Fig. 10). The diminishment of biosensor response could be ascribed to the following possible reasons: (1) the deterioration of the electronic conductivity of PPY film caused by H_2O_2 oxidation; (2) partial inactivity of HRP induced by H_2O_2; and (3) leaching of enzymes from the swollen polymer matrix.[38,39]

Table I represents a comparative evaluation of mono and bienzymatic biosensor performance. It has been found that bienzymatic electrodes GOx-HRP/MWCNT/PPY/ITO exhibit better performance in terms of linearity, shelf life, and sensitivity as compared with single enzyme-based GOx/MWCNT/PPY/ITO electrode. This improved results offered by GOx-HRP/MWCNT/PPY/ITO is due to the presence of HRP which enhances overall biochemical process by the faster decomposition of hydrogen peroxide. In Table II the characteristics of the present nano bioelectrodes are shown with the reported values for GOx-HRP system in the literature. Table II

TABLE I
A Comparative of Evaluation of Single and Bienzymatic Biosensor Performance

Sl No	Characteristics	GOx/ MWCNT/ PPY/ITO	GOx-HRP/ MWCNT/ PPY/ITO
1	Linearity	1–6 m*M*	1–10 m*M*
2	Detection limit	0.3 m	0.1 m*M*
3	Response time	15 s	10 s
4	Sensitivity	4.4 μA/m*M*	13.8 μA/m*M*
5	K_m	0.52 mA/m*M*	0.42 mA/m*M*
6	Shelf life	2.5 weeks	5 weeks

TABLE II
Characteristics of GOx-HRP/MWCNT/PPY/ITO Nano Bioelectrodes Including Reported in the Literature

Sl No	Components of biosensor	Characteristics	Reference
1	[Mat]: GOx-HRP/MWCNT/PPY/ITO [E]: GOx, HRP [M]: Ampero. vs. Ag/AgCl	[L]:1 to 10 m*M* [S]: 13.8 mA/μ*M* [RT]:10 s [DL]:0.1 m*M* [SL]: 5 weeks	Present investigation
2	[Mat]: MWCNT/poly(O-toludene)/GC [E]: GOx, HRP [M]: Ampero. vs. Ag/AgCl	[DL]:0.1 m*M*	[27]
3	[Mat]: GPTMS-CS/Au [E]: GOx, HRP [M]: Ampero. vs. Ag/AgCl	[L]: 1 to 351 μmol/L [DL]: 0.3 μmol/L]	[21]
4	Mat]: MWNT/TB/GOD/HRP/Nf [E]: GOx, HRP [M]: Ampero. vs. Ag/AgCl	[L]: 1.4×10^{-7}–1.6×10^{-3} *M*	[22]
5	[Mat]: AuNPs/SBA-15	[L]: 1 to 48 μ*M* [S]:0.42 mA/m*M*	[20]
6	[Mat]:AuNP/CS/GMB [M]: Electroluminescence	[SL]:5 weeks	[33]
7	[Mat]: CNT-PAMAM E]: GOx, HRP [M]: Ampero. vs. Ag/AgC	[L]:4μM to 1.2 m*M* [S]:2200 nA/m*M* [DL]:2.5 μ*M* [RT]:1 s	[19]

[Mat], material; [E], enzyme; [M], method; [DL], detection limit; [L], linearity; [SL], shelf life; [RT], response time; CS, chitosen; Nf, Nafion; SBA-15, mesoporous silica; PAMAM, polyamidoamine; TB, toluidine blue; GPTMS, glycidoxypropyltrimethoxysiloxane.

shows the characteristics of GOx-HRP/MWCNT/PPY/ITO nano bioelectrodes including reported in the literature for GOx-HRP system. In this context, it can be mentioned that Jeykumari et al. have developed a similar type of bienzymatic glucose biosensor based on Toluidine blue (TB) nafion functionalized multiwalled carbon nanotubes (MWCNTs).[41] They have observed much lower detection limit of 0.14 μ*M*/L as compared with the present electrode system but the upper range is only upto 1.6 mm/L. They have neither reported the K_m value or performance of the electrode for the detection glucose in actual blood serum.

Blood serum testing

The response of the GOx/MWCNT/PPY/ITO and GOx-HRP/MWCNT/PPY/ITO nano-bioelecectrodes to the glucose in human blood serum was investigated. Two serum samples obtained from pathological lab were analyzed. The respective nano bioelectrode is dipped into the reaction mixture containing 100-μL blood serum (after dilution) in phosphate buffer (50 m*M*, pH 7.0, 0.9% NaCl, 5 m*M* $[Fe(CN)_6]^{3-/4-}$) and DPV measurements have been carried out using three electrode system. The glucose content of the blood serum has been calculated from the DPV calibration curve (Fig. 5). The results were matched with referenced values obtained by the automated standard colorimetric technique in the lab. Table III shows the comparison of the determined values and the referenced values. Both the nanobioelectrodes provide excellent performance in evaluation of bold glucose in blood serum samples which may be due to the permselectivity of the polypyrrole matrix and high electrocatlytic effect of MWCNT-polypyrrole nano composite. The results obtained from GOx-HRP/PPY/MWCNT/ITO are

TABLE III
Results from Blood Serum Samples Using GOx/MWCNT/PPY/ITO and Bienzymatic GOx-HRP/MWCNT/PPY/ITO Biosensors

Glucose content in blood serum serum as obtained from lab (mg/dL) using optical method (mg/dL)	Glucose content in bloodserum as calculated from GOx/MWCNT/PPY/ITO electrotrode (mg/dL)	Glucose content in blood serum as calculated from GOx-HRP/MWCNT/PPY/ITO electrotrode (mg/dL)
259	240	249
76	69	72

very close to the actual values (within 3.8–5% error) while GOx/PPY/MWCNT/ITO shows comparatively higher deviation from the actual values (7.3–9% error).

CONCLUSIONS

The carboxy-modified MWCNT and PPY multilayer electrodes have been fabricated for the development of glucose biosensor. Both single GOx and GOx-HRP-based nano biosensors are developed using covalent bonding through gluteraldehyde. The bi-enzyme-based nanobioelectrodes (GOx-HRP/MWCNT/PPY/ITO) offer better performance in terms of linearity, sensitivity, response time, and shelf life than single enzyme system. This is attributed to the presence of HRP along with GOx to enhance the overall biochemical reaction. It has been shown that this nanobiocomposite electrode can be used to estimate glucose in blood serum samples. The unique features of the GOx-HRP/MWCNT/PPY/ITO nanobioelectrode lie with the novelty of fabrication, minimum interference, very low K_m value, low response time, and the its usefulness for blood serum samples. It should be interesting to utilize this nano composite electrodes for development of other biosensors.

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