RESEARCH PAPER

SOD1 coupled biosensor for the sensitive detection of neurodegenerative biomarker dopamine

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ABSTRACT

In this paper, we have described the construction of a covalently coupled Cu,Zn superoxide dismutase (SOD1) enzyme based electrochemical biosensor for the sensitive determination of a neurodegenerative biomarker dopamine (DA) in complex biological samples. The recognition enzyme SOD1 was successfully bonded on Pt surface modified by cysteamine (CA) self-assembled monolayer (SAM) bonded gold nanoparticles (GNPs) on polypyrrole (PPy) matrix by using EDC and NHS. Scanning electron microscopy (SEM) was utilized to study the morphological changes occurred on the electrode surface while electrochemical modifications were studied through cyclic voltammetry (CV). Direct voltammetric response of SOD1 biofunctionalised electrodes revealed a well-defined quasi reversible voltammetric peaks at 0.06 *vs.* Ag/AgCl. Further, in the presence of dopamine, SOD1 modified electrode promptly shows catalytic currents, well-defined peak appeared at about 0.08–0.10 V (vs. Ag/AgCl) for all dopamine concentrations, indicating that the entrapped SOD1 molecules are participated in the electrochemical reaction *via* direct electron transfer. At optimal conditions, the minimum detectable range is 1 nM. The sensor sensitivity is 0.096 μA μM^{-1} within a linear range of 1 nM–100 μM. The SOD1-SAM-GNP-PPy-Pt electrochemical biosensor was further applied to quantify the DA in biological samples, the results agreed well with standard reference assay.

KEYWORDS

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INTRODUCTION

Dopamine (DA) is the most dominant catecholamine in the brain and acts as a neurotransmitter playing essential roles in the cardiovascular and central nervous systems (CNS), including those for regulate mood, arousal, brain's reward system and endocrine functions [1, 2, 3, 4]. It plays a key role in abundant pathologies, such as high DA levels indicates the cardiotoxicity leading to rapid heart rates, hypertension, and further heart attack [5]. On the other hand, low levels of dopamine in the CNS are concerned as a major cause of numerous neurological diseases viz., stress, depression, Alzheimer's disease, Parkinson's disease and schizophrenia [6, 7, 8].

Further, DA dysfunction can lead to abnormalities in prolactin levels in the pituitary gland. In certain cases, drugs that affect DA levels are used diagnostically [9]. For example, the administration of a DA agonist (such as levodopa) or a DA antagonist can help differentiate between Parkinsonian syndromes and other movement disorders [10, 11]. Hence, the measurements of DA plays an imperious in human physiology as it gives valuable information regards to cardiovascular and neurological diseases [12]. Moreover,

determinations of DA are also important in CNS management [13]. Several methods were used for analysis of DA includes spectroscopy, liquid chromatography, mass spectrometry, flow injection analysis technique, capillary electrophoresis, with optical or electrochemical detection. However, the most straight forward, sensitive, prompt, and cost-effective method is the use of electrochemical sensing techniques for detection of electroactive neurotransmitters, particularly dopamine [14, 15, 16].

Complementary materials for high performance electrochemical biosensors have been developed using conducting polymers (CPs) and metal nanoparticles (MNPs) [17, 18]. CPs like polyaniline (PANI), polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) serve as an electrically active and chemically tunable framework that allows for electron transport and provides functional groups for the immobilization of biomolecules [19, 20]. Their porous and flexible surface can enhance the availability of analytes to the sensing interface, thus enabling better analytical performance. Further supporting this sensing platform are the excellent catalytic properties, the great electrical conductivity and the large surface to volume ratio of MNPs, especially gold (GNP), palladium, platinum, and silver nanoparticles. In the presence of CPs, MNPs can form synergistic interfaces, in which the polymer forms a stable and biocompatible matrix, while the nanoparticles (NPs) aid in rapid electron transfer (ET) and provide increased active sites [21]. CPs/MNPs based composites are promising for the detection of biological molecules, disease biomarkers, and other clinically relevant analytes and can have improved sensitivity, selectivity, response time, and detection limits [22]. PPy/GNP composites have attracted considerable attention in biosensor applications because they combine the excellent electrical conductivity and biocompatibility of PPy with the high surface area, catalytic activity, and favourable ET properties of GNPs. The incorporation of GNPs into the PPy nanocomposite increases the active surface area available for binding of biomolecules such as enzymes, antibodies, DNA, and aptamers [23]. In addition, the strong interaction between GNPs and biomolecules, particularly through Au–S bonds with thiol-containing groups, provides stable and efficient bioreceptor immobilization [24]. The conductive network formed by PPy and GNPs facilitates rapid ET between the recognition layer and electrode, thereby enhancing the electrochemical response and lowering the detection limit of biosensors [25].

Their porous and nanostructured architecture provides more active sites and improves analyte diffusion, while the conductive PPy/GNP interface amplifies the electrochemical signal. Selectivity can be achieved by incorporating specific recognition elements such as enzymes, aptamers, antibodies, or molecularly imprinted polymers onto the composite surface. Consequently, PPy/GNP based biosensors have demonstrated significant potential for the sensitive and selective detection of hydrogen peroxide, glucose, pathogens, DNA, proteins, and various environmental pollutants [26].

The metalloenzyme Cu/Zn superoxide dismutase (SOD1) is an essential first-line defense against oxidative stress in biological systems, and is a highly efficient enzyme [27, 28]. It has an active site that consists of copper and zinc ions in a small protein structure in which the copper ion changes its oxidation–reduction state during the process, enabling the formation of molecular oxygen and hydrogen peroxide from the superoxide radical. Zinc does not participate directly in the redox reaction, but it does help to maintain the proper structural and functional organization of the active site. In addition to its classical antioxidant function, the SOD1 may be considered as a molecular regulator linking the metal-coordinating function, the structure of the protein, its electron transfer, and the redox balance in the cells. It is crucial for its catalytic activity that the copper centre, the zinc-coordinating structural elements, the neighbouring amino-acid residues and the conformational fluctuations of the protein play in perfect harmony. This means that changes in metal binding, protein conformation, or the oxidation/reduction status of the metal can affect the activity of SOD1 and could have biologically significant effects. The combination of these properties renders SOD1 an interesting subject for study for biosensors, monitoring of oxidative stress, biomolecular recognition and building analytical systems based on nanostructured surfaces and electrodes [29].

In this paper, we propose the fabrication of the truly non-mediated voltammetric biosensor for the sensitive and selective determination of a neurodegenerative biomarker, DA in complex biological samples. This sensor is depends on the electrochemical activities of SOD1, covalently bonded with SAM-GNP-PPy matrix modified Pt electrode by EDC and NHS couple. Cyclic voltammetry (CV) measurements are used to evaluate the electrochemical nature of the fabricated sensor. SEM and impedance techniques are used to characterize modified electrode surfaces. The fabricated biosensor utilize CV, a

powerful analytical technique that measures the current response of an electrochemical system as the potential is scanned linearly over time.

EXPERIMENTAL

CHEMICALS AND REAGENTS

Cu,Zn superoxide dismutase, sodium dihydrogen phosphate, disodium hydrogen phosphate, pyrrole, potassium chloride, sodium nitrate, dopamine, auric chloride trihydrate, cysteamine (CA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), were purchased from Sigma Aldrich (USA). All the solutions were prepared through doubly distilled water.

INSTRUMENTS

The morphological studies of the fabricated electrodes were obtained using FEI Quanta FEG High Resolution scanning electron microscope (SEM). Entire CV studies were carried out using CHI 1200B electrochemical workstation (CHI, USA). Impedance studies were made using a VMP3-Modular 16 Channel Potentiostat/Galvanostat/EIS from Bio-Logic Science Instruments, France. Data processing and analysis of the impedance measurements were studied in 40 mV perturbation over a frequency range from 10 KHz to 1 MHz.

SURFACE MODIFICATION AND BIOFUNCTIONALIZATION OF THE GNP-PPy-Pt ELECTRODE TO FORM THE SOD1-SAM-GNP-PPy-Pt BIOSENSOR PLATFORM

SAM functionalization on GNPs electrodeposited onto a PPy-Pt electrode was fabricated as per our previous report [10, 11, 12]. The resultant SAM-GNP-PPy-SPCE surface was further cleaned with doubly distilled water to remove excess/unbound cysteine moieties. Then, SOD1 was covalently bind to the SAM-GNP-PPy-Pt by incubation of SOD1 in PBS for minimum 12 hours in 4 °C. 5 µL of glutaraldehyde (2.5٪) cross-linking agent was incubated onto the surface of SAM-GNP-PPy-Pt. After the incubation of SOD1 on the SAM-GNP-PPy-Pt, the biofunctionalised surface was washed with PBS to remove any excess/unbound, SOD1. Fig. 1 represents the schematic representation of each and every step of SOD1 biofunctionalisation on SAM-GNP-PPY-Pt surface.

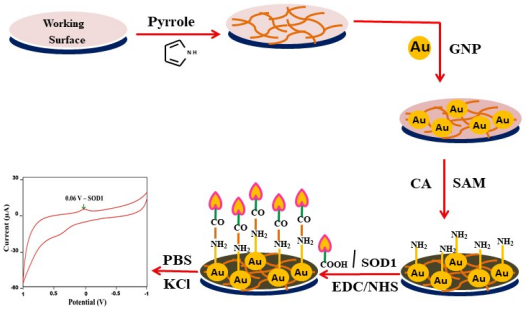

Figure 1: Schematic representation of SOD1 biofunctionalisation on SAM-GNP-PPy-Pt surface.

RESULTS AND DISCUSSION

CHARACTERIZATION OF CONSTRUCTED SOD1 BIOSENSOR

Surface morphologies of the fabricated biosensor was characterized by SEM. It was used to examine the morphological changes of the Pt-based biosensor during each step of the modification process [13]. In fig. 2 curve a shows the homogenous bare surface of the Pt however, curve b reveals the typical highly porous surface morphology of PPy on Pt. Accordingly the high surface area and porous nature of the PPy affords large sites for ensuing further GNP and SOD1 binding than the bare Pt alone.

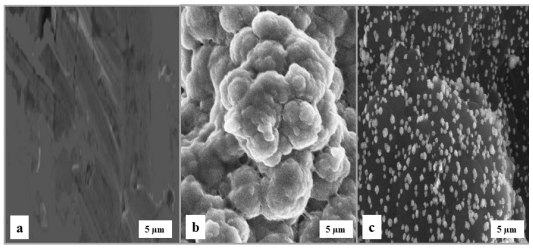

Figure 2: SEM images of bare Pt, PPy-Pt and GNP-PPy-Pt electrodes respectively.

SEM exposes an even distribution of GNPs with average diameter of ~20 nm and moderately symmetric spreading over the microporous environment of the PPy-Pt, curve c [10, 11]. This GNP nanostructure also enables additional binding sites for SAM layer and SOD1 binding.

ELECTROCHEMICAL CYCLIC VOLTAMMETRIC CHARACTERIZATION

CV studies were performed to know the electrochemical characteristics of the Pt conductive surface after each electrode functionalization step. Fig. 3 shows the characteristics CVs of SOD1-SAM-GNP-PPy-Pt (curve d), GNP-PPy-Pt (curve c), PPy-Pt (curve b), and bare Pt (curve a) electrodes. As expected, the bare Pt (curve a), PPy-Pt (curve b), and GNP-PPy-Pt (curve c), sensors showed a well-defined characteristic broad peaks in the CV possibly because of the complex redox

features, high capacitive currents and fast charge transport kinetics. Such fast transport kinetics is most possible due to the high electrical conductivity and large surface area [10, 11].

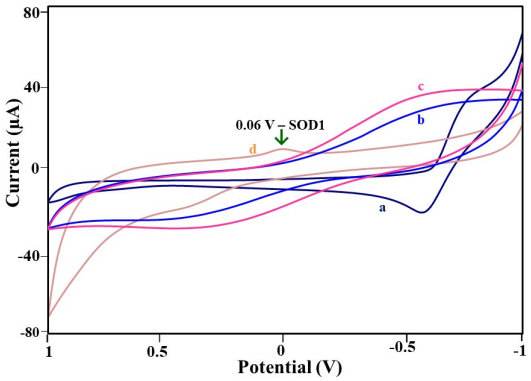

Figure 3: Cyclic voltammograms of 0.1 M PBS (pH 7.0) at (a) bare Pt, (b) PPy/Pt, (c) GNP/PPy/Pt, and (d) SOD1/SAM/GNP/PPy/Pt; scan rate 50 mV/s.

ELECTROCHEMICAL IMPEDIMETRIC CHARACTERIZATION

Electrochemical impedance spectroscopy (EIS) analyzes the impedance characteristics of a modified electrode at various frequencies, revealing information about the electrode-solution interface, charge transfer resistance, and diffusion processes [14]. This technique is used to characterize modified electrode surfaces [15, 16]. The Nyquist plot of bare Pt showed low R_{et} (Fig. 4, curve a) as a result of the highly conducting nature of Pt. The increasing diameter of the semicircle portion of curve b (2400 Ω), as related to curve a (300 Ω) in Fig. 4, confirms the electro chemically changing effect of changing the Pt interface with the semiconducting nature of PPy nonomatrix. However, the GNP deposited PPy-Pt shows a much lower Ret (1700 Ω) compared to curve b in Fig. 4 for the redox probe (Fig. 4, curve c) inferring that the GNP is an outstanding electric conducting material accelerated the electron transfer properties of the amended electrode interface. On the other hand, the R_{et} value significantly amplified to 3600 (Fig. 4, curve d) and blocked the electron exchange between the redox probe and electrode surface when SOD1 was successfully biofunctionalised on the SAM-GNP-PPy-Pt. This phenomenon is due to the accumulation of an insulating enzyme layer on the GNP matrix, which results in a higher R_{et} and thereby substantially enlarges the diameter of the semicircle. The results obtained from EIS are in a noble correlation with the results extracted from the CV (Fig. 3) and thus confirms clearly the successful biofunctionalisation of SOD1 on the surface of GNP functionalized electrode surface.

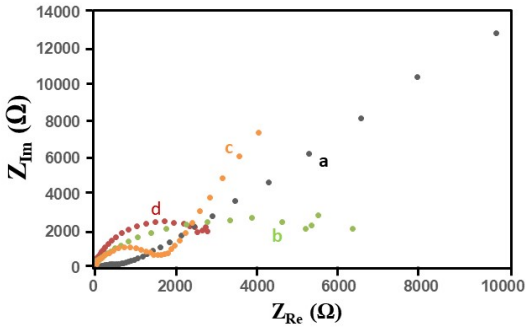

Figure 4: Nyquist plot (Z vs. Z) for Faradaic impedance measurements in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$ redox couple containing 0.1 M KCl at (a) bare Pt, (b) PPy/Pt, (c) GNP/PPy/Pt and (d) SOD1/SAM/GNP/PPy/Pt.

ELECTROCHEMICAL RESPONSE TO DOPAMINE

Differential pulse voltammogram (DPV) was utilized to determine the electrochemical sensing ability of the SOD1 biofunctionalised electrodes for the sensitive determination of DA. The potential window selected was in the range from +0.5 to -0.5 V with a fixed scan rate of 50 mV/s. A rise in the peak current was observed with the increase of concentration of DA at a fixed scan rate of 50 mV/s. A single, well-defined peak appeared at about 0.08–0.10 V (vs. Ag/AgCl) 1 nM to 100 µM (Fig. 5) DA concentrations, with a stable baseline at more negative potentials and no change in peak position, which indicates a reproducible electrode process.

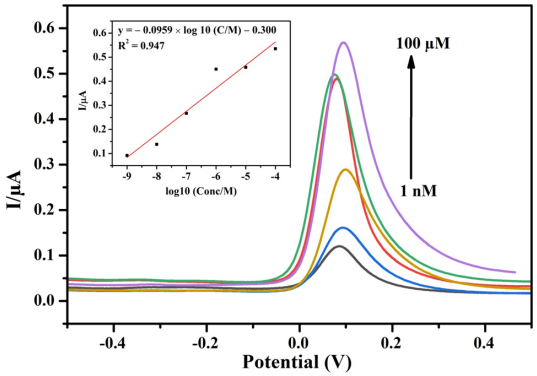

Figure 5: Differential pulse voltammograms of various concentrations of dopamine from 1 nM to 100 µM in 0.1 M PBS (pH 7.0) at SOD1/SAM/GNP/PPy/Pt; scan rate 50 mV/s. Inset figure represents the linear calibration plot of $\log_{10}$[C/M] vs current value in µA.

The peak current varied systematically with DA concentration, decreasing from about 0.57 µA at 1 nM to about 0.12 µA at 100 µM. The net peak current (peak minus baseline) showed a linear relationship with the logarithm of the DA concentration, described by y = −0.0959x − 0.300

($R^2 = 0.947$), where y is the net current (μA) and x is $\log_{10}$[C/M]. From the slope of this calibration plot, the sensor sensitivity was 0.096 μA per decade of concentration. The detection limit, calculated as 3σ/S (σ = standard deviation of the blank, S = sensitivity), was 1 nM, and a clear peak was still obtained at the lowest concentration tested (1 nM). LoD was getting in catalyzes dopamine by extracting the two electrons cum two protons from the hydroxyl groups and oxidizes it to dopamine-o-quinone.

By optimizing the electrode surface, selectivity can be enhanced to minimize interference from other biological substances. DPV is capable of providing real-time data, which is crucial for understanding dopamine fluctuations in biological contexts. The SOD1-SAM-GNP-PPy-Pt biosensor was further applied to quantify the DA in biological samples, the results agreed well with standard reference assay.

Biological matrices can introduce noise and interfere with the detection of dopamine, which can complicate analysis. Nafion like membranes eliminate uric like interferences. The fabrication reproducibility of five electrodes of SOD1, made independently, showed an acceptable reproducibility with a R.S.D.s of 5.1% for the current determined at a DA concentration of 10 μM. Thus, SOD1-SAM-GNP-PPy-Pt biosensors were very efficient for retaining the electrocatalytic activity of DA and preventing SOD1 from leaking out of the electrode surface.

The storage stabilities of DA biosensors stored in air at 4 °C were examined by checking periodically their relative response currents (the ratios of the catalytic currents detected at different times to the initial current value) in 0.1 M PBS (pH 7.0) containing 10 μM DA. After a storage period of 5 weeks at 4 °C the SOD1 based biosensor showed on 13% loss of activity for DA. Thus, the biosensors were stored in air at 4 °C when not in use. Our results of determining DA from biological samples like urine and other biological fluids match with other studies. But in some cases, rapid release of DA and reuptake in the brain make continuous monitoring difficult.

CONCLUSION

DAs significance in diagnostics is vast, affecting a wide range of disorders, especially those related to movement, mood, and reward systems. While direct measurement of DA levels is not always feasible, understanding its role in these conditions helps inform diagnosis, treatment planning, and therapeutic interventions. Electrochemical biosensors are valuable tools in both research and clinical settings for monitoring dopamine levels. Ongoing advancements in material science, electrode design, and data analysis techniques are likely to enhance the performance and applicability of SOD1 biofunctionalized electrochemical biosensor for the selective and sensitive detection of DA in real samples in the future. The sensor exhibited a wide linear range, low detection limit, high stability and good reproducibility.

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DECLARATION OF CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest to declare.

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No funding is received for this work.

AUTHOR CONTRIBUTION STATEMENT

All authors have accepted responsibility for the content of the manuscript, reviewed all results, and approved the final version.

Conceptualization, data collection, and statistical supervision: **P.R.A.S., R.V.;** Data analysis: **R.V., P.G., P.S., S.D., M.D.K.;** Data interpretation: **R.V., P.G., P.S., G.A.;** Writing original draft: **R.V.;** Writing – review and editing: **P.R.A.S., R.V., A.M..**

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are not publicly available as the study is ongoing; however, they are available from the corresponding author upon reasonable request.

Declaration of Generative AI and AI-Assisted Technologies in the Manuscript Preparation Process Statement

The authors declare that ChatGPT was used only for language improvement and grammar checking. All scientific content, analysis, and interpretations were developed solely by the authors.

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