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A new PANI biosensor based on catalase for cyanide determination

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Abstract

Cyanide is one of the most widespread of compounds measured in environmental analysis due to their toxic effects on environment and health. We report a highly sensitive, reliable, selective amperometric sensor for determination of cyanide, using a polyaniline conductive polymer. The enzyme catalase was immobilized by electropolymerization. The steps during the immobilization were controlled by electrochemical impedance spectroscopy. Optimum pH, temperature, aniline concentration, enzyme concentration, and the number of scans obtained during electropolymerization, were investigated. In addition, the cyanide present in artificial waste water samples was determined. In the characterization studies of the biosensor, some parameters such as reproducibility and storage stability, were analyzed.

Keywords: catalase, cyanide, electrochemical impedance spectroscopy, inhibitory biosensor, PANI

Introduction

Catalase (E.C. 1.11.1.6) (CAT) is present in almost all aerobic living organisms. CAT is a heme enzyme with a ferritoporphyrin IX at its redox center, which belongs to the oxidoreductase family class (Nicholls et al. 1963). It catalyzes the disproportionation of H_2O_2 , to produce oxygen and water. This reaction is mainly catalyzed by CAT present in the red blood cells of mammals. CAT serves to protect cells from the toxic effects of H_2O_2 , by catalyzing its decomposition into molecular oxygen and water without the production of free radicals, and is therefore known as an antioxidative enzyme (Murthy et al. 1981, Fita and Rossmann 1985, Jouve et al. 1991).

CAT from bovine liver is a tetramer consisting of four equal subunits with a molecular weight of 60 kDa each. Each subunit contains iron bound to a protoheme IX group. CATs have a wide range of industrial applications. The enzyme is used in the dairy industry for milk and cheese sterilization, and in textile for the degradation of peroxide used for bleaching, to avoid any problems in next dyeing process (Betancor et al., 2003). It is one of the first enzymes to have been purified and

crystallized. In recent years, its importance has gained more attention because of its relation to cancer, diabetes and ageing, both in humans and animals (Melov et al. 2000, Preston et al. 2001).

An important regulatory mechanism of some biological pathways is the interaction between the enzyme and different small molecules present in the enzyme's surrounding. Some of them may act as inhibitors, and some of them as activators. CAT activity is inhibited by 2-mercaptoethanol, 3-amino-1-H-1,2,4 triazole, cyanide, hydroxylamine, azide (Loewen and Switala 2002), nitrite, nitrate, and S-nitroso-cysteine (Titov et al. 2007).

Cyanide is one of the most remarkable and competitive inhibitors of CAT. Cyanide is a chemical known to be a hazardous and toxic environmental contaminant. It is related to industrial waste from activities like gold mining, metal electroplating, nitrile manufacturing, herbicide production, and plastic industries (Behnamfard and Salarirad 2009, Yu et al. 2010, Yeddou et al. 2011). Cyanide also pollutes the water in areas which are used for fishing exotic fish (Mak et al. 2005). Cyanide is a component of chemical warfare agents (Kim et al. 2011). The maximum level for cyanide contamination in drinking water has been set by the United States Environmental Protection Agency at 0.1 ppm. Due to these deleterious effects of cyanide, developing selective, sensitive sensors for its monitoring is exceptionally important.

Approaches to detect cyanide include spectrophotometry (Geetha and Balasubramanian 2001, Deep et al. 2003), titrimetry (Pohlandt et al. 1983, Sun et al. 2012), the use of optical sensors (Jawad and Alder 1991), ion-selective electrodes (Clysters and Adams 1976), chromatographic (Christison and Rohrer 2007), fluorimetry (Lee et al. 2008) and flow injection (Surleva et al. 2007), electrochemical (Lindsay and O'Hare 2006) and luminescent analyses techniques (Varela et al. 2008). All these techniques currently exist for cyanide detection. However, some of them are time-consuming or require expensive equipments or complicated protocols. For the detection of various chemicals and ions, the electrochemical techniques prove to be faster, cheaper, reliable and reproducible, involving cathodic

reduction and anodic oxidation of analytes at various solid electrodes (Mehta et al. 2012).

In the present work, an amperometric biosensor has been fabricated for the determination of cyanide, based on cyanide's inhibitory effect on the activity of CAT, and the enzyme immobilization on the surface of the electrode was controlled by electrochemical impedance spectroscopy. The studies were based on the measurement of the differences in the responses of the biosensor, both in the absence and in the presence of cyanide. This biosensor includes three electrodes (working, reference and counter electrodes), and an enzyme component (CAT). There are a lot of materials for immobilization of enzyme on the electrode surface. Some of the materials easily crack and fall from the electrode surface, and this leads the loss of enzyme stability. When conducting polymers, such as polypyrrole (PPY), polyaniline (PANI), and polythiophene (PTH) arrived, they began to be used for immobilizing the enzyme to form reliable biosensors (Foulds and Lowe 1986, Bartlett and Whitaker 1988).

The biosensor uses the CAT on a cross-linked redox polymer like PANI, which may act as a mediator to decrease interference in the H_2O_2 signal, or a PANI film, which blocks interference from endogenous reducing agents. PANI is one of the conducting polymers used in optical sensors, chemical sensors, and biosensors, which has optical and electrochemical properties. It shows changes in the electrical and optical properties when chemically treated with oxidizing or reducing agents (Virji et al. 2004). PANI is an attractive material, due to its low cost, environmental stability, and reversible redox activity. Moreover, it is easily processed from solution to create uniform thin films. Its conductivity depends on the ability to transport charge carriers, both along the polymer backbone and between the polymer chains (Oh et al. 2004, Harsha et al. 2005, Li and Kaner 2006).

Among the various biosensors, the inhibition-based biosensors are very appealing for use in environmental monitoring, especially for field applications. Some of them have been used successfully to determine the concentration of inhibitors like toxic compounds, pollutants such as insecticides, and heavy metals at lower concentrations (Evtugyn et al. 1998, Chen et al. 2008). The advantages of inhibition-based biosensors are the savings in time and cost, reduction in the need for pretreatment of the sample, high selectivity, and sensitivity.

Methods

Chemicals and apparatus

All reagents used were of analytical grade, unless stated otherwise, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catalase (CAT) E.C. 1.11.1.6, from bovine liver ($\geq 10,000$ units/mg protein), was purchased from Sigma-Aldrich (St. Louis, MO, USA). CAT portions were prepared at certain concentrations and were stored at -20°C until use. The composition of the synthetically concocted waste water was as follows: $(\text{NH}_4)_2\text{SO}_4$ (0.5g), MgSO_4 (0.1 g), MnSO_4 (0.01 g), FeSO_4 (0.0005 g), and known amounts of cyanide in tap water (100 mL), at a pH of 7.2 (Livingston 1993).

In this study, the Palmsense potentiostat/galvanostat (the Netherlands), a triple-electrode system containing a platinum working electrode (with a surface area of 2.01 mm^2), Ag/AgCl reference electrode, and a platinum wire counter electrode (Basi, W. Lafayette, USA), were used for amperometric detection of cyanide. To prepare the buffer solution, a magnetic stirrer (RCA, UK) and a pH meter with an electrode (Schott Handylab, Spain) were used. All the measurements were carried out at a constant temperature maintained using a thermostat (Nüve BM302, UK). Electrochemical impedance experiments were performed using a Gamry Interface 1000

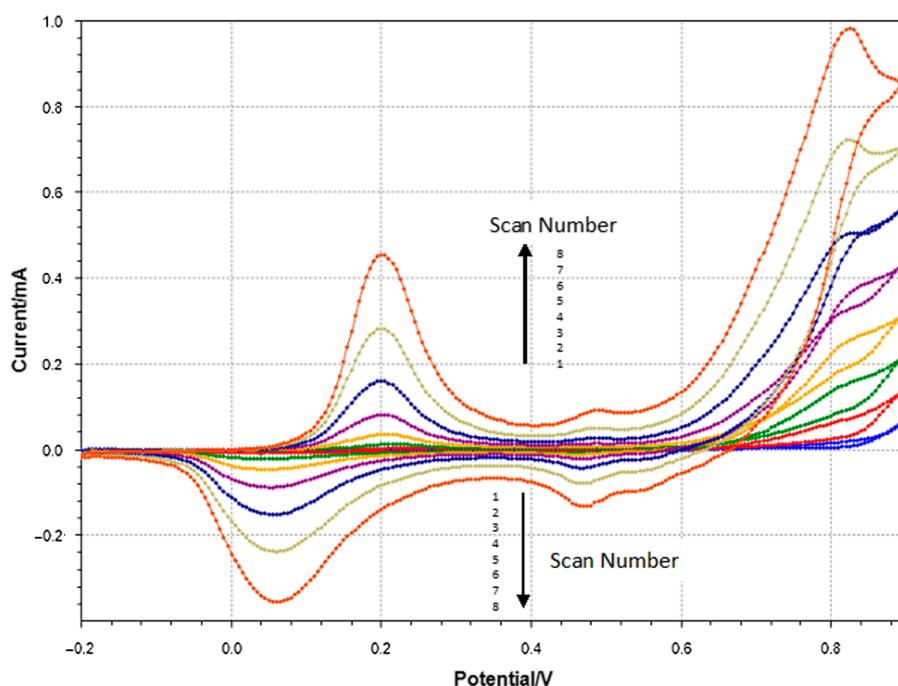


Figure 1. Cyclic voltograms of electropolymerization of aniline.

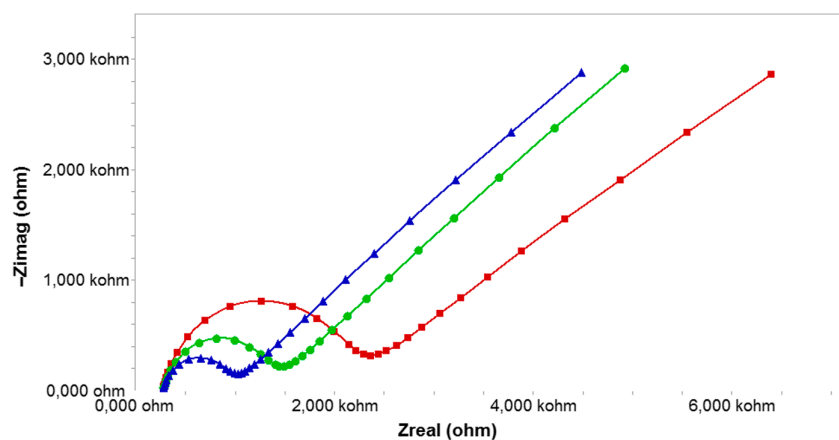


Figure 2. Impedance spectra of the immobilization steps [a) $\blacktriangle$ - $\blacktriangle$ -(blue): PANI electrode, b) $\bullet$ - $\bullet$ -(green): PANI-GLT electrode, $\blacksquare$ - $\blacksquare$ -(red): PANI-GLT-CAT electrode].

potentiostat/galvanostat (Gamry Instruments, Warminster, USA), interfaced with a PC via an EChem Analyst containing software for physical electrochemistry and electrochemical impedance spectroscopy (Gamry Instruments, Warminster, USA). In all electrochemical experiments, a Faraday cage (from iBAS, Warwickshire, UK) was used to block out external static electric fields.

Preparation of the biosensors

Prior to the electrochemical polymerization of aniline, the working surface of the Pt electrode was polished on a micro-fiber cloth using alumina powder, until a shining surface was obtained. After that, it was thoroughly rinsed with distilled water and then by sonication, first with absolute ethanol for 10 min and finally with distilled water for 5 min, to remove undesirable adsorbed particles. Then, it was electrochemically cleaned by cycling the electrode potential between -0.4 and $+1$ V vs Ag/AgCl, at a scan rate of 250 mVs^{-1} in 1 M HCl , until a constant current-voltage relationship was observed.

In the electrochemical polymerization step, a layer of polyaniline film was grown on the surface of the Pt electrode by cyclic voltammetry, in a cell containing 0.1 M aniline, 0.1 M HCl and 0.1 M KCl . The electrochemical polymerization of aniline is shown in Figure 1. The peaks due to the

oxidation and reduction of the film increase in intensity as the film grows. Eight cycles were scanned, with a scan rate of 50 mV/s at a potential ranging from -0.2 V to 0.9 V , until the electrode surface was changed to dark green. This clearly indicated the formation of a conductive polyaniline film on the surface of the Pt electrode. In the next step, the PANI-Pt electrode was washed with distilled water and dried with an ultra-pure argon stream.

After polymerization, 2.5% glutaraldehyde was first poured onto the surface of the modified PANI/Pt electrode and incubated for 30 min at room temperature, to form a bond between the free NH_2 groups of the polyaniline film and the aldehyde groups of the cross-linker.

The treated electrode was then washed with distilled water to remove the loosely-bound glutaraldehyde from the surface, and dried by using the ultra-pure argon stream. For physically adsorbed CAT, the PANI/Pt electrode was immersed in a 1 mL solution containing CAT for 1 h, immediately washed to remove unbound enzyme, and again dried by using an ultra-pure argon stream. Thus, the fabricated biosensor system was used for the experimental measurements and stored at 4°C until further use.

Principle of the measurement

CAT is a very fast biocatalyst that uses hydrogen peroxide as a substrate, forming oxygen and water. The enzyme can also act on alkyl hydrogen peroxides, and several organic

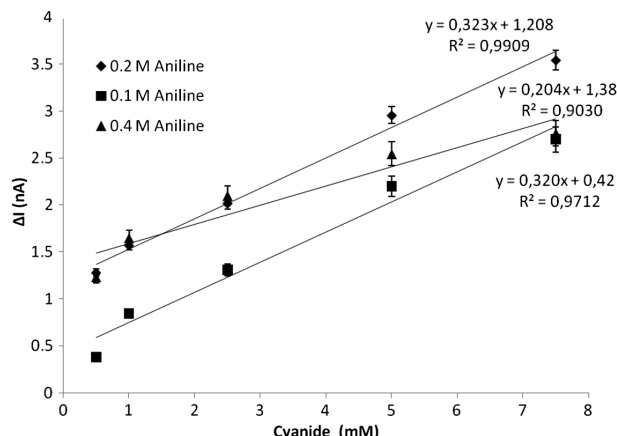


Figure 3. The effect of aniline concentration on the biosensor response.

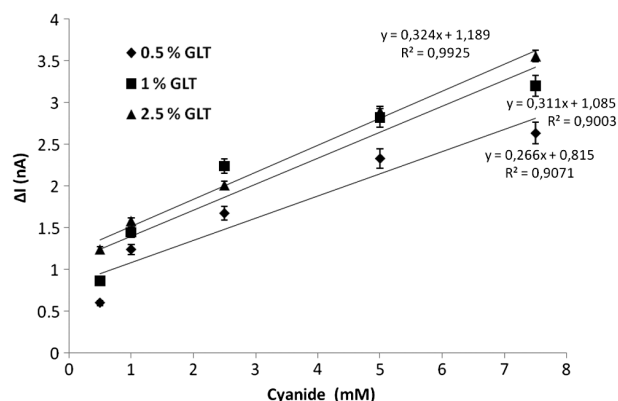


Figure 4. The effect of GLT concentration on the biosensor response.

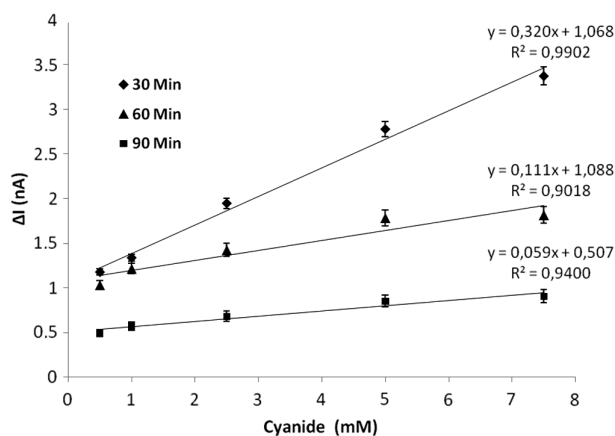
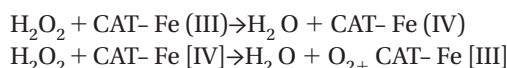


Figure 5. The effect of GLT incubation period on the biosensor response.

substances can replace the second hydrogen peroxide molecule as the hydrogen donor (ethanol, thiol, etc.). Cyanide is one of the competitive inhibitors of CAT, and was added to inhibit the enzyme activity.

The determination of hydrogen peroxide is based on the measurement of the oxygen produced during the enzymatic reaction according to the accepted mechanism (López et al. 2006):



Amperometric measurements were performed with the PalmSense Electrochemical Analyzer, with a three electrode system – a modified Pt electrode as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (3.0 M KCl) electrode as the reference electrode. Measurements were done in an electrochemical thermostatic cell, with stirring at 30°C. A series of H_2O_2 solutions were diluted from the stock solution, with different concentrations. All solutions were prepared with a phosphate buffer (0.1 M, pH 7); other chemicals were of analytical grade and used without further purification.

The measurements were carried out in 15 mL of 0.1 M phosphate buffer solution. A solution with a known concentration of H_2O_2 was added as substrate, and the steady

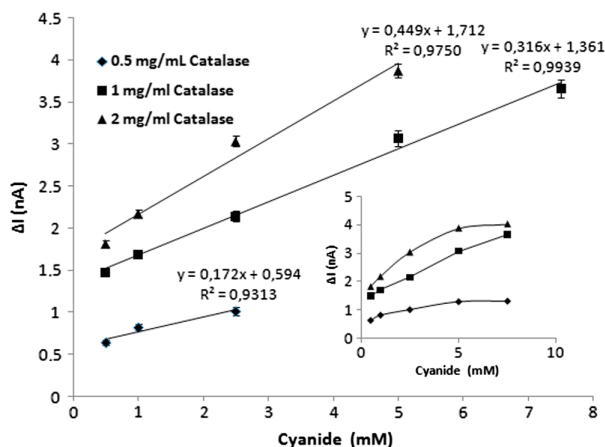


Figure 6. The effect of enzyme amount on the biosensor response.

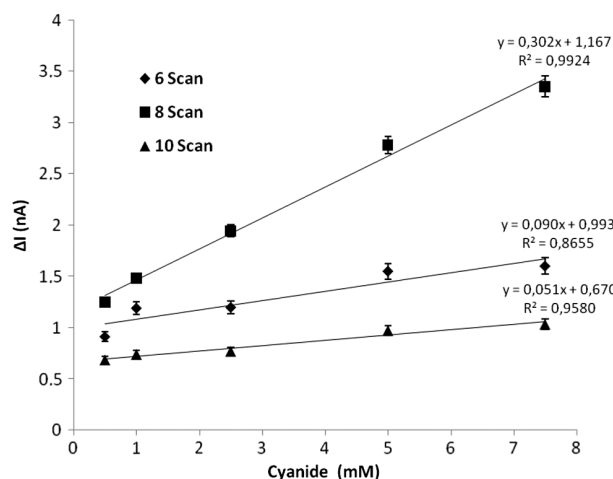


Figure 7. The effect of the number of scans on the biosensor response.

state current was recorded and noted as I_0 . Standard working solutions of cyanide were prepared daily from potassium cyanide (KCN) in phosphate buffer (0.1 M, pH 7). Finally, each cyanide concentration was added to inhibit the enzyme activity, and the decreasing values of current were noted as I_1 . This current is proportional to the final concentration of inhibitor in the solution. The inhibition percentage (I %) due to selected levels of cyanide was calculated using the following equation (Nwosu et al. 1992):

$$I(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

Impedance measurements

Electrochemical impedance spectra provide an effective method to analyze the interface properties of surface-modified electrodes. The impedance measurements of electrodes were carried out at the open circuit potential in a phosphate buffer (pH 7.0) containing 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ over a frequency range of 1×10^{-2} to 1×10^5 Hz. It can be seen that curve (a) in Figure 2 has a small semicircle, implying very low electron-transfer resistance (R_{ct}). The lowest R_{ct} (curve (a)) indicates the fastest electron-transfer rate between the Pt electrode and the PANI film. According to the impedance spectra, the R_{ct} values were calculated for

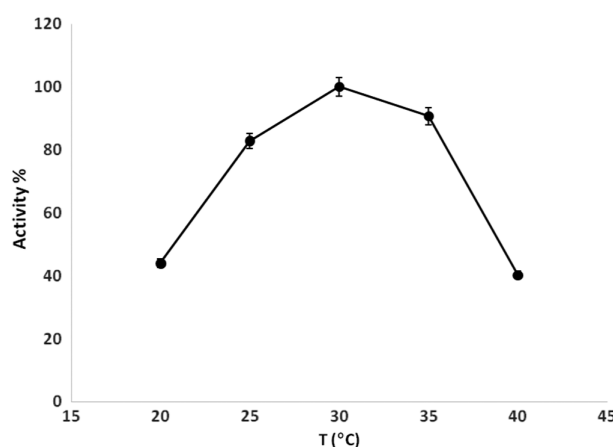


Figure 8. Effect of temperature on the biosensor response.

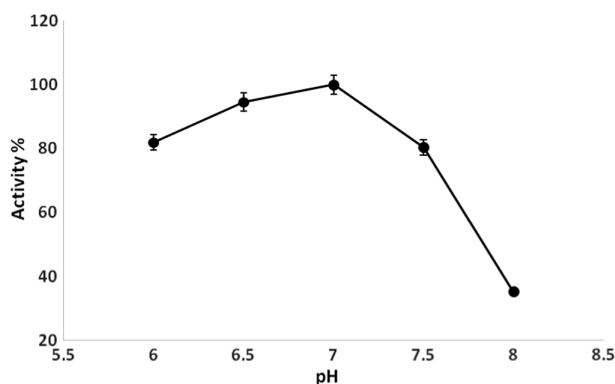


Figure 9. Effect of pH on the biosensor response.

the PANI electrode, the PANI- Glutaraldehyde (PANI-GLT) electrode, and the PANI-Glutaraldehyde-Catalase (PANI-GLT-CAT) electrode, and were 0.245, 1.031 and 2.049 kohm, respectively. The PANI-GLT-CAT electrode shows a higher R_{ct} value, which may be ascribed to the bond of non-conductive glutaraldehyde on the polyaniline surface. After enzyme immobilization, an obvious increase in the interfacial resistance is observed (curve (c)). This result is very similar to that of the polyaniline-uricase biosensor (Arora et al. 2007). The increase in the R_{ct} might be attributed to the non-conductive properties of PPO, which hinders electron transfer of the electrochemical probe. It confirms successful immobilization of the enzyme on PANI.

Results and discussion

Optimization of construction of the biosensor

Effect of the amount of aniline on the biosensor response

To determine the effect of the amount of aniline on the response of the biosensor, different amounts of aniline were used. Standard cyanide solutions of concentrations ranging from 0.25–7.5 mM were separately analyzed by biosensors prepared with different concentrations of aniline (0.1, 0.2 and 0.4 mM). Polymerization of aniline in an acidic solution yields conductive polyaniline. The conductivity

of polyaniline decreases with an increase in the pH of the solution (Garjonyte and Malinauskas 2000). The results are given in Figure 3. According to the linear response of biosensors, the optimal biosensor responses were achieved by the biosensor which contained aniline at a concentration of 0.2M. When we used 0.4M aniline, responses were very low, because the increase in aniline leads to thicker diffusion barriers in between the surface of the electrode and the bioactive layer. A thicker diffusion barrier hinders the effective working of the enzyme as well as the diffusion of substrate and inhibitor from the bioactive layer to interval surface. An aniline concentration of 0.1M showed a swerve on the curve along with a decrease in the response of the biosensor. The most important reason for this effect was the escape of enzyme from the surface of the electrode, due to insufficient tightness of the electropolymerization.

The effect of the percentage of glutaraldehyde on the biosensor response

In the study to determine the effect of the percentage of glutaraldehyde on the biosensor response, biosensors were prepared with 0.5%, 1%, and 2.5% glutaraldehyde solutions for cross-linking. The results are given in Figure 4. The values of R^2 were calculated as 0.9071, 0.9003 and 0.9925, respectively.

Due to the linear response profile of the biosensor, a concentration of 2.5% glutaraldehyde was chosen as the optimum value. At this concentration, the responses of the biosensor are higher than those obtained using 0.5% and 1.0% glutaraldehyde. Lower concentrations of glutaraldehyde were not sufficient to allow adequate cross-linking. Therefore, the most useful and best responses were obtained by using the biosensor prepared using 2.5% glutaraldehyde.

Effect of the incubation time of glutaraldehyde on the biosensor response

To study the effect of incubation period of glutaraldehyde on the biosensor response, the amount of CAT, the percentage of glutaraldehyde, and concentration of aniline were kept constant at 1mg/mL, 2.5%, and 0.2M, respectively, and the

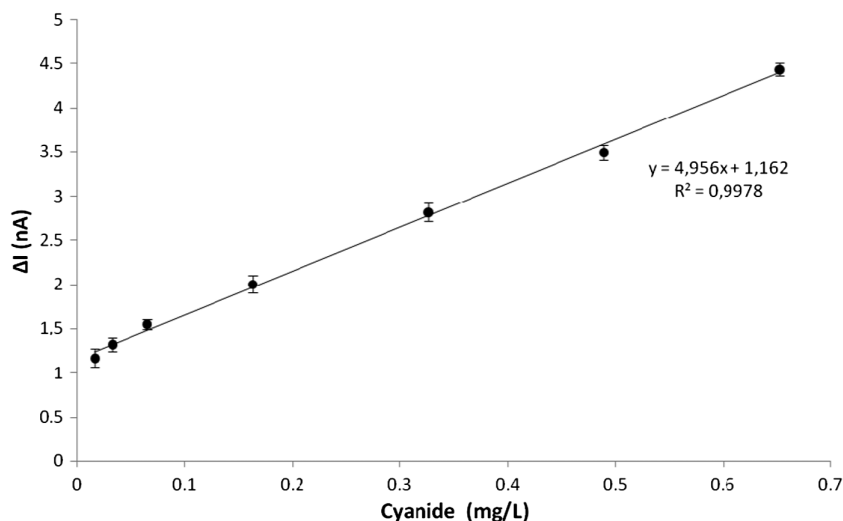


Figure 10. Calibration curve of the biosensor.

Table I. Comparison of the methods developed and some of the reported methods for the cyanide determination.

Technique	Sample	Linear range (mg/L)	Detection limit ($\mu\text{g/L}$)	References
HF-HS-CE-UV	Urine	0.0056–0.52	2.08	Jermak et al. (2006)
FI fluorimetry	Water	0.001–0.2	0.26	Infante et al. (2008)
Spectrophotometry	Water	0.05–2	0.5	Hamza et al. (2010)
FI spectrophotometry	Water	0.01–0.04	1.5	Santelli et al. (2006)
GD-SI spectrophotometry	Water	0.0075–0.2	2.5	Themelis et al. (2009)
PANI biosensor	Water	0.0136–0.65	2	This work

HS-SDME, headspace-single-drop microextraction; CE-UV, capillary electrophoresis with UV detection; FI, flow injection; GD-SI, gas diffusion-sequential injection; PANI, polyaniline

incubation period of GLT immobilization was changed 30, 60 and 90 min. The results can be seen in Figure 5. R^2 values were calculated as 0.9902, 0.9018 and 0.9400, respectively. According to the linearity of the response, the incubation period of 30 min was chosen as the optimum incubation period for GLT immobilization.

Effect of the amount of enzyme on the biosensor response

Different amounts of CAT were studied to determine the effect of the amount of enzyme on the biosensor response. Three different biosensors were prepared with CAT concentrations of 0.5, 1, and 2 mg/mL, using GLT (2.5%) and aniline (0.2 M). The results are shown in Figure 6.

The results indicate that the best responses and linear range were obtained by the biosensor prepared with a CAT concentration of 1 mg/mL. A narrow linear response region was observed with the biosensor which contained CAT amounting to 0.5 mg/mL. On the other hand, when an enzyme concentration of 2 mg/mL was used in biosensor construction, some deformations and diffusion barriers occurred on the surface layer. According to the response profile of the biosensor, the CAT amount of 1 mg/mL was chosen as the optimum amount of enzyme for construction of the biosensor.

Effect of the number of electropolymerization scans on the biosensor response

To determine the effect of the number of electropolymerization scans on the biosensor response, the amount of CAT, the percentage of glutaraldehyde, and the concentration of aniline were kept constant at 1 mg/mL, 2.5%, and 0.2 M, respectively, and the number of scans was changed to 6, 8, and 10. It can be seen in Figure 7 that the highest biosensor response and best linearity were obtained at 8 scans. Therefore the optimum number of scans was chosen as 8.

Optimization of experimental conditions

Effect of temperature on the biosensor response

The electrocatalytic activity of enzymes or proteins is strongly affected by temperature. In this part of the optimization studies, measurements of the prepared biosensor were carried out in media of different temperatures (20°C, 25°C, 30°C, 35°C, 40°C). The determination of the effect of temperature on the biosensor response is very important, due to the nature of the biocomponents, and also to obtain the best biosensor responses. The results obtained from the experiments are shown in Figure 8. It can be said that the highest biosensor responses were obtained at a temperature of 30°C.

Effect of pH on the biosensor response

Another parameter affecting the amperometric response of the biosensor is the pH of the solution. The experiments were carried out in different buffer systems to determine the effect of pH value on the biosensor response. For this purpose, 100 mM concentrations of phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0) buffers were prepared and used. The results obtained from these experiments are shown in Figure 9. According to the findings, the optimum pH value was determined as 7.0. Apparently, the response current increases as the pH changes from 6.0 to 7.0. At the pH range from 7.0 to 8.0, the response current decreases as the pH value increases. However, the change in the response current of the PANI biosensor may not be ascribed to the decrease of conductivity of the PANI with increasing pH, because the PANI synthesized in an acidic solution containing HCl has good electroactivity and high conductivity at high pH (Singh et al. 2009).

Analytical characteristics of the biosensor

Linear range of the biosensor

After optimization of the biosensor, experiments were carried out to achieve a calibration curve for cyanide at optimal conditions. From the results obtained, it can be said that the biosensor responses correlated linearly at a concentration of potassium cyanide ranging between 16.3×10^{-3} –0.65 mg/L. From the experiments, a linear regression equation and R^2 value were obtained as $y = 4.956x + 1.162$ and 0.9978, respectively. The calibration curve and comparison of some methods reported for cyanide detection are given in Figure 10 and Table I.

Reproducibility

The reproducibility of the biosensor was tested by 12 average standard solutions containing equal amounts of cyanide

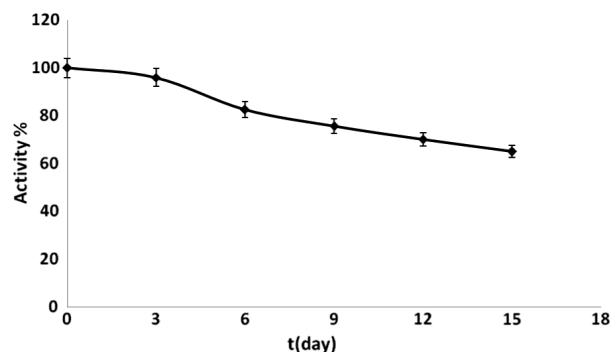


Figure 11. Storage stability of the biosensor.

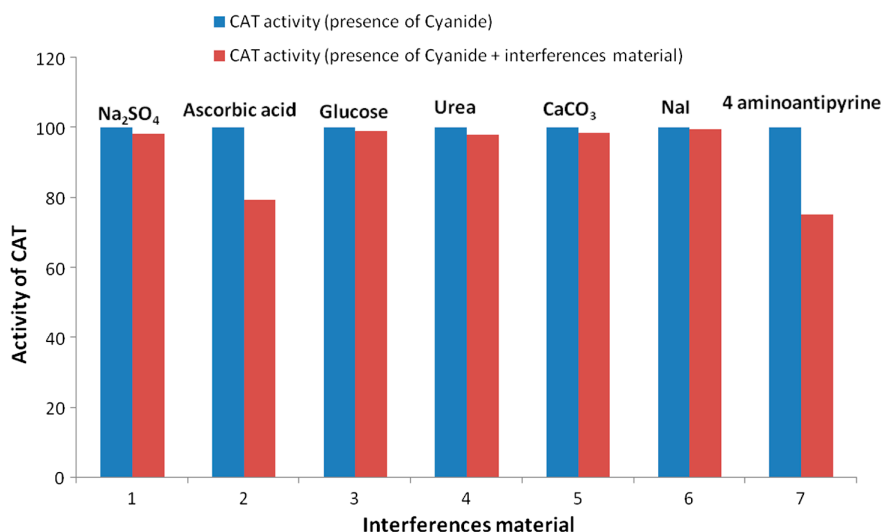


Figure 12. Effect of interference materials on biosensor response.

(0.32 mg/L). The standard deviation (SD), variation coefficient (CV), and average value (X) were calculated as ± 0.0077 mg/L, 2.43% ($n = 12$), and 0.3179 mg/L respectively.

Storage stability

The new PANI biosensor was stored at -4°C , to observe the storage stability of the biosensor. Measurements were carried out in the presence of cyanide and 5mM H_2O_2 on every third day, for a period of 15 days. The results are shown in Figure 11. Although 9 days passed, there was only a 24.5% loss in the activity. After 15 days, there was a 35% loss in the activity and also in the responses of the biosensor to cyanide.

Interference studies

In order to determine the effect of different compounds on the biosensor response, some experiments were carried out using various substances such as Na_2SO_4 , ascorbic acid, glycose, urea, CaCO_3 , NaCl, KCl and 4-aminoantipyrine, at concentrations of 5 mM, in the presence of 5 mM hydrogen peroxide. Average activities were calculated from five measurements for each of the compounds.

Figure 12 surveys the observations obtained by studying selected ions and salts as species that could interfere with the biosensor. As expected, ascorbate and 4-aminoantipyrine caused an interference effect. This result agreed with the data in the literature, because ascorbate and 4-aminoantipyrine had an inhibitory effect on CAT (Akyılmaz and Kozgus 2009). Besides these, the other substances such as glucose and urea which were tested, caused no or negligible interference effect, as seen in Figure 12.

Application in artificial waste water

In addition, the biosensor was applied to the artificial waste water samples spiked with cyanide. The results from 5 different measurements which were averaged can also be seen in Table II. The results presented here show that cyanide levels in the artificial waste water samples analyzed by the present biosensor agreed with that of the artificial waste water samples with the spiked amounts.

Conclusions

In this paper, a new inhibition-based biosensor for cyanide detection has been described. The biosensor was prepared by using CAT/PANI film-modified Pt electrode which can be used for the immobilization of CAT. This proposed new biosensor is economical due to the low cost, high sensitivity, good selectivity, simple and fast fabrication of the sensor.

We described immobilization steps by using electrochemical impedance spectroscopy for inhibition-based biosensors for the first time in literature. Also, some parameters such as optimum temperature, pH, and the effect of the number of electropolymerization scans, the amount of aniline, amount of enzyme, and the effect of the incubation time of glutaraldehyde, were investigated in the optimization studies of the biosensor. We found that the best conditions were determined to be as follows: temperature: 30°C , phosphate buffer: pH 7.0, 100 mM, scan number: 8, aniline: 0.2 M, CAT: 1mg/mL and incubation period: 30 min.

Thus, this work, based on the inhibition of cyanide, has good analytical properties such as fast response time (6 min), long-time stability, and a good detection range, and

Table II. Results of analysis of artificial waste water samples (*Average values of 5 measurements).

Added cyanide (mg/L)	*Found by biosensor (mg/L)	Recovery (%)	Relative differences (%)	*Found by spectrophotometric method (mg/L)
0.065	0.06350 ± 0.0012	97.70	2.30	0.06420 ± 0.0054
0.13	0.13136 ± 0.0021	101.05	1.05	0.1308 ± 0.0015
0.195	0.18817 ± 0.0071	96.50	3.50	0.1910 ± 0.0025
0.26	0.25875 ± 0.0064	99.52	0.48	0.2592 ± 0.0051
0.325	0.33306 ± 0.0017	102.48	2.48	0.3284 ± 0.0045

it is suitable for routine analysis of H_2O_2 and cyanide at the same time.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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