Polypyrrole/MWCNT nanobiocomposite based electrochemical urease biosensor

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Abstract

Fabrication of nanocomposite film of electrically conducting polypyrrole (PPy) and functionalized multi-walled carbon nanotubes (MWCNTs) on a stainless steel electrode by electro-deposition method and immobilization of urease onto the nanocomposite film to obtain a nanobiocomposite electrode as a sensitive electrochemical urease biosensor is reported. Cross-linking by glutaraldehyde (0.1%) method for the immobilization of urease (2 mg/mL) in a phosphate buffer solution of 0.1 molarity at a pH of 7.0 was used. The Characterization of the nanocomposite and nanobiocomposite film thus obtained was done by Scanning Electron Microscopy (SEM), Fourier Transform Infrared spectroscopy (FTIR), Cyclic Voltammetry (CV), and Electrochemical Impedance Spectroscopy (EIS). The increased size of the Cyclic voltammogram and shifting of anionic peaks towards the lower voltage indicates the incorporation of MWCNTs into the growing film during the electro-deposition of PPy on electrode. Reduction of the oxidation potential due to MWCNTs leads to lowering of potential for the electro-catalytic reduction of urea. The incorporation of functionalized MWCNT also made possible increased amount of enzyme concentration, an extended lifetime, long time stability and improved response times of the enzyme electrode. This modified nanobiocomposite electrode showed a good linear response to the urea concentration change in the range of 10 mM to 50 mM. The results obtained from Michaelis–Menten constant K'_m, maximum current (I_{max}), detection limit, sensitivity, response time and shelf-life of electrochemical biosensor indicating good sensing for urea detection.

Keywords: Electrochemical biosensor, conducting polymers, polypyrrole, multi-walled carbon nanotubes, urease.

Introduction

Recently research on electrochemical biosensors has increased many folds as they are proving to be a promising tool in food, environment and clinical diagnosis. Electrochemical biosensors are modified electrode devices consisting of a bio-molecule (most often an enzyme) and an electrode transducer that converts a specific biological recognition event into a quantifiable electrical signal. Construction of effective biosensors involves immobilization of the bio-molecule on a substrate while retaining its biocatalytic activity for longer time periods, as well as its affinity towards the target analyte [**1-3**]. Now a day Conducting Polymers are playing important role as a transducer for a biosensing element because of their ability to efficiently transfer the biological reaction into an electrical signal to the electronic devices and also their compatibility to provide a neutral environment for the bio-molecules. Among the various conducting polymers, Polypyrrole (PPy) is highly compatible for the association between a biological recognition element and supporting electron transfer mechanism as it gets easily deposited on electrode by electrochemical methods in neutral environment. Recently it has been found that the incorporation of multi-walled carbon nanotubes (MWCNTs) to conducting polymer matrix results in the increased charge-transfer mechanism and enlarged surface area for the catalysis of the biological reaction [**4, 5**]. Studies have demonstrated that Carbon nanotubes (CNTs) have become the best fillers for the improved polymeric nanocomposites for biosensor applications [**6, 7**]. Conducting Polymers/Carbon Nanotubes nanocomposites are the most sought out materials as a biosensor, not only because the CNTs improves the strength and electrically conductive properties of original polymer matrix, but also the nanocomposites possessed properties of each of the individual element with a collaborative effect of both the components [**8**]. Han *et al.* reported electrochemical synthesis of PPy-MWCNT composite films [**9**]. This high conductive property of Polymer/CNT nanocomposites has given rise to new openings for chemical biosensors [**10**]. PPy-MWCNT composites have opened new opportunities due to the CNTs becoming a potential candidate for wiring material

[**11**]. Wang *et al.* reported a sensitive PPy/Glucose oxidase films for glucose detection [**12**]. Korkut *et al.* reported the working electrode constructed in one-step by the electro polymerization process of multi-walled CNT and pyrrole for the bio analytical applications [**13**]. Porras-Gutiérrez *et al.* reported the potentiodynamic electro polymerization of PPy films by cyclic voltammetry, incorporating in the polymeric matrix, MWCNTs to prepare composite electrode materials [**14**]. Determination of NO using CuZnSOD immobilized on carbon nanotubes (CNTs) in polypyrrole (PPy) matrix was reported [**15**]. Tam and co-worker investigated CNT/PPy/goat IgGs film synthesis using one step immobilization process [**16**]**.** Hieu and co-worker presented the sensing properties of the PPy/SWCNTs (single walled CNTs) nanocomposite thin film, toward low concentration of NH_3 (Ammonia). [**17**]. Lata and Pundir used a new hybrid material consisting of polypyrrole, nickel hexacyanoferrate and carboxylated multiwalled carbon naotubes, for better analytic performance of the biosensor [**18**]. Manisankar and co-worker prepared PPY/MWCNT /GCE (Glassy Carbon Electrode) electrode to investigate electrochemical behavior of three pesticides. Based on this, a convenient, simple, fast and accurate procedure for the determination soil pollutants was determined with good results [**19**]. Valentini and co-worker reported Electrophoresis Deposition Process technique with the electrochemical one-step deposition of polypyrrole-Glucose Oxidase on the SWCNT/Au microelectrodes [**20**].

Urea biosensing involves hydrolysis of this compound in the presence of urease enzyme as catalysts. The urea analysis is of significant importance in clinical analysis, in food industry and in agriculture pollutants. Excess urea concentration, than its permissible range causes dysfunction of the kidney [**21**]. Adultery in food industry, especially in dairy products, study of urea concentration becomes of utmost importance [**22**]. Also, it is widely known that urea act as a pollutant due to the fertilizers used excessively in agricultural land. Guilbault *et al.* were the first to develop a urea biosensor [**23, 24**]. To construct a urea biosensor, urease is immobilized on a substrate. Urease enzyme catalyzes the urea into ammonium and bicarbonate ions based on enzyme substrate reaction which is then detected by the transducer for the further analysis. A large number of urease biosensor has been developed till date but only few are available based on PPy/CNTs composite. Ivanova *et al.* reported amperometric urea biosensor based on nanostructured polypyrrole. They found that the sensitivity of biosensors was increased with CNT and PPy [**25**]. In the previous studies done on biosensors based on CNT/PPy modified electrodes, the CNT was physically entrapped within the growing film by using some mediator which can alter the environment for the biomolecule to be immobilized. However, in this case the PPy/MWCNT films modified steel electrode have been formed by using a neutral solution of PBS and hence the possibility of any change in the biological catalytic activity is reduced which increases the selectivity of the biosensor. Cyclic Voltammogram indicates the incorporation of MWCNT within the growing polymeric film such that an anion is attached as dopant to PPy. Such a PPy/MWCNT film does not compromise with the electro-catalytic activity of CNT and facilitates a highly sensitive biosensing of urease, and this presents a simplest and effective method for preparing biosensor electrode.

Facile synthesis of a urease biosensor, PPy/c-MWCNT/Urease modified steel electrode by electro-deposition method for urea detection have been reported here in this paper. Most of previous studies involve mediators or chemical treatment with modified electrode which can denature the enzyme and also the possibility of arising limitations from employment of mediators. In this study no mediator has been employed and the urea hydrolysis is possible under a very low potential. The urease biosensor thus obtained has been tested analytically for the range, sensitivity, response time, shelf life and stability.

Experimental

Materials / chemicals details

Pyrrole with 98% purity was procured from Sigma-Aldrich and purified by distillation and stored below 5^{o}C for further use. Phosphate buffer solution (PBS) was also obtained from Sigma-Aldrich and used as it was received. Urease was procured from Fluka. The required solutions were prepared with the use of de-ionized water.

Characterizations

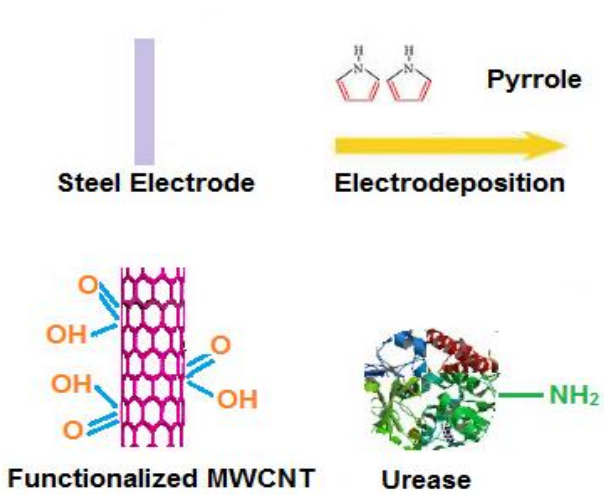
Electrochemical Analyzer (CHI600D) was used for Cyclic Voltammetry (CV). A 1000 Gamry model interface Potentiostat with a three electrodes system has been used for the measurement of Electrochemical Impedance Spectroscopy (EIS). In the three electrodes cell, A thin wire of platinum was utilized as the counter electrode, steel foil of dimension 5cm × 0.5cm × 0.1cm was used as working electrode, and for the reference electrode Ag/AgCl (3 M/saturated KCl) was used. Scanning electron microscope (JEOL JSM-6360) was used for surface morphology of PPy/CNTs nanocomposite and the urease immobilized PPy/CNT nanobiocomposite. Fourier transform infrared spectrometer (Bruker Alpha) study was done to identify the presence of characteristic functional groups in PPy/CNTs.

Synthesis of PPy and PPy/c-MWCNT nano-composites

PPy was electrochemically synthesized by using well-polished and ultrasonicated stainless steel foil as a working electrode. The electrochemical synthesis was performed in an aqueous solution of 0.5 M pyrrole in 0.1M phosphate buffer solution (PBS) of pH 7 at 27°C with a potential window -1.6 to +0.8V and scan rate 50 mV/s. Repeated cycling of the potential resulted in continuous deposition of PPy film onto the electrode surface. PPy film coated working electrode was dried under vacuum. MWCNTs were functionalized to attach the carboxylic (-COOH) and the hydroxyl (-OH) groups on CNTs by a chemical reaction in the presence of nitric acid (HNO_3) and sulfuric acid (H_2SO_4) [**26**]. 5wt% functionalized carbon nanotubes (c-MWCNTs) were dispersed in 15 ml 0.1M PBS buffer and sonicated for 1 hour. 0.5 M pyrrole was mixed in above solution and stirred for 15 minutes to obtain the nanocomposite film of PPy/c-MWCNT. The solution was then electrolyzed in continuous stirring condition for 10 cycles by applying the sweep potential in between the range from −1.2 to +1.0 V against Ag/AgCl and at a scan rate 50mV/s.

Urease immobilization on PPy/c-MWCNT nano-composites

Functionalized MWCNTs were ultrasonicated in de-ionized water for 1 hour and then 2 mg/mL urease mixed in a phosphate buffer solution of 0.1 M and pH 7 was added to ultrsonicated solution. 0.5M Pyrrole was added in above solution and stirred for 15 minutes. The solution was electrolyzed for 10 cycles by keeping the sweep potential in the range from −1.6 to +1.2 V maintaining the scan rate at 50 mV/s. A film of PPy/c-MWCNT/Urease is obtained on the steel electrode which was used as biosensor. The schematic of construction of PPy/c-MWCNT/urease nanobiocomposite on steel electrode for use as a biosensor is shown in **Scheme 1**.



Scheme 1. Preparation of PPy/c-MWCNT/urease nanobio-composite electrode.

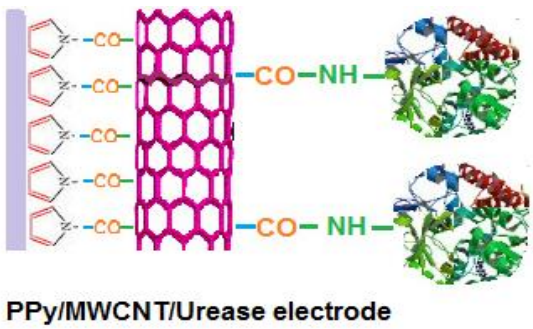
Electrochemical measurements of PPy/c-MWCNT / Urease electrode

Cyclic Voltammetry of PPy, PPy/c-MWCNT nanocomposite and PPy / c-MWCNT / Urease nanobiocomposite electrodes was performed by using 0.1M PBS solution at a pH of 7 by applying potential +1.2 V to -1.2 V at scan rate 50mV/s with respect to Ag/AgCl electrode for NH_4^+ (Ammonium) ion detection. Electrochemical Impedance Spectroscopy (EIS) was performed by applying potential of +0.01 V versus Ag/AgCl, the signal amplitude was kept at 5 mV and frequency range was maintained at 100 kHz–0.01 Hz. The potentio-dynamic response of the constructed biosensor electrode PPy/c-MWCNT/Urease, were studied for urea by taking the solution of urea in PBS. The response was measured by using Cyclic Voltammeter with an electrochemical cell of 20 mL capacity containing a 0.1 M PBS having pH 7 by using a potential selected between the range from +1.2 V to -1.2 V and maintaining at a scan rate of 50mV/s in the absence and presence of urea in PBS solution. Responses were recorded between working electrode and the reference electrode with the addition of 0.1ml of 10 mM urea in successive steps. Urease hydrolyzes urea to NH_4^+ ions and hence this brings a change in the pH of the solution that can be detected as a change in current at the electrode. Lineweaver–Burk plot was used for the calculation of K'_m and I_{max} value for urea.

Results and discussion

Morphology

SEM images of PPy, PPy/c-MWCNT, and PPy/c-MWCNT/Urease are shown in **Fig. 1**. High porosity of pure PPy coated uniformly on the steel electrode is observed from **Fig. 1(a)**. This highly porous surface morphology of PPy and availability of larger surface area provides an enlarged possibility for the entrapment of more MWCNTs and the enzyme immobilization at the surface of electrode.



It is clearly seen from **Fig. 1(b)** that the surface morphology of nanocomposite film is much uniform and homogeneous. The PPy enwraps the carbon nanotubes which forms the surfaces of the nanocomposite film. Nano-sized MWCNTs supports for the formation of a very thin, porous and homogeneous film of PPy on the electrode surface [**27, 28**]. In **Fig. 1(c)** the change in the surface morphology and a bright spot of globe like structure on the film can be observed. From this immobilization of urease is confirmed on PPy/c-MWCNT nanocomposite film.

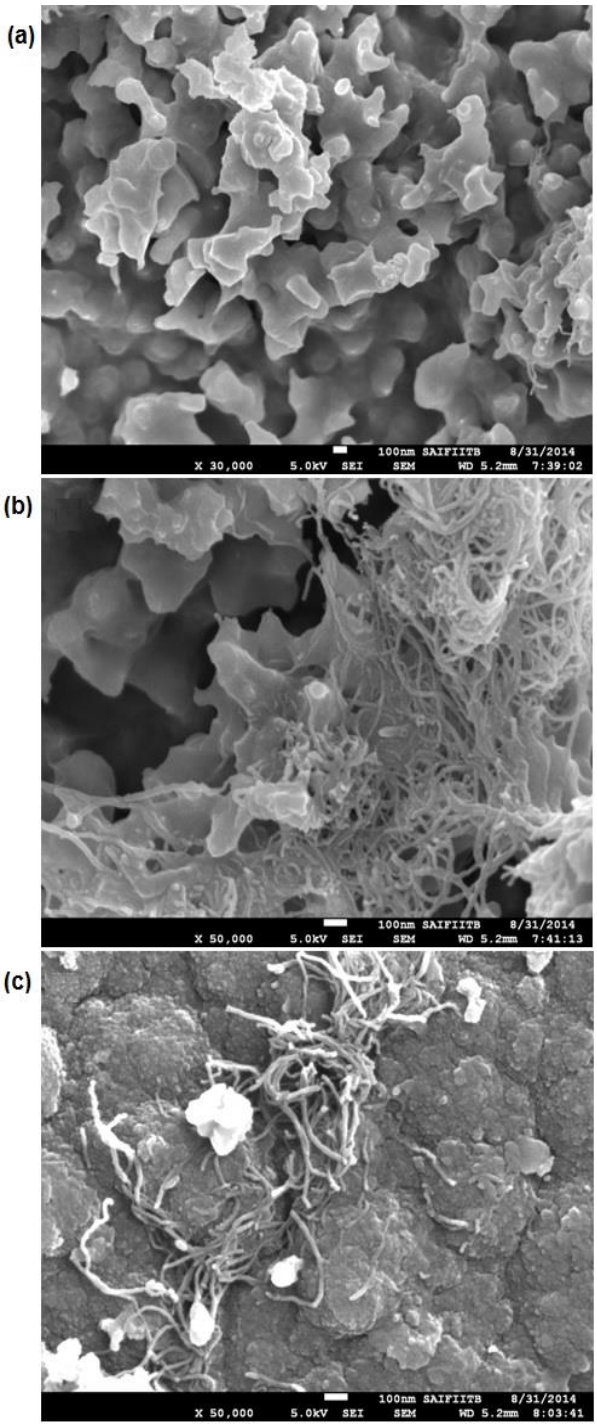


Fig. 1. SEM images of (a) PPy, (b) PPy/c-MWCNT, and (c) PPy/c-MWCNT/Urease.

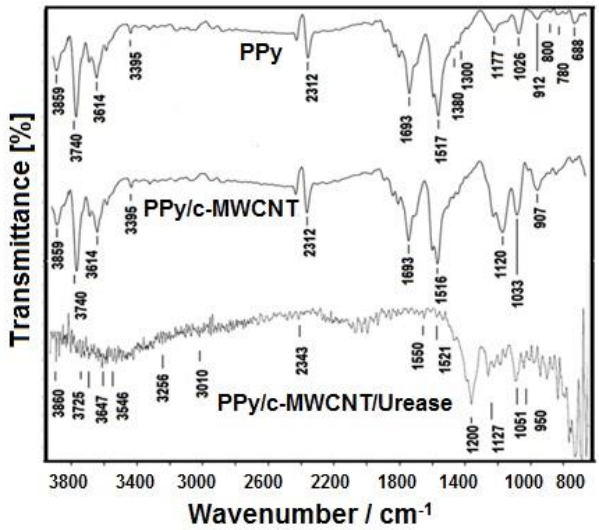


Fig. 2. FTIR Spectra of PPy, PPy/c-MWCNT, and PPy/c-MWCNT/Urease.

FT-IR spectroscopy

FTIR spectra recorded for PPy/c-MWCNT and PPy/c- MWCNT/Urease were compared to that of spectra recorded for pure PPy. As can be seen from **Fig. 2**, for pure PPy the characteristic bands at 800, 912 , 1026 and 1177 cm^{-1} are due to N-H bending, C-H bending, C=C stretching, respectively. Also C-N stretching at 1300 cm^{-1}, C-C vibration at 1380 cm^{-1}, N-H bending in secondary amines at 1517 cm^{-1} and C = N stretching at 1693 cm^{-1} of PPy are prominently observed [**29**]. There are clear differences between spectrum of PPy/c-MWCNT and the spectrum of PPy. The N-H stretching region near 3740 cm^{-1} showed broad peak. The bands have been shifted towards lower frequencies in the nanocomposites spectra which suggest that an interaction has been occurred between MWCNTs and PPy. This confirms the formation of PPy/c-MWCNT nanocomposite [**30-32**]. It is observed from spectra of PPy/c-MWCNT/Urease nanobiocomposite that characteristics peaks appear almost at the same region in except few peaks of the urease enzyme.

Electrochemical characterization

Cyclic Voltammetric study was performed on Electrochemical Analyzer. The cyclic voltammogram of PPy/c-MWCNT and PPy/c-MWCNT/Urease are compared to that of pure PPy. It can be seen in **Fig. 3**, that the oxidation peak observed for pure PPy are at 0.7973V is related to the conjugation length of the polymer. The pyrrole monomer gets oxidized at 0.7973V potential as the anodic current rises at this range of potential which then shifts toward more positive potentials indicating the deposition of a polymer film on the surface of electrode [**33**]. For PPy/c-MWCNT, the oxidation peak is observed at the potential of 0.5197V. On comparison, it was

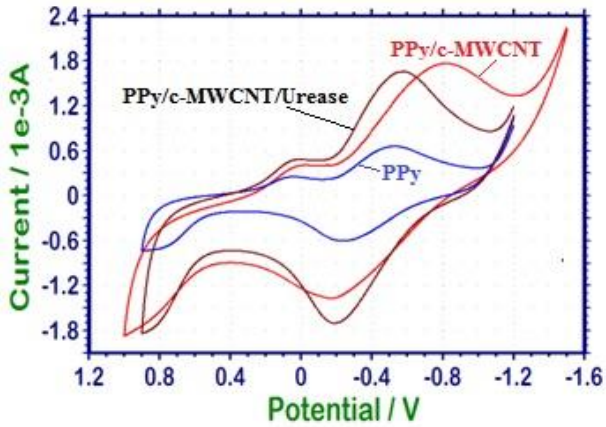


Fig. 3. Cyclic voltammograms of PPy, PPy/c-MWCNT, and PPy/c-MWCNT/Urease in 0.1M PBS (pH 7).

found that the oxidation peak obtained for nanocomposite is shifted towards the lower potential as compared to PPy (0.7973V). The shift of oxidation peak towards lower potential suggests that the conjugation length of composite is higher than the individual PPy. The increase in conjugation length results the higher value of electrical conductivity [**34, 35**]. In case of PPy/c-MWCNT/urease nanobiocomposite, only one oxidation peak is obtained at voltage 0.7516V. The oxidation peak corresponding to PPy/c-MWCNTs/Urease nanobiocomposite is found to shift towards the lower potential as compared to PPy (0.7973V) and higher as compare to composite of PPy/c-MWCNTs (0.5997V). The shift of oxidation peak towards higher potential suggests that the conjugation length of nanobiocomposite of PPy/c-MWCNTS/Urease is higher than the individual PPy and lower than the composite of PPy/c-MWCNTs. The decrease in conjugation length results the lower value of electrical conductivity of PPy/c-MWCNT/Urease nanobiocomposite as compare PPy/c-MWCNTs nanocomposite. [**36-38**]. In case of PPy/c-MWCNT, the increased in anodic current from 0.7 to 1.7 mA as compared to that of pure PPy is due to enlarged surface area of electrode after the c-MWCNTs incorporated in it. The good catalytic activity of enzyme urease is seen from the significant increase in the current responses of the PPy/c-MWCNT/Urease biosensor.

Electrochemical Impedance Spectroscopy (EIS)

The electronic and charge-transfer control properties of PPy, PPy/c-MWCNT and PPy/c-MWCNT/Urease films are compared with the help of EIS plots. The Nyquist plots shows the imaginary (Z´´) versus the real (Z´) part of the impedance of the circuit. The resistance of circuit (R_{CT}) values for bare steel electrode (R_{CT} 290 Ω), PPy (R_{CT} 210 Ω), PPy/c-MWCNT film (R_{CT} 150 Ω) are shown in **Fig. 4.** The impedance of PPy/c-MWCNT electrode was found to be much smaller than that of bare steel electrode and pure PPy electrode. This can be associated with the enlarged surface area and also with the conducting nature of CNTs [**39**]. But for the PPy/c-MWCNT/Urease electrode, the impedance was found to be comparatively more than that of PPy/c-MWCNT electrode due to poor electrical conduction of molecules of enzymes at low frequencies [**40-42**].

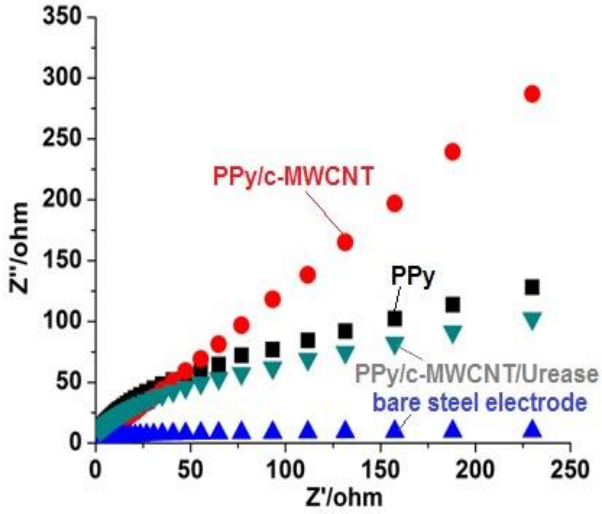


Fig. 4. Impedance spectra of modified electrodes.

Biosensor responses

PPy/c-MWCNT/Urease electrode is used as urease biosensor for different concentrations of Urea. From potentiodynamic response of the biosensor in 0.1M PBS at 50 mVs^{-1} scan rate for the different concentrations of Urea (mM) as shown in **Fig. 5**, the response of the biosensor was observed to be maximum at 0.12V. The working potential in the biosensor has been lowered. This shows the possibility of the role played by synergistic action of c-MWCNT and PPy which provides enhanced electro-catalytic effect for urea detection and improved rate of electron-transfer. This suggests the presence of c-MWCNTs in the nanocomposite have great promise for urease based biosensors.

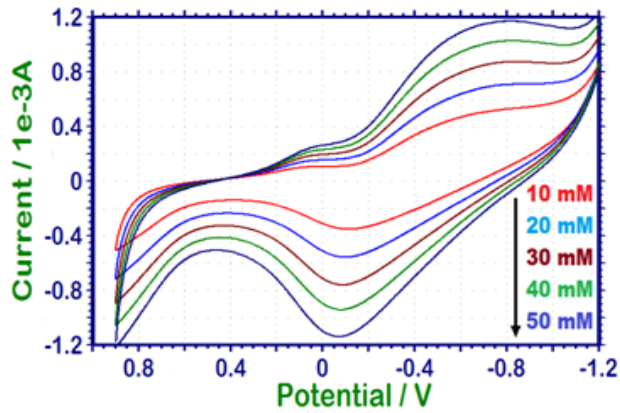


Fig. 5. Potentiodynamic response of PPy/c-MWCNT/Urease electrode for different concentrations of Urea.

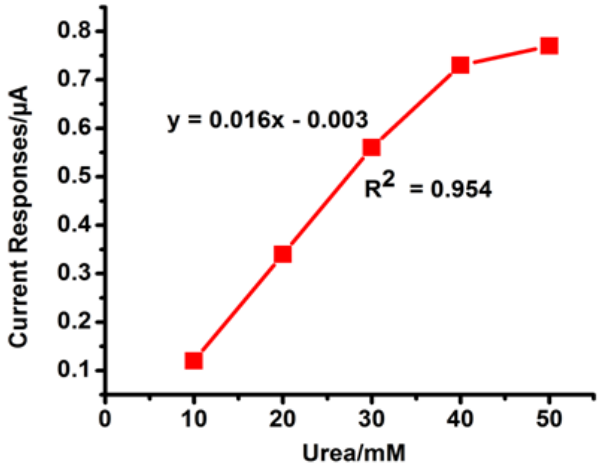


Fig. 6. Calibration curve of PPy/c-MWCNT/Urease biosensor.

Calibration curve

From the curve, for calibration of PPy/MWCNT/urease biosensor as shown in **Fig. 6**, it was found that the response current increased linearly with the increase in concentration of urea. The enzymatic reaction taking place produces ammonium ions which increase the response current. PPy/MWCNT/urease biosensor has exhibited an excellent response for urea. The detection limit and sensitivity of the biosensor were found to be 0.43 mM/l and 0.0235µA/mM, respectively. I (µA) = 0.003+ 0.016 [urea] (mM) is the linear regression equation obtained from calibration curve of biosensor and the value of correlation coefficient (R^2) is 0.954 which confirms the linearity of the curve. Lineweaver–Burk plot as shown in **Fig. 7** is used to study the urea concentration effect on the response of PPy/c-MWCNT/Urease electrode**.** The lower value obtained for Michaelis–Menten constant (K'_m) = 0.33 mM from the plot indicates the higher affinity of enzyme towards urea after immobilization due to enhanced diffusion of urease. [**43**].

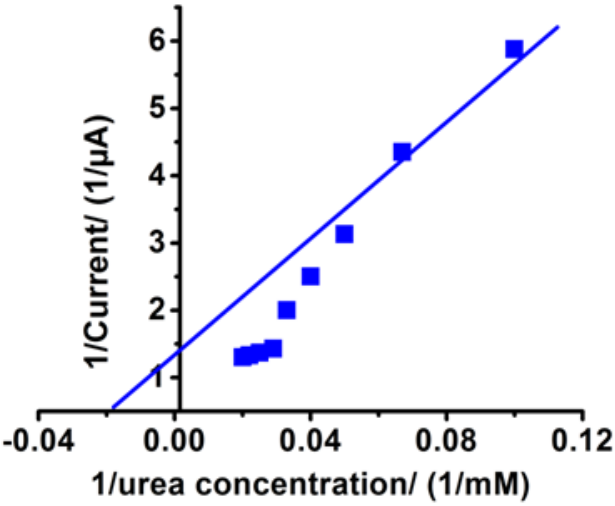


Fig. 7. Lineweaver–Burk plot for PPy/c-MWCNT/Urease biosensor.

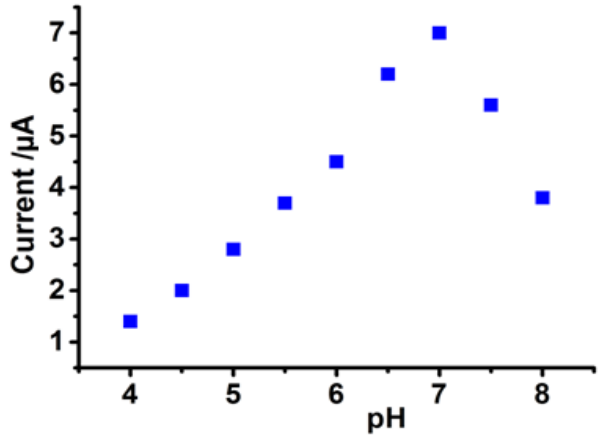


Fig. 8. Response of biosensor for change in pH.

Effect of pH on Urease Biosensor

Optimum pH values were determined for enzyme activity since pH changes in the medium cause denaturation of enzyme. The variation in responses of PPy/c-MWCNT/Urease biosensor for change in pH of 0.1M PBS solution with 10 mM urea is shown in **Fig. 8**. The maximum steady state current at potential +1.2 V to -1.2 as a function of pH was found to be at pH = 7.

Response time and stability

PPy/c-MWCNT/Urease biosensor was found to be highly sensitive for urea. As can be seen from **Fig. 9**, the biosensor was found to have the response time of about 7 seconds for 20 mM. The shelf life of the PPy/c-MWCNT/Urease biosensor was also studied. It is clearly seen from the **Fig. 10** that in the first few days electrode showed a maximum response and then after nearly tenth day the biosensor response slowly starts decreasing. The decrease in the response after first few days may be due to the slow detachment of the enzyme from the biosensor surface. After the tenth day a gradual decrease in biosensor response is observed. The biosensor response is still significant up to fifteenth day indicating the suitability of the biosensor for urea detection up to 15 days.

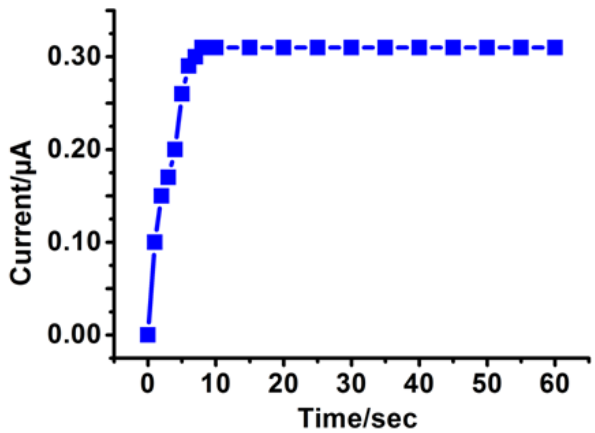


Fig. 9. Response of PPy/c-MWCNT/Urease biosensor for urea.

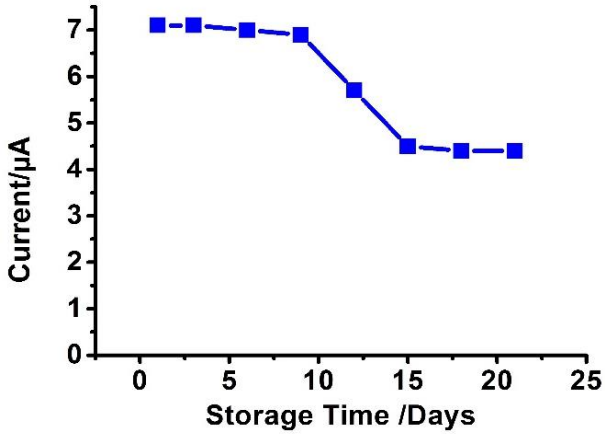


Fig. 10. Stability of PPy/c-MWCNT/Urease biosensor.

Conclusion

Successful fabrication of a urease biosensor PPy/c-MWCNT/Urease nanobiocomposite film was carried out by electrochemical method on stainless steel electrode. Other than being cost effective this facile electrochemical method of fabrication of urease biosensor provided an easy method of entrapping of enzyme molecules. PPy/c-MWCNT/Urease biosensor exhibited an excellent response for urea. The lowered value of Michaelis–Menten constant obtained for this urease biosensor indicates improved response of the modified electrode to enzyme. The greater sensitivity of PPy/c-MWCNT/Urease biosensor was due to conductive path provided by MWCNTs. Also for this urease biosensor response time was found to be very fast with the storage stability of about 20 days.

Acknowledgements

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Author's contributions

Conceived the plan: SBK; Performed the expeirments: BHM; Data analysis: SBK, BHM; Wrote the paper: BHM, SBK. Authors have no competing financial interests.

Supporting information

Supporting informations are available from VBRI Press.

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