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Enzymatic rotating biosensor for cysteine and glutathione determination in a FIA system

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Abstract

The high sensitivity that can be attained using an enzymatic system and mediated by catechols has been verified by on-line interfacing of a rotating biosensor and continuous flow/stopped-flow/continuous-flow processing. Horseradish peroxidase, HRP, [EC 1.11.1.7], immobilized on a rotating disk, in presence of hydrogen peroxide catalyzed the oxidation of catechols, whose back electrochemical reduction was detected on glassy carbon electrode surface at −150 mV. Thus, when L-cysteine (Cys) or glutathione (GSH) was added to the solution, these thiol-containing compounds participate in Michael addition reactions with catechols to form the corresponding thioquinone derivatives, decreasing the peak current obtained proportionally to the increase of its concentration. Cys was used as the model thiol-containing compound for the study. The highest response for Cys was obtained around pH 7. This method could be used to determine Cys concentration in the range 0.05–90 μM ($r = 0.998$) and GSH concentration in the range 0.04–90 μM ($r = 0.999$). The determination of Cys and GSH were possible with a limit of detection of 0.7 and 0.3 nM, respectively, in the processing of as many as 25 samples per hour. Current response of the HRP-rotating biosensor is not affected by the oxidized form of GSH and Cys (glutathione disulfide, GSSG, and L-cystine, respectively), by sulfur-containing and alkyl-amino compounds such as methionine and lysine, respectively. The interferences from easily oxidizable species such as ascorbic acid and uric acid are lowest.

Keywords: Cysteine; glutathione; Glassy carbon; Biosensor; Peroxidase; Catechol; FIA

1. Introduction

Thiol-containing compounds such as cysteine (Cys) and glutathione (GSH, reduced form) play an important role in many biochemical processes. Cys is an important thio-containing compound and involved in a variety of important cellular functions, such as protein synthesis, detoxification and metabolism, etc. Disorders of Cys metabolism include cystinosis, an autosomal recessive disease produced by a defect in lysosomal transport and cystinuria, a common heritable disorder of amino acids transport. More and more researches have been focused on the determination of Cys in pharmaceuticals, urine, serum and plasma, since altered levels of Cys have been implicated in hyperhomocysteunemia and in a number of pathological conditions, including Alzheimer's and Parkinson's disease as well as autoimmune deficiency syndrome. Furthermore, plasma thiol groups have been shown to be susceptible to oxidative damage and, therefore, can serve as diagnostic markers for cardiovascular diseases [1]. Studies have also indicated that low GSH concentration in HIV-infected patients may contribute to their immune deficiency [2,3]. Therefore, the sensitive determination of Cys and GSH in biological matrices are highly desirable.

Many analytical methodologies have been proposed for the determination of thiols taking into account its chemical role, including spectrophotometry [4–9], mass spectrometry [10,11], fluorimetry [12,13], gas chromatography [14], chemiluminiscence [15–19], and electrochemistry.

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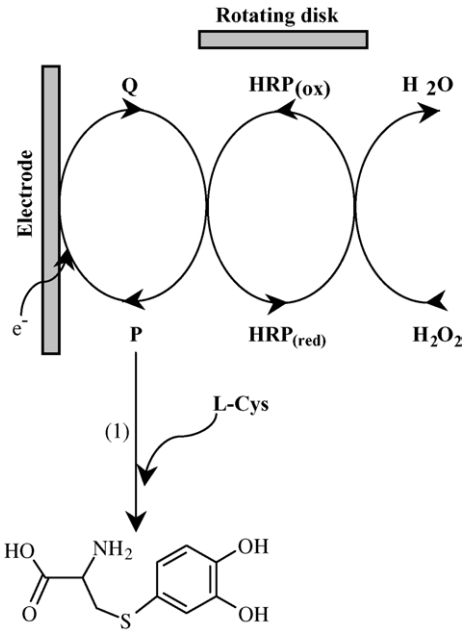


Scheme 1.

Whilst the application of chromatographic [20–24] or electrophoretic [25–27] separation will ultimately provide superior resolution, sample preparation and calibration is a non trivial task [28] and, as such, there is considerable merit in the potential simplicity offered by the direct application of electrochemical techniques [29–34]. Numerous strategies have been developed to alleviate some of the problems that afflict both the selectivity and sensitivity of electroanalytical procedures. Cathodic stripping techniques based upon the accumulation and subsequent reduction of mercury–thiol complexes at mercury electrodes have been extensively studied [35–43] and can often provide significant enhancements in sensitivity. The oxidation of thiols at solid electrodes has found fewer advocates but increasing interest in electrocatalysts such as phtalocyanine derivatives [44–48], and organic mediators [49,50]. A less labor-intensive way for thiol measurement is the sensor method. A variety of amperometric electrodes systems for thiol detection has been described [51,52] (and references cites herein). These systems generally do not require extensive sample preparation and have fast response.

The measuring principle of this biosensor for the detection of thiol compounds is shown in Scheme 1. Horseradish peroxidase (HRP) in the presence of H_2O_2 catalyses the oxidation of catechols (Q) [53] whose electrochemical reduction back was obtained at peak potential of −150 mV. Nevertheless, when thiol-containing compound is added to the solution, readily undergo reaction with quinone derivative (P), through the Michael addition, decreasing the peak current obtained proportionally to the increase of thiol-containing compound concentration.

The initial reaction in the sequence (Q ⇄ P) is well established [54–56] with NMR, pulse radiolysis [54] and a number of electrochemical techniques [56] used to probe the mechanism. A substantial body of research has also been compiled which documents the possible physiological consequences of such reactions [57,58]. The potential analytical utility offered by the second step (1) as a method of detecting Cys and GSH is explored in this paper.

2. Experimental

2.1. Reagents and solutions

All reagents used, were of analytical reagent grade. The enzyme horseradish peroxidase, HRP, [EC 1.11.1.7] Grade II, were purchased from Sigma Chemical Co., St. Louis. The concentration of HRP was determined spectrophotometrically using the Soret extinction coefficient of 102 mM^{-1} cm^{-1} at 403 nm (181 IU mg^{-1}). Glutaraldehyde (25% aqueous solution) and hydrogen peroxide were purchased from Merck, Darmstadt. 3-Aminopropyl-modified controlled-pore glass, 1400 Å mean pore diameter and 24 m^2 mg^{-1} surface area, was from Electro-Nucleonics (Fairfield, NJ) and contained 48.2 μmol g^{-1} of amino groups. Hydroquinone, catechol, 4-*tert*-butylcatechol, glutathione (reduced, GSH and oxidized, GSSG), cysteine, cystine, methionine, lysine, ascorbic acid, uric acid were purchased from Sigma Chemical Co., St. Louis. Hidroquinone (0.1 M), catechol (0.1 M), 4-*tert*-butylcatechol (0.1 M), glutathione (0.01 M) cysteine (0.01 M), cystine (0.001 M), methionine (0.005 M), lysine (0.005 M), ascorbic acid (0.01 M), uric acid (0.1 M) were prepared through the dissolution of the appropriate salt in deionized water and generally used within 1 h. All other reagents employed were of analytical grade and used without further purifications. All solutions were prepared with ultra-high-quality water obtained from a Barnstead Easy pure RF compact ultra pure water system, and the samples were diluted to the desired concentrations using a 10 ml Metrohm E 485 burette.

2.2. Flow-through sensor/detector unit

The main body of the cell was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the rotating enzyme biosensor and the detector system. Glassy carbon electrode (GCE) is on the top of the rotating biosensor. The rotating biosensor is a disk of Teflon in which a miniature magnetic stirring bar (Teflon-coated Micro Stir bar from Markson Science, Inc., Phoenix, AZ) has been embedded. Typically, a sensor disk carried 1.4 mg of controlled-pore glass on its surface. Rotation of the lower sensor was effected with a laboratory magnetic stirrer (Metrohm E649 from Metrohm AG Herisau, Switzerland) and controlled with a variable transformer with an output between 0 and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina.). Amperometric detection was performed using a BAS LC-4C potentiostat and BAS 100 B/W (electrochemical

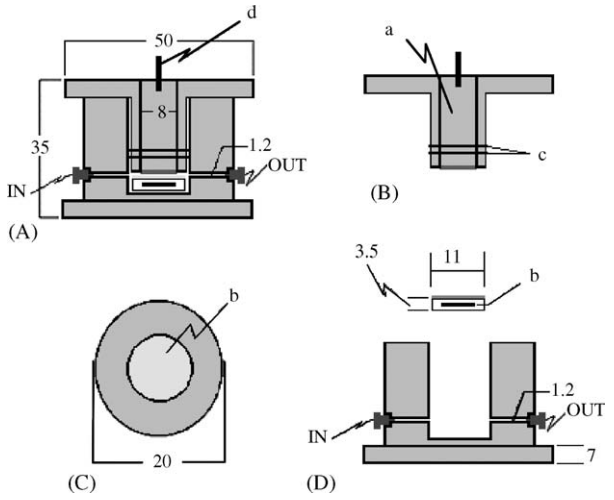


Fig. 1. Schematic representation of components in the biosensor flow cell. (A) Assembled sensor, (B) upper cell body, (C) top view of lower cell body; (b) rotating biosensor (with immobilized HRP), (D) lower cell body; (a) glassy carbon electrode; (b) rotating biosensor; (c) O-ring; (d) electrical connection. All measurements are given in millimeters.

analyzer Bioanalytical System, West Lafayette IN) was used to voltammetric determinations. The potential applied to the GCE for the functional group detection was −150 mV versus Ag/AgCl, 3.0 M NaCl reference electrode BAS RE-6, and a Pt wire counter electrode. At this potential, a catalytic current was well established.

A pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc., Middleton, WI) was used for pumping, sample introduction, and stopping of the flow. Fig. 2 illustrates schematically the components of the single-line continuous-flow setup. The pump tubing was Tygon (Fisher AccuRated, 1.0 mm i.d., Fisher Scientific Co., Pittsburgh, PA) and the remaining tubing used was Teflon, 1.00 mm i.d. from Cole-Parmer (Chicago, IL).

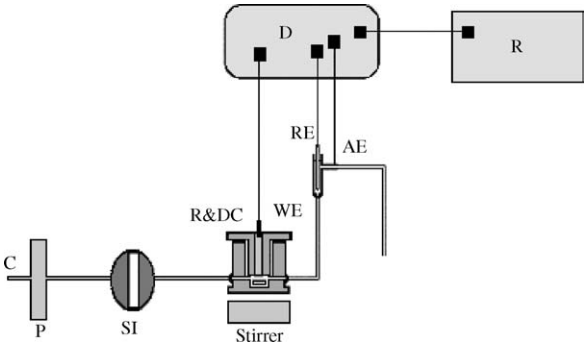


Fig. 2. Block diagram of the continuous-flow system and detection arrangement. P: pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc., Middleton, WI); C: carrier buffer line; SI: sample injection; W: waste line; R&DC: sensor and detector cell; WE: glassy carbon electrode; RE: reference electrode (Ag/AgCl, 3.0 M NaCl); AE: auxiliary electrode (platinum); D: potentiostat/detection unit (CV27, Bioanalytical Systems, West Lafayette, IN); R: recorder (Varian, Model 9176, Varian Techtron, Springuale, Australia).

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc., Cambridge, MA). This pH-meter was calibrated with two buffers: biphthalate buffers, prepared by dissolving 2.53 g of potassium biphthalate in 250.0 ml of deionized water for pH 4.0 and tetraborate buffer, prepared by dissolving 0.95 g of sodium tetraborate in 250.0 ml of deionized water for pH 9.0.

2.3. Horseradish peroxidase immobilization

The rotating disk biosensor (bottom part) was prepared by immobilizing HRP on 3-aminopropyl-modified controlled-pore glass (APCPG). The APCPG, smoothly spread on one side of a double-coated tape affixed to the disk surface, and was allowed to react with an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washing with purified water and 0.10 M phosphate buffer of pH 7.00, the enzyme (10.0 mg of enzyme preparation in 0.50 ml of 0.10 M phosphate buffer, pH 7.00) was coupled to the residual aldehyde groups in phosphate buffer (0.10 M, pH 7.00) overnight at 5 °C. The immobilized enzyme preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C between uses. The immobilized HRP preparations were perfectly stable for at least one months of daily use.

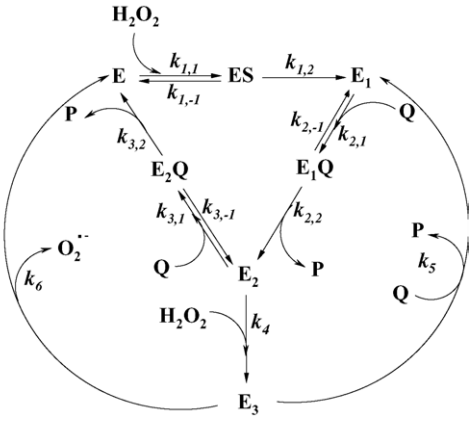
3. Results and discussion

3.1. Broad features of the amperometric detection of HRP in the presence of peroxide

Reactions catalyzed by enzymes have long been used for analytical purposes in the determination of different analytes, such as substrates, inhibitors and also the enzymes. Biosensors, which combine the selectivity of enzymes with the high sensitivity of electrochemical measurements, provide an excellent tool for analytical chemistry [59].

The mechanisms of HRP catalyzed reactions can be represented as follows [60].

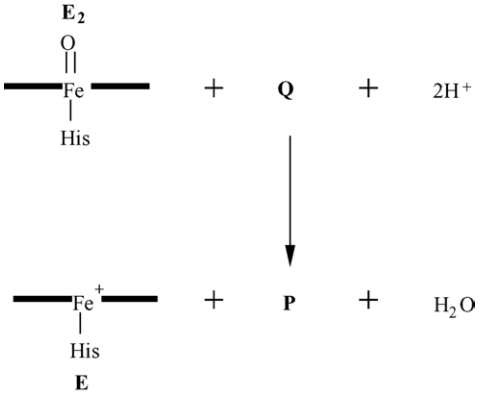
In the primary catalytic cycle of HRP (Scheme 2), the kinetics of the reaction of E with H_2O_2 to yield E_1 has been investigated extensively [61,62]. Although evidence has been previously gathered that the kinetics follows a Michaelis–Menten behavior [63], it is only recently that its characteristics have been unambiguously determined [64], leading to $K_{1,M} = (k_{1,-1} + k_{1,2})/k_{1,1})$ 128 μM and to a confirmation of the k_1 value, i.e., $k_1 = k_{1,1}k_{1,2}/(k_{1,-1} + k_{1,2}) = 1.7 \times 10^7\ M^{-1}\ s^{-1}$. The reduction of E_1 and E_2 by several electron donors has been reported, where they are in most cases both electron and proton donors. Scheme 2 indicates the possibility of a Michaelis–Menten behavior also for the reduction of E_1 and E_2 in view of the fact that such behavior has been reported for several cosubstrates [64].



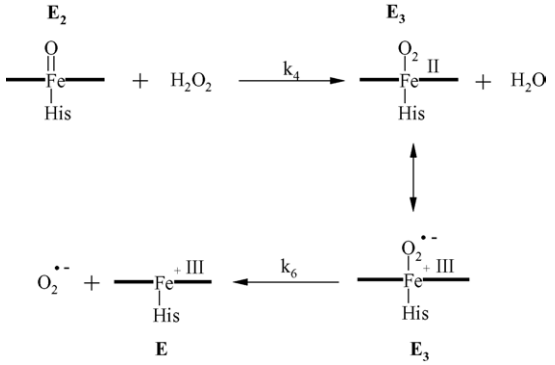
Scheme 2.

Concerning the Michaelis–Menten behavior observed for the E_2/E reaction, it should be emphasized that the reduction of E_2 is not a mere outersphere electron-transfer reaction but rather involves the exchange of one electron and two protons and the cleavage of the iron–oxygen bond (Scheme 3). These reactions, or maybe other mechanistic peculiarities to be uncovered, might be the cause of the observed kinetics showing saturation behavior upon increasing the reactant concentration, which therefore does not necessarily reflect a true Michaelis–Menten mechanism such as the one depicted in Scheme 2.

Inhibition by conversion of the initial enzyme by H_2O_2 into inactive oxyperoxidase, E_3, may occur even in the presence of the oxidized form of the cosubstrate. H_2O_2 may indeed reduce E_1 into E_2, albeit slowly [61], thus opening a route to the conversion of E_2 into E_3. Two pathways for this inactivation have been previously identified. One is an irreversible set of reactions finally yielding a verdohemoprotein (also designated as P670) [65]. This irreversible inactivation pathway of HRP is insignificant under our experimental conditions. The second pathway involves the formation of oxyperoxidase [66], usually designated as compound III or E_3. This compound, which does not normally participate in the peroxidase activity of HRP, has a structure similar to that of oxyhemoglobin [67] (Scheme 4). In the presence of



Scheme 3.



Scheme 4.

H_2O_2, the formation of E_3 from the reaction of H_2O_2 with E_2 occurs with a rate constant k_4 ranging from 16 to 40 $M^{-1}\,s^{-1}$, depending on pH and temperature [68]. E_3 is not necessarily a dead end to the catalytic cycle of HRP. It is indeed converted back to E by spontaneous decomposition, yielding superoxide ion.

This fact can be observed easily in experimental form varying H_2O_2 concentration from 2.5×10^{-5} M to 5.0×10^{-3} M, for 20 μM Q solutions and several concentrations of HRP, maintaining constant the 4-TBC concentration (Fig. 3). Low H_2O_2 concentration, 0.025 mM, a lineal relation can be seen only when the enzymes concentrations are low, losing this linearity as increases the enzymatic concentration. That is observed because this H_2O_2 concentration is insufficient to generate maximum catalytic activity. To 0.1 mM H_2O_2 concentration, a perfect linearity in all concentrations range studied is obtained. To 0.5 mM H_2O_2 concentration, linearity is lost to low concentrations. That is because the HRP is inactivated in excess of H_2O_2. At higher H_2O_2 concentration, 5 mM, inhibition by conversion of the initial

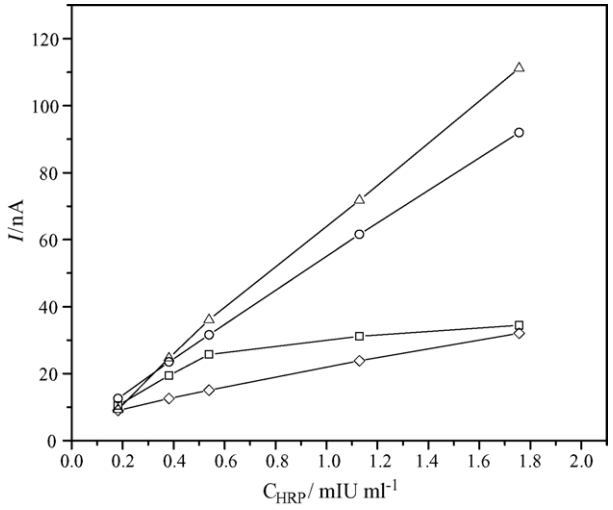


Fig. 3. Catalytic current as a function of the HRP concentration. Cell volume: 200 μl; flow rate: 1 ml min^{-1}; potential: −150 mV vs. Ag/AgCl 3 M NaCl in a phosphate buffer (pH 7.00) containing 1.2 μM 4-TBC, and 0.025, 0.1 (○), 0.5 (△), and 5 (◊) mM H_2O_2.

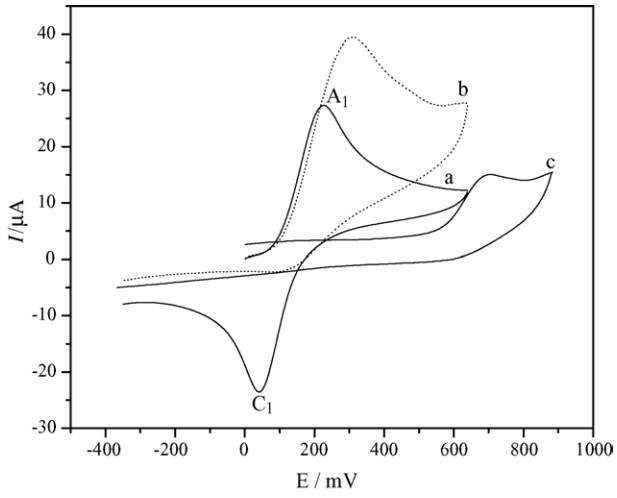


Fig. 4. Cyclic voltammograms of 1 mM 4-TBC: (a) in the absence; (b) in the presence of 2.5 mM Cys; and (c) 1 mM Cys in the absence of 4-TBC, at glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.1 M phosphate buffer (pH 7.00). Scan rate: 100 mV s^{-1}; T: 25 ± 1 °C.

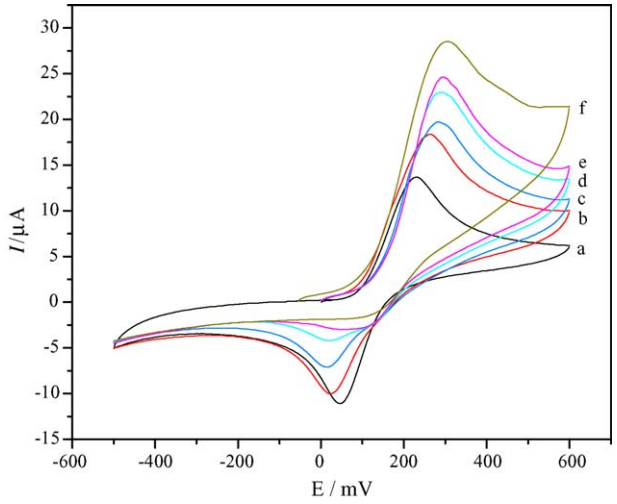


Fig. 5. Typical voltammograms of 0.5 mM 4-TBC at a glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.1 M phosphate buffer (pH 7.00) at various Cys concentrations, C_{Cys}: (a) 0.0, (b) 0.09, (c) 0.17, (d) 0.29, (e) 0.37 and (f) 0.67 mM; scan rate: 100 mV s^{-1}, T: 25 ± 1 °C.

enzyme by H_2O_2 into E_3 is observed in all HRP concentration range studied. In this case linearity is observed but the catalytic current obtained is less significant than in the optimal case.

3.2. Electrooxidation of 4-tert-butylcatechol in the absence and presence of L-Cys

In blood and serum, most of the free sulfhydryl groups reside in compounds such as the reduced form of GSH, Cys, and homocysteine [69]. Cys is used as the model compound although its concentration in blood serum is 20–50 times lesser (in μM range) than GSH (1–3 mM). Therefore, Cys concentration is more critical for determination.

Cyclic voltammetry of a 1 mM solution of 4-*tert*-butylcatechol (4-TBC) in an aqueous solution containing 0.10 M phosphate buffer pH 7.0 as supporting electrolyte, shows one anodic (A_1) and a corresponding cathodic peak (C_1) which corresponds to the transformation of 4-TBC to *o*-benzoquinone and vice-versa within a quasi-reversible two-electron process (Fig. 4, curve a). A peak current ratio ($I_p^{C_1}/I_p^{A_1}$) of nearly unity, particularly during the repetitive recycling of potential, can be considered as a criterion for the stability of *o*-quinone produced at the surface of the electrode under the experimental conditions. In other words, any hydroxylation [70–73] or dimerization [74,75] reactions are too slow to be observed on the time scale of cyclic voltammetry. The oxidation of 4-TBC in the presence of L-Cys as a nucleophile was also studied. Fig. 4, curve b, shows the cyclic voltammogram obtained for a 1 mM solution of 4-TBC in the presence of 1 mM L-Cys. The voltammogram exhibits an anodic peak at 220 mV versus Ag/AgCl 3 M NaCl, and the cathodic counterpart of the anodic peak A_1 has disappeared.

The influence of increasing Cys concentration on the electrochemical behavior of 4-TBC was investigated and the responses recorded are shown in Fig. 5. The height of the oxidation peak was found to increase with increasing additions of L-Cys with the loss of the corresponding reduction peak consistent with the ECE type mechanism proposed in Scheme 1. The increase in the oxidation peak height is attributed to the oxidation of 4-TBC–cysteine adducts that have arisen through the electrochemically initiated reaction previously detailed in Scheme 1. The successive decrease in the height of the 4-TBC reduction peak can be ascribed to the fact that increasing concentration of Cys serve to scavenge the oxidized form of 4-TBC such that on the reverse sweep there is little available for electro-reduction.

Given that the direct oxidation of this thiol at the electrode does not occur within the potential window studied (Fig. 4, curve c), the increase in the magnitude of the 4-TBC oxidation peak can be attributed solely to the re-oxidation of the 4-TBC–Cys adduct.

The influence of pH on peak potential (E_p) of the reaction was assessed through examining the electrode response to 4-TBC–Cys obtained in solutions buffered between pH 4 and 8. The position of the redox couple was found to be dependent upon pH with a shift of 61 mV pH^{-1} indicative of an n electron n proton behavior with n likely to be two [76]. A quantitative evaluation of the 4-TBC change peak current (ΔI) response to increasing additions of Cys as a function of solution pH is highlighted in Table 1. The ΔI reported hereafter is the difference between the reduction current (from addition of 4-TBC) and the current due to the addition of Cys. The response was found to decrease steadily as the acidity of the solution is increased. This can be attributed to the fact that as the solution pH is lowered, the thiol functionality will be increasingly protonated (Cys RSH, pK_a ~8.4) and hence the nucleophilic character of the thiol moiety diminished. Increasing the pH clearly improves the response but an operational limit is reached once neutral

Table 1
Influence of pH on the magnitude of the oxidation signal for 1.00 mM 4-TBC in the presence of several Cys concentrations

Cysteine added (μM)	ΔI (μA) ± S.D.			
	pH 4.00	pH 5.00	pH 6.00	pH 7.00
0	0	0	0	0
20	3.46 ± 0.02	5.59 ± 0.01	8.50 ± 0.01	10.85 ± 0.01
40	5.95 ± 0.02	11.44 ± 0.01	16.73 ± 0.02	21.67 ± 0.02
60	8.69 ± 0.03	17.65 ± 0.02	24.99 ± 0.01	33.73 ± 0.01
80	10.75 ± 0.01	22.13 ± 0.02	34.14 ± 0.02	44.26 ± 0.03
100	13.17 ± 0.02	26.57 ± 0.03	43.14 ± 0.03	51.81 ± 0.03
120	15.23 ± 0.01	30.73 ± 0.03	49.31 ± 0.03	61.13 ± 0.02
140	17.29 ± 0.01	33.47 ± 0.02	54.82 ± 0.03	68.97 ± 0.03
160	19.35 ± 0.02	36.91 ± 0.03	59.63 ± 0.03	75.58 ± 0.04

conditions prevail. Alkaline solution severely compromises the enzyme stability as well as the response as the increased presence of nucleophilic hydroxyl ions (and amine groups where complex physiological media are employed) compete with the less prevalent thiol. Therefore, the pH value used was 7.00 in 0.1 M phosphate buffer in concordance with the steadier pH of the enzyme.

3.3. Redox indicator selection

The enzymatic generated reaction clearly represents a sensitive and selective method for the determination of sulphydryl thiols but the true strength of the protocol lies in the generic nature of the indicating process. This was highlighted through the examination of a further three derivatives of varying chemical functionality. The compounds investigated are shown in Table 2 along with a summary of their electrochemical properties. While the chemical composition has been varied, each derivative retained the capacity for electrochemical conversion to a quinoid intermediate and hence was generally amenable to reaction with thiol. Changing the chemical composition of the parent indicator species can alter the electrochemical properties of the system and hence the nature of the resulting analytical signal. As can be seen from this table, the 4-TBC increases the technique sensibility, therefore, is the selected compound for this work.

3.4. Effect of biosensor rotation and continuous-flow/stopped-flow operation

The implementation of continuous-flow/stopped-flow programming and the location of two facing independent biosensors (Fig. 1), permits: (a) utilization of relatively low enzyme loading conditions (b) instantaneous operation under high initial rate conditions, (c) easy detection of accumulated products, and (d) reduction of apparent Michaelis–Menten constant, K'_M. A more complete reagent homogenization is achieved, because the cell works as a mixing chamber by facilitating the arrival of substrate at the active sites and the release of products from the same sites. The net result is high values of current (see Table 3). The main advantages of this system are its simplicity, and the ease with which it can be applied to the determination of Cys and GSH at low levels.

If the sensor in the cell is devoid of rotation, there is practically no response. If a rotation of 900 rpm is imposed on the sensor located at the bottom of the cell (with immobilized HRP), the signal is dramatically enlarged. As shown in traces *d* in Fig. 6, if the lower sensor is devoid of rotation, the response is lower because diffusional limitations control the enzyme-catalyzed reaction. The trend indicates that, up to velocities of about 900 rpm, a decrease in the thickness

Table 3
Values of K'_M (apparent Michaelis–Menten constant)

Rotation velocity (rpm)	K'_M (mM)[a]	Linear regression, standard deviation
170	22.97	±0.42
240	16.35	±0.30
420	10.14	±0.70
600	6.42	±0.10
840	3.39	±0.28
900	2.98	±0.68
[b]	280.03	±0.33

Determinated as discussed in the text (temperature 20 ± 1 °C).
[a] Each value of K'_M based on triplicate of six different substrate concentrations.
[b] Estimated free enzyme in solution.

Table 2
Electrochemical detection of Cys and GSH via HRP-rotating biosensor with redox co-substrate: analytical parameters

Compounds investigated	E_{ox} (V)	Cys		GSH	
		Range (μM)	DL[a] (nM)	Range (μM)	DL (nM)
Hidroquinone	0.324	0.10–60	1.6	0.14–70	1.4
Catechol	0.248	0.20–60	2.5	0.15–65	1.9
4-*tert*-Butylcatechol	0.227	0.05–90	0.7	0.04–90	0.3

[a] 95% confidence interval; $n = 6$.

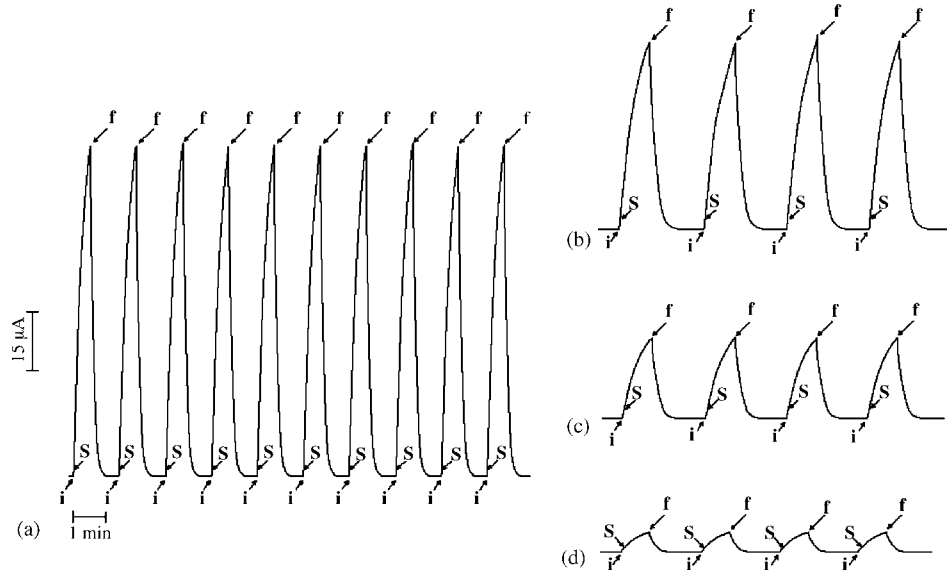


Fig. 6. Effect of sensor rotation under continuous- and stopped-flow conditions. (a) Stopped flow with rotation, (b) continuous flow with rotation, (c) stopped flow without rotation, (d) continuous flow without rotation. The solution containing 0.1 mM H_2O_2 and 1.0 mM 4-TBC in 0.1 M phosphate buffer, pH 7.00. Flow rate: 1.00 ml min^{-1}; cell volume was 200 μl; potential: −150 mV vs. Ag/AgCl 3 M NaCl. The flow was stopped for 60 s during measurement.

of the stagnant layer improves mass transfer to and from the immobilized enzyme active sites. Beyond 900 rpm, the current is constant, and chemical kinetics controls the overall process. As observed earlier [77], although the mass transfer is being realized under conditions similar to a thin-layer bounded diffusion with imposed turbulence, the dependence seems to agree better with the response at a rotating disk electrode. Fig. 6 shows the effect of rotation under continuous and stopped flow conditions. Response to 1.0 mM 4-TBC under continuous flow is relatively small but comparatively larger if the sensor is rotated (compare traces *b* and *c* in Fig. 6). A significant signal that increases almost linearly as time develops when the disk is rotated. Under stopped flow conditions there is a response, but smaller than with rotation. These responses indicate that the utilization of the biocatalytic action of the immobilized enzyme preparations is better under rotation of the sensor at the bottom of the cell.

The current developed at the detector should be directly proportional to the concentration of analyte in the bulk of solution and should increase with increasing rotation velocity. If the flow is stopped when the sample plug transported by continuous flow reaches the center of the sensor, detection take place under conditions similar to those of batch detection [78].

3.5. Effect of cell volume and sample size

Depending on the volume of the cell in contact with the sensors, the overall process becomes controlled by diffusion (large volumes) or by the chemical kinetics of the enzyme-catalyzed reactions (small volumes). The cell volume was changed from 200 μl to 1 ml by removing the O-rings between the upper and lower half of the cell. The measured current, as expected, decreased linearly with an increase in cell volume, due to the dilution effect favored by rotation, and the fact that the measured current is directly proportional to bulk concentration. The smallest cell volume of 200 μl was adopted for further studies.

The measured current increased lineally with sample size up to 200 μl in a cell with a volume of 200 μl. For convenience a sample size of 200 μl was used. Sensitivity is almost tripled in the range between 50 and 200 μl (Table 4).

3.6. Cys measurement with HRP-rotating biosensor

The working potential was selected using the same cyclic voltammogram showed before (Fig. 4a) for the couple P/Q at a GCE in phosphate buffer. For potentials values below −150 mV, the cathodic current became independent of the applied potential; therefore, this value was chosen as working

Table 4
Effect of sample size[a]

Sample size (μl)	*I* (μA)	Linear regression, standard deviation
50	5.82	±0.34
75	14.20	±0.14
100	22.38	±0.19
125	34.31	±0.24
150	52.80	±0.08
175	68.94	±0.31
200	74.60	±0.19
225	74.41	±0.22
250	74.85	±0.32
300	74.51	±0.23

Solution containing 0.1 mM H_2O_2, 1.0 mM 4-TBC in 0.1 M phosphate buffer, pH 7.00; flow rate, 1.00 ml min^{-1}.

[a] The current was measured under stopped-flow conditions. Each value of current is based on triplicate of six determinations.

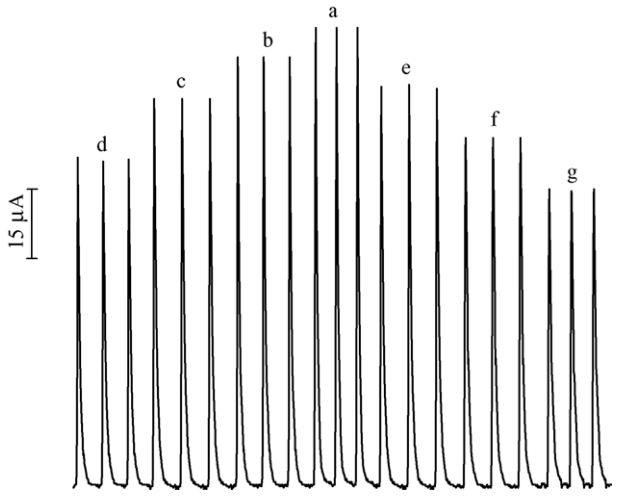


Fig. 7. Response of the HRP-rotating biosensor for Cys determination. (a) 4-TBC 1.0 mM, and H_2O_2 0.1 mM, without addition of Cys solution. From (b–g) is show the response for several Cys concentrations: (b) 13.74, (c) 32.90, (d) 60.85, (e) 26.43, (f) 50.88 and (g) 75.48 μM. Flow rate: 1.00 ml min^{-1}; cell volume was 200 μl; potential: −150 mV vs. Ag/AgCl 3 M NaCl. The Flow was stopped for 60 s During Measurement.

potential. Furthermore, at this potential, a less contribution of the electroactive interferences present in serum is expected.

The performance of the HRP-rotating biosensor for the measurement of reduced thiols concentrations was characterized. For Cys measurement, the following procedure was used: (a) a baseline current was established with the buffer solution; (b) a solution containing 1 mM 4-TBC and 0.1 mM H_2O_2 were injected in the rotating biosensor; (c) the flow was detained and the disk was rotated to 900 rpm, thus, a large reduction current was observed due to the quinone derivative and after 1 min the flow was started again; then (d) a solution containing 1 mM 4-TBC, 0.1 mM H_2O_2, and several Cys concentrations were injected in the rotating biosensor; (e) the flow was detained and the disk was rotated to 900 rpm and the reduction current was measurement. The addition of Cys resulted in a current decrease. After 1 min the flow was started again. A Cys calibration plot was obtained by plotting ΔI versus Cys concentration.

The current versus time profiles obtained with this method of Cys measurement is shown in Fig. 7. The background solution was buffer phosphate pH 7.0. A linear relation (Eq. (1)) was observed between the ΔI and the Cys concentration in the range of 0.05 and 90 μM (rotation 900 rpm).

$$\Delta I\,(\mu A) = -0.024 + 0.474\,[C_{Cys}] \quad (1)$$

The correlation coefficient for this type of plot was typically 0.998. Detection limit (DL) is the minimal difference of concentration that can be distinguished from the signal of the pure 4-TBC solution. The DL was calculated as the amount of Cys required to yield a net peak that was equal to three times the SD of the pure 4-TBC signal. In this study, the minimal difference of concentration of Cys and GSH were, ca. 0.7 and 0.3 nM, respectively. Reproducibility assays were made using repetitive standards solutions ($n = 5$) containing 1.0 mM 4-TBC, 0.1 mM H_2O_2 and 10 μM Cys; the percentage standard error was less than 4%.

The stability of the biosensor was tested for nearly 3 h of continuous use in the FIA system. In this experiment, after every four samples, a standard solutions containing 1.0 mM 4-TBC, 0.1 mM H_2O_2 and 10 μM Cys was injected to test the electrode response. In the FIA system using an enzymatic sensor, there is practically no decay in the catalytic current after eight samples.

The effect of the various compounds on the response of the HRP-rotating biosensor was tested. The oxidized form of Cys and GSH and sulfur-containing compound such as methionine showed little or no effect on the current response of the electrode; similar behavior was observed for lysine and uric acid. The addition of 10 μM ascorbic acid resulted in ca. 4% decrease vs. the reductive current obtained with 1 mM 4-TBC. The data show that the reaction of the quinones with the free SH group is quite fast compared with that with ascorbic acid. Despite the fact that ascorbic acid can also react with the quinones, and because the physiological concentration of ascorbic acid (in plasma) is 40–100 times less than GSH, but is in the same concentration than Cys, we believe that the interference for ascorbic acid is minimal for GSH and can be important for Cys determination. This fact can be avoided for Cys if standard addition method is used instead of calibration curve. To check this fact in the determination of Cys, an experiment using the standard addition method was carried out in the same biosensor system. Different known amounts of Cys were added to the synthetic sample containing a known amount of Cys and ascorbic acid proportion (in the same rate like plasma) prior to testing and injection; from this calibration curve the recovery was 100.4%. Therefore, the interference is avoided on the response to Cys. By the other way, the effect of oxidizing interferents on the response of the HRP-rotating biosensor was tested. For this matter a solution containing 1 mM 4-TBC and 0.1 mM H_2O_2 were injected in the rotating biosensor without immobilized enzyme on the disk surface, the flow was detained and the disk was rotated to 900 rpm, thus, current was not observed due to the quinone derivative and after 1 min the flow was started again. Then, solutions containing 1 mM 4-TBC, 0.1 mM H_2O_2, and several sample concentrations were injected in the rotating biosensor; the flow was detained and the disk was rotated to 900 rpm. The reduction current was not observed. After 1 min the flow was started again. Therefore, oxidizing interferents have not been founded in this kind of sample.

4. Conclusions

The usefulness of enzyme biosensor used for the determination of very low concentrations of Cys and GSH was demonstrated. The biosensor developed in this work is found to be more sensitive than another biosensor previously developed. This type of detection (addition reaction

on co-substrates) shows good promise in biological sensing, particularly in plasma and whole blood samples. Also, this biosensor is able to operate as a fast, selective and sensitive detection unit when is incorporated into a FIA system, and provides a fast and cost effective solution to the realization of quantitative information at extremely low levels of Cys and GSH concentrations.

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