Biosensors and Bioelectronics 35 (2012) 302–307

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Functionalization of carbon buckypaper for the sensitive determination of hydrogen peroxide in human urine

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ARTICLE INFO

Article history:
Received 14 January 2012
Received in revised form 1 March 2012
Accepted 2 March 2012
Available online 10 March 2012

Keywords:
Biosensor
Buckypaper
Horseradish peroxidase
Hydrogen peroxide
Human urine

ABSTRACT

Here we report on a new approach for the electrochemical detection of hydrogen peroxide (H_2O_2) based on the co-immobilization of horseradish peroxidase and methylene blue on the functionalized carbon buckypaper supported by a titanium substrate. Cyclic voltammetry was used to study and optimize the performance of the resulting electrochemical biosensor. The proposed biosensor exhibited high analytical performance towards the quantification of H_2O_2 at the physiological pH 7.4. Under optimized conditions, the biosensor shows a wide linear response range from 0.1×10^{-6} to 5×10^{-4} M concentrations of H_2O_2. The detection limit was determined to be 7.5×10^{-8} M (based on S/N = 3). Reproducibility and stability of the fabricated biosensor were examined with satisfactory results. The biological relevance of the developed electrochemical biosensor has been further studied by the determination of H_2O_2 in human urine samples of normal volunteers prior to and following the ingestion of coffee. Increased levels of urinary H_2O_2 concentration suggest that oxidative stress is induced by coffee drinking in humans. There is considerable interest in oxidative stress as relates to human physiology. The sensitive determination of H_2O_2 in human urine may serve as a valuable biomarker to effectively elucidate specific levels of oxidative stress in vivo.

1. Introduction

Carbon-based nanomaterials such as nanotubes and graphene are currently at the forefront of materials research as they exhibit outstanding electrocatalytic activity due to their unique electrical and chemical properties. Recently, much attention has been given to macroscopic assemblies of carbon nanotubes (CNTs), including buckypapers, fibers, pellets and thin films, in order to exploit the characteristic properties of single carbon nanotubes at the macroscopic scale. Buckypaper (BP) consists of a macroscopic aggregate of CNTs or buckytubes as a thin film. Since the concept of buckypaper was first introduced by Rinzler et al. (1998), BPs and derivative composite nanostructures have been under investigation by many researchers. In BPs, nanotube network structures are performed during the fabrication process (Wen et al., 2011) BP has several advantages over CNT based films prepared by other methods, as it is highly porous, flexible and electrically conductive. It can form a uniform film of almost any shape and size (Smajda et al., 2007; Whitby et al., 2008) and it can also be prepared from functionalized CNT (Anson-Casaos et al., 2010). Although the factors that govern the morphology and physical properties of buckypaper are not well understood, applications based on BPs have recently become commonplace. Owing to its unique properties, it might be an excellent candidate for use as radio frequency filter (Prokudina et al., 2005) and could be potentially useful as a substrate in nano-bio interface research (Zanello et al., 2006) for nerve cell growth (Mattson et al., 2000), and artificial muscles (Vohrer et al., 2004). Buckypaper is a self-supported mat of entangled assemblies of CNTs forming a well-defined membrane-like black film which makes handling of the CNT very easy (Kukovecz et al., 2007). It can be utilized as a suitable electrode support material since it is highly conductive, flexible and mechanically stable (Hussein et al., 2011). Furthermore, high levels of doping with metallic nanoparticles or decorating with enzymes may be achieved with BP, due to its high specific surface area, which enhances the electroactive surface area, while a low electrode thickness is still maintained (Li et al., 2005). In order to expand the potential and applicability of electroanalysis via BP modified electrodes, the immobilization of functional entities such as enzymes on the surface of the BP have been investigated (Ahmadalinezhad et al., 2011). The extremely attractive properties of BP for the electrochemical detection of hydrogen peroxide (H_2O_2) have led to the development of novel enzymatic biosensors, where rapid electron transfer at the electrode substrate is required. The immobilization of enzymes is an important step in the fabrication of biosensors. Biosensors developed for medical biodiagnostic applications typically utilize enzymes in order to catalytically generate a redox active product, such as hydrogen peroxide.

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0956-5663/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2012.03.005

Hydrogen peroxide is one of the most important analytes in the field of electroanalysis, as it is cytotoxic, an indicator of oxidative stress, and the product of several enzyme-catalyzed reactions. Thus, the detection of H_2O_2 with high sensitivity and low detection limits is desirable for the food industry, in environmental waste management, and in medical diagnostics (Tripathi et al., 2006; Chen et al., 2007). Cholesterol, glucose and triglyceride may be determined by means of the quantitation of hydrogen peroxide production in enzymatic or enzymatic-like reactions. Many methods such as titrimetry, spectrophotometry and chemiluminescence have been developed for this purpose (Armstrong and Lannon, 1987; Xu and Dong, 1999). Electrochemical methods have proved to be an inexpensive and effective means for the determination of hydrogen peroxide. The direct reduction or oxidation of hydrogen peroxide at a bare electrode is not suitable for analytical applications due to sluggish kinetics and high overpotentials, which are required for redox reactions of H_2O_2 on many electrode materials. For this reason, redox mediators have found wide use in decreasing overpotential and increasing electron transfer kinetics. Furthermore, the immobilization of different peroxidase enzymes has been employed for the fabrication of hydrogen peroxide biosensors (Song et al., 2006; Wang et al., 2009). In view of the importance of H_2O_2 in human physiology, an attempt has been made to develop a simple and robust hydrogen peroxide biosensor that is based on horseradish peroxidase (HRP), which is immobilized by chitosan film in the presence of methylene blue (MB) as a redox indicator. The high sensitivity of the biosensor was due to the efficiency of electron transfer between the immobilized horseradish peroxidase and the electrode, via methylene blue. Titanium (Ti) was selected as the substrate because of its good conductivity, biocompatibility and high corrosion resistance. The strong affinity of the buckypaper modified titania layer for the positively charged HRP enzyme in conjunction with the high reactivity of the substrate toward the carboxylic group of enzymes, have been utilized to accomplish the immobilization of the HRP enzyme.

Currently, there is considerable interest in oxidative stress as relates to human physiology. One important reactive oxygen species that is generated in the human body is H_2O_2. Therefore, the determination of H_2O_2, especially in human urine, might serve as a valuable biomarker, which may elucidate specific levels of oxidative stress in vivo (Varma and Devamanoharan, 1989). A literature survey reveals that coffee drinking leads to increased levels of urinary H_2O_2, thereby suggesting that oxidative stress is induced within the human body by coffee drinking (Long and Halliwell, 1999; Hiramoto et al., 2002; Halliwell and Whiteman, 2004). Therefore, efforts were made in this paper to electrochemically determine H_2O_2 concentrations in normal human urine samples, prior to and following the intake of coffee. To the best of our knowledge, titanium interfaced buckypaper is explored, for the first time in this work, for the determination of H_2O_2 in real samples. The proposed H_2O_2 biosensor exhibits a wide linear detection range, high reproducibility and stability in operation and storage.

2. Experimental

2.1. Chemicals and reagents

Hydrogen peroxide solution (30%) was purchased from Fluka, from which stock solutions of H_2O_2 were diluted. Horseradish peroxidase (type VI, 330 units/mg) was purchased from Sigma, was used as received. Methylene blue was obtained from the British Drug Houses Ltd., Poole, in the UK and chitosan from Aldrich. All other reagents were of analytical grade and used as supplied. The buckypaper utilized in this study was produced by the High-Performance Materials Institute of Florida State University with a thickness of 0.035 mm and the titanium foils (99.2%) were obtained from Alfa Aesar. The experimental solutions were prepared on a daily basis by the appropriate dilution of stock solutions. The double distilled water (18.2 MΩ cm) used throughout the experiments was purified with the Nanopure® water system. All experiments were performed in 0.1 M phosphate buffer solutions (PBS) at pH 7.4. Urine samples were obtained from healthy human volunteers and were subsequently used after 10 times dilution with PBS to reduce the matrix complexity.

2.2. Instrumentation

The voltammetric experiments were accomplished using a three electrode single compartment cell that was equipped with platinum coil as the counter electrode and an Ag/AgCl (3 M KCl) as the reference electrode. The platinum coil was flame-annealed prior to each experiment. The fabricated titanium/buckypaper based biosensors were employed as working electrodes. Experiments were carried out using an electrochemical workstation (CHI 660, CH Instrument Inc., USA). All the potentials quoted are versus an Ag/AgCl electrode at an ambient temperature of $22 \pm 2\,^{\circ}C$. Field-emission scanning electron microscopy (FE-SEM) (Hitachi, SU-4800) and transmission electron microscopy (TEM) (JEOL 2010F) were utilized to characterize the morphology and structure of the buckypaper.

2.3. Fabrication of H_2O_2 biosensor

The titanium plates (0.8 cm × 1.25 cm × 0.5 mm) were ultrasonically cleaned in acetone followed by pure water. The surface of titanium plate was then coated with a gold (Au) thin film that was sputtered by argon plasma for 30 s. The Au thin film enhances conductivity along with the provision of a mechanical support for BP. A section of BP was attached to the Au coated titanium plate with the aid of 5 μL of 2 mg mL^{-1} chitosan. The BP was electrochemically activated to functionalize the carboxylic groups present within. The activated surface was then ready for the immobilization of enzymes along with a mediator. For this purpose, a mixed solution of 10 μL of 2 mg mL^{-1} chitosan, 10 μL of 2 mg mL^{-1} MB and 50 μL of 2 mg mL^{-1} HRP were cast onto the buckypaper. The amount of enzymes immobilized on the electrode was calculated to be 33 units. Chitosan was selected as the matrix for the immobilization of the enzyme because of an unusual combination of its properties, which includes an excellent membrane-forming ability, high permeability toward water, good adhesion, biocompatibility, non-toxicity and high mechanical strength. The abundant amino groups present in chitosan provide a biocompatible environment for immobilization of HRP. The charged functional groups on chitosan molecules induce strong adsorption of HRP and MB on the surface of buckypaper. Therefore, the interconnectivity of CNTs in the functionalized buckypaper, its compatibility and strong interactions with chitosan on one hand and chitosan interaction with the enzymes on the other hand significantly enhance the interfacial adhesion and mechanical strength of the biosensor (Ahmadalinezhad et al., 2011). The prepared biosensor is referred to as Ti/Au/BP/HRP-MB, which was stored in a 0.1 M PBS with a pH of 7.4 at 4 °C when not in use. FE-SEM and TEM were used to characterize the morphology and composition of the BP. Fig. 1A shows a FE-SEM image and a TEM image of the BP is displayed in Fig. 1B, which clearly shows that the nanotubes were aggregated in a rope-like structure.

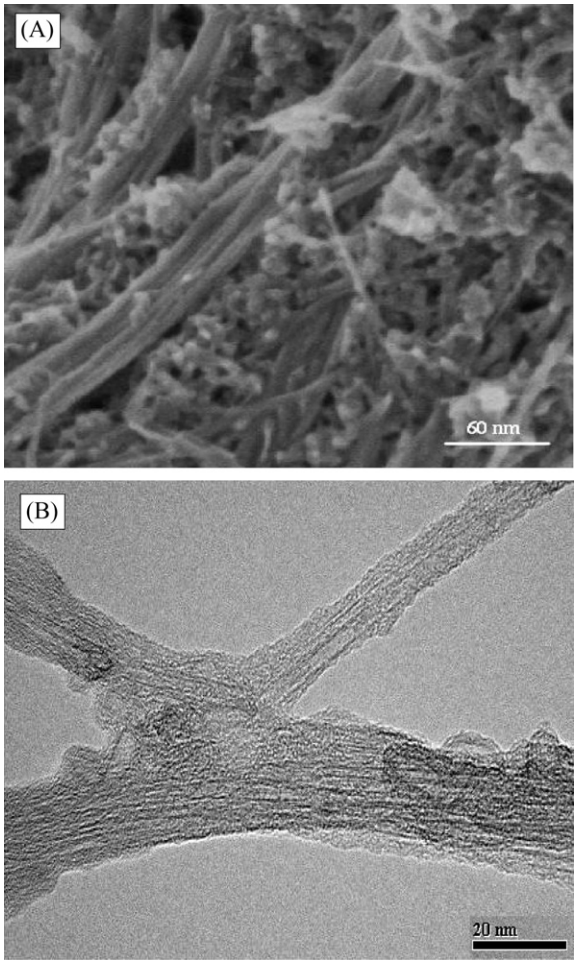

Fig. 1. (A) FE-SEM image and (B) TEM image of the buckypaper.

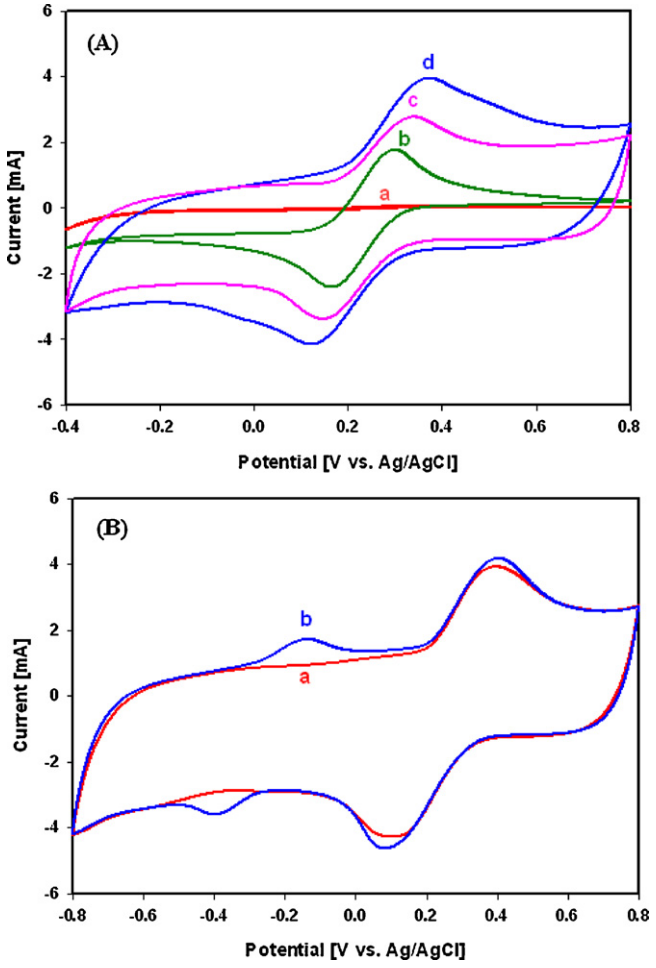

Fig. 3. (A) Cyclic voltammograms recorded at (a) Ti, (b) Ti/Au, (c) Ti/BP and (d) Ti/Au/BP electrodes at a scan rate of 20 mV s^{-1} in 0.1 M $K_3Fe(CN)_6$ and 0.1 M KCl solution in PBS of pH 7.4. (B) Voltammetric response at (a) Ti/Au/BP/HRP and (b) Ti/Au/BP/HRP-MB electrodes at a scan rate of 20 mV s^{-1} in 0.1 M $K_3Fe(CN)_6$ and 0.1 M KCl solution in PBS of pH 7.4.

3. Results and discussion

3.1. Electrochemical activation of buckypaper

The BP was electrochemically activated in order to engender its structure with utility as a hydrogen peroxide biosensor. Fig. 2 depicts the comparative response obtained in 0.1 M PBS at pH 7.4 at a scan rate of 20 mV s^{-1} after the activation of Ti/Au/BP using three different procedures. In the first electrode (electrode 1), cyclic voltammetry was performed in the range of −0.8 to 0.8 V followed by a range of −0.8 to 1.5 V until a steady cyclic voltammogram was obtained. A potential of 1.5 V was then applied for 90 s employing amperometry. Electrode 2 was activated by applying the cyclic voltammetry range of −0.8 to 0.8 V followed by −0.8 to 1.5 V, excluding the amperometry activation. In contrast, for the case of electrode 3, a range of only −0.8 to 0.8 V was applied by cyclic voltammetry. It is clearly observed in Fig. 2 that electrode 1 was endowed with the maximum functionalization of the BP with carboxylic groups, displaying a distinct redox couple. Therefore, the activation procedure, as applied to electrode 1 is followed throughout the paper for the preparation of the biosensors.

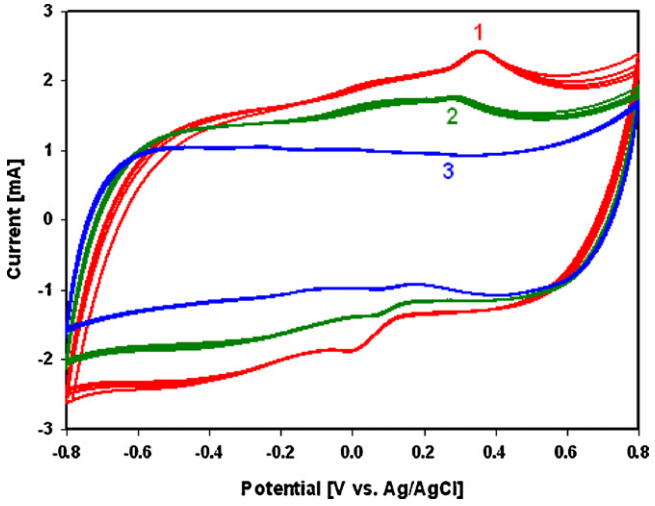

Fig. 2. Comparison of cyclic voltammograms of electrodes 1, 2 and 3 in PBS at 20 mV s^{-1} after different activation procedures.

3.2. Electrochemical characterization

The direct electrochemistry of the enzyme immobilized biosensor was investigated using cyclic voltammetry. Fig. 3A compares the voltammetric response of the Ti, Ti/Au, Ti/BP and Ti/Au/BP electrodes recorded in 0.1 M $K_3Fe(CN)_6$ and 0.1 M KCl in 0.1 M PBS of pH 7.4 at a scan rate of 20 mV s^{-1}. Neither oxidation nor a reduction peak is observed in the cyclic voltammogram of the Ti electrode. In contrast, a well-defined redox couple is observed at all the

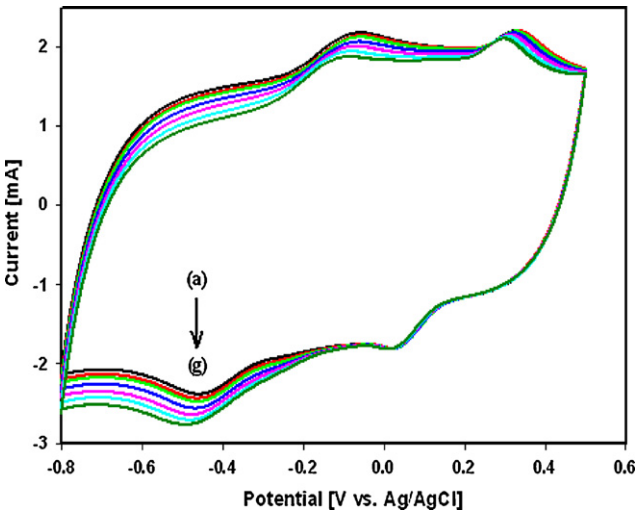

Fig. 4. Cyclic voltammograms recorded for increasing concentrations of H_2O_2 at the Ti/Au/BP/HRP-MB electrode. Curves are recorded at (a) 0.0, (b) 0.1, (c) 0.2, (d) 0.4, (e) 0.6, (f) 0.8 and (g) 1.0 μM concentration at the scan rate of 20 mV s^{-1} in PBS of pH 7.4.

other three electrodes. The enhancement in peak current at the Ti/Au/BP electrode in comparison to the Ti/Au and Ti/BP electrodes reveals that the Au thin film significantly increases the electrical conductivity at the interface between the BP and the Ti substrate. A comparison of voltammograms on the electrodes Ti/Au/BP/HRP and Ti/Au/BP/HRP-MB are shown in Fig. 3B under identical conditions. It has been clearly demonstrated that along with an increase in the oxidative and reductive current in the presence of the MB mediator, there is also an additional redox couple that is observed at potential ~0.14 and −0.40 V. The increase in the peak current values in the presence of MB indicates that methylene blue acts as an electron mediator to promote the electrochemical reaction between the immobilized HRP and the electrode surface. The effect of scan rates on the response of the Ti/Au/BP/HRP-MB electrode was then investigated in the range 5–100 mV s^{-1} in PBS at pH 7.4. It was observed that both the anodic and cathodic peak currents increased linearly, indicating that the pair of redox waves originates from the surface confined molecules (Fig. S-1). The anodic and cathodic peak potentials also change as a function of the scan rate. The peak to peak separation is found to increase along with the increase of the scan rate. The electron transfer rate constant was calculated to be 0.12 s^{-1} using the method developed by Laviron (1979) for a surface-controlled electrochemical system.

3.3. Electrochemical response of the biosensor to H_2O_2

Cyclic voltammograms were recorded at the Ti/Au/BP/HRP-MB electrode in 0.1 M PBS at pH 7.4 in the absence and presence of hydrogen peroxide, as shown in Fig. 4. In the absence of H_2O_2, a pair of small redox peaks is observed due to the direct electron transfer between the immobilized HRP and the Ti/Au/BP electrode surface. Subsequent additions of hydrogen peroxide to the solution provoked a remarkable increase in the reductive, and decrease in the oxidative current, indicating that the electrocatalytic reduction of hydrogen peroxide was apparently taking place at the electrode and that the biocatalytic activity of the HRP enzyme was effectively maintained on the substrate. The presence of MB plays an important role as an electron mediator thereby increasing the sensitivity of the proposed biosensor. The possible reaction mechanism can be explained by the following scheme (Kafi et al., 2008):

$$HRP(red) + H_2O_2 \rightarrow HRP(ox) + H_2O$$

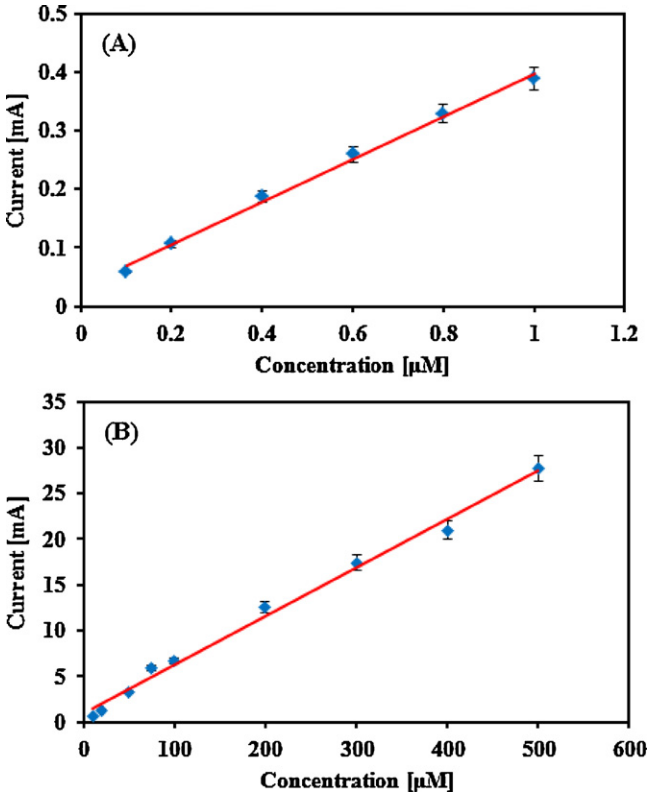

Fig. 5. (A) The observed calibration curve in the range 0.1–1.0 μM. (B) Linear calibration plot of H_2O_2 in the range 10–500 μM observed at Ti/Au/BP/HRP-MB electrode.

$$HRP(ox) + MB(red) \rightarrow HRP(red) + MB(ox)$$

$$MB(ox) + e^- \rightarrow MB(red)$$

Initially, hydrogen peroxide is reduced in the electrolyte by the HRP (red), forming HRP (ox) followed by regeneration of HRP (red) with the aid of mediator MB, while the MB (red) is oxidized in the enzymatic reaction. Finally, the MB (ox) is electrochemically reduced on the electrode surface.

Fig. 4 represents a set of voltammograms depicting the systematic increase in the cathodic peak current values with increasing concentrations of hydrogen peroxide in the range 0.1–1 μM at the Ti/Au/BP/HRP-MB electrode. The linear calibration plot in this range is shown in Fig. 5A. The cathodic current at the potential ~−0.46 V vs. Ag/AgCl was found to increase with the subsequent addition of H_2O_2 to the electrolyte, thereby indicating that the cathodic current was based on the reduction of H_2O_2.The quantitative analysis of H_2O_2 is based on the dependence of the peak current on the concentration of the analyte. Hydrogen peroxide was safely estimated in the concentration range of 0.1–500 μM at pH 7.4. Fig. 5B depicts the linear calibration plot in the range of 10–500 μM, with error bars. The current values are obtained by subtracting the background current and are reported as an average of at least three replicate determinations. The detection limit of the proposed method has been calculated by using $3\sigma/b$, where σ is the standard deviation of the blank and b is the slope of the calibration curve, which has been found as 7.5×10^{-8} M. The sensitivity is estimated to be 54 μA μM^{-1} with a correlation coefficient of 0.994.

3.4. Stability and reproducibility of the biosensor

A good immobilization method should provide satisfactory stability of the biosensors, aside from good sensitivity. Consequently,

Table 1
Urinary H_2O_2 concentration before and after 60 min of intake of coffee at the Ti/Au/BP/HRP-MB biosensor at pH 7.4.

Urinary H_2O_2 concentration (μM)		Recovery (%)
Spiked	Detected	
Before intake of coffee:		
Sample A1		
0.00	24.56	–
20.00	42.34	95.02
40.00	66.25	102.62
60.00	88.09	104.17
Sample B1		
0.00	15.32	–
20.00	36.13	102.29
40.00	58.04	104.92
60.00	72.75	96.59
Sample C1		
0.00	31.64	–
20.00	50.79	98.35
40.00	73.81	103.03
60.00	89.27	97.41
After intake of coffee:		
Sample A2		
0.00	41.23	–
20.00	63.47	103.66
40.00	78.41	96.53
60.00	104.15	102.88
Sample B2		
0.00	31.08	–
20.00	48.98	95.89
40.00	70.76	99.55
60.00	94.14	103.36
Sample C2		
0.00	58.24	–
20.00	81.70	104.42
40.00	99.69	101.47
60.00	115.48	97.66

stability tests were performed for the developed biosensor. In spite of the fact that HRP has been found to be very stable in solution, the storage stability of the HRP enzyme electrode is directly associated with the methodology for HRP enzyme immobilization. Hence, the storage stability of the biosensor was examined by measuring the biosensor response in the presence of 10 μM of hydrogen peroxide over a one-month period (Fig. S-2). When not in use, the biosensor was stored in 0.1 M PBS (pH 7.4) at 4 °C. During the first three days the response current has a ~1.6% decrease, whereas in the next two weeks the current response decreases to ~3.5% of its initial response. After 30 days, the residual response current of the biosensor retained was about 96%. Consequently, the electrode favors the retention of the bioactivity of HRP, which may be attributed to the biocompatibility of the titanium substrate and the mediator-buckypaper nanocomposites. In addition, the interaction between the Au thin film that was sputtered on the Ti substrate and the buckypaper, with the aid of chitosan, is so strong that it is almost impossible to remove the buckypaper from the substrate without breaking the buckypaper film.

The reproducibility of the biosensor was investigated at an H_2O_2 concentration of 10 μM. The relative standard deviation for 10 successive determinations was 2.9%. The electrode-to-electrode reproducibility was tested with four electrodes that were prepared under the same conditions. The relative standard deviation was 3.1%, which reveals that the proposed system is able to provide a satisfactory reproducibility in the determination of hydrogen peroxide. This can be attributed to the efficient immobilization of the enzymes along with the unique physical and chemical properties of the activated buckypaper, which indicates the feasibility of the application of the proposed methodology for the detection of H_2O_2.

3.5. Real sample assay

A literature survey reveals that a substantial amount of H_2O_2 is excreted in normal human urine, thus it follows that its determination might be a valuable biomarker of the extent of oxidative stress in vivo (Kuge et al., 1999; Long et al., 1999; De Zwart et al., 1999). Urine contains autooxidizable molecules that, upon exposure to 21% O_2, undergo rapid superoxide dependent autooxidation reactions to generate H_2O_2 (Long et al., 1999). In the present paper, an attempt has been made for the first time to determine the concentration of H_2O_2 in excreted human urine employing cyclic voltammetry. For this purpose, freshly voided human urine was collected from healthy volunteers (samples A–C) and diluted ten times with phosphate buffer solution of pH 7.4 to reduce the matrix complexity. The concentration of hydrogen peroxide was determined using the regression equation; and the results obtained for different urine samples, prior to and 60 min following the intake of coffee, are tabulated in Table 1. The data presented in Table 1 clearly show that in all cases coffee drinking was followed by a detectable rise in urinary H_2O_2 levels. One possible explanation for the rise in urinary H_2O_2 levels subsequent to the intake of coffee is due to the presence of 1,2,4-benzenetriol (hydroxy hydroquinone) in coffee beans which acts as a major hydrogen peroxide generating component (Halliwell et al., 2004). Pure 1,2,4-benzenetriol autoxidizes, generating reactive oxygen species, superoxide, hydrogen peroxide and hydroxyl radical (Halliwell and Whiteman, 2004; Hiramoto et al., 1998). Therefore, one can conclude that the hydroxy hydroquinone in coffee is absorbed into the body, excreted into the urine and autooxidizes therein to produce H_2O_2.

4. Conclusions

A procedure for the fabrication of an enzymatic electrode has been described that is based on the effective co-immobilization of horseradish peroxidase and methylene blue on the functionalized BP supported by an Au sputtered titanium substrate. The direct electrochemistry and analytical performance of this biosensor were investigated, and our experimental results have revealed that the carbon buckypaper based biosensor developed in the present work possesses a very low detection limit (7.5×10^{-8} M), a broad linear calibration range (0.1–500 μM) and high sensitivity (54 μA μM^{-1}) for hydrogen peroxide determination, superior to those obtained through the use of other modified electrodes (Periasamy et al., 2011; Kafi et al., 2008, 2011; Tang et al., 2011; Liu and Chen, 2005; Cao and Zhou, 2006; Arvinte et al., 2009; You et al., 2011). The efficient functionalization of the buckypaper, coupled with the biocompatibility of its components leads to a biosensor with enhanced electrocatalytic properties. The designed biosensor has been satisfactorily applied for the quantitative determination of urinary hydrogen peroxide concentration prior to and following the intake of coffee. It exhibits many advantages such as remarkable catalytic activity, good reproducibility, a facile preparation procedure and long term stability of signal response for the determination of hydrogen peroxide. The sensitive determination of H_2O_2 in human urine may serve as a valuable biomarker to effectively elucidate specific levels of oxidative stress as relates to human physiology.

Acknowledgments

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). We greatly appreciate the provision of the buckypaper samples by Prof. B. Wang and Prof. Z. Liang of the High-Performance Materials Institute, Florida State University. A.C. acknowledges the NSERC and

the Canada Foundation for Innovation (CFI) for the Canada Research Chair Award in Materials & Environmental Chemistry.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.03.005.

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