Int. J. Electrochem. Sci., 4 **(2009)** 895 - 905

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Direct Electron Transfer of Polyphenol Oxidase on Carbon Nanotube Surfaces: Application in Biosensing

Ali Mohammadi[1,2], *Abdolmajid Bayandori Moghaddam* [2,*], *Rassoul Dinarvand* [2], *Saeed Rezaei-Zarchi*[3]

[1] Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[2] Nanotechnology Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, P.O. Box 14155-6451, Iran
[3] Department of Biology, Payam-e-Noor University, Yazd, Iran
[*]E-mail: bayandori@farabi.tums.ac.ir, bayandori@gmail.com

Received: 28 May 2009 / *Accepted:* 19 June 2009 / *Published:* 19 July 2009

In this work, the adsorption of polyphenol oxidase (PPO) on single-walled carbon nanotubes (SWCNTs) and its electrocatalytic behavior were investigated. To construct the biosensor, polyphenol oxidase was immobilized on the SWCNTs/GC electrode. Scanning electron microscope (SEM) and atomic force microscope (AFM) images were obtained for used SWCNTs. Direct electrochemistry of immobilized PPO was extensively investigated. Immobilized enzyme displayed a couple of stable redox peaks with a formal potential ($E^{\circ'}$) of 0.102 V with respect to reference electrode in 0.05 M phosphate buffer solution (pH 7.0). The experimental results demonstrated that the immobilized PPO on SWCNTs could catalyze the oxidation of HCA. Additionally, apparent Michaelis-Menten constant (K_m), a reflection of enzymatic affinity, for HCA and stability of PPO/SWCNTs/GC electrode were estimated. The proposed method exhibits good sensitivity, stability and reproducibility.

Keywords: Polyphenol oxidase; Biosensing

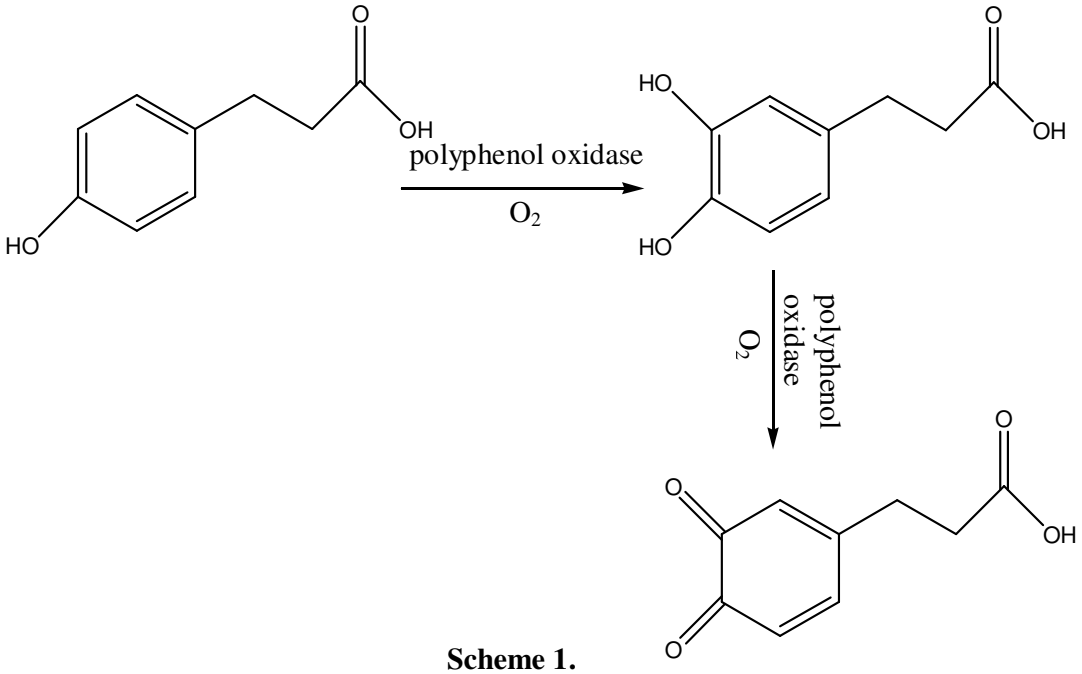
1. INTRODUCTION

Because of their many potential applications, there has been great interest in the novel properties discovering of nanostructured materials. Because of their large surface-to-volume ratio and quantum limitation effect, nanomaterials can be applicable in many fields and have been extensively studied during the past decade [1-7]. Nanomaterials were analyzed because of their size dependent properties [8]. A range of nanostructure materials prepared from carbon, polymers, metals, and

semiconductors have been greatly examined [9,10]. Carbon nanotubes (CNTs) are promising nanoscale building blocks and many potential applications for CNTs have been reported [11,12]. In order to the application of CNTs in biosensors, biomolecules must be connected to the CNTs. The immobilization of biomolecules on CNTs can be non-covalent interaction or covalent bonding. The most of reports use non-covalent interaction [13,14].

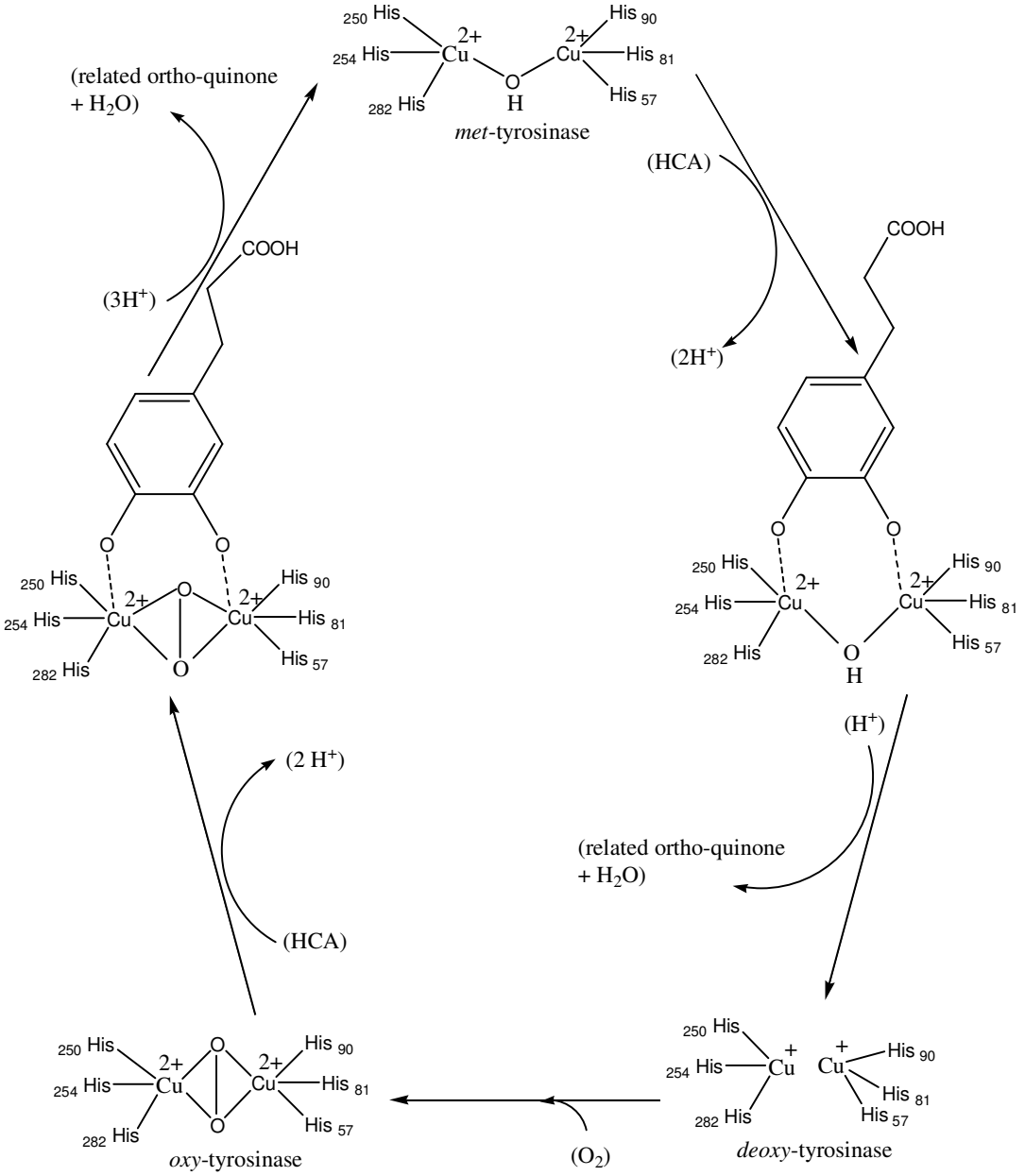
Natural enzymes have been extensively used in biochemistry. They have significant practical applications in food and chemical industry, agriculture and medicine [15]. In recent years, electrochemical study of redox enzymes has attracted widely attending, because of the significance of bioscience and their potential applications in biosensors and biocatalysts. A penicillamine biosensor based on polyphenol oxidase immobilization on nano-Au/PAMAM dendrimer and direct spectrophotometric assay of monooxygenase and oxidase activities of polyphenol oxidase were reported [16,17]. Additionally, direct electrochemistry of redox proteins and enzymes on a variety of modified electrodes has been reported [18].

Polyphenol oxidase, also known as tyrosinase, is a dinuclear copper containing protein in which active site can be prepared in several forms: *met*-tyrosinase, *oxy*-tyrosinase, and *deoxy*-tyrosinase [19]. Polyphenol oxidase is an extensively distributed enzyme and is involved in the biosynthesis of melanin in animals and in the browning of plants. It catalyzes the oxidation of phenol derivatives in the presence of O_2, to respective *ortho*-diphenol derivatives (monophenolase activity, scheme 1), that are further oxidized by enzyme to respective quinones (diphenolase activity, schemes 1 and 2) [20-23]. This enzyme which produced from mushrooms with an isoelectric point value of 4.5 and takes a negative charge at pH > 4.5 in aqueous solution [24]. Recent reports addressed optical methods to detect PPO activity in which providing an optical assay for the enzyme [25]. It has been reported that PPO can catalyze formation of covalent bonds between proteins [26].



Scheme 1.

Various methods have been reported for the immobilization of PPO on diverse substrates [27-31]. In this work, we presented an assembled PPO immobilized layer on a SWCNTs/GC electrode. SWCNTs connect enzyme molecules and glassy carbon surface. The redox behavior of PPO/SWCNTs/GC electrode was investigated by electrochemical approach.



Scheme 2. Diphenolase activity of polyphenol oxidase

2. EXPERIMENTAL PART

2.1. Reagents and materials

Polyphenol oxidase (Tyrosinase, T 7755, from mushroom) [9002-10-2] was purchased from Sigma and used without further purification. Other chemicals were of analytical reagent grade and were purchased from Merck and used as supplied. Phosphate buffer solution (PBS) were prepared by mixing the stock standard solutions of consisted of a potassium phosphate solution KH_2PO_4 and K_2HPO_4. Single-walled carbon nanotubes (SWCNTs) were purchased from Research Institute of the Petroleum Industry (Iran). SWCNTs were prepared by chemical vapor deposition, with up to 80% yield of high quality. Removing of metallic impurity from CNTs was performed by washing in HCl. Distilled water was used in all experiments.

2.2. Instrumentation

Electrochemical experiments were done by Autolab potentiostat PGSTAT 30 (Eco Chemie B.V., the Netherlands), operational with GPES 4.9 software. A conventional three-electrode cell was also used with an Ag|AgCl|KCl (sat.) electrode as a reference, a narrow platinum rod was applied as counter electrode and a glassy carbon (GC, 2 mm in diameter) electrode or modified-GC electrodes, acting as working electrode. All experiments were performed at 25 ± 2 °C. The sonication was performed by using an ultrasonic bath system TECNO-GAZ, Tecna 6 (50–60 Hz, 230±10% V, 0.138 KW). Furthermore, scanning electron microscopic images were recorded using a ZEISS DSM 960.

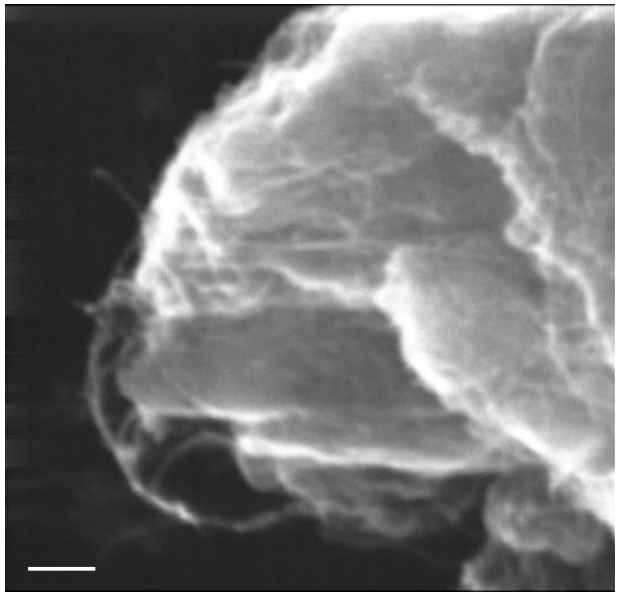


Figure 1. SEM image of SWCNTs, scale bar is 500 nm.

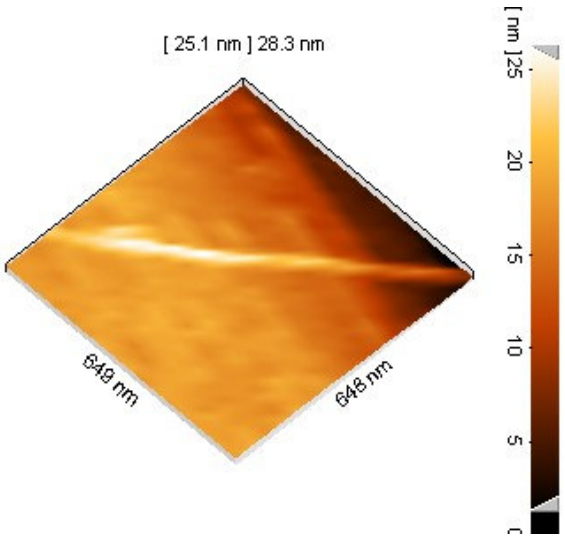


Figure 2. AFM image of SWCNTs.

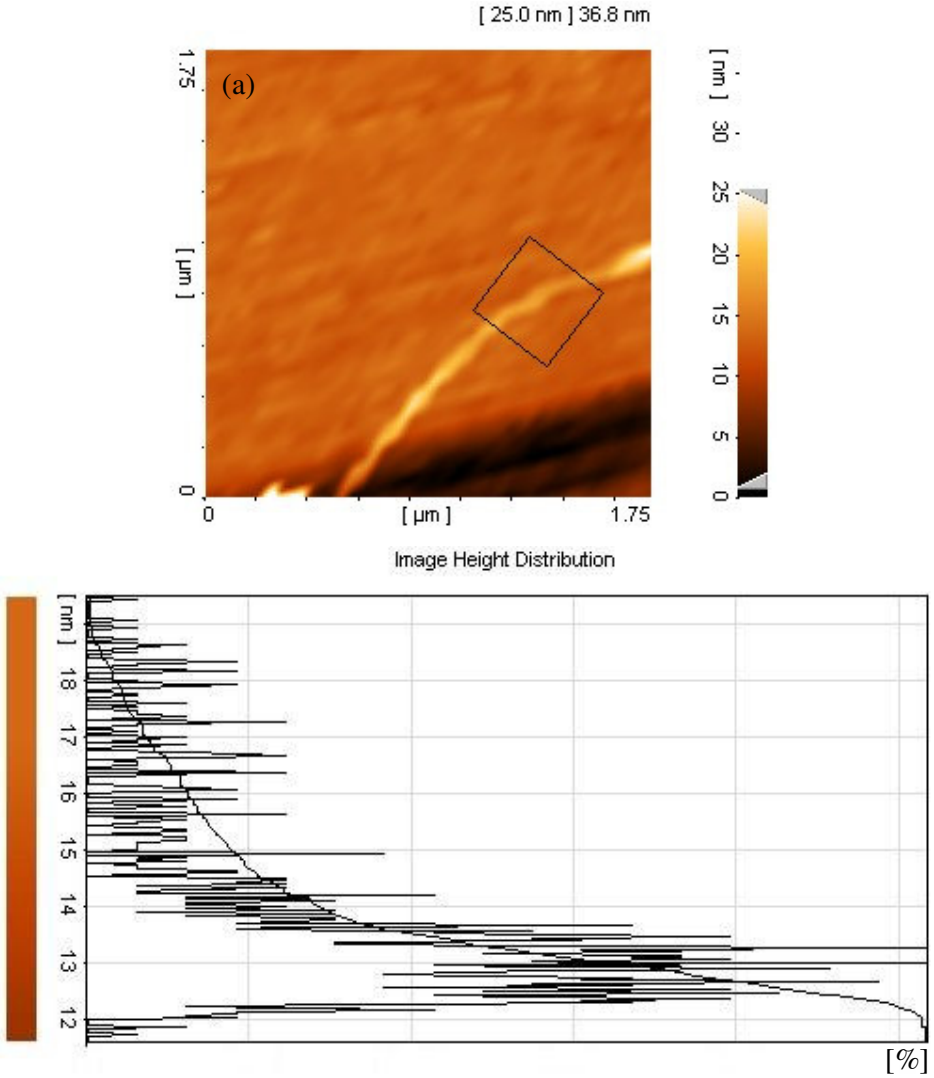


Figure 3. (a) AFM image of SWCNTs, (b) Image height distribution for selected area.

2.3. Procedure

Microscopic image of SWCNTs were obtained by SEM (Figure 1) and AFM (Figures 2 and 3). Figure 2 and part (a) of Figure 3 show the AFM images of bundles of SWCNTs. Part (b) of Figure 3 shows an image height distribution for selected area of related AFM image.

Before the modification of glassy carbon surface by SWCNTs, surface was polished with alumina powder (0.3 and 0.05 μm, respectively) on polishing cloth. The electrode was then successively sonicated in ethanol (5 min.) to remove the adsorbed particles. The polished electrode was treated electrochemically in PBS, cycling between -500 and 1000 mV at 150 mV/s, until a clean GC electrode was obtained. 10 μl of acetone solution in which SWCNTs dispersed (2 mg/ml) and sonicated, dropped on the electrode surface and dried. Afterwards, modified electrode was dipped into a PBS with 5 mg/ml of enzyme. Then, cyclic voltammetry was applied (40 scans) from -1000 to 1500 mV at 100 mV/s, washed with distilled water and stored in 0.05 M PBS (3-5 °C) when not in use.

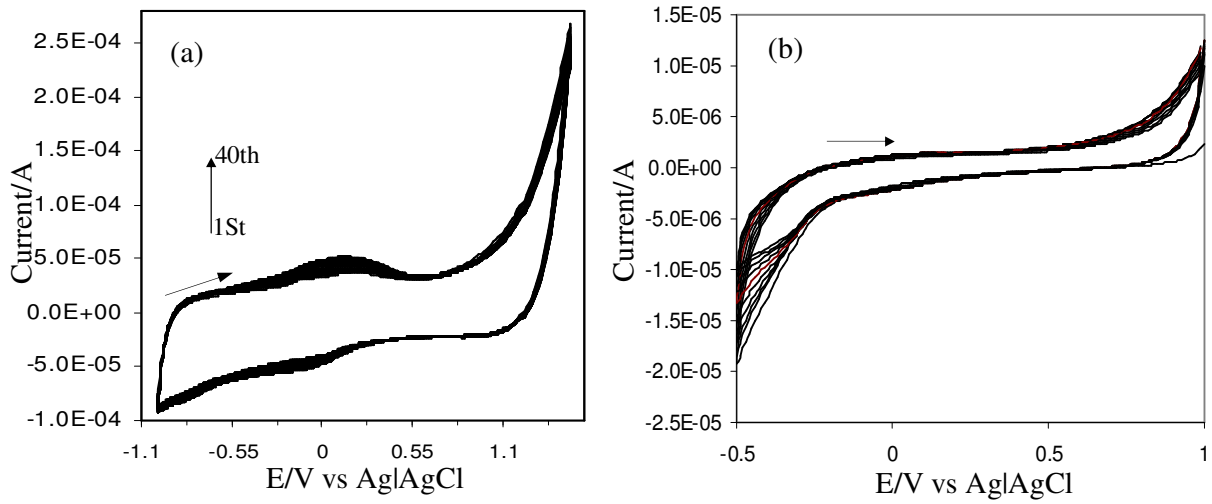


Figure 4. Continuouse cyclic voltammograms observed upon cycling a glassy carbon electrode (a) modified by SWCNTs and (b) bare electrode, in 0.05 M PBS containing of 5 mg/ml of polyphenol oxidase, scan rate; 100 mV/s.

3. RESULTS AND DISCUSSION

3.1. Direct voltammetric behavior

Parts (a) and (b) of Figure 4 are representative cyclic voltammograms observed upon SWCNTs/GC and bare GC electrodes in 0.05 M PBS, respectively. These voltammograms recorded in the presence of 5 mg/ml of enzyme (polyphenol oxidase). It was obvious from part (a) of Figure 4 that obtained current augmented with the augment of cycles, which corresponding to the direct electron

transfer of immobilized enzyme on modified electrode. The enzyme on SWCNTs increased during cycles. Afterwards, enzyme enclosed SWCNT surfaces and stable CVs were obtained. As we declared, increasing of electrochemical responses connected to the increasing of immobilized enzyme. The integrity of immobilized enzyme construction and its ability to electrochemical response were assessed by voltammetry and scan rate effect on PPO/SWCNTs/GC voltammetric behavior was studied in detail. However, parts (a) and (b) of Figure 5 show a pair of redox peaks at a variety of scan rates. The PPO/SWCNTs/GC electrode presented reductive peak at 0.067 V, corresponding oxidative peak at 0.137 V (scan rate: 20 mV/s). The difference of anodic and cathodic peak potential was $\Delta E = 0.070$ V. These redox peaks were attributed to the redox reaction of enzyme electroactive centers. Formal potential $E^{\circ'}$ (estimated as the midpoint of cathodic and anodic peak potentials) is about 0.102 V against the reference electrode (0.301 V vs. NHE). The formal potential of PPO on graphite electrode was 0.550 V vs. SCE (0.794 V vs. NHE) [27]. This value was 0.415 V vs. NHE for adsorbed PPO on silver electrode [28]. Reductive and oxidative peak currents vs. scan rate (v) and square root scan rate ($v^{1/2}$) are shown in parts (b) and (c) of Figure 5. It clear that redox peak currents augmented linearly with scan rate, correlation coefficient was 0.998 (i_{pc} = -207.6 v – 2.749) and 0.998 (i_{pa} = 187.01 v + 3.761), respectively. This fact introduced that redox process was an adsorption-controlled and immobilized enzyme was stable.

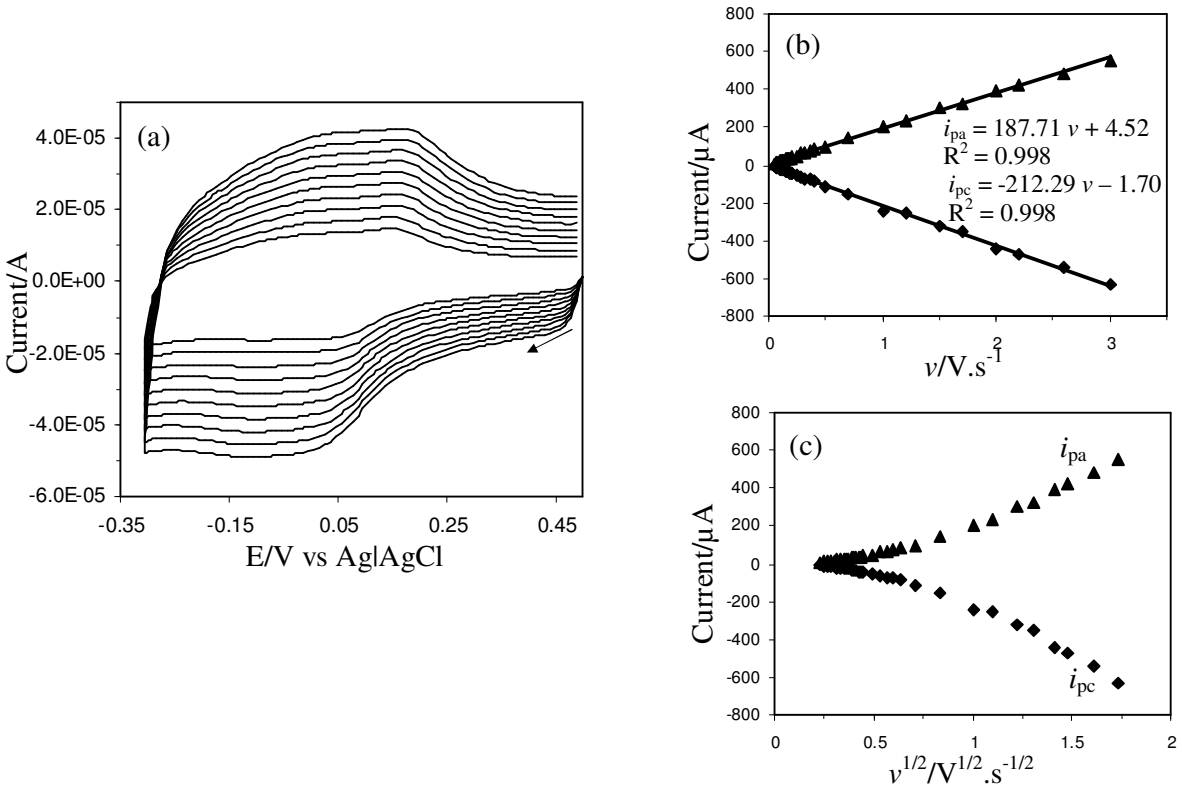


Figure 5. (a) Representative cyclic voltammograms observed upon PPO/SWCNTs/GC electrode immersed in 0.05 M PBS at various scan rates, from inner to outer; 60, 70, 80, 90, 100, 110, 120, 130, 140, and 150 mV/s. The relationship between peak currents (i_{pa}, i_{pc}) vs. (b) scan rates and (c) square root of scan rates.

Figure 6 shows plot of E vs. Q vs. time. Q is the amount of consumed charge during continuous CVs. In this figure, distances among lines remained more or less stable with consumed charge (Q) increases.

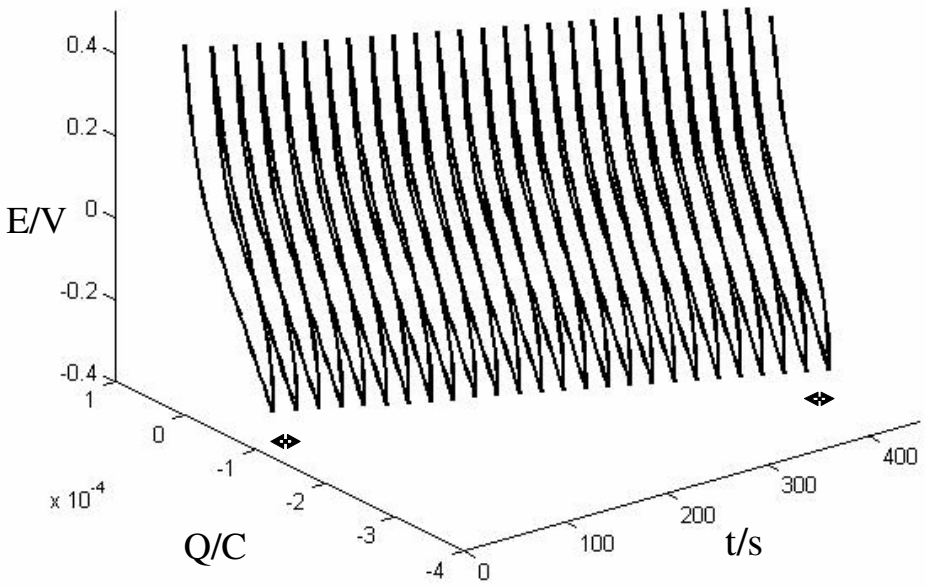


Figure 6. Potentials (E) vs. charge passed through electrochemical cell (Q) vs. time (s) during the 1st-25th CVs of PPO/SWCNTs/GC electrode in PBS.

3.2. Electrode response characteristics

Phenolics are associated with bitterness, astringency, color stability and could be inhibitors for microbiological growth-avoiding process spoilages [32,33]. Selective antiproliferative effect of caffeic acid esters, such as caffeic acid phenethyl and benzyl esters, versus some kinds of cancer cells were reported [34]. In other hand, PPO is bifunctional enzyme, which catalyzes two different reactions involving O_2: the *ortho*-hydroxylation of monophenols to *ortho*-diphenols (cresolase activity) and the subsequent oxidation of *ortho*-diphenols to *ortho*-quinones (diphenolase activity) [35]. So, we examined the catalysis of HCA through PPO/SWCNTs/GC electrode for electrochemical response characteristics.

Part (a) of Figure 7 (0 µM HCA) shows a background differential pulse voltammogram (DPV) corresponding to the PPO/SWCNTs/GC electrode in PBS. The addition of HCA results in a bioelectrocatalytic current related to the oxidation of HCA by PPO/SWCNTs on the GC electrode surface. Responses for HCA (catalytic current vs. HCA concentration) are shown in part (b) of Figure 7. Three correlation coefficients of R_1 = 0.994, R_2 = 0.997 and R_3 = 0.992 with %R.S.D. values ranging from 1.9–5.2% across the concentration range studied were obtained following linear regression analysis. Detection limit (3σ) of electrode towards HCA was found to be 0.03 µM.

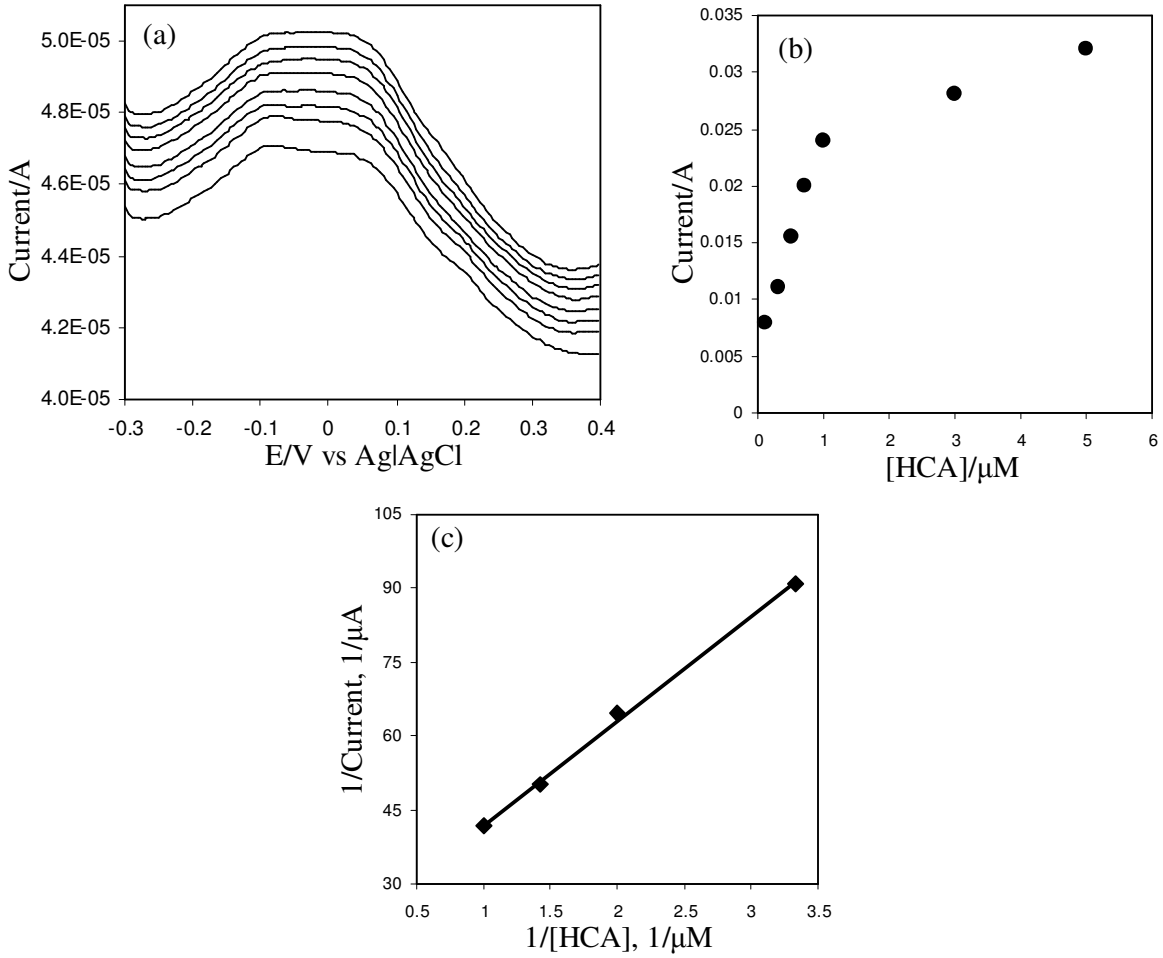


Figure 7. (a) Representative differential pulse voltammograms observed upon PPO/SWCNTs/GC electrode in a 0.05 M PBS in the presence of different concentrations of HCA, from inner to outer; 0, 0.1, 0.3, 0.5, 0.7, 1.0, 3.0 and 5.0 μM with the step potential and modulation amplitude values of 5 mV and 25 mV, respectively. (b) Calibration curve corresponding to the electrochemical analysis of DPVs. (c) Corresponding Lineweaver-Burk plot.

The modern relationship between substrate and enzyme was planned in 1903 by Victor Henri. An explanation was after that proposed in 1913 by Leonor Michaelis and Maud Menten, following earlier work by Archibald Vivian Hill [36]. It postulated that enzyme and substrate are in rapid equilibrium with their complex, which then decouples to yield product and enzyme. This model is related to conditions where very simple kinetics can be assumed. The Michaelis-Menten kinetic constant (K_m) as an estimation of apparent dissociation constant, telling the speed at which the enzyme-substrate intermediate dissociation without change of substrate to product. So, a larger value of K_m shows a lower probability of product formation per binding event. K_m calculated by construction of a Lineweaver-Burke, i.e., a double-reciprocal, plot from signal intensity against substrate

concentration. Lineweaver-Burke plots for data in part (a) of Figure 7 are shown in part (c) of this figure. The line of best fit found by linear regression analysis is explained by equation 1 [37]:

$$i_p^{-1} = i_{max}^{-1} + (K_m\, i_{max}^{-1})\, [HCA]^{-1} \qquad (1)$$

Where, K_m can be obtained by dividing the slope of line by its *y*-intercept (i_{max}^{-1}). The data showed in part (c) of Figure 7 yield K_m value of 1.035 μM, demonstrating that PPO/SWCNTs/GC electrode shows a high affinity for HCA. The correlation coefficient was 0.998 (i_p^{-1} = 21.255 $[HCA]^{-1}$ + 20.528). It is well known that Michaelis-Menten kinetic constant changes with carrier and immobilization method used. To conclude the storage stability, the performance of enzyme electrode checks during 6 weeks. It stored at 3-5 ºC in PBS, only a small loss of enzyme activity observed.

4. CONCLUSIONS

PPO can be strongly adsorbed on the surface of SWCNTs to form a PPO/SWCNTs modified electrode. It showed a well-defined and quasi-reversible redox peaks, which is characteristic of the Cu(II)/Cu(I) redox couples. The data from this study show that such modified electrodes can be useful for biological sensing and bioelectrochemical studies, while immobilized enzyme exhibited good catalytic activity.

ACKNOWLEDGEMENT
Financial support from the Tehran University of Medical sciences Research Affairs is gratefully acknowledged.

References

1. J. Wang, *Analyst* 130 (2005) 421.
2. A. K. Wanekaya, W. Chen, N. V. Myung, A. Mulchandai, *Electroanalysis* 18 (2006) 533.
3. R. Baron, F. W. Campbell, I. Streeter, L. Xiao, R. G. Compton, *Int. J. Electrochem. Sci.* 3 (2008) 556.
4. M. R. Nabid, M. Golbabaee, A. Bayandori Moghaddam, R. Dinarvand, R. Sedghi, *Int. J. Electrochem. Sci.* 3 (2008) 1117.
5. P. Xiao, W. Wu, J. Yu, F. Zhao, *Int. J. Electrochem. Sci.* 2 (2007) 149.
6. S. Zhao, B. Guo, G. Han, Y. Tian, *Mat. Lett.* 62 (2008) 3751.
7. A. Mohammadi, A. Bayandori Moghaddam, M. Kazemzad, R. Dinarvand, J. Badraghi, *Mat. Sci. Eng. C* 29 (2009) 1752.
8. C. B. Murray, C. R. Kagan, M. G. Bawendi, *Annual. Rev. Mat. Sci.* 30 (2000) 545.
9. S. G. Penn, L. He, M. J. Natan, *Curr. Opin. Chem. Biol.* 7 (2003) 609.
10. M. S. Dresselhaus, G. Dresselhaus, P. C. Eklund, Science of Fullerenes and Carbon Nanotubes, Academic Press, 1996, New York.
11. R. H. Baughman, A. A. Zakhidov, W. A. De Heer, *Science* 297 (2002) 787.

12. K. A. Williams, P. T. M. Veenhuizen, B. G. de la Torre, R. Eritja, C. Dekker, *Nature* 420 (2002) 761.
13. B. F. Erlanger, B.-X. Chen, M. Zhu, L. Brus, *Nano Lett.* 1 (2001) 465.
14. R. J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* 123 (2001) 3838.
15. D. L. Nelson, M. M. Cox, Lehninger Principles of Biochemistry, 4th ed.; W. H. Freeman & Co Ltd., 2005, New York, Chapter 6.
16. N. B. Li,a, J. Kwaka, *Electroanalysis* 19 (2007) 2428.
17. K. Haghbeena, E. W. Tan, *Anal. Biochem.* 312 (2003) 23.
18. Y. Cheng, Y. Liu, J. Huang, K. Li, Y. Xian, W. Zhang, L. Jin, *Electrochim. Acta* 54 (2009) 2588.
19. N. Makino, H. S. Mason, *J. Biol. Chem.* 248 (1973) 5731.
20. E. J. Land, C. A. Ramsden, P. A. Riley, *Acc. Chem. Res.* 36 (2003) 300.
21. C. Gardemann, C. Eicken, B. Krebs, *Acc. Chem. Res.* 35 (2002) 183.
22. E. I. Solomon, U. M. Sundaram, T. E. Machonkin, *Chem. Rev.* 96 (1996) 2563.
23. A. De, S. Mandal, R. Mukherjee, *J. Inorg. Biochem.* 102 (2008) 1170.
24. C. Vedrine, S. Fabiano, C. Tran-Minh, *Talanta* 59 (2003) 535.
25. R. Gill, R. Freeman, J. P. Xu, I. Willner, S. Winograd, I. Shweky, U. Banin, *J. Am. Chem. Soc.* 128 (2006) 15376.
26. S. Halaouli, M. Asther, K. Kruus, L. Guo, M. Hamdi, J.-C. Sigoillot, M. Asther, A. Lomascolo, *J. Appl. Microbiol.* 98 (2005) 332.
27. A. Liu, I. Honma, H. Zhou, *Biosens. Bioelectron.* 21 (2005) 809.
28. B. Ye, X. Zhou, *Talanta* 44 (1997) 831.
29. L. Chen, B. Gu, G. Zhu, Y. Wu, S. Liu, C. Xu, *J. Electroanal. Chem.* 617 (2008) 7.
30. S. Liu, J. Yu, H. Ju, *J. Electroanal. Chem.* 540 (2003) 61.
31. Z. Liu, B. Liu, J. Kong, J. Deng, *Anal. Chem.* 72 (2000) 4707.
32. M. M. Cowan, *Clin. Microbiol. Rev.* 12 (1999) 564.
33. G. A. Spanos, R. E. Wrolstad, *J. Agric. Food Chem.* 40 (1992) 1478.
34. T. Nagaoka, A. H. Banskota, Y. Tezuka, I. Saiki, S. Kadota, *Bioorg. Med. Chem.* 10 (2002) 3351.
35. M. Jimenez, F. Garcia-Carmona, *J. Agric. Food Chem.* 47 (1999) 56.
36. L. Michaelis, M. Menten, *Biochem. Z*, 49 (1913) 333.
37. T. Nakaminami, S. Ito, S. Kuwabata, H. Yoneyama, *Anal. Chem.* 71 (1999) 1068.