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MWCNT-Chitosan-ZrO₂ Nanocomposite Modified Sensor for Sensitive and Selective Electrochemical Determination of Serotonin

Harsha Devnani¹, Soami Piara Satsangee¹ and Rajeev Jain²

University Science Instrumentation Centre, Dayalbagh Educational Institute, Dayalbagh, Agra, India¹

School of Studies in Chemistry, Jiwaji University, Gwalior, India²

ABSTRACT: A novel sensitive electrochemical sensor has been developed by modification of glassy carbon electrode (GCE) with multi-walled carbon nanotube (MWCNT), chitosan (CHIT) and zirconium oxide (ZrO₂) nanoparticles. The morphological characteristics of nanocomposite (MWCNT-CHIT-ZrO₂ or MCZ) were studied by scanning electron microscope and atomic force microscopy. The electrochemical behavior of serotonin at nanocomposite modified GCE (MCZ/GCE) was investigated in pH 6.5 Britton Robinson buffer solution using cyclic voltammetry and square wave voltammetry. MCZ/GCE showed an enhancement in the current response as compared to bare GCE and showed a linear response to serotonin in the range 800 to 5000 nM. The limit of detection was found to be 0.6 nM, which is lower than many other sensors reported for serotonin in literature. The modified sensor showed high sensitivity (9 nA/nM) and selectivity for serotonin. The proposed method was successfully employed for quantification of serotonin in human blood serum samples.

KEYWORDS: Serotonin, MWCNT, Chitosan, ZrO₂, Glassy carbon electrode, Voltammetry

I. INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important catecholamine neurotransmitter which plays a vital role in the regulation of mood, sleep, emesis, sexuality and appetite. Of the total serotonin content, the central nervous system contains only less than 2% of it, but still plays an important role in various brain functions. It is synthesized from amino acid tryptophan. It acts as a pacemaker in various regions of the brain at times of alertness while co-ordinating sensory and motor activity. It also contributes to proper execution of feeding, sleeping and reproductive behaviours [1]. Fluctuations in the normal serotonin level are associated with a number of diseases. While low levels of 5-HT can cause depression, anxiety and migraines, high levels can result in toxicity and in extreme cases fatal effects such as serotonin syndrome, obsessive compulsive disorder, and autism [2-6].

Thus, detection and determination of 5-HT are important and research thrust is to work out simple, sensitive, selective and fast analytical methods for its determination. Electrochemical method is given preference in this context over traditional methods due to their simplicity and sensitivity for determination of 5-HT [7]. Traditionally, neurotransmitter's concentrations are determined by chromatographic, spectrophotometry, fluorescence, chemical luminescence and capillary electrophoresis methods [8-11]. These techniques are complex, time-consuming, inconvenient for real-time and in-situ monitoring [12]. Since, 5-HT is electrochemically active it can be determined using electrochemical sensors which are fast and inexpensive. Some major problems are associated with the determination of 5-HT. Presence of dopamine (0.22 V) poses interference as it has oxidation potential close to that of 5-HT (0.38 V). Ascorbic acid and uric acid also have a similar oxidation potential and thus interfere with determination of 5-HT. Thus, the focus is on developing faster, cost-effective, sensitive and selective electrodes for the determination of 5-HT. Electroanalytical techniques fulfill these criteria but with bare electrodes they offer low sensitivity. Using modified electrodes, low sensitivity and poor selectivity can be overcome [5].

The modification of electrodes with electron transfer mediators is gaining impetus in field of analytical chemistry. The importance of using any mediator is to cause an effect in reduction of the overpotential required for the electrochemical

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reaction, which results in sensitivity enhancement and an improvement of selectivity [13-14]. CNTs have attracted much attention in recent years in the field of electrochemical biosensing as they offer high effective surface area, mass transfer, catalysis and control over local microenvironment. CNTs are porous nanostructured carbon materials, which hold promise as immobilization substances due to their significant mechanical strength, excellent electrical conductivity and good chemical stability. A decrease in overpotential can be observed with CNTs owing to their catalytic property, producing a more reversible voltammetry than that displayed by same material in macroelectrode form [15,16]. Thus, CNTs can efficiently enhance electron transfer reactions.

Chitosan, a natural biopolymer obtained from chitin, possesses significant film-forming and adhesion properties. It has good biocompatibility and is non-toxic. Chitosan provides a hydrophilic environment to biomolecules as it has abundant amino and hydroxyl groups. Thus, it offers to improve hydrophilicity as well as biocompatibility via covalent grafting techniques. The co-existence of chitosan and CNTs creates a positive charge which is favorable for the further immobilization of biomolecules that are negatively charged [17-19].

Further, metal nanoparticles can be used to enhance the amount of immobilized biomolecules in fabrication of a sensor. This capability is taken advantage of in biosensor design as metal nanoparticles have been used to catalyze biochemical reactions. In this context, zirconia has emerged as an important catalyst [20,21]. In the present communication, we have fabricated a nanocomposite comprising MWCNT, Chitosan and ZrO_2 for the sensitive and selective determination of 5-HT. Electrochemical analysis was carried out under the optimized conditions using cyclic voltammetry (CV) and square wave voltammetry (SWV) in BR buffer; pH 6.5. MCZ/GCE showed a remarkable enhancement in the current response of oxidation process of 5-HT in comparison to the bare GCE which was described on the basis of the interlayer mass transport regime within the porous layer of MCZ [22-25]. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were employed for the structural and morphological analysis of the nanocomposite. Electrochemical response characteristics of 5-HT at MCZ/GCE were found to be a function of pH, scan rate and the concentration. The fabricated sensor was further successfully applied for the estimation of 5-HT in the human blood serum.

II. MATERIALS AND METHODS

Instrumentation:

All electrochemical studies were performed at a PC-controlled AUTOLAB PGSTAT 302N (Eco-Chemie B.V., Utrecht, The Netherlands) potentiostat-galvanostat with IME663 and software NOVA 1.8. EIS was carried out using FRA 2 module. A standard three electrode electrochemical assembly was used in the study that contained GCE and MCZ/GCE as working electrode, platinum wire as counter and $Ag | AgCl$ (3M KCl) as reference electrode that were fitted in one compartment cell connected with electrochemical workstation through Metrohm 663VA stand. The electrochemical cell was fitted with the nitrogen gas bubbler. All pH measurements were made on a Mettler Toledo pH meter fitted with a glass electrode and a $Ag | AgCl$ electrode as reference which was pre-standardized with buffers of known pH. All measurements were carried out at room temperature. A DL-180 ultrasonic apparatus was used for sonicating the suspension of MWCNT, MWCNT-CHIT and MCZ for modifying GCE. AFM study was carried out at Nanosurf Easyscan (Switzerland) with software Nanosurf 1.8. SEM was performed at Tescan (7718) involving the software, Mira TC.

Materials and Reagents:

ZrO_2 (<100 nm) was procured from Sigma Aldrich (India) and Chitosan was purchased from MP Biomedicals (India). MWCNT (10 nm) was procured from Drop Sens. Serotonin standard ($\geq 99\%$) was obtained from the Sigma Aldrich. Ultra pure water (Milli-Q water with resistivity 18M Ω .cm) was obtained from ELGA purification system (U.K). Standard solution of 5-HT (10 mM) was prepared by dissolving pure compound in 0.1 M perchloric acid and was further diluted with phosphate buffer to get the concentration in the working range. Solutions at all the stages of the study were prepared by using analytical grade reagents and were used without further purification.

Fabrication of MWCNT, MWCNT-CHIT and MCZ modified sensor:

Prior to modification, the bare working electrode (GCE) was polished against the alumina slurry (particle size-0.05–0.3 μ m) spread over the Buehler cloth, followed by gently blowing under the nitrogen stream to remove the residual alumina particles and then sequentially rinsed with de-ionized water. For modification, MWCNT-CHIT was

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obtained by dispersing 0.5 mg of MWCNT in 1mL of 0.5 (wt) % of CHIT solution (in DMF) and another suspension of MCZ was obtained by dispersing 0.5 mg of ZrO_2 in 1mL of the suspension containing 0.5 mg of MWCNT and 0.5 (wt) % of CHIT solution with ultrasonication for 5 hours. MWCNT (0.5 mg in DMF) alone was also used as a modifier. 1 μ L of each of the modifiers was withdrawn and drop casted over the surface of GCE to get MWCNT/GCE, MWCNT-CHIT/GCE and MCZ/GCE. The modified electrodes were kept for drying at room temperature.

Analytical Procedure:

The stock solution of 5-HT (10 mM) was prepared in 0.1 M $HClO_4$. Working solutions were prepared by further dilution with the supporting electrolyte to get the desired concentration range. Initially, a series of BR buffer (2.5-12 pH) was prepared in ultrapure water and used as supporting electrolytes. About 10 mL of electrolyte solution containing appropriate amount of standard 5-HT or sample were added to the electrolytic cell. Prior to all the electrochemical measurements, all the solutions were purged with the pure N_2 gas to remove the interference due to O_2 . Then the electrodes were immersed and the SWV and CV were recorded with potential between -0.2 to 0.8 V. The surface of GCE was renewed prior to the modification by abrading the surface smoothly against the alumina slurry spread over the Buehler cloth. All voltammetric measurements were carried out at ambient temperature.

III. RESULTS AND DISCUSSION

Characterization of the modifiers:

The surface morphology of the modifiers was studied using SEM technique. The modifier suspension was drop-casted over the surface of ITO (Indium Tin Oxide) section for carrying out analysis. The SEM images of MWCNT (A) MWCNT-CHIT (B) and MCZ (C) are depicted in Fig. 1. The MWCNT and MWCNT-CHIT modifiers show a rough surface owing to random dispersion of MWCNT in the within the chitosan matrix as well as the solvent (DMF). The MCZ nanocomposite SEM image (C) exhibits a different surface morphology due to the presence of globular ZrO_2 nanoparticles with porous topology within the chitosan and graphene matrix. This is outcome of the electrostatic interactions between the cationic chitosan and the surface charged ZrO_2 nanoparticles [26]. The formation of a co-dispersion of MWCNT, CHIT and ZrO_2 resulting in a nanocomposite is thus explained.

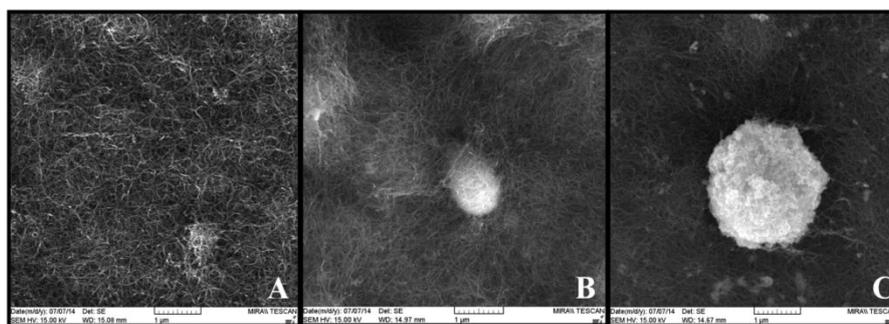


Fig. 1. Scanning electron microscopic images of MWCNT (A), MWCNT-CHIT (B) and MCZ (C) at 35 KX

The surface topology which is indication of roughness parameters of the electrode surface was interpreted using AFM. The surface area was found to be 26.8 μm^2 ; roughness average, 123.7 nm; root mean square, 150.2 nm; peak-valley height, 960.45 nm; peak height, 377.35 nm; valley depth, 529.1 nm and mean value to be 120.53 μm The data implies that the modifier is well-coated onto the surface of GCE. The nanocomposite coatings were not removed from the surface of GCE by several flushings with deionized water (Fig. 2).

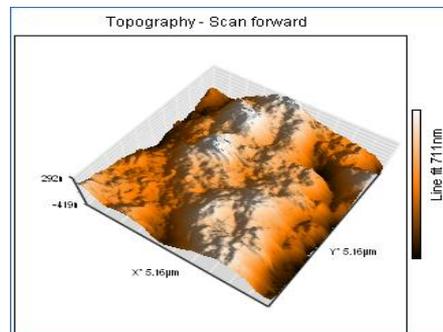


Fig. 2. 3D AFM image of MCZ/ITO

Electrochemical characterization of modified electrodes:

The electrochemical response of $K_3Fe(CN)_6$ at bare and modified GCE (Fig. 3) was also checked to examine their electrocatalytic properties. In comparison to bare GCE, better electrochemical response was observed for $K_3Fe(CN)_6$ at modified electrodes. The maximum enhancement was seen in case of MCZ/GCE with decreasing over potential. The peak separation was seen to be considerably reduced at the MCZ/GCE. At bare GCE the anodic peak potential (E_{pa}) was found to be 266 mV and cathodic peak potential (E_{pc}) 149 mV. The separation of redox potential peaks (E_p) 111 mV and the ratio of peak current (I_{pa}/I_{pc}) was 1.06. At MCZ/GCE, a pair of redox peak is obtained with strong increase in both anodic and cathodic peak current. The E_{pa} was found at 239 mV and E_{pc} at 81 mV. The separation of redox potential peaks E_p was found to be 117 mV and the I_{pa}/I_{pc} was 1.02.

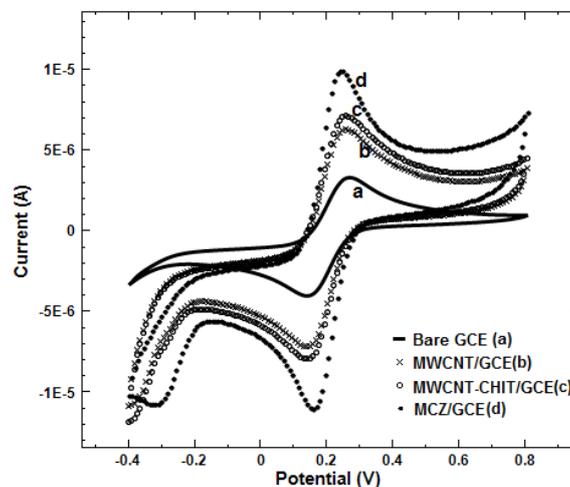


Fig. 3. Cyclic voltammetric response of the standard redox system ($K_3Fe(CN)_6$) (1 mM) at GCE (a), MWCNT/GCE (b), MWCNT-CHIT/GCE (c) and MCZ/GCE (d)

The electro-active area of the MWCNT/GCE, MWCNT-CHIT/GCE, MCZ/GCE and bare GCE were obtained by the CV using 1 mM $K_3Fe(CN)_6$ as a redox indicator at different scan rate. The peak current I_p can be calculated using Randles Sevcik equation at 25 °C [27]:

$$I_p = (2.69 \times 10^5) n^{3/2} A C_0 D^{1/2} \nu^{1/2} \quad (1)$$

For $K_3Fe(CN)_6$, $n=1$ and $D=7.6 \times 10^{-6} \text{ cm}^2/\text{s}$. From slope of I_p (μA) vs $\nu^{1/2}$ the area were determined to be 0.028 cm^2 for bare GCE, 0.051 cm^2 for MWCNT/GCE, 0.085 cm^2 for MWCNT-CHIT/GCE and 0.213 cm^2 for MCZ/GCE. Thus after modification the surface area of the electrodes was considerably increased.

Electrochemical behavior of 5-HT at the modified electrodes:

The electrochemical determination of 5-HT was analyzed at bare GCE and at the modified electrodes using CV and SWV techniques. Fig. 4A and 4B respectively represent the CV and SWV of 5-HT (1 μM) at bare GCE (a), MWCNT/GCE (b), MWCNT-CHIT/GCE (c) and MCZ/GCE (d).

A well-defined oxidation peak is observed at bare GCE and also at each of the modified electrodes that can be ascribed to the oxidation of 5-HT to its respective quinone (serotonin quinoneimine) [28,29]. While at MWCNT/GCE and MWCNT-CHIT/GCE the current response is enhanced but the base line current is also considerably raised. At MCZ/GCE, maximum current enhancement is observed and base line current was also not raised much. The enhancement in current response is attributed to increase in the surface area owing to porosity of the modified layer. The enhanced anodic peak current intensity is due to the trapping of the solution containing 5-HT within the multiple layer of the porous nanocomposite [22,25]. Thus, MCZ/GCE proved to be highly sensitive towards the oxidation of 5-HT and thereby it was applied for rest of the electrochemical measurements.

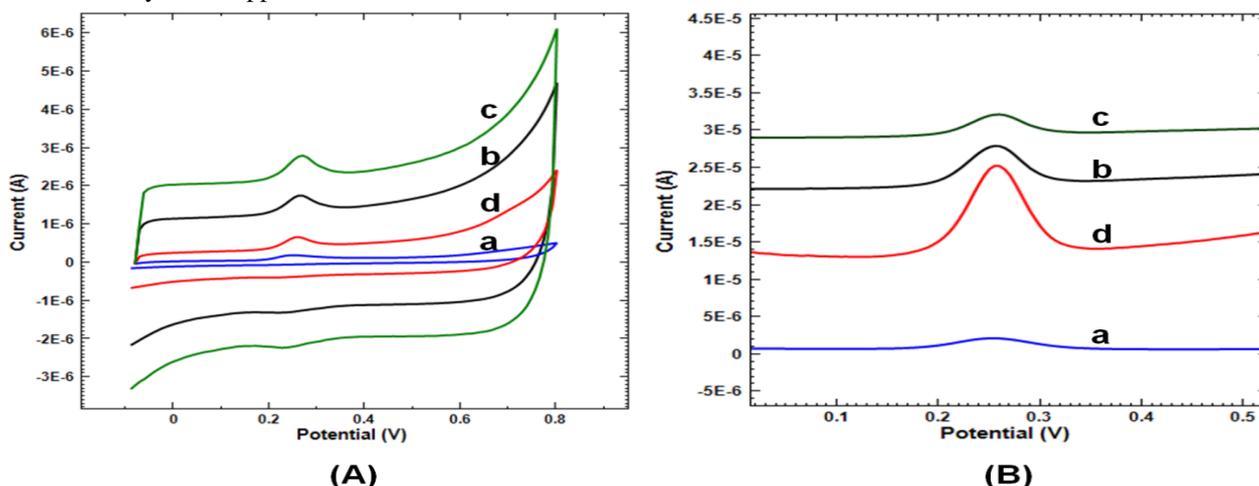


Fig. 4. Cyclic voltammograms (A) and square wave voltammograms (B) for determination of 5-HT (1 μM) in pH 6.5 BR buffer at bare GCE (a), MWCNT/GCE (b), MWCNT-CHIT/GCE (c) and MCZ/GCE (d)

Effect of supporting electrolyte and pH:

The nature of electrolyte and pH of the medium affect the peak shape, height and stability of voltammograms (SWV). The influence of various supporting electrolytes such as KCl, phosphate buffer, acetate buffer and Britton Robison (BR) buffer on the electro-oxidation of 5-HT (1 μM) was investigated to optimize the experimental condition. BR buffer (0.1 M) gave maximum current response accompanied with optimum peak shape and stability. The influence of pH on the 5-HT oxidation (1 μM) at MCZ/GCE was examined over the pH range 2.5–9 by SWV. The anodic peak current intensity was found to be sensitive towards the pH and it gave a maximum response at pH 6.5. The sensor is thus suited for physiological applications as it shows best response in pH 6.5 while losing its sensitivity in both highly acidic and basic conditions. On the basis of these results phosphate buffer at pH 6.5 was selected as the optimum supporting electrolyte. A sequential shift of the anodic peak potential with the augmentation of solution pH was also observed (Fig. 5), indicating that the protons participate in the electrode reaction process of 5-HT [30,31]. A linear dependence of peak potential on pH of the supporting electrolyte was obtained following a linear equation: $E_p/mV = 787.4 - 62.64 \text{ pH}$; $R^2 = 0.997$. The peak potential at 25 °C is expressed as [32]:

$$E_p = E^\circ - (m/n) 59 \text{ pH} \quad (2)$$

where, E° is the standard potential, m and n are the number of protons and electrons respectively. Since the plot of E_{pa} (mV) vs. pH gave a slope of 62.64 mV/pH which is close to the expected value of 59 mV/pH, we can say m/n is 1. Thus, in accordance with Nernst equation (2) it can be proposed that the no. of protons and electrons participating in the electrode process of 5-HT oxidation are equal [28].

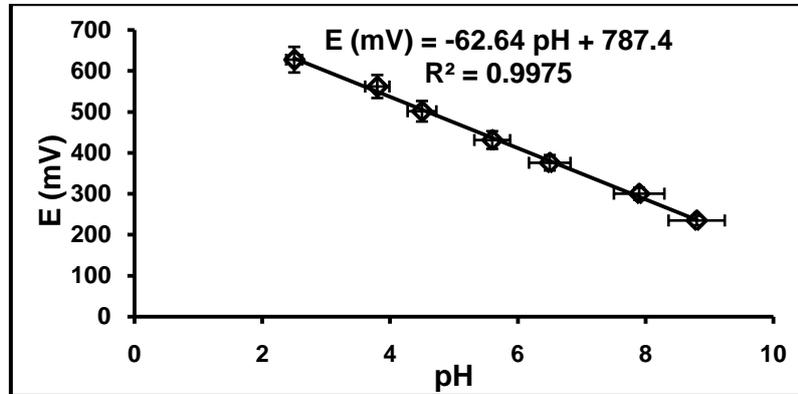


Fig. 5. Dependence of peak potential of 5-HT (1 μM) on the pH of supporting electrolyte (BR buffer) at MCZ/GCE

Effect of scan rates:

The influence of varying scan rates (40-180 mV/sec) on the electro-oxidation process of 5-HT (1 μM) at MCZ/GCE is shown in Fig. 7. The anodic peak current intensity was found to increase with increasing scan rate. A linear plot of peak current intensity (I_p) vs. square root of scan rate (v) was obtained (Fig. 6), following the linear equation: I_p (μA) = 0.0524 v (mV/s) - 0.2488; $R^2 = 0.99$, which implies that the redox process of 5-HT at the MCZ/GCE is diffusion-controlled [33]. Also, the difference between the anodic peak potential and the cathodic peak potential, ΔE_p was observed to increase with increasing scan rate. No reverse peak was observed at lower scan rates while relatively small cathodic peaks were observed at higher scan rates which implies irreversible or quasi-reversible electrode process for 5-HT.

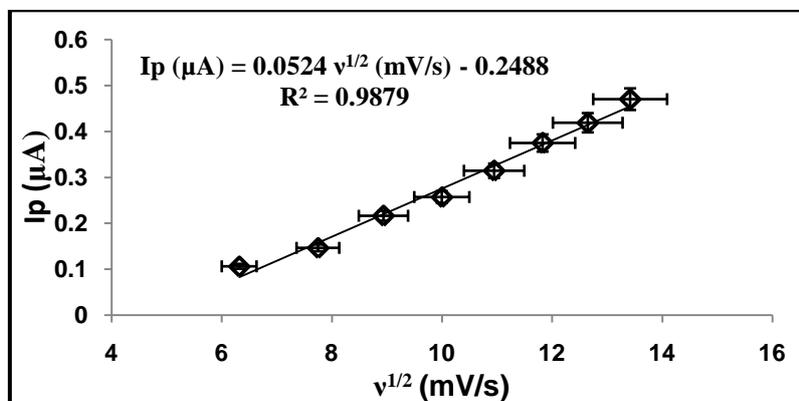


Fig. 6. Linearity plot of anodic peak current [I_p (μA)] obtained for 1 μM of 5-HT at various scan rates (40-180 mV/s) in pH 6.5 BR buffer vs. square root of scan rate [$v^{1/2}$ (mV/s)]

Determination of electrode dynamics parameters (α and n) and proposed mechanism of 5-HT:

The relationship between E_{pa} and $\ln v$ in the cyclic voltammetry is given by Laviron as follows [34]:

$$E_p = E^0 + [RT/(\alpha nF)] \ln [(RTk_s)/(\alpha nF)] - [RT/(\alpha nF)] \ln v \tag{3}$$

where k_s is the standard rate constant of the surface reaction; n , F , α , R and T have their usual significance. Substituting the respective values in Eq. (3), $\alpha n = 1.14$ is calculated from the slope of E_{pa} (V) = 0.0225 $\ln v$ + 0.3208 ($r = 0.99$). In most cases α is found to lie between 0.3 and 0.7, and it can usually be approximated by 0.5 [35]. So, the number of electrons (n) involved in the reaction is calculated to be $2.28 \approx 2$. It has been already described that protons participate in the reaction mechanism. So using the linearity, $E_{pa} = -0.0626 \text{ pH} + 0.7874$ ($r = 0.997$) and Eq. (4) [36], the number of protons part in the electrode reaction is calculated to be $m = 2.12 \approx 2$.

$$E_{pa} = E^0 - 2.303(mRT/nF) \log H^+ \tag{4}$$

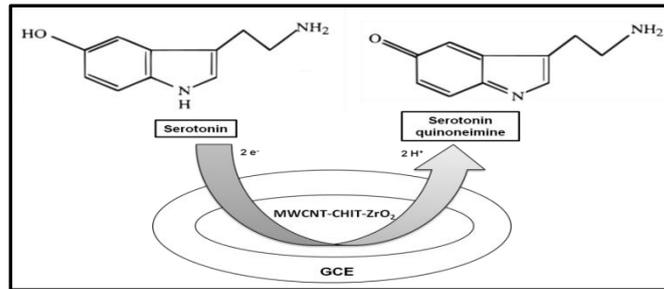
where E^0 is the formal potential, m is number of the protons involved in the reaction and n is 2 as calculated. Other symbols have their usual meaning. The 5-HT oxidation mechanism thus involves 2 electrons and 2 protons as depicted

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in scheme 1. The electrochemical oxidation of 5-HT is believed to occur at the phenol group in the molecule to form the corresponding ketone. The absence of the corresponding reverse peak indicates the instability of the oxidation product, which then undergoes chemical reaction to form a product that is easily oxidizable. [28, 29].



Scheme 1. 5-HT oxidation mechanism

Calibration curve, detection limit and method validation:

SWV recorded with the increasing amounts of 5-HT under the optimized conditions exhibited linearity of the peak currents with the increasing concentration (Fig. 7A). A linear relationship between the peak current and the 5-HT concentration was observed in the range of 800 to 5,000 nM. The calibration plot of I (μA) vs. concentration (nM) (Fig. 7B) is described by the equation:-

$$I (\mu\text{A}) = 0.009 (\text{nM}) + 1.5001; R^2 = 0.9986$$

Limit of detection (LOD) and limit of quantification (LOQ) (estimated as $3 S/m$ and $10 S/m$, respectively, 'S' being the standard deviation and 'm' being the slope of the calibration curve) were obtained as 0.6 nM and 2 nM. Various statistical parameters for the linear regression equation have been evaluated and reported in table 1.

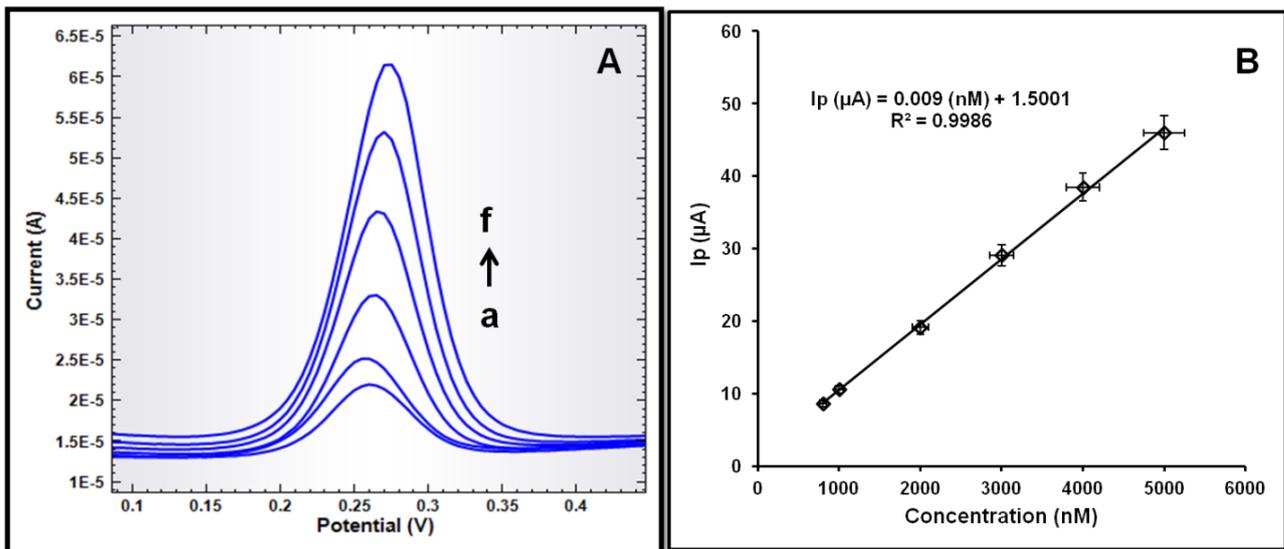


Fig. 7. (A) Square wave voltammograms of 5-HT (BR buffer; 6.5 pH) at different concentration levels (a – f: 800 – 5,000 nM) at MCZ/GCE; (B) Plot of concentration vs. current

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Table 1. Square-wave voltammetric method validation parameters for standard linearity

Linearity parameters	Results
Slope	0.009
Intercept	1.5001
Standard deviation	0.0018
Correlation coefficient	0.9986
Standard error of estimation	0.0677
Limit of detection (nM)	0.6
Limit of quantification (nM)	2

Reproducibility and stability of fabricated sensor:

The reproducibility of the modified electrode was investigated by the successive measurement of 5-HT (1 μ M) at MCZ/GCE by SWV (N = 6). The corresponding relative standard deviation of 1.7 % showed that the results are satisfactorily reproducible.

Another important attraction of the proposed modified electrode is that the resulting modified electrode is of long term stability. Stability of the proposed modified electrode was tested by measuring the decrease in voltammetric current during repetitive SWV measurements of 5-HT (1 μ M) with MCZ/GCE stored in air over a period of two weeks. After two weeks, it was found that the corresponding current response fell less than 2 % and showed a RSD of 1.94 %. The reproducibility results are reported in table 2.

Table 2. Reproducibility results at MCZ/GCE in the presence of 1 μ M 5-HT

Sensor Reproducibility			Sensor Repeatability	
Sensor	Average Current, I (μ A)	R.S.D (%)	Average Current, I (μ A)	R.S.D (%)
Sensor 1	0.98 ^a	1.7	0.96 ^a	1.94
Sensor 2	0.95 ^a	1.6		
Sensor 3	0.96 ^a	1.82		
Average	0.96 ^b	1.7		

^aAverage of 6 replicates

^bAverage of three sensor

Interference Study:

The influence of various compounds that are expected potentially to interfere with the determination of 5-HT (1 μ M) were studied under the optimum conditions in pH 6.5 BR buffer. The tolerance limit was defined as the maximum concentration of the interfering substance that caused an error of less than ± 5 % for the determination of 5-HT. The interfering species were chosen from substances commonly found with 5-HT in pharmaceutical formulations and/or in biological fluids. In biological environments, the main interference is the presence of high concentration of ascorbic acid and uric acid. While ascorbic acid didn't pose any interference up to 150 folds, uric acid was tolerated up to 300 folds. Dopamine also interferes with determination of 5-HT as they have close oxidation potentials. At MCZ/GCE, peak separation of 82 mV among these interferents was observed and thus allowing reliable and selective determination of 5-HT. The results depicted the high selectivity of the MCZ/GCE for the determination of 5-HT.

Assay of 5-HT in human blood serum:

For the purpose of the practical applicability of the proposed method and the modified sensor, determination of 5-HT in human blood serum were performed by spiking diluted samples with known amounts of 5-HT. The human blood serum was obtained from a blood bank. To evaluate the accuracy of the method, standard addition method was used by spiking different concentration of 5-HT in the sample solution. SWV of unspiked and spiked samples was performed under optimized conditions at MCZ/GCE. Percentage recoveries have been reported in table 3. The good recoveries confirm that the detection of 5-HT at the modified sensor, MCZ/GCE is a reliable method for direct determination of 5-HT in human blood samples.

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Table 3. Determination of 5-HT in spiked human blood serum samples using the proposed method at MCZ/GCE

Sample	Standard added (nM)	Standard found* (nM)	Recovery (%) [#]	R.S.D. (%)
A	800	789.23	98.65	0.22
	900	892.8	99.2	0.21
	1000	1003.78	100.38	0.24
B	900	893.25	99.25	0.2
	1000	998.72	99.87	0.24
	2000	1972.4	98.62	0.26

[#] [standard found/standard added] x 100

*Amount found represents the average of six observations (n = 6)

IV. CONCLUSION

A novel MWCNT-CHIT-ZrO₂ nanocomposite modified sensor was fabricated to study the voltammetric behavior of 5-HT. The resulting MCZ/GCE sensor exhibited higher sensitivity to serotonin as compared to the MWCNT/GCE, MWCNT-CHIT/GCE and bare GCE due to the synergistic effect of MWCNT, CHIT and ZrO₂ nanoparticles facilitating the electron transfer process. The surface modifier was successfully characterized by SEM and AFM. Effect of pH of the supporting electrolyte and varying scan rates on the response characteristics of 5-HT exhibited that its electro-oxidation process at MCZ/GCE is a proton coupled and diffusion controlled process. Ascorbic acid and uric acid were found not to pose interference to quite a good extent. The practicable applicability of the proposed method with higher sensitivity, specificity, reproducibility and accuracy was applied for the estimation of 5-HT in human blood samples.

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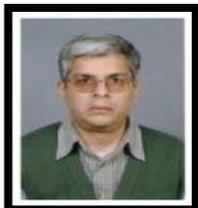


Harsha Devnani (born October 1989) completed her B.Sc, M.Sc and M.Phil in Chemistry from Dayalbagh Educational Institute, Agra and is currently pursuing Ph.D in Chemistry from the same university as DST INSPIRE JRF. Her research interests includes electrochemical sensors and biosensors, supercapacitors, green nanotechnology and environmental electrochemistry.

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Prof. Soami Piara Satsangee (born March 1961) completed his M.Sc from IIT Kanpur (India) and Ph.D in Chemistry from State University of New York at Stony Brook (New York). He is currently a Professor of Chemistry and Incharge of University Science Instrumentation Centre at Dayalbagh Educational Institute, Agra (India). He is also working on MHRD Virtual Labs project. His research interests include electrochemical sensors and biosensors, environmental electrochemistry, nanocomposites and 3D printing.



Prof. Rajeev Jain (born August 1954) completed his B.Sc. and M.Sc. from C.C.S. University, Meerut and Ph.D. in Chemistry from the university of Roorkee (now Indian Institute of Technology, Roorkee), India, in 1978 .He worked as Post Doctoral Fellow and Research Associate at the same institute. He was awarded D.Sc. degree by Jiwaji University, Gwalior, India in 1990. Currently he is working as Professor of Chemistry, Head of Environmental Chemistry and Dean College Development Council. He has published over 270 research papers and has worked over on 15 research projects sanctioned by CSIR, UGC, MOEF, DRDO etc. His research interests include electroanalytical behaviour of pharmaceuticals, micellar catalysis, electrocatalysed reactions, method development and validation, environmental engineering chemistry and wastewater treatment.