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Polypyrrole/carbon nanotubes/lactate oxidase nanobiocomposite film based modified stainless steel electrode lactate biosensor

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Abstract

Highly sensitive electrochemical lactate biosensor was constructed by one-step preparation route of amperometric enzyme electrode based on carbon nanotubes (CNT) and biocatalyst within an electropolymerized polypyrrole (PPy) film electrodeposited on stainless steel electrode. The nanobiocomposite film was electrochemically synthesized by electro-oxidation of pyrrole in a neutral pH solution containing appropriate amounts of lactate oxidase (LOD) and functionalized multi-walled carbon nanotubes (c-MWCNT). The nanobiocomposite film was characterized by FTIR, SEM and EIS. The incorporation of CNT facilitated high enzyme loading, increase in lifetime, stability and fast response time of the enzyme electrode. In present work artificial sweat was used as a non-invasive analyte for detection of lactate. When PPy/c-MWCNT/LOD modified electrode tested for lactate in artificial sweat, the apparent Michaelis–Menten constant (K’m) and maximum current (Imax) were found to be 0.833mM and 2.5μA respectively. The sensitivity and the detection limit were found to be 0.0778μA/μM and 5.61μM/l respectively with a linear response range from 5 to 60μM (R2 =0.95). The response time and shelf-life were found to be 8 sec and 2 weeks, indicating good sensing results.

1. Introduction

Electropolymerization represents an attractive well controlled one-step avenue for preparing amperometric enzyme electrodes (Wang et al., 2005). In this methodology, the enzyme is mixed with a monomer, which is electropolymerized at an inert electrode, whereupon the enzyme becomes embedded into the polymer matrix (Brânzoi et al., 2009). Polypyrrole (PPy) has been particularly useful for this task since it can be electropolymerized at neutral pH which allows the entrapment of a wide range of biocatalysts (Gutiérrez et al., 2013). Recently,
nanomaterials have been employed in the construction of electrochemical biosensors to improve their analytical performance (Christopher et al., 2010). It has been shown that the introduction of carbon nanotubes (CNTs) into a polymer matrix can improve the electrical conductivity and the mechanical properties of the original polymer matrix (Martina et al., 2011, Ghica et al., 2014). Multi-walled CNTs consist of several concentric tubes of graphite inside one another. These nanotubes have been employed in biosensors as effective catalyst supports due to their large surface area, unique structural and electromechanical properties, good biocompatibility, ease of preparation and surface renewability (Chauhan et al., 2012). Recent studies demonstrated that CNT can enhance the electrochemical reactivity of important biomolecules, and can promote the electron-transfer reactions of enzymes (Peng et al., 2012, Sahoo et al., 2010). A large number of Biosensors are available in medical analysis all based on in-vivo method. Outcome through a non-invasive method is the main aim to be achieved in health care. The advantage of measurement of metabolite in media other than blood has become increasingly significant. In present work we are using sweat as a non-invasive analyte for detection of Lactate. There are several molecules and ions in human sweat that, when excreted in abnormal amounts, may be an indication of a separate pathological disorder. Lactate are analytes present on the skin and excreted in sweat that may be detected and evaluated for diagnostic purposes. (Weber et al., 2006) Lactate detection is helpful for monitoring respiratory insufficiency, shocks, heart failure, metabolic disorder and Psychological disorder (Rahman, 2010). The most common analytical method for determination of lactate concentration is high-performance liquid chromatography (HPLC) (Nesakumar et al., 2014) with either ultraviolet (UV)–spectrophotometric or refractive index detection. However, this method is time-consuming because of the requirement for pretreatment and the complicated procedure involved. Therefore, the demand for a sensitive, simple, and accurate method has arisen (Rahman et al., 2009).

In the lactate oxidase (LOD) based amperometric biosensor, LOD catalyzes the conversion of lactate to pyruvate and hydrogen peroxide (H$_2$O$_2$), which can be oxidized at the electrode surface (Romero et al., 2008). However, the LOD based biosensors have some common drawbacks, including the fact that they are unstable and that the electrochemical oxidation of the hydrogen peroxide commonly occurs at high potentials, which can allow interference from other electroactive compounds usually present in real samples. Moreover, the intermediates produced during the oxidation reaction can lead to electrode fouling. Therefore, there is a demand for the development of a biosensor that is stable, reproducible, and sensitive for lactate detection in real samples (Loaiza et al., 2015). In this paper, we report a simple, selective, and sensitive biosensor for lactate detection. The nanobiocomposite film was electrochemically synthesized by electro oxidation of pyrrole in a phosphate buffer solution (PBS) containing appropriate amounts of LOD and functionalized multi-walled CNTs (c-MWCNT) on a steel electrode to obtain PPy/c-MWCNT/LOD modified steel electrode. In contrast to most of previous studies that rely on employment of the mediators or chemical treatment, with our proposed modified electrode, the H$_2$O$_2$ oxidation would be possible under a very low potential without limitations arising from employment of mediators. Analytical performances of the lactate biosensor in terms of the dynamic linear range, sensitivity, and stability of the electrode were determined.

2. Experimental

2.1. Materials

Lactate Oxidase from Pediococcus lyophilized powder ≥20 units/mg solid, Pyrrole (98% purity), Lactic acid and Phosphate buffer solution were procured from Sigma-Aldrich. Multi-walled Carbon nanotubes (MWCNT) with diameter ~20nm synthesized by chemical vapor deposition made available from National Physical Laboratory New Delhi, India. All the other reagents were of AR grade and all solutions were prepared using double distilled water.

2.2. Characterizations

Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) were recorded on Potentiostat/Galvanostat (Model CHI600D Electrochemical Analyzer, Interface 1000 Gamry Potentiostat) with a three electrode system consisting of a Pt wire as a counter electrode, an Ag/AgCl (3 M/saturated KCl) electrode as reference electrode and modified steel electrode (5cm x 0.5cm) as a working electrode. Morphological characterization of PPy/c-MWCNT nanocomposite and PPy/c-MWCNT/enzyme nanobiocomposite were obtained
from Scanning electron microscope (Model JSM-7600F). Fourier transform infrared (FTIR) spectra were recorded on a FTIR spectrometer (Model Bruker Alpha).

2.3. Fabrication of PPy/c-MWCNT/LOD electrode

Electrochemical synthesis of PPy/c-MWCNT/LOD nanobiocomposite film was performed in one-compartment three-electrode glass cell at room temperature (27 °C) using the potentiostat-galvanostat under computer control. To perform the experiment, at first MWCNTs were functionalized by sonication using 3:1 sulfuric-acid/nitric-acid mixture for 3 hours. Subsequently, the pretreated MWCNTs were washed with water, then with 0.1M NaOH (to reach neutrality of pH 7.0), filtered, and dried overnight at 80°C (Ribeiro et al., 2013). The functionalized MWCNTs (15% by weight of pyrrole) were dispersed in distilled water by sonication for 1 hour. The selected amount of LOD in 0.1M PBS (pH 7) was then added to the c-MWCNTs solution. In the end, pyrrole was added (at a concentration of 0.5 M) to the LOD-c-MWCNT mixture and this solution was again stirred for 15 minutes. Nanobiocomposite film was electrochemically synthesized by electrolyzing this medium on the steel electrode in continuous stirring conditions by sweeping the potential on the working electrode, from −0.8 to +0.8 V versus a standard counter electrode, at room temperature with a scan rate 50 mV/s. PPy/c-MWCNT/LOD film thus obtained was washed repeatedly with double distilled water and then dried at room temperature under vacuum. The enzyme electrode prepared was stored at 4 °C in a refrigerator immersed in a phosphate buffer solution of pH 7 for further studies. PPy/c-MWCNTs film was also obtained for comparison. Its preparation was done in a similar manner to that of PPy/c-MWCNTs/LOD film, but in absence of LOD.

2.4. Electrochemical measurements of PPy/c-MWCNT/LOD electrode

EIS and CV experiments for PPy/c-MWCNT and PPy/c-MWCNT/LOD characterization were performed in electrolyte support solution of 0.1 M PBS of pH 7. Applied potential of +0.01 V against Ag/AgCl, signal amplitude of 5 mV and frequency range from 100 kHz – 0.01 Hz were used for EIS measurements. Cyclic voltammetry studies were carried out using PPy/c-MWCNTs/LOD modified steel electrode as working electrode. Cyclic voltammograms of bare steel electrode, PPy/c-MWCNT electrode and PPy/c-MWCNT/LOD electrode were recorded in 0.1 M PBS (pH 7) at a scan rate of 50mV/s. To measure the response of PPy/c-MWCNT/LOD biosensor, the three-electrode system was immersed in 15 ml of 0.1M PBS (pH 7) and the reaction was started by adding 0.1 ml of lactic acid (1 μM), which was oxidized to pyruvate, producing an electroactive H2O2. Formation of H2O2 was detected by its oxidation to generate electrons i.e., current at the electrode. The response measurement of PPy/c-MWCNT/LOD electrode was obtained at different lactic acid concentrations ranging from 5 to 60 μM. The calibration curve for the responses was plotted and K´m and Imax value for lactic acid was calculated from a Lineweaver–Burk plot.

2.5. Optimization and storage stability of PPy/c-MWCNT/LOD electrode

To optimize working conditions of the biosensor, effects of pH, temperature, and time on biosensor response were studied. To determine the optimum pH for the PPy/c-MWCNT/LOD electrode, the pH of the reaction buffer was tested from pH 6.0 to 10.0 at intervals of 0.5 pH units using PBS solution. The storage and stability of the biosensor was investigated for over 2 weeks. The activity of PPy/c-MWCNT/LOD electrode was measured every alternate day and it was washed by the PBS solution every time before use. PPy/c-MWCNT/LOD electrode was stored in a refrigerator at 4°C when not in use.

2.6. Determination of lactate in artificial sweat by PPy/c-MWCNT/LOD electrode

Artificial sweat was used for lactate determination using the PPy/c-MWCNT/LOD electrode. The procedure was same as described for response measurement of electrode except that lactic acid was replaced by artificial sweat.
3. Results and discussion

3.1. Morphology

Scanning electron microscopy (SEM) was used for examining the morphology of the PPy/c-MWCNT nanocomposite and PPy/c-MWCNT/LOD nanobiocomposite. It is seen from Fig. 1(a) that the surface of pure polypyrrole is highly porous useful for adsorption of enzyme to be immobilized. From Fig. 2(a) it is observed that the diameter of the individual PPy/c-MWCNT fibril has increased to nearly 40-50 nm which is larger than that of the corresponding MWCNT fibril alone (~20 nm). This suggests that the nanocomposite exhibits well dispersed carbon nanotubes over Polyppyrole film (Lien et al., 2010, Paul et al., 2010). The PPy/c-MWCNT layer is characterized by a very fibrous structure of interlocking pores. Such morphology is in agreement with earlier studies of conducting-polymer/CNT nanocomposites (Valentini et al., 2013). Fig. 3(a) shows incorporation of enzyme on PPy/c-MWCNT nanocomposite film, as one can see the appearance of spotted globular structure on the film, indicating that enzyme was successfully immobilized on the surface of PPy/c-MWCNT nanocomposite film.

3.2. FT-IR spectra

FT-IR spectra of PPy, PPy/c-MWCNT nanocomposites and PPy/c-MWCNT/LOD nanobiocomposites are shown in Fig. 1(b), Fig. 2(b) and Fig. 3(b) respectively. The characteristic bands at 1517, 1177, 1033, 907 and 650 cm\(^{-1}\) corresponds to C=C vibration, C–O stretching vibration, C–H bonding and ring deformation in PPy (Ramya et al., 2012). Significant differences between IR spectra of pure PPy and PPy/c-MWCNT nanocomposites appear for the bands ascribed to pyrrole ring vibrations, located around 688, 912, 1177 cm\(^{-1}\). These absorption bands are sensitive to the oxidation level and to the conjugation length of the PPy chain. The shift of these bands to lower frequencies in the nanocomposites spectra as compared with PPy suggests that an interaction between the polymer and CNT occurs (Zou et al., 2008). In the FTIR spectra of PPy/c-MWCNT/LOD nanobiocomposite the appearance of additional absorption bands at 1550 and 1521 cm\(^{-1}\), represents carbonyl stretch (amide I band) and N-H bending (amide II band), respectively. Additionally, a broad band is observed at approximately 3278 cm\(^{-1}\) which can be attributed to an amide bond present in LOD. This result confirms the incorporation of LOD during the electropolymerization of PPy/c-MWCNTs film on steel electrode (Lata et al., 2013).

![Fig. 1: (a) SEM image and (b) FTIR spectrum of PPy](image-url)
3.3. Construction of lactate biosensor

Fig. 4 shows the schematic diagram of electrodeposition of PPy, c-MWCNT and LOD simultaneously on steel electrode. During the electrodeposition the enzyme LOD gets incorporated into the PPy/c-MWCNT matrix. The incorporation of LOD includes the formation of an intermediate ester (the product of condensation of the free –COOH groups of PPy/c-MWCNT/LOD). The active ester intermediate reacts with the amide (–NH₂) groups on the surface of enzyme to yield the final amide bond, confirming the enzyme on the surface of PPy/c-MWCNT/LOD nanobiocomposite film. This incorporation of the enzyme onto PPy/c-MWCNT/LOD nanobiocomposite film does not allow the leaching of the enzyme during repeated washing of the enzyme electrode for its reuse.
3.4. EIS measurements

To characterize the electronic and transport properties of PPy/c-MWCNT and PPy/c-MWCNT/LOD film, electrochemical impedance experiments were performed. Fig. 5 show Nyquist plots of the imaginary vs. the real part of the impedance for bare steel electrode (RCT 430 $\Omega$) Fig. 5 (a) and the PPy/c-MWCNT film (RCT 220 $\Omega$) Fig. 5 (b). For both systems, the intersection at high frequencies on the real axis corresponds to the electrolyte resistance. The impedance values attained for the PPy/c-MWCNT electrode are smaller than for bare steel electrode but increases for PPy/c-MWCNT/LOD electrode (RCT 240 $\Omega$) Fig. 5(c). The increased RCT value of PPy/c-MWCNT/LOD electrode was due to the incorporation of enzyme into PPy/c-MWCNT electrode. This increase in RCT is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer (Lata S., Pundir C., 2013, Li et al., 2013).

3.5. Cyclic Voltammetry of electrodeposition

Fig. 6 illustrate the cyclic voltammograms of bare steel electrode (a), PPy/c-MWCNT (b) and PPy/c-MWCNT/LOD (c). The bare steel electrode (curve a) shows no redox Peak. The conducting polymer films were deposited on steel electrode between −0.8 and +0.8 V in 0.1 M PBS of pH 7 as described in experimental section. It can be seen that a polymer growth, with increasing current is observed in the presence of c-MWCNT (curves b). The rise in the anodic current at −0.6 V potential range corresponds to the oxidation of the pyrrole monomer to form methylpyrrole radical cation, subsequent the radical reacts with another monomer to form dimerization of radical cation and coupling will result in the formation of an insoluble polymer, positively charged on the surface. In this case the growth of Ppy/c-MWCNT is performed without additional other dopants. Thus, the CNTs acts as the counter ion incorporated within the polymer film to balance the cationic charge of the oxidized polymer (Han et al., 2005, Moyo et al., 2012, Tam et al., 2011). The PPy/c-MWCNT film also offers a more symmetrical voltammogram, with redox activity starting at a lower potential (−0.6 versus −0.2 V), indicating that the presence of the CNT promotes the electron-transfer of the oxidation–reduction process. The LOD incorporated to the PPy/c-MWCNT films by entrapment and then diffuse in the nanocomposite film leading to decrease the current density due to the negative charge on the film. The peak current increased from 0.9 to 1.8 by the introduction of LOD into the film, indicating the synergy effect between LOD and CNTs similar to the previous study (Korkut et al., 2008). In addition to this, the electrocatalytic sites placed in the active centre of LOD would join into the PPy/c-MWCNT nanobiocomposite film. The well-defined peaks indicate that films are highly homogeneous.
3.6. Cyclic Voltammetric responses of biosensors

Fig. 6(d) shows potentiodynamic response of PPy/c-MWCNT/LOD biosensor, with successive addition of 0.1ml of 1 μM Lactic acid in 0.1 M PBS (pH 7) at a scan rate of 50 mV s$^{-1}$ and potential of -0.8 V. As soon as the lactate was introduced into the buffer, the lactate was converted into pyruvate and H$_2$O$_2$ by PPy/c-MWCNT/LOD. The electrons thus generated from H$_2$O$_2$ were detected at the applied potential utilizing PPy/c-MWCNT/LOD as electron transferring medium. It can be seen from Fig. 6 that the response current increases linearly with increasing concentration of lactate in the range of 5 - 60 μM, which is due to the produced ammonium from the enzymatic reaction, then reached saturation.

![Graph 5: Impedance spectra of (a) bare steel electrode, (b) PPy/c-MWCNT and (c) PPy/c-MWCNT/LOD electrode](image)

![Graph 6: Cyclic voltammograms of (a) bare steel electrode, (b) modified PPy/c-MWCNT electrode, (c) modified PPy/c-MWCNT/LOD electrode and (d) Cyclic voltammograms of PPy/c-MWCNT/LOD electrode in PBS (0.1M, pH 7) at a scan rate of 50 mV s$^{-1}$ at different concentrations of Lactic acid (in μM)](image)

3.7. Calibration curve of lactate Biosensor

The calibration lines as shown in Fig. 7 were calculated by taking the maximum current reading for each concentration in Fig. 6 subsequently plotting current value versus lactate concentration. The detection limit and
sensitivity was calculated to be 5.61 μM/l and 0.0778 μA/μM. The greater sensitivity was due to the incorporation of c-MWCNTs deposited PPy film along with LOD by one-step electrodeposition method.

3.8. Lineweaver–Burk plot

Lineweaver–Burk plot between 1/current (μA) and 1/lactate concentration (μM) is shown in Fig. 7(b). The value of Michaelis–Menten constant (Km) for lactate was obtained as 0.833 mM and maximum current (Imax) were found to be 2.5 μA from the plot. This Km value is lower than that for free enzyme (1.6 mM) indicating increased affinity of enzyme toward lactate after immobilization, which might be due to enhanced diffusion of lactate through PPy/c-MWCNT/LOD nanobiocomposite film (Devi et al., 2013)

![Lineweaver-Burk plot](image)

Fig. 7: (a) Calibration curve of PPy/c-MWCNT/LOD lactate biosensor and (b) Lineweaver–Burk plot for effect of Lactate concentration on current response of PPy/c-MWCNT/LOD electrode

3.9. Effect of pH and Response time of biosensor

Enzyme sensor response depends on the working pH of the sampling solution. The effect of pH on the behaviour of the PPy/c-MWCNT/LOD electrode was studied with 0.1 M phosphate buffer solution with 1 μM lactic acid. The steady state currents at -0.8 V as a function of pH values is shown in Fig. 8(a). The electrochemical response is quite good at pH ranging from 4.0 to 8.0 and the maximum current occurred at pH 7.2 after which it starts decreasing. The response time was recorded for PPy/c-MWCNT/LOD electrode. Fig. 8(b) shows a response time of 8 seconds for lactate biosensor in the presence of 1 μM of lactic acid.

3.10. Interference study

The interference study of the lactate biosensor was carried out by comparing the current response before and after adding some interferents such as citric acid, ascorbic acid, and glucose along with 1 μM of lactic acid in 0.1M PBS pH 7 at their physiological concentrations. The results showed negligible interference of these substances.

3.11. Storage stability of enzyme electrode

Fig. 9 shows the response of PPy/c-MWCNT/LOD electrodes as a function of storage time in the presence of lactate 1 μM and 5 μM in phosphate buffer (0.1 M, pH 7). PPy/c-MWCNT/LOD electrodes were tested for stability over 2 weeks. When not in use, the electrodes were stored at 4°C. It can be seen that there is an initial sharp decline in response followed by a gradual decrease. After about 10 days the sensor response is still significant and thus can be
used for lactate determination for about 15 days.

![Graph](image1.png)

Fig. 8: (a) The effect of pH on the response of the biosensor and (b) The response time of the biosensor

![Graph](image2.png)

Fig. 9: Response of PPy/c-MWCNT/LOD electrode as a function of storage

4. Conclusion

A simple, selective, and sensitive biosensor for lactate detection has been successfully fabricated. The nanobiocomposite film was electrochemically synthesized by electro oxidation of pyrrole in a phosphate buffer solution (PBS) containing appropriate amounts of LOD and functionalized multi-walled CNTs on a steel electrode to obtain PPy/c-MWCNT/LOD modified steel electrode. PPy/c-MWCNT/LOD electrode when tested for lactate, have shown detection limit of 5.61 μM/l and sensitivity of 0.0778μA/μM, response time of about 8s, shelf-life of about 2 weeks, linear response range from 5 to 60 μM. The simple fabrication method of the biosensor has many advantages such as ease of fabrication, enhanced electrocatalysis, and efficiently preserving the activity of biomolecules. This biosensor can be tested simultaneously to detect several analytes in human sweat.

References
