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Virtual electrochemical nitric oxide analyzer using copper, zinc superoxide dismutase immobilized on carbon nanotubes in polypyrrole matrix

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ABSTRACT

In this work, we have designed and developed a novel and cost effective virtual electrochemical analyzer for the measurement of NO in exhaled breath and from hydrogen peroxide stimulated endothelial cells using home-made potentiostat. Here, data acquisition system (NI MyDAQ) was used to acquire the data from the electrochemical oxidation of NO mediated by copper, zinc superoxide dismutase (Cu,ZnSOD). The electrochemical control programs (graphical user-interface software) were developed using LabVIEW 10.0 to sweep the potential, acquire the current response and process the acquired current signal. The Cu,ZnSOD (SOD1) immobilized on the carbon nanotubes in polypyrrole modified platinum electrode was used as the NO biosensor. The electrochemical behavior of the SOD1 modified electrode exhibited the characteristic quasi-reversible redox peak at the potential, +0.06 V vs. Ag/AgCl. The biological interferences were eliminated by nafion coated SOD1 electrode and then NO was measured selectively. Further, this biosensor showed a wide linear range of response over the concentration of NO from 0.1 μM to 1 mM with a detection limit of 0.1 μM and high sensitivity of 1.1 μA μM^{-1}. The electroanalytical results obtained here using the developed virtual electrochemical instrument were also compared with the standard cyclic voltammetry instrument and found in agreement with each other.

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1. Introduction

NO is involved in signaling in the central and peripheral nervous systems [1]. It also plays a regulatory role in physiological processes such as smooth muscle relaxation and anticoagulation [2]. Further, it has been implicated in Parkinson's disease [3] and in the pathogenesis of several cardiovascular diseases i.e., impairment of NO production leads to hypertension or atherosclerosis [4] and excess of NO may participate in ischemia reperfusion injury [5]. The measurement of NO in exhaled breath is increasingly important in clinical settings to monitor airway inflammation and oxidative stress implicated in the pathogenesis of various respiratory conditions [6] and diagnosis of diseases such as asthma, primary cilia dyskinesia [7]. Moreover, the measurement of NO release from endothelial cells gives an idea to know mechanistic perspective in the oxidant-induced regulation of signal transduction processes involved in cell survival and cell death [8]. Based on its important functions in the physiological processes, there has been a substantial growth in the development of methods for the measurement of NO at biologically relevant concentrations.

Various methods, such as electron paramagnetic resonance [9,10], spectrophotometry [11,12] and chemiluminescence [13,14] have been reported for the determination of NO. However, these methods are not succeeded in providing adequate sensitivity and specificity. Until now, specific determination of NO in biological sample has been a big challenge, since NO is an unstable compound with half-life time of about five seconds and also it reacts rapidly with oxygen (O_2) to form NO_2^- and NO_3^-. Electrochemical techniques are most promising for their simplicity, high sensitivity (particularly with the use of modified electrodes), good selectivity, fast response and long term stability for the direct determination of NO [15–18].

Recently, electrochemical determination of various electroactive substrates using cyclic voltammetry (CV) have been reported [19–21]. However, the physical instrument CV involves high cost and the hardware instrumentation systems are made up of pre-

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defined hardware components that are completely specific to their measurement function. Because of their hard-coded function, these stand-alone physical instruments are more limited in their versatility. The recent development of software and hardware technology opens a new way to overcome these drawbacks with the help of virtual instruments. Virtual instrument is a program that implements the functions of physical instruments and the program written in the LabVIEW (Laboratory Virtual Instrumentation Engineering Work bench) or in other programming languages.

LabVIEW is a suitable multifunction graphical programming environment for the development of virtual instruments. It has been reported that using LabVIEW, the virtual instruments were developed to demonstrate the instrumentation principles [22], study the oxidase reactions [23], measure the heart and lung sounds [24] and also for the determination of rate constant [25], trace metals [26], morphine [27]. Economou et al. developed a virtual instrument for the electroanalytical measurements [28] and also reported the square wave voltammetry for the determination of riboflavin in multivitamin tablets [29]. Shi et al. fabricated an electrochemical instrument using MATLAB environment and validated with the ferricyanide solutions [30]. Until now in the literature, development of virtual CV for the measurement of NO using Cu, ZnSOD (SOD1) has not been reported. The electrochemical oxidation of NO mediated by SOD1 is a selective since the active site channel of the SOD1 is so narrow that it can allow selectively only small substrates [31].

So, a LabVIEW-based virtual CV for the determination of NO using SOD1 immobilized on carbon nanotubes (CNT) in polypyrrole (PPy) matrix for the first time has been reported. Highly flexible electrochemical control programs (graphical user-interface software) were created within short time periods using LabVIEW 10.0. Further, this virtual electrochemical analyzer was used to measure the NO level present in the exhaled breath and also applied to measure the NO release from hydrogen peroxide (H_2O_2) stimulated endothelial cells. Thus, inexpensive electronic components, small dimensions and battery operation make this virtual electrochemical analyzer suitable for the determination of NO and ideal for in situ or field applications.

2. Materials and methods

2.1. Chemicals and reagents

Copper, zinc superoxide dismutase (Cu,ZnSOD) from bovine erythrocytes, sodium ascorbate, uric acid, sodium hydrogen phosphate, disodium hydrogen phosphate, pyrrole, nafion, citric acid, NaOH, H_2SO_4, $NaNO_2$, KCl and glutaraldehyde were purchased from Sigma Company (Milwaukee, WI, USA). Single walled carbon nanotubes were purchased from Carbon solutions Inc., CA, USA. Human aortic endothelial cells and fetal bovine serum (FBS) were obtained from Lonza (Walkersville, MD). All the solutions were prepared with doubly distilled water.

2.2. Endothelial cell culture

Endothelial cells were grown in endothelial cell basal medium-2 (EBM-2 bullet kit) containing 10% FBS and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Then, the cultured endothelial cells were treated with 250 μM and 500 μM H_2O_2. After 8 h treatment the media was taken for the NO measurement.

2.3. Preparation of saturated NO solution

Saturated NO solution (2 mM) was prepared as per earlier report [17]. Briefly, NO gas was produced by dropping 1.5 M sulfuric acid solution in saturated solution of sodium nitrite ($NaNO_2$). Then, the produced NO gas was forced to bubble into a NaOH solution (30%) to remove the NO_2 (formed as a result of its reaction with O_2) and the other gases. This filtered pure NO gas was bubbled in water for 10 min. Further, this saturated NO solution was used for the various concentration study by making serial dilutions. All the solutions used for the preparation of standard (including water for dilutions) and buffer solutions were deoxygenated with argon for 15 min.

2.4. Equipments

MyDAQ from National Instruments provides analog input (AI), analog output (AO), digital input and output (DIO) functions with USB connectivity. LabVIEW 10.0 software from National Instruments was used to develop the electrochemical control programs. PC running windows 2007 and XP was used for monitoring the results. Standard physical instrument CHI 1200B electrochemical workstation (CH Instruments, USA) was used to validate the results obtained using virtual electrochemical analyzer. All the electrochemical experiments were performed with a conventional three electrode system which consisted of an Ag/AgCl wire as reference electrode, Pt wire as auxiliary or counter electrode and SOD1 immobilized on CNT-PPy modified Pt electrode as the working electrode.

2.5. Construction of the home-made potentiostat and NO biosensor

The potentiostat circuit for the virtual electrochemical analyzer was constructed using an operational amplifier LM358. The potentiostat was powered by a ± 5 V regulated power supply constructed using two 9 V batteries and IC voltage regulators. Further, this potentiostat was connected to the NI MyDAQ for the data acquisition as shown in Fig. S1. The dual operational amplifier (LM358) was employed to sweep the potential between the working and reference electrodes. Further, the potentiostat consists of a feedback resistor (R_f), acting as a current to voltage (I to V) converter placed parallel to a 0.1 μF capacitor to arrest the noise thereby producing noiseless voltammogram. Graphical user interface (GUI) software was developed using LabVIEW 10.0 to control the potentiostat, acquire the current response and process the acquired current signal (Supplementary material). The overall electrochemical setup for the electrochemical analyzer is shown in Fig. 1.

SOD1 modified CNT-PPy-Pt electrode was constructed as per earlier report [19] and first time it was used as the NO biosensor based on the electrochemical oxidation of NO mediated by SOD1. Briefly, pyrrole was electropolymerized on the Pt electrode by the irreversible oxidation of 0.4 M pyrrole in 0.1 M KCl by applying the potential of 0 V to 0.9 V vs. Ag/AgCl at a scan rate of 50 mV s^{-1} for 10 complete cycles. Then, CNT modified PPy-Pt electrode was made by dropping 25 μL of CNT solution (1 mL of 0.5 wt% nafion-ethanol solution containing 2 mg of CNT) on PPy-Pt electrode and dried at the room temperature. After that, 10 μL of SOD1 solution was dropped onto the CNT-PPy-Pt electrode by employing the 5 μL of glutaraldehyde as a cross-linking agent to obtain SOD1-CNT-PPy-Pt electrode (Scheme 1). This SOD1 modified electrode was immersed in 0.1 M PBS to remove the loosely adsorbed SOD1 and stored at 4 °C when not in use.

3. Results and discussion

Recently, virtual instrumentation is rapidly replacing the costly bench top instrumentation because it offers flexible, fast and cost effective solutions [32]. Development of virtual instrument with the

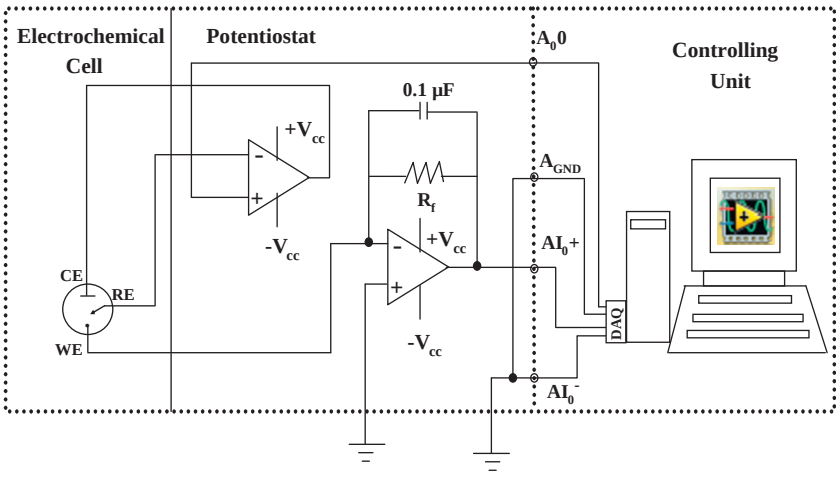
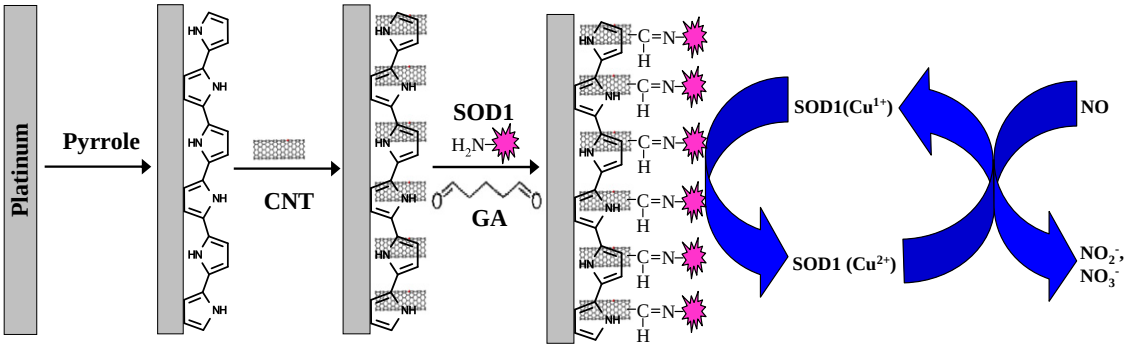


Fig. 1. Electrochemical setup for the virtual electrochemical NO analyzer.



Scheme 1. Schematic representation of the fabrication of SOD1-CNT-PPy-Pt electrode and the illustration of the reactions during the determination of NO.

help of user-friendly graphical programming languages allows fast program development and its execution speed is quite comparable with the conventional languages c and c++. LabVIEW is the suitable graphical language used to replace the conventional programming languages. LabVIEW-based virtual instrument has been developed for pH measurement in biological system [33], calcium buffer calculations [34] and to control the instrument [35]. The main objective of our work is to design the virtual instrument using LabVIEW 10.0 for the electrochemical determination of NO. Moreover, it reduces the cost and achieves flexibility for sensitive and selective NO measurement. In order to test the performance of the developed virtual electrochemical NO analyzer, sensitivity, linearity, detection limit were investigated and validated with the biological samples.

3.1. Electrochemical characterization of the SOD1-CNT-PPy-Pt electrode using the virtual electrochemical analyzer

Fig. 2 shows the electrochemical response of the PPy-Pt (curve a), CNT-PPy-Pt (curve b) and SOD1-CNT-PPy-Pt (curve c) electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 100 mV s^{-1} vs. Ag/AgCl. There were no significant peaks obtained on the range from −0.2 V to 1.0 V for PPy-Pt and CNT-PPy-Pt electrodes. However, the SOD1-CNT-PPy-Pt electrode exhibited a quasi-reversible peak at the potential of +0.06 V vs. Ag/AgCl. This observed quasi-reversible peak is attributed to the Cu^{2+}/Cu^{+} redox changes at the active site of SOD1 in agreement with the previously reported data [19]. Hence, it clearly reveals that the SOD1 was immobilized on the electrode surface.

3.2. Electrochemical response to NO

Fig. 3 shows the comparison of the electrochemical responses of the SOD1-CNT-PPy-Pt electrode obtained using virtual electrochemical analyzer (curve a and c) and the standard physical instrument CHI 1200B (curve b and d) in the absence and presence of 500 µM NO solution in 0.1 M PBS (pH 7.0) at a scan rate of 100 mV s^{-1} vs. Ag/AgCl. Before the addition of NO solution into 0.1 M PBS, no significant changes were observed on both virtual (curve a) and physical instruments (curve b) CV. After the addition of 500 µM NO solution into the 0.1 M PBS, a new irreversible anodic oxidation peak was observed at the potential of 0.8 V on both the virtual (curve c) and physical (curve d) instrument CVs, which may be attributed to the electrochemical oxidation of NO mediated by SOD1 at the electrode surface (Scheme 1). It is clearly seen that from Fig. 3, both the virtual electrochemical analyzer and physical instrument CHI 1200B exhibited almost similar electrochemical responses under the same conditions.

3.3. Linearity, stability, reproducibility and selectivity

Typical electrochemical responses were obtained using virtual electrochemical analyzer for the various concentrations of NO in 0.1 M PBS (pH 7.0) at a scan rate of 100 mV s^{-1} for SOD1-CNT-PPy-Pt electrode and are shown in Fig. 4A. The observed anodic currents vs. NO concentrations are plotted as shown in Fig. 4B exhibits a linear range of response for the NO concentrations from 0.1 µM to 1 mM (r^2 = 0.999, n = 3) with a detection limit of 0.1 µM.

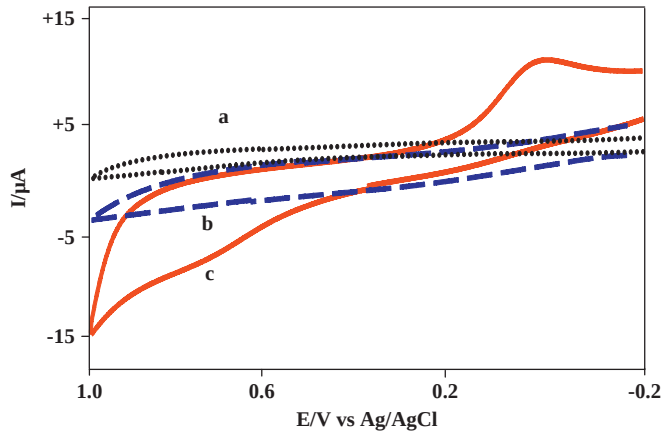


Fig. 2. Electrochemical response of the (a) PPy-Pt, (b) CNT-PPy-Pt and (c) SOD1-CNT-PPy-Pt electrodes in 0.1 M PBS (pH 7.0) at scan rate: 100 mV s^{-1} vs. Ag/AgCl.

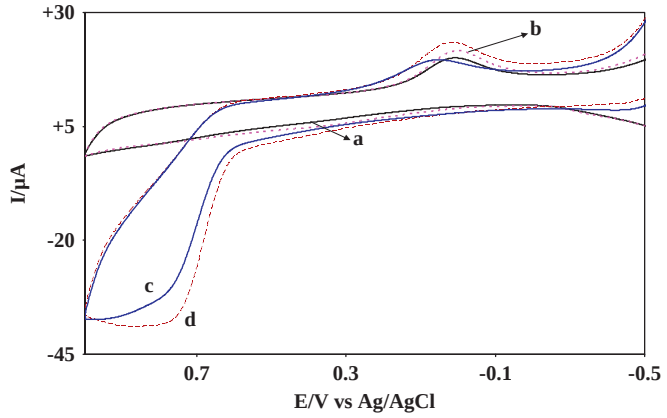


Fig. 3. Comparison of the electrochemical response of the SOD1-CNT-PPy-Pt electrode measured using virtual electrochemical NO analyzer (curve a and c) and the standard physical instrument CHI 1200B (curve b and d) in the absence (a and b) and presence (c and d) of 500 μM NO solution in 0.1 M PBS (pH 7.0) at scan rate: 100 mV s^{-1} vs. Ag/AgCl.

For the stability and reproducibility tests, anodic responses for NO were recorded three times daily and the current responses were found to be constant for two months, which holds relatively longer stability.

Earlier, superoxide anion radical, NO_2^- and cysteine biosensors were developed by us using SOD1 [19,20]. The SOD1 modified electrode exhibited the peak potentials at −0.37 V and +0.06 V for cysteine and superoxide anion radical measurements, respectively. So, there may not be any interference due to them in the measurement of NO in biological samples. However, the NO_2^- was measured at the potential of 0.68 V; it is very near to the electrochemical oxidation potential of NO. Thus, the NO_2^- may be interfering with the NO measurement. Besides NO_2^-, the effect of other coexisting biological substrates such as ascorbic acid (AsA), uric acid (UA) and NO_3^- were investigated by the time vs. current response of the biosensor using chronoamperometry. Upon addition of 250 μM each of UA and NO_3^- into 0.1 M PBS containing 500 μM NO, no current changes were observed (data not shown). Therefore, these substrates were (UA and NO_3^-) not interfering with the NO measurement. However, after the addition of 250 μM each of AsA and NO_2^-, noticeable current changes were observed (Fig. 5A). In order to eliminate these interferences (AsA and NO_2^-), nafion membrane was coated onto the SOD1-CNT-PPy-Pt electrode by dipping the electrode into nafion solution (5% solution (w/w) was diluted to a final concentration of 1% (w/w) using ethanol) and dried at room temperature.

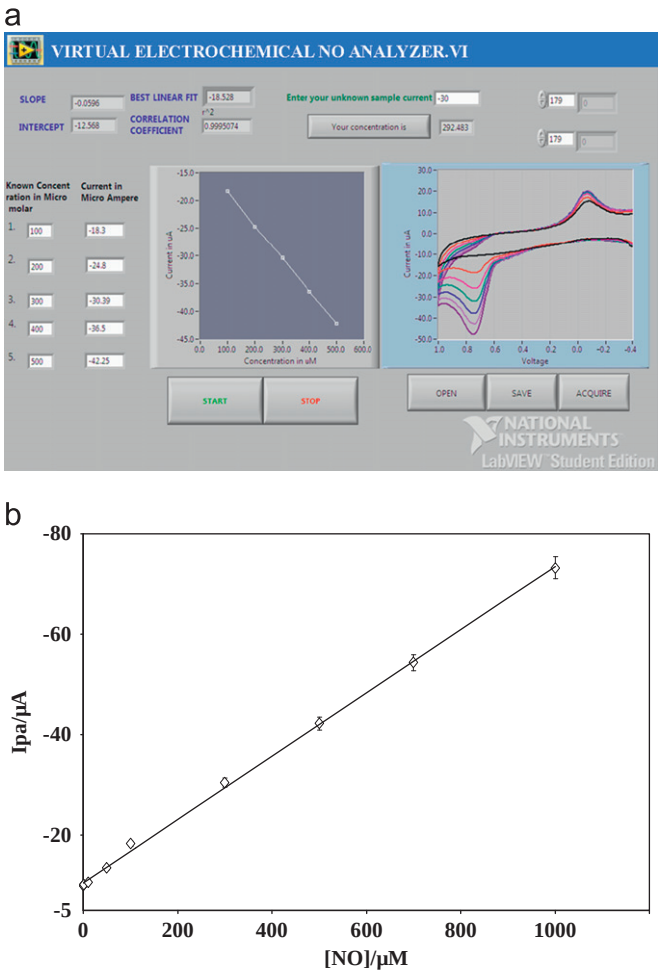


Fig. 4. (A) Front panel of the virtual electrochemical NO analyzer show the typical cyclic voltammetric response of the SOD1-CNT-PPy-Pt electrode at control, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM and 600 μM NO solution in 0.1 M PBS (pH 7.0) at scan rate: 100 mV s^{-1} vs. Ag/AgCl and the linear plot for the above concentrations and (B) A linear calibration plot for the anodic peak currents I_{pa}/μA vs. [NO]/μM. (I_{pa} = −0.063 [NO] − 10.49, r^2 = 0.999). Each point represents the average of three measurements.

Again the similar chronoamperometric study was carried out upon addition of AsA and NO_2^-. Now, no current changes were observed on the time vs. current response of the biosensor (Fig. 5B). Thus, the nafion membrane coated SOD1-CNT-PPy-Pt electrode is highly selective to the NO measurement.

3.4. Effect of pH and scan rate

The electrochemical behavior of the NO biosensor was studied using virtual electrochemical analyzer in the pH range of 3.0–10.0. For the pH study, a mixture of disodium hydrogen phosphate and citric acid buffer was used. The current response was decreased from pH 7.0 to 3.0 and also from pH 7.0 to 10.0, perhaps due to the denaturation of immobilized SOD1. However, the maximum current response was observed at pH 7.0 (Fig. 6A). Further, the influence of the scan rate on the virtual CV performance of the SOD1-CNT-PPy-Pt electrode was also investigated (Fig. 6B). It was found that the anodic peak currents negatively increased linearly with increasing the scan rate from 100 mV s^{-1} to 350 mV s^{-1} and as well the characteristic CV remains unchanged. This indicates the favorable orientation of SOD1 at the CNT-PPy-Pt electrode leading to a facilitated electron transfer of SOD1.

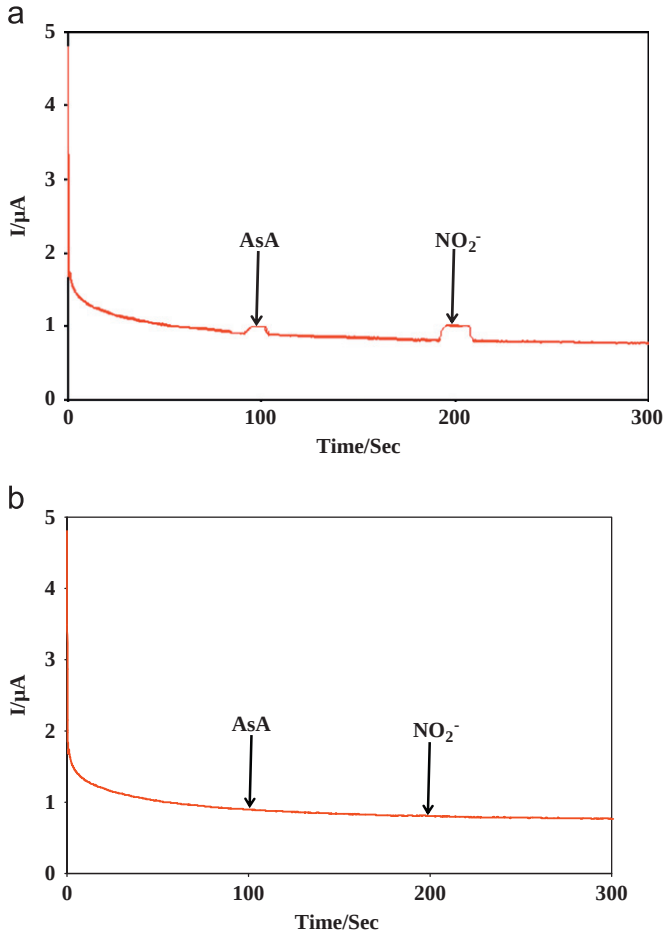


Fig. 5. (A) The chronoamperometric response obtained for the SOD1-CNT-PPy-Pt electrode (before nafion coating) upon addition of 250 μM each of AsA and NO_2^- in 0.1 M PBS containing 500 μM NO at scan rate: 100 mV s^{-1} vs. Ag/AgCl and (B) The time vs. current response obtained for the SOD1-CNT-PPy-Pt electrode (after nafion coating) upon addition of 250 μM each of AsA and NO_2^- in 0.1 M PBS containing 500 μM NO at scan rate: 100 mV s^{-1} vs. Ag/AgCl.

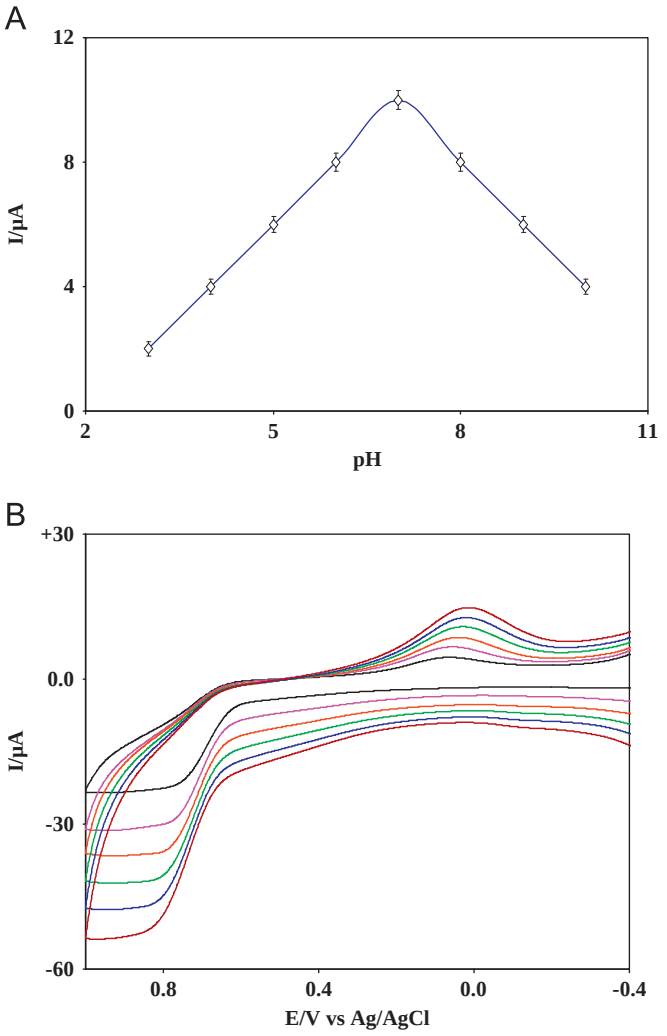


Fig. 6. (A) Effect of pH on peak current of SOD1-CNT-PPy-Pt electrode in 0.1 M PBS at scan rate: 100 mV s^{-1} vs. Ag/AgCl. Each point represents the average of three measurements and (B) Effect of increasing scan rate from 100 mV s^{-1} to 350 mV s^{-1} on SOD1-CNT-PPy-Pt electrode in 0.1 M PBS containing 100 μM of NO solution.

3.5. Determination of NO in exhaled breath

The human exhaled breath consists of NO, acetone, ammonia, ethanol, isoprene, CO, CO_2 and O_2 [13]. Among them, NO, a marker of inflammation in the lung [36] augmented in inflammatory respiratory diseases such as bronchiectasis, lower respiratory tract infection [37] and asthma [38,39]. We have measured here the NO levels present in the exhaled breath of four healthy individuals of different ages from 24 to 32 years using virtual electrochemical NO analyzer by following the American Throcic Society guidelines [40]. The subject exhaled through the mouth using mouthpiece by slow exhalation for 20 s at a constant flow rate, 50 mL s^{-1} into an electrochemical cell containing 1 mL of deaerated 0.1 M PBS. During this process, the exhaled NO was dissolved in PBS due to its solubility reported by Zacharia et al. [41]. Further, approximately 20% of exhaled breath NO may spontaneously react with the co-exhaled O_2 (rate constant, 2×10^{-11} ppm^{-2} s^{-1}) to form NO_2 [42], which is also completely trapped in PBS [43]. It has been reported that both the NO and NO_2 (NO_X) gave oxidation peaks at the same potential [44]. Therefore, the virtual electrochemical analyzer measures here the exhaled NO in the form of dissolved NO_X. The measured values are shown in Table 1. Each reading represents the average of three measurements. The mean ± standard deviation (SD) value of the NO present in the exhaled breath of normal human was 24.5 ± 0.5 ppb. The measured values are in good agreement with the reported data [45] indicating the complete entrapment of NO_X in PBS.

3.6. Measurement of NO release from H_2O_2 treated endothelial cells

Endothelial cell injury is an early oxidative injury in several vascular diseases [46]. It has been reported that endothelial cells treated with higher concentration of H_2O_2 increased the expression of transferrin receptor (TfR) levels leading to enhanced iron uptake and cellular apoptosis by decreasing the NO-mediated proteosomal activity and lower concentration of H_2O_2 enhanced eNOS protein phosphorylation and NO-mediated proteosomal activation [47].

Hence, in this study, we attempted to measure the NO release from H_2O_2 treated endothelial cells by using our highly sensitive and cost effective virtual electrochemical NO analyzer. Nafion membrane coated SOD1 electrode was used here to measure NO selectively. After 8 h stimulation by 250 μM H_2O_2 the anodic peak current at 0.8 V clearly increased compared to control and found that the concentration of NO released from the endothelial cell was 20.3 ± 0.4 μM. The anodic peak current of the 500 μM H_2O_2 stimulation was also investigated and found that 50.2 ± 1.0 μM of NO was released. Thus H_2O_2 dose dependently stimulated NO

Table 1
Determination of NO level present in the exhaled breath.

Age (years)	Weight (kg)	Concentration of NO ± SD (ppb)
25	70	23 ± 0.46
26	55	20 ± 0.40
29	55	25 ± 0.50
32	65	30 ± 0.60

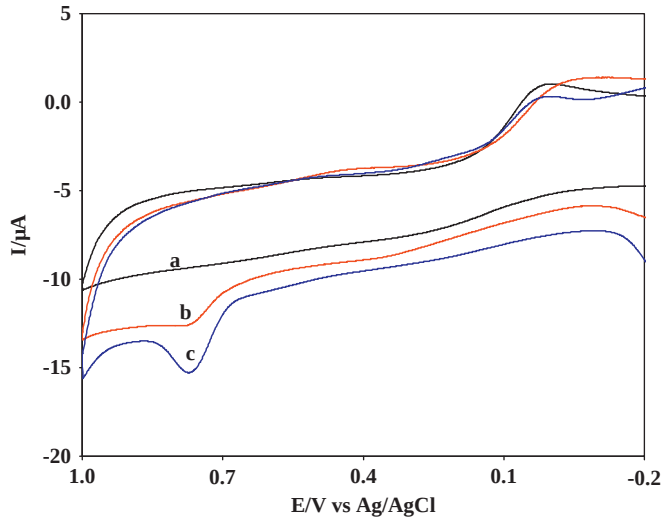


Fig. 7. Typical electrochemical response of the SOD1-CNT-PPy-Pt electrode for endothelial cells treated with (a) control, (b) 250 μM H_2O_2 and (c) 500 μM H_2O_2 in 0.1 M PBS (pH 7.0) at scan rate: 100 mV s^{-1} vs. Ag/AgCl.

release from the endothelial cells (Fig. 7). The concentrations of the NO levels generated from the endothelial cells determined were in agreement with the reference method [8].

3.7. *Benefits of the virtual electrochemical NO analyzer*

The developed virtual electrochemical user-friendly software draws the linear plot and measures the concentration of NO present in the unknown sample. The electrochemical current responses obtained with respect to the known concentrations of NO can be entered simultaneously into the column given in the left side of the main.VI front panel and can plot the linear calibration curve by pressing the start button. The mathematical parameters like slope, intercept, correlation co-efficient and best linear fit values are displayed as soon as the linear graph plotted. Further, with the help of the above parameters, the concentration of NO present in the real samples can also be determined and displayed immediately. The linearity (0.1 μM to 1 mM), sensitivity (1.1 μA μM^{-1}), and detection limit (0.1 μM) of the NO biosensor investigated by the virtual electrochemical analyzer is quiet comparable with those obtained using standard cyclic voltammetry instrument. Commercially available NO analysers are functioning based on the chemical reaction of NO with ozone (O_3) and the concentration of NO is measured with respect to the luminescent intensity [13]. These analysers involve high cost, corrosive chemicals and also not a selective method for the determination of NO. In an effort to diminish the cost of the measuring unit, make it environment and user friendly and also to achieve the specific determination of NO, we have introduced here a compact, flexible and low cost electrochemical NO analyzer.

4. Conclusion

In this report, a cost effective and flexible virtual electrochemical instrument for the determination of NO using home-made potentiostat has been described. It is based on the graphical user interface (GUI) electrochemical software developed by using LabVIEW 10.0 interfaced to MyDAQ. The SOD1 modified CNT-PPy-Pt electrode was used as NO biosensor and applied for the selective determination of NO from exhaled breath and H_2O_2 stimulated endothelial cells. The common biological interferences were eliminated using the nafion membrane. Further, it showed a wide linearity, high sensitivity, good reproducibility and long term stability. The electroanalytical performance of the successfully developed virtual electrochemical NO analyzer was compared well with the physical instrument CV and concluded that it is suitable for the biological applications.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.033.

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