Tyrosinase-Conjugated Prussian Blue-Modified Nickel Oxide Nanoparticles-Based Interface for Selective Detection of Dopamine

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In this paper, we have reported fabrication of a label free dopamine biosensor with improved sensitivity and selectivity using an interface based on Prussian blue (PB) modified nickel oxide (NiO) nanoparticles (NPs) and tyrosinase enzyme conjugates. A wet chemical sol-gel method was used to synthesize NiO NPs followed by surface modification with PB and utilized as a matrix to immobilize tyrosinase. The structural and morphological studies of the prepared NPs were conducted using X-ray diffraction (XRD), high resolution transmission electron microscopy (HR-TEM), Raman and UV-Vis spectroscopy and purity of the sample was confirmed through X-ray photo-electron spectroscopy (XPS), energy dispersive X-ray spectroscopy (EDX) and Fourier transform infrared spectroscopy (FTIR) studies. Subsequently, the PB modified NiO NPs were deposited onto a flexible screen printed carbon electrode (SPCE) substrate and tyrosinase enzyme molecules were immobilized onto PB-NiO NPs functionalized SPCE by covalent immobilization for selective and sensitive detection of neurotransmitter dopamine. The enzyme immobilization was confirmed through scanning electron microscopy (SEM) and FTIR studies and the fabricated electrode was used for electrochemical detection of dopamine using cyclic voltammetry and chrono-amperometric methods. The results of the electrochemical response studies revealed high sensitivity of 60.459 μA/nanomoles in a broad detection range (0.0075-1.5 nanomoles) with a detection limit of 3.117 picomoles, whereas sample volume was as low as 15 μL. The proposed sensor exhibited fast response time of 24 seconds; good selectivity in presence of interferents ascorbic and uric acid; descent shelf life of 50 days with excellent reusability (> 30 times with 78% residual response). The sensor was also successfully validated with spiked real serum samples.

Introduction

With recent advances in nanotechnology, the use of nanomaterials has produced increased interests among researchers to develop miniaturized bioelectronic devices especially biosensors system with improved sensing methodology. A wide variety of nanomaterials in particular nanoparticles with unique physical, chemical and electronic properties can be effectively used to interface biological recognition elements with electronic signal transducer for constructing a new generation biosensor with unique functions.[1] For designing a highly sensitive electrochemical biosensor, nanoparticles can provide an excellent platform to immobilize biomolecules, act as a catalyst in electrochemical reactions, facilitate direct electron transfer at biointerface and enhance electron communication.[1,2] In recent years, metal oxide nanoparticles have attracted huge attention as immobilization matrices owing to wide adsorption capabilities, high surface reactivity and efficient electron transfer between redox centers of the enzymes and substrate.[2,3] Moreover the metal oxide nanoparticles are often used to immobilize biomolecules due to their biocompatibility and effective surface area for immobilization with desired orientation and better conformation that leads to high biological activity of the immobilized bio-sensing molecules.[3,4] Among metal oxide nanoparticles, nickel oxide (NiO) nanoparticles exhibit fascinating features in bio-sensing application in terms of high electro-catalytic activity, fast electron transport property, chemical stability, low cost and biocompatibility.[5] In particular, the availability of variable oxidation states in NiO nanoparticles promotes easy mobility of the electrons.[6] Additionally, the high isoelectric point (IEP 10.8) of NiO nanoparticles make it favorable for immobilization of proteins with low IEPs viz. enzymes. However the oxidation and agglomeration of NiO nanoparticles restrict its functions and...
applications in biosensor development. In this context, the surface of NiO nanoparticles can be modified with a suitable surface modification agent to prevent oxidation and agglomeration and to increase efficacy of the sensing system. Prussian blue (PB) or ferric hexacyanoferrate \([\text{Fe}_{6}\text{[Fe}^{3+}\text{(CN)}_6]\]) is an well-known surface modification agent that can form an electroactive layer by chemical or electrochemical methods. In addition, PB shows an excellent prospect to mediate and accelerate electron transfer process in sensing system in the form film or nanoparticles. The formation of film reveals the advantages of high stability and ease in preparation, whereas nanoparticles can form hybrid structures with other materials. In amperometric biosensor PB has been widely used as an electron transfer mediator for its excellent electrocatalysis and extraordinary electrochemical behavior owing to reversible redox reactions between Fe (II) and Fe (III). Hence the incorporation of PB in nanostructured NiO film promotes direct electron transfer across the biointerface due to high electrocatalytic activity and may result in improved biosensing performance. Therefore, in present work, we have used PB as surface modification agent on NiO NPs and work as immobilization matrix for biosensing purpose.

Detection of neurochemicals is a much challenging task, due to low availability of such chemicals in blood stream, as the neurochemicals cannot easily cross blood-brain barrier. Amongst these, dopamine has been extensively studied due its prominent role in central nervous, cardiovascular, renal and hormonal systems. It is one of the most important excitatory neurotransmitter that influences several physiological conditions such as behavior, learning, memory, mood, attention and movement. Chemically dopamine belongs to catecholamine family and form due to the decarboxylation of 3,4-dihydroxyphenylalanine and act as a precursor in the synthesis of other important neurotransmitters epinephrine and norepinephrine. The abnormalities of dopamine level in body fluids are the symptoms of several diseases namely Parkinson’s disease, epilepsy, schizophrenia, senile dementia and attention deficit hyperactivity disorder (ADHD). Beside this, dopamine has been used as intravenous medication that acts on sympathetic nervous system to increase heart rate and blood pressure during acute heart surgery and other emergency medical conditions. Hence, the precise and accurate determination of the dopamine concentration in biological system is of great interest in medical diagnostics.

In recent past, several approaches have been made to detect dopamine such as laser-induced native fluorescence, high performance liquid chromatography, capillary electrophoresis, flow injection analysis with spectrophotometric detection, colorimetric detection based on silver nanoparticles, UV spectrometry, liquid chromatography-electrospray tandem mass spectrometry and electrochemical analysis. Compared with other methods, the electrochemical detection technique has gained much interest due to fast detection, simplicity in design, high sensitivity, reproducibility and cost-effectiveness. Moreover, the easy operation of electrochemical techniques based on oxidation and reduction of the desirable species on electrode surface make it favorable for easy integration and real time detection. In particular, being an electrochemically active element, dopamine can be detected successfully by applying appropriate potential across the electrodes. However its electrochemical detection is largely affected due to the presence of interfering compounds mainly ascorbic acid and uric acid. Generally ascorbic acid and uric acid co-exist with dopamine in biological environment and due to nearer oxidation potential of dopamine with those interfering substances, it results poor selectivity and sensitivity during dopamine detection. In this regards, an enzyme can be used for specific and selective detection of dopamine with enhanced sensitivity. Tyrosinase (EC 1.14.18.1) is a copper containing oxidase enzyme that catalyzes oxidation of the phenolic compounds into their corresponding o-quinones in a two-step reaction and o-quinones further reduce to form original phenol at appropriate redox potential. Being a catechol like phenolic compound, dopamine acts as a substrate for tyrosinase enzyme and can be detected selectively by its redox reactions. Based on these principles, our group in past have developed a dopamine sensor using tyrosinase enzyme physically adsorbed on NiO NPs surface, which was then deposited onto ITO coated PET substrate for selective detection of dopamine by using cyclic voltammetry. In addition, a layer of polyvinyl alcohol (PVA) was also coated on this surface to prevent desorption of enzyme. The developed sensor exhibited good sensitivity (0.06 μA/μM), short response time (45 sec), intermediate LOD (1.04 μM) and long shelf life (45 days). However, it was still insufficient to detect the level of neurochemical actually present in neuropsychiatric patient. This prompted us to further stabilize the tyrosinase enzyme on the matrix by using covalent immobilization and use additional electron mediator to enhance detection sensitivity. These steps, along with several other refinement in interfacial chemistry and sensor measurement techniques yielded highly improved sensor parameters, which have been discussed in this manuscript.

Results and Discussion

Structural and Morphological Studies of Nanoparticles

A schematic of the step-wise fabrication of bio-sensing platform is demonstrated in Scheme 1. The X-ray diffraction (XRD) pattern of the synthesized NiO NPs shows cubic (fcc) crystal system with space group Fm-3 m and lattice parameter \(a = b = c = 4.18\) Å. The diffraction planes (111), (200), (220), (311), (222) are obtained at 20 positions 37.27°, 43.35°, 62.92°, 75.44° and 79.47° respectively as shown in Figure 1(A), curve (i). All the diffraction peaks are consistent with standard XRD data for NiO phase (JCPDS card no. 73–1523) and confirm the formation of highly crystalline, cubic structure of NiO NPs. No other phases are detected suggesting phase purity of the sample. The average crystal size for NiO NPs is calculated as 13.52 nm for the most intense peak (200) using Debye-Scherrer formula. After modification of NiO NPs with PB, additional diffraction planes (220), (311), (400), (420), (440), (600), (620), (640), (642) are observed at 20 positions 24.62°, 29.17°, 35.33°, 39.66°,
50.71°, 54.14°, 57.41°, 66.27° and 69.19° respectively (Figure 1(A), curve ii) and the subsequent diffraction peaks are matched with PB phase (JCPDS card no. 73–0687). The average crystallite size for PB-NiO NPs for the same peak (200) is calculated using Debye-Scherrer formula and found as 14.27 nm. The slight increase in average crystallite size indicates modification of NiO NPs with PB.
The Raman spectra of the synthesized NPs were recorded at room temperature (25°C) with 514 nm laser. From the Raman spectra of NiO NPs (Figure 1(B), curve i), the peak obtained at 502 cm⁻¹ is due to first-order longitudinal optical (1LO) phonon mode and the peaks at 730 and 1087 cm⁻¹ are associated with second-order transverse optical (2TO) and longitudinal optical (2LO) phonon modes. The vibrational band for two magnon excitation can be observed at 1580 cm⁻¹. After addition of PB, a strong vibrational band is obtained at 2151 cm⁻¹ due to stretching vibration of carbon nitrogen triple bond group of PB (Figure 1(B), curve ii). The peak shifts for PB-NiO NPs from 502 to 551 cm⁻¹, 730 to 758 cm⁻¹, 1087 to 1092 cm⁻¹ and 1580 to 1603 cm⁻¹ from previous NiO NPs indicate the interaction of PB on NiO NPs surface.

The optical absorption spectrum for NiO NPs obtained from UV-visible spectroscopy studies reveals a strong peak at 308 nm due to intra-3d transition of Ni²⁺ in NiO structure and confirms the formation of NiO NPs [Figure S1 in Supporting Information (SI)]. After modification with PB, an additional mixed valence charge transfer band of the polymeric complex [Fe(II)-C≡N-Fe(III)] is attained at 700 nm confirming the formation of PB over NiO NPs surface.[33]

The shape, size and structure of the as-synthesized NPs were determined by high-resolution transmission electron microscopy (HR-TEM) studies (Figure 2). The HR-TEM images of the NiO NPs show randomly oriented, nearly-spherical structure and the presence of clearly visible lattice fringes indicates highly crystalline nature of the sample (Figure 2, images i-iii). The d-spacing of the lattice fringes are found as 0.202 nm and 0.232 nm (Figure 2, image iii) and matched with d-value.

Figure 1. (A) X-ray diffraction spectra of (i) NiO NPs and (ii) PB-NiO NPs; (B) Raman spectra of (i) NiO NPs and (ii) PB-NiO NPs.

Figure 2. TEM and HR-TEM images of (i-iii) NiO NPs and (v-vii) PB-NiO NPs; size distribution histogram of (iv) NiO NPs and (viii) PB-NiO NPs.
corresponding to (200) and (111) planes respectively (JCPDS card no. 73–1523). The mean particle size for NiO NPs has been calculated as 11.34 nm from the histogram analysis of particle size distribution graph fitted with Gaussian function (Figure 2, image vii). After modification with PB, the particles show same spherical shape but tend to be a little larger than NiO NPs due to deposition of the PB (Figure 2, images v-vii). The center of the particle reveals highly crystalline structure of NiO (d-spacing 0.152 nm, corresponding to (220) plane) and the periphery shows dark layer of less crystalline PB structure (Figure 2, image vii). The mean particle size for PB-NiO NPs enhances to 15.1 nm (Figure 2, image viii), confirming the surface modification of NiO NPs with PB. The scanning electron microscopy (SEM) images reveal agglomeration of the NiO NPs with uniformity in size but after surface modification with PB, comparatively less aggregation can be observed (Figure S2 in SI).

The elemental confirmation and distribution in the synthesized NPs were investigated using energy-dispersive X-ray spectroscopy (EDX) analysis. The EDX spectrum of unmodified NiO NPs (Figure S3, curve i in SI) shows consistent peaks for nickel and oxygen indicates absence of any impurities in the prepared sample. After modification, further peaks for iron, carbon and nitrogen are detected due to the presence of PB on NiO NPs surface (Figure S3, curve ii in SI). Table S1 and S2 in SI display the weight and atomic percentages of subsequent elements obtained for NiO and PB-NiO NPs respectively.

Moreover the composition of the prepared NPs were verified by X-ray photoelectron spectroscopy (XPS) studies. The wide range XPS spectrum for NiO NPs reveals the presence of Ni2p and O1s band and after modification, the wide range XPS spectrum for PB-NiO NPs exhibits the band for Fe2p, C1s and Ni2p and O1s band and after modification, the wide range XPS spectrum of NiO NPs (Figure 2, image vii) and the peak at 531.1 eV can be ascribed to C1s are obtained at binding energy 397.4 and 284.4 eV and Fe2p

The deconvoluted spectrum for Ni2p shows five peaks in that region. The peaks at binding energy 853.7, 854.9 and 860.7 eV are associated with Ni2p3/2 and the binding energy 872.4 and 879 eV are attributed with Ni2p1/2 peak. The Ni2p1/2 are raised due to Ni(II) ions in the NiO structure, and the positions of the particular peaks are near to the value of pure NiO (854.2 eV) with a little shift to the lower binding energy due to the oxygen vacancy existing on the surface. For deconvoluted spectrum of O1s, two distinct peaks are detected at binding energy 528.9 and 531.1 eV. The peak at 528.9 eV is due to O– (metal oxide bond) and the peak at 531.1 eV can be ascribed to H2O (chemically and physically bounded water molecules at the surface of NP). After modification with PB, the deconvoluted spectrum of Fe2p exhibits a broad peak and a small peak at binding energy 708.1 and 721.7 eV corresponding to Fe2p3/2 and Fe2p1/2 respectively. Apart from that, the peaks of Ni1s and C1s are obtained at binding energy 397.4 and 284.4 eV respectively due to the presence of –CN functional group in the PB structure.

The Fourier transform infrared (FTIR) spectra of blank SPCE, PB-NiO/SPCE and Tyrosinase/PB-NiO/SPCE electrodes are shown in Figure 3 in the range of 400 to 4000 cm\(^{-1}\). The FTIR spectrum of blank SPCE (Figure 3, curve i) exhibits absorption bands at 1526 and 1702 cm\(^{-1}\) for C=C and C=O stretching vibration mode and the broad peak at 2341 cm\(^{-1}\) is due to C≡C stretching vibration mode. The peaks arise at 3742 and 3849 cm\(^{-1}\) are for OH\(^{-}\) stretching vibration mode of the physically adsorbed water. For PB-NiO/SPCE, the FTIR spectrum (Figure 3, curve ii) shows absorption band at 448 cm\(^{-1}\) due to the formation of [Fe(II)-CN–Fe(III)], indicates the presence of PB. The characteristic absorption band at 670 cm\(^{-1}\) is due to the stretching vibration mode of the metal oxide (M–O) bond revealing the formation of NiO NPs. The two strong absorption bands present at 1013 and 1163 cm\(^{-1}\) are associated with C–O stretching mode of the amide bond used for covalent attachment PB-NiO NPs on SPCE surface and the peak seen at 1641 cm\(^{-1}\) is assigned to N–H bending mode of the same amide bond. The absorption band observed at 1523, 1714 and 2339 cm\(^{-1}\) are assigned to stretching vibration mode of C=C, C=O and C≡C bond respectively of the substrate SPCE. The sharp peak observed at 2091 cm\(^{-1}\) is for C–N stretching vibration mode revealing the formation of PB on NiO NPs surface. The peak at 2934 cm\(^{-1}\) and the weak band at 3346 cm\(^{-1}\) are attributed to C–H stretching and N–H stretching vibration mode of the amide bond used for attachment of PB-NiO NPs on the SPCE substrate and the peaks at 3744 and 3843 cm\(^{-1}\) can be observed due to O–H stretching vibration of the physically adsorbed water. However after immobilization of the enzyme, the FTIR spectrum of Tyrosinase/PB-NiO/SPCE (Figure 3, curve iii) exhibits a strong band at 1643 cm\(^{-1}\) corresponds to N–H bending of the amide bond present in the enzyme. Moreover, the intensity of the absorption band for N–H stretching vibration mode at 3289 cm\(^{-1}\) increases rapidly after introduction of the enzyme from previous PB-NiO/SPCE suggesting successful immobilization of the enzyme. The presence of other characteristics peaks obtained in previous electrode are also detected viz. absorption band at 442 cm\(^{-1}\) due to formation of [Fe(II)-CN–Fe(III)], stretching vibration
mode of Ni–O metal oxide bond at 664 cm$^{-1}$, C–O stretching vibration mode of the amide bond at 1015 and 1153 cm$^{-1}$, C=C stretching vibration mode at 1551 cm$^{-1}$ and C≡C stretching vibration mode at 2335 cm$^{-1}$ in SPCE and C–N stretching vibration of the PB at 2093 cm$^{-1}$. The peaks for O–H stretching vibration due to physically adsorbed water can be seen at 3748 and 3847 cm$^{-1}$.

The surface morphological studies of the blank SPCE, PB-NiO/SPCE and Tyrosinase/PB-NiO/SPCE were conducted by SEM measurements and shown in Figure S5 in SI. The blank SPCE (Figure S5, images i & ii in SI) shows mesoporous rough surface with homogeneous distribution of fine graphite particles. The rough surface facilitates settlement of the NPs on SPCE. After deposition of the PB-NiO NPs on the SPCE surface by covalent attachment, the surface morphology of the PB-NiO/SPCE (Figure S5, images iii & iv in SI) exhibits rough nanoporous surface with the presence of granular particles. The NPs are agglomerated in some places due to the high surface charge. However, after immobilization of the tyrosinase enzyme (Figure S5, images v & vi in SI), the rough granular morphology of the PB-NiO/SPCE electrode changes to smooth, globular morphology and the macromolecular structure of the enzyme is clearly visible. It appears that the rough nanoporous morphology of the PB-NiO/SPCE helps in immobilization and higher loading of the enzyme on working electrode surface.

**Electrochemical Studies**

The electrodes fabricated along with NPs were characterized using cyclic voltammetric (CV) technique prior to biosensor calibration. As shown in Figure 4(A), the cyclic voltammogram of bare SPCE (Figure 4(A), curve i) exhibits no definite redox peak in the mediator free electrolyte. The CV spectra of the PB-NiO/SPCE (Figure 4(A), curve ii) shows well defined oxidation peak at 0.34 V due to reversible redox reaction of Fe(II)/Fe(III) redox couple of PB, which acts as a mediator for direct electron transfer towards the substrate. Moreover, the high electrocatalytic activity, higher surface area and charge of PB-NiO NPs facilitate electron transfer leading to an increased current. After immobilization of the enzyme, CV spectrum of Tyrosinase/PB-NiO/SPCE (Figure 4(A), curve iii) reveals broader oxidation peak at 0.39 V due to insulating nature of the biomolecules that restrict electron mobility. However, after addition of 1.5 nanomoles dopamine solution, the definite redox peaks for dopamine can be observed for Tyrosinase/PB-NiO/SPCE (Figure 4(A), curve iv). Dopamine can be oxidized in presence of tyrosinase to form o-...
dopamine and at lower potential the reduction of α-dopquinone executes. The oxidation of dopamine for Tyrosinase/PB-NiO/SPCE has been detected at the potential of 0.35 V and the anodic peak current (Ipa) increases rapidly due to the availability of active-binding sites in enzyme and enhanced electron transfer between the active sites of the enzyme and electrode. The oxidation of dopamine is a quasi-reversible electron transfer process and the first redox couple is obtained for redox reactions of dopamine, where dopamine is oxidized at 0.351 V to α-dopquinone and the α-dopquinone again reduced to form dopamine at 0.019 V. But some of the α-dopquinone is transformed into more easily oxidizable leucodopaminechrome. The second redox couple at lower potential is attained due to the oxidation of leucodopaminechrome to dopaminechrome at −0.231 V and reduction of dopaminechrome at −0.348 V. The presence of tyrosinase enzyme can make the electrochemical detection of dopamine highly specific and selective and avoid the interference effect of the other compounds.

To check interfacial kinetics of the Tyrosinase/PB-NiO/SPCE surface, the CV spectra were recorded as a function of scan rate varying from 10−250 mV/s (Figure 4(B)). With increasing scan rate the oxidation peak shifts towards more positive potential, indicating a uniform facile charge transfer due to high electrocatalytic activity of PB-NiO NPs. As shown in Figure 4(C) the magnitudes of anodic peak currents (Ipa) increase almost linearly with square root of scan rate (ν1/2), suggesting reversible electron transfer between Fe(II) and Fe(III) of PB that act as a mediator and accelerate electron transfer between enzyme and substrate of the fabricated Tyrosinase/PB-NiO/SPCE. The values of the slope, intercept and regression coefficient are given in eq. (1)

\[ I_{pa} = \frac{-17.80 \mu A + 7.98 ([\mu A^2 s^{-1}(mV)]^{-1/2} \times \sqrt{\text{scan rate (mV.s^{-1})}}{2}} \]

The diffusion co-efficient value (D) of electrolyte solution to the corresponding electrode surface has been calculated using Randles-Sevcik equation (eq. 2)

\[ I_p = (2.69 \times 10^{5}) n^{3/2} A^{1/2} D^{1/2} C^{1/2} \]

where, Ip is the peak current of the electrode (Ipa anodic and Ipc cathodic), n is the number of electrons transferred (1), A is the surface area of the electrode (0.125 cm²), D is the diffusion co-efficient, C is the surface concentration in mol (50 mM) and ν is the scan rate (50 mV/s). The diffusion co-efficient value for Tyrosinase/PB-NiO/SPCE (1.33 × 10^{-8} cm².s^{-1}) is higher as compared to PB-NiO/SPCE (3.12 × 10^{-9} cm².s^{-1}) (Table S3 in SI). The electroactive surface area (Aele) of the Tyrosinase/PB-NiO/SPCE has been determined using Randles-Sevcik equation and calculated diffusion co-efficient value (D).

\[ A_{el} = S/ (2.99 \times 10^{9}) n^{3/2} CD^{1/2} \]

where, S is the slope of straight line obtained from eq. (1). The Ael for Tyrosinase/PB-NiO/SPCE has been calculated as 0.463 mm². Moreover the peak potential reveals a linear relationship with logarithmic function of scan rate [Figure 4(D)]. The value of electron transfer co-efficient (α) and n (number of electrons involved in the redox reaction) can be calculated from slope of the straight line equal to 2.3RT/(1-α)nF for the anodic peak using Laviron’s equation. For Tyrosinase/PB-NiO/SPCE, electron transfer co-efficient (α) has been calculated as 0.585.

For a surface controlled process, the change in surface concentration of the ionic species due to surface modification has been estimated from Brown-Anson model (eq. 4).

\[ I_p = n^2F^2/νA \sqrt{RT} \]

where, γ is the surface concentration of ionic species of the corresponding electrode (mol cm⁻²), F is the Faraday constant (96485 C/mol), R is the gas constant (8.314 Jmol⁻¹ K⁻¹) and T is room temperature (25°C). As shown in Table S3 in SI, the surface concentration of ionic species for Tyrosinase/PB-NiO/SPCE (7.41 × 10⁻⁹ mol cm⁻²) is higher than that of the PB-NiO/SPCE (3.58 × 10⁻⁹ mol cm⁻²).

All the electrochemical measurements were conducted in triplicate under identical condition. The optimum pH for tyrosinase enzyme has been found 6.5 from the previous studies. Hence pH of the reaction medium (electrolyte) has been kept 6.5 for all the experiments. Furthermore the working electrode of each strip has contained with an enzyme loading of 12.66 Units of tyrosinase when prepared afresh. After immobilization, the value of Michaelis–Menten constant (Km) for the immobilized enzyme at fabricated Tyrosinase/PB-NiO/SPCE has been estimated as 54.1 μM for dopamine from Hanes-Wolf plot. The obtained low Km value indicates high affinity of the Tyrosinase/PB-NiO/SPCE towards the substrate dopamine and the matrix PB-NiO NPs facilitate enzymatic reaction between tyrosinase and dopamine with higher enzyme activity. For the CV studies (Figure 4(A)), the 10th cycle of CV spectra for each curve have been plotted.

Sensor Response Studies

The amperometric response studies of the fabricated Tyrosinase/PB-NiO/SPCE for dopamine were investigated by chronoamperometry method. As shown in Figure 4(A) dopamine can be oxidized at 0.35 V by Tyrosinase/PB-NiO/SPCE, so that the potential was kept fixed at oxidation potential of dopamine (0.35 V) and the resulting current from faradaic processes at the electrode were recorded as a function of time. The response time of the fabricated Tyrosinase/PB-NiO/SPCE was determined for available highest dopamine concentration (3.75 nanomoles) in PBS (50 mM, pH 6.5, 0.9% NaCl) using chronoamperometry method in the time range of 0 to 120 seconds. From Figure S6 in SI, it can be observed that 90% of the sensor response has been obtained within 24 seconds, suggesting this as the response time for the developed sensor. For all the successive chronoamperometry measurements, sensor response was denoted at 24 sec.
The electrochemical response studies of the fabricated Tyrosinase/PB-NIO/SPCE were conducted as a function of dopamine concentration (0.0015-3.75 nanomoles) in PBS (50 mM, pH 6.5, 0.9% NaCl) by chronoamperometry method. The results of the studies (Figure 5(A)) show the magnitude of the current enhances with increasing concentration of dopamine due to the transfer of more electrons during enzymatic reaction with higher concentrations of substrate. It has been observed from the corresponding calibration curve (Figure 5(B)) that \( \delta I \) (response current at particular concentration of the analyte - response current for blank) maintains a linear relationship with dopamine concentration in the range of 0.0075 to 1.5 nanomoles with linear regression co-efficient (\( R^2 \)) of 0.985. The following equation is attained from calibration curve (eq. 5)

\[
\delta I \ [\mu A] = 60.459 \frac{[\mu A](\text{nanomoles})^{-1}}{\text{concentration (nanomoles)}} \times \]

\( R^2 = 0.985 \) (5)

The sensitivity of the fabricated Tyrosinase/PB-NIO/SPCE has been calculated as 60.46 \( \mu A/\text{nanomoles} \) from slope of the curve. The standard deviation and low detection limit (LOD) have been found as 0.032 \( \mu A \) and 3.117 picomoles, respectively. However, the calibration curve for entire concentration range (0.0015-3.75 nanomoles) for dopamine detection has been fitted with Hill equation (inset of Figure 5(B)), with regression coefficient 0.998 and Chi²/DoF value 5.237. The sensing parameters of the fabricated Tyrosinase/PB-NIO/SPCE are given in Table 1 along with those reported in literature. From the comparison, it can be seen that the present method exhibits better sensing characteristics for dopamine with simple fabrication strategy. Among the other reported results, few differential pulse voltammetry (DPV) based studies\(^{[41,42]} \) yielded better LOD. But DPV system requires more sophisticated platform for detection with costly instrumentation. Furthermore, our method utilized a simpler and easy to fabricate matrix for tyrosinase immobilization in comparison to the complex matrix such as bilayer poly-[\( \beta \]-CD-pyrrole/reduced graphene oxide/poly-[\( \text{NEt}^+ \)-pyrrole], as reported by Fritea et al\(^{[43]} \) and it still shows one of the lowest LOD value.

**Figure 5.** (A) Amperometric response studies of the fabricated Tyrosinase/PB-NIO/SPCE at different concentration of dopamine (0-3.75 nanomoles); (B) Calibration curve of the biosensor at linear concentration (0.0075-1.5 nanomoles) range for dopamine, (inset) shows calibration curve of the biosensor at entire concentration range (0.0015-3.75 nanomoles).

**Selectivity, Reusability and Shelf-life Studies**

The effect of potential interferents viz. ascorbic acid (AA) and uric acid (UA) on the performance of the fabricated Tyrosinase/PB-NIO/SPCE during dopamine detection was analysed because AA and UA present with dopamine in human serum and other real samples and show interference effect in electrochemical detection of dopamine due to presence of a nearer oxidation potential\(^{[29,30]} \). In order to avoid the interference from AA and UA, the electrochemical detection of dopamine was performed by chronoamperometry method at fixed potential of 0.35 V, which is the oxidation potential of dopamine for the fabricated Tyrosinase/PB-NIO/SPCE electrode. Moreover the immobilized tyrosinase enzyme was efficient in selective detection of dopamine. The interference effect of the AA and UA on Tyrosinase/PB-NIO/SPCE was determined in PBS (50 mM, pH 6.5, 0.9% NaCl) solution containing equal amount (1:1) of dopamine (1.5 nanomoles) and either normal physiological concentration or 10 times of normal physiological concentration of interferents, such as AA (0.05 and 0.5 mM), UA (0.3 and 3 mM) and mixture of two interferents (AA 0.05 mM and UA 0.3 mM) and (AA 0.5 mM and UA 3 mM). The change in current response for the interferent solution has been compared and measured with respect to control sample (1.5 nanomoles of dopamine) and as illustrated in Figure 6(A), no considerable change (RSD value ~2-14%) has been observed due to the presence of interfering agents. The details of the relative standard deviation with control sample for each solution have summarized in Table S4 in SI.

The reproducibility of the Tyrosinase/PB-NIO/SPCE was analysed by measuring the current response in presence of 1.5 nanomoles dopamine solution using chronoamperometry method for five different electrodes prepared in the same batch. The results reveal (Figure S7 in SI) good reproducibility for the five different electrodes with standard error less than 3%. The reusability of the same electrode was investigated by repetitive measurements with 1.5 nanomoles dopamine sol-
It has been found that the sensor can retain 78.15% of the initial response even after 30th measurements (Figure 6[B]). This is attributed to the covalent immobilization of the enzymes which can sustain repetitive usage during successive dopamine detections while minimizing enzyme leaching and denaturation under the sensor measurement conditions.

The shelf-life and storage stability of the fabricated Tyrosinase/PB-NiO/SPCE was studied by observing the current response using chronoamperometry method for 1.5 nanomoles dopamine solution at a regular interval of 2 days up to 11 days and then after a gap of 5 days. As shown in Figure 6(C), the developed sensor can retain its response by up to 97% after 5 days, 92% after 10 days, 87% after 30 days, 82% after 46 days and falls to 80% after 50 days (extrapolation) when stored under refrigerated condition (4°C). The results reveal high storage stability due to strong covalent immobilization of the tyrosinase enzyme on PB-NiO NPs matrix, which can effectively preserve the enzyme activity for a long period of time.

Table 1. Sensing performance of the fabricated Tyrosinase/PB-NiO/SPCE for dopamine along with those reported in literature.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Method</th>
<th>Linearity (µM)</th>
<th>Sensitivity (µA/µM)</th>
<th>Detection limit (µM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase/Biocomposite gel (agar-guar gum)/GCE</td>
<td>DPV</td>
<td>2-10</td>
<td>-</td>
<td>0.9</td>
<td>[44]</td>
</tr>
<tr>
<td>Tyrosinase/MWNT/Nafion/GCE</td>
<td>Amperometric</td>
<td>5-23</td>
<td>0.012</td>
<td>0.52</td>
<td>[45]</td>
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<tr>
<td>Tyrosinase/Glutaraldehyde/Egg-shell membrane</td>
<td>DPV</td>
<td>50-250</td>
<td>-</td>
<td>25</td>
<td>[46]</td>
</tr>
<tr>
<td>Tyrosinase/Poly(vinyl alcohol)/Ferrocene/Pd/sol-gel</td>
<td>Amperometric</td>
<td>-</td>
<td>0.000125</td>
<td>50</td>
<td>[47]</td>
</tr>
<tr>
<td>Tyrosinase/Carbon paste</td>
<td>Amperometric</td>
<td>15-250</td>
<td>0.0063</td>
<td>15</td>
<td>[48]</td>
</tr>
<tr>
<td>Tyrosinase/NiO/ITO</td>
<td>CV</td>
<td>2-100</td>
<td>0.06</td>
<td>1.04</td>
<td>[49]</td>
</tr>
<tr>
<td>Bilayer poly-[l]-CD-pyrrole/RGO/Poly-[NEt4+]-pyrrole/Tyrosinase</td>
<td>Amperometric</td>
<td>0.027-38.6</td>
<td>0.012</td>
<td>0.027</td>
<td>[50]</td>
</tr>
<tr>
<td>Graphene-Chitosan/GCE</td>
<td>CV</td>
<td>5-200</td>
<td>-</td>
<td>-</td>
<td>[51]</td>
</tr>
<tr>
<td>CD-MWCNT-poly(luminal)/AuNP/GCE</td>
<td>DPV</td>
<td>1-56</td>
<td>-</td>
<td>0.19</td>
<td>[52]</td>
</tr>
<tr>
<td>AuNP</td>
<td>UV-Visible</td>
<td>0.54-5.4</td>
<td>-</td>
<td>0.36</td>
<td>[53]</td>
</tr>
<tr>
<td>CNP/functionalyzed-silicate particles/ITO</td>
<td>DPV</td>
<td>0.3-18</td>
<td>-</td>
<td>0.36</td>
<td>[54]</td>
</tr>
<tr>
<td>Polycresol red/GCE</td>
<td>LSV</td>
<td>10-100</td>
<td>-</td>
<td>1.5</td>
<td>[55]</td>
</tr>
<tr>
<td>MWCNT/GCE</td>
<td>DPV</td>
<td>3-300</td>
<td>-</td>
<td>0.8</td>
<td>[56]</td>
</tr>
<tr>
<td>Exfoliated flexible graphite paper (e-FGP)</td>
<td>DPV</td>
<td>0.3-35</td>
<td>-</td>
<td>0.01</td>
<td>[57]</td>
</tr>
<tr>
<td>(Gold nanofilm/NPG/ITO)</td>
<td>DPV</td>
<td>1.5-27.5</td>
<td>-</td>
<td>1.5</td>
<td>[58]</td>
</tr>
<tr>
<td>Electrochemically reduced graphene oxide (ERGO)/GCE</td>
<td>DPV</td>
<td>0.5-60</td>
<td>0.482</td>
<td>0.5</td>
<td>[59]</td>
</tr>
<tr>
<td>Graphene/GCE</td>
<td>DPV</td>
<td>4-100</td>
<td>-</td>
<td>2.64</td>
<td>[60]</td>
</tr>
<tr>
<td>Tyrosinase/PB-NiO/SPCE</td>
<td>Amperometric</td>
<td>0.5-100</td>
<td>0.907</td>
<td>0.208</td>
<td>Present work</td>
</tr>
</tbody>
</table>
Real Sample Analysis

To demonstrate the potential applications and to investigate the matrix effect of biological fluids on the developed sensor, the detection of dopamine was conducted by spiking known concentration of dopamine in fetal bovine serum samples using standard addition method. In this regards, the fetal bovine serum sample was diluted 10 times with PBS (pH 6.5) and five different concentrations (0.0015, 0.015, 0.15, 1.5 and 3.75 nanomoles) of dopamine were added for electrochemical measurements. The current responses for standard samples, serum samples added with particular concentrations of dopamine with or without the presence of excess amount of interferents ascorbic acid (0.5 mM) and uric acid (3 mM) were recorded by chronoamperometry method. The obtained results [Figure 6(D)] show the current responses for serum samples with or without the presence of interferents are in reasonable agreement with equivalent standard samples. The satisfactory recovery values found for both serum samples with or without interferents are illustrated in Table S5 in SI. In our experiment, fetal bovine serum was chosen as a matrix for real sample analysis as it comprises with high content of protein and the protein content in cerebrospinal fluid (CSF) and other body fluids is much less than fetal bovine serum sample. The proposed sensor exhibits satisfactory performance in terms of low relative standard deviation for fetal bovine serum and standard solutions. Hence the developed sensor can precisely determine the dopamine concentration in real samples such as human serum and CSF.

Conclusions

In summary, we reworked our strategy for developing a sensitive and selective biosensor for dopamine and from our previous work, wherein we immobilized tyrosinase through physical adsorption onto NIO NPs and kept it onto ITO coated PET substrate with an additional layer of PVA to prevent desorption of enzyme. However, it was still insufficient to detect the low levels of neurochemical actually present in neuropsychiatric patients. This prompted us to further stabilize the tyrosinase enzyme on the matrix by using covalent immobilization and use additional electron mediator to enhance detection sensitivity. These, and additional sensor optimization steps yielded significantly improved sensor parameters. The sensitivity has been enhanced more than 15 times to reach 0.91 µA/µM, (60,459 µA/nanomoles), whereas LOD decreased 5 times to 0.208 µM (3.117 picomoles). The fabricated Tyrosinase/PB-NIO/SPCE also showed reduced response time of 24 seconds from previous 45 seconds, broader linear detection range of 0.5-100 µM (0.0075-1.5 nanomoles) and lower sample volume of 15 µL. The sensing performance of the developed Tyrosinase/PB-NIO/SPCE for dopamine has been well comparable with seventeen previously reported work on dopamine sensor.

Moreover, the developed sensor was used to estimate dopamine in spiked real samples and in presence of potential interfering substances ascorbic acid and uric acid with reason-

able RSD value (2-15%). Apart from that, the sensor exhibited a descent storage stability (50 days) with good reusability (> 30 times with 78% residual response) which makes it an effective biosensor. The sensor fabrication strategy provides a simple, sensitive, relatively specific, ease in operation and cost-effective method to prepare dopamine biosensor and the observed results offer bio-functionalized PB-NIO NPs as a new promising platform in sensor development and other biomedical applications. Efforts are being made to incorporate the developed biosensing platform in point-of-care and microfabricated devices in order to get even more sensitive detection of neurotransmitters and other metabolites in lesser sample, shorter response time and low interference, so as to effectively detect the neurochemicals in serum of neurodegenerative disease patients.

Supporting Information (SI)

Materials and experimental methods used in present study, tables showing percentage of elemental distribution of the prepared NIO NPs and PB-NIO NPs, values of interfacial kinetics parameters for PB-NIO/SPCE and Tyrosinase/PB-NIO/SPCE electrodes, interfering effect of ascorbic acid and uric acid during dopamine detection, response of dopamine detection in fetal bovine serum samples and figures containing UV-vis absorption spectra, scanning electron microscopy (SEM) images, energy dispersive X-ray (EDX) spectra, X-ray photoelectron spectroscopy (XPS) of NIO NPs and PB-NIO NPs, SEM images of SPCE, PB-NIO/SPCE, Tyrosinase/PB-NIO/SPCE electrodes, calculation of sensor response time of the developed Tyrosinase/PB-NIO/SPCE for dopamine detection, reproducibility studies of Tyrosinase/ PB-NIO/SPCE electrodes prepared in same batch.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Dopamine biosensor · Electrochemical sensor · NIO nanoparticles · Prussian blue · Tyrosinase
