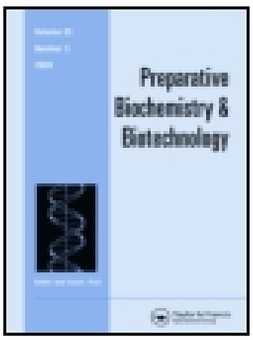

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A New Polyaniline-Catalase-Glutaraldehyde Modified Biosensor for Hydrogen Peroxide Detection

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Abstract

A new biosensor based on catalase enzyme immobilized on electrochemically constructed polyaniline (PANI) film modified with glutaraldehyde (GA) has been developed for the determination of hydrogen peroxide (H_2O_2) in milk samples. Assembly processes of polyaniline and immobilization of the enzyme were monitored with the help of electrochemical impedance spectroscopy (EIS). Amperometric measurements have been carried out at cathodic peak (– 0.3 V vs. Ag/AgCI) which was attributed to reduction of PANI. Hydrogen peroxide was determined by using amperometric method at –0.3 V. The biosensor responses were correlated linearly with the hydrogen peroxide concentrations between 5.0×10^{-6} and 1.0×10^{-4} M by amperometric method. Detection limit of the biosensor is 2.18×10^{-6} M for H_2O_2. In the optimization studies of the biosensor, some parameters such as optimum pH, temperature, concentration of aniline, amount of enzyme and number of scans during electropolymerization were investigated.

Keywords: Biosensor, Catalase, Polyaniline, Glutaraldehyde, Hydrogen peroxide

INTRODUCTION

Organic conducting polymers have attracted interest in recent years, due to exhibiting a wide range of novel electrochemical properties [1]. PANI has been studied extensively to be an important conducting material that possesses interesting electrical, electrochemical, and optical properties [2]. Properties of polyaniline and also permeability to electroactive substances could be controlled through the polymerization conditions such as time, potential and concentration [3]. H_2O_2 plays very important roles as a signalling molecule in the regulation of different biological processes [4] . It is also an effective factor implicated in the free radical theory of aging. The theory is based on how hydrogen peroxide can decompose into a hydroxyl radical and also how superoxide radical byproducts of cellular metabolism can react with water to form H_2O_2. Hydroxyl radicals react with cellular components and damage the mitochondria of the cells [5–7] . H_2O_2 is involved in many physiological processes such as hypoxic signal transduction and especially oxidative processes [8]. There are some studies about the function of H_2O_2 in oxidative processes such as lipid peroxidation in chronic alcoholism [9], metabolic oxidative stress [10], response to an antioxidant treatment [11], and also apoptosis and intracellular oxidative stress [12].

Hydrogen peroxide also plays very important roles in regulating diverse biological processes [13]. Some of them are DNA repair [14], oxidative activity [15], host defense [16], and apoptosis [17].

The detection of hydrogen peroxide has become extremely important because of its wide and varied applications in different industrial processes. As a well-known oxidizing agent, H_2O_2 is employed in textiles, cleaning products, organic compounds, the food industry, and for some environmental treatments[18]. Several analytical methods have been developed for the determination of hydrogen peroxide including, titrimetric [19,20], spectrometric [21], chemiluminescence [22,.23] and electrochemistry [24–34] . Hydrogen peroxide is used as a preservative for milk and milk products so to determine its level is very important in this industry. There are some analytical methods for determination of H_2O_2 in milk. A highly sensitive fluorimetric method was described for the determination of H_2O_2 in milk[35]. Some electrochemical biosensors were also developed for the determination of H_2O_2 in milk analysis [36–39] .

Catalase (EC 1.11.1.6) is a hemoprotein which can function both in the hydrogen peroxide catabolism and peroxidatic oxidations of small compounds like ethanol, methanol or elemental mercury (HgO) [40]. Furthermore catalase is implicated in inflammation, apoptosis, aging and cancer. Loss of catalase leads to the human genetic disease known as Acatalasemia or Takahara's disease [41]. Substrat selection and the proposed catalytic mechanism of catalase has been reported by Prajapati et al. [42]. Some amperometric biosensors based on catalase enzyme has been reported. These biosensors involve dissolved oxygen probes [43,44] or glassy carbon electrodes [45,46]. Also entrapment of catalase in polyacrylamide gel has been studied [47]. Catalase is not only used for determination of hydrogen peroxide, but also for alcohols [48] and for some anions, such as nitrite, iodate and periodate [49], as well as for calcium in milk and water samples [50].

The aim of this project is to construct a new biosensor based on PANI-GA-CAT modified Pt electrode for sensitive determination of hydrogen peroxide.

EXPERIMENTAL METHODS

Chemicals

Catalase (Cat) (12 U/mg protein) from bovine liver, hydrogen peroxide, aniline, glutaraldehyde (25%), and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions used in the experiments were prepared just before their use.

Apparatus

In the experiments PalmSens potentiostat (Netherlands), a three-electrodes system from CH Instruments (USA) that contains a CHI 112 model Pt working electrode (2mm diameter), a CHI 111 model Ag/AgCl reference electrode and a CHI 115 model platinum wire counter electrode, Eppendorf automatic pipettes (Germany), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used.

Preparation Of The Biosensor

Before the preparation of the bioactive layer, Pt electrode (PtE) surface was polished with alumina slurries on microfiber cloth to obtain a mirror surface. After that, it was thoroughly rinsed with double distilled water and sonicated first in absolute ethanol and then in double distilled water for 10 min. to remove undesired adsorbed particles. At the

final step, the electrode was cleaned by taking twenty successive cyclic voltammetric sweeps between −1.0 and +1.0 V in the 0.1M HCI.

After the initial cleaning procedure, in order to form the conductive polyaniline layer on the cleaned platinum electrode, polyaniline was electrochemically grown on the bare Pt electrode from 0.1 M aniline solution (prepared in 0.1 M HCI) by taking eight cyclic voltammetric sweeps at a potential range between –0.3 and 1.0 V with a scan rate of 20 mVs^{-1}. After the electropolymerization, the PANI modified Pt electrode (PtE-PANI) was rinsed with double distilled water to remove unbounded molecules from the electrode surface. Then, the PtE-PANI was immersed into the % 1.0 glutaraldehyde (GA) solution (prepared in pH 7.5 Pi buffer) for 2 h at room temperature. After that, the PANI-GA modified Pt electrode was rinsed with double distilled water and dried by applying the nitrogen gas gently on the modified electrode surface. Finally, immobilization of the enzyme onto the PANI-GA modified Pt electrode was carried out as follows: 5 µL of the 1 mg mL^{-1}. Catalase enzyme was dropped on the PANI-GA modified PtE and left to dry. At the end of the process PANI-GA-CAT modified platin electrode has been obtained. Scheme 1 shows the prepartion steps of the biosensor.

Measurements

In order to choose the potential in which the amperometric detections have been carried out, cyclic voltammograms were carried out at a potential range between (– 0.75) and (+0.75) V by using PANI-GA-CAT modified Pt electrode in 50 mM pH 7.0 phosphate buffer. Differential pulse (DP) voltammograms were carried out at a potential range

between (0.0) – (– 0.6) V.–0.3 V potential which was attributed to reduction of polyaniline was chosen to be working potential for amperometric studies. Concentration of hydrogen peroxide was detected amperometrically at – 0.3 V potential by observing the differentiations in the current values.

RESULTS AND DISCUSSION

Immobilization

Electropolymerization of aniline in acidic solution yields to conductive polyaniline [12]. Hence, cyclic voltammograms were carried out at a potential range between -0.3 and 1.0 V by using bare Pt electrode in 0.1 M Aniline (prepared in 0.1 M HCI solution) to form the polyaniline conductive polymer on the Pt electrode surface. Fig. 1 shows the cyclic voltammograms appeared during the formation of the conductive polyaniline layer. Electropolymerization stage was followed by immersing of the electrode into the cross-linker agent, glutaraldehyde, to provide the covalent attachment between the enzyme and PANI.

After the immobilization of catalase on the PANI - GA modified Pt electrode, cyclic voltammograms were carried out at a potential range between – 0.75 and 0.75 V by using PANI-GA-CAT modified PtE. According to the voltammograms a clear cathodic peak current was monitored related to enzymatic reaction at nearly -0.3 V potential. So, amperometric measurements have taken at this potential.

Impedimetric characterization of the biosensor has also been described in order to demonstrate whether the immobilization procedure had been successful or not. For this purpose, impedimetric measurements with the Pt electrode at different stages of immobilization have been taken, including bare Pt electrode, PANI – GA modified Pt electrode and PANI – GA – CAT modified Pt electrode.

It can be obviously seen from Fig. 2, modification of Pt electrode with polyaniline has decreased its impedance dramatically and has enhanced its conductivity when compared with bare Pt electrode (1). Considering the curve (3), there was a little increasing in electrochemical impedance when compared with curve (2) and this outcome shows that immobilization of the enzyme onto PANI – GA modified Pt electrode is successful.

After the determination of the response potential of the PANI-GA-CAT modified PtE, amperometric measurements were taken to show the responses of the biosensor to the substrates. The amperometric current responses of the biosensor to the different concentrations of the substrate were clear, and the biosensor was appropriate for determining the amount of hydrogen peroxide usually added to the commercial milks up to 0.6 % to provide the sterilization.

Optimization Of The Bioactive Layer

To optimize the components of the bioactive layer of the biosensor some parameters such as number of scans during the electropolymerization , amount of enzyme, concentration

of aniline, duration time in glutaraldehyde and concentration of glutaraldehyde were investigated.

Effect Of Number Of Scans During The Electropolymerization On The Biosensor Responses

Numbers of scan employed during the electropolymerization was investigated. The results obtained from the experiments showed that in the case of 8 scans the current responses were the highest. The current responses obtained from the calibration curve of electropolymerization constructed with 11 numbers of scan were dramatically low because high thickness of polyaniline led to diffusion restrictions. According to the results, electropolymerization during the construction of polyaniline were carried out with 8 numbers of scan.

Effect Of Amount Of The Enzyme On The Biosensor Responses

Different amounts of enzyme were used in order to investigate the effect of catalase amount on the biosensor responses. In the case of 0.5 $mgmL^{-1}$ enzyme low current responses were obtained because the amount of the enzyme used was insufficient for high catalytic activity. In the case of both 1.0 $mgmL^{-1}$ and 2.0 $mgmL^{-1}$ enzyme, linearities of both calibration curves were acceptable but current responses of 1.0 $mgmL^{-1}$ were higher than that of 2.0 $mgmL^{-1}$. This can be explained that 2.0 $mgmL^{-1}$ enzyme amount showed a diffusion barrier for substrate diffusion into the bioactive layer of the biosensor. From these results, 1.0 $mgmL^{-1}$ enzyme concentration was chosen in the formation of the bioactive layer of the biosensor.

Effect Of Aniline Concentration On The Biosensor Responses

To investigate the effect of aniline concentration on the biosensor responses 0.05, 0.1 and 0.2 M aniline were used in the electropolymerization step. According to the results, the current responses and also linearities of the calibration curves were very low for both 0.05 and 0.2 M aniline concentration. 0.05 M aniline was not adequate for the formation of polyaniline layer, furthermore, 0.2 M aniline concentration led to undesired bondings between the amino groups of the polyaniline layer. Therefore, the regression coefficient and especially current responses obtained by using 0.1 M aniline were considerably higher from those of 0.05 and 0.2 M aniline. As a result, 0.1 M aniline concentration was chosen in the construction of the conductive polymer of the biosensor.

Effect Of Duration Time In Glutaraldehyde On The Biosensor Responses

For this purpose, PANI-modified Pt electrodes were crosslinked with 1.0 % glutaraldehyde solution for 1 h, 2 h and 4 h. From the results, it can be said that 1 h duration of the electrode in glutaraldehyde was not satisfactory in terms of both linearity of the calibration curve and current responses. Because, the duration time was not enough for glutaraldehyde to attach completely on the modified electrode surface and this lead to insufficient binding of catalase as well. Considering 2 h and 4 h durations, there was no big difference between the current responses of both electrodes. But the regression coefficient obtained in the case of 2h duration time was relatively higher than 4h duration . So, 2h was chosen to be optimum duration time in glutaraldehyde solution.

Effect Of Concentration Of Glutaraldehyde On The Biosensor Responses

To investigate the effect of glutaraldehyde concentration on the biosensor responses different concentrations of glutaraldehyde were used such as 0.5 %; 1.0 % and 2.0 % in the biosensor construction. According to the results, current responses observed in the case of 1.0 % glutaraldehyde concentration were better than those of 0.5 % and 2.0 %. The lowest current responses were obtained in the case of 2.0 % concentration, and this outcome is owing to the fact that high concentrations of glutaraldehyde lead to overmuch binding and consequently, give rise to some deformations on the enzyme molecules.

Optimization Of Working Conditions

Effect Of Working Temperature And Ph On The Biosensor Responses

The experiments were made by using DP method between (0.0) - (-0.7) V potentials vs. Ag/AgCl. To investigate the effect of working temperature on the biosensor responses experiments were made at 15, 20, 25, 30, 35 and 40 ^{0}C. Increasing in temperature from 15 ^{0}C to 30 ^{0}C resulted in increases in the biosensor responses. As the temperature increases from 30 ^{0}C to 40 ^{0}C decreases on the biosensor responses were observed related to the lower activities of the enzyme. From the results, 30 ^{0}C was chosen to be the working temperature. To investigate the effect of the working pH on the biosensor responses, experiments were carried out using 50 mM phosphate buffers with different pH values (pH 6.0 ; 6.5 ; 7.0 ; 7.5 and 8.0). From the experiments it can be said that the cathodic peak current increased with pH changes from 6.0 to 7.0, then, the biosensor responses decreased when the pH was further raised to 8.0. The maximum biosensor responses was obtained at pH 7.0, therefore, it was chosen as the optimal pH value for the

biosensor. This pH value was consistent with that reported for catalase enzyme [51]. If we consider the electrocatalytic effect of pH on the modified biosensor it can be said that optimum pHs of the free and immobilized enzymes does not differ much.

Cyclic Voltammetric Response Of Hydrogen Peroxide At PANI-GA-CAT Modified Biosensor

Cyclic voltammetric responses of different concentrations of hydrogen peroxide at the PANI-GA-CAT modified electrode are shown in Fig. 3. According to the figure, the cathodic peak currents vary linearly with the increase in concentration of hydrogen peroxide in the ranges from 5.0×10^{-6} and 100.0×10^{-6} M. Cathodic peaks were obtained in a potential range between -0.5 and -0.25 V potentials. This result was supported and also verified with DP measurement results shown in Fig. 3.

Analytical Characteristics Of The Biosensor

Linear Range For Hydrogen Peroxide

In order to detect the linear range for hydrogen peroxide experiments were made by using differential pulse (DP) and amperometric methods. Linear ranges for hydrogen peroxide were obtained between $5.0x10^{-6}$ M and $100.0x10^{-6}$ by amperometric method and $5.0x10^{-6}$ M and $75.0x10^{-6}$ by differential pulse method, respectively. DP voltammograms and amperometric results of hydrogen peroxide as functions of the concentration, with calibration straight line inserted, are shown in Fig. 4 and Fig. 5. Limit of detection of the biosensor for hydrogen peroxide was determined using the $3S_b/m$ where m is the slope of

the calibration curve and S_b is the standard deviation. From the calculations, limit of detection was found to be 2.18 µM.

Comparison Of The Analytical Performance Of The Biosensor

The analytical performance of the biosensor was compared with that of some other H_2O_2 biosensors (Table 1).

From the Table it can be said that all biosensors developed are sensitive for hydrogen peroxide. APTMS – colloidal Au modified ITO based peroxidase biosensor exhibited fast amperometric response (within 5 s) to H_2O_2 [52]. In another study, methlene green and HRP co-immobilized in montmorillonite-modified bovine serum albumin-glutaraldehyde matrix. This biosensor showed a wide linear range ($2.0 – 3000x10^{-6}$ M) for H_2O_2 [53]. The best detection limit for hydrogen peroxide was obtained by Cysteamine – Glutaraldehyde – Cysteamine – Colloidal gold modified gold electrode [54] but the best response time has been obtained by our biosensor.

Reproducibility

The reproducibility of the biosensor was determined for 50 µM hydrogen peroxide (n = 9). From the experiments, average value (*X*), standard deviation (SD) and coefficients of variation (CV %) were calculated to be 53.47 µM, ±1.66 µM, and 3.01 %, respectively.

Storage Stability Of The Biosensor

In order to investigate the storage stability of the biosensor, measurements were taken with the same electrode during 9 days. The biosensor was stored in the phosphate buffer (50 mM, pH: 7.0) at 4 ^{0}C when not in use. After the 9 days of storage in the phosphate buffer, 76.98 % of initial sensitivity to hydrogen peroxide was observed.

Real Sample Analysis

In order to demonstrate the application of the biosensor to real samples, the hydrogen peroxide concentrations in ultra-high-temperature (UHT) processed milk samples were amperometrically determined by the standard addition method. Milk samples were purchased from the local markets. Before the analyses, the milk samples were diluted with phosphate buffer (pH 7.0). According to the standard addition method, a known concentration of hydrogen peroxide was added to the diluted milk samples. Amperometric results obtained from the measurements were compared with those determined by the classical potassium permanganate titration method. This method utilizes the reduction of potassium permanganate ($KMnO_4$) by hydrogen peroxide in sulfuric acid. Before titration, 2.0 mL of 3.0 M H_2SO_4 was added to 2.0 mL of milk sample and centrifuged for 15 min, followed by filtration with a micropore membrane filter. The results obtained by the biosensor were in satisfactory agreement with those given by the titration method (Table 2)

From the results, it is obvious that hydrogen peroxide concentration was found higher with titration method than the biosensor. In addition, if we consider the standard

deviation and also coefficient of variance (CV %) values of the two methods it can be said that the biosensor is more sensitive than titration method.

In this study, an amperometric biosensor for the determination of hydrogen peroxide was described. In the biosensor construction, polyaniline electrochemically grown on Pt electrode surface and then catalase was immobilized on modified Pt electrode with the help of glutaraldehyde. Impedimetric characterization of the biosensor proved that the immobilization procedure was successful. In the cyclic voltammetric studies, -0.3 V was determined as the working potential, and amperometric measurements were carried out at this potential. Using the biosensor the hydrogen peroxide could be measured with high sensitivity. In addition, the hydrogen peroxide content of the milk samples was measured with the novel biosensor and the results obtained were compared with the titrimetric method.

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REFERENCES

[1] Gerard, M.; Chaubey, A.; Malhotra, B.D. Application of conducting polymers to biosensors, *Biosens. Bioelectron.* **2002**, *17*, 345–359.

[2] Mu, S.;Chen, C.; Wang, J.The kinetic behavior for the electrochemical polymerization of aniline in aqueous solution, *Synth Met.* **1997,** *88*, 249–254.

[3] Garjonyte, R.; Malinauskas, A. Amperometric glucose biosensors based on Prussian Blue– and polyaniline–glucose oxidase modified electrodes, *Biosens. Bioelectron.* **2000**, *15*, 445–451.

[4] Veal, E.A.; Day, A.M.; Morgan, B.A. Hydrogen peroxide sensing and signaling, *Mol. Cell* **2007**, *26,* 1–14.

[5] Giorgio, M.; Trinei, M.; Migliaccio, E.; et al. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev. Mol. Cell Biol.* **2007**, *8,* 722–728.

[6] González, D.; Bejarano, I.; Barriga, C.; et al. Oxidative Stress-Induced Caspases are Regulated in Human Myeloid HL-60 Cells by Calcium Signal, *Curr. Signal Trans. Therapy*, **2010,** *5***,** 181-186.

[7] Bejarano, I.; Espino, J.; González-Flores, D.; et al. Role of Calcium Signals on Hydrogen Peroxide-Induced Apoptosis in Human Myeloid HL-60 Cells, *Int. J.of Biomed. Sci*. **2009,** *5,* 246-256.

[8] López-Lázaro, M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy, *Cancer Lett*. **2007**, *252***,** 1–8.

[9] Pace, M.C.; Passavanti, M.B.; Aurilio, C. et al. Polydatin administration improves serum biochemical parameters and oxidative stress markers during chronic alcoholism: a pilot study, *In Vivo*. **2015**,*29*,405-408.

[10] Federico, A.; Dallio, M.; Di Fabio; G. et al. Silybin-Phosphatidylcholine Complex Protects Human Gastric and Liver Cells from Oxidative Stress, *In Vivo*. **2015**,29, 569-575.

[11] Stiuso, P.; Scognamiglio, I.; Murolo, M. et al. Serum oxidative stress markers and lipidomic profile to detect NASH patients responsive to an antioxidant treatment: a pilot study, *Oxid Med Cell Longev.* **2014**, *2014*, 1-8.

[12] Lamberti, M.;, Porto, S.; Marra, M. et al. 5-Fluorouracil induces apoptosis in rat cardiocytes through intracellular oxidative stress, *J.Exp Clin Cancer Res*. **2012**,*31*, 1-8.

[13] Finkel, T. and Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing, *Nature*, **2000,** *408*, 239–247.

[14] Yaffe, M.B. and Cantley, L. C. Signal transduction: Grabbing phosphoproteins, *Nature*, **1999,** *402*, 30–31.

[15] Scott, J. D. and Pawson, T. Cell signaling in space and time: where proteins come together and when they're apart. *Science*, **2009**, *326*, 1220–1224.

[16] Trachootham, D.; Alexandre, J. and P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach, *Nat. Rev. Drug Discovery*, **2009,** *8*, 579–591.

[17] Chang, M. C. Y. ; Pralle, A.; Isacoff, E.Y. et al. A selective, cell-permeable optical probe for hydrogen peroxide in living cells. *J. Am. Chem. Soc*., **2004**, *126*, 15392–15393.

[18] Lin, K.C.; Yin, C.Y.; Chen, S.M. An electrochemical biosensor for determination of hydrogen peroxide using nanocomposite of poly(Methylene Blue) and FAD hybrid film, *Sens Act B: Chem*. **2011**, *157*, 202-210.

[19] Hurdis, E.C. and Romeyn JR, H. Accuracy of determination of hydrogen peroxide by cerate oxidimetry, *Anal. Chem*. **1954,** *26*, 320-325.

[20] Brestovisky,A. and Kirowa-Eisner, E. Direct and titrimetric determination of hydrogen peroxide by reverse pulse polarography, *Anal. Chem*. **1983,** *55*, 2063-2066.

[21] Fernandes, K. F.; Lima, C. S.; Lopes, F. M.; et al. Hydrogen peroxide detection system consisting of chemically immobilized peroxidase and spectrometer, *Proces. Biochem* **2005,** *40*, 3441–3445.

[22] Janasek, D.; Spohn, U. An enzyme-modified chemiluminescence detector for hydrogen peroxide and oxidase substrates, *Sens Act B: Chem*. **1997**; *(38-39)*, 291-294.

[23] Pehrman, R. and Amme, M. Comparison of chemiluminescence methods for analysis of hydrogen peroxide and hydroxyl radicals, *Czec. J. Phy.* **2006**, *56*, D373-D379.

[24] Garcia, M. A. V.; Blanco, P. T.; Ivaska, A. A poly(o-aminophenol) modifed electrode as an amperometric hydrogen peroxide biosensor, *Elec. Chim. Acta* **1998**, *43*, 3533-3539.

[25] Nakabayashi, Y.; Yoshikawa, H. Amperometric Biosensors for sensing of hydrogen peroxide based on electron transfer between horseradish peroxidise and ferrocene as a mediator, *Anal. Sci.* **2000**, *16*, 609-613.

[26] Wang, B.; Zhang, J.; Cheng, G.; et al. Amperometric enzyme electrode for the determination of hydrogen peroxide based on sol–gel/hydrogel composite film, *Anal. Chim. Acta* **2000,** *407*,111–118.

[27] Jia, J.; Wang, B.; Wu, A. et al. A method to construct a third-generation horseradish peroxidase biosensor: Self-assembling gold nanoparticles to three-dimensional sol-gel network, *Anal. Chem.*, **2002,** *74*, 2217-2223.

[28] Liu, S.Q. and Ju, H.X., Renewable reagentless hydrogen peroxide sensor based on direct electron transfer of horseradish peroxidase immobilized on colloidal gold-modified electrode, *Anal. Biochem.* **2002**, *307*, 110-116.

[29] Xu, Q.; Mao, C.; Liu, N. et al. Direct electrochemistry of horseradish peroxidase based on biocompatible carboxymethyl chitosan-gold nanoparticle nanocomposite, *Biosens. Bioelectron.* **2006**, *22*, 768-773.

[30] Shan, D.; Li, Q.B.; Ding, S.N. et al. Reagentless biosensor for hydrogen peroxide based on self-assembled films of horseradish peroxidase/laponite/chitosan and the primary investigation on the inhibitory effect by sulfide , *Biosens. Bioelectron*. **2010**, *26*, 536-541.

[31] Mathew, M. and Sandhyarani, N. A novel electrochemical sensor surface for the detection of hydrogen peroxide using cyclic bisureas/gold nanoparticle composite, *Biosens. Bioelectron.* **2011**, *28*, 210-215.

[32] Wei, N.; Xin, X.; Du, J.,et al. A novel hydrogen peroxide biosensor based on the immobilization of hemoglobin on three-dimensionally ordered macroporous (3DOM) gold-nanoparticle-doped titanium dioxide (GTD) film, *Biosens. Bioelectron*. **2011**, *26*, 3602-3607.

[33] Shen, J.; Yang, X.; Zhu, Y. et al. Gold-coated silica-fiber hybrid materials for application in a novel hydrogen peroxide biosensor , *Biosens. Bioelectron.* **2012**, *34*, 132-136.

[34] Radhakrishnan, S. and Kim, S.J. An enzymatic biosensor for hydrogen peroxide based on one-pot preparation of CeO_2-reduced graphene oxide nanocomposite. *RSC Adv.*, **2015**, *5*, 12937-12943.

[35] Abbas, M.E.; Luo, W.; Zou, J. et al. Fluorometric determination of hydrogen peroxide in milk by using a Fenton reaction system, *Food Chem*., **2010**, *120*, 327–331.

[36] Alpat, S.; Kilinc, S.; Dursun, Z. et al. Development of a new biosensor for mediatorless voltammetric determination of hydrogen peroxide and its application in milk samples, *J.Appl. Electrochem.*,**2009**, *39*, 971-977.

[37] Silva, R A.B.; Rodrigo, H.O.M.; Eduardo, M. et al. Rapid and selective determination of hydrogen peroxide residues in milk by batch injection analysis with amperometric detection, *Food Chem.* **2012**,*133*, 200-204.

[38] Campuzano, S.; Pedrero, M.; Pingarrón, J.M. .A peroxidase-tetrathiafulvalene biosensor based on self-assembled monolayer modified Au electrodes for the flow-injection determination of hydrogen peroxide. *Talanta.* **2005**, *66*, 1310-1319.

[39] Liang, K.Z. and Mu, J.W. ZrO_2/DNA-derivated polyion hybrid complex membrane for the determination of hydrogen peroxide in milk. Ionics, 2008, 14, 533-536.

[40] Sichak, S. P. and Dounce, A. L. Analysis of the peroxidatic mode of action of catalase, *Arc. Biochem. Biophys.* **1986,** *249*, 286-295.

[41] Putnam, C. D.; Arvai1, A. S.; Bourne, Y.; et al. Active and Inhibited Human Catalase Structures: Ligand and NADPH Binding and Catalytic Mechanism, *J. Mol. Biol.* **2000,** *296*, 295-309.

[42] Prajapati, S.; Bhakuni, V.; Babu, K. R.;et al. Alkaline unfolding and salt-induced folding of bovine liver catalase at high pH, *Euro. J. Biochem.* **1998**, *255*, 178-184.

[43] Choi, M.M.F.; Yiu, T. P. Immobilization of beef liver catalase on eggshell membrane for fabrication of hydrogen peroxide biosensor, *Enz. Micro. Technol.* **2004,** *34*, 41–47.

[44] Akgöl, S. and Dinçkaya, E. A novel biosensor for specific determination of hydrogen peroxide: catalase enzyme electrode based on dissolved oxygen probe, *Talanta* **1999,** *48*, 363–367.

[45] Ertaş, N.; Timur, S.; Akyilmaz, E.; et al. Specific determination of hydrogen peroxide with a catalase biosensor based on mercury thin film electrodes, *Turk. J. Chem* **2000,** *24*, 95 – 99.

[46] Dandan, C.; Baohong, L.; Zhengjiu, L.; et al. An amperometric biosensor for hydrogen peroxidase based on the co-immobilization of catalase and methylene blue in an Al_2O_3 sol-gel modified electrode, *Anal. Lett.* **2001,** *34*, 687 – 699.

[47] Varma, S. and Mattiasson, B. Amperometric biosensor for the detection of hydrogen peroxide using catalase modified electrodes in polyacrylamide, *J.Biotechnol.* **2005**, *119,* 172–180.

[48] Akyilmaz, E. and Dinçkaya, E. Development of a catalase based biosensor for alcohol determination in beer samples, *Talanta* **2003**, *61*, 113-118.

[49] Salimi, A.; Noorbakhsh, A.; Ghadermarzi, M. Amperometric detection of nitrite, iodate and periodate at glassy carbon electrode modified with catalase and multi-wall carbon nanotubes, *Sens. Act. B: Chem.* **2007**, *123*, 530–537.

[50] Akyilmaz, E. and Kozgus, O. Determination of calcium in milk and water samples by using catalase enzyme elecytrode, *Food Chem.* **2009**, *115*, 347–351.

[51] P. Rahimi, H.; Rafiee-Pour, H.; Ghourchian, P. et al. Ionicliquid/NH2-MWCNTs as a highly sensitive nano-composite for catalase direct electrochemistry, *Biosens. Bioelectron.* **2010**, *25*, 1301-1306.

[52] Wang, L. and Wang, E. A novel hydrogen peroxide sensor based on horseradish peroxidase immobilized on colloidal Au modified ITO electrode, *Electrochem. Commun* . **2004**, *6*, 225–229.

[53] Lei, C. and Deng, J. Hydrogen Peroxide Sensor Based on Coimmobilized Methylene Green and Horseradish Peroxidase in the Same Montmorillonite-Modified Bovine Serum Albumin-Glutaraldehyde Matrix on a Glassy Carbon Electrode Surface, *Anal. Chem.* **1996,** *68*, 3344-3349.

[54] Xiao, Y.; Ju, H.-X.; Chen, H.-Y. Hydrogen peroxide sensor based on horseradish peroxidase-labeled Au colloids immobilized on gold electrode surface by cysteamine monolayer, *Anal. Chim. Acta*,**1999**, *391*, 73-82.

Table 1. Comparison of performances of hydrogen peroxide biosensors.

Method	**Linear range (mol/L)**	**Detection limit (mol/L)**	**Response time (s)**	**Reference**
Beef liver catalase – egg shell membrane coated oxygen electrode	No data	3×10^{-6}	60	[43]
Catalase – Gelatin coated oxygen electrode modified with glutaraldehyde	$(1 – 300) \times 10^{-5}$	No data	30	[44]
HRP immobilized onto APTMS – colloidal Au modified ITO	$(2 – 800) \times 10^{-5}$	8×10^{-6}	5	[52]
Methlene green and HRP coimmobilized in the same montmorillonite-modified bovine serum albumin (BSA)-glutaraldehyde matrix	$(2 – 3000) \times 10^{-6}$	4×10^{-7}	20	[53]
HRP immobilized to Cysteamine – Glutaraldehyde – Cysteamine – colloidal Au modified Au electrode	$(3.9 – 3300) \times 10^{-7}$	1.5×10^{-7}	5	[54]
Catalase immobilized onto PANI-GA modified Pt electrode	$(5- 100) \times 10^{-6}$	2.18×10^{-6}	3	Present work

Table 2. H_2O_2 determination in milk samples by using the biosensor and the reference method.

Method	Added Hydrogen Peroxide (µM)	Found Hydrogen Peroxide by the biosensor (µM)[a]	Standard Deviation (S.D.) (µM) [b]	Coefficient of Variance (C.V. %) [b]	Found Hydrogen Peroxide by titration method (µM)[a]	Standard Deviation (S.D.) (µM) [c]	Coefficient of Variance (C.V. %) [c]
Milk 1	57.9	58.7	± 0.652	1.11 %	62.7	± 1.624	2.59 %
Milk 2	57.9	59.4	± 0.829	1.39 %	63.8	± 1.862	2.92 %

[a] *Averages of obtained five measurements*

[b] *for biosensor*

[c] *for titration method*

Scheme 1. Preparation procedure of the biosensor (Akyilmaz E. et al.)

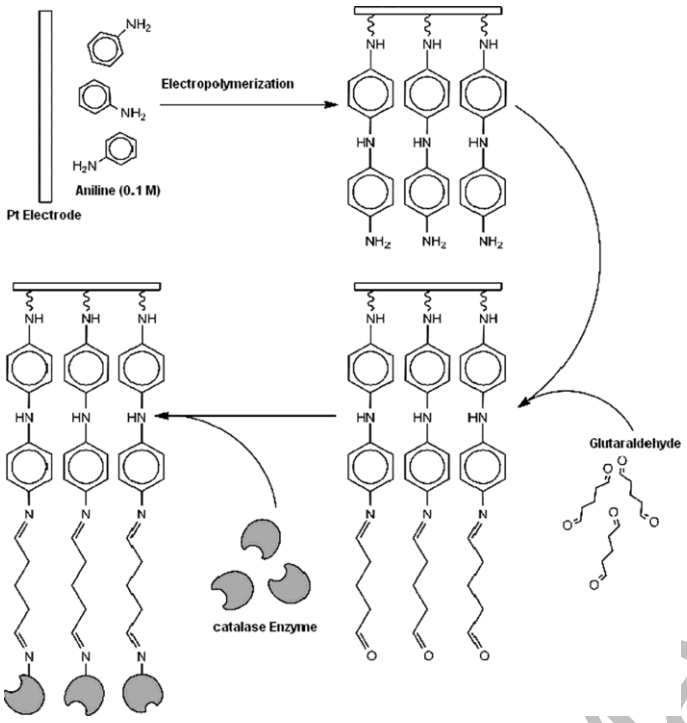

Figure 1. Cyclic voltammograms during electropolymerization of aniline on the Pt electrode. PANI was electropolymerized after 8 cycles from 0.1 M aniline solution (prepared in 0.1 M HCI solution) at a scan rate of 20 mV s^{-1} in the potential range -0.3 – 1.0 V.

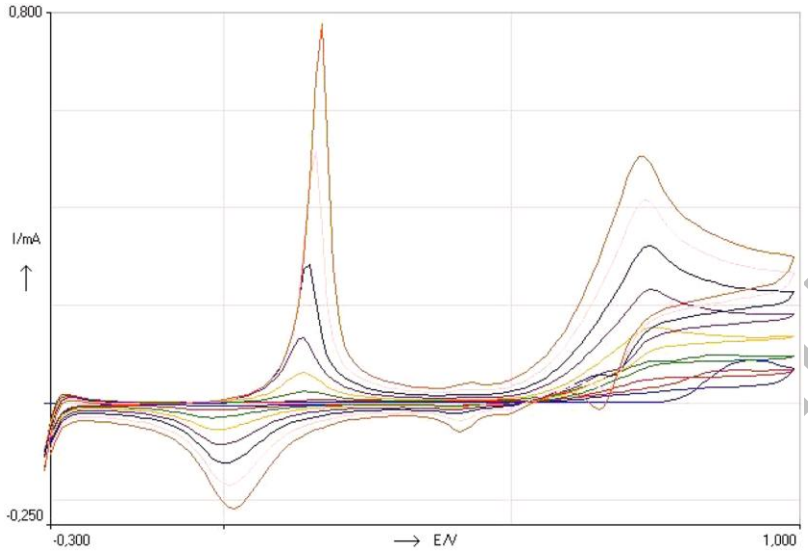

Figure 2. Impedimetric characterization of the biosensor at different stages of immobilization. (1) Bare Pt electrode; (2) PANI – GA modified Pt electrode and (3) PANI – GA– CAT modified Pt electrode. In phosphate buffer solution (50 mM, pH 7.4) with 5 mM $Fe(CN)_6^{3-/4-}$.

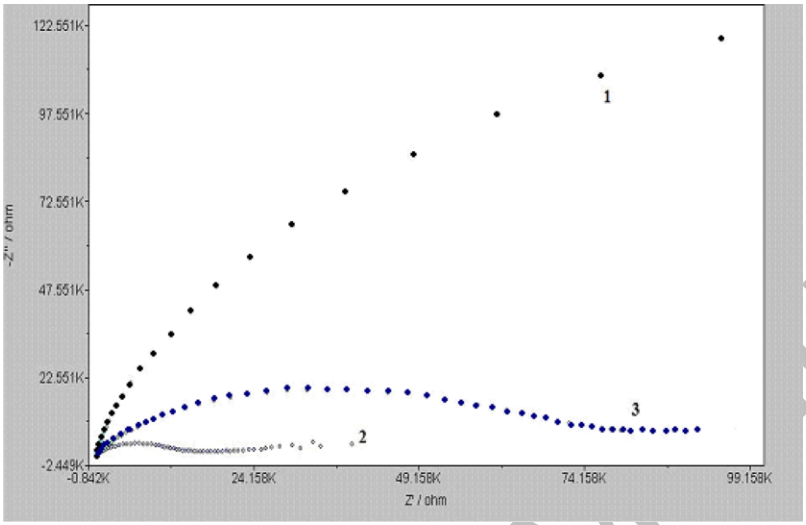

Figure 3. Cyclic voltammograms of different concentrations of H_2O_2; a) 5.0×10^{-6}, b) 10.0×10^{-6}, c) 30.0×10^{-6}, d) 60.0×10^{-6} and e) 100.0×10^{-6} M. . In phosphate buffer solution (50 mM, pH 7.0) vs Ag/AgCl with a scan rate of 50 mV.

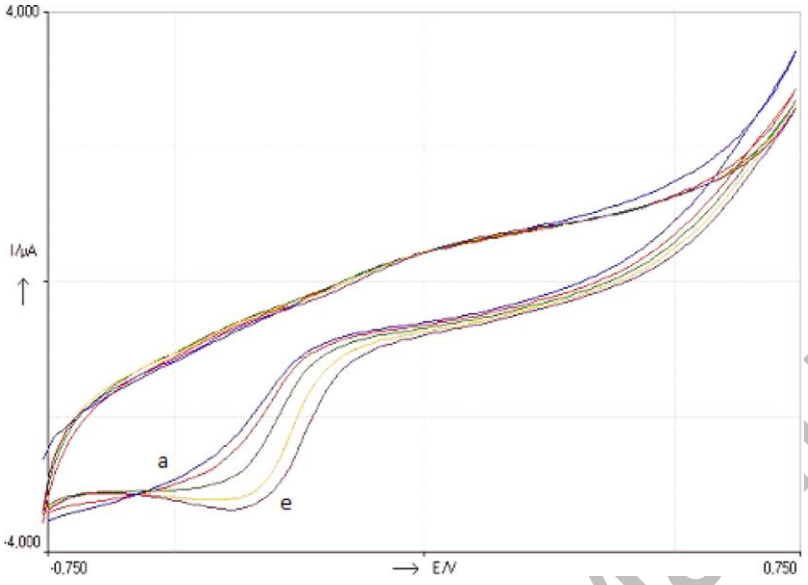

Figure 4. DP voltammograms and corresponding calibration curve (inset) of various concentrations of H_2O_2 .Concentration (curve a–f): 0 , 5.0×10^{-6}, 10.0×10^{-6}, 25.0×10^{-6}, 50.0×10^{-6} and 75.0×10^{-6} M. . In phosphate buffer solution (50 mM, pH 7.0) vs Ag/AgCl

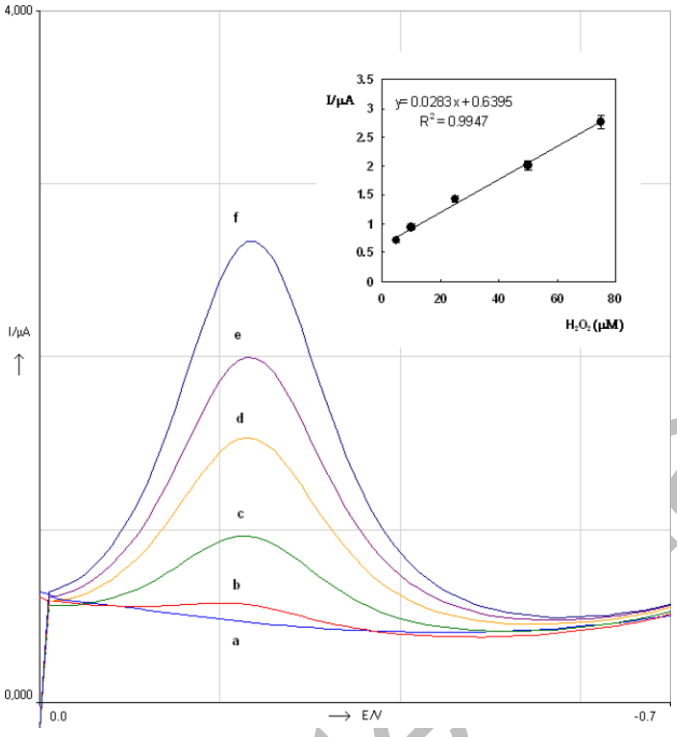

Figure 5. Amperometric measurements and corresponding calibration curve (inset) of various concentrations of H_2O_2 .Concentration (curve a–f): 5.0×10^{-6}, 10.0×10^{-6}, 25.0×10^{-6}, 50.0×10^{-6}, 75.0×10^{-6} and 100.0×10^{-6} M.