Talanta 120 (2014) 17–22

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Polymelamine modified edge plane pyrolytic graphite sensor for the electrochemical assay of serotonin

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ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form
25 November 2013
Accepted 26 November 2013
Available online 3 December 2013

Keywords:
Polymelamine
Serotonin
Pyrolytic graphite
Human blood serum

ABSTRACT

A sensitive and novel electrochemical method has been developed for the determination of an important neurotransmitter, serotonin, using a polymelamine modified edge plane pyrolytic graphite sensor (EPPGS). Melamine was used for the modification of sensor by electropolymerizing it at the surface of EPPGS in acidic medium to form a layer of conducting polymer. Field emission scanning electron microscopy (FE-SEM) and electrochemical impedance spectroscopy (EIS) were used for the characterization of the surface of polymer modified sensor. The electrochemical measurements were carried out using square wave voltammetry and cyclic voltammetry. The polymelamine modified sensor exhibited excellent electrocatalytic activity towards the electrochemical oxidation of serotonin, exhibiting a larger peak current and shift of peak potential to less positive potentials as compared to the unmodified sensor. The dynamic range for the serotonin determination was found between 1–100 μm and 0.1–100 μm with detection limit of 492 nM and 30 nM for unmodified and polymer modified sensors, respectively. The determination of serotonin in human blood serum and urine has been carried out. The common metabolites such as ascorbic acid, dopamine, xanthine and hypoxanthine do not interfere in the determination up to 10-fold concentration, revealing good selectivity of the proposed sensor.

1. Introduction

In recent years, modification of the electrode surface by special layers has been the major innovative area in electrochemical sensors [1–3]. Conducting polymer-modified electrodes (PMEs) have received attention due to their wide applications in chemical and biosensors [4]. The better chemical stability of polymeric film, high sensitivity, selectivity, reproducibility, more active sites, homogeneity and strong adherence to electrode surface, have been found to stimulate the field of bioanalytical electrochemistry [5–7]. Conducting polymers have an additional interest because of their combined properties of organic polymer and electronic properties of semiconductor, three dimensional molecular structures which control the electronic properties and nanostructured shape. Small dimension and large surface area of the nanocomposites allow fast electron transfer and significant increase in the current response. The conducting polymers have the genuine reason for use as a surface modifier in voltammetric sensors because they significantly increase the electrocatalytic activity, decrease the over potential and increase the reaction rate [8–12]. The intrinsic conductivity of these polymers is due to the substantial π-electron delocalization along their backbone, due to which they show good electronic conductivity during oxidation and reduction. These features of conducting polymers are expected to increase the selectivity and sensitivity and should increase the limit of detection [13,14]. Among the different methodologies for the preparation of polymer-modified electrode, electropolymerization has been demonstrated as a very convenient method to immobilize polymer on the electrode surface because the thickness, permeation and charge transport characteristics of the modified polymeric film can be well defined by the controlled electrochemical parameters [15].

5-Hydroxytryptamine (serotonin, 5-HT) is an important and major biogenic monoamine neurotransmitter as well as neuromodulator [16],widely distributed in human brain and makes an important contribution in wide variety of biological, physical, psychopathological processes including sleep regulation, depression, eating disorder, alcoholism, infantile autism, anxiety disorders, muscle contraction, liver regeneration, endocrine regulation, obsessive-compulsive disorder and psychosis [17–20]. Serotonin was found in gastrointestinal (GI) tract, platelets, and in central nervous system (CNS). The major amount of serotonin in body is located in the GI tract; 90% is in the enterochromaffin cells (EC) in the alimentary canal (gut) and 5% in serotonergic neurons of myentric plexus, whereas, the rest amount is found in the brain. Overflowing serotonin from EC cells is stored in blood platelets. Serotonin is synthesized from essential amino acid tryptophan by tryptophan hydroxylase-I [21,22]. The determination of 5-HT is informative in the diagnosis of various diseases and hence makes it the subject of biologically and pharmacologically oriented research [23].

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0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.11.075

Several techniques have been employed for the determination of serotonin such as high performance liquid chromatography, capillary electrophoresis, mass and fluorescent spectrometry, and capillary liquid chromatography coupled with mass spectrometry [24–28]. However, most of these techniques require heavy and expensive instruments with sophisticated process, tedious time consuming pretreatment and derivatization. These techniques also required organic solvents for separation and thus cause environment pollution. Electrochemical techniques on the other hand are considered ecofriendly and have high selectivity, sensitivity, reproducibility rapid response and are low cost [29–31]. In recent years, organic conducting polymers such as polypyrrole, polyaniline, polydiaminonapthalene and some other polymers along with carbon or gold nanoparticles have attracted considerable attention to modify the surface of the electrodes for the determination of neurotransmitters and biomolecules [32–35]. In this study, we have used melamine (1,3,5-triazine-2,4,6-triamine) monomer as a modifier because of its high catalytic current, better sensitivity, film stability, low cost and easy availability. The polymer was electrochemically synthesized at the surface of edge plane of pyrolytic graphite [36–38]. After optimizing the experimental conditions the application of the developed sensor has been demonstrated in the determination of serotonin in biological samples and pharmacological formulations.

2. Experimental

2.1. Apparatus

All the voltammetric experiments were performed with a computerized Bio Analytical System (BAS, West Lafayette, USA) Epsilon voltammetric analyzer. A conventional single compartment three electrode cell assembly equipped with bare edge plane pyrolytic graphite sensor (EPPGS) or polymelamine modified EPPGS as a working electrode, Ag/AgCl (3 M NaCl) (BAS Model MF-2052 RB-5B) as reference electrode and platinum wire as a counter electrode was used for the electrochemical measurement. The pH measurement of the buffer solutions was carried out using a Thermo Fisher Scientific, Singapore Digital pH meter (Eutech pH 700). The pyrolytic graphite pieces were obtained from Pfizer Inc. New York, USA as a gift. Field Emission Scanning Electron Microscopy (FE-SEM) instrument (JEOL, JSM-7400) was used to characterize the surface morphology of the bare and melamine modified sensor.

2.2. Chemical and reagents

Serotonin, melamine, sulfuric acid, dopamine, xanthine and hypoxanthine were purchased from Sigma-Aldrich (USA) and used as received without further purification. Phosphate buffers of ionic strength (1.0 M) and appropriate pH were prepared according to the method of Christian and Purdy [39] by mixing the solutions of H_3PO_4, Na_2HPO_4 and NaH_2PO_4. Biological samples of human serum and urine were collected from Vaisnavi Pathology Lab., Roorkee, and used after suitable dilution. All other chemicals and solvents used in the experiment were of analytical grade and double distilled water was used throughout the experiments.

2.3. Fabrication of polymelamine on the surface of EPPGS

Prior to modification, in order to remove adhered particles from the edge plane surface (area 3 mm^2) of pyrolytic graphite, it was rubbed on an emery paper (P-400) and then washed thoroughly with double distilled water and dried. The electropolymerization of melamine was carried out on the surface of EPPGS in the solution of 0.1 M H_2SO_4 containing 1.0 mM melamine by scanning the potential between 0.1 and 1.6 V vs. Ag/AgCl (3 M NaCl) reference electrode at the scan rate of 100 mV s^{-1} for 20 cycles. After modification, the modified electrode was carefully rinsed with distilled water in order to remove soluble product and monomer of melamine and then cyclic voltammograms of modified electrode were recorded between −1.2 and +1.2 V at 100 mV s^{-1} in pH 7.2 buffer solution (0.5 M), after deaeration of buffer solution by bubbling high purity nitrogen for 10–15 min. until stable voltammograms were obtained.

2.4. Voltammetric procedure

Stock solution of serotonin (1 mM) was prepared by dissolving the required amount in double distilled water. For voltammetric experiments, desired volume of 5-HT solution was taken in an electrolytic cell and mixed with 2.0 ml phosphate buffer of pH 7.2 (1.0 M). The total volume was made to 4.0 ml by the addition of double distilled water. The voltammograms were then recorded by using the voltammetric analyzer under optimized parameters. The optimized square wave voltammetric parameters used were square wave amplitude (Esw): 25 mV, step potential (E): 4 mV and square wave frequency (f): 15 Hz. Optimum conditions for cyclic voltammetry (CV) were initial (E): 0 mV, switching potential (E): 600 mV, final (E): 0 mV, and scan rate (ν): 100 mV/s. Solution was used after deaeration by high purity nitrogen for 10–12 min. All potential were referred to the Ag/AgCl electrode at an ambient temperature of 25 ± 2 °C.

3. Result and discussion

3.1. Fabrication of polymelamine film and characterization

Fig. 1 displays the successive cyclic voltammograms recorded during the electropolymerization of melamine at the surface of EPPGS in the solution of 1.0 M melamine in 0.1 M H_2SO_4. During the first cycle, a well-defined anodic peak at 0.64 V was observed towards the positive potential. A cathodic peak at 0.56 V was observed in the reverse sweep. In the consecutive scan, peak current continuously increased with increasing number of potential cycles and became practically stable after 16 cycles. Finally in the 20th cycle well-defined anodic and cathodic peaks are observed at about 0.67 V and 0.57 V, respectively. The increase in peak current with increase in number of potential scanning indicates the subsequent growth of the polymer film on the EPPGS

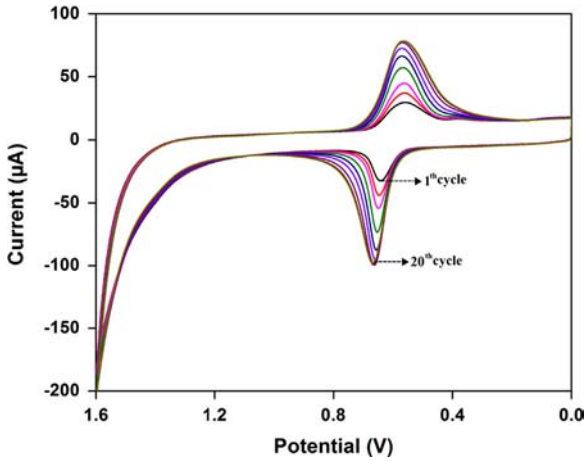

Fig. 1. Effect of consecutive cyclic voltammograms on the growth of polymelamine film at the surface of EPPGS. Polymerization was carried out in 0.1 M H_2SO_4 solution containing 1 M melamine monomer at a scan rate 100 mV s^{-1}.

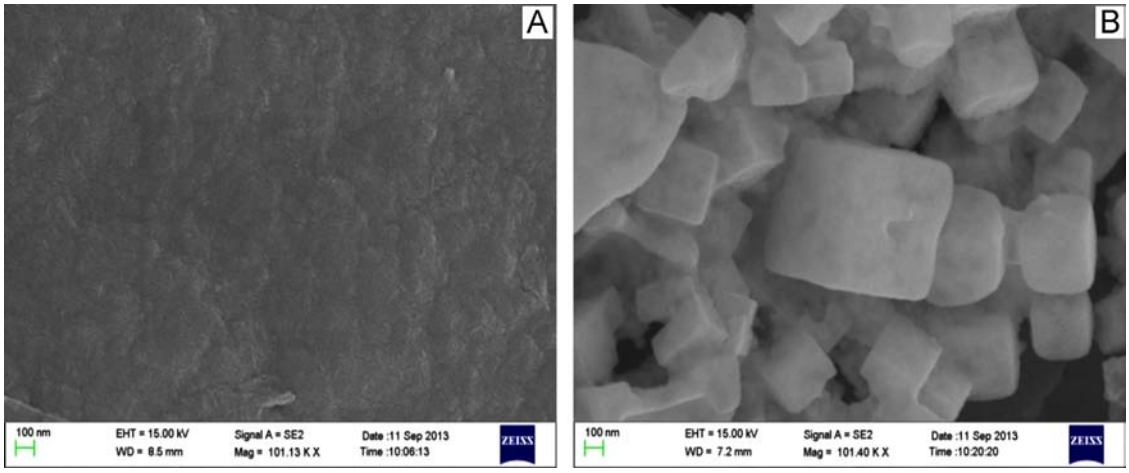

Fig. 2. Typical FE-SEM images of (A) unmodified EPPGS and (B) polymelamine/EPPGS.

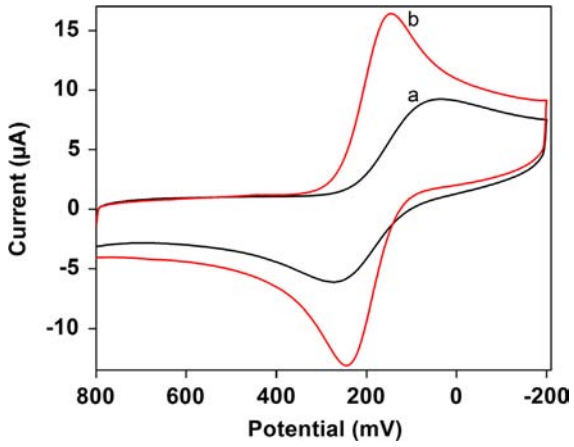

Fig. 3. A comparison of cyclic voltammograms of 5 mM $K_3Fe(CN)_6$ in 0.1 M KCl at (a). unmodified and (b) polymelamine/EPPGS.

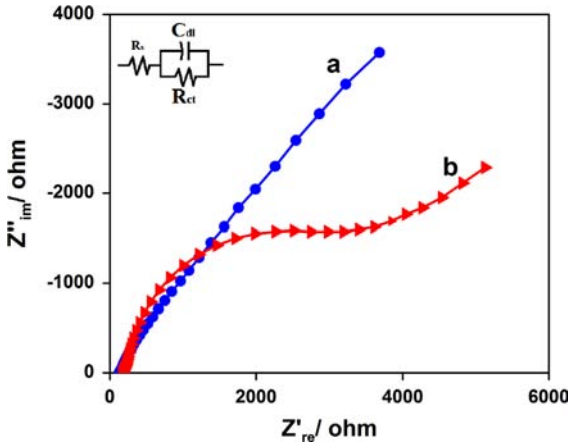

Fig. 4. Nyquist-diagram (imaginary part Z_{im} vs. real part Z_{re}) for the electrochemical impedance measurement at (a) unmodified and (b) polymelamine modified EPPGS. The inset represents Randle's equivalent circuit.

surface and finally a uniform adherent film was developed at the pyrolytic graphite surface. This cyclic voltammetric behavior of melamine was similar to that reported in literature [36,38].

The surface morphology of the polymelamine film prepared was characterized by the scanning electron microscope (SEM). Fig. 2 presents a comparison of FE-SEM images of unmodified and modified EPPGS. The typical image of polymer film (Fig. 2B) indicates that the film has a cube, crystalline image and it appears that they are bonded to each other. On the other hand the unmodified EPPGS (Fig. 2A) did not show such cubes. The nanocluster and microporous structure of polymelamine film, which was formed via layer to layer deposition, provides an effective large surface area that affects the electrode surface properties towards defusing electroactive species and electrochemical sensing.

In order to confirm the efficacy of the surface modification procedure, surface area of unmodified and polymelamine modified EPPGS were calculated by recording the cyclic voltammograms (CVs) of 5 mM $K_3Fe(CN)_6$ at different scan rates using 0.1 mM KCl as supporting electrolyte. At both the surface a redox couple was observed due to Fe^{+2}/Fe^{+3}; however, an increment in peak current of Fe^{+2}/Fe^{+3}couple at the polymelamine modified sensor, as compared to the unmodified electrode, was observed and ΔE_p value decreased to 130 mV from 235 mV showing improvement in the reversibility of Fe^{+2}/Fe^{+3}redox couple at the modified surface as shown in Fig. 3. The surface area was calculated from the slope of i_p vs. $\nu^{1/2}$ using the Randles–Sevcik equation and found as 0.081 and 0.320 cm^2 for bare and modified EPPGS, respectively. Thus, the polymelamine modified sensor had an area nearly four times larger than the unmodified EPPGS surface.

The surface properties of unmodified and melamine modified EPPGS were also characterized by electrochemical impedance spectroscopy. Randle's equivalent circuit was used to obtain data, where parallel combination of resistance to charge transfer (R_{ct}) and interfacial capacity (C_{dl}) gave rise to a semicircle and semicircle diameter was equal to the R_{ct}. The experiment was carried out in 1:1 solution of 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl solution in the frequency range of 0.1–100 KHz. The results are shown in Fig. 4. At unmodified EPPGS, a charge transfer resistance for the Fe $[CN]_6^{3-/4-}$ redox process was observed as 5554 Ω (curve a). However for modified EPPGS, it can be seen that the charge transfer resistance decreases significantly to about 1500 Ω (curve b), implying that melamine plays an important role in increasing the rate of electron transfer between the sensor and electrolyte and shows a conducting wire like behavior. These data showed that the melamine film had been successfully attached to the electrode surface

3.2. Cyclic voltammetry

Initially, cyclic voltammograms of 5-HT were recorded to get information about the oxidation behavior. Fig. 5 represents cyclic voltammograms, recorded for 50 μM serotonin in phosphate buffer

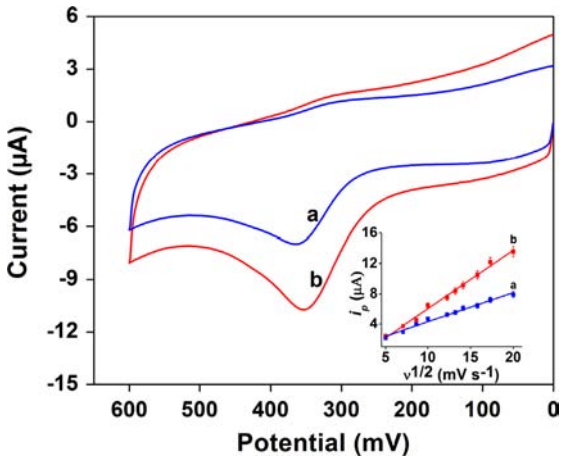

Fig. 5. Observed cyclic voltammograms of 50 μM serotonin in phosphate buffer of pH 7.2 using (a) unmodified and (b) polymelamine/EPPGS at a scan rate of 100 mV s^{-1}. Inset is the (b) graph between i_p and scan rate ($\nu^{1/2}$).

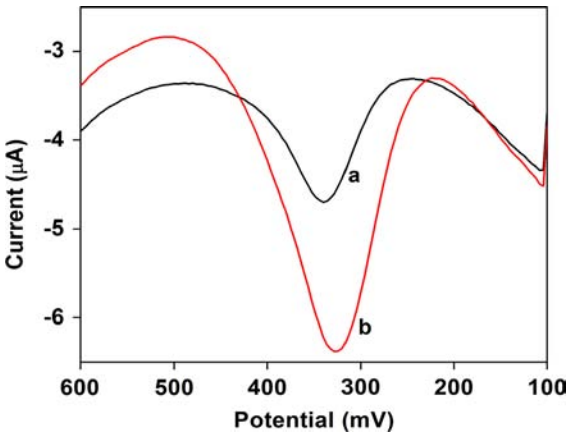

Fig. 6. A comparison of SWVs observed for 20 μM serotonin at (a) unmodified and (b) polymelamine modified EPPGS.

solution of pH 7.2 at a scan rate of 100 mV s^{-1} using unmodified and polymelamine modified EPPGS. At both the sensors, anodic peaks were obtained for the oxidation of serotonin. E_p of the oxidation peak at unmodified and modified sensor was 366 and 352 mV, respectively as shown in Fig. 5. The absence of reduction peak in the reverse sweep clearly reveals that the oxidation of serotonin is irreversible. The large oxidation peak current and shift in E_p to less positive potential as compared to the unmodified EPPGS indicates the electrocatalytic activity of polymelamine/EPPGS. To ascertain the effect of scan rate on the anodic peak current, scan rate studies were carried out in the range 25–400 mV s^{-1}. The anodic peak current of serotonin was found to increase with increasing sweep rates and the dependence of the peak current on scan rate can be expressed by the following linear relationships:

$$i_p = 0.3848[\nu^{1/2}] + 0.455 \quad \text{for bare EPPGS}$$

$$i_p = 0.7724[\nu^{1/2}] - 1.695 \quad \text{for modified EPPGS}$$

having a correlation coefficient (R^2) of 0.987 and 0.993 for unmodified and modified EPPGS, respectively. In the relations ν is the scan rate in mV $s^{-1}$and i_p is the peak current in μA. The linear relationship between i_p vs. $\nu^{1/2}$ (inset of Fig. 5) suggests that the oxidation of serotonin at the polymelamine modified EPPGS is a diffusion controlled process.

3.3. Square wave voltammetry

The square wave voltammograms of 20 μM of serotonin at unmodified and polymelamine modified EPPGS were recorded in phosphate buffer solution of pH 7.2 using the optimized parameters of SWV. On scanning the potential from 0 to 600 mV, a well-defined peak was observed at unmodified (E_p ~ 340 mV) and modified (E_p ~ 328 mV) sensors corresponding to the oxidation of serotonin. A remarkable enhancement in the peak current with less positive oxidation potential of 5-HT in case of modified EPPGS in Fig. 6 clearly reveals that the polymelamine modified sensor has excellent electrocatalytic properties to enhance the kinetics of the electrochemical process towards the oxidation of serotonin as compared to the unmodified surface.

3.3.1. Effect of pH

pH of the supporting electrolyte is an important factor that affects the electrochemical behavior of biomolecule and drugs. The effect of the pH of phosphate buffers on the anodic peak current of serotonin was evaluated in the pH range of 2.1–10.0 for polymelamine/EPPGS. It was observed that the peak potential of 5-HT shifted to less positive potentials with increase in the pH. The E_p vs. pH plot was linear and dependence of E_p of the analyte on the pH of supporting electrolyte can be expressed by the relation

$$E_p/\text{mV (pH 2.1} - \text{10.0)} = -39.68\ \text{pH} + 612.2$$

having a correlation coefficient of 0.995. The value of dE_p/dpH for serotonin indicates that the number of protons and electrons involved in oxidation of 5-HT are not the same.

3.3.2. Effect of square wave frequency

The dependence of anodic peak current (i_p) of serotonin on the square wave frequency (f) was studied in the range of 10–200 Hz at pH 7.2 for the melamine modified sensor. The anodic peak current of 20 μM serotonin was found to increase linearly with increasing square wave frequency. The linear relation between peak current (i_p) and square root of ($f^{1/2}$) can be presented by the following equation:

$$i_p\ (\mu A) = 3.966 f^{1/2}\ (\text{Hz}) - 12.98$$

having correlation coefficient 0.990. The above voltammetric response further confirmed that the oxidation of serotonin is a diffusion controlled pathway, which supported the results obtained using cyclic voltammetry.

3.3.3. Concentration study

SWVs were recorded at different concentrations of serotonin (0.1–100 μM) in the phosphate buffer of pH 7.2 and it was observed that with increasing concentration of 5-HT, oxidation peak current increased. The peak current (i_p) was found to be linearly dependent on the concentration in the range of 0.1–100 μM as depicted in Fig. 7. The values of the anodic current were obtained by subtracting the background current of buffer solution and an average of at least three replicate measurements was used to plot the calibration curve. The linear relationship between the anodic peak current and concentration of serotonin can be expressed by the relation:

$$i_p\ (\mu A) = 0.088C + 0.715$$

with a correlation coefficient 0.972, where C is the concentration of serotonin.

The sensitivity of the proposed method is found to be 0.088 μA μM^{-1}. The detection limit is calculated by using the relation $3\sigma/b$, where σ is the standard deviation of the blank solution and b is the slope of the calibration and is found to be 30 nM.

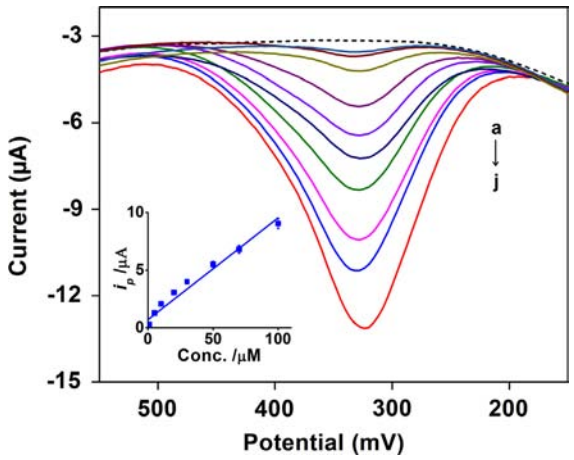

Fig. 7. Square wave voltammograms observed for increasing concentration of serotonin. Curves were recorded at (a) 0.2; (b) 0.8; (c) 1; (d) 5; (e) 10; (f) 20; (g) 30; (h) 50; (i) 70 and (j) 100 μM concentrations using polymelamine/EPPGS in phosphate buffer of pH 7.2. The observed calibration plot for serotonin between [*C*] and i_p shown in inset. The background curve is shown by dotted lines.

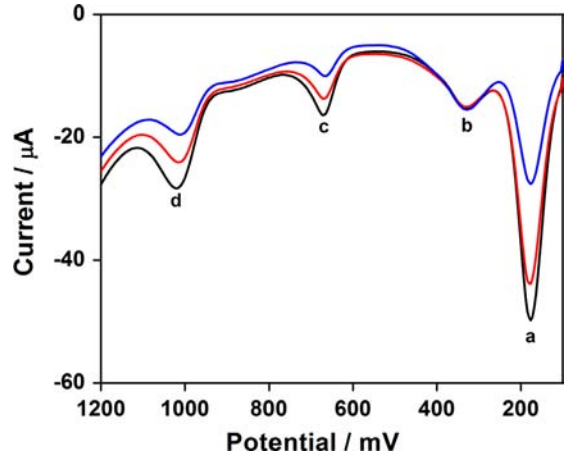

Fig. 8. Square wave voltammograms showing interference of dopamine (peak a), xanthine (peak c), and hypoxanthine (peak d) and fixed serotonin concentration 20 μM (peak b).

3.4. Interference study

The concentration of several metabolites present in the blood and urine may alter the electrochemical signal of a sensor and consequently affect selectivity. Hence, selective determination is an important goal in biological and chemical research. In order to examine the selectivity of polymelamine/EPPGS, influence of metabolites commonly present in urine such as dopamine, xanthine and hypoxanthine was evaluated. Interference study was carried out at pH 7.2 keeping the concentration of serotonin fixed at 20 μM and varying the concentration of interferents up to 10-fold excess. In the SWVs it was found that four well separated peaks at 176, 328, 671, 1018 mV were observed corresponding to the oxidation of dopamine, serotonin, xanthine and hypoxanthine, respectively (Fig. 8). The experimental results suggest that no significant change in anodic peak current and peak potential of serotonin was observed for the entire range of the concentration of these interferents. Hence, it is concluded that the developed method can be safely applied for the determination of serotonin in human body fluids as well as in pharmaceutical preparations without facing any difficulty due to the interferents.

3.5. Stability and reproducibility of polymelamine/EPPGS

The stability of the polymelamine/EPPGS sensor was evaluated by measuring the anodic peak current response at the fixed concentration of 20 μM serotonin in phosphate buffer of pH 7.2. The modified sensor was used daily up to 10 consecutive days and stored in air. The experimental results revealed that the voltammetric current response of the modified sensor deviated with the RSD of ± 2.43%. Thus, the current response of the modified sensor did not change significantly and indicated that the polymelamine/PGE sensor possesses excellent stability. In order to check the reproducibility of the modified sensor, six repetitive measurements were carried out at the same concentration (20 μM) in phosphate buffer solution of pH 7.2 on the same day to show intraday reproducibility. The RSD recorded for the six replicate runs was ± 3.21%, indicating excellent reproducibility of the results of polymelamine/EPPGS.

3.6. Real sample analysis

In order to evaluate the execution of a polymelamine modified sensor and the stability of serotonin in biological samples, an experiment was carried out via the recovery study of 5-HT in the urine sample of healthy persons. Prior to the analysis, urine sample was diluted 100 times with phosphate buffer of pH 7.2 to reduce the matrix complexity and the sample was spiked by adding known concentration (10, 20, and 35 μM) of serotonin. Under optimized parameters, Square wave voltammograms with anodic current response for urine sample at polymelamine/EPPGS were recorded. The concentration of serotonin in the urine sample was calculated from the standard addition plot and the results are summarized in Table 1. Excellent recoveries have been achieved for serotonin with low RSD values indicating reproducibility of the developed method.

The altered concentration of serotonin in human system affects the function of central nervous system, hence the proposed method has also been implemented for the evaluation of serotonin level in the blood serum. Polymelamine/EPPGS was applied for the determination of serotonin in the blood serum of patients undergoing treatment with dopamine. The serum was diluted 30 times with buffer and square wave voltammograms were recorded. By the standard addition technique the concentration of serotonin in serum sample was detected as 2.01×10^{-6} μM (Fig. 9). The negative value at *X*-axis is insignificant. The serum sample was then spiked with exogenous serotonin and SWVs were recorded. The recoveries were found in the range of 99–101% for serotonin (Table 2).

4. Conclusions

The proposed method deals with the preparation of a novel electrochemical sensor and provides an extremely sensitive and selective method for the determination of serotonin. The formation of polymelamine layer significantly increases the surface area. The change in peak potentials towards less positive potentials is relatively small (about 20 mV). The increase in the current levels of

Table 1
Recovery data for serotonin determination in human urine samples at polymelamine/EPPGS.

Sr. no.	Amount added (μM)	Amount detected (μM)	Recovery (%)
1	10	9.63	96.30
2	20	19.32	96.62
3	35	34.16	97.61

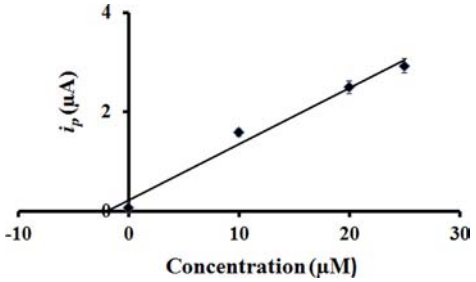

Fig. 9. Calibration plot of spiked serotonin concentration in patient blood serum sample.

Table 2
Recovery results observed for serotonin in human serum sample at polymelamine/EPPGS.

Sr. no.	Amount added (μM)	Amount detected (μM)	Recovery (%)
1	0	2.01	–
2	10	9.92	99.25
3	20	20.23	101.65
4	25	25.16	100.65

Table 3
Comparison of the working range and detection limit of dopamine by polymelamine/EPPGS with recently reported methods.

Reference no.	Linear dynamic range (M)	Detection limit (M)	Determination in real sample
[40]	5.0×10^{-6}–3.5×10^{-5}	1.7×10^{-6}	No
[41]	0.5×10^{-6}–130×10^{-6}	0.08×10^{-6}	Serum and urine
[42]	1×10^{-6}–1×10^{-4}	0.16×10^{-6}	No
[43]	1×10^{-7}–1×10^{-5}	0.03×10^{-6}	Serum
[44]	0.1×10^{-6}–100×10^{-6}	0.08×10^{-6}	No
[45]	0.1×10^{-6}–10×10^{-6}	0.25×10^{-6}	No
Proposed method	0.1×10^{-6}–100×10^{-6}	0.03×10^{-6}	Serum and urine

5- HT oxidation is likely due to the increase in the effective surface area of the electrode. This modified surface avoids the superficial poisoning effects of EPPGE, by which the peak potential shifted only 10–20 mV towards the less positive region. The sensor was capable of successfully separating the voltammetric signal of 5-HT from the metabolites commonly present in biological fluids. A comparison of the detection limit of 5-HT observed at polymelamine modified EPPGS with detection limit recently reported at other electrodes (Table 3) clearly indicated that the detection limit observed in the present study is lower or comparable [40–45]. The application of the developed sensor for the determination of serotonin in urine and serum has also been demonstrated. The method provides an ecofriendly procedure for using the detrimental compound (melamine) for sensor preparation for the detection of biomolecules and neurotransmitters. The wide linear range, low detection limit, long term stability and high reproducibility with essentially no pretreatment offers a successful approach for extending the proposed method for the clinical analysis of 5-HT.

Acknowledgments

One of the authors (PG) is thankful to the Department of Biotechnology, Ministry of Science and Technology, New Delhi, for the award of Junior Research Fellowship. The financial assistance for this work was provided by the Department of Biotechnology, New Delhi, with Grant no. BT/PR13954/MED/32/143/2010.

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