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Nanobiocomposite Based Electrochemical Sensor for Sensitive Determination of Serotonin in Presence of Dopamine, Ascorbic Acid and Uric Acid In Vitro

M. Satyanarayana,[a] K. Koteshwara Reddy,[a] and K. Vengatajalabathy Gobi*[a]

Abstract: A multiwalled carbon nanotube – chitosan (MWCNT-Chit) composite modified glassy carbon electrode (GCE) was fabricated and applied for sensitive and selective determination of serotonin also known as 5-hydroxytryptamine (5-HT). Electroanalytical response of the fabricated electrode towards the detection of 5-HT was studied by using cyclic voltammetry and electrochemical impedance spectroscopy. The results show that the MWCNT-Chit/GCE has greatly enhanced the oxidation peak current and that the nanocomposite film enhanced the electron transfer to the analyte. Applying differential pulse voltammetry method, a linear calibration plot has been achieved over the concentration range 5×10^{-8}–1.6×10^{-5} M and the low-detection-limit is 5×10^{-8} M. Selective determination of serotonin in the presence of dopamine, ascorbic acid and uric acid was established. The fabricated biosensor detects 5-HT in-vitro directly from artificial urine samples and from diluted serum samples.

Keywords: Serotonin · Carbon nanotubes · Chitosan · Electrocatalysis · Voltammetric determination · Artificial urine.

1 Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine neurotransmitter widely present in the brain and plays a significant role in various pharmacological, physical and biological processes including temperature regulation, muscle contraction, liver regeneration, and depression. The analysis of neurotransmitters in urine is critical in the case of psychic disorder, neurological disease and tumour diagnoses. Also, in order to evaluate the physiological investigations, it is very important to determine the serotonin alone in biological fluids from a variety of interferences. Therefore, quantitative analysis of 5-HT in human urine and plasma is essential because of its coexistence in biological systems and regulating several physiological functions. Various sensing techniques have been examined for the determination of 5-HT which were equipped with high performance liquid chromatography [1–3], mass spectroscopy [4], coulometry [5], capillary electrophoresis [6] and flow injection analysis [7], however these techniques require expensive instruments and are time consuming for sample preparation and derivatization processes which result in low recoveries and very high detection times compared to electroanalytical methods. Electrochemical techniques based on various methodologies have been developed to overcome these difficulties. However, determination of 5-HT by electrochemical methods remained a challenge due to the interference of other biomolecules like ascorbic acid, uric acid and dopamine present in biosystems [8–12], because these interferents oxidize at electrode potentials close to that of 5-HT, resulting in an overlapping voltammetric response.

Carbon nanotubes (CNTs) have been recognized as an important material in recent years in various fields due to their unique electrical, mechanical and structural properties. The remarkable electron-transfer of CNTs to the surface adsorbates permits the use of CNTs in the fabrication of highly sensitive nanoscale sensors [13]. The electrocatalytic amplification of CNTs increases the current generated by the analytes of interest, which improves sensitivity. The electrocatalytic effect of CNTs has been attributed to the activity of edge-plane-like graphite sites at the CNT ends and it would be further increased by functionalization of CNTs [14,15]. CNTs also reduce the electrode fouling which can greatly improve the reuse of such sensors. The low solubility of CNTs in most solvents is the major problem to control for their use as modifiers in the fabrication of biosensors. In order to overcome it, several strategies have been proposed for effective immobilization of CNTs on electrochemical transducers, like dispersion in different solvents or polyelectrolytes or incorporation in composite matrices using distinct binders [16–19].

Chitosan (Chit) is a linear β-1,4-linked polysaccharide (similar to cellulose) that is obtained by the partial deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi [20]. It possesses many advantages such as excellent strong film forming ability, high permeability towards water, biocompatible, good adhesion and high mechanical strength. It also acts as a very good

[a] M. Satyanarayana, K. Koteshwara Reddy, K. Vengatajalabathy Gobi
Department of Chemistry, National Institute of Technology Warangal, Andhra Pradesh 506004, India
tel: +91-870-2462674
*e-mail: drkvgobi@gmail.com

matrix for immobilization of analyte molecules. Chitosan was used as a dispersant to bind with the electrode surface and to form a stable CNT–chitosan composite film on the electrode surface.

In this work, the electrochemical sensor was fabricated by using functionalized multi-walled carbon nanotubes (MWCNT) in combination with chitosan. MWCNTs were dispersed in chitosan solution to form stable thin films on glassy carbon electrode. The experimental results showed that MWCNT-Chit film had good electrocatalytic activity towards the detection of serotonin at biological pH in the presence of main biological interferents. Optimum conditions for sensitive determination of serotonin were established using the MWCNT-Chit modified glassy carbon electrode, and this method could be effectively applied to determine serotonin in biological fluids like urine and blood serum.

2 Experimental

2.1 Chemicals and Materials

Serotonin and ascorbic acid were purchased from Tokyo chemical industry, Japan. Chitosan (from crab shells, minimum 85% deacetylation) and uric acid were obtained from Sigma Aldrich, USA. MWCNTs (95% purity, 20–50 nm outer diameter and 2–5 μm length) were purchased from Sisco research laboratories, India. All other chemicals were of analytical grade (>99.5% purity) and were used without further purification. Phosphate buffer solution (PBS) of 50 mM was prepared by using 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4. The desired solution pH was obtained by adding 0.1 M NaOH. Artificial urine solution was prepared according to the procedure provided by Brooks and Keevil [21]. Typically, it was prepared using a mixture of 1.1 mM lactic acid, 2.0 mM citric acid, 25 mM sodium bicarbonate, 170 mM urea, 2.5 mM calcium chloride, 90 mM sodium chloride, 2.0 mM magnesium sulfate, 10 mM sodium sulfate, 7.0 mM potassium dihydrogen phosphate, 7.0 mM dipotassium hydrogen phosphate and 25 mM ammonium chloride in distilled water. The pH of this artificial urine solution was adjusted to 7.4 through the addition of 1.0 M hydrochloric acid. Blood collected from a healthy human was clotted, centrifuged and sterile–filtered, and the resultant human serum was used for analysis. All aqueous solutions were prepared using double distilled water.

2.2 Functionalization of MWCNT

MWCNT (120 mg) was treated with 10 mL of 3 M nitric acid for 24 h at 60 °C to functionalize it with –COOH groups, as reported elsewhere [22]. Nitric acid oxidizes CNTs and introduces –COOH groups at the ends and at the sidewall defects of the nanotube structure. The resultant black solid product was filtered and then washed several times with double distilled water until the filtrate solution became neutral (pH 7), and the obtained solid product was collected in petri dish and dried in an oven at 80 °C for 24 h.

2.3 Preparation of MWCNT-Chitosan Modified Electrodes

At first, GCE (3 mm diameter, BAS) was polished with alumina slurry (0.04 μm), washed thoroughly with double distilled water, sonicated in 1:1 aq.HNO_3, ethanol and double distilled water consecutively and finally dried at room temperature. A solution of chitosan (1% w/v) was prepared by dissolving 1 g of chitosan powder in 100 mL aq. acetic acid (1% v/v) solution and sonicated for 30 min. 4 mg of functionalized MWCNTs was added to 1 mL chitosan solution and sonicated for 30 min for homogeneous dispersion. Then, 10 μL of the resulting homogeneous suspension was cast on the surface of cleaned GCE and dried over night at room temperature and it was denoted as MWCNT-Chit/GCE.

2.4 Instrumentation

Cyclic and differential pulse voltammetry measurements were carried out using CHI electrochemical analyzer (Model 619d, USA), and electrochemical impedance measurements were carried out using Zahner-elektrik workstation (Model IM6e, GmbH, Germany) equipped with Thales 3.08 USB software. Impedance measurements were carried out at the open circuit potential in the frequency range from 100 kHz to 10 mHz under an excitation of sinusoidal wave of ±5 mV amplitude. A sine wave with an amplitude of 10 mV was employed to perturb the system with 64 sine waves per decade. Differential pulse voltammograms (DPVs) were optimized by changing the parameters, step increment in the range of 5–20 mV, pulse amplitude 50–250 mV, pulse width 50–250 ms and cycle period 400–800 ms, so as to record DPVs with high peak currents, low noise and low background current. All the electrochemical measurements were performed in a conventional electrochemical cell of 20 mL volume with bare or modified GCE as working electrode, Pt spiral wire as auxiliary electrode and Ag|AgCl (3 N KCl) electrode as reference. All the potentials were referred against Ag|AgCl (3 N KCl) electrode throughout the manuscript. The electrochemical experiments were carried out in aqueous phosphate buffer solution (PBS) of pH 7.4 which is equivalent to biological fluid pH, and experimental solutions were purged with nitrogen gas for 10 min to de-aerate the solution. All electrochemical measurements were performed at room temperature.

Surface morphology studies have been carried out using scanning electron microscope (SEM: TESCAN; model: VEGA 3 LMU). MWCNT-chitosan nanocomposite film was formed by casting 1% w/v chitosan solution dispersed with MWCNT at 4 mg/mL level. A thin layer of gold was sputtered on the film to avoid charging during SEM analysis.

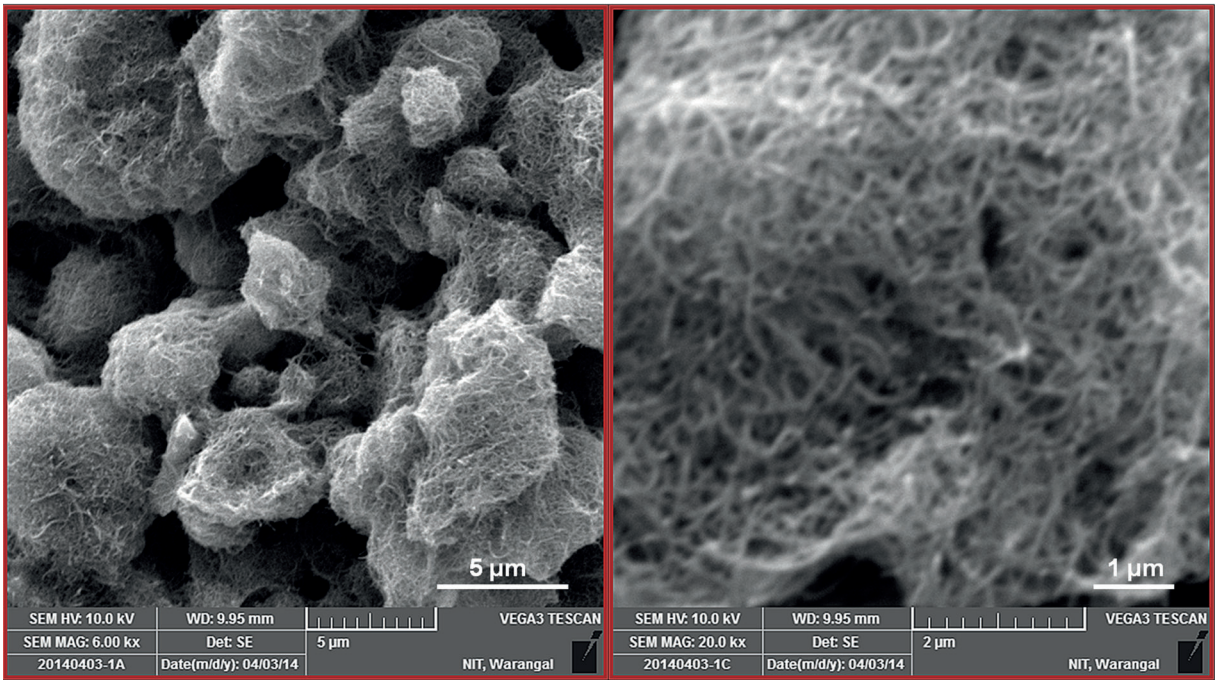

Fig. 1. SEM images of MWCNT-chitosan nanocomposite film.

3 Results and Discussion

3.1 Electroactive Surface Area of MWCNT-Chit/GCE and Surface Morphology

Active surface area of bare GCE and MWCNT-Chit/GCE was determined by electrochemical method. Cyclic voltammograms of 10 mM $K_3[Fe(CN)_6]$ in aq. 0.1 M KCl were recorded at both MWCNT-Chit/GCE and bare GCE at different scan rates. Further calculations have been carried out using Randles–Sevcik equation for a reversible process:

$$i_p = 2.69 \times 10^5\ A\ n^{3/2}\ D^{1/2}\ c\ \nu^{1/2}$$

where i_p refers to the peak current, n is the number of electrons transferred, A is the surface area of electrode, D is diffusion coefficient, c is the concentration of $K_3[Fe(CN)_6]$ and ν refers to the scan rate. The surface area can be calculated from the slope of the i_p vs. $\nu^{1/2}$ plot, using the D value of $K_3[Fe(CN)_6]$, 6.7×10^{-6} $cm^2 s^{-1}$ [23]. Active surface area for bare GCE was found to be 0.071 cm^2 which is equal to the geometrical surface area of 3 mm diameter electrode, whereas it is 0.262 cm^2 for MWCNT-Chit/GCE. From these observations, we can conclude that the surface area of MWCNT-Chit/GCE was nearly four times higher than that of bare GCE, and it is due to the distribution of nanostructural MWCNT on the surface of GCE.

The morphological features of the MWCNTs with chitosan matrix have been examined by scanning electron microscope (SEM) analysis. The SEM profile of MWCNT-Chit composite (Figure 1) shows densely populated and uniformly distributed MWCNTs all over the surface with high porosity, which could increase the active surface area. It clearly shows that the carbon nanotubes are well and homogeneously dispersed in the film and that individual ribbon-like structures of MWCNT with a diameter of ~20–50 nm are seen. Hence, the increase in oxidation peak current at MWCNT-Chit/GCE in electrochemical measurements could be attributed to both chitosan and MWCNT, where chitosan helps in excellent distribution of MWCNT and the surface area increased due to the nanostructured MWCNT.

3.2 Electrocatalytic Oxidation of Serotonin

Cyclic voltammetry is the most important technique to establish basic redox processes of a compound. Cyclic voltammograms of 0.2 mM serotonin in PBS of pH 7.42 at MWCNT-Chit/GCE and bare GCE were recorded at a sweep rate of 100 $mV s^{-1}$ and are shown in Figure 2. Serotonin is irreversibly oxidized showing an anodic peak at +250 mV at both MWCNT-Chit/GCE and bare GCE, whereas no peak was observed in blank PBS. At MWCNT-Chit/GCE, the oxidation peak current of 5-HT was very high compared to that at bare GCE. As can be seen, a poorly defined oxidation peak of serotonin with very low peak current was observed at bare GCE. However, at the MWCNT-Chit/GCE, the electrodic response of serotonin has improved considerably, and the peak current has greatly enhanced. A significant increase in peak current could be due to the high surface area, because of the nanostructural morphology of MWCNT.

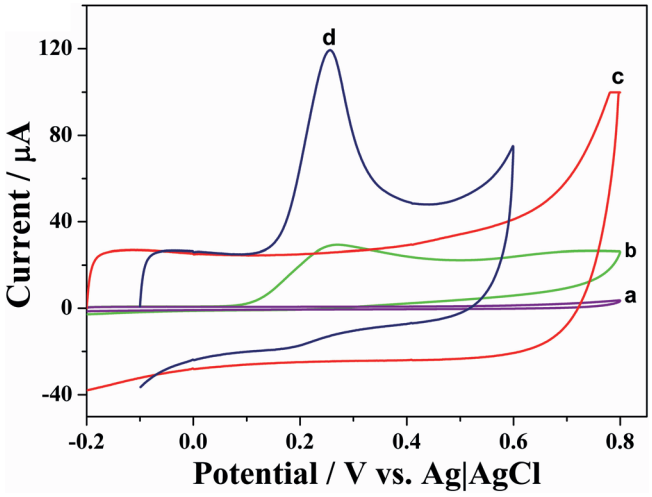

Fig. 2. Cyclic voltammograms recorded at bare glassy carbon electrode (a, b) and at MWCNT-Chit/GCE (c, d) in pure PBS (a, c) and in PBS with 0.2 mM serotonin (b, d). Scan rate = 100 mV s^{-1}.

The influence of scan rate on the electrochemical response of 5-HT at MWCNT-Chit/GCE was investigated by cyclic voltammetry, and the respective results are shown in Figure 3. The oxidation peak currents gradually increased with increasing scan rate (Figure 3A), in the range of 10 to 200 mV s^{-1}. When the peak current values were plotted against the square root of scan rate ($v^{1/2}$), a linear relationship with a regression coefficient of 0.990 was obtained (Figure 3B). This behaviour suggests that the oxidation of 5-HT at the nanobiocomposite MWCNT-chitosan modified electrode is diffusion controlled. A positive shift of the anodic peak potential ($E_{p,a}$) with the increase of the scan rate also observed in Figure 3A. It was found that $E_{p,a}$ increased linearly with ln(scan rate) (Figure 3C). The number of electrons involved in the reaction can be calculated according to the Laviron's equation [24]. Accordingly, the slope of the E_p vs. ln(scan rate) plot, $b = RT/\alpha n_\alpha F$, where b is the slope and α for the totally irreversible electrode process is assumed as 0.5. The obtained value for n_α is around 2, which indicates that two electrons are involved in the oxidation of serotonin.

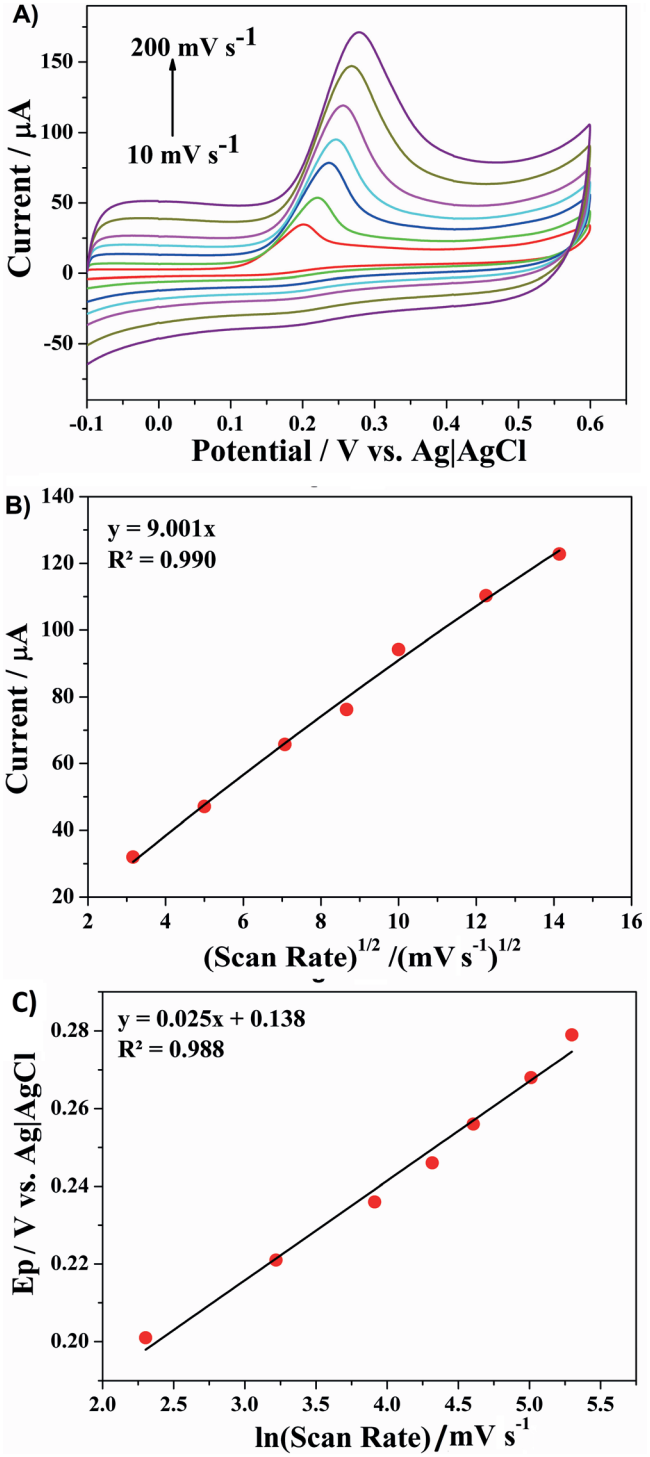

Fig. 3. A) Cyclic voltammograms of 0.2 mM serotonin in PBS using MWCNT-Chit/GCE at scan rates 10, 25, 50, 75, 100, 150 and 200 mV s^{-1}. B) Plot of i_p vs. square root of scan rate. C) Plot of E_p vs. ln(scan rate).

3.3 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) is a powerful tool for studying the interfacial properties of surface-modified electrodes. The electron-transfer resistance (R_{CT}) of the electrode surface is an important parameter, which is obtained from the Nyquist plot. Figure 4 shows the Nyquist plots of the MWCNT–chitosan modified electrode and bare GCE in aqueous 1 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl. The Nyquist plot of MWCNT-Chit/GCE (Figure 4a) is nearly a straight line and represents Warburg impedance alone, which indicates the good conductivity of the modified electrode whereas a large electron-transfer resistance was observed at bare GCE (Figure 4b).

The cyclic votammetric and EIS experimental results show that the CNT–chitosan composite film might form a better electron conduction pathway on the electrode. It

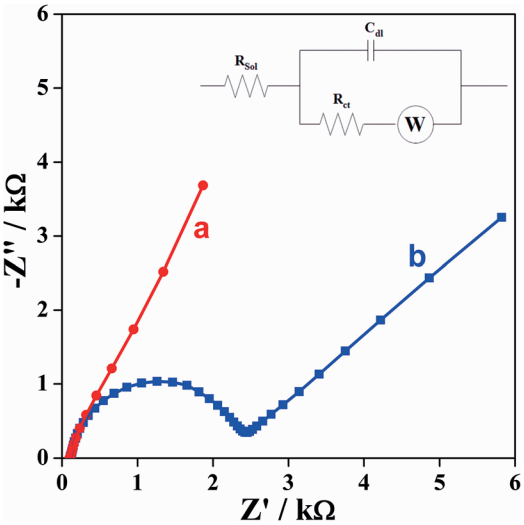

Fig. 4. The Nyquist plots of EIS measurements of MWCNT-Chit/GCE (a) and bare GCE (b) in 1 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl. Inset: Randles equivalent circuit.

seems that the CNTs played an important role as electron-transfer promoter thus made the electron-transfer easier. This is due to the nanolevel structural and morphological features of the modified CNTs with excellent electrical conductivity and due to the extensive uniform dispersion of CNTs resulted in the chitosan matrix.

3.4 Determination of Serotonin by Differential Pulse Voltammetry (DPV)

The voltammetric response of the MWCNT-Chit/GCE electrode to the presence of 5-HT was investigated by DPV. At the MWCNT-chitosan modified electrode, the anodic peak at +0.25 V was monitored and a good voltammetric profile was obtained with the optimum parameters of 100 mV pulse amplitude, 200 ms pulse width, 10 mV step increment and 500 ms cycle period. After optimizing the operating conditions, differential pulse voltammetric measurements were carried out using the MWCNT-Chit/GCE in PBS buffer containing 5-HT in the concentration range of 0.05–16 μM and the results are shown in Figure 5A. The dependence of the oxidation peak currents on the concentration of 5-HT is shown in Figure 5B. The anodic peak current increased parabolically as the concentration of serotonin increases, and two linear regions are seen in Figure 5B. Such a parabolic increase could be attributed to a slow heterogeneous electron-transfer process at the interface, and thus the peak current could not increase proportionately with the concentration of serotonin. Similar results were reported previously for electrochemical biosensors based on chemically modified electrodes [25,26]. The slope of the linear region in the low concentration range of 0.05 to 1.0 μM is high compared to the other, and the linear regression equations are expressed as: i (μA) = 9.92 $C_{serotonin}$ (μM) with the regression coefficient R^2 = 0.9912 in 0.05–1 μM

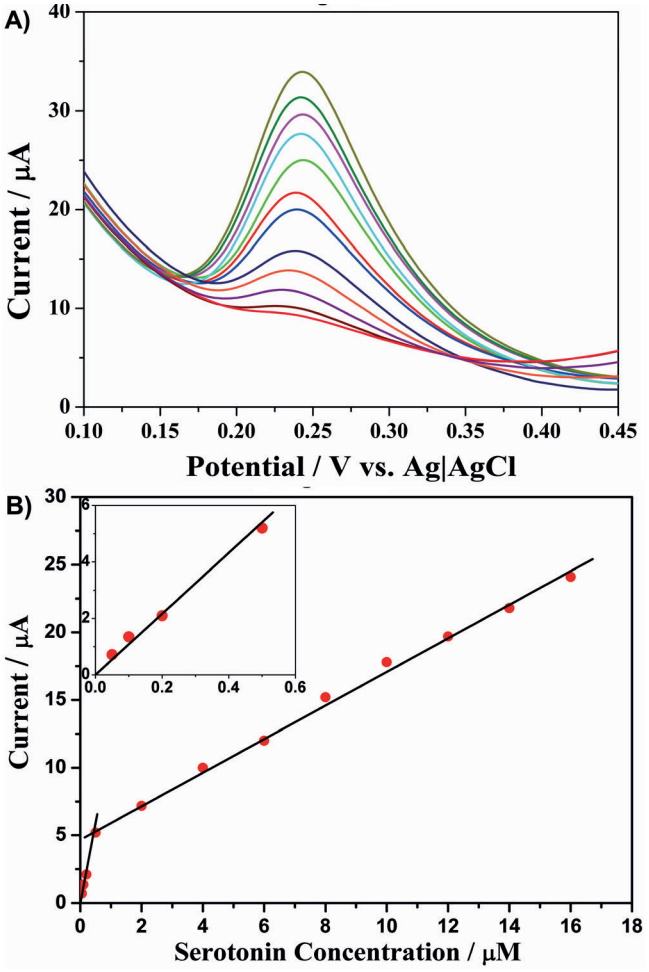

Fig. 5. A) DPVs of serotonin of different concentrations (0.05–16 μM) in PBS recorded at MWCNT-Chit/GCE. B) Plot of the peak current against the concentration of serotonin.

range and i (μA) = 4.3214 + 1.2726 $C_{serotonin}$ (μM) with the regression coefficient R^2 = 0.988 in 2–16 μM range. Considering that the change in peak current by three relative standard deviations (*RSDs*) as the detection limit, the low-detection-limit of the present sensor was determined from the plot to be 50 nM with a linear determination range of 5×10^{-8}–1.6×10^{-5} M. The performance of the present electrochemical sensor is compared with those reported in the literature (Table 1). The low-detection-limit obtained in the present study using MWCNT-Chit/GCE by DPV is comparable to the low-detection-limits reported previously by using various electrochemical methods with different modified electrodes [8–10,27–29]. Since the concentration of serotonin in healthy human is in the range of 0.5 to 1.4 μM [9], the present sensor system with a low-detection-limit of 50 nM is well applicable for the analysis of serotonin in diluted physiological samples. The present sensor system was then subjected to interference studies and for the determination of serotonin directly from physiological samples.

Table 1.
Electrochemical detection of 5-HT using different modified electrodes. CILE: carbon ionic liquid electrode; NE: norepinephrine.

Electrode	Method	Linear range (μM)	Limit of detection (nM)	Interferents overcame	Reference
Graphene oxide grafted poly(lactic acid)	Amperometry	0.1–100	80	AA, DA, UA, H_2O_2	[27]
MWNT-intercalated graphite electrodes	DPV	1–15	200	AA, DA	[28]
Nafion/$Co(OH)_2$–MWCNTs/CILE	DPV	0.05–75	23	levodopa	[9]
Carbon Nanotube/Ionic Liquid	DPV	5–900	3.2×10^3	NE	[8]
Cyclodextrin host–guest recognition	DPV	2–200	Sub-μM level	–	[10]
Acetylcholine/GCE	DPV	1–30	500	AA, DA	[29]
MWCNT-chitosan/GCE	DPV	0.05–16	50	AA, DA, UA	Present Work

3.5 Interference Studies

Electrochemical response of serotonin in the presence of major electroactive biological components such as ascorbic acid (AA), uric acid (UA), and an other important neurotransmitter such as dopamine (DA) has been carried out at MWCNT-Chitosan modified electrode using DPV method. Figure 6 shows the DPVs at both bare GCE and MWCNT-Chitosan modified electrode in the mixture of 0.02 mM of AA, UA and DA each and different concentrations of serotonin at micromolar levels (4–16 μM) in PBS (pH 7.4). At bare GCE, only one peak with a very low peak current was observed to the presence of 16 μM 5-HT together with AA, UA and DA 0.02 mM each in PBS (Figure 6b). At the modified MWCNT-Chit/GCE, distinct peaks for AA, DA, UA and 5-HT each were observed and the peak currents for 5-HT increased as the concentration of 5-HT increases. Even higher concentrations of AA, UA and DA together did not influence the current response of serotonin in PBS and also a good peak separation was observed between these interferences and serotonin. From the experimental results, it is clearly evident that the proposed electrochemical sensor using MWCNT-Chit/GCE can be used effectively for the detection of serotonin even in the presence of various possible interferents.

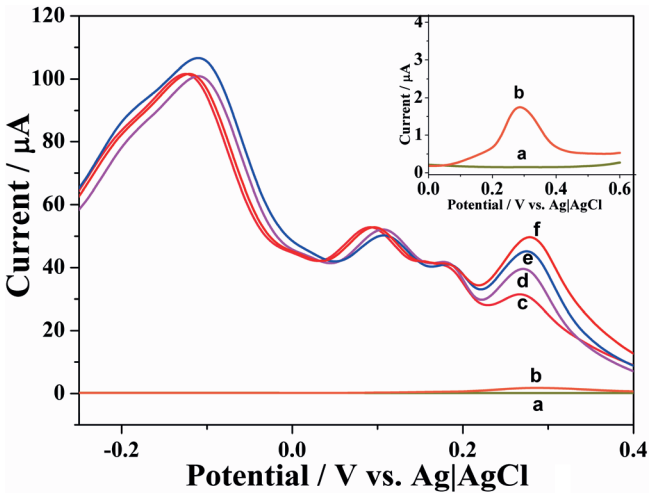

Fig. 6. DPVs recorded at bare GCE (a, b) and at MWCNT-Chit/GCE (c–f) in pure PBS alone (a) and in a mixture AA, DA and UA 0.02 mM each in PBS (b–f) together with the presence of serotonin at different concentrations (4 (c), 8 (d), 12 (e), 16 (b, f) μM). Inset: Enlarged view of the DPVs (a, b).

3.6 Repeatability and Reproducibility

Reproducibility of the MWCNT-Chit/GCE was examined by recording differential pulse voltammograms of 0.02 mM serotonin repetitively using a same electrode. During several successive measurements for 25 times in a time period of 7 days, only a slight decrease in the peak current by ~4.5% was observed, suggesting that the MWCNT-Chit/GCE has excellent stability and repeatability. Further, no much change is observed in the oxidation peak potential of 5-HT at the nanocomposite modified electrode even after one month of its fabrication, though the modified electrode kept at ambient conditions. The *RSD* of the anodic peak currents for the determination of 5-HT in multiple experiments using six independent MWCNT-Chit/GCE electrodes was found to be only 2.6%, indicating good reproducibility of the fabricated electrode. From these observations, we could conclude that the MWCNT-chitosan composition formed a reproducible and reusable electroactive nanobiocomposite film on GCE for the determination of 5-HT.

3.7 Determination of 5-HT in Serum and Artificial Urine Samples

Determination of 5-HT was investigated in serum and in artificial urine samples using DPV analysis at MWCNT-Chit/GCE by standard addition method. Recovery of 5-HT from artificial urine was determined from the DPV responses obtained to the direct addition of 5-HT (0.2–3 μM) in artificial urine without any buffer (Figure 7A). The DPVs recorded in the presence of 5-HT showed a well-defined anodic peak at ~+0.25 V, while that recorded in artificial urine alone did not show any peak. The recovery of 5-HT from artificial urine samples at different concentrations was listed in Table 2, which varies from 97.3% to 104.0%. The results of the recovery analy-

Table 2.
Determination of 5-HT in artificial urine and blood samples using MWCNT-Chit/GCE.

Sample	5-HT ($\times 10^{-6}$ M)	Found ($\times 10^{-6}$ M) [a]	Average recovery (%)	*RSD* (%) [a]
Urine sample	0.5	0.52	104.0	0.9
	1	0.97	98.0	1.3
	3	2.92	97.3	2.9
Blood samples	0.5	0.51	102.0	1.2
	2	1.89	101.4	0.7
	4	3.98	99.5	1.7

[a] Mean value of six measurements.

sis showed that the proposed method can be used efficiently for the determination of trace amounts of 5-HT directly from urine samples. Electrochemical response of the MWCNT-Chit/GCE towards 5-HT in serum samples was investigated by standard addition method, and serum samples were diluted 25 times using PBS (pH 7.4) buffer in this study. DPVs recorded in the presence of 5-HT at different concentrations (0–6 μM) in diluted serum are shown in Figure 7B. In the absence of 5-HT, a weak anodic peak was observed at ~ +0.15 V, which could be attributed to the presence of uric acid (vide supra, Figure 6). In the presence of as low as 0.5 μM 5-HT, the anodic peak corresponding to the oxidation of 5-HT appears at ~ +0.25 V, and the peak current increases with the concentration of 5-HT. The DPVs obtained for 0.5 μM serotonin in diluted blood samples show a very prominent peak. From the results in Figures 7A and 7B, it can be readily assumed that the present biosensor can detect as low as 0.1 μM serotonin from artificial urine and blood samples. The recovery limits for the determination of 5-HT directly from serum samples at various concentrations (0.5, 2 and 4 μM) were given in Table 2, which varies from 99.5% to 102.0%. All these investigations clearly show that the present nanobiocomposite electrode could be used efficiently for the determination of 5-HT directly from serum and urine samples.

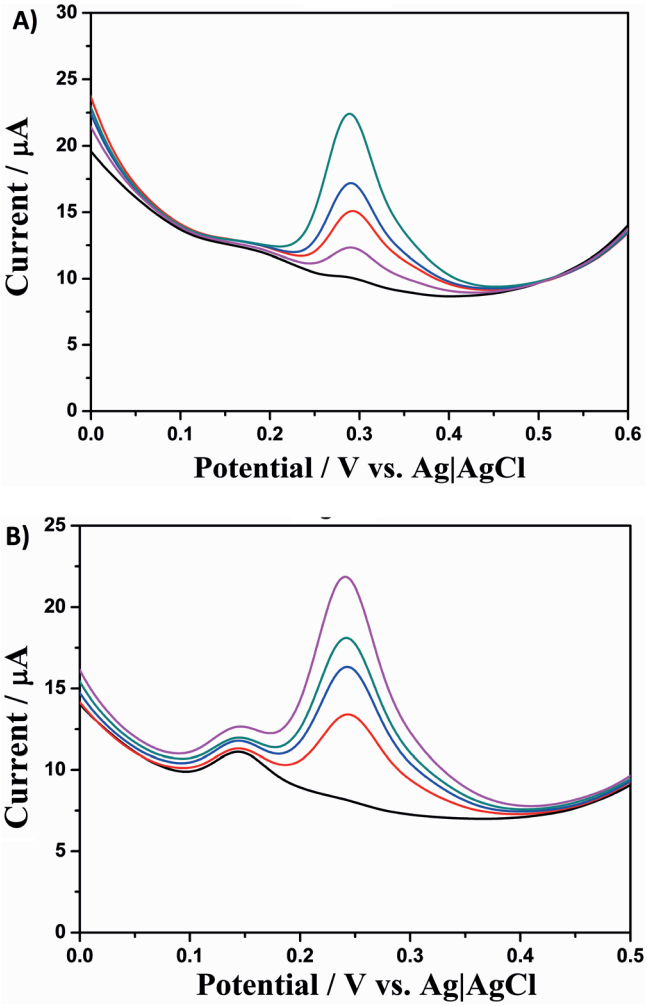

Fig. 7. DPVs of serotonin recorded at MWCNT-Chit/GCE A) in artificial urine (pH 7.4) at various concentrations (0, 0.2, 0.5, 1, 3 μM) and B) in diluted human serum (25 times) at various concentrations (0, 0.5, 2, 4 and 6 μM).

4 Conclusions

A highly sensitive electrochemical sensor was fabricated for selective determination of serotonin at biological pH. Serotonin showed a well defined oxidation peak at MWCNT-Chit/GCE. The linear range was found to be 5×10^{-8}–1.6×10^{-5} M and the low-detection-limit was found to be 5×10^{-8} M. MWCNT–chitosan composite film formed an efficient electron-transfer linkage between analyte and electrodic surface for the oxidation of serotonin, characterized by the enhancement of the peak current. The nanocomposite film was highly stable for multiple analysis over a long period due to the unique binding character of chitosan biopolymer. The fabricated sensor was successfully applied for the determination of serotonin in pure form in the presence of various possible biological interferences. The present sensor system exhibited very good recovery limits for the determination of 5-HT directly from urine and blood serum samples.

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