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Copper, zinc superoxide dismutase and nitrate reductase coimmobilized bienzymatic biosensor for the simultaneous determination of nitrite and nitrate

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ABSTRACT

This work presents a novel bienzymatic biosensor for the simultaneous determination of nitrite (NO_2^-) and nitrate (NO_3^-) ions using copper, zinc superoxide dismutase (SOD1) and nitrate reductase (NaR) coimmobilized on carbon nanotubes (CNT)–polypyrrole (PPy) nanocomposite modified platinum electrode. Morphological changes of the PPy and CNT modified electrodes were investigated using scanning electron microscopy. The electrochemical behavior of the bienzymatic electrode (NaR–SOD1–CNT–PPy–Pt) was characterized by cyclic voltammetry exhibiting quasi-reversible redox peak at +0.06 V and reversible redox peaks at −0.76 and −0.62 V vs. Ag/AgCl, for the immobilized SOD1 and NaR respectively. The electrocatalytic activity of SOD1 towards NO_2^- oxidation observed at +0.8 V was linear from 100 nM to 1 mM with a detection limit of 50 nM and sensitivity of 98.5 ± 1.7 nA μM^{-1} cm^{-2}. Similarly, the coimmobilized NaR showed its electrocatalytic activity towards NO_3^- reduction at −0.76 V exhibiting linear response from 500 nM to 10 mM NO_3^- with a detection limit of 200 nM and sensitivity of 84.5 ± 1.56 nA μM^{-1} cm^{-2}. Further, the present bienzymatic biosensor coated with cellulose acetate membrane for the removal of non-specific proteins was used for the sensitive and selective determinations of NO_2^- and NO_3^- present in human plasma, whole blood and saliva samples.

1. Introduction

Nitric oxide (NO) is an essential messenger molecule regulating the biological processes viz. blood vessel relaxation, neuronal cell-to-cell communication and immune function. (Bredt and Snyder, 1994; Lowenstein et al., 1994). Due to the rapid metabolism and short half-life time of NO, it is readily oxidized into its metabolites nitrite (NO_2^-) and nitrate (NO_3^-) (Szabo, 1996). Therefore, the NO level is commonly assessed by determining plasma concentrations of NO_2^- and NO_3^- or that of total NO_x ($NO_2^- + NO_3^-$) (Vitturi and Patel, 2011). Further, NO_2^- and NO_3^- are recently emerged as an endocrine reservoir capable of producing NO within hypoxic, ischemic or injured tissue (Lundberg et al., 2008, Lundberg et al., 2009). More prominently, the blood concentrations of NO_2^- and NO_3^- are possibly altered in physiological hypoxic signaling, vasodilation, modulation of cellular respiration and cellular response to ischemic stress (Kevil and Lefer, 2011; Butler and Feelisch, 2008). Hence, the measurements of NO_2^- and NO_3^- are imperative in human physiology as it provides valuable information with regard to in vivo NO production, bioavailability and prognostic of various diseases including oxidative stress. Moreover, determinations of NO_2^- and NO_3^- ions are also important in environmental pollution, agriculture, marine cycles, water management and food analysis.

Numerous strategies have been reported for the determination of NO_2^- and NO_3^- including the Griess colorimetric assay (Guevara et al., 1998; Miranda et al., 2001; Nataliya and Andrei, 2005), fluorometry (Fang et al., 2009) and chemiluminescence methods (Nagababu and Rifkind, 2010). Separation based methods viz. GC–MS (Tsikas et al., 2012; Kage et al., 2002) and HPLC (Jobgen et al., 2007) with variety of detection system have also been reported. Based on the spectrophotometric determination, some commercial assay kits are also available in the market (Sigma, Catalog no. 06239; Cayman Chemical Company, Prod. no. 850-001-KI01). Most of these techniques are indirect, not suitable for in vivo measurement and require sophisticated instruments. Usually, they have tedious detection procedures and therefore are time-consuming. Recently, electrochemical biosensor techniques are proved to be a powerful tool for the measurement of NO_2^- and NO_3^- by providing practical advantages, such as operation simplicity, low expense of fabrication and suitability

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for real-time detection. In addition, it provides fast response, more sensitive (particularly with the use of modified electrodes) and selective determination.

In the literature, several electrochemical biosensors were reported for the independent determinations of NO_2^- and NO_3^- (Badea et al., 2001; Boo et al., 2007; Gamboa et al., 2009; Geng et al., 2008; Quan et al., 2005; Rocha et al., 2002; Sohail and Adeloju, 2008; Sun and Wang, 2009; Wang et al., 2006; Zhang et al., 2009). Recently, there has been emergent interest in the direct determination of NO_2^- and NO_3^- simultaneously without any separation steps to save time and cost of the experiments. In this exertion, researchers have developed the electrochemical sensors for the simultaneous determination of NO_2^- and NO_3^- using silver-doped zeolite (Manea et al., 2010), copper complex (Shiddiky et al., 2006), graphite (Kaminskaya et al., 2004) and copper electrodes (Shariar and Hinoue, 2010). However, non-selectivity of these chemically modified electrodes limited their applications to biological samples for the reason that the co-existing biological substrates probably interfere with the measurement and also the concentrations of NO_2^- and NO_3^- are in low molar level. Moreover, its operation under normal physiological conditions is also uncertain. Therefore, there is a real need for the simultaneous measurement of NO_2^- and NO_3^- in biological samples with high specificity and sensitivity.

In order to overcome these limitations, for the first time we have developed here a novel bienzymatic biosensor system for the simultaneous determination of NO_2^- and NO_3^- using copper, zinc superoxide dismutase (SOD1) and nitrate reductase (NaR) coimmobilized electrode. It is based on our previous research works for the independent determinations of NO_2^- using SOD1 (Rajesh et al., 2010) and NO_3^- using NaR modified electrodes (Madasamy et al., 2013). The stable coimmobilization of two enzymes on an electrode surface with complete retention of its biological activity is a crucial problem for the development of biosensors. The electrode surface modified with polypyrrole (PPy) provides porous host matrix for the immobilization of SOD1 and NaR. It also affords well-ordered conductive polymer chain with good environmental stability (Reiter et al., 2001). Further, modification of PPy matrix with carbon nanotubes (CNT) forms CNT–PPy nanocomposite which access additional surface area to immobilize more SOD1 and NaR and also act as molecular wires to accelerate electron transfer between underlying electrode and active sites thereby increasing the sensitivity of the biosensor. Further, to eliminate all the possible interferences during the NO_2^- and NO_3^- measurements in biological samples, cellulose acetate (CA) membrane was used. The analytical applicability of this highly sensitive and selective bienzymatic biosensor was investigated for human plasma, whole blood and saliva samples under normal physiological conditions.

2. Materials and methods

2.1. Chemicals and reagents

Copper, zinc superoxide dismutase from bovine erythrocytes, nitrate reductase from *Aspergillus niger*, cellulose acetate, acetone, sodium ascorbate, uric acid, sodium hydrogen phosphate, disodium hydrogen phosphate, pyrrole, $NaNO_3$, $NaNO_2$, KCl and glutaraldehyde were purchased from Sigma Company (Milwaukee, WI, USA). Single walled carbon nanotubes were purchased from carbon solutions Inc., CA, USA. All the solutions were prepared with doubly distilled water.

2.2. Preparation of Human plasma, Blood and saliva samples

The protocol for the collection of human saliva and blood was approved by the Institutional ethical committee. A written informed consent was obtained from the donors before salivary and blood collection. A total of six healthy male volunteers (23–28 years) were recruited for the study. All the participants were free from fever, cold, non-smokers and had good oral hygiene. The saliva samples were collected between 9 am and 11 am and the participants were asked to refrain from eating and drinking one hour prior to saliva collection. The participants rinsed their mouth with water prior to collection, and waited 10 min before commencing with the collection. Resting drooling (minimal oral movements) method was used to collect whole mouth saliva from the oral cavity. Participants were asked to sit comfortably in an upright position and tilt their heads down slightly to pool saliva in the mouth. The first expectoration was discarded to eliminate food debris and unwanted substance contaminating the sample that may cause analytical inaccuracy. The subsequent sample was then expectorated into a pre-labelled sterile container. Around 2 mL saliva was collected and stored at −80 °C. All the stored salivary samples were subjected to freeze-thaw cycles to break down mucopolysaccharides to reduce viscosity and to minimize pipetting errors. All thawed saliva samples were centrifuged at 10,000*g* for 10 min at 4 °C to remove cellular debris and to minimize the salivary turbidity which can negatively impact on the accuracy of analysis (Worthman et al., 1990; Schipper et al., 2007; Mohamed et al., 2012). Blood was drawn by standard venipuncture into 4 ml Vacutainer tubes using EDTA as anticoagulant (BD Worldwide, NJ, USA). Plasma was obtained by centrifugation of whole blood for 15 min at 380*g*. The plasma samples were passed through 10 kDa cutoff membranes and further used for nitrate/nitrite estimation.

2.3. Instrumentations

All the electrochemical experiments were performed using CHI 1200B electrochemical workstation (CHI, USA) with a conventional three electrode system which consisted of an Ag/AgCl wire as reference electrode, a Pt wire as auxiliary or counter electrode and a Pt electrode with SOD1 and NaR coimmobilized on CNT–PPy nanocomposite as working electrode. The size of Pt wire working electrode was 0.5 mm in diameter. The morphological images were obtained using a FEI Quanta FEG 200-High Resolution Scanning Electron Microscope (FEI Co., Netherlands).

2.4. Construction of bienzymatic (NaR–SOD1–CNT–PPy–Pt) biosensor

CNT modified PPy–Pt electrode was constructed as per our earlier report (Madasamy et al., 2012). Briefly, pyrrole was electropolymerized onto the Pt electrode by the irreversible oxidation of 0.4 M pyrrole in 0.1 M KCl as supporting electrolyte by applying potential from 0 V to 0.9 V vs. Ag/AgCl at a scan rate of 50 mV s^{-1} for 10 complete cycles. Then, CNT integrated 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) onto the PPy–Pt electrode and dried at room temperature. The enzyme solutions were prepared in 0.1 M PBS (pH 7.0) with working concentration of 300 U/ml for SOD1 and 10 U/ml for NaR. Then, these enzyme solutions were mixed in the ratio of 1:1 and 10 μL of the mixture was dropped onto the CNT–PPy–Pt electrode by employing 5 μL of glutaraldehyde as a cross-linking agent. During this process, SOD1 and NaR were coimmobilized on CNT–PPy–Pt electrode. Further, it was immersed in 0.1 M PBS to remove loosely affixed SOD1/NaR and dried at room temperature, stored at 4 °C when not in use. The above fabricated bienzymatic biosensor is represented in Scheme 1.

3. Results and discussion

In a single experiment, measurement of various analytes using multienzymatic biosensor is a challenging research area. Asav et al.