

Bioelectrochemical Detection of Alanine Aminotransferase for Molecular Diagnostic of the Liver Disease

Lara F. Paraíso¹, Lucas F. de Paula², Diego L. Franco², João M. Madurro², Ana G. Brito-Madurro^{1,*}

¹ Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, Brazil

² Institute of Chemistry, Federal University of Uberlândia, Uberlândia, Brazil.

*E-mail: agbrito@iqufu.ufu.br

Received: 9 October 2013 / Accepted: 27 November 2013 / Published: 5 January 2014

This paper reports a new bioelectrode for detection of alanine aminotransferase (ALT), a biomarker of hepatic disorders, using pyruvate oxidase immobilized onto graphite electrode modified with poly(4-aminophenol) and 4-aminoantipyrine as electrochemical indicator. Assays through cyclic voltammetry and morphological analysis by atomic force microscopy indicated that the enzyme was successfully incorporated onto the graphite electrode modified with poly(4-aminophenol). The biocatalytic process used on the bioelectrode to evaluate ALT by voltammetry was based on the fact that the target alanine aminotransferase, in presence of L-alanine and α -ketoglutarate, produces pyruvate, which is a substrate for the enzyme pyruvate oxidase (PyO) incorporated onto the modified electrode. The H_2O_2 produced from the reaction pyruvate/PyO oxidizes chemically 4-aminoantipyrine (4-APP), leading to a decrease in oxidation current of this compound. This decrease is associated with the consumption of 4-APP by competitive chemical reaction with H_2O_2 , decreasing the availability of 4-APP to oxidation in the electrode, showing that the 4-APP oxidized electrochemically is inversely proportional to the amount of ALT. The bioelectrode showed attractive characteristics such as short response time (about 200 s), low detection limit to ALT (2.68×10^{-5} U/L) and good stability after storage (97% of response after 30 days), indicating to be a promising approach for diagnosis of hepatic diseases.

Keywords: alanine aminotransferase, bioelectrode, modified electrode, pyruvate oxidase, poly(4-aminophenol)

1. INTRODUCTION

Liver diseases are a growing public health problem that affects million people worldwide [1, 2]. The evaluation of hepatic function is important for the diagnosis of a number of clinical disorders, such as hepatitis A, B, C, cirrhosis, steatosis and hepatitis induced by drug [3, 4]. The measurement of

alanine aminotransferase (ALT) in blood is frequently used to determine these diseases or only to evaluate liver function [5-9].

ALT is found primarily in the liver and kidneys, with smaller amounts in the heart and in skeletal muscles [10-12]. It has a catalytic activity of reversible conversion of alanine and α -ketoglutarate to pyruvate and glutamate. Under normal circumstances, this enzyme resides within the cells of the liver, but when the liver is injured, it is spilt into the blood. Elevated levels of ALT are a signal of liver damage such as hepatitis and jaundice [13]. Recent studies found alteration in ALT concentrations in individuals with metabolic syndrome [14, 15], insulin resistance [16], diabetes mellitus and obesity [17], revealing that the measurement of this enzyme concentration is an important tool for the diagnosis of these diseases too. Normal levels of ALT in the blood are 5 to 35 U/L⁻¹. Following severe liver damage, ALT levels increase to >50 times the normal level [18].

Some of the main detection strategies, such as colorimetry, chemiluminescence, chromatography and electrochemical techniques have been employed for ALT determination [18]. Methodologies based on conventional spectrophotometric assay, routinely performed in clinical laboratories, are costly, requiring complex reagents and trained operators. Therefore, there is a growing demand for the development of healthcare devices such as electrochemical sensors, which have proven advantages for such applications [19].

Usually the determination of ALT is carried out by colorimetric test [20, 21]. In particular, the colorimetric determination of ALT is carried out directly as phenylhydrazon, which is the product developed by reacting in acid medium pyruvate with 2,4-dinitrophenylhydrazine, which gives, in alkaline medium, a dark brown colour monitored at 520-550 nm versus a blank and extrapolating the corresponding value of enzyme activity from a titration curve. The method is time-consuming (about 1 hour), needing many reagents, and preparation of a standard curve and analytical conditions (basic pH), which results in a colorimetric (570 nm)/ fluorometric ($\lambda_{ex}= 535/\lambda_{em} = 587$ nm) product, proportional to the pyruvate generated.

A number of biosensors for ALT monitoring has been reported using electrode without modification or on modified platforms [22], based on glutamate oxidase [23-26] and pyruvate oxidase [2], or using indirect electrochemical detection with mediators [27-29]. Most of these devices were developed by using expensive transducers such as gold [25] or platinum [25]. Xuan *et al.* [2] used monoclonal antibodies to human recombinant ALT. The anti-ALT antibody immunosensor system showed sensitivity of 26.3 nA/(ng ml⁻¹) with detection limit of 10 pg/ml. In other work, Chang *et al.*, (2007) [23] demonstrated an electrochemical biosensor using palladium electrode modified by cation exchanger membrane based on glutamate oxidase. The rate of signal increase obtained by sensor for ALT activity was 0.596 nA/minU⁻¹ and linear range from 8 to 250U/L. Jamal *et al.* [27] described a sensor for ALT using platinum electrodes and the current response from either the oxidation of hydrogen peroxide or the re-oxidation of the mediator ferrocene carboxylic acid. The linear range was from 10 to 1000U/L and limit of detection of 3.29U/L using amperometry. Song *et al.*, [28] developed a biosensor for ALT using platinum electrodes and a polydimethylsiloxane (PDMS) microchannel. The sensitivities derived from a semi-logarithmic plots were 0.145 μ A/(U/L) for ALT and linear range from 1.3U/L to 250U/L.

Electrodes electrochemically modified by polymeric films offer advantages in the construction of biosensors, helping in the interaction of the analyte with the target and increasing the electric conductivity [30]. The development of polymeric films using 4-aminophenol have already been reported [31-35]; however, no study using poly(4-aminophenol) as matrix for immobilization of pyruvate oxidase, aiming to detect alanine aminotransferase was found in the literature. In this way, we report the development of an electrochemical bioelectrode for ALT detection, based on pyruvate oxidase immobilized on graphite surface modified with 4-aminophenol, using 4-aminoantipyrine as electrochemical indicator.

2. MATERIAL AND METHODS

2.1. Apparatus

All electrochemical experiments were carried out using a potentiostat CH Instruments, model 760 C connected to a serial output program. Surface morphology was assessed through atomic force microscopy (AFM) (*Shimadzu* SPM 9600). Electrochemical polymerization was performed in a three-compartment cell using a graphite disk (6 mm diameter, 99.9995%) from Alfa Aesar as working electrode and a platinum plate as counter electrode. All potentials refer to a silver/silver chloride reference electrode (Ag/AgCl , $\text{KCl } 3.0 \text{ mol.L}^{-1}$).

2.2. Chemicals

All reagents used were of analytical grade. The monomer 4-aminophenol and α -ketoglutarate were purchased from Acros Organics. L-alanine was obtained from Vetec. 4-aminoantipyrine (4-APP), thiamine pyrophosphate (TPP), *flavin* adenine dinucleotide (FAD), *porcine* heart alanine aminotransferase (E.C. 2.6.1.2) and bacterial pyruvate oxidase (E.C.1.2.3.3) were purchased from Sigma. Ultra-high purity water (Master System, Gehaka, Brazil) was used for the preparation of all solutions.

2.3. Electrode surface modification

Prior to electropolymerization, bare graphite electrode was mechanically polished with alumina ($0.3 \mu\text{m}$) slurry, ultrasonicated, washed with deionized water and dried in the air. 4-Aminophenol solution ($2.5 \times 10^{-3} \text{ mol.L}^{-1}$ in perchloric acid 0.5 mol.L^{-1}) was deaerated with ultra pure nitrogen for ca. 45 minutes, prior to electropolymerization. The monomer 4-aminophenol was electropolymerized on graphite electrode through continuous potential scans, according to Vieira and col. [35]. After the electropolymerization, the modified electrode was rinsed with deionized water to remove non-reacting monomers.

2.4. Stability of the modified electrode

Graphite electrodes modified with poly(4-aminophenol) were maintained at 8 ± 1 °C protected from light, and its stability was evaluated by cyclic voltammetry in HClO_4 solution (0.5 mol.L^{-1}), every 5 days, during 30 days.

2.5. Immobilization of the pyruvate oxidase (PyO) and detection of ALT

For the pre-conditioning of the surface of the graphite electrode modified with poly(4-aminophenol), it was subjected to successive potential scans from 0 to +1.0 V vs. Ag/AgCl, in phosphate buffer 0.1 mol.L^{-1} (pH 7.4) until voltammograms remained constant. Next, 15 μL of PyO (2 U ml^{-1}) in phosphate buffer pH 5.9 (0.15 mol.L^{-1}) and the cofactors of the PyO (6 μL thiamine pyrophosphate 3.0 mmol.L^{-1} in deionized water and 6 μL of FAD 0.15 mmol.L^{-1} in MgSO_4 0.15 mol.L^{-1}), were dropped onto the electrode. The electrode was dried at room temperature and then kept at 8 °C before use. After, 10 μL of substrates (alanine, 0.1 mol.L^{-1} and α -ketoglutarate 0.01 mol.L^{-1}), 15 μL of indicator 4-aminoantipyrine (1.0 mmol.L^{-1}) and 10 μL of ALT (0.003 U/L) were added onto poly(4-AMP)/PyO. This system was maintained at 37 °C for 25 minutes before detection, carried out in phosphate buffer, pH 7.4. The concentrations of the enzyme (PyO), substrates (alanine and alpha-ketoglutarate), cofactors [thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD)] and the indicator 4-aminoantipyrine (4-APP) were adapted from literature [27, 28, 36, 37].

2.6. Calibration curve

To evaluate the sensitivity and detection limit of the bioelectrode, 10 μL of different concentrations of ALT, 0.00003U/L, 0.003U/L, 0.03U/L; 0.3U/L; 3U/L were added to the bioelectrode. The reaction was conducted at 37 °C, during 25 minutes. For the detection, 4-aminoantipyrine (1.0 mmol.L^{-1} , 15 μL) was added to the electrode surface.

2.7. Interference studies

Substances found in serum or urine were studied for evaluation of the possible interfering effects. 1 mg/dL uric acid (UA), 1 mmol.L^{-1} glutamate (Glut), 1 mmol.L^{-1} glucose (Glu) or 3.6 mg/dL ascorbic acid (AA) was added to ALT 0.003 U/L. All experiments were performed at 37°C.

2.8. Bioelectrode stability

Operational stability may be defined as the retention of the activity of biomolecules when in use [38]. In order for the commercialization of a biosensor to be feasible, it should have good selectivity and stability during storage to assure reproducibility of measurements. Long-term lifetime is

beneficial to transport and storage of biosensor and also presents a critical importance in pharmaceutical and industrial applications [39].

In order to evaluate bioelectrode stability, the modified electrodes containing pyruvate oxidase were stored at 8 ± 1 °C, protected from light, during 30 days.

3. RESULTS AND DISCUSSION

3.1. Stability studies of the electrode modified with poly(4-aminophenol)

Studies of electrodeposition and characterization of polymers derived from 4-aminophenol have been described by our group [31-34] but no study on the stability in function of storage at low temperature has been reported. Figure 1 shows the stability of graphite electrode modified with poly(4-aminophenol), during 30 days, at 8°C, protected from light. The experiment was conducted in triplicate.

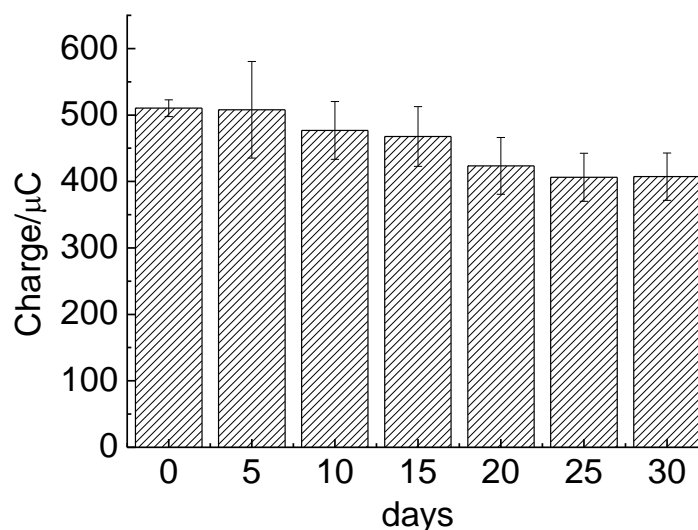


Figure 1. Stability study of graphite electrode modified with poly(4-aminophenol), by storage at 8°C. Cyclic voltammetries were realized in HClO₄ solution (0.5 mol.L⁻¹).

The electrode modified with polymer film, submitted to low temperature (8°C), during 30 days, kept about 75% of its electroactivity, indicating maintenance of the polymer structure, after this treatment. The partial loss of the stability of the conjugated polymers can be caused by presence of oxygen and/or energy (light or heat), breaking the conjugated bonds and resulting in reduction of its electrochemical response. The severity of this degradation depends of the oxygen concentration and level of energy that the polymer was exposed, decreasing the electrochemical response of the modified electrode.

3.2. Immobilization of pyruvate oxidase on the modified electrode

One way to demonstrate the adsorption of a biomolecule on the surface of transducer is conducting studies using electroactive complexes such as the redox pair potassium ferrocyanide/ferricyanide or hexaammineruthenium chloride [36, 40, 41] and mediators [27-29].

In order to evaluate the enzyme immobilization and the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry technique was employed, using as indicators of this immobilization the 4-aminoantipyrine (Fig. 2A) or $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Fig. 2B). Pyruvate Oxidase and cofactors (TPP and FAD) were immobilized onto graphite electrode modified with poly(4-aminophenol), freshly prepared. After immobilization, the modified electrode containing the enzyme was evaluated in presence of the redox probes.

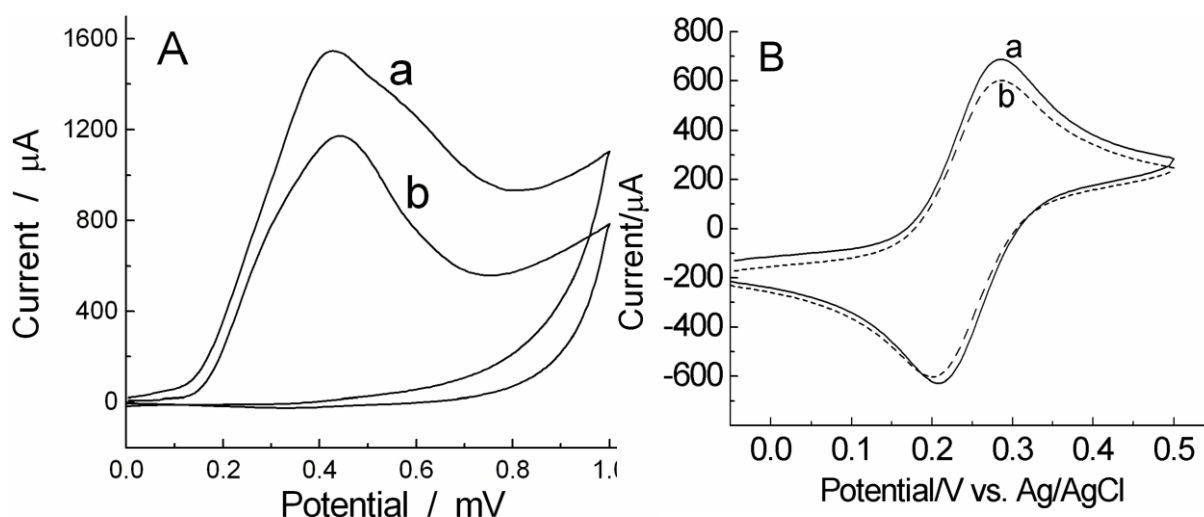


Figure 2. Cyclic voltammograms of graphite electrode modified with poly(4-aminophenol), $100 \text{ mV}\cdot\text{s}^{-1}$, in absence (a) or presence of pyruvate oxidase (b), containing: (A) 4-aminoantipyrine in phosphate buffer pH 7.4 ($0.1 \text{ mol}\cdot\text{L}^{-1}$). (B) $K_3Fe(CN)_6$ ($5 \text{ mmol}\cdot\text{L}^{-1}$), $K_4Fe(CN)_6$ ($5 \text{ mmol}\cdot\text{L}^{-1}$) in KCl ($0.1 \text{ mol}\cdot\text{L}^{-1}$) solution.

Figure 2A shows an irreversible electron transfer of 4-aminoantipyrine to the electrode surface, suggesting the occurrence of others processes in sequence, such as chemical reaction of the indicator oxidized. It is also observed that the current signal of 4-aminoantipyrine electrooxidation, in presence of the PyO, decreased 1.5 times and its oxidation peak shifted slightly to more anodic potentials, when compared with the modified electrode in absence of the biomolecule, indicating that the enzyme was immobilized on surface of the electrode. In addition, Figure 2B shows that the electron transfer of the redox pair $K_3Fe(CN)_6/K_4Fe(CN)_6$ to the modified electrode occurs without significant thermodynamic barriers, with $\Delta E_p=75\text{mV}$ and I_{pa}/I_{pc} near unity, indicating a reversible system. Figure 2B shows also a decrease in the current values in the modified electrode in presence of PyO, caused by reducing of the electron transfer of the redox couple to the electrode. This result is in accordance with the net charge negative value of pyruvate oxidase (isoelectric point: 4.3, solution pH: 5.7) causing repulsion of the redox pair.

3.3. Morphological characterization of the bioelectrode using atomic force microscopy

AFM measurements were carried out to characterize the morphological changes of the electrode modified, with or without biomolecules. Fig. 3 shows representative 2D and 3D AFM images of these surfaces.

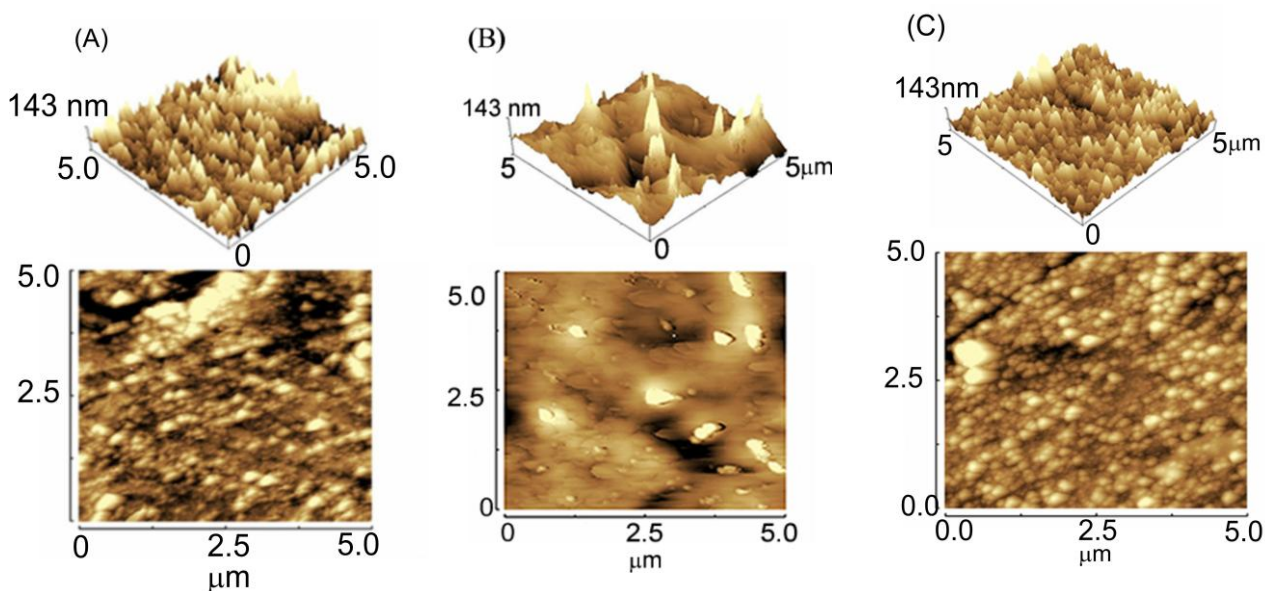


Figure 3. AFM images of graphite (A), graphite/poly(4-aminophenol) (B), graphite/poly(4-aminophenol)/pyruvate oxidase (C).

Images of bare graphite, graphite/poly(4-aminophenol), graphite/poly(4-aminophenol)/PyO, presents roughness values of 312 nm, 1133 nm, 306 nm, respectively. After electropolymerization, the surface of the graphite electrode became rougher, indicating that the surface modification with poly(4-aminophenol) was effective (see Figures 3A and 3B). The comparison between the surfaces of the graphite electrode modified with poly(4-aminophenol) before (Fig. 3B) and after immobilization of the pyruvate oxidase (Fig. 3C) shows significant change in surface, being observed formation of numerous clusters and decrease in roughness value, indicating that the enzyme was incorporated on the modified graphite electrode, in accordance with the voltammetric studies (see Fig.2).

3.4. Detection of alanine aminotransferase

Graphite electrode modified with polymeric film, containing PyO, was applied for detection of ALT, using 4-aminoantipyrine as indicator of enzymatic reaction (Fig. 4). The biocatalytic scheme to evaluate ALT is illustrated in Fig. 4. The enzyme ALT, in the presence of L-alanine and α -ketoglutarate, produces pyruvate, which is a substrate for the second enzyme, pyruvate oxidase, producing H_2O_2 . The peroxide oxidizes 4-APP_{red} and leads to a decrease in oxidation current. The quantity of 4-APP_{ox} electrochemically produced is inversely proportional to the amount of ALT. The system proposed is compatible with the results obtained in Fig. 5.

Linear voltammetry (Fig. 5A) of graphite electrode modified with poly(4-aminophenol)/PyO showed that the presence of ALT causes decrease in the oxidation current of 4-APP_{red}. This decrease is associated with the consumption of 4-APP_{red} by competitive chemical reaction with H₂O₂, decreasing the availability of 4-APP_{red} to the oxidation in the electrode and, consequently, decreasing the oxidation current. These results are consistent with the amperometric response obtained in the presence or absence of ALT (Fig. 5B), where a decrease in the charge values was obtained for the bioelectrode in the presence of ALT (5.65C) compared with the bioelectrode in the absence of ALT (6.19 C).

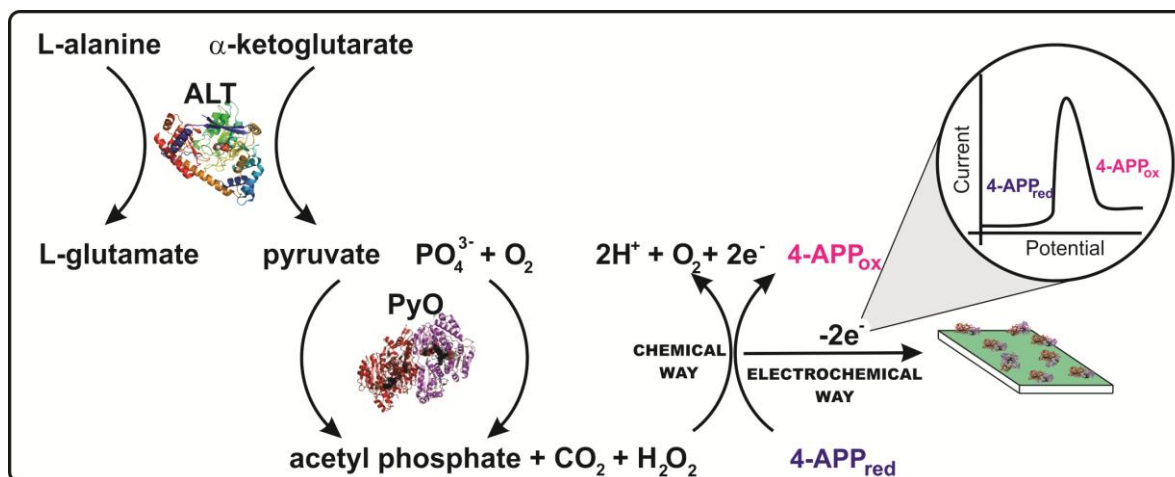


Figure 4. Schematic diagram displaying the enzyme and electrode reactions involved in the ALT activity onto graphite electrode modified with poly(4-aminophenol). PyO: pyruvate oxidase, ALT: alanine aminotransferase; 4-APP: 4-aminoantipyrine.

Also, it was determined by chronoamperometry that the response time was less than 200s when the current was stable (Figure 5B).

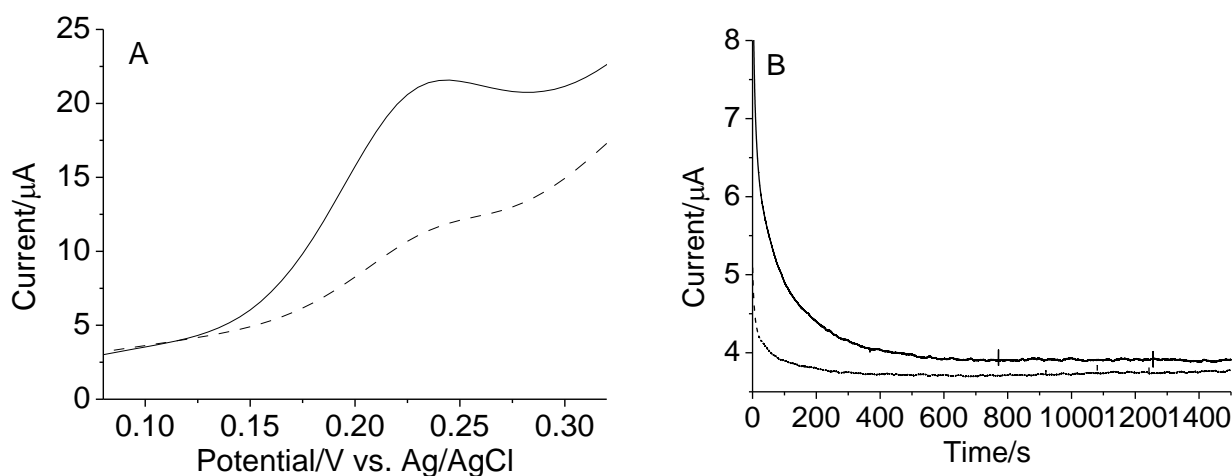


Figure 5. Alanine aminotransferase (ALT) detection based on graphite electrode modified with poly(4-aminophenol) using 4-aminoantipyrine as electrochemical indicator. (A) Linear voltammogram, (B) Amperometric response at +0.24V. Absence of ALT (—) and presence of ALT (---). All detection was done in phosphate buffer, pH 7.4.

3.5. Sensitivity of bioelectrode for ALT

The bioelectrode was evaluated using samples of ALT prepared in buffer phosphate, monitoring 4-APP_{red} through linear voltammetry (Fig. 6A). Fig. 6B shows the oxidation charge of 4-APP_{red} in function of the variation of ALT quantity.

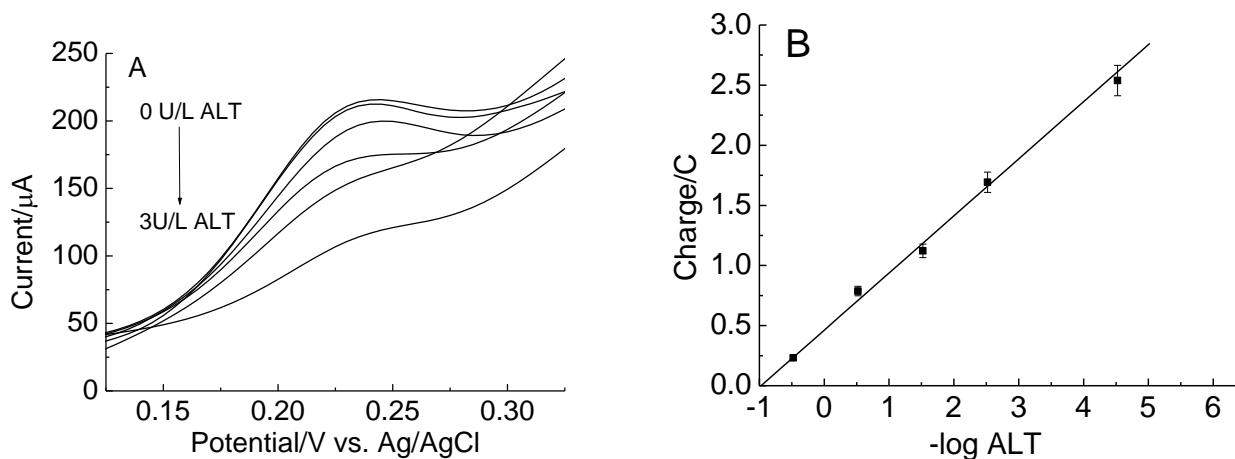


Figure 6. (A) Linear voltammograms of bioelectrode for alanine aminotransferase (ALT), based on pyruvate oxidase immobilized on graphite electrode modified with poly (4-aminophenol) and 4-aminoantipyrine as electrochemical indicator, in absence or presence of different concentrations of alanine aminotransferase. (B) Calibration curves of bioelectrode for ALT. Electrolyte: phosphate buffer, pH 7.4.

The sensitivity, determined from the semi-logarithmic plot, was 2.68×10^{-5} U/L for ALT, in linear range from 3.0×10^{-5} U/L to 3.0 U/L (correlation coefficient $r = 0.998$). The normal concentrations of ALT in the blood are from 5 to 35 U/L and ALT levels >50 times the normal level indicate hepatic discords. After severe damage, ALT can reach higher levels (up to 50 times greater than normal). The bioelectrode proposed in these study presents the advantage of using low blood volume, where the plasma solution containing ALT should be diluted about 1000-fold for the analysis, since that the bioelectrode presents linear range from 0.0003U/L to 3U/L.

3.6. Interference studies

In analysis of biological fluids, background signals due to physiological levels of electroactive species, such as ascorbic acid and uric acid, create selectivity challenges [25]. Effect of some common interfering substances in ALT determination, such as uric acid, glutamate, glucose, ascorbic acid in the response of the bioelectrode has been studied (Fig. 7). The experiment was conducted in triplicate.

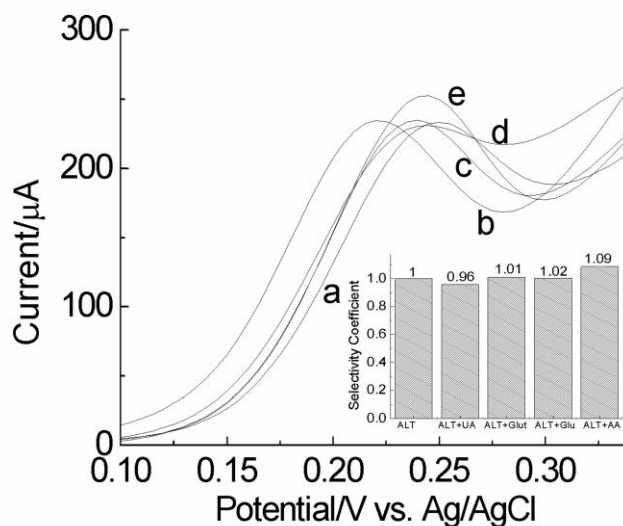


Figure 7. Linear voltammograms of alanine aminotransferase detection, in absence (a) or presence of the interfering substances: uric acid, 1mg/dL (b), glutamate, 1m mol.L⁻¹ (c), glucose, 1m mol.L⁻¹ (d) ascorbic acid, 3.6 mg/dL (e). Electrolyte: phosphate buffer (0.1 mol.L⁻¹), pH 7.4, 100 V/s. Inset: selectivity coefficient of bioelectrode.

Selectivity coefficient (SC) of the bioelectrode for each interferent was calculated using the equation, $SC = I_{c+i}/I_c$, where I_{c+i} and I_c are the bioelectrode response for ALT (0.003U/L) in the presence and absence of each interferent, respectively [42]. Results obtained indicate that the response of the bioelectrode is not significantly affected in the presence of these interfering substances, indicating high selectivity towards the determination of ALT.

3.7. Bioelectrode stability

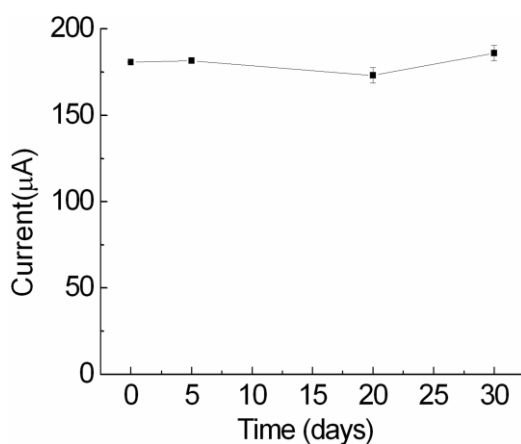


Figure 8. Operational stability of the bioelectrode to alanine aminotransferase detection.

The storage stability of bioelectrodes is a critical feature in the context of potential pharmaceutical and industrial applications [43]. Fig. 8 depicts the stability of the bioelectrode for ALT, stored in dry state. The experiment was conducted in triplicate.

Figure 8 indicates that the bioelectrode response was still 97% of the initial value after 30 days of storage (8°C). This result was considered as an indication that the microenvironment of the modified electrode is a stable platform for PyO immobilization, preventing its leaching and preserving its stability and biological activity.

4. CONCLUSIONS

The present report describes the development of a new bioelectrode to alanine aminotransferase detection, obtained by immobilization of pyruvate oxidase onto poly(4-aminophenol). Cyclic voltammetry, using as indicators the 4-aminoantipyrine or $K_3Fe(CN)_6/K_4Fe(CN)_6$, and AFM images confirmed the modification of the surface, after immobilization of the pyruvate oxidase. The graphite electrode modified with poly(4-aminophenol) showed a favorable effect onto the bioactivity of the immobilized pyruvate oxidase in a 30-days storage. This bioelectrode was evaluated to alanine aminotransferase detection, presenting fast response, high selectivity, linear range from 3.0×10^{-5} U/L to 3.0 U/L and detection limit of 2.68×10^{-5} U/L.

The combination of enzymatic assay for ALT and modified electrode with poly(4-aminophenol) showed to be a promising approach towards the development of a diagnosis kit for hepatic diseases, based on electrochemical detection.

ACKNOWLEDGMENTS

The authors are grateful for the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

1. E. Vezali, A. Aghemo, M. Colombo, *Clin. Therap.*, 32 (2010) 2117.
2. G.S. Xuan, S.W. Oh, E.Y. Choi, *Biosens. Bioelectron.*, 19 (2003) 365.
3. G. Li, J.M. Liao, G.Q. Hu, N.Z. Ma, P.J. Wu, *Biosens. Bioelectron.*, 20 (2005) 2140.
4. T. J. Liang, M.G. Ghany, *N. Engl. J. Med.*, 368 (2013) 1907.
5. T. Poynard, Y. Ngo, M. Munteanu, D. Thabut, V. Ratziu, *Curr. Hepat. Rep.*, 10 (2011) 87.
6. H. Kuroda, K. Kakisaka, Y. Tatemichi, K. Sawara, Y. Miyamoto, K. Oikawa, A. Miyasaka, Y. Takikawa, T. Masuda, Suzuki K, *Hepatogastroenterology*, 57 (2010) 1203.
7. B. Rietz, G.G. Guilbault. *Anal. Chim. Acta*, 77 (1975) 191.
8. S. Reitman, S. Frankel, *Am. J. Clin. Pathol.*, 28 (1957) 56.
9. A. Karmen, *J. Clin. Invest.*, 34 (1955) 131.
10. L. Adolph, R. Lorenz, S. Karger, *Enzyme Diagnosis in Diseases of the Heart, Liver, and Pancreas*. Pub, Basel, Switzerland, 1982.
11. K. Jung, D. Mildner, B. Jacob, D. Scholz, K. Precht, *Clin. Chim. Acta*, 115 (1998) 1105.

12. F. Wroblewski, *Adv. Clin. Chem.*, 1 (1958) 313.
13. D.R. Dafour, J.A. Lott, F.S. Nolte, D.R. Gretch, R.S. Koff, L.B. Seeff, *Clin. Chem.*, 46 (2000) 2027.
14. C.H. Saely, A. Vonbank, P. Rein, M. Woess, S. Beer, S. Aczel, V. Jankovic, C. Boehnel, L. Risch, H. Hexel, *Clin. Chim. Acta* 397 (2008) 82.
15. W. Goessling, J.M. Massaro, R.S. Vasan, R.B. D'Agostino, R.C. Ellison, C.S. Fox, *Gastroenterology.*, 135 (2008) 1935.
16. C-C. Wang, W-W. Wu, C-S. Hsu, P-C. Wang, H.H. Lin, J-H. Kao, Tzu Chi, *Med. J.*, 20 (2008) 275.
17. S-Y. Oh, Y-K. Cho, M-S. Kang, T-W. Yoo, J-H. Park, H-J. Kim, C-I. Sohn, W-K. Jeon, B-I. Kim, B-H. Son, J-H. Shin, *Metabol. Clin. Experiment*, 55 (2006) 1604.
18. X.J. Huang, Y.K. Choi, H.S. Im, O. Yarimaga, E. Yoon, H.S. Kim, *Sensors* 6 (2006) 756.
19. K.W. Plaxco, H.T. Soh, *Trends Biotechnol.*, 29 (2011)1.
20. S. Reitman, S. Frankel. *Am. J. Clin. Pathol.*, 28 (1957) 56.
21. H.U. Bergmeyer, *Clin. Chim. Acta*, 105 (1980) 147.
22. Y.N. He, H.Y. Chen, *Anal. Chim. Acta*, 353 (1997) 319.
23. K-S. Chang, C-K. Changa, S-F. Chou, C-Y. Chen, *Biosens. Bioelectron.*, 22 (2007) 2914.
24. S. Pan, M.A. Arnold, *Talanta*, 43 (1996) 1157.
25. B.C. Ye, Q.S. Li, Y.R. Li, X.B. Li, J.T. Yu, *J. Biotechnol.*, 42 (1995) 45.
26. W.H. Oldenzien, B.H.C. Westerink, *Anal. Chem.*, 77 (2005) 5520.
27. M. Jamal, O. Worsfold, T. McCormac, E.M. Dempsey, *Biosens. Bioelectron.*, 24 (2009) 2926.
28. M-J. Song, D-H. Yun, N-K. Min, S-I. Hong, *J. Biosci. Bioeng.*, 103 (2007) 32.
29. S. Suman, R. Singhal, A.L. Sharma, B.D. Malthotra, C.S. Pundir, *Sens. Actuators B*, 107 (2005) 768.
30. A. Zhu, R. Romero, R.P. Howard, *Anal. Biochem.*, 396 (2010) 146.
31. A.G. Brito-Madurro, L.F. Ferreira, S.N. Vieira, L.R. Goulart Filho, J.M. Madurro, *J. Mater. Sci.*, 42 (2007) 3238.
32. D.L. Franco, A. S. Afonso, L.F. Ferreira, R.A. Gonçalves, J.F.C. Boodts, A.G.B. Madurro, J.M. Madurro, *Polym. Eng. Sci.*, 48 (2008) 2043.
33. A.S. Afonso, L.R. Goulart, I.M.B. Goulart, A.E.H. Machado, J.M. Madurro, A.G. Brito-Madurro, *J. Appl. Pol. Sci.*, 118 (2010) 2921.
34. S.N. Vieira, A.S. Afonso, A.G.B. Madurro, L.F. Ferreira, R.G. Ariza, L.R. Goulart Filho, J.M. Madurro, *Macromol. Symp.*, 245-246 (2006) 236.
35. T.V.S. Santos, R.R. Teixeira, D.L. Franco, J.M. Madurro, A.G. Brito-Madurro, F.S. Espindola. *Mater. Sci. Eng., C*, 32 (2012) 530.
36. B. Sedewitz, K.H. Schleifer, F. Götz, *J. Bacteriol.*, 160 (1984) 273.
37. X. Hu, J. Yang, C. Yang, J. Zhang, *Chem. Eng. J.*, 161 (2010) 68.
38. T.D. Gibson, *Analysis*, 27 (1999) 630.
39. J. Rubio-Retama, E. Lopez-Cabarcos, B. Lopez-Ruiz, *Talanta*, 2005 (68) 99.
40. R.P. Janek, W.R. Fawcett, A. Ulman, *Langmuir*, 14 (1998) 3011.
41. M. Steichen, T. Doneux, C. Buess-Herman, *Electrochim. Acta*, 53 (2008) 6202.
42. U. Saxena, M. Chakraborty, P. Goswami, *Biosens. Bioelectron.*, 26 (2011) 3037.
43. J. Rubio-Retama, E. López-Cabarcos, B. López-Ruiz, *Talanta*, 68 (2005) 99.