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Tailored interfacial architecture of chitosan modified glassy carbon electrodes facilitating selective, nanomolar detection of dopamine

Chiranjeevi Srinivasa Rao Vusa[#], Venkatesan Manju[#], K. Aneesh, Sheela Berchmans[*], Arumugam Palaniappan

Electrodics and Electrocatalysis Division, CSIR-Central Electrochemical Research Institute, Karaikudi, Tamilnadu-630006, India.

Abstract

Herein, it is demonstrated that the sulfonation of chitosan (CS) on glassy carbon surface (GCE) facilitates the making of a new sensing platform for the selective, nanomolar detection of dopamine. The surface functionalisation of CS was carried out using sulphamic acid (SA) by simple glutaraldehyde (GA) cross-linking to yield the sulfonated derivative (GCE-CS-GA-SA). The sulfonated chitosan possesses sulfonic acid functionalities which provide an electrostatic barrier, thereby discriminating dopamine from ascorbic acid. Electrochemical techniques, branded for their accuracy and fast response, are employed to determine dopamine concentrations down to few nanomoles in the presence of high concentrations of AA. In addition to nanomolar detection, the reported sensing methodology exhibits a low dopamine oxidation potential of 210 mV vs normal calomel electrode (NCE) and wide linear ranges of 50 nM – 10 μM and 10 - 400 μM in chronoamperometry and differential pulse voltammetry respectively. These results reveal that an inexpensive, simple and facile functionalization of chitosan like polymers on carbon surfaces can open up new avenues in the creation of perm-selective membranes that can find application in novel biosensors fabrication, especially in electrophysiology.

Key words: chitosan, glutaraldehyde, cross-linking, sulphamic acid, perm selective membrane, dopamine, nanomolar detection.

*the author to whom correspondence should be addressed

E mail sheelaberchmans@yahoo.com or sheelab@cecri.res.in

Tel +91(4565)241485 Fax +91(4565)227779

1. INTRODUCTION:

Most of the neurogenic disorders such as Parkinson's, Alzheimer's, Schizophrenia, epilepsy and addictions like cocaine and amphetamine addiction are associated with dopaminergic pathways which transmit the dopamine (DA) from one region of the brain to another[1-4]. Hence, the monitoring of dopamine level is essential in assessing the normal and pathological neural system functions. Specifically, early and trace level measurement of DA becomes crucial in understanding the role of the DA in neurophysiology, pathology, behavioral effects, disease diagnostics and treatment[5]. Therefore, the development of an accurate probe to detect nanomolar level of DA in brain environment is of great significance.

Since, 1970's researchers have explored different methods such as Flourometry[6], UV-Vis Spectrophotometry[7], Chromatography[1], Chemiluminescence[8] and Electroanalytical techniques[9-13] to detect DA. Among them, electroanalytical techniques were proven to be rapid, direct, simple, sensitive and can extensively be used in the detection of DA in *vivo*[9, 14]. But, the main difficulty in the detection of DA proficiently *in-vivo* is the interference of ascorbic acid (AA)[11, 15], whose oxidation potential is close to that of DA. Hence, its voltammetric response overlaps with that of the DA response making it difficult to detect DA selectively. To exclude the voltammetric response of AA, electrodes have usually been coated with anionic polymers such

as Nafion[15], poly(styrene sulfonic acid)[12], poly(p-aminobenzene sulfonic acid)[16], etc. which eliminate the interference of AA by electrostatic interactions. However, none of these polymer coated electrodes detect DA in nanomolar levels. In addition, these polymers are synthetic and their biocompatibility is limited compared to the natural biopolymers such as chitin and chitosan[17, 18]. In this study, the electrode material is modified using sulfonated chitosan. The sulfonated chitosan is proved to be an excellent material for selective detection of DA even in the presence of high concentration of AA. The electrostatic repulsion between AA and sulfonated chitosan make this electrode selective towards DA while at the same time extending the detection level to nanomolar concentration.

Chitosan is the second most available natural biopolymer after cellulose[18]. Owing to its unique properties such as rapid membrane forming ability, excellent adhesion, non-toxicity, biocompatibility, high mechanical strength and ease of chemical modifications[18], it finds a variety of applications such as cell and enzyme immobilization matrix[19], artificial skin[20], in wound healing[21], in surgery[22], etc. Especially, sulfonated derivatives of chitosan were well explored as drug delivery systems, anti-bacterial materials and blood anti-coagulant systems[23, 24].

Herein, the chitosan overlayer on carbon electrode is sulfonated with sulphamic acid by glutaraldehyde chemistry and is used to detect DA selectively in the presence of AA. The sensor shows nanomolar level detection and high selectivity towards DA. The efficiency of the sensor is investigated using cyclic voltammetry (CV), differential pulse voltammetry (DPV) and chronoamperometry (AA).

2. EXPERIMENTAL SECTION

2.1. Materials

Chitosan from shrimp cells (deacetylated), sulphamic acid, Acetic acid (glacial), $K_2H_2PO_4$, NaOH, KCl, $K_4[Fe(CN)_6]$, $[Ru(NH_3)_6]Cl_3$, Dopamine hydrochloride, L-ascorbic acid, uric acid, serotonin hydrochloride were purchased from Sigma-Aldrich. Glutaraldehyde (20 wt % solution in H_2O) was purchased from Loba chemicals and H_2O_2 was purchased from Nice chemicals. Cholesterol and Glucose were procured from Hi-media. All the chemicals were used as received. Phosphate buffer solution of pH 7.2 (PBS 7.2) was prepared using $K_2H_2PO_4$ and NaOH while water from Siemens water purification system was used throughout the experiments.

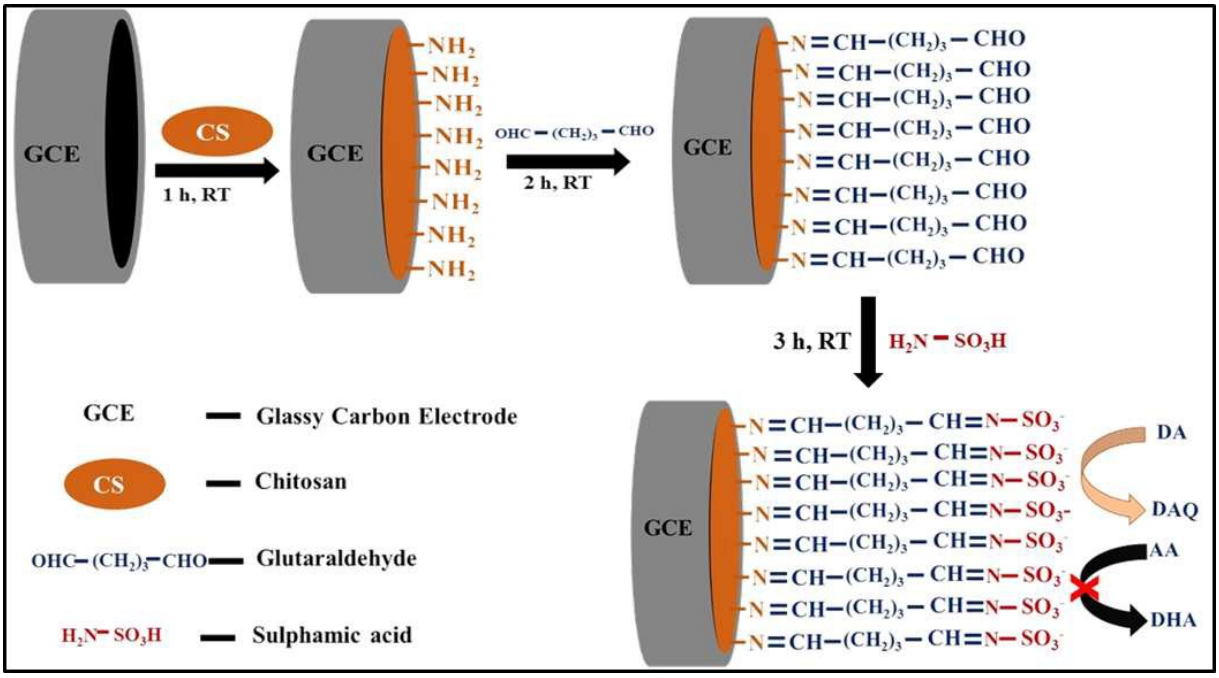
2.2. Characterizations and electrochemical measurements

All the electrochemical studies were carried out on Autolab PGSTAT 302N using conventional three electrode system which comprised of GCE (glassy carbon electrode) or modified GCE as a working electrode, a platinum wire as a counter and calomel (1 M KCl) as a reference electrode. Electrochemical impedance spectra were recorded using PARSTAT-4000 from AMETEK and analyzed using Z view software from Scribner associates Inc. All the electrochemical experiments were made at room temperature. Fourier transform infrared (FT-IR) spectra were recorded using a Tensor 27 FT-IR spectrophotometer (Bruker optics) between 4000 and 400 cm^{-1} and scanning electron microscopy (SEM) images were obtained using a Tescan SEM VEGA 3 XM. EDS data were collected using Bruker X-ray detector attached to Tescan SEM and analyzed using Quantax software.

2.3. Preparation of functionalized chitosan matrix on GCE: (GCE-CS-GA-SA)

Prior to surface modification, GCE was polished with 1.0, 0.5 and 0.05 μm alumina slurries which was followed by ultra-sonication in ethanol and deionized water (1:1 V/V) for 5 min. The resulting pretreated GCE surface was dried using N_2 gas. Then, 7 μL of 0.01% chitosan in 0.1 M acetic acid was drop casted on GCE and dried at room temperature for 1 h, the obtained

GCE-CS was dipped in 1% GA for 2 h to activate the chitosan film (GCE-CS-GA) for further functionalization. The activated GCE-CS (GCE-CS-GA) was washed with distilled water repeatedly in order to remove unreacted GA from the surface of GCE-CS-GA. The activated GCE-CS-GA was incubated in aqueous solution of sulphamic acid (10 mg/mL) for 3 h. The obtained sulfonic acid terminated GCE-CS-GA-SA surface was washed with distilled water to remove unreacted SA and dried at room temperature. A schematic representation of the electrode modification stages is given in scheme 1.



Scheme 1. A schematic illustration of glassy carbon electrode surface modification using chitosan, glutaraldehyde and sulphamic acid

3. RESULTS AND DISCUSSION

By using the unique properties of chitosan membranes and adopting the well-established glutaraldehyde chemistry[25, 26], a thin film of chitosan was allowed to react and crosslink with sulphamic acid via the glutaraldehyde linker. The surface derivatisation was well characterized

by FTIR and SEM. Further electrochemical techniques were employed to demonstrate the selective, nanomolar detection of DA aided by modified chitosan layer on GCE.

3.1. Characterization of GCE-CS-GA-SA by FT-IR spectroscopy SEM and EDS

To confirm the functionalization of chitosan on GCE, FT-IR measurements were carried out and the results are presented in Figure 1. The disappearance of the peaks at 3475 and 1020 cm^{-1} and the appearance of new peaks at 1648 and 1070 cm^{-1} in the FT-IR spectra of the GCE-CS-GA-SA (solid line) represents the formation of Schiff base and the imine (C=N) bonds[27, 28]. In addition, the appearance of new peaks in GCE-CS-GA-SA at 682 and 600 cm^{-1} corresponds to SO_3^- deformation which confirms the sulfonation of chitosan on GCE.

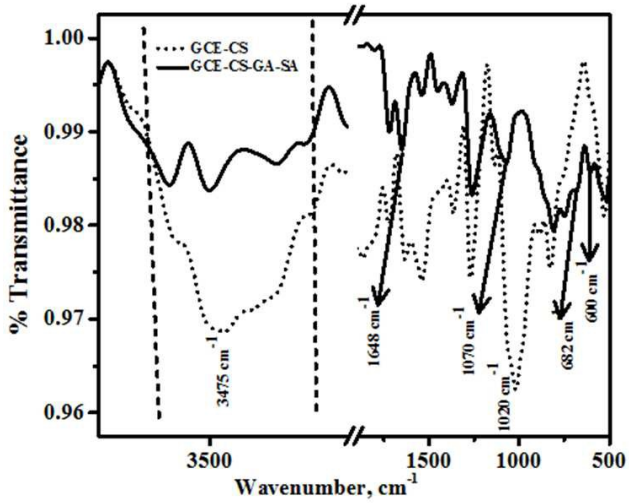


Figure 1. FT-IR spectrum of GCE-CS (dotted line) and GCE-CS-GA-SA (solid line).

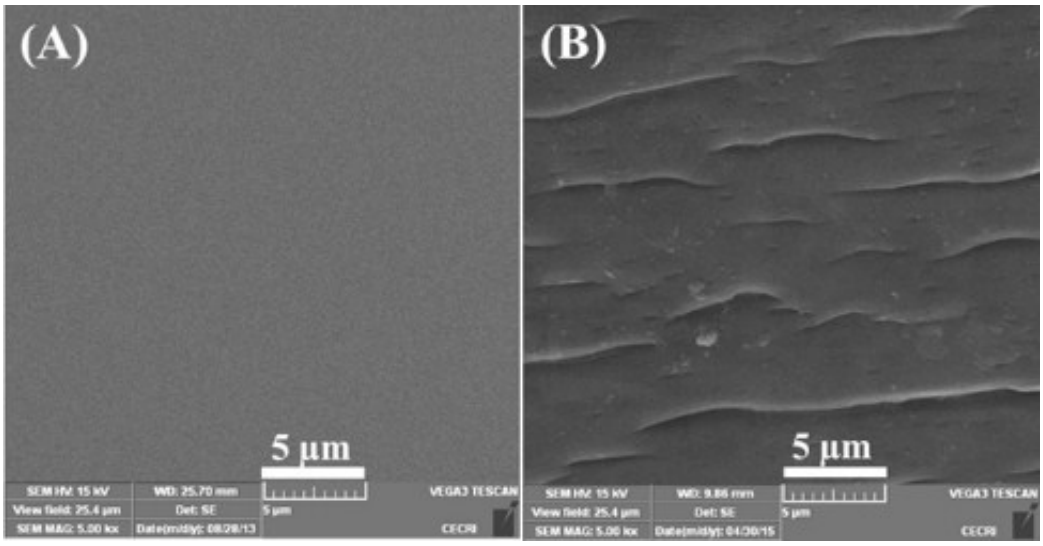


Figure 2. Scanning electron micrographs of thin film of chitosan on GCE before (A) and after functionalization with sulphamic acid (B)

From Figure 2A and 2B it is evident that the microscopically smooth surface of GCE-CS becomes rough after functionalization with sulphamic acid. This could be attributed to the formation of imine bond between GA and sulphamic acid making the chitosan matrix relatively stronger and stiffer.

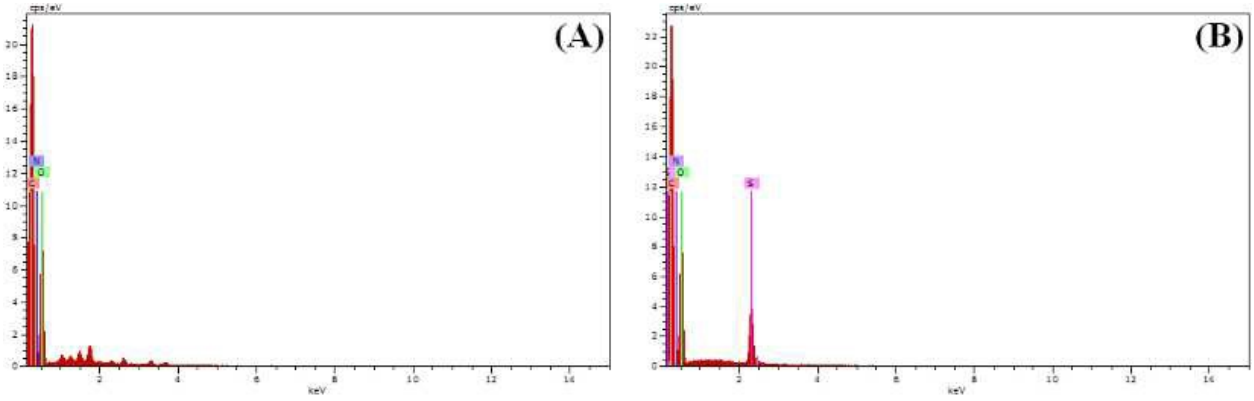


Figure 3. EDS data of thin film of chitosan on GCE before (A) and after functionalization with sulphamic acid (B)

Table 1. EDS Analysis of GCE-CS and GCE-CS-GA-SA

Weight % of the elements	GCE-CS	GCE-CS-GA-SA
C	48.21	49.80
N	10.30	11.09
O	41.49	36.07
S	—	3.04

It is evident from EDS analysis, (Figure 3A and Figure 3B) that no sulfur is present in GCE-CS before functionalization with SA while the presence of elemental sulfur can be detected in sulphamic acid terminated GCE-CS-GA-SA. The elemental analysis of electrodes GCE-CS and GCE-CS-GA-SA using EDS is given in table 1. The presence of sulfur and nitrogen confirms the effective functionalization of GCE by chitosan, GA and sulphamic acid.

3.2. Electrochemical characterizations of GCE-CS-GA-SA

It is a well-known fact that the selectivity and/or sensitivity of a sensor can be improved by charge discrimination if the analytes of interest have similar redox potential. In this work, the interfacial sulfonic acid functionalities created by glutaraldehyde linkage aid selective sensing of dopamine. The charge discrimination property of the sensor was initially assessed using two oppositely charged redox species viz., $[Fe(CN)_6]^{3-}$ and $[Ru(NH_3)_6]^{3+}$ of concentration 5 mM each in 0.5 M KCl. As shown in Figure 4, the response to negatively charged $[Fe(CN)_6]^{3-}$ species decreased to a lesser extent on GCE-CS (dashed line) due to the film resistance resulting from CS while no redox behavior (dotted line) could be observed for $[Fe(CN)_6]^{3-}$ on sulfonated surface, GCE-CS-GA-SA due to the electrostatic repulsions between the negatively charged sulfonate functionalities and the $[Fe(CN)_6]^{3-}$ ions. In the case of positively charged $[Ru(NH_3)_6]^{3+}$, the quasi-reversible behavior was observed on GCE-CS-GA-SA. But the current response

decreased compared to GCE-CS which may be attributed to the increase in film resistance during each modification. These results indicate that the sulfonated functionalities present on chitosan film are effective in discriminating the negatively charged $[Fe(CN)_6]^{3-}$ and positively charged $[Ru(NH_3)_6]^{3+}$ redox probes.

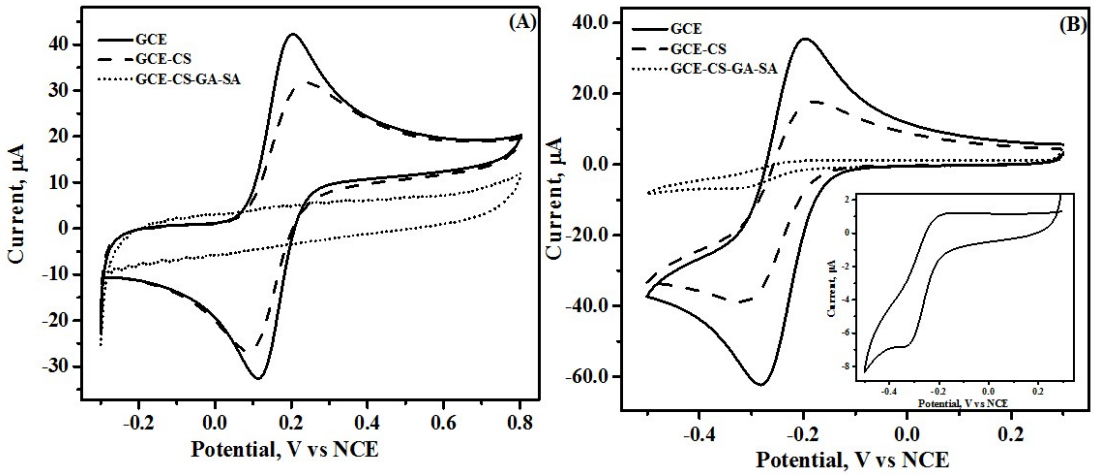


Figure 4. Cyclic voltammograms of 5 mM solutions of (A) $[Fe(CN)_6]^{3-}$ and (B) $[Ru(NH_3)_6]^{3+}$ in 0.5 M KCl at GCE (solid line), GCE-CS (dash line) and GCE-CS-GA-SA (dotted line) at the scan rate of 50 mV s^{-1}. Inset shows the quasi reversible behavior of $[Ru(NH_3)_6]^{3+}$ on GCE-CS-GA-SA.

Electrochemical impedance spectroscopic measurements were carried out further to explore the changes in the impedance and the electron transfer properties of the electrodes during the modification protocol. Figure 5 A illustrates the typical Nyquist plot obtained with the bare GCE (a), GCE-CS (b) and GCE-CS-GA-SA (c) in 10 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 100 mM KCl. The gradual increase in the R_{ct} from GCE (335 Ω) to GCE-CS (411 Ω) and from GCE-CS (411 Ω) to GCE-CS-GA-SA (512 Ω) , observed in Figure 5 A result from the electrode passivation by the successive modification steps and charge based repulsion between the GCE-CS-GA-SA and the negatively charged $[Fe(CN)_6]^{3-}$. This is in good agreement with the cyclic voltammetric results. In the case of positively charged $[Ru(NH_3)_6]^{3+}$, GCE, GCE-CS and GCE-CS-GA-SA show no apparent charge transfer resistance as the process is often controlled

entirely by diffusion. As shown in Figure 5 B, the GCE-CS-GS-SA has much lower imaginary impedance value than GCE which may be attributed to the increased interfacial capacitance of the GCE-CS-GA-SA. But when compared to the GCE-CS, the imaginary impedance value of GCE-CS-GA-SA is slightly higher representing a low interfacial capacitance, that would have resulted from the charge based interactions between the GCE-CS-GA-SA and $[Ru(NH3)_6]^{3+}$.

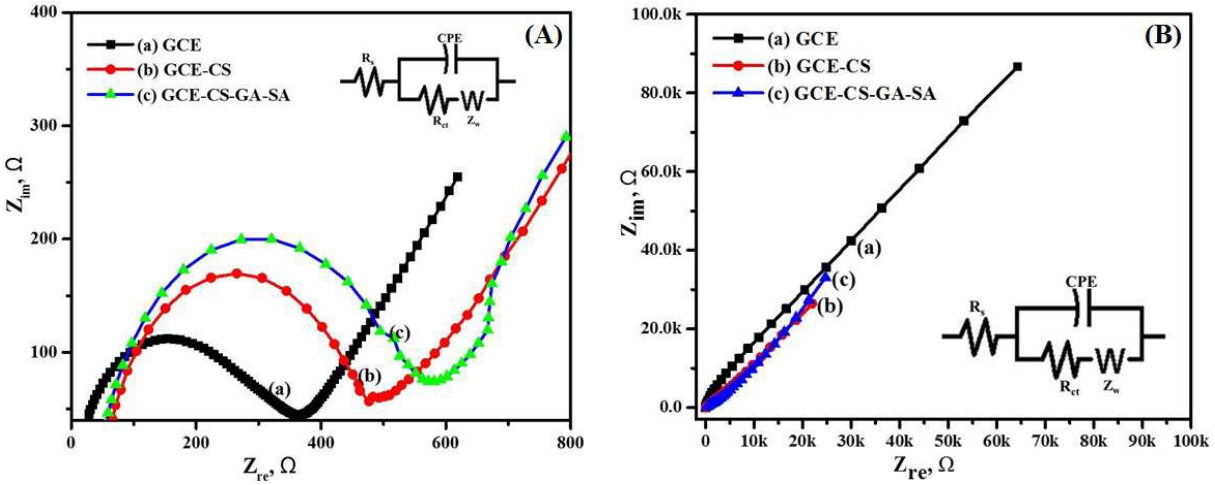


Figure 5. Electrochemical impedance spectra of (a) bare GCE, (b) GCE-CS and (c) GCE-CS-GA-SA in (A) 10 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 100 mM KCl and (B) 5 mM $[Ru(NH_3)_6]^{3+}$ in 0.5 M KCl. Inset shows the typical equivalent circuits.

3.3. Electrochemical studies of dopamine on GCE-CS-GA-SA electrode

To investigate the electroactivity of GCE, GCE-CS and GCE-CS-GA-SA towards DA, cyclic voltammetric studies were carried out and the corresponding voltammograms are given in Figure 6A and 6B. The Figure 6A shows that the anodic current of GCE-CS-GA-SA towards DA (400 µM) oxidation decreased compared to GCE and GCE-CS. This may be attributed to the increase in the film resistance resulting from the successive modification steps. On the other hand the anodic peak potential of DA is lower in GCE-CS-GA-SA (260 mV) than GCE (360 mV). These results indicate that there is better interaction between the sulfonated chitosan film and DA which in-turn promotes the oxidation of DA at lower potential. In addition to this, the AA

response (1 mM) was completely blocked on GCE-CS-GA-SA possibly due to the electrostatic repulsions between the negatively charged AA and sulfonate functionalities present on sulfonated chitosan as shown in Figure 6B which might be following the same principle and mechanism reported[15, 16, 29].

The high specificity of the sensor towards DA is also proved by a more sensitive electrochemical technique known as differential pulse voltammetry (DPV) as shown in Figure 7. The DPV were recorded in PBS solution containing 400 μM DA and 1 mM AA using three different electrodes, GCE, GCE-CS and GCE-CS-GA-SA (See Figure 7A, 7B and 7C). It is evident from Figure 7A that the electrochemical oxidation of AA occurs approximately at the same potential as that of the electrochemical oxidation of the DA on bare GCE. Because of the unfavorable interactions between the GCE-CS electrode surface and the AA, the AA is getting oxidized at slightly more positive potential than DA on GCE-CS electrode (Figure 7B). From Figure 7C it is evident that the GCE-CS-GA-SA has less interaction with AA and hence, unable to detect the electroactivity of AA thus making the electrode surface very specific for dopamine detection. Even when the concentration of AA is higher than the levels found in biological fluids, no electroactivity of AA was noticed on GCE-CS-GA-SA. Thus, the GCE-CS-GA-SA provides the selectivity towards the detection of DA against AA by simple functionalization of chitosan with negatively charged sulfonate functionalities.

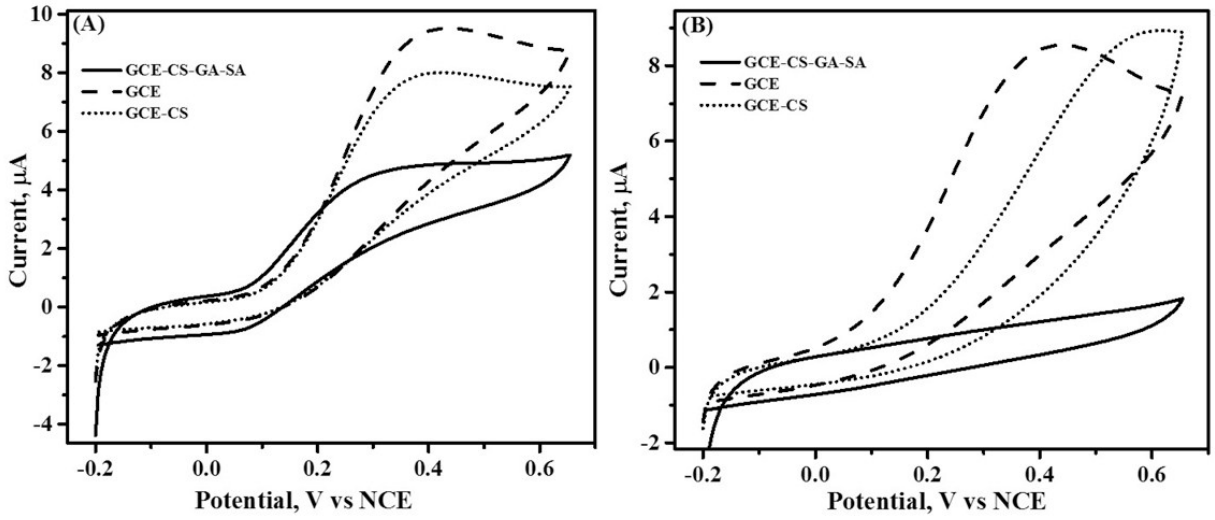


Figure 6. Cyclic voltammograms recorded in PBS-7.2 solution containing (A) DA (400 µM) and (B) AA (1 mM) at GCE (dash line), GCE-CS (dotted line) and GCE-CS-GA-SA (solid line) with scan rate of 50 mV s^{-1}.

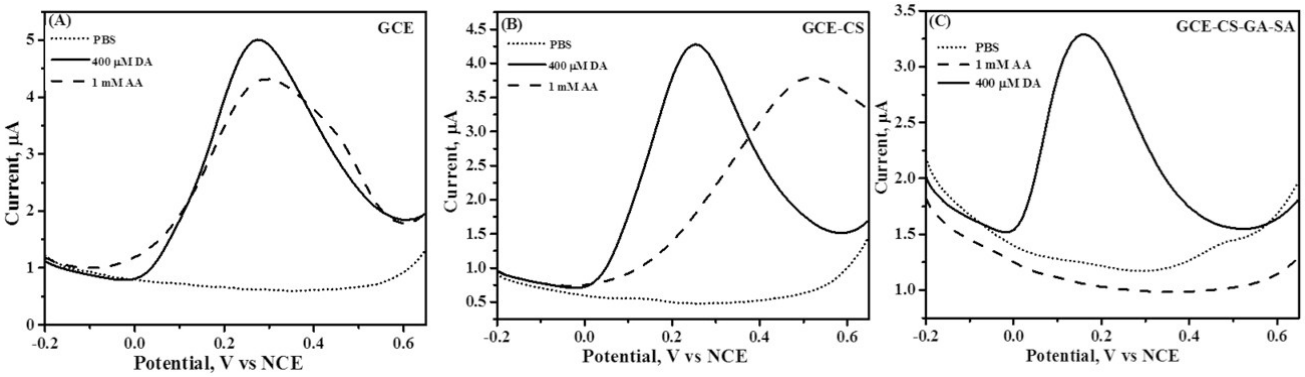


Figure 7. Differential pulse voltammograms of (A) GCE, (B) GCE-CS and (C) GCE-CS-GA-SA recorded in PBS (pH 7.2, dotted line) solution containing DA (400 µM, solid line) and AA (1 mM, dash line).

3.4. Electrochemical detection of dopamine on GCE-CS-GA-SA using DPV and chronoamperometry

To demonstrate the efficiency of the sensor towards DA determination, chronoamperometry and differential pulse voltammetry were used as these techniques are more sensitive than CV. DPV was recorded in PBS (pH=7.2) solution containing 1 mM AA along with the addition of DA ranging from 10 µM to 400 µM at a scan rate of 50 mV s^{-1}. As shown in Figure 8A, the

oxidation peak current increases linearly with increasing concentration of DA in presence of 1 mM AA indicating that the probe has high selectivity towards DA. The anodic peak current (I_{pa}) of DA exhibits good linear relationship from 10 µM to 400 µM (shown in Figure 8B). The linear regression equation is I (µA) = 0.00212 C (µM) +1.1651 with R^2= 0.98, where I and C represents the anodic peak current and the concentration of DA respectively.

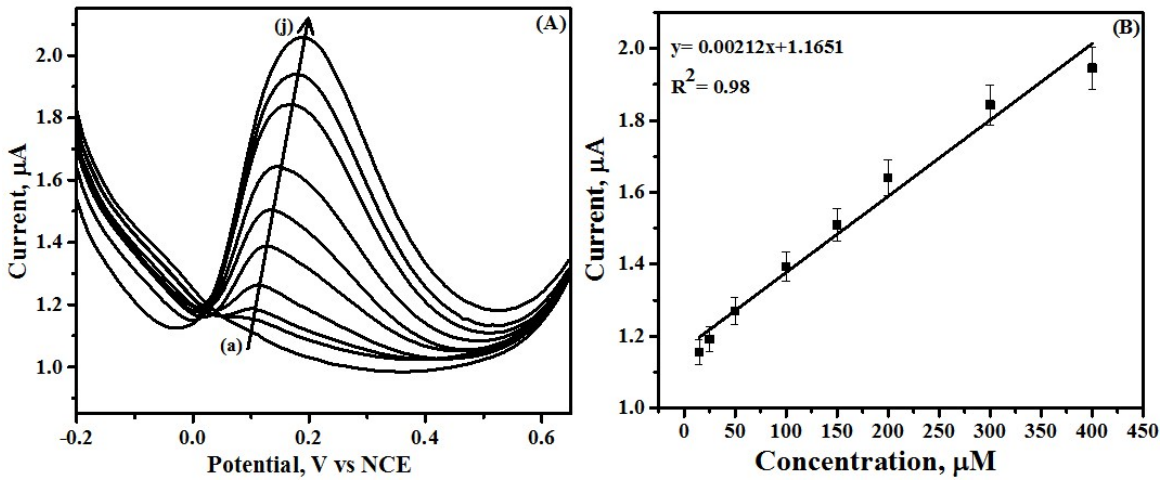


Figure 8. (A) DPV curves of (a) PBS (b) 1 mM AA and (c-j) different concentrations of DA (10, 25, 50, 100, 150, 200, 300 & 400 µM) on GCE-CS-GA-SA in PBS (pH-7.2) in presence of 1 mM AA at the scan rate of 50 mV s^{-1}. (B) The linear relationship between anodic peak current and DA concentration.

In addition to DPV, chronoamperometric measurements were also carried out on the fabricated sensor which is still more powerful and sensitive than DPV. The chronoamperometric signal for dopamine on GCE-CS-GA-SA surface was recorded at a fixed potential of 210 mV vs NCE by successive additions of DA into a PBS (pH 7.2) solution and the results are shown in Figure 9A. The GCE-CS-GA-SA has shown an exceptional linear range from 50 nm -10 µM with a correlation coefficient (R^2) of 0.9958 as shown in the inset of Figure 9A. The detection limit (LOD) and the sensitivity of GCE-CS-GA-SA was found to be 9.5 nM and 14.6 nA μM^{-1} respectively.

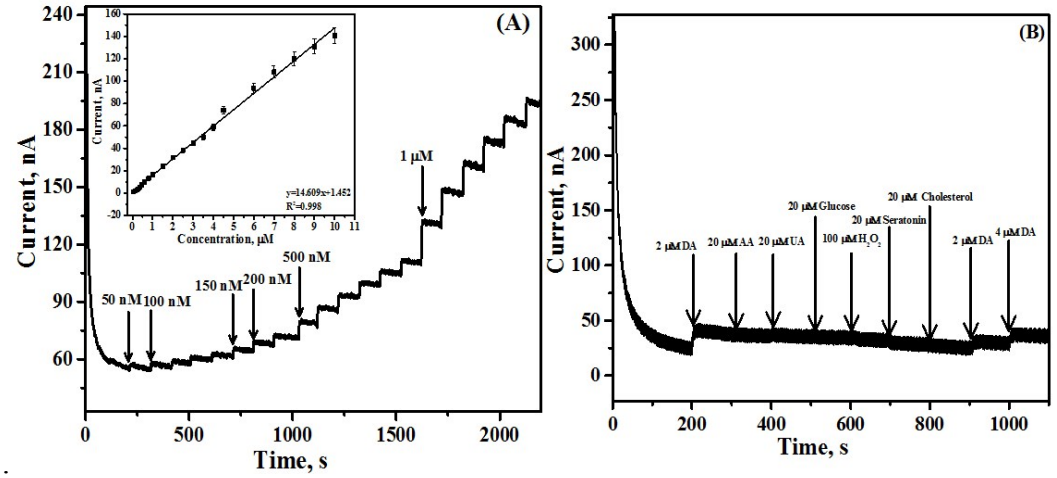


Figure 9. (A) Chronoamperometric response of successive addition of DA in PBS (pH-7.2) on GCE-CS-GA-SA at a fixed potential of 210 mV Vs calomel (1 M KCl) (Inset shows the linear relationship between current and DA concentration with regression equation as I (nA) = 14.609C+1.452 and R^2=0.9958). (B) The interference study using modified GCE-CS-GA-SA electrode.

3.6. Investigation of stability and interference

To investigate the selectivity of GCE-CS-GA-SA towards DA detection interference studies were carried out using various analytes such as AA, UA, Glucose, Hydrogen peroxide, Serotonin and Cholesterol which are usually present in biological fluids. No considerable chronoamperometric response was observed for analytes other than DA (Figure 9B). This indicates that there is no interference from the electroactive species that are normally present in physiological fluids. The complete elimination of interferences arising from ascorbic acid (AA) and uric acid (UA) is also evident from Figure 9B. The concentrations of the interfering analytes were taken at the physiological levels. Besides selectivity, sensitivity and interference studies, the stability of the sensing platform was also investigated using 200 μM DA in PBS between -0.1 and 0.6 V at the scan rate of 50 mV s^{-1} as shown in Figure 10. The electrode was cycled 25 times continuously at the above conditions and at the end of 25th cycle, 96.7% of its electrochemical

response towards dopamine is retained, which confirms the stable electrochemical response of DA on fabricated sensor.

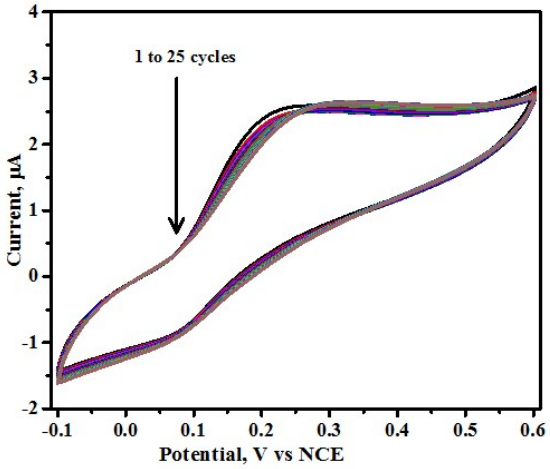


Figure 10. Stability of the GCE-CS-GA-SA electrode was investigated in PBS (pH-7.2) containing DA of 200µM by cyclic voltammograms.

Table 2 compares the performance of the sensor with other reported sensors in which anionic membranes and or nanomaterials were used to detect DA by using same electrochemical techniques namely DPV and CA. It may be observed from the table that few materials give better current response but lack wide linear range and low detection levels. In comparison, simple and cost effective techniques are used in current work for surface functionalization of the electrodes. The GCE-CS-GA-SA electrode shows comparable linear range and limit of detection towards DA sensing thus making it possible to build prototype dopamine sensors at the next level.

Table 2. Comparison of electrochemical response of various modified electrodes with GCE-CS-GA-SA platform towards dopamine sensing.

Electrode and Modification	Method of detection	pH	Linear range (μM)	LOD (μM)	Sensitivity nA/μM	(Ref)
GCE/Poly(m-ABSA)	DPV	7.0	0.1-100	0.005	-	[29]
GCE/Poly(p-ABSA)	CA	7.0	0.1-100	0.02	-	[16]
GCE/GO/SiO_2/AgNP	DPV	7.0	2-80	0.26	-	[30]
GCE/Nafion-ferrocene	CA	7.0	250-5 mM	22.7	1.1	[31]
Pt/poly eugonol	DPV	7.0	4-50	0.1	7.9	[32]
ITO/MWCNTs/Nafion	CA	7.0	0.1-10	0.20	200	[33]
GCE/GO/PANI	DPV	7	2-18	0.50	2000	[34]
GCE/Pectin-AuNP/Nafion	CA	7.	0.02-0.9	0.0061	0.033	[13]
GCE/ERGO/PoPD	DPV	7.1	10-400	7.5	-	[35]
GCE/ZnO-Au	DPV	7.0	0.1-300	0.02	-	[36]
GCE/PANI-AuNPs	CA	4.0	3-115	0.8	26.9	[37]
GCE-CS-GA-SA	CA	7.2	0.05-10	0.0095	14.6	This work
GCE-CS-GA-SA	DPV	7.2	10-400	3	2.12	This work

Poly(m-ABSA) = ploy(m-aminobenzene sulphonic acid), Poly(p-ABSA) = ploy(p-aminobenzene sulphonic acid), AgNP = Silver nanoparticles, GO = Graphene oxide, ITO = indium tin oxide, MWCNT = Multi wall carbon nanotube, PANI = Polyaniline, AuNP = Gold nanoparticles, ERGO = Electrochemically reduce graphene oxide, PoPD = Poly(o-phenylenediamine).

4. Conclusion

The sulfonation of chitosan on glassy carbon electrode using sulphamic acid as functionalizer and glutaraldehyde as linker provides nanomolar level detection of DA. The constructed sensor shows high selectivity towards DA even in the presence of high concentrations of possible interferents such as AA, UA, Glucose, Cholesterol, Serotonin and H_2O_2. The fabricated sensor shows an exceptional detection limit of 9.5 nM and a wide linear range between 50 nM and 10 μM DA as established by amperometry. In the case of DPV, the sensor exhibited a detection

limit of 3 μM and a linear range of 10 μM - 400 μM. These results show that the simple sulfonation of chitosan directly on GCE provides a novel, cost-effective and highly selective dopamine sensing platform. The developed methodology may open a new window towards the fabrication of sensors for dopamine-like molecules using naturally occurring biopolymer like chitosan.

Author Information

Corresponding Author

*Sheela Berchmans. E-mail sheelaberchmans@yahoo.com or sheelab@cecri.res.in

#Authors contributed equally.

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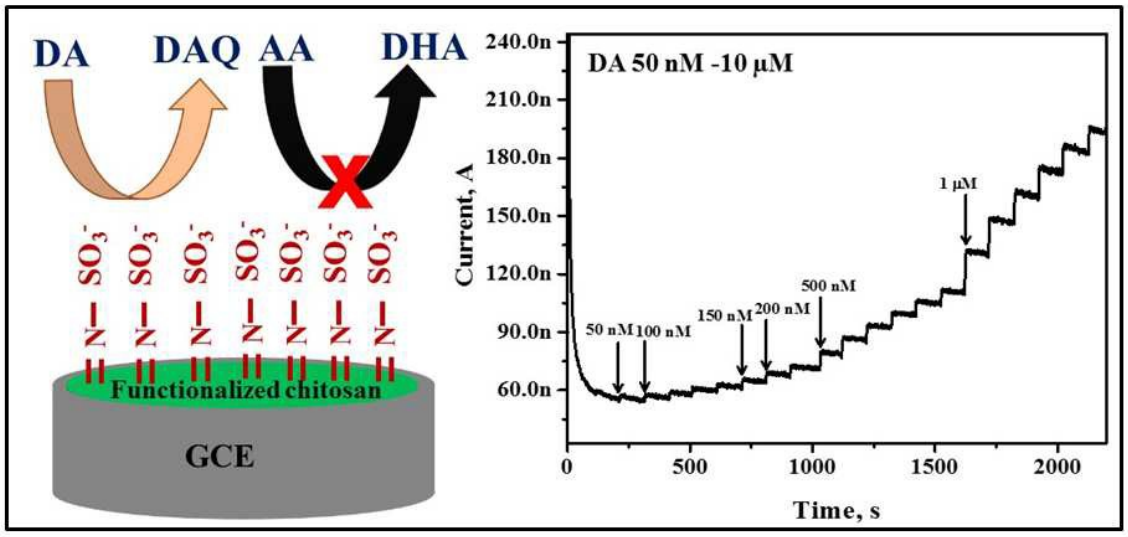
Tailored interfacial architecture of chitosan modified glassy carbon electrodes facilitating selective, nanomolar detection of dopamine

Chiranjeevi Srinivasa Rao Vusa[#], Venkatesan Manju[#], K. Aneesh, Sheela Berchmans[*], Arumugam Palaniappan

Electrodics and Electrocatalysis Division, CSIR-Central Electrochemical Research Institute, Karaikudi, Tamilnadu-630006, India.



Chitosan was tailored directly on the electrode surface to detect DA selectively in nanomolar level at physiological pH.