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HIGHLY SENSITIVE AND RAPID DETECTION OF PUTRESCINE BY USING AN PLATINUM ELECTRODE MODIFIED WITH ZINCOXIDE NANOPARTICLES-POLYPYRROLE COMPOSITE

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Keywords: *Diamine oxidase, putrescine, putrescine, polypyrrole 3-carboxylic acid and zinc oxide nanoparticles.*

Abstract

A highly selective, sensitive, specific and rapid electrochemical putrescine biosensor was constructed by covalently immobilizing diamine oxidase purified from *Pisum sativum* leaves on polypyrrole 3-carboxylic acid (PPy-3C) with zinc oxide nanoparticles (ZnONps) using N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) modified platinum electrode.Transmission electron microscopy (TEM), X-ray diffraction (XRD), UV spectroscopy and Dynamic light scattering (DLS) were used for characterization of ZnONps. The modified enzyme electrode was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS).The experimental variables that can affect the putrescine amperometric response, such as the applied potential, pH and temperature have been optimized to perform a selective determination of putrescine.The optimized putrescine biosensor showed linear response range of 0.1–750µM and rapid response within 4s at a potential of 0.4V vs Ag/AgCl. The sensitivity was approximately 40µA/mM/cm^2 with a detection limit of 0.1µM. Proposed putrescine biosensor was successfully applied to cancer patients urine sample, which correlated well with a standard enzymic colorimetric method (r = 0.98).

Introduction

Polyamines such as putrescine (Put.), spermidine and spermine are biologically active amines, which are essential for many cellular processes such as cell growth, cell proliferation and cell differentiation malignant and normal cells (Hoet et al., 1996). Concentrations of these compounds in physiological fluids are low or undetectable under normal conditions, elevated in patients with metastatic cancer, and are thought to reflect growth (Put. concentrations) and cell turnover (spermidine concentrations) of the organism. Cancers are a broad spectrum of diseases in which there are altered growth fractions and cell-turnover fractions, and therefore cancer chemotherapeutic agents have been developed to take tumor kinetics into account. Because changes in polyamines in physiological fluids reflect cell kinetics compiles evidence of their efficacy as biochemical markers of cancer and suggests their possible usefulness to clinicians in rapidly assessing tumor response to chemotherapy or to multimodality therapy. Abnormally high levels of Put. have been also associated with Menky's Kinky hair disease, a heritable copper deficiency disorder (Rennert et al.,1980). Put., when present at high concentrations in the mammalian brain, has an influence in modulating. Put. levels in plasma of the renal failure patients have been shown to be elevated (Sakata et al.,2003), since Put. plays a very important role in the metabolism of various biological tissues, its detection even at very low concentrations is critical. A number of studies have indicated that the concentrations of polyamines in urine or serum samples of almost all cancer patients are higher than those of healthy humans (Russell, 1971, Lee et al., 1997). Even though polyamines have some limitations as markers for malignant tumors (Seiler et al., 1981, Hiramatsu et al., 1995), they are now considered to be suitable tumor markers in humans (Bachrach, 1992). The clinical importance of rapid assessment of tumor kinetics in relation to treatment and the fact that changes in the

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concentrations of Put. in body fluids appear to accurately reflect growth and cell loss point to the importance of developing rapid, sensitive tests for the polyamines. Lipton et al., (1975) found increase of polyamines in body fluids to be highest in the early stages of tumor growth. Because growth and tumor cell loss are probably greatest during tumorigenesis, there is always the possibility that a rapid assay procedure could at least be used to screen that population.

Various methods are accessible for determination of Put. in biological samples. In addition, many improved techniques and separations based on high-performance liquid chromatography (HPLC) (Hunter et al., 1990), HPLC mass spectrometry (Palma et al., 1997), gas chromatography-mass spectrometry (Khuhawar and Qureshi, 2001) and immunoassay (Fujawara et al., 2001) have been developed. However, many of these methods involve the use of sophisticated equipment and are complicated. On the other hand, a few enzymatic methods have been developed for the assay of polyamines in urine, but they are rarely applied to blood samples because of requirement for much larger sample volumes and cumbersome procedures (Isobe et al.,1987). Most of these methods are precise and suitable for many applications, however, they do not satisfy the requirements for a simple, fast, accurate and specific analysis; as these are complicated, require time-consuming sample pre-treatment, expensive instrumental set-up and skilled person to operate. Enzyme based nanobiosensor are considered a viable alternative to the earlier methods for polyamines determination for on-site analysis. Besides being specific and sensitive, they are portable, less expensive and do not require tedious sample pretreatment.

Various electrochemical biosensors have been reported for put analysis (Bóka et al., 2012, Keow et al., 2012, Hernandez-Cazares et al., 2012, Shanmugam et al., 2011, Fusco et al., 2011, Alonso-Lomillo et al., 2010, Piermarini et al., 2010, Keow et al., 2010, Carelli et al., 2007, Compagnone et al., 2001, Carsol & Mascini, 1999, Estiet et al.,1998, Bouvrette et al., 1997). However, these biosensors also had some problems like low stability, low sensitivity, reproducibility and slow electron transfer,which is still to be improved. This problem of low stability was overcome by immobilizing the diamine oxidase (Dox) covalently onto the platinum electrode by watersoluble coupling agents N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) on activation of 3-carboxylated group of polypyrrole, Pyrrole is conducting polymer (CP) and after eletropolymerzation its have a good film formation abilty provide good environmental stability and biocompatibility support for immobilization of enzyme, while other problems can be overcome by using nanomaterials, In this context, different types of nanoparticles such as gold (AuNPs), Iron-oxide (Fe_3O_4-NPs) (Devi et al., 2013), have been suggested as promising matrices for enzyme immobilization to improve stability and sensitivity of biosensor. Among various metal oxide nanoparticles, ZnO nanoparticles have been considered as interesting for immobilization of desired biomolecules due to biocompatibility, high isoelectric point and non toxicity. A very important property of this type of nanoparticles for electrochemical biosensors is their ability to provide a favourable microenvironment for biomolecules to exchange electrons directly with an electrode, thus improving the sensitivity of electrochemical biosensors.

The present report describes the construction and application of a 3-carboxylated polypyrrole / PPy-3C) with zinc oxide nanoparticles (ZnONps) nanocomposite based Put. biosensor which provides covalent linkage between DOx and electropolymerized PPy-3C/ZnONps composite film on Pt electrode using standard watersoluble coupling agents N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) with an overall aim of improving the put biosensor performance.

Metarials and Methods

Sources of chemicals

Spermine, spermidine, histamine, Put., pyrrole 3-carboxylic acid, zinc nitrate ($Zn(NO_3)_2.4H_2O$), Sephacryl S-100, DEAE-Sep 4-aminophenazone, Horse radish peroxidase (RZ 3.0), succinic acid, phenol, sodium dodecyl sulphate (SDS), ammonium sulphate (enzyme grade), sodium hydroxide, copper sulphate, ammonium persulphate, N-ethyl-

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N'-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Folin Ciocalteau's (FC) reagent, potassium hydrogen phosphate, dipotassium hydrogen phosphate, sodium hypochlorite were purchased from Sigma Aldrich, St. Louis USA. Pyrrole was stored below 5ºC in dark Pvt. Ltd, Mumbai. All other chemicals were of analytical reagent (AR) grade.

Preparation of zinc oxide nanoparticles (ZnONps)

ZnONps were prepared as described (Devi et al., 2011) using a 0.45M aqueous solution of zinc nitrate ($Zn(NO_3)_2 \cdot 4H_2O$) and 0.9M aqueous solution of sodium hydroxide (NaOH). NaOH (100 ml) solution was heated at 55ºC. Zinc nitrate solution (50 ml) solution was added drop wise (slowly for 40 min) to the heated NaOH solution under high speed stirring. The beaker was sealed during this process for 2 h. The precipitated ZnONps were cleaned with deionized water and ethanol, dried in air at 60◦C. The morphology, particle size and structure of the ZnONps were determined by a XRD, TEM, DLS and UV and visible spectra in a UV–vis spectrophotometer and diffraction light scattering.

Deposition of ZnONps on Pt electrode

The surface of the Pt electrode (2.15cm×1mm) was manually polished successfully by 0.05M of alumina slurry on a polishing cloth, followed by a thorough distilled water rinsing. The electrode was then sonicated in ethanol and distilled water to remove the adsorbed particles. To electropolymerized 0.1M pyrrole-3-carboxylic acid (4 ml), 50 mg ZnONps were added in 100 mm $NaClO_4$ (10 ml) and 0.1 M phosphate buffer (pH 7.0). The electropolymerization of polypyrrole (PPy) was carried out by applying potential -0.1 to 0.9 Pt working electrode at100 mV/s. For control, a pure pyrrole-3-carboxylic acid was also polymerized under the similar conditions as mentioned above, but no ZnONps were added in the polymerization electrolyte. The electropolymerization of pyrrole-3-carboxylic acid was studied by SEM, FTIR and EIS.

Immobilization of Dox onto ZnONps /PPy-3C/Pt electrode.

The Scheme 1 summarizes reaction involved mechanism of the Put., biosensor based on immobilization of Dox onto ZnONps/PPy-3C/Pt electrode.The procedure for covalent immobilization of Dox was performed after the electropolymerization of PPy-3C film with ZnoNps. Carboxylic groups on the surface of the ZnONps/PPy-3C/Pt electrode were activated with 1:1 activator solutions, 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) in deionized water and then ZnONps/PPy-3C/Pt electrode was dipped into a 10µl solution of DOx (0.1U) and kept undisturbed for about 12 h at 4◦C. Finally, the electrode was immersed into 0.05M sodium phosphate buffer (PBS) (pH 7.0) to wash out any unbound enzyme from the electrode, surface. To measure activity of enzyme electrode a solution of Put. (0.1 ml) was added to 3ml PBS (pH 7.0). Dox/ZnONps// PPy-3C/Pt electrode was immersed into this solution and incubated for 5 min at 25ºC. The electrode was taken out and the absorbance of the solution was taken at 520 nm.

Electrochemical measurement/ Construction and testing of enzyme electrode

Electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the Dox/ZnONPs/PPy-3C/ Pt electrode as the working electrode, a Pt wire as the auxiliary electrode and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode.The electrode system was pre-equilibrated at +0.4 V before each detection run and steady current readings were obtained at +0.4 V (vs. Ag/AgCl). After 100 µL of an put (0.05 M) solution was injected into the cell containing 20 mL electrolyte [2.5 mM $K_3Fe(CN)_6$–$K_4Fe(CN)_6$ (1 : 1)], a low voltage of +0.4 V was applied. All electroanalytical measurements were carried out at room temperature.The detection of Put. are based on the following enzyme reactions:

Putrescine + O_2+ H_2O Dox 4-aminobutanal + H_2O_2 +NH_3

H_2O_2 0.4V $2H^+ + O_2 + 2e^-$

2e- through ZnO-NPs/ PPy-3C Pt electrode

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The current generated by the oxidation of hydrogen peroxide, is linearly related to Put. is converted to 4-aminobutanal enzymatically using the enzyme Dox; the oxidation current is in turn proportional to Put.concentration.

Optimization of enzyme electrode / biosensor

Optimization of Put. measurements with the Dox/ZnONPs/PPy-3C/ Pt electrode was accomplished by testing sodium phosphate buffers at different pH 6.0–8.0 at an interval of 0.5 at a concentration of 0.1 M. To determine the optimum temperature, the reaction mixture was incubated at every 5°C interval between 25°C to 55°C. To study the effect of substrate concentration, different Put. concentrations were tested from 0.1µM to 1200µM by appling potential +0.4 V vs. Ag/AgCl reference. The amperometric response was also measured in the presence of interfering species viz. spermine, spermidine, histamine, ascorbic acid, uric acid, urea, bilurubin, glucose and triglyceride.

Urinary putrescine determination by putrescine biosensor

First morning urine samples from apparently healthy individual and cancer patent of different age groups and sex from civil hospital panchkula were collected in tubes and stored at 4°C until use and then maintained at pH 6.0 after addition of drop by drop NaoH (0.1M). To determine urinary Put., the same procedure was used, as described above for testing of biosensor under its optimal working conditions except that the partition was replaced by urine sample. The concentration of Put. in urine was extrapolated from a standard curve of partition between Put. concentration and current (mA) prepared under optimal assay conditions (Fig. 1).

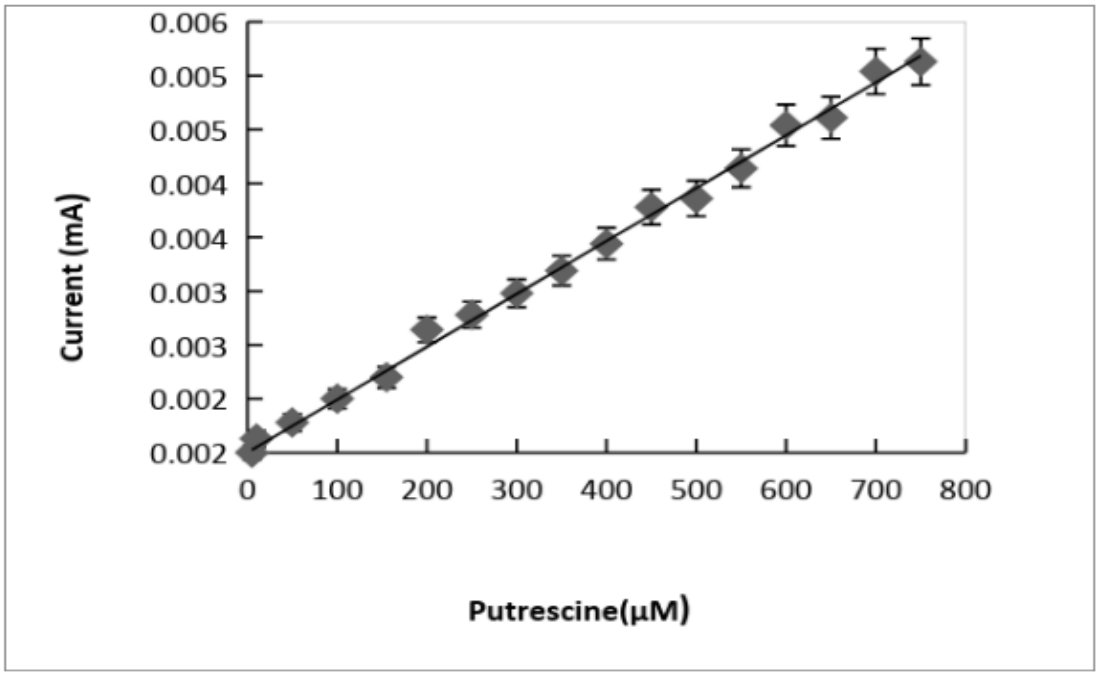

Fig.1 Calibration curve of putrescine by putrescine biosensor based composite film electrodeposited on Pt electrode.

Stability and reproducibility of the biosensor

To reuse the working electrode, it was washed by dipping it in 5 mL of 0.1 M phosphate buffer pH 7.0. The long-term storage and stability of the biosensor was investigated over a 3-month time period, during which the Dox/ZnONps/PPy-3C/ Pt electrode was stored dry in a refrigerator at 4 °C. The activity of the enzyme electrode was measured once a week

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Results and discussion

3.1. Characterization of ZnONps and ZnONps /PPy-3C nanocomposite

The characterization of ZnONps was carried out by DLS, XRD and TEM. The structure of ZnONps was also characterized by XRD (Fig. 2A). All peaks were consistent with the peaks of ZnONps with high crystallinity. The X-ray diffraction data were recorded using Cu Ka radiation (1.5406A). The intensity data were collected over a 2q range of 25–75. A definite line broadening of the diffraction peaks is an indication that the synthesized materials are in the nanometre range. The grain size was found to be in the range of 10–43 nm depending on the growth condition. TEM measurements were used to determine the size, shape, and morphological study of ZnONps. A TEM micrograph (Fig. 2B) showed well distribution of spherical ZnONps, with average height of particles is 44.26 nm. ZnONps were analyzed by DLS using particle size analyzer (Fig. 2C).Histogram shows particle size ranges from 10 to 100nm and possess an average size of 43.67 nm. The Polydispersityindex (PDI) of 0.275 shows high monodispersity of the particle. Zeta potential of ZnONps was -40.7mV with conductivity of 1.15Ms/cm(Fig. 2C). The characterization of ZnONps /PPy-3C was carried out by UV spectrophotometric spectra. Fig. 3 shows UV–visible light absorption pattern of ZnONps (Fig. 3a), PPy-3C(Fig. 3b), and ZnONps /PPy-3C(Fig. 3c), nanocomposite monitored in the range of 200–1000 nm. ZnONps shows transition in the region of 300-400 nm and in this case maximum absorbance were observed at 388 nm and PPy-3C showed maximum absorbance at 562. In case of ZnONps/PPy-3C solution appearance of the characteristic absorbance maxima at 378 and 572 nm, indicated the successful preparation of ZnONps /PPy-3C nanocomposites.

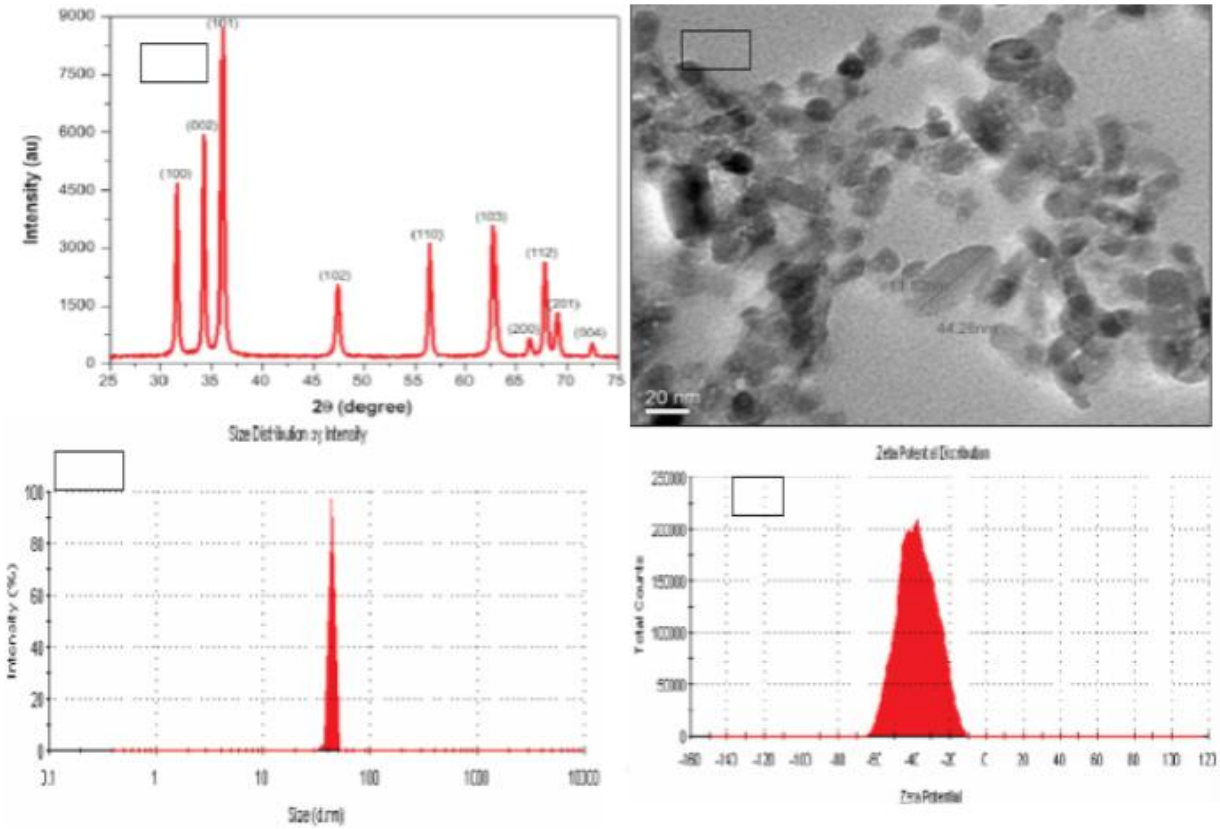

Fig.2 X-ray diffraction (XRD) pattern of ZnoNps(A) TEM image of ZnoNps (B) Size study (C) Zeta potential study of ZnoNps by DLS.

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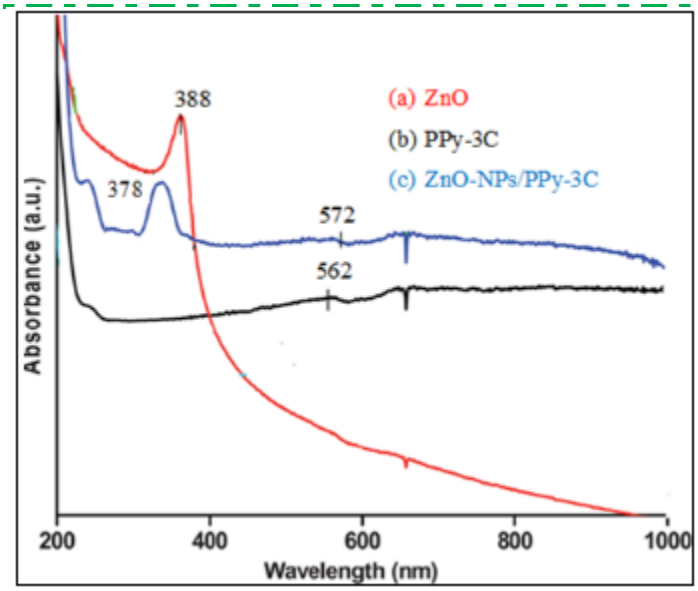

Fig.3 UV spectrophphometry of ZnoNPs (a)PPy-3C(b) and ZnONPs/PPy-3C nanocomposite on Pt electrode.

Surface characterization by SEM

The surface morphologies of ZnONps, ZnONps/PPy-3C and Dox/ZnONps/PPy-3C on Pt electrode were studied by SEM (Fig. 3). The SEM images for ZnONps (Fig. 4A) show a different granular cluster morphology whereas the ZnONps/PPy-3C had uniform granular porous morphology attributed to the homogeneous dispersion of ZnONps in PPy-3C (Fig. 4B) network. On immobilization of Dox, the globular structural morphology of ZnONps in PPy-3C changes into the regular form due to the electrostatic and covalent interaction between ZnONps in PPy-3C with enzyme (Fig. 4C).

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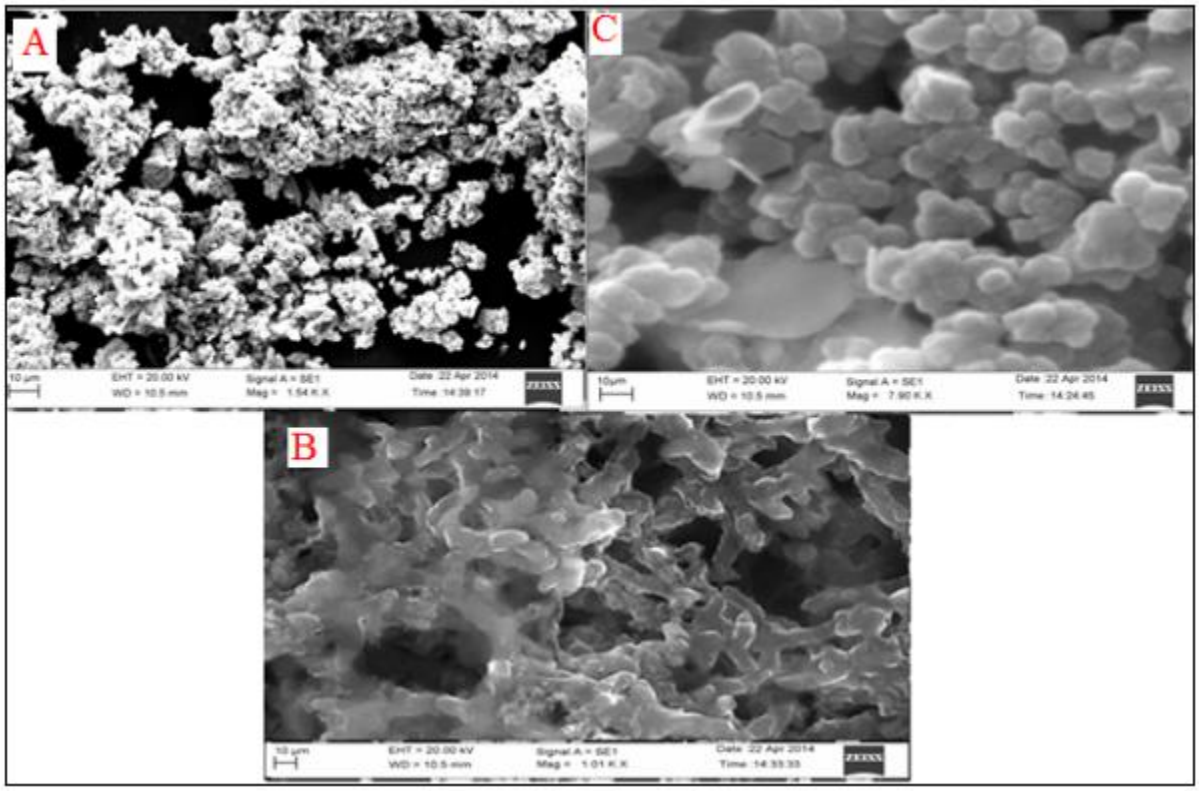

Fig. 4 SEM images of ZnONps (A) ZnONps /PPy-3C (B) and DOx/ ZnONps /PPy-3C on Pt electrode.

FTIR

The FTIR transmission spectra of PPy-3C(i), ZnONp(ii) ZnONps/PPy--3C(iii) and Dox/ZnONps/PPy-3C(iv) using KBr pellets were recorded in the range of 500–4000 cm^{-1} (Fig. 5). The FTIR of PPy-3C, in the stretching vibration peak at 3115 cm^{-1} can be attributed to the N–H stretching vibrations. For pure PPy-3C, all the characteristics peaks are observed at 794 cm^{-1} (=C-H wagging), 926 cm^{-1} (C-C out of phase), 1048 cm^{-1} (=C-H in plane vibration), 1292 cm^{-1} (C-C bond), 1478 cm^{-1} (vibration of the pyrrole ring), 1558 cm^{-1} (C=C bond) and 1705 cm^{-1} (C=N bond). The peaks observed in FTIR of PPy-3C matches well with the ones available in literature, confirming the formation of polypyrrole. The ZnONPs/PPy-3C nanocomposites showed some shift in the wavenumber as well as change in the intensity of peaks as compared to PPy. The most changes are shift of =C-H wagging, C=C and C=N bonds to lower values from 794 cm^{-1}- 791 cm^{-1}, 1558 cm-^{1} -1555 cm^{-1} and 1705 cm^{-1}-1701 cm^{-1} respectively. It is clear that, the peak shifts in ZnONPs/PPy-3C nanocomposite which illustrated that PPy-3C had incorporated with ZnO successfully and the addition of ZnONPs there is strong localization of electrons over PPy ring and gets highly reduced from oxidized form. The Dox binding on ZnONPs/PPy-3C/Pt electrode was indicated by the appearance of additional absorption bands at 1652.9 and 1559.6 cm^{-1} which were assigned to carbonyl stretch (amide-1 band) and –N–H bonding (amide-11 band), respectively.

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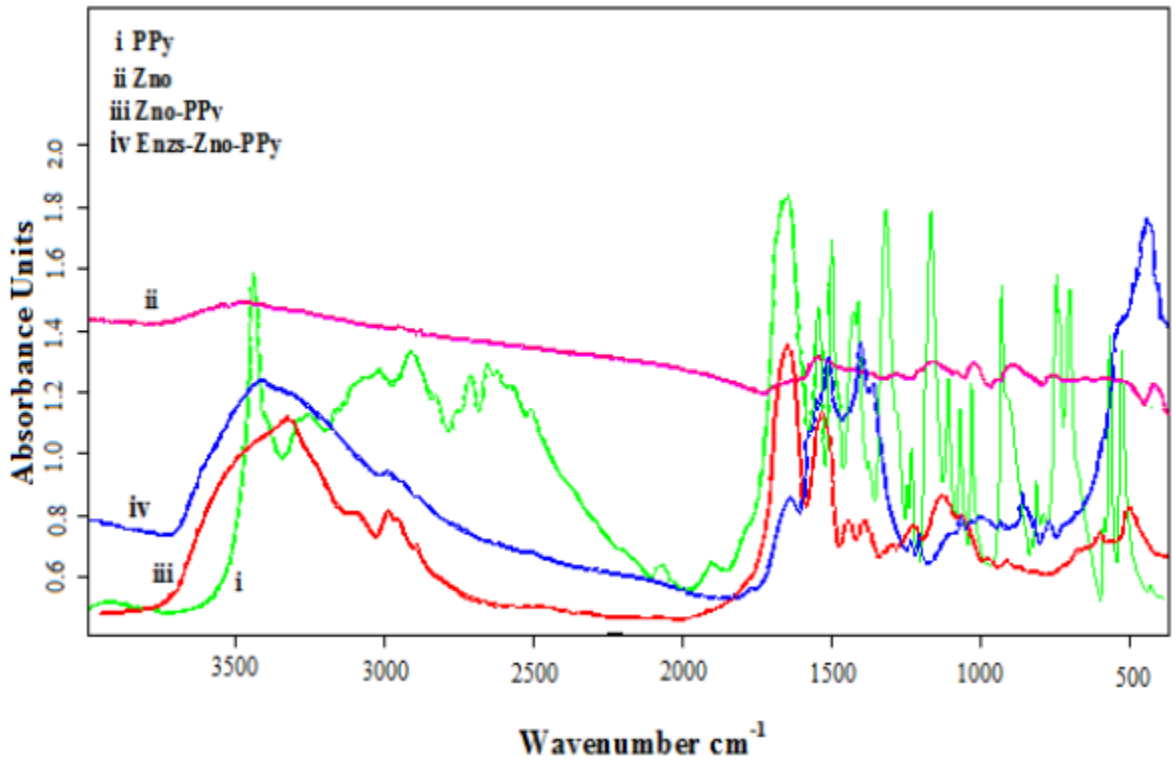

Fig.5 The FTIR transmission spectra of PPy-3C(i), ZnONp(ii) ZnONps/PPy-3C(iii) and Dox/ZnONps/PPy-3C(iv).

Cyclic voltammetry study

To evaluate the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry using a potassium ferricyanide–potassium ferrocyanide solution as the electrolyte was employed. Cyclic voltammograms (Fig. 6A) were recorded in 2.5 mM $K_3Fe(CN)_6$–$K_4Fe(CN)_6$ [(1 : 1)] and 0.1 M phosphate buffer (pH 7.0)with 100mM $NaClO_4$. No peak was observed for the bare Pt and PPy-3C/Pt electrode (curve a,b). An oxidation peak at 0.4 V (vs. Ag/AgCl) (curve c) was observed in the cyclic voltammogram of the ZnONps/PPy-3C/Ptelectrode. The electrodeposition of ZnONps/PPy-3C(curve c) onto the surface of the Pt electrode led to an increase in current intensity as compared to electrodeposition PPy-3C onto Pt electrode(curve b) and bare Pt electrode(curve a).

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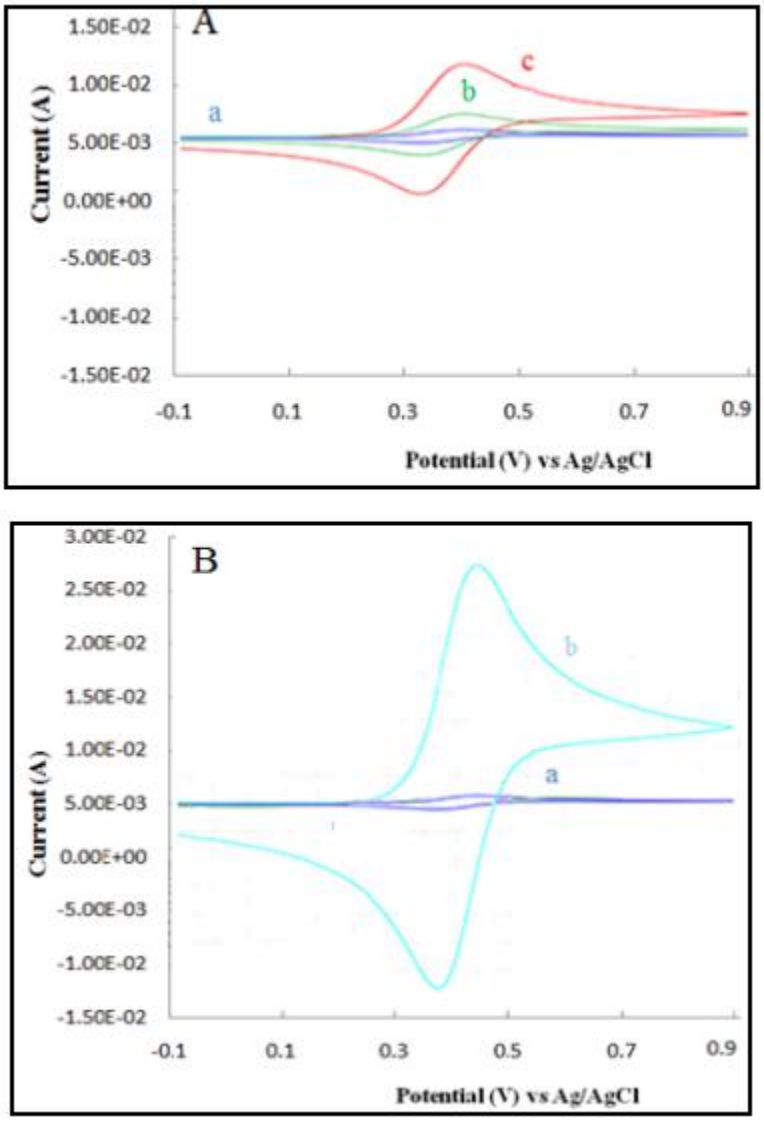

Fig. 6. Cyclic voltammogram(A) of electrodeposition for bare Pt(a) pure PPy-3C/Pt (b) and ZnO-NPs/PPy-3C/Pt electrode (B) Dox/ZnONps/PPy-3C/Pt without (curve a) and with 0.5 mM Put. (curve b) solution (100 µL) in 0.05 M sodium phosphate buffer pH 7.0; scan rate:100 mV s^{-1}.

Cyclic voltammetry of the Dox/ZnONps/PPy-3C/ Pt electrode in the presence of Put.

To evaluate the catalytic activity of Dox when immobilized on the Dox/ZnONPs/PPy-3C, the modified Pt electrode was characterized using cyclic voltammetry in the presence of put in the potential range from −0.1 V to +0.9 V. Fig. 6B showed a cyclic voltammogram (CV) of Dox/ZnONPs/PPy-3C in 0.05 M phosphate buffer (pH 7.0) without (curve a) and with (curve b) an put solution at a scan rate 100 mV s^{-1}. It was observed that with the addition of 0.5 mM put, the oxidation current increased, revealing the improved catalytic properties of the modified electrode for the oxidation of Put. A well-defined oxidation peak (0.4V vs. Ag/AgCl) was observed, which clearly indicates the catalytic properties of the modified electrode.

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Electrochemical impedance measurements (EIS)

The electrode surface during the fabrication process was carried out to investigate immobilization of Dox onto ZnONps/PPy-3C/Pt electrode. The diameter of the semicircle portion at higher frequencies of the Nyquist plot was equal to the charge transfer resistance (R^{CT}), which controled the electron transfer kinetics of the redox probe at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process (Devi et al., 2010). The Nyquist plot (Fig. 7) displayed EIS studies of PPy-3C/Pt (curve a), ZnONps/PPy-3C/Pt (curve b), and Dox/ZnONps/PPy-3C/Pt (curve c) in 0.05 M PB (pH 7.0) containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) as a redox probe. It was observed that the R^{CT} of ZnONps/PPy-3C/Pt electrode (curve b)(170 Ω) was lower than of PPy-3C/Pt electrode (curve a) (210 Ω), revealing its decreased resistance and high electron transfer efficiency. However, the R^{CT} of Dox/ZnONps/PPy-3C/Pt (curve c) (223 Ω) bioelectrode increased compared with that of ZnONps/PPy-3C/Pt electrode. This increase in R^{CT} can be attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies and cause hindrance to electron transfer. These results also indicated the binding of Dox onto ZnONps/PPy-3C/Pt composite.

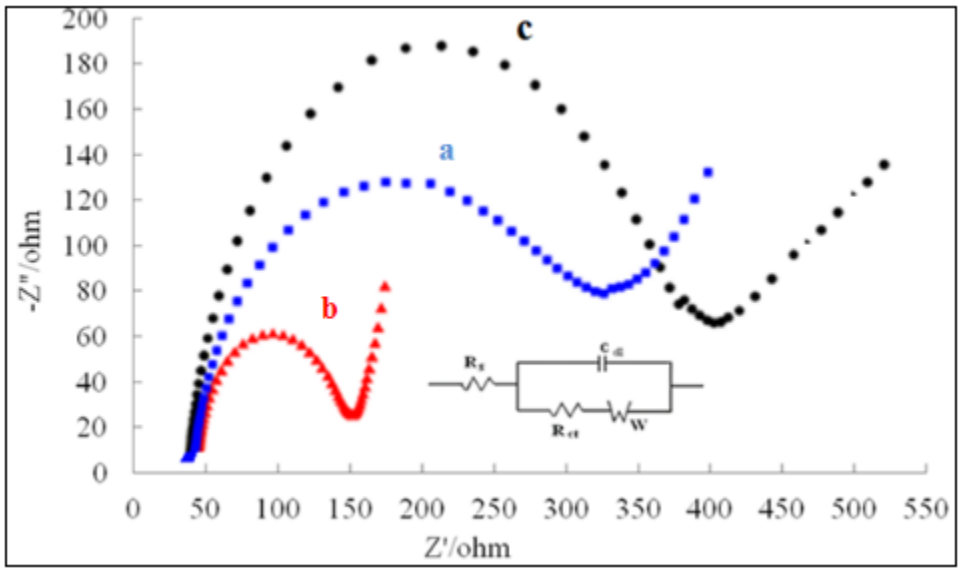

Fig.7 Nyquist plot of electrochemical impedance spectra for PPy-3C/Pt electrode (curve a), ZnONps/PPy-3C/Pt electrode (curve b) and Dox/ZnONps/PPy-3C/electrode (curve c) in 0.05 M PB (pH 7.0) containing 5 mM $K_3Fe(CN)_6$/ $K_4Fe(CN)_6$ (1:1) as a redox probe

Optimization of the experimental conditions of the biosensor

CV of the Dox/ZnONps/PPy-3C/Pt electrode was recorded in the potential range of -0.1 to +0.9 V at a scan rate of 100mV s^{-1} versus Ag/AgCl as the reference electrode in 15 mL of 0.1 M phosphate buffer (pH 7.0) containing 1.0 mL of 0.5 mM Put. The maximum response was observed at+0.4 V hence, subsequent studies were carried out at this voltage. The biosensor showed a maximum response at pH 7.0 , which is slightly lower than that of the platinum electrode based on screen-printed carbon strips (pH 7.4) (Fusco et al., 2011) and screen-printed carbon electrode (C-SPE) based crosslinking bovine serum albumin (BSA)–glutaraldehyde (GA) (Piermarini et al., 2010). The optimum temperature of the biosensor was determined to be 35 °C . This optimum temperature of the biosensor is higher than that of the free enzyme (30°C) and earlier biosensors (30°C) (Bóka et al.,2012). These minor changes in the kinetic properties of the Dox after immobilization might be due to the change in the conformation and microenvironment of the Dox after immobilization. Hence, the subsequent experiments were carried out at pH 7.0 and 35°C. The biosensor showed an optimum response within 4 s.

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Evaluation of biosensor

The time required to attain the 95% of the steady-state response was within 4 s, which indicated a very fast diffusion process. The difference between the baseline and the maximum value of the current reached a plateau at 4s, which was used to build the calibration curve of the biosensor. A linear relationship between current (μA) and Put. concentration ranging from 0.1–1200 μM was observed (Fig. 8A). A K_m for Dox was noticed by 2.5 μM (Fig. 8B).The detection limit of the present sensor was determined to be 0.1 μM (S/N = 3), which is lower/better than that of Dox C-SPE modified by crosslinking (BSA-GA) (0.1mM)((Piermarini et al., 2010), an Pt electrode modified by crosslinking (GA-Gel) 250μM) (Carelli et al.2007) and a Pt electrode modified by pore glass beads (50μM) (Carsol & Mascini, 1999). The mean analytic recoveries of added Put. at 0.5 and 1.0 μM (final concentration in urine) as determined by the current biosensor were 98.47±1.1 and 97.91±1.8%, respectively. To test the reproducibility and reliability of the current Put. biosensor, Put. content in six urine samples (real samples) was determined on a single day (within a batch) five times and again after storage at 4°C for 1 week (between batches). The determinations of Put. content were consistent: the coefficients of variation (CVs) within and between batches were observed to be 3.6% and 4.1%, respectively. These results indicate the reproducibility and consistency of the current method. The good stability, repeatability and reproducibility observed for the proposed biosensor could be attributed to the excellent immobilization of Dox onto the ZnONps/PPy-3C/Pt electrode.

The Put. values of our method are in good agreement with those determined by the standard standard chemical spectrophotometric method with a good regression coefficient (r = 0.998) (Fig.9). Practically no interference was observed during measurements of Put. content by this biosensor in the presence of interfering species viz. spermine, spermidine, histamine, ascorbic acid, uric acid, urea, bilurubin, glucose and triglyceride. Efforts were made to study the effects of individual interferents at a 0.1 mM concentration.

The value of Imax did not vary significantly in the presence of interferents, indicating the lack of influence of the individual interferents . The earlier Put.biosensors showed a considerable decrease in their activity by histamine, spermine and spermidine (Alonso-Lomillo et al.,2010) (Hernandez-Cazares et al., 2012).

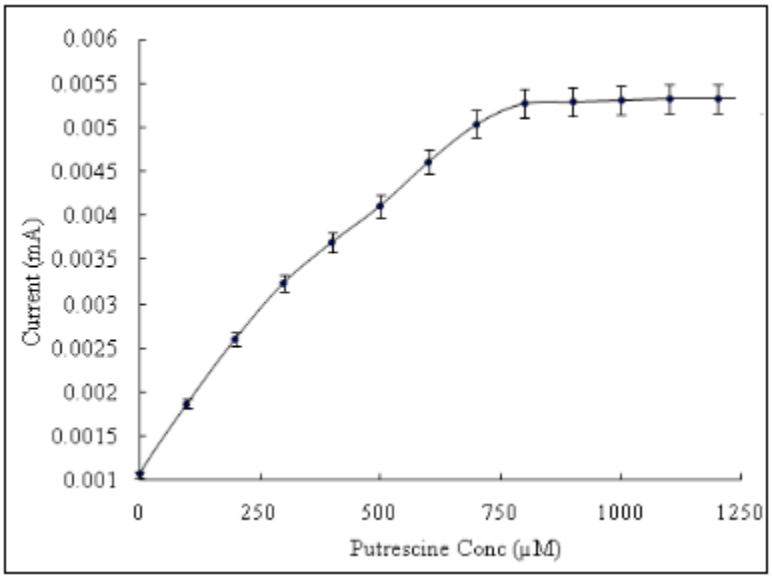

Fig.8A Effect of substrate concentration on current response of ZnO-NPs/PPy-3C composite film based putrescine biosensor. Standard assay conditions were used except varying the putrescine concentration from 0.1-1200 μM

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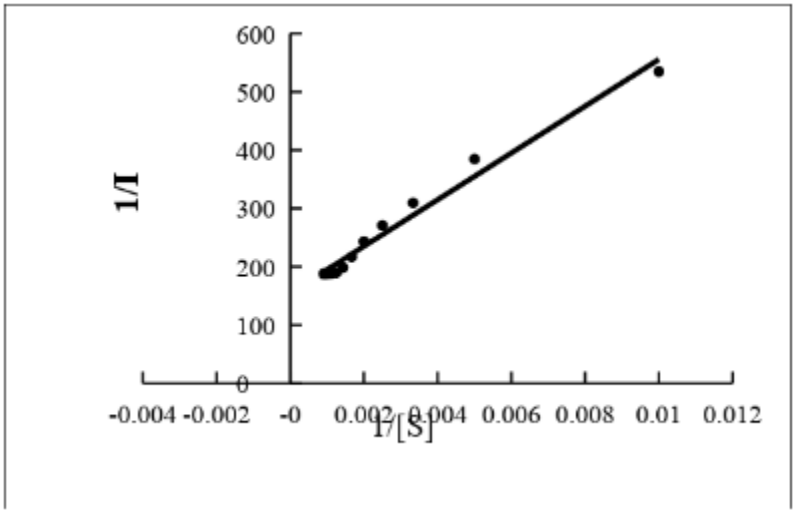

Fig.8B. Lineweaver-Burk plot for effect of putrescine concentration on current response of putrescine ZnO-NPs/PPy-3C composite film based putrescine biosensor

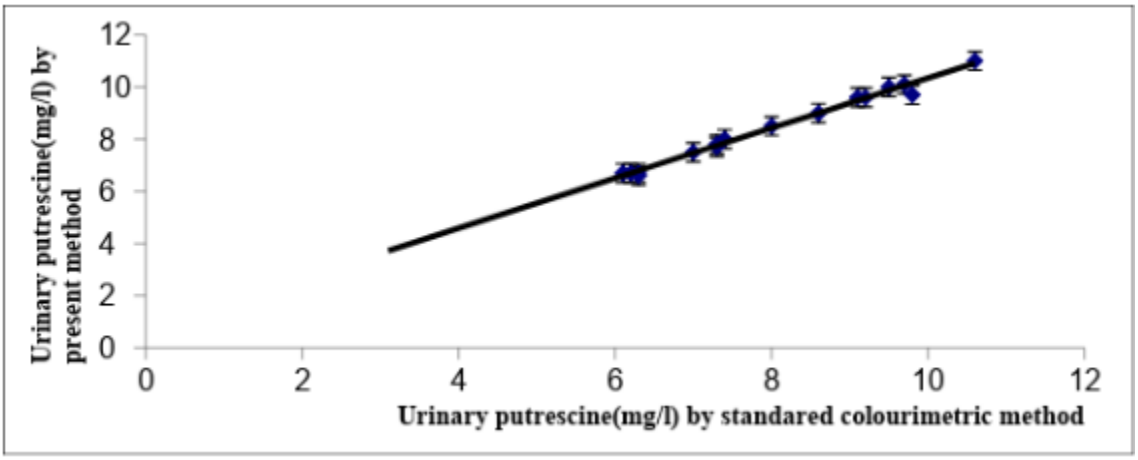

Fig.9 Correlation between urine putrescine values as determined by standard chemical spectrophotometric method (x-axis) and present biosensor (y-axis) based on ZnO-NPs/PPy-3C composite film.

Detection of Put. in urine samples

The Put. values in urine sample of apparently healthy individuals (n =6) were measured to be in the range of 0.071±0.141to 0.152±0.132µM (with a mean of 1.39 µM) in our experiment. The normal established concentration of Put. in urine has been reported to be 0.03 ± 0.34 µM(Paik et al., 2008). However, in cancer patients (n =6) (S.Table.3), Put. concentrations between 2.542±0.131 to 3.921±0.163 (with a mean of 18.4 µM) were found, which is significantly higher than those in healthy individuals.

Stability and reusability of the biosensor

Our enzyme electrode lost 50% of its initial activity after being used 100 times in a span of 4 months and stored at 4 °C. This amount of time during which half of the initial activity is retained is greater than that for earlier biosensors .Our enzyme electrode was constructed thrice in similar ways and the effect of storage at 4°C was studied for each of them. The results showed practically no variation in storage stability of the three electrodes. The better shelf life

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of the present enzyme electrode over previously reported sensors (Shanmugam et al., 2011) may be attributed to the presence of ZnONps/PPy-3C/Pt.

Conclusions

The use of a composite film of ZnONps/PPy-3C in the construction of a biosensor for Put. has led to its improved analytical performance in terms of low working potential (0.4 V), short response time (4 s), low detection limit (0.1μM) and high storage stability (3 months). Based on these observations, this composite could be employed for the of improvement other biosensors.

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