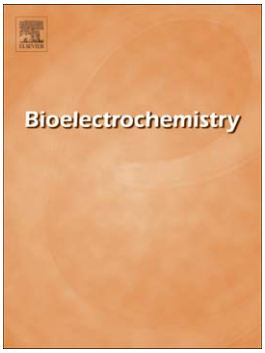

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Amperometric inhibition biosensors based on horseradish peroxidase and gold sononanoparticles immobilized onto different electrodes for cyanide measurements

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Abstract

New biosensors based on inhibition for the detection of cyanide and the comparison of the analytical performances of nine enzyme biosensor designs by using three different electrodes: Sonogel-Carbon, glassy carbon and gold electrodes were discussed. Three different horseradish peroxidase immobilization procedures with and without gold sononanoparticles were studied. The amperometric measurements were performed at an applied potential of −0.15 V vs. Ag/AgCl in 50 mM sodium acetate buffer solution pH = 5.0. The apparent kinetic parameters (K_{mapp}, V_{maxapp}) of immobilized HRP were calculated in the absence of inhibitor (cyanide) by using caffeic acid, hydroquinone, and catechol as substrates. The presence of gold sononanoparticles enhanced the electron transfer reaction and improved the analytical performance of the biosensors. The HRP kinetic interactions reveal non-competitive binding of cyanide with an apparent inhibition constant (K_i) of 2.7 μM and I_{50} of 1.3 μM. The determination of cyanide can be achieved in a dynamic range of 0.1–58.6 μM with a detection limit of 0.03 μM which is lower than those reported by previous studies. Hence this biosensing methodology can be used as a new promising approach for detecting cyanide.

Keywords: cyanide; enzyme biosensor; horseradish peroxidase; Sonogel-Carbon; gold sononanoparticles.

1. Introduction

Cyanide is known to be an extremely poisonous substance and a highly toxic pollutant of surface and ground waters. However, it is widely used, due to its excellent properties, in different applications such as chemical industry (1.5 million tons/year), ores extraction (e.g. gold and silver), and plastics production [1-3]. Cyanide is an ion readily absorbed by living organisms by inhalation, oral and dermal routes of exposure [4]. The main toxic effects of cyanide are attributed to its high affinity toward the iron atoms of haemoproteins such as peroxidase, ferric haemoglobin and myoglobin, as well as catalase. It also binds to the ferric form of cytochrome-c and inhibits the mitochondrial electron-transport chain [2], thus inhibiting the oxygen transference of the main parts of human body such as brain, heart and lungs, and consequently disrupts the mechanism of the whole organism [1]. Therefore, strict environmental regulations are applied to cyanide in waters, e.g. in the UE countries the upper limit for cyanides in drinking water is 50 µg/L [5]. The more restrictive Australian and New Zealand Environmental and Conservation Council water quality criterion of total cyanide in freshwater is 4 µg/L [6], hence requiring highly sensitive procedures for environmental monitoring.

Cyanide determination is of great interest in environmental control and usually it is carried out by using various conventional instrument-based methods, such as titration [7-9], chromatography [10], and atomic absorption spectrometry [11,12]. Although all these methods are currently available for cyanide detection, some of them present complicated and time-consuming protocols and also they can hardly be used for in-situ analysis and/or low-cost screening procedures. On the contrary, these tasks can be successfully accomplished by biosensors.

Electrochemical sensors and biosensors show important advantages: simplicity of analysis, good reproducibility, low-cost, high sensitivity and short response time. Inhibition based enzyme biosensors have been widely developed for the environmental monitoring [13]. Indeed, sensitivity and selectivity of enzymatic biosensors against chemicals can be improved by using inhibition based enzyme detectors [14,15]. Amine et al. [16] developed an inhibition sensor by immobilization of cytochrome oxidase in a carbon paste/lipid matrix with a detection limit of 0.5 µM for cyanide. Gayet et al. [17] demonstrated that properly chosen immobilization methods could considerably improve the inhibitor sensitivity of the immobilized enzyme compared to that of the free enzyme (in solution). Moreover, covalent immobilization of enzyme can modify the protein conformation and, thus, lead to better biomolecule activity and improved stability [18,19]. The use of nanoparticles might also enhance the sensitivity of these biosensors by increasing the binding site on the electrode surface [20]. Indeed, nanoparticles have emerged as nanomaterials that are receiving considerable interest owing to their ability to promote electron transfer between electrodes and the active site of the enzyme because of their large surface-to-volume ratio, high surface reaction activity and strong adsorption ability [21]. Therefore the enzyme immobilization into or onto various nanoparticles including, magnetic nanoparticles [22], gold nanoparticles [23], or platinum nanoparticles [24] have been proposed and reported.

In the present work, gold sononanoparticles (AuSNPs) and horseradish peroxydase (HRP) enzyme-based electrodes are proposed for the first time for inhibitive determination of cyanide. The aim of the research was to develop new inhibition biosensors for detecting cyanide and to compare the performances of several enzyme (HRP) biosensor designs, with and without gold sononanoparticles, deposited onto different kind of electrodes.

2. Experimental

2.1. Reagents and equipment

Sodium acetate buffer solution (50 mM, pH = 5.0) was prepared from sodium acetate (Riedel-de-Haen) and acetic acid (Riedel-de-Haen) using Milli-Q nanopure water (resistivity > 18 MΩ cm, Millipore, Bedford, MA, USA). The sodium acetate buffer was used as supporting electrolyte for all the electrochemical measurements.

Horseradish peroxidase (HRP) (E.C. 1.11.1.7, 274 U/mg), catechol, glutaraldehyde (GA) (25% (v/v) in water) and bovine serum albumin (BSA) used in this work were purchased from Sigma.

Methyltrimethoxysilane (MTMOS) was from Merck (Darmstad, Germany) and caffeic acid from Fluka. Hydroquinone was acquired from Panreac (Barcelona, Spain). All reagents were of analytical grade and used as received without further purification.

The synthesis of the Sonogel-Carbon materials as well as the ultrasonic synthesis of AuSNPs were carried out sonicating with a high power ultrasonic generator, SONICATOR 3000, from MISONIX (MISONIX, Inc. Farmingdale, NY, USA) (equipped with a 13 mm titanium tip), that provides a maximum power of 600 W. Graphite powder natural, high purity-200 Mesh, 99.9999% (metal basis), was from Alfa-Aesar (Johnson Matthey GmbH, Germany). Glass capillary tubes, i.d. 1.15 mm (A = 0.0415 cm^2), were used as the bodies for the composite electrodes.

2.2. Preparation of the biosensors

2.2.1. Glassy carbon (GCE) and gold (AuE) electrodes

The glassy carbon electrode (3 mm diameter) and gold electrode (0.0177 mm diameter) were mechanically polished before each experiment with 0.3 and 0.05 μm alumina powders on a polishing cloth, successively rinsed thoroughly with Milli-Q water and ethanol for 10 min in ultrasonic bath and dried in air. After mechanical cleaning, the gold electrode was subjected to a chemical treatment, by immersion in a 'piranha solution' (98% H_2SO_4 / 30% H_2O_2, 3:1 v/v), and to an electrochemical treatment by cyclic voltammetry, carrying out 20 cycles between -0.4 and +1.5 V at 100 mV s^{-1} until stable scans were recorded. Then, the electrodes were rinsed with Milli-Q water before modification.

2.2.2. Sonogel Carbon (SNGCE) electrode

The Sonogel-Carbon material was prepared using the method described in previous papers [25,26]. A mixture of 500 μL of methyltrimethoxysilane (MTMOS) and 100 μL of 0.1 M HCl was insonated for 10 s. Then, 0.5 g of graphite powder were added to the obtained sonosol, and adequately dispersed. After several minutes, the resulting material acquires enough consistency to filled the glass capillary tubes. 24 h later, the surface of the electrodes could be polished, before modification, with emery paper No. 1200 to remove extra composite material, wiped gently with weighing paper, thoroughly washed with Milli-Q water, and allowed to dry at room temperature. Finally a copper wire was inserted as the electrical contact into the electrodes, being ready to be used.

2.2.3. Synthesis of gold sononanoparticles

The gold sononanoparticles were synthesized following the method described previously [27,28]. Firstly, 1.25 mL of 1.5 mM $KAuCl_4$ aqueous solution was placed in a cylindrical glass vessel and sonicated with a high-power ultrasound generator. After 1.5 min of sonication, 250 μL of 38.8 mM dihydrate sodium citrate aqueous solution were added to the vessel, the solution turning immediately into grey colour. After 4 min of sonication, the colour

of the solution turns into dark red wine, indicating the formation of AuSNPs. The total time of synthesis is 5.5 min. The sample vessel was maintained during the entire process in a water bath at ambient temperature to prevent local heating produced by the sonication. The sononanoparticles obtained by this route, predominantly with spherical projection, are stable in solution for more than 30 days and a homogeneous size distribution within the range 5–17 nm, with an average diameter of 10 ± 1 nm. Moreover, 90% of the particles have a diameter ranging from 7 to 13 nm. The sononanoparticles solution obtained in this way, like those for normal gold nanoparticles, shows optical properties due to surface plasmon resonance, which depends on the dielectric constant of the surrounding media. In our case, the surface plasmon resonance peak can be observed between 520 and 525 nm, considered typical for small or relatively small gold nanoparticles (5 – 20 nm).

2.2.4. Enzyme immobilisation procedures.

The enzymes were immobilised on three different electrodes by three established procedures:

a. Immobilization of HRP on the working electrodes by cross-linking method

The first type of biosensors developed in this study was obtained by the immobilization of the Horseradish peroxidase (HRP) onto the surface of three electrodes (AuE, GCE and SNGCE) by cross-linking with glutaraldehyde and bovine serum albumin (BSA) as previously described for several enzyme biosensors [13, 29] in order to aid the retention of the enzyme and increase its stability [30,31]. A mixture of HRP with BSA and glutaraldehyde (GA) was prepared. The enzyme mixture contained 3 µL of BSA (0.5 % in Milli-Q water), 17 µL of HRP solution (1 mg mL^{-1} in Milli-Q water), 25 µL of Milli-Q water and 5 µL GA (0.5% in Milli-Q water). 7 µL, 28 µL, 4.1 µL of this mixture were dispersed respectively over the surfaces of the AuE, GCE and SNGCE and the electrodes were dried at room temperature for 1 to 2 h. The volumes of the mixture deposited were proportional to the area of the electrodes. The electrodes must be kept in sodium acetate buffer solution pH = 5.0 at 4 °C until needed. Those enzyme electrodes were called HRP/AuE, HRP/GCE and HRP/SNGCE.

b. Immobilization of the HRP enzyme onto AuSNPs modified electrodes

For the fabrication of the second type of biosensors, adequate quantities of AuSNPs (7 µL, 28 µL and 4.1 µL of solution, also proportional to the electrode areas) were dropped respectively on the top of AuE, GCE, SNGCE and allowed to dry under room conditions. Then, 7 µL, 28 µL, 4.1 µL of the mixture of HRP, BSA and glutaraldehyde were spread coated over the surfaces of the AuE, GCE and SNGCE, respectively, and allowed to dry for 1 to 2 h. Those enzyme electrodes were called HRP/AuSNPs/AuE, HRP/AuSNPs/GCE and HRP/AuSNPs/SNGCE.

c. Immobilization of AuSNPs-entrapped HRP enzyme on the electrodes

The third type of biosensors developed in this study was obtained by the immobilization of a mixture of HRP enzyme and AuSNPs, onto the three electrodes surface (AuE, GCE and SNGCE). A mixture of HRP with, AuSNPs, BSA and glutaraldehyde was prepared. The enzyme mixture contained 3 µL of BSA (0.5 %), 17 µL of HRP solution (1 mg mL^{-1} of HRP dissolved in 5.4 µL of Milli-Q water and 11.6 µL of AuSNPs), 25 µL of AuSNPs and 5 µL of GA (0.5 % in water). 7 µL, 28 µL, 4.1 µL of this mixture were dispersed respectively over the surfaces of the AuE, GCE and SNGCE and were dried at room temperature for 1 to 2 h. Those enzyme electrodes were called (HRP+AuSNPs)/AuE, (HRP+AuSNPs)/GCE and (HRP+AuSNPs)/SNGCE.

2.3. Electrochemical measurements

The electrochemical measurements were carried out using an AUTOLAB potentiostat/galvanostat with GPES software (Ecochemie, Utrecht, Netherlands). A conventional three-electrode system comprising either AuE (1.5 mm diameter), GCE (3 mm diameter), or SNGCE (1.15 mm diameter) as the working electrode, a Pt counter electrode and an Ag/AgCl as the reference electrode was employed for all electrochemical experiments in an electrochemical cell containing 10 mL of 50 mM sodium acetate buffer at room temperature. In steady-state amperometric experiment, the potential was set at -0.15V vs. Ag/AgCl under gently magnetic stirring.

2.4. Procedure for enzyme inhibition measurements

The modified electrodes were immersed into acetate buffer solution (pH = 5.0) containing 1 mM H_2O_2. A given caffeic acid (substrate) concentration was added to record a steady-state current (I_0) before adding inhibitor (cyanide). The concentration of added cyanide was increased stepwise, and the current decrease (I_1), which was proportional to the final concentration of inhibitor in solution, was recorded. The percentage of inhibition (I (%)) due to the cyanide was evaluated according to the following equation (1) [32]:

$$I\ (\%) = \frac{I_0 - I_1}{I_0} X\ 100 \qquad (1)$$

where I_0 and I_1 are the currents recorded before and after inhibition, respectively.

3. Results and discussion

3.1 Response of the different biosensors to caffeic acid

HRP utilizes hydrogen peroxide to oxidize aromatic phenols and phenolic acids [33]. The role of the phenolic substrate (QH_2) is to shuttle electrons efficiently between electrode and enzyme. In the presence of phenolic substrate, the possible catalytic mechanism of HRP can be expressed as summarized in Scheme. 1, where Fe^{3+} is the cofactor of HRP, compounds I and II denote intermediates in the reactions, and QH_2 and Q represent phenol and its oxidized form (quinone), respectively [15].

Typical current–time response of the biosensor was obtained by successive additions of caffeic acid into a stirred cell containing 1 mM H_2O_2. The data were registered for the different biosensor designs: (HRP+AuSNPs)/SNGCE, HRP/AuSNPs/SNGCE and HRP/SNGCE.

With increasing concentration of caffeic acid the amperometric response increased linearly and then tended to a plateau value (Fig. 1), indicative of a typical Michaelis–Menten process. The apparent Michaelis constants, K_{Mapp}, and maximal rates, j_{max}, values were determined from caffeic acid calibration curves (Fig. 1). Each calibration curve was repeated three times for the same electrode; the calculated average values of kinetic parameters (K_{Mapp} and j_{max}) were reported in Table 1.

The same procedure was done using glassy carbon and gold electrodes. Calibration plots for caffeic acid, obtained with different configurations, showed the analytical characteristics summarized in Table 1. The lowest apparent Michaelis–Menten constant K_{Mapp} values achieved with AuE, GCE and SNGCE were 2.5 µM for (HRP+AuSNPs)/SNGCE, 3.8 µM for HRP/AuSNPs/GCE and 5.2 µM for (HRP+AuSNPs)/AuE (Table 1). These values were lower than those obtained with all other configurations, indicating that the immobilized HRP on the AuSNPs-modified GCE and the immobilized mixed HRP+AuSNPs on SNGCE and AuE surfaces had a greater affinity to caffeic acid.

The limits of detection (LODs) were calculated on basis of the definition given by the International Union of Pure and Applied Chemistry (IUPAC) ($C_{LOD} = 3.3S_b/m$, where S_b is the standard deviation of the blank and m the slope of the calibration curve) [34], while the sensitivities were calculated from the slope of the linear part of the calibration curve obtained from plotting density current (j (A/cm^2)) versus the substrate concentration (Fig.1).
From Table 1, it can be seen that higher sensitivity was achieved with the HRP/AuSNPs/GCE configuration, if compared to the calibration slope values obtained with all the other configurations. However, a good sensitivity was also obtained with the (HRP+AuSNPs)/AuE, HRP/AuSNPs/AuE and (HRP+AuSNPs)/SNGCE. From Fig. 1, it is evident that the Sonogel-Carbon electrodes based on AuSNPs enhance analytical performance in comparison with the Sonogel-Carbon electrodes without AuSNPs (higher sensitivity and lower LOD values), thus showing the catalytic effect of AuSNPs on the enzymatic reaction. A wider range of linearity as well as greater sensitivity were obtained with (HRP+AuSNPs)/SNGCE.
Moreover, when comparing the values of LOD, it can be observed that the lowest LOD was obtained with (HRP+AuSNPs)/SNGCE electrode, although it was similar to those obtained with HRP/AuSNPs/GCE, (HRP+AuSNPs)/GCE and (HRP+AuSNPs)/AuE. Nevertheless, comparing these four biosensors in terms of affinity ((HRP+AuSNPs)/SNGCE, HRP/AuSNPs/GCE, (HRP+AuSNPs)/GCE and (HRP+AuSNPs)/AuE), it seems obvious that the greater affinity (low K_{Mapp} value) is achieved when using the (HRP+AuSNPs)/SNGCE and HRP/AuSNPs/GCE biosensors. According to these results, these two biosensors ((HRP+AuSNPs)/SNGCE and HRP/AuSNPs/GCE) were selected for the determination of cyanide by inhibition.

3.2 Optimisation of experimental conditions

3.2.1 Influence of applied potential
Investigation of the influence of the potential on the response of the biosensor was performed in the range from −0.05 to −0.25 V vs. Ag/AgCl (Fig. 2). With the decreasing potential from −0.05 to −0.15 V vs. Ag/AgCl, the biosensor response increased considerably. By increasing the potential (in terms of absolute value) from −0.15 to −0.25 V vs. Ag/AgCl, the results showed small changes. Hence, a potential of −0.15 V vs. Ag/AgCl was selected as the applied potential for amperometric measurements in order to avoid the interferences at more negative potential values.

3.2.2 Effect of buffer pH
The sensitivity of the enzymatic biosensor can vary significantly depending on the different pH of the medium.
The effect of buffer pH on the biosensor response is illustrated in Fig. 3. As it can be seen, the degree of inhibition of the electrode for 1.3 µM of cyanide increases from pH = 4 to pH = 5 where the maximum was achieved, and then begins to decrease slightly. Thus, a pH = 5.0 was selected as the more suitable pH buffer for amperometric measurements.

3.2.3 Influence of substrate concentration
Fig. 1 illustrates the variation of the density current response versus the substrate (caffeic acid) concentration profile. As shown in this figure, initially, the current response generated by the biosensors increases rapidly with the caffeic acid concentrations. However the current signal reaches a stable value from 20 µM caffeic acid, showing almost no change in the current response.
It was observed that the high substrate concentration (> 10 µM) caused relatively high background current response. This phenomenon may have negative effect on current

measurements. Besides, at low concentration of substrate (around 1 µM), the current response generated by the biosensor was small. In order to get high sensitivity of the response and lower background effect, a concentration of 5 µM caffeic acid was selected as the optimum concentration for further experiments.

3.3 Analytical performance of the biosensor for cyanide

A typical inhibition response for the determination of cyanide using (AuSNPs+HRP)/SNGCE and HRP/AuSNPs/GCE biosensors is shown in Fig. 4a and Fig. 5a. Following stabilisation of baseline, caffeic acid is injected in the solution causing a fast response. Successive additions of cyanide solution in the range of 0.1 µM - 30 µM are then done and a rapid decreasing of enzymatic activity occurs, reaching approximately a steady-state within 100 s.

Fig. 4b and Fig. 5b reports inhibition percentage versus inhibitor concentration (KCN/µM) for (HRP+AuSNPs)/SNGCE and HRP/AuSNPs/GCE biosensors, respectively, using 5 µM caffeic acid as fixed substrate concentration. The inhibition mechanism was also studied in the presence of hydroquinone and catechol. The calibration curves of cyanide (inhibitor), for the HRP-based bioelectrodes, recorded in the presence of fixed concentration of caffeic acid, hydroquinone and catechol were interpreted and the parameters describing the inhibition process were estimated using I_{10} and I_{50} values (Table 2). The I_{10} and I_{50} are related to the concentrations of the inhibitor corresponding to 10 % and 50 % of the inhibition signal respectively. The detection limit, using (HRP+AuSNPs)/SNGCE biosensor, is 0.03 µM based on 10 % inhibition degree considering the error of the measurement [35]. Indeed, the European Community fixed the maximum acceptable level of cyanide in drinking water to 50 µg/L (0.7 µM KCN) [5]. Hence, the developed detection system (biosensor) is highly sensitive and is able to detect cyanide in the range of 0.1 µM to 58.6 µM with a detection limit of 0.03 µM. It should be mentioned that this detection limit is not only much lower than detection limits of other biosensors developed in this work (Table 2), but also much lower than the most reported cyanide biosensors which have been developed by Ghanavati et al. [1], Ketterer and Keusgen [36] and Volotovsky et al. [37].

The experimental results showed a notable difference in I_{50} towards cyanide ions for the two sensors (HRP+AuSNPs)/SNGCE and HRP/AuSNPs/GCE at different substrates (caffeic acid, hydroquinone and catechol) (Table 2). When using 5 µM of caffeic acid as fixed substrate concentration and HRP/AuSNPs/GCE as biosensor, the calibration curve for the cyanide determination covered the inhibitor range from 0.1 µM up to 80.6 µM with 50 % enzyme inhibition at 17.3 µM KCN (Fig. 4b and Table 2). But when using (HRP+AuSNPs)/SNGCE as a biosensor, the detection range of cyanide is from 0.1 µM to 58.6 µM with 50 % enzyme inhibition at 1.3 µM KCN (Fig. 5b and Table 2). This value is much lower than the value determined for HRP biosensor developed by Ghanavati et al. [1] and that obtained by Volotovsky et al. [37].

3.3 Study of the mechanism of inhibition

It is well known that the preparation procedure of a sensor affects the substrate as well as inhibitor kinetics of an enzyme. Thus, in the present work, the kinetic study of the inhibition of the HRP-based biosensor for cyanide was also carried out.

In order to assess the type of inhibition by cyanide, the data on kinetics were analyzed according to Dixon and Cornish-Bowden. The Dixon plot (representation of the inverse of the enzyme activity vs. inhibitor concentration) is often used for the evaluation of the inhibition constant and for the differentiation between different types of reversible inhibition [38].

Dixon plot (Fig. 6a) was made for two different substrate (caffeic acid) concentrations: 5 and 20 µM. The two linear plots converged on the X-axis, suggesting the non-competitive nature of the cyanide inhibition process. Analyzing the Cornish-Bowden plot [39], where the ratio of

substrate concentration and enzyme activity is plotted vs. inhibitor concentration (Fig. 6b), the HRP inhibition by cyanide was confirmed to be of full non-competitive type since the straight lines drawn through the experimental points also converge on the X-axis. The apparent inhibition constant (K_i = 2.7 µM) was calculated from the Dixon plot as the point where lines cross the X-axis.

To estimate the inhibition mechanism, a plot of % inhibition versus [KCN] can be also constructed at two different substrate concentrations as described by Amine et al. [40]. Fig. 6c shows the effect of two different caffeic acid concentrations on I_{50} obtained using HRP/AuSNPs/GCE. The inhibition curve remains unvaried suggesting a non-competitive inhibition. Indeed the shift of the inhibition curve to the right and left should be observed in the case of competitive and uncompetitive inhibition respectively [40].

From Table 2, it can be also seen that, the I_{50} values of cyanide measured at 5 µM and 10 µM of caffeic acid, hydroquinone and catechol using two different HRP immobilization techniques are not dependent on the substrate concentration. Hence, it can be concluded that cyanide inhibits horseradish peroxidase activity in a non competitive way. This was confirmed by the fact that when increasing the substrate concentration (caffeic acid, hydroquinone or catechol) the inhibition does not vary for all the substrates tested, since they do not compete with the inhibitor [40].

Shan et al. [41] suggested that cyanide acts as a non-competitive inhibitor of HRP. When using other enzymes, such as catalase [19] and polyphenol oxidase the inhibition was also non-competitive [42]. Ghanavati et al. [1] reported uncompetitive cyanide inhibition process of HRP.

3.4 Interferences and stability

Selectivity is a critical factor in the performance of an inhibition based enzyme biosensor. It is reported in literature that sulfide and azide are the most potent inhibitors of peroxidase [1,16,43]. In order to demonstrate the selectivity of the biosensor developed in this work, the cross-sensitivity towards sodium azide and sodium sulfide was also investigated under the same experimental conditions described for cyanide using (HRP+AuSNPs)/SNGCE biosensor in the presence of 5 µM of caffeic acid. The I_{10} and I_{50} were determined and compared to those of cyanide. The biosensor was able to detect sulfide but with a higher detection limit of 1.8 µM (corresponding to an inhibition degree of 10 %), than cyanide which was 0.03 µM. The I_{50} value for sulfides was 3.8 µM while the I_{50} for cyanide was 1.3 µM indicating that sulfides had a significant interference on the cyanide detection. This interference has been also reported by Ghanavati et al. [1] for HRP based inhibition biosensor.

However, azide interference was negligible and does not interfere significantly, the 10 % inhibition in the presence of azide was equal to 46.4 µM, and considerably higher in comparison with the I_{10} of cyanide. The I_{50} for azide was 132.0 µM and also noticeably higher than the one obtained for cyanide. This means that (HRP+AuSNPs)/SNGCE biosensor is less sensitive to azide than cyanide.

Selective determination of cyanide in mixture of cyanide and sulfides can be performed by removing sulfides by precipitation with lead carbonate $Pb(CO_3)$ [16] prior to any measurements for obtaining accurate assays with this method.

The diagnosis of the type of HRP inhibition that sulfides and azides cause, was also investigated in the presence of two different concentrations of substrate (5 and 10 µM of caffeic acid). The I_{50} increased significantly when increasing the caffeic acid concentration. This rising of the I_{50} values indicates a decrease in the enzyme/substrate interaction and also illustrates the characteristic behaviour of competitive inhibitors [40].

The (HRP+AuSNPs)/SNGCE biosensor was tested in terms of operational and storage stabilities. The amperometric response was compared with freshly prepared electrode. It was

noticed that this biosensor can be used for cyanide determination 15 times per day for at least one week after which a decrease of about 10 % from the initial response was observed. Besides, the (HRP+AuSNPs)/SNGCE biosensor exhibited a good storage life stability. The current response maintained 95 % of the initial value over a storage period of 60 days. These indicated that the reusability and the stability of the developed electrodes were both rather satisfactory.

Conclusions

Successful detection devices for environmental monitoring frequently require high sensitivity and low LOD. The data presented in this paper focused on the development of enzyme inhibition-based biosensors for the detection of cyanide. We employed three different electrodes, three different enzyme immobilization procedures and three different substrates. The results reveal that gold sononanoparticles (AuSNPs) have the capacity to achieve improvements in kinetic assays. This fact has important implications for improved cyanide determination in terms of sensitivity and detection limit since lower values of I_{50} and I_{10} were obtained by using AuSNPs. It was further demonstrated that the use of SNGCE modified with a mixture of AuSNPs and HRP ((HRP+AuSNPs)/SNGCE) allows obtaining significantly low LOD and low I_{50} values. Thus, the AuSNPs modified SNGCE could be a very good probe, since can give rapid and easy information to evaluate the presence of small amounts of cyanide. SNGCE biosensors offer other several advantages like an easy immobilization of the HRP enzyme, fast measurement, possibility of being integrated in portable instrumentation suitable for in situ measurements (i.e., in the field of screening analysis of toxic compounds), and cost effective.

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Figure captions

Scheme 1. Schematic diagram of the possible mechanism of the catalytic system based on the biosensor for detecting phenols

Fig. 1. Caffeic acid (CA) calibration curves corresponding to the different HRP modified Sonogel-Carbon electrodes in 50 mM of sodium acetate buffer pH = 5.0 containing 1.0 mM of H_2O_2 at -0.15 V vs. Ag/AgCl.

Fig. 2. Effect of the applied potential on the enzyme electrode response to 5 µM of caffeic acid. Supporting electrolyte: 50 mM of sodium acetate buffer pH = 5.0.

Fig. 3. Effect of pH on the degree of inhibition *I* (%) of 1.3 µM of cyanide. Applied potential: -0.15 V vs. Ag/AgCl. Supporting electrolyte: 50 mM of sodium acetate buffer pH = 5.0 containing 1.0 mM of H_2O_2 and 5µM of caffeic acid.

Fig. 4. a. Inhibition by KCN; b. Calibration curve obtained with (HRP+AuSNPs)/SNGCE at -0.15 V vs. Ag/AgCl in 50 mM of sodium acetate buffer pH = 5.0 in the presence of 5 µM of caffeic acid (CA).

Fig. 5. a. Inhibition by KCN; b. Calibration curve obtained with HRP/AuSNPs/GCE at -0.15 V vs. Ag/AgCl in 50 mM of sodium acetate buffer pH = 5.0 in the presence of 5 µM of caffeic acid (CA).

Fig. 6. a. Dixon plot; b. Cornish-Bowden plot of the cyanide inhibition at (HRP+AuSNPs)/SNGCE electrode; c. Calibration curves obtained with HRP/AuSNPs/GCE at -0.15 V vs. Ag/AgCl in 50 mM of sodium acetate buffer pH = 5.0 in the presence of 5 and 10 µM of caffeic acid (CA).

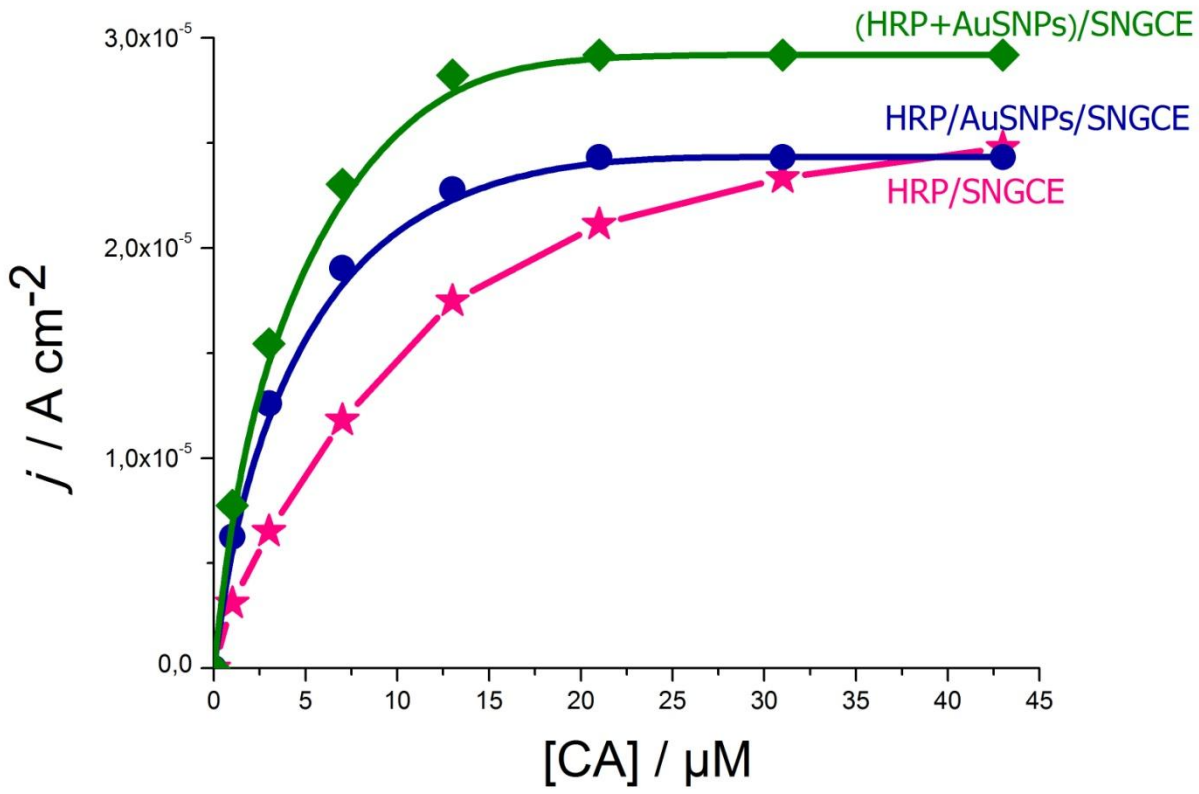


Figure 1

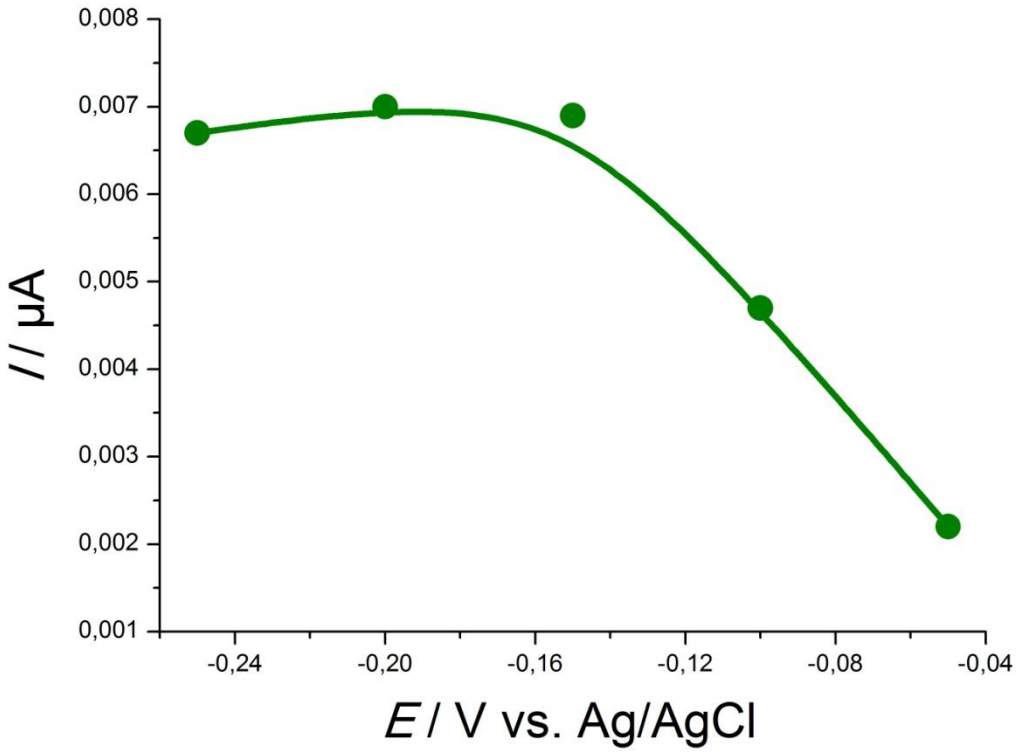


Figure 2

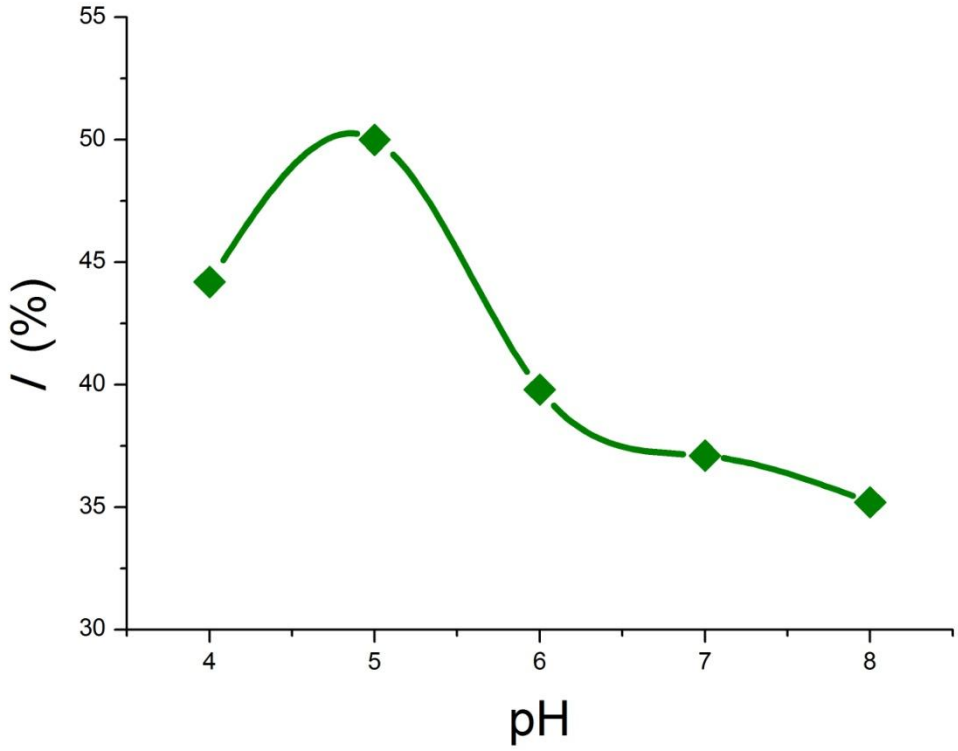


Figure 3

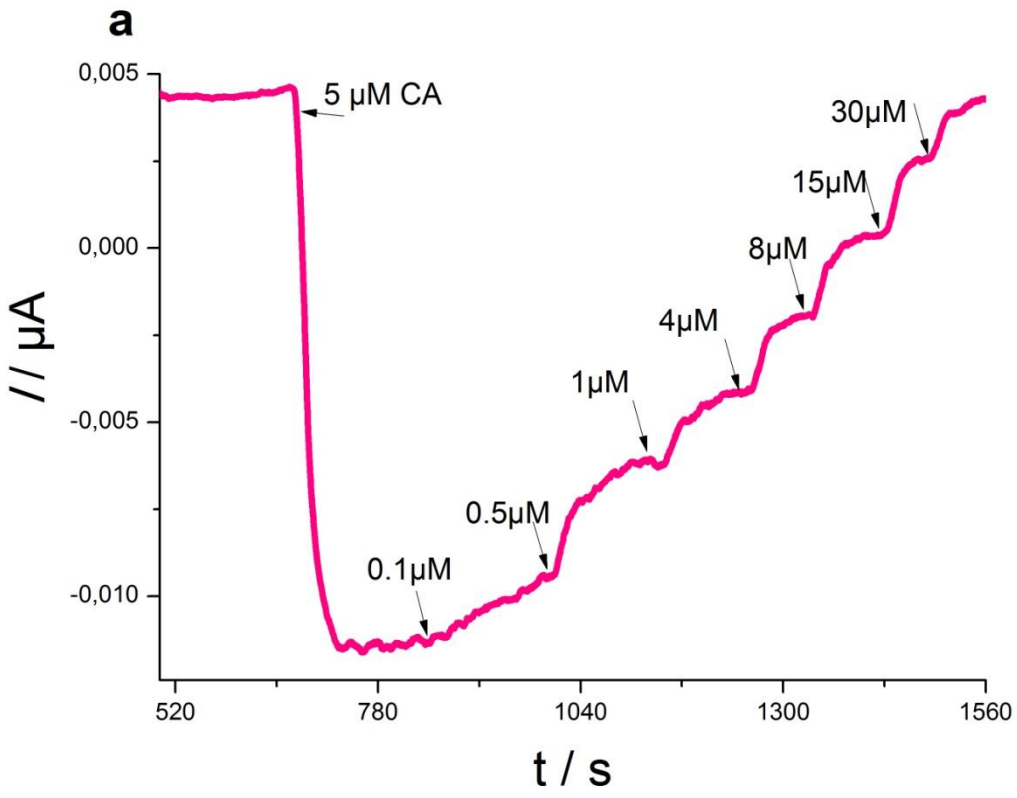


Figure 4a

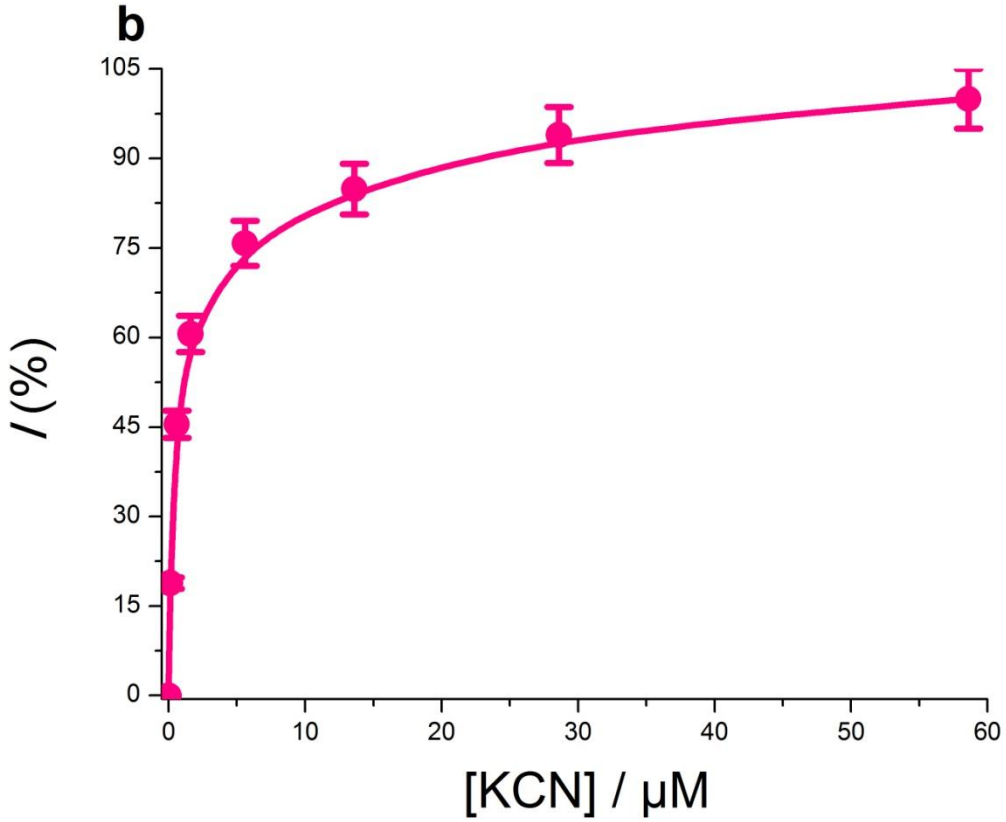


Figure 4b

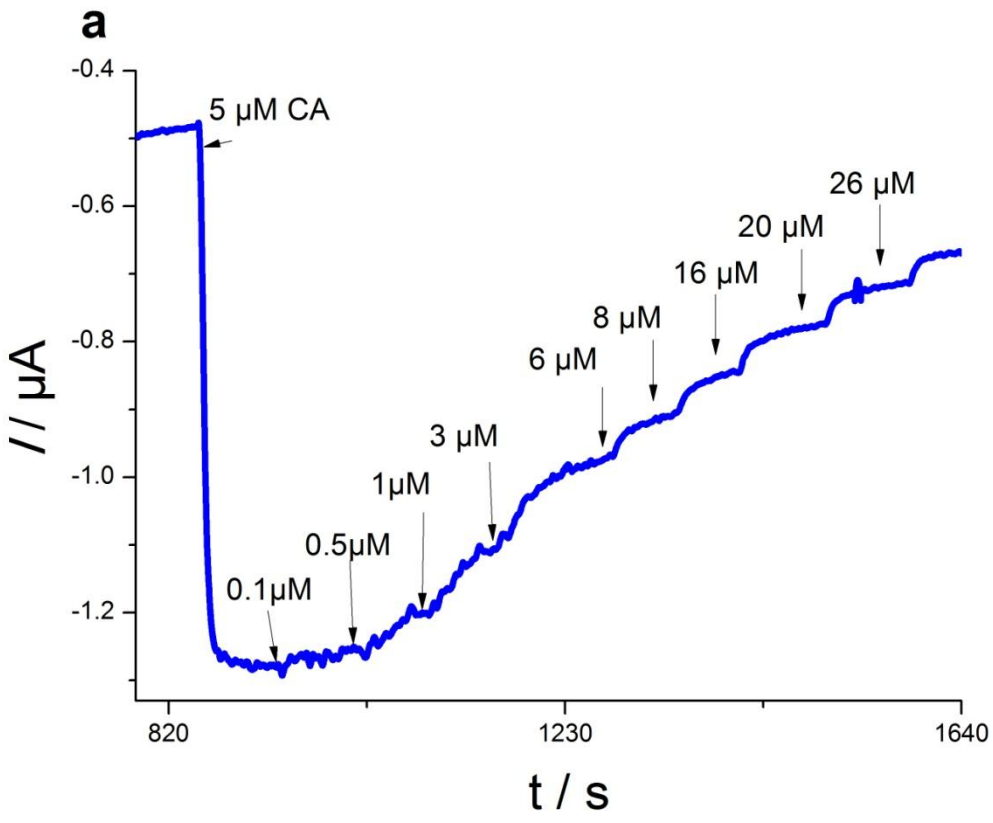


Figure 5a

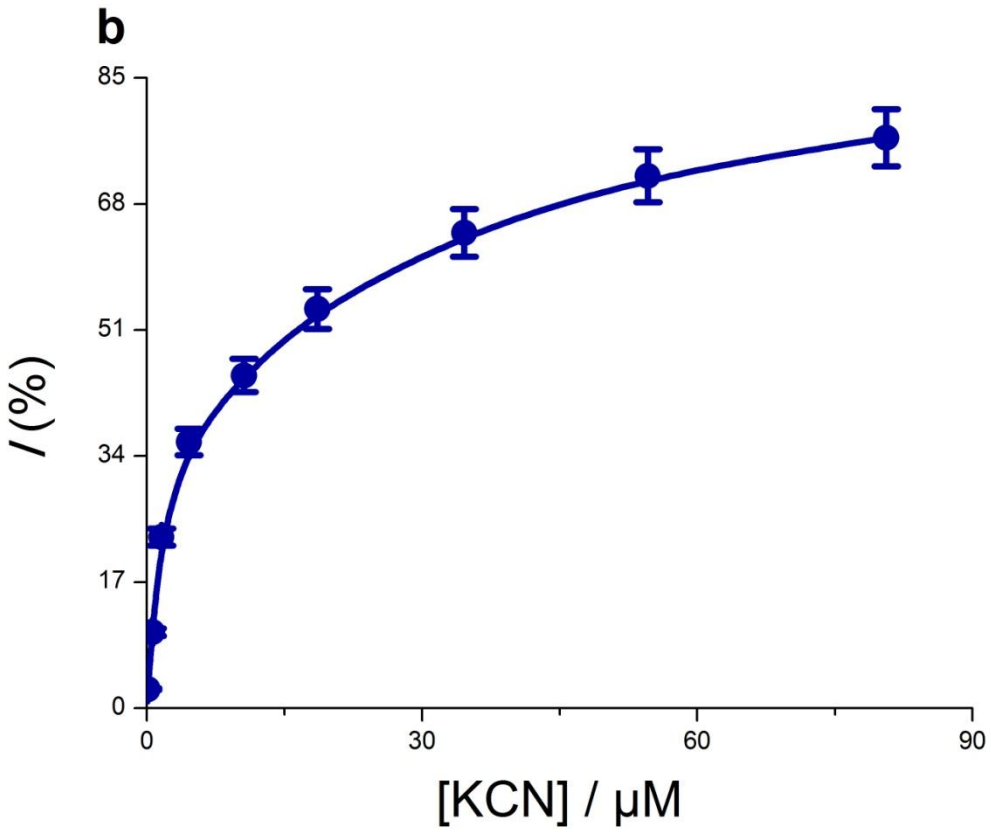


Figure 5b

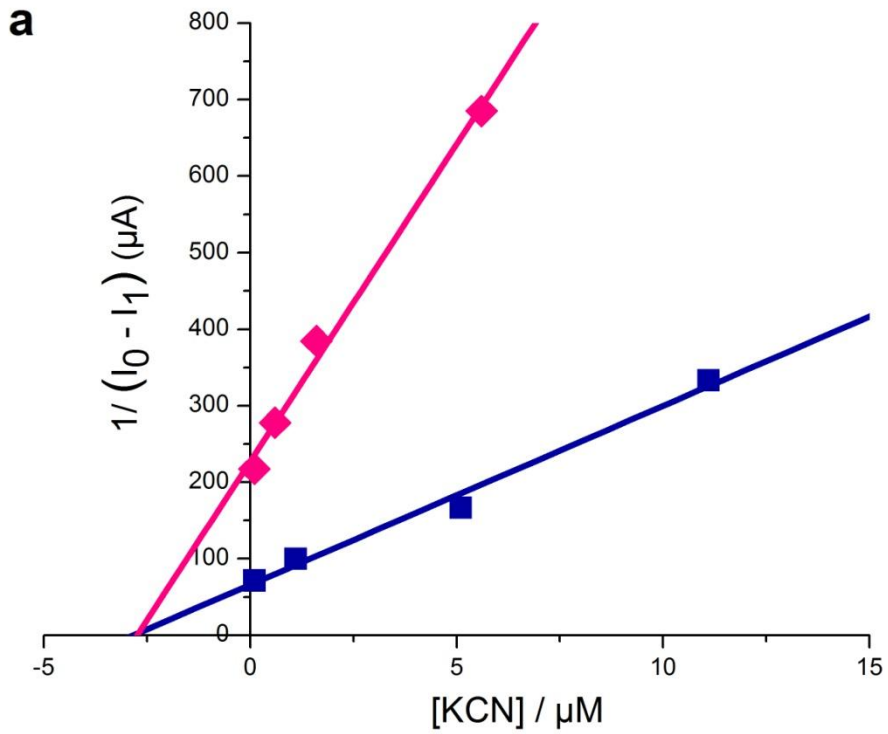


Figure 6a

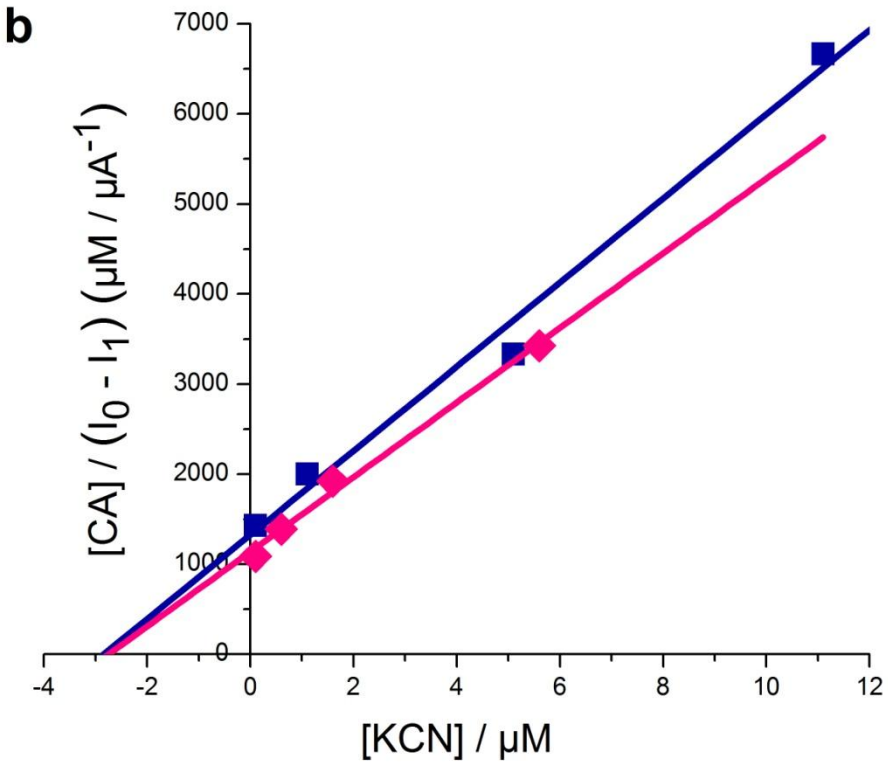


Figure 6b

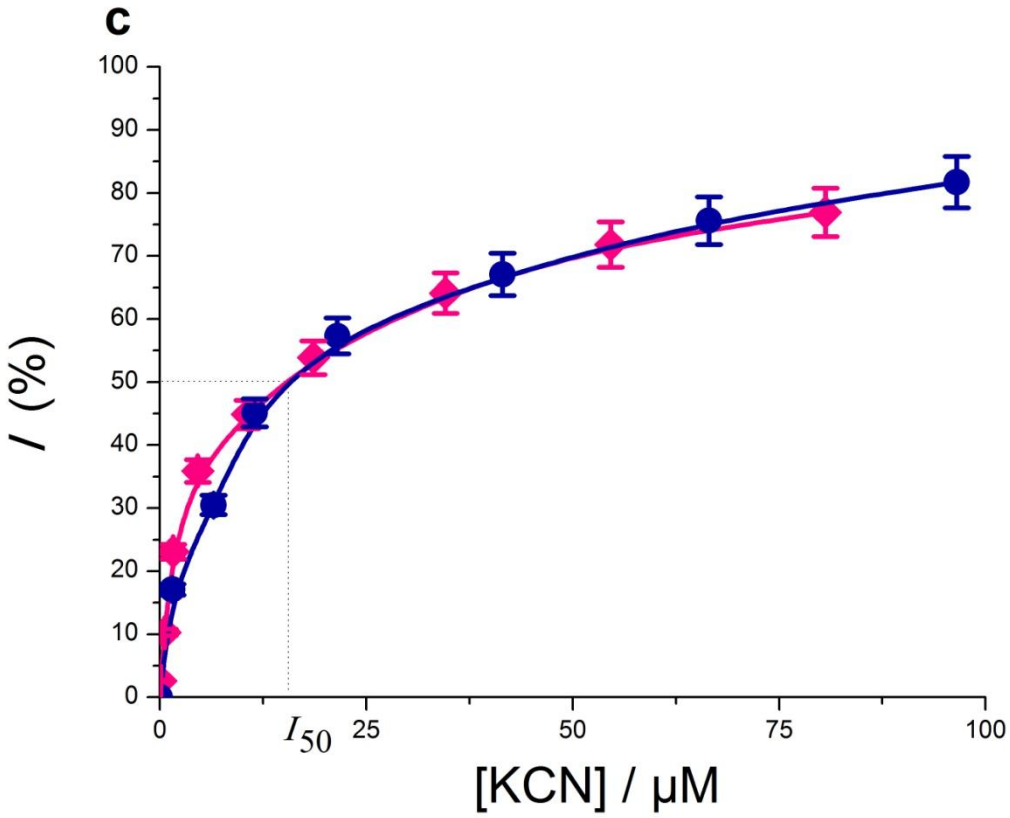
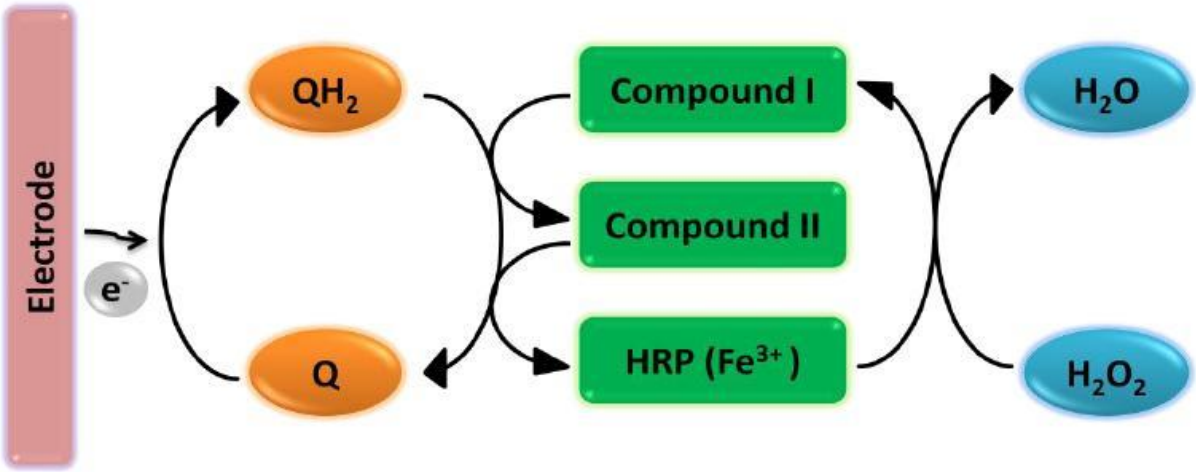


Figure 6c



Scheme 1

Tables

Table 1
Calculated values of kinetic and analytical parameters for the nine developed biosensor designs.

Modified Electrode	j_{max} (A cm^{-2} × 10^{-5})	K_{Mapp} (μM)	j_{max} / K_{Mapp}	S (A M^{-1} cm^{-2})	LOD (nM)
HRP/SNGCE	2.4	6.9	0.3	1.4	72
HRP/AuSNPs/SNGCE	2.2	3.3	0.6	2.0	60
(HRP+AuSNPs)/SNGCE	3.0	2.5	1.2	3.5	36
HRP/GCE	3.8	5.2	0.7	3.1	132
HRP/AuSNPs/GCE	5.5	3.8	1.4	6.7	38
(HRP+AuSNPs)/GCE	2.9	5.2	0.5	3.1	34
HRP/ AuE	4.1	10.2	0.4	2.1	817
HRP/AuSNPs/ AuE	7.9	6.5	1.2	4.3	62
(HRP+AuSNPs)/ AuE	1.2	5.2	0.2	4.5	40

HRP: Horseradish peroxidase; AuSNPs: Gold sononanoparticles; SNGCE: Sonogel-Carbon electrode; GCE: Glassy carbon electrode; AuE: Gold electrode. S: sensitivity (slope of the calibration curve); LOD: limit of detection.
*Each experiment was repeated three times with the use of the same electrode and the average values were reported.

Table 2

Principal analytical data referred to the calibration curves for the amperometric response of (HRP+AuSNPs)/SNGCE and HRP/AuSNPs/GCE biosensors and comparison with cyanide inhibition biosensors reported in the literature.

Biosensors	[Substrates]	Inhibited enzyme	I_{10} (μM)	I_{50} (μM)	Ref.
(HRP+AuSNPs)/SNGCE	5 μM CA	HRP	0.03	1.3	This work
	10 μM CA		0.05	1.3	
	5 μM QH_2		0.3	26.7	
	20 μM QH_2		0.5	25.5	
	30 μM CAT		0.45	12 .0	
	60 μM CAT		0.53	12.2	
HRP/AuSNPs/GCE	5 μM CA	HRP	0.39	17.3	This work
	10 μM CA		0.87	18.7	
	5 μM QH_2		0.73	33.7	
	20 μM QH_2		0.8	31.0	
	30 μM CAT		2.25	20.0	
	60 μM CAT		2.3	23.1	
Chitosan/HRP/GCE	1.25 mM QH_2	HRP	0.43	7.0	[1]
GCE; Remote sensing	0.5 mM CAT	Tyrosinase	2	NS*	[44]
PPO/[Zn-Al-Cl]	20 μM DOPAC	Polyphenol oxidase	0.04	0.2	[42]
PVPy /HRP/ISFET	15 mM L-ascorbic acid	HRP	0.1	0.6	[37]
CP electrode matrix	2 % Cytochrome c	Cytochrome oxidase	0.5	12	[16]

PPO: Polyphenol oxidase; PVPy: Poly(4-vinylpyridine-co-styrene); ISFET: Ion Sensitive Field Effect Transistor; CP: Carbon paste; CA: caffeic acid; QH2: hydroquinone; CAT: catechol; DOPAC: 3,4-dihydroxyphenylacetic acid.
*NS-not specified

HIGHLIGHTS

Amperometric inhibition biosensors based on horseradish peroxidase and gold sononanoparticles immobilized onto different electrodes for cyanide measurements

- New amperometric inhibition biosensors based on HRP and AuSNPs for inhibitive determination of cyanide.
- Three different enzyme immobilization procedures with and without AuSNPs were compared.
- Improved analytical performances compared to previous inhibition biosensors.
- The inhibiting action of cyanide on the horseradish peroxidase biosensors was confirmed to be non-competitive.