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Direct Electron Transfer Kinetics of Horseradish Peroxidase on Self-Assembled Monolayer/Gold Nanoparticles Decorated Multi-walled Carbon Nanotubes

Saimon Moraes Silva, Delton Martins Pimentel, Rita de Cássia Silva Luz and Flavio Santos Damos[*]

Department of Chemistry, UFVJM, 39100-000, Diamantina, MG, Brazil
[*]E-mail: flavio.damos@ufvjm.edu.br

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This study analyzed the percentage of direct electron transfer (DET) of Horseradish Peroxidase on multi-walled carbon nanotube (HRP/MWCNT) and mercaptopyridine self-assembled monolayer/gold nanoparticles decorated multi-walled carbon nanotube (MPy/AuNP/MWCNT), since nanostructured materials has been widely used as support for immobilization of enzymes. The kinetic studies for H_2O_2 reduction were carried out with rotating-disk electrode (RDE) system in phosphate buffer solution, pH 7, in the absence and presence of dopamine. In order to found the percentage of TED of HRP on MPy/AuNP/MWCNT was applied the Koutecky-Levich equation, as well as the kinetic current expressions for TED and simultaneous TED/MET, respectively. Finally, the expressions for the kinetic currents were used to estimate of TED percentages for HRP immobilized on following obtained materials, respectively: 51% of TED for MWCNT/HRP, and 68% of TED for MPy/AuNP/MWCNT. These results demonstrate for the first time that a kinetic investigation can improve the control of the TED for HRP in development of nanostructured materials as platform to the enzyme immobilization.

Keywords: HRP; Multi-walled carbon nanotube; Gold nanoparticles; Self-assembled monolayer; Direct electron transfer; Mediated electron transfer

1. INTRODUCTION

Nowadays, the electroanalytical chemistry turns to the development of new nanomaterials in order to improve the quality of the electrochemical biosensors exploiting the bioelectrocatalysis. One of the most investigated phenomena involved in bioelectrocatalysisis the particular case of direct electron transfer (DET) between the electrode surface and redox active biomolecules. The phenomenon of DET has significant importance from both practical and fundamental points of view since first reported by Yaropolov et al. in 1978 [1].

Depending on the practical needs, different redox enzymes have been used in biosensor design, including oxidases, dehydrogenases, peroxidases, and others. Among them, horseradish peroxidase (HRP) is one of the most used enzymes in biosensors construction due to their biological role, their wide spectrum of activity and their participation in various physiological processes [2,3].Thus, detailed investigations of their molecular and structural characteristics and reaction mechanisms have been investigated by several research groups. Indeed, the mechanisms of peroxidase-catalyzed reactions have been widely studied using horseradish peroxidase (HRP) as a model of the common mechanism of peroxidase action [3-5].

On the other hand, the access to the HRP active site is difficult due to the existence of a narrow channel, which naturally affects the mechanism of electron transfer (ET) between the electrode material and the peroxidase active site. As a result, an extensive investigation about the DET on a number of materials has been carried out [3-6]. However, a few amounts of these works have given attention to quantification of the percentage of molecule able to exhibit direct electron transfer on these electrode materials. An interesting approach developed to calculate the percentage of DET, exploiting graphite as electrode material, has been carried out by Gorton research group [7] which has found that the percentage of active native horseradish peroxidase molecules in direct ET is about 40%. In this sense, the development of novel electrode materials for enzyme immobilization is of high importance in development of peroxidase based biosensors looking for the optimization of the direct electron transfer of the immobilized HRP.

Carbon nanotubes (CNTs) have caused a profound impact on many areas of science and technology, including the biosensor development. The properties and applications of carbon nanotubes have been well reviewed in the literature and a number of reviews with an electrochemical emphasis have been published [8,9]. CNTs are excellent electrode materials due to their good electrical conductivity, thermal stability, flexibility, and mechanical strength, as well as being relatively inert in most electrolyte solutions, retaining a high surface activity in a wide potential operational window [10].

These unique properties of CNTs essentially allow them the versatility to be non-covalently functionalized without destroying their electronic and chemical structures with some organic and inorganic compounds, through π-π electronic interactions [11]. The use of CNTs increases the sensitivity of the electroanalytical method due to their specific electronic, chemical and mechanical properties, and can facilitate electron transfer between the electroactive species and electrodes. Indeed, electrochemical studies have clearly demonstrated that CNTs based modified electrodes could exhibit a sensitive and highly selective electrochemical detection and that CNTs could minimize the electrode fouling [12]. However, the MWCNT modification with some molecules such as thiol compounds become difficult and it demands covalent linking.

Gold nanoparticles are also of great interest due to their potential applications in microelectronics, optoelectronics, catalysis, information storage, as well as in sensors [13]. It is a versatile building block for the construction of hybrid materials by combining the AuNPwithnanostructured materials. In this sense, carbon nanotube (CNT)has been used as supports for preparing different CNT/gold nanoparticle composites, which have potential applications in catalysis, sensing, electroanalysis, light harvesting, and development of electronic nanodevices [14]. Indeed,

hybrid materials may have collective properties that are dramatically different from individual component. Therefore, there is an ever-increasing interest in synthesizing CNT-metal NP composites [15]. For instance, the composites of CNTs and some nanoparticles expand their applications from CNT-assisted catalytic systems to complex conductor networks in microelectronics [16]. In particular, gold nanoparticle-deposited CNTs are important due to their potential applications as the electrode system of solar cell devices[17], electroanalysis [18].

The ability of gold nanoparticles to provide a stable immobilization of biomolecules retaining their bioactivity is a major advantage for the preparation of biosensors [19]. Furthermore, gold nanoparticles permit direct electron transfer between redox proteins and bulk electrode materials, thus allowing electrochemical sensing to be performed with no need for electron transfer mediators. Characteristics of gold nanoparticles such as high surface-to-volume ratio, high surface energy, ability to decrease proteins–metal particles distance, and the functioning as electron conducting pathways between prosthetic groups and the electrode surface, have been claimed as reasons to facilitate electron transfer between redox proteins and electrode surfaces [20]. Indeed, conjugation of gold nanoparticles with other nanomaterials and biomolecules is an attractive research area within nanobiotechnology. Hybrid nanoparticles/nanotubes materials have shown to possess interesting properties, which can be profited for the development of electrochemical biosensors [19, 20].

The aim of the present work is study the kinetics of direct and mediated electron transfer of horseradish peroxidase on self-assembled monolayer-modified gold nanoparticles decorated multi-walled carbon nanotubes in comparison to non hybrid materials in order to improve the control on the direct and mediated electron transfer on using a new electrode material.

2. EXPERIMENTAL PART

2.1. Reagents and solutions

All chemicals used were of analytical grade. Peroxidase from horseradish (EC 1.11.1.7 type VI) was obtained from Sigma. 4-mercaptopyridine was acquired from Aldrich (Milwaukee, USA). Ethanol (99%) from Synth (São Paulo, Brazil) was used as received. The CNTs (10-20 nm diameter, 2-5 μm length) were acquired from NanoLab Inc. (Brighton, MA, USA). Disodium and monosodium phosphates (Na_2HPO_4 and NaH_2PO_4) were acquired from Synth, São Paulo, Brazil. Working standard solutions were prepared daily with appropriate dilution of the stock solutions with deionized water. All solutions were prepared with water purified in a Milli-Q Millipore system and the actual pH of the buffer solutions was determined with a Corning pH/Ion Analyser 350 model. Phosphate buffer solutions (0.1 mol L^{-1}) were prepared from 0.1 mol L^{-1} H_3PO_4–NaH_2PO_4, and the pH was adjusted with 0.1 mol L^{-1} H_3PO_4 or 2.0 mol L^{-1}NaOH.

2.2. Electrochemical measurements

Electrochemical measurements were performed with an Autolab PGSTAT 128N potentiostat/galvanostat from Eco chemie (Utrecht, Netherlands) coupled to a PC microcomputer with

GPES 4.9 software. A three electrode electrochemical cell was employed for all electrochemical measurements. The working electrode was a glassy carbon mounted in Teflon®. The counter and reference electrodes were a platinum electrode and Ag/AgCl (sat.), respectively. Oxygen was removed by bubbling nitrogen through the solution.

The glassy carbon electrode was fitted into a rotating disk electrode holder (RDE; Model Number AFMSRCE, PINE Instrument Company, Grove City, USA), which was then placed in a three electrode cell with an Ag/AgCl (3 M KCl) reference electrode and a platinum wire as the auxiliary electrode. All RDE measurements were performed at an applied potential of -0,25 mV versus Ag/AgCl with an electrolyte of 0.1 M phosphate buffer, pH 7.0. Prior to the experiment the solution was bubbled with nitrogen for 10 min. Nitrogen was passed over the solution during the experiments.

2.3. Sensor Construction

Previously to the electrode modification, the glassy carbon (GC) electrode surface was polished and then cleaned by sonication to remove any adhesive [21]. Immediately after, a dispersion prepared by mixing 1.0 mgMWCNTand 1ml of gold nanoparticle (AuNP) colloidal with the aid of sonication. Then 30µL of this suspension was placed directly onto the GC electrodesurface and allowed to dry at 80 ^{O}C for 10 min to form a AuNP/MWCNT nanocomposite at the GC electrode surface.Finally it was added 20µL of a solution of 4-mercaptopyridine dispersed in 1 mL of alcohol and it was also left to dry.Finally it was added 20µL of a solution of 4-mercaptopyridine dispersed in 1 mL of alcohol and it was also left to dry to produce the self-assembled monolayer and gold nanoparticle grafted multwalled carbon nanotube modified glassy carbon electrode (MPy/AuNP/MWCNT). HRP-modified MPy/AuNP/MWCNT electrodes were prepared by placing 30 µL of asolution of 10 mg mL^{-1} HRP (dissolved in phosphate buffer) on thetop and keeping in a beaker covered with a sealing film at +4^{o}C. After adsorption for 20 h, the electrodes werewashed with Millipore water and stored in 0.1 M phosphatebuffer at + 4^{o}C until required.

To comparison, a MWCNT and HRP modified GC electrode was also constructed. Thus, a dispersion prepared by mixing 1.0 mg MWCNT and 1ml of dimethylformamide with the aid of sonication. Then30µL of this suspension was placed directly onto the GC electrodesurface and allowed to dry at 80 ^{O}C for 10 min to form aMWCNT film at the GC electrode surface [22]. After this, the previous procedure to HRP immobilizatio was carried out. In every cases the enzyme solutions contained 0.5% of the agent glutaraldehyde. All modified electrodes were thoroughly washed with distilled water and they were placed inside the chemical cell.

2.4. Electrochemical measurements

Linear sweep voltammetry and amperometry were carried out at different concentrations of hydrogen peroxide in phosphate buffer, varying the speed of rotation of the modified electrode in order to obtain the kinetics parameters involved in the direct electron transfer electrode to the enzyme.

Studies were also performed with affixed volume of hydrogen peroxide in phosphate buffer and adding dopamine to achieve the mediated electron transfer parameters for the enzyme.

3. RESULTS AND DISCUSSION

Initially, hydrodynamic voltammetry and amperometry were obtained for GC electrode modified with HRP/MWCNTat different rotation rates of the electrode in phosphate buffer pH7, with 20μmolL^{-1} hydrogen peroxide (Figure 1a-b).

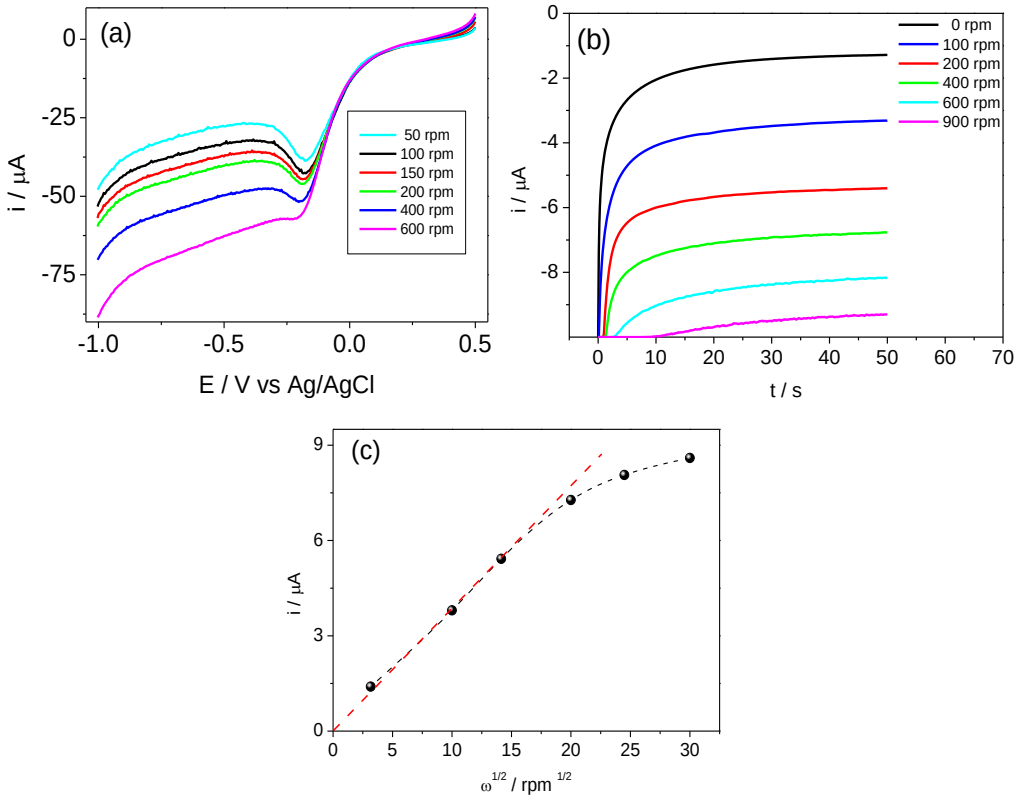


Figure 1. (a) Linear sweep voltammograms for glassy carbon electrode modified with HRP/MWCNT in 50μmolL^{-1} of H_2O_2 at various rotation rates of the electrode and **(b)** Hydrodynamic amperometry for a glassy carbon electrode modified with HRP/MWCNT in 10μmolL^{-1} of H_2O_2 at various rotation rates of the electrode. Applied potential, -0.25 V/ Ag/AgCl. **(c)** Levich Plot from plots in (b).

Under the mass-transport-limited condition, the solution of the convective-diffusion equation shows that the limiting cathodic current ($i_{l,c}$) is proportional to the bulk concentration, C_0*, as well as the square root of rotation speed of the electrode, $\omega^{1/2}$, as determined by the well-known Levich equation as follows [23]:

$$i_{l,c} = -0{,}62nFAD_o^{2/3}\omega^{1/2}v^{-1/6}C_o^* \qquad (1)$$

where n represents the number of electrons to the cathodic reaction, F is the Faraday constant ($Cmol^{-1}$), A is the electrode area (cm^2), $D_0$is the diffusion coefficient of electroactive species (cm^2s^{-1}), ν is the kinematic viscosity of the medium (cm^2s^{-1}), C_0^* is the concentration of electroactive species and ω is the angular frequency of rotation of the electrode.

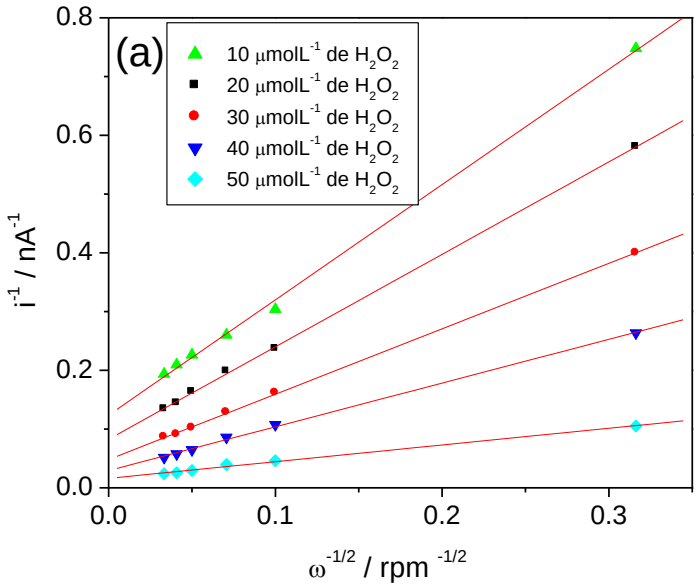
On the other hand, a deviation of a plot of current *vs.* $\omega^{1/2}$ from a straight line intersecting the origin suggest that a kinetic limitation is involved in the electron transfer reaction (Figure 1c). Under kinetic control, the electric current is called kinetic current, i_K, and expresses the current in the absence of any effect of mass transport. In other words, this is the electric current that will flow under kinetic limitation if the mass transport is efficient enough to maintain concentration on the surface as high as the concentration in the bulk of the solution.

In this context, the reduction current of hydrogen peroxide on an electrode modified with the enzyme HRP can be limited by transport of peroxide to the electrode surface and/or electron transfer kinetics of the enzymatic reaction. The measured current, i, is a combination of current limited by mass transport, i_l, and the current limited by the kinetics of the process, i_k, according to the Koutecky-Levich equation [23-25]:

$$\frac{1}{-i} = \frac{1}{-i_k} + \frac{1}{-i_{l,c}} = \frac{1}{-i_k} + \frac{1}{-0{,}62nFAD_0^{2/3}\omega^{1/2}\nu^{-1/6}C_0^*} \qquad (2)$$

where all terms have their usual meanings, as previously defined.

In this sense, the catalytic currents for hydrogen peroxide reduction on a rotating disk (a) HRP/MWCNT and (b) HRP/MPy/AuNP/MWCNT electrodes were measured in 0.1 $molL^{-1}$ phosphate buffer solution (pH 7.0) from Koutecky-Levich plots for six different concentrations of H_2O_2 (10, 20, 30, 40 and 50 $\mu molL^{-1}$) under different rotation rates of the modified electrode (ω = 10, 100, 200, 400, 600 e 900 rpm) (Figure 2a-b).



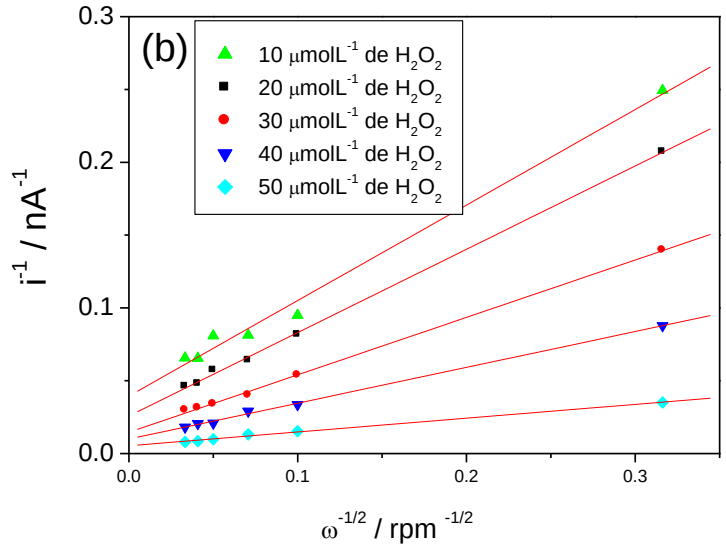


Figure 2. (a) Koutecky-Levich plots of H_2O_2 reduction at: (a) HRP/MWCNT and (b) HRP/MPy/AuNP/MWCNT modified electrode at various H_2O_2 concentrations: (1) 10 μmolL^{-1}; (2) 20 μmolL^{-1}; (3) 30 μmolL^{-1}; (4) 40 μmolL^{-1} and (5) 50 μmolL^{-1} in 0.1 M phosphate buffer solution, (pH 7.0). Applied potential, -0.25 V/ Ag/AgCl.

It can be seen that the cathodic current depends on electrode rotation rate. Clearly, $\omega^{-1/2}$C (from Eq. 2) should be constant if $i_k$is high, and the graph I vs $\omega^{1/2}$ should be curved and tending to a limit value i = i_k,when $\omega^{1/2} \rightarrow \infty$. Consequently, the plot 1/i vs $1/C_o^* \omega^{1/2}$ is linear and can be extrapolated to $\omega^{-1/2} = 0$ it is found out $1/i_k$. Thus, kinetically limited currents of the reduction of H_2O_2, were evaluated from the intercepts of the Koutecky-Levich plotspresented in Fig. 2(a) and Fig. 2(b).

In order to evaluate the percentage of HRP involved indirect ET, Koutecky-Levich theory was used to obtain the kinetic current in presence of H_2O_2 as well as H_2O_2 and Dopamine, simultaneously, based on mechanisms for HRP electron transfer.

The data analysis was conducted by exploring two different mechanistic approaches: (i) direct electron transfer and (ii) and simultaneous, direct and mediated electron transfer. The peroxidase catalytic cycle occurs through a multistep reaction that involves, first, the reaction of the active site with hydrogen peroxide.

In the DET, the reduction of the peroxide is conducted by the enzyme accordingto reaction Eq. (3)and the reduction of the active center of the enzyme occurs directly on the electrode (Eq. (4)), as follows [26]:

$$[\text{heme } (Fe^{III}), R] + H_2O_2 \rightarrow [\text{heme}(O = Fe^{IV}), R^{+*}] + H_2O, \; k_1 \qquad (3)$$

$$[\text{heme } (O = Fe^{IV}), R^{+*}] + 2e^- + 2H^+ \rightarrow [\text{heme } (Fe^{III}), R] + H_2O, \; k_s \qquad (4)$$

where$[\text{heme}\ (Fe^{III}), R]$is the native ferric enzyme HRP and $[\text{heme}\ (Fe^{III}), R]$is itsoxidised intermediate compound I, k_1 and k_s represent the rate constants referring to Eqs (3) and (4), respectively.

In the case of direct ET, I_{kin} depends onthe H_2O_2 concentration according to Eq. (5) [24-27]:

$$\frac{1}{i_{kin}} = \frac{1}{nFA\Gamma_{TED}}\left(\frac{1}{k_1[H_2O_2]} + \frac{1}{k_s}\right) \qquad (5)$$

where n is the number of electrons transferred in reaction (1), and Γ_{DET} is the amount of enzymeactive in direct ET.

On the other hand, the reduction of the oxidised intermediate (compound I) can proceeds via two steps in the case of mediated ET by an electron donator. In the high oxidation state of the protein, compound I,itcontains an oxyferrylcenter, with the iron in the ferryl state (Fe^{IV}=O), and anorganiccation radical which is an oxidizableporphyrinπcation-radical. Then, compound Ioxidizes a substrate (S) to give a substrate radical and compound II ($[\text{heme}\ (O = Fe^{IV}), R]$), where the organic cation radical is reduced by a second substrate molecule, regenerating the iron (III) state [28].

$$[\text{heme}(O = Fe^{IV}), R^{+*}] + S + H^+ \rightarrow [\text{heme}(O = Fe^{IV}), R] + S^*,\ k_2 \qquad (6)$$

$$[\text{heme}(O = Fe^{IV}), R] + S + H^+ \rightarrow [\text{heme}(Fe^{III}), R] + S^* + H_2O,\ k_3 \qquad (7)$$

where$[\text{heme}(O = Fe^{IV}), R]$is the compound II.

In this sense, when an electron donor (S) is present in a peroxidase electrode system, the direct and mediated processes can occur simultaneously, with the reduction of oxidized donorS^* by an electrode (Equation 8).

$$S^* + e^- H^+ \rightarrow S, \quad K_{s,m} \qquad (8)$$

As proposed by Lindgren et al [6], under the presence of saturating concentrationof S all peroxidase molecules work in mediated ET, I_{kin}can be expressed according to the simplified Eq. (9):

$$\frac{1}{I_{kin}} = \frac{1}{2n_1 FA\Gamma}\left(\frac{1}{K_1[H_2O_2]} + \frac{1}{K_S + K_s[S]}\right) \qquad (9)$$

whereΓrepresents the total amount of active enzyme at the electrode surface and n_1 is the number of electrons transferred per donor molecule.

In Figure 3 the reciprocal kinetically limited current (I_{kin}^{-1}) from intercept of the plots presented in Fig 2 was plotted versus the reciprocal H_2O_2 concentration ($[H_2O_2]^{-1}$). According to the equations (5) and (9) for I_{kin}, the dependence of kinetic current on the hydrogen peroxide concentration are similar in direct and simultaneous direct and mediated electron transfer of HRP.

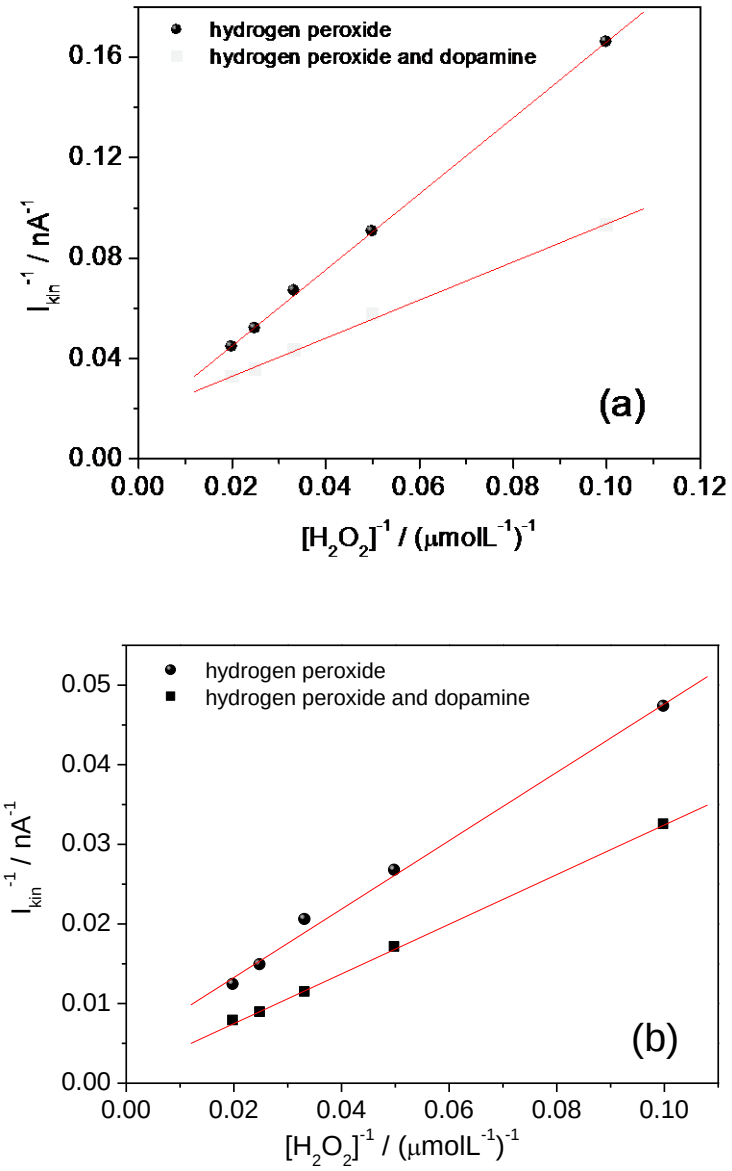


Figure 3. The kinetically limited currents (from the intercepts of Koutecky–Levich plots of Figure 2a-b) plotted versus the hydrogen peroxide concentration in inverted co-ordinates for: 0; 100μ molL^{-1}dopamine using (a) the HRP/MWCNT and (b) HRP/MPy/AuNP/MWCNT modified electrode.

In this sense, the ratio of the slopes of the equations for DET (Eq. 6) and simultaneous DET and MET (Eq. 9) offer the percentage of active enzymes on the surface active in direct electron transfer.

$$\frac{\text{Slope for DET/MET}}{\text{Slope for DET}} = \frac{\frac{1}{2n_1FA\Gamma_{DET/MET}}}{\frac{1}{nFA\Gamma_{DET}}} = \frac{\Gamma_{DET}}{\Gamma_{DET/MET}} \qquad (10)$$

Thus, the Equation 10 was used in estimative of the percentage of enzymes HRP participating in DET on the constructed materials respectively: 51% of DET for MWCNT/HRP, and 68 % of DET on HRP/MPy/AuNP/MWCNT. These results demonstrate that the modified electrodes show themselves as excellent materials to explore the DET for the enzyme HRP with a reduction of MET on nanostructured materials.

4. CONCLUSION

In this study, it was possible to explore the different parameters that cause variations in the percentages of DET in an enzymatic biosensor containing HRP as biological material. It was verified the influence of the platform on the DET percentage. This may occurs due to the HRP orientation at the modified electrode surface. Comparison of the dependence of the kinetically limited currents on the peroxide concentration in the absence and presence of p-cresol (mediator) in solution shows that the DET increases from 51% in HRP/MWCNT to 68% in HRP/MPy/AuNP/MWCNT. These results demonstrate for the first time that a kinetic investigation can improve the control of the TED for HRP in development of nanostructured materials as platform to the enzyme immobilization.

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References

1. A.I. Yaropolov, M.R. Tarasevich,and S.D. Varfolomeev, *Bioelectrochem. Bioenerg.*, 5 (1978) 18.
2. T. Ruzgas, E. Csoregi, J. Emneus, L. Gorton and G. Marko-Varga, *Anal. Chim.Acta* 330 (1996) 123.
3. A.Lindgren, J. Emnéus, T. Ruzgas, L. Gorton and G. Marko-Varga, *Anal. Chim.Acta*. 347 (1997) 51.
4. J.-L. Huang and Y.-C. Tsai, *Sensors and Actuators B* 140 (2009) 267.
5. E. E. Ferapontova, V. G. Grigorenko, A. M. Egorov, T. Borchers, T. Ruzgas and L. Gorton, *Biosens. Bioelectron*. 16 (2001) 147.
6. A.Lindgren, F. D. Munteanu, I. G. Gazaryan, T. Ruzgas and L. Gorton, J. *Electroanal. Chem*. 458 (1998) 113.
7. T. Ruzgas, L. Gorton, J. Emnéus and G. Marko-Varga, J. *Electroanal. Chem.* 391 (1995) 41.
8. M. Pumera, *Chem. Eur. J.* 15 (2009) 4970.
9. I.Dumitrescu, P. R. Unwin and J. V. Macpherson, *Chem. Commun*., (2009) 6886.
10. R. L. McCreery, *Chem. Rev*, (2008) 2646.
11. R. C. S. Luz, F. S. Damos, A. A. Tanaka, L. T. Kubota and Y. Gushikem, *Talanta* 76 (2008) 1097.
12. B. E. K. Swamy and B. J. Venton, *Analyst* 132 (2007) 876.
13. D.L. Feldheim and C.A. Foss, *Metal Nanoparticles: Synthesis, Characterization and Applications*, Dekker, New York, 2000.
14. K.R. Gopidas, J.K. Whitesell and M.A. Fox, *Nano Lett*. 3 (2003) 1757.

15. J. Kim, M. M. Rabbani, D. Kim, M. Reeb, J. H. Yeumc, C. H. Ko, Y. Kim, J.S. Bae and W. Oha, *Current Appl. Physics* 10 (2010) S201.
16. V. Georgakilas, D. Gournis, V. Tzitzios, L. Pasquato and D.M. Guldi, *J. Mater. Chem*. 17 (2007) 2679.
17. G. G. Wildgoose, C. E. Banks and R. G. Compton, *Small* 2 (2006) 182.
18. S. Hrapovic, E. Majid, Y. Liu, K. Male and J.H.T. Luong, *Anal. Chem*. 78 (2006) 5504.
19. C. Ou, R. Yuan, Y. Chai, M. Tang, R. Chai and X. He, *Anal. Chim. Acta* 603 (2007) 205.
20. J. M. Pingarrón, P. Yáñez-Sedeño and A. González-Cortés, *Electrochim. Acta*, 53 (2008) 5848.
21. J. Pillay and K.I. Ozoemena, *Electrochem. Commun*. 9 (2007) 1816.
22. R. C. S. Luz, F. S. Damos, A. A. Tanaka, L. T. Kubota and Y. Gushikem, *Electrochim. Acta* 53 (2008) 4706.
23. A.J. Bard and L.R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, second ed., John Wiley & Sons, Hoboken, NJ, 2001.
24. T. Ruzgas 1, L. Gorton, J. Emneus and G. Marko-Varga, *J. Electroanal. Chem.* 391 (1995) 41.
25. A.Lindgren, F. D. Munteanu, I. G. Gazaryan, T. Ruzgas and L. Gorton, J. *Electroanal. Chem.* 458 (1998) 113.
26. S. Ledru, N. Ruille and M. Boujtita, *Biosens. Bioelectron.* 21 (2006) 1591.
27. L. Gorton, A. Lindgren, T. Larsson, F.D. Munteanu, T. Ruzgas and I. Gazaryan, *Anal. Chim. Acta* 400 (1999) 91.
28. H.B. Dunford, *Heme Peroxidase*. Wiley, New York; (1999).