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Amperometric biosensing of ethanol based on integration of alcohol dehydrogenase with a Pt/PPy–PVS/MB electrode

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Abstract: A novel amperometric ethanol biosensor with alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD^+) on polypyrrole–polyvinylsulfonate (PPy-PVS) film was accomplished. Meldola's blue (MB) was used as a mediator; it can facilitate the electron transfer that involves a chemical interaction between the electrode surface and NADH. Determination of ethanol was carried out by the oxidation of MB at –0.072 V vs. Ag/AgCl. The effects of pH and temperature were investigated and optimum parameters were found to be 7.0 and 40 °C, respectively. There are 2 linear parts at 1.0 μM–10.0 μM ($R^2 = 0.993$) and 0.01 mM–0.1 mM ($R^2 = 0.996$). The storage stability and operational stability of the enzyme electrode were also studied. The results showed that 95.7% of the response current was retained after 17 activity assays. The ethanol biosensor gave perfect reproducible finding with 4.6% relative standard deviation. The prepared biosensor retained 55.8% of initial activity after 24 days when stored in 0.1 M phosphate buffer solution at 4 °C. Effects of the several possible interfering substances such as ascorbic acid, citric acid, and methanol on the ethanol biosensor were investigated and the developed biosensor was tested in alcoholic beverages.

Key words: Ethanol, alcohol dehydrogenase, biosensor, polypyrrole, polyvinylsulfonate

1. Introduction

Ethanol is the most common toxic compound consumed by humans involved in clinical diagnostic analyses such as blood, serum, and urine analysis and it is frequently a contributory factor in a variety of accidents.[1–3] The determination of alcohol content for control of the fermentation process and product quality and freshness is crucial in the food and beverage industries.[4,5] Besides these applications, determination of ethanol is also important in agricultural and environmental analyses.[5,6] Finally, the detection and quantification of ethanol with high accuracy, sensitivity, and selectivity in a variety of samples has received much attention, due to its toxicological and psychological effects.[7]

Various analytical methods have been reported for the sensing of ethanol, including gas chromatography (GC),[8] GC/mass spectroscopy (MS),[9] high performance liquid chromatography,[10] capillary electrophoresis,[11] Raman spectrometry,[12] enzymatic colorimetric method,[13] and refractometry.[14] However, these methods are relatively expensive, time consuming, complex to perform, and require laborious sample pretreatment. An alternative approach to facilitate the analyses could be by biosensors, especially those employing amperometric biosensors, which have been regarded as promising because of their high selectivity, sensitivity, simplicity, portability, relative low cost of construction and storage, potential for miniaturization, and fast analysis.[1,5,15]

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The 2 kinds of enzymes majorly used for the construction of amperometric biosensors for ethanol detection are alcohol dehydrogenase and alcohol oxidase (AOD).[2] Almost all AOD-based ethanol biosensors depend on the monitoring of O_2 consumption or H_2O_2 formation.[6] In an ADH-based biosensor, the enzyme catalyzes the oxidation of ethanol to acetaldehyde in the presence of NAD^+ and reduction of NAD^+ to NADH, which can be oxidized by releasing electrons and protons and can be detected amperometrically.[1] ADH biosensors have some important properties for ethanol monitoring, such as not being oxygen dependent and being more selective to ethanol.[1,3] However, ADH-based biosensors have some drawbacks, including the fact that they are unstable[1] and the direct electrochemical oxidation of NADH on a bare electrode needs a considerable overpotential and often occurs only at potentials higher than 0.4 V vs. a Ag/AgCl reference electrode depending on the surface properties of the electrode.[16] Great attention has been focused on decreasing the overpotential by using a mediator as a suitable electrocatalyst that can facilitate the electron transfer between the cofactor of redox enzymes and electrodes.[17] As a result, when mediators are used for construction of biosensors, the overpotential is decreased and fouling of the electrode is prevented and since the working potential is decreased, the number of possible interferences is also reduced, permitting a higher selectivity.[18,19]

Many different compounds have been used as redox mediators such as quinones,[20] fluorenones,[21] organic salts,[22] organometallic complexes,[23] NAD-oxidation products,[24] conducting polymers,[25] adenine derivatives,[26] phenazines,[27] phenothiazines,[28] or phenoxazines.[29] Being the most suitable mediator for facilitating NADH oxidation, Meldola's blue (MB) has been extensively used.[30]

Conducting polymers have been widely used for construction of biosensors since they can be used as both immobilization matrices and redox systems for the transfer of electrical charge.[31] Polypyrrole is one of the most commonly employed conducting polymers for biosensor applications, because it has good biocompatibility and environmental and chemical stability, and high conductivity and can be easily polymerized.[32] Polyvinylsulfonate is a porous polymer widely used as support material for composite membranes and can increase the conductivity, stability, and mechanical strength of the PPy matrix.[33]

In the present study, we report a new ADH and MB based amperometric ethanol biosensor for the determination of ethanol. ADH and NAD^+ were immobilized onto a PPy-PVS film surface by cross-linking with glutaraldehyde (GA) and bovine serum albumin (BSA). The amperometric response was based on the electrocatalytic properties of MB to oxidize NADH, which was generated in the enzymatic reaction of ethanol with NAD^+ by catalysis of ADH. Optimum working conditions of the biosensor with respect to the substrate concentration, pH, and temperature were investigated. The storage stability and operational stability of the biosensor were also studied.

2. Results and discussion

We report a new ADH and MB based amperometric biosensor including NAD^+ for determination of ethanol. We used as a mediator, MB, a phenoxazine dye, increasing the rate of electron transfer with NADH. This approach allowed us not only to shift the equilibrium of the enzymatic reaction to the product side but also to decrease the overpotential for the oxidation of NADH. Ethanol was determined by measuring the anodic current of MB on the modified electrode surface. The amperometric response was based on the electrocatalytic properties of MB to oxidize NADH, which was produced in the enzymatic reaction of ethanol with NAD^+ under catalysis of ADH (Figure 1). The changes in anodic current at –0.072 V vs. Ag/AgCl produced by MB are proportional to the alcohol concentration changes in the sample. The parameters affecting the performance of the biosensor and optimum working conditions were investigated.

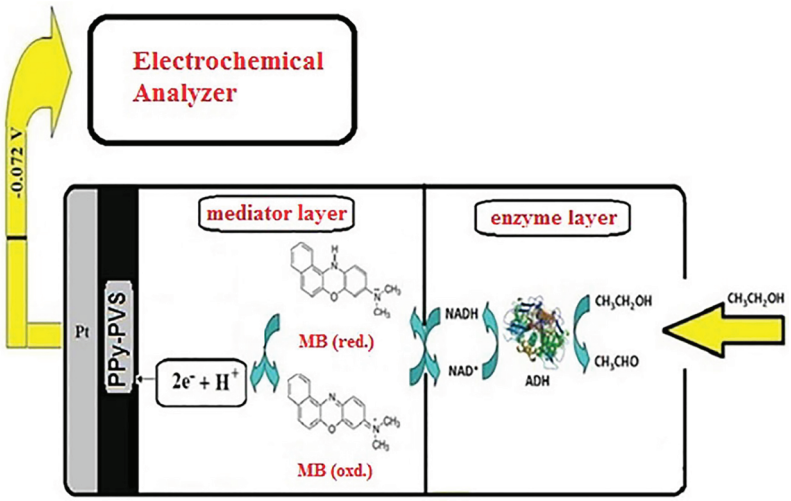

Figure 1. Reaction scheme for ethanol determination.

2.1. Surface morphological characterization using SEM

Scanning electron microscopy (SEM) was used to observe the surface changes of the Pt/PPy-PVS film (Figure 2a) when the MB (Figure 2b) and the MB, enzyme, and NAD^+ (Figure 2c) were immobilized. The surface morphology of the Pt/PPy-PVS/MB/ADH-NAD^+ (Figure 2c) film was completely different compared to the films produced in the absence of ADH and NAD^+ (Figures 2a and 2b). It can be seen that the surface morphology of the PPy-PVS film is a cauliflower-like structure (Figure 2a).[33] For the Pt/PPy-PVS/MB/ADH-NAD^+ film, the cauliflower-like structure was significantly damaged (Figure 2c). Figure 3 presents the elemental compositions of Pt/PPy-PVS (Figure 3a) and Pt/PPy-PVS/MB film electrodes (Figure 3b) as obtained from energy-dispersive X-ray spectroscopy (EDS) analysis. As can be seen in Figures 3a and 3b, peaks for Pt corresponding to the platinum electrode were observed. In the EDS results, the signature peak for Zn (Figure 3b) confirmed that the Pt/PPy-PVS was coated by MB.

2.2. Determination of working potential

After preparing the Pt/PPy-PVS electrode, differential pulse voltammetry (DPV) was applied between –0.6 V and 0.6 V using varying concentrations (0.25 mM, 0.5 mM, 0.75 mM) of MB for identification of the peak of oxidation of the MB. The oxidation peak of MB was observed at –0.072 V (Figure 4). In the following studies –0.072 V was taken as the working potential.

2.3. Obtained Pt/PPy-PVS/MB electrode's sensitivity to ethanol

After preparing the Pt/PPy-PVS electrode, its sensitivity to ethanol was investigated using DPV between –0.4 V and 0.4 V. Ethanol solutions were added to the cell containing MB (5 mM), alcohol dehydrogenase (270 U), and 0.1 M phosphate buffer at a pH of 7.0. Following the addition of ethanol solutions (0.25 mM, 0.5 mM, 0.75 mM) and NAD^+ solutions (0.25 mM, 0.5 mM, 0.75 mM) simultaneously, the obtained increase in the anodic peak currents can also be seen on the corresponding differential pulse voltammograms (Figure 5).

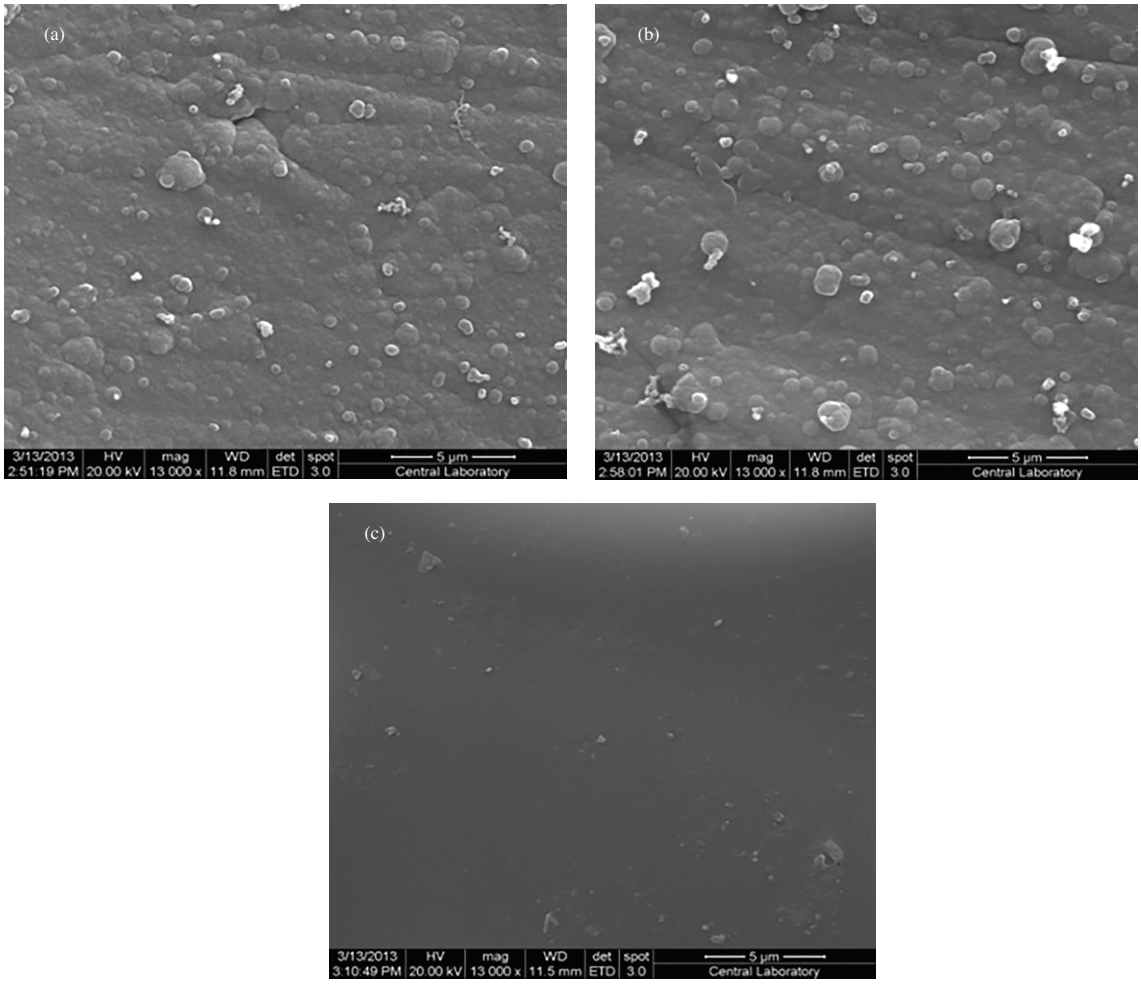

Figure 2. Scanning electron micrograph of electrode surfaces (**a**) Pt/PPy-PVS, (**b**) Pt/PPy-PVS/MB, (**c**) Pt/PPy-PVS/MB/ADH-NAD^+.

2.4. Effect of pH

Since enzyme activity depends on the ionization state of the amino acids in the active site, pH plays an important role in maintaining the proper conformation of an enzyme.[34] The effect of pH on the response of the ethanol biosensor was determined in 0.1 M phosphate buffer at a pH range of 6.0–9.0. The effect of pH on the behavior of the enzyme electrode was studied by controlled potential electrolysis (CPE) with 0.1 M phosphate buffer solution containing 0.1 mM ethanol. Figure 6 shows that the maximum response was obtained at pH 7.0. For the ethanol biosensor, pH values employed in the literature were 7.0,[5,30] 7.5,[2] and 7.2.[35]

2.5. Effect of temperature

Temperature has a great effect on enzyme activity and it is important to investigate temperature's dependence on the response of the enzyme electrode. Temperature's influence on the response of the ethanol enzyme electrode was tested by CPE between 20 °C and 50 °C at pH 7.0 using a constant ethanol concentration of 0.1 mM. As illustrated in Figure 7, current response of the biosensor gradually increased with increasing temperature and reached a maximum at 40 °C. For the ethanol biosensor, temperature values employed in the

literature were 25 °C[30] and 40 °C.[5] In terms of practical usage of the biosensor, 25 °C was chosen as the working temperature for all further experiments.

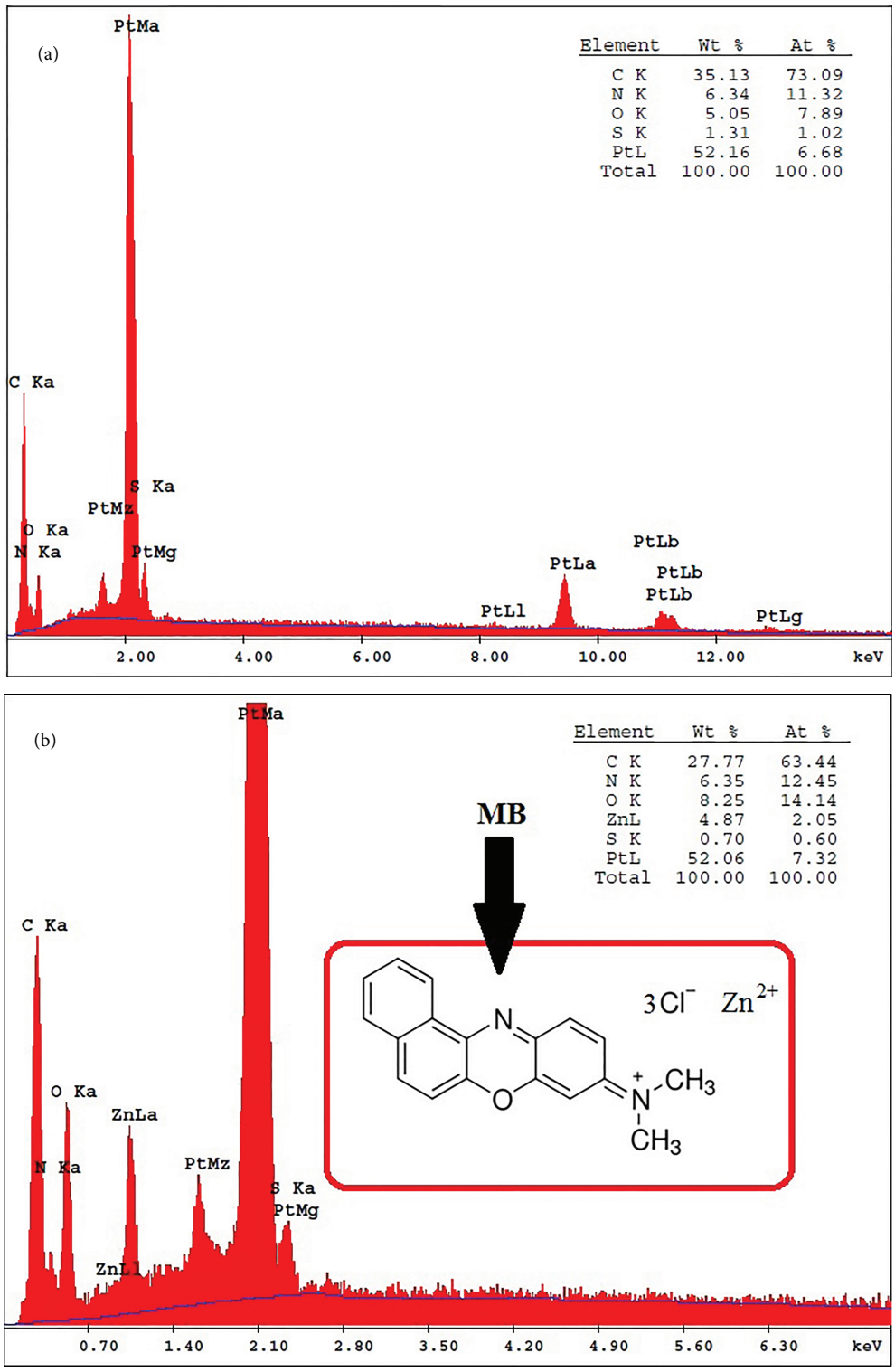

Figure 3. Elemental analysis of electrode surfaces with EDS (**a**) Pt/PPy-PVS, (**b**) Pt/PPy-PVS/MB.

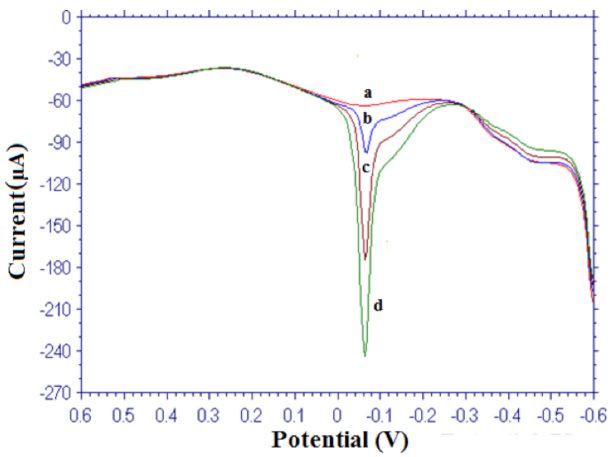

Figure 4. The differential pulse voltammogram of the Pt/PPy-PVS electrode **(a)** in the absence of MB and in the presence of **(b)** 0.25 mM, **(c)** 0.5 mM, **(d)** 0.75 mM MB, in 0.1 M phosphate buffer pH 7.0.

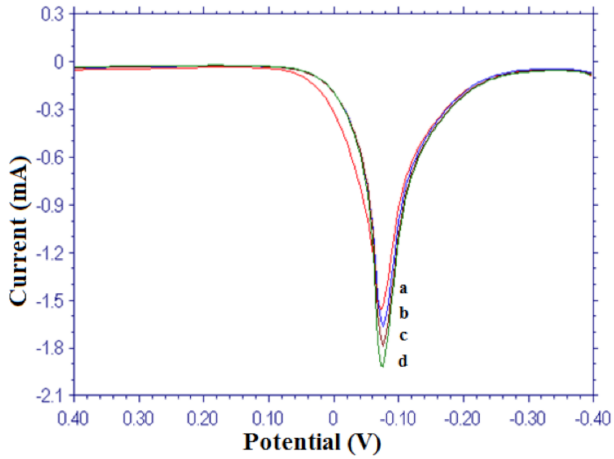

Figure 5. The differential pulse voltammogram of the Pt/PPy-PVS electrode **(a)** in the absence of ethanol and NAD^+, presence of MB (5 mM), in the presence of **(b)** 0.25 mM, **(c)** 0.5 mM, **(d)** 0.75 mM ethanol and NAD^+ in 0.1 M phosphate buffer pH 7.0.

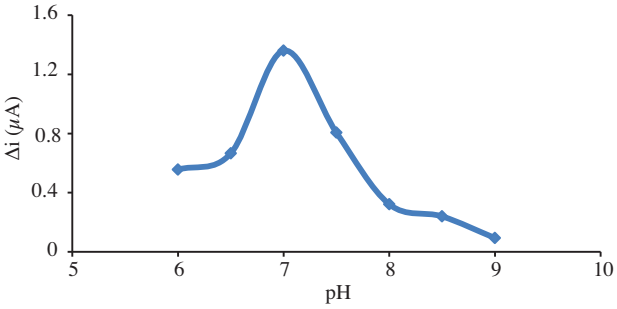

Figure 6. The effect of pH on the response of the biosensor (phosphate buffer, 0.1 mM ethanol at –0.072 V operating potential, 25 °C).

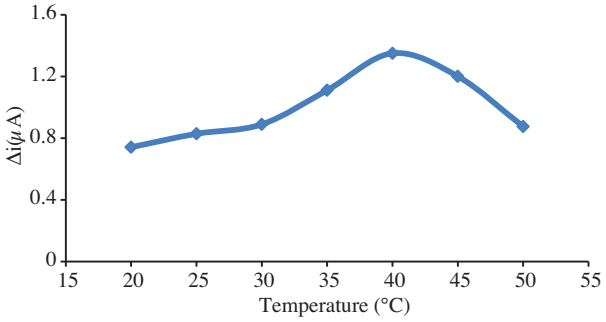

Figure 7. The effect of temperature on the response of the biosensor (at pH 7.0, 0.1 mM ethanol at –0.072 V operating potential).

2.6. Substrate concentration and calibration curves

In order to determine the linear working range of the ethanol biosensor, the current values obtained from solutions containing 1.0 μM–1.0 mM ethanol were recorded. There are 2 linear parts in the region at 1.0 μM–10.0 μM (R^2 = 0.993) (Figure 8) and 0.01 mM–0.1 mM (R^2 = 0.996) (Figure 9). When compared with the literature (1 mM–5 mM and 1.0 μM–30 μM, respectively, for Luo et al.[36] and Hasebe et al.[37]), the linear working range of the biosensor is wider. The low detection limit of the biosensor was found to be 0.1 μM and its response time was 200 s. The detection limit of the biosensor is lower than values in the literature (11 μM and 30 μM, respectively, for Luo et al.[36] and Wen et al.[38]). Because the prepared ethanol biosensor has a wide linear working range, this range includes low concentrations, and the determination limit is low, ethanol determination can be conducted with a variety of different samples with our biosensor.

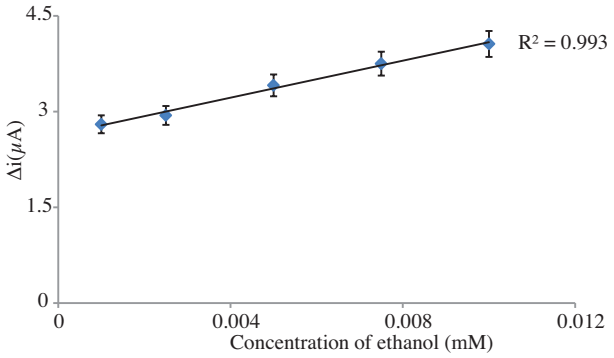

Figure 8. The calibration curve of the ethanol biosensor ranging between 1.0 μM and 10.0 μM ethanol (at 0.1 M, pH 7.0 phosphate buffer, 25 °C).

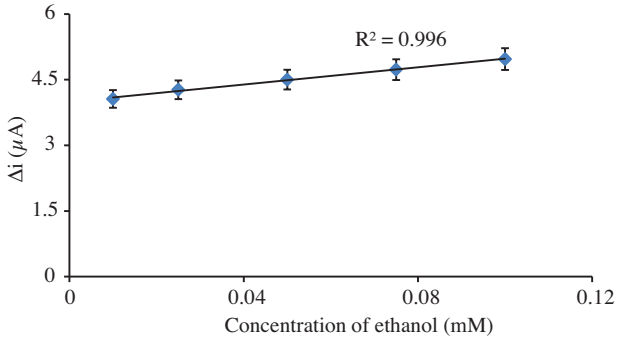

Figure 9. The calibration curve of the ethanol biosensor ranging between 0.01 mM and 0.1 mM ethanol (at 0.1 M, pH 7.0 phosphate buffer, 25 °C).

2.7. Operational stability and storage stability of the ethanol biosensor

The stability of an enzyme is of significant importance for scheduling its application in a particular reaction. In general, an enzyme is not stable in aqueous solution during storage and the activity is gradually reduced.[39] Immobilized enzymes have enhanced stability compared to soluble enzymes, and can easily be separated from the reaction. Long-term stability is one of the significant features required for the satisfactory application of a biosensor. The biosensor was used at optimum activity conditions in 17 activity assays in 1 day to determine the operational stability. The response of the ethanol biosensor exhibited good reproducibility with a relative standard deviation (RSD) of 4.57% at constant ethanol concentration of 0.1 mM (Figure 10). Storage stability of the biosensor was determined by performing activity assays over 24 days. The response of the enzyme electrode prepared under optimum conditions was measured for a period of 24 days at constant ethanol concentration (0.1 mM). An activity loss of 44.16% was observed on day 24 (Figure 11). These results as to both the storage and operational stability of the sensor are extremely promising for assembly of an ethanol biosensor.

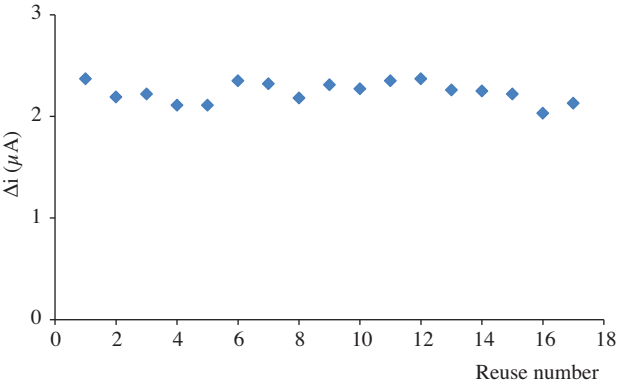

Figure 10. Operational stability of the biosensor in pH 7.0 phosphate buffer, at a –0.072 V operating potential, 25 °C.

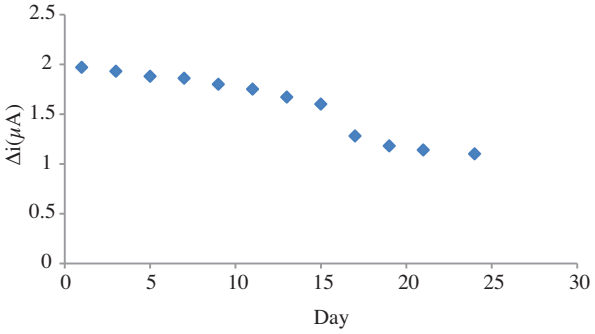

Figure 11. Storage stabilization of the biosensor in pH 7.0 phosphate buffer, at a –0.072 V operating potential, 25 °C.

2.8. Effect of interferences

Electroactive substances, such as methanol, ascorbic acid, and citric acid, can cause problems in the accurate determination of ethanol. The influences of interferences on determination of ethanol such as ascorbic acid,

uric acid, paracetamol, citric acid, methanol, n-butanol, propanol, and isopropyl alcohol were investigated at 0.1 mM ethanol concentration. No interference effect of ascorbic and uric acid was observed but paracetamol and citric acid showed some slight interferences (Figure 12). For a biosensing system, the operation potential range of –100 to 0 mV is regarded as the ideal region in which most electroactive biological substances do not interfere.[5,40] The use of a low operating potential (–0.072 V (vs. Ag/AgCl)) greatly reduced the interference, and thus a highly selective response to ethanol was achieved without the use of a permselective membrane. However, an interference effect of other alcohols, mostly methanol, was observed since the enzymatic reaction of a biosensor involves the oxidation of the alcohol class of substrates to their corresponding aldehydes (Figure 12). This can be minimized by increasing the selectivity of the biosensor in the following studies. Moreover, it is known that methanol and other alcohols are present in very small amounts in alcoholic beverages.

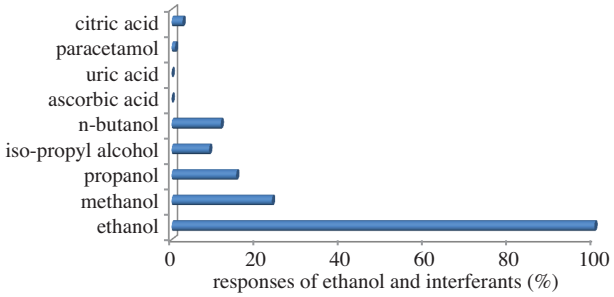

Figure 12. Effect of interferences to amperometric response of ethanol.

2.9. Determination of ethanol in alcoholic beverages

To investigate its practical ability, the developed biosensor was employed for the determination of ethanol in commercial alcoholic beverages, i.e. beer, vodka, and white wine, under optimized conditions (Table 1). Samples of alcoholic beverages were diluted with 0.1 M phosphate buffer solution (pH 7.0) depending on their ethanol concentration. Analyses were carried out by amperometric measurements in stirred solution at potential of –0.072 V vs. Ag/AgCl. In order to eliminate possible matrix effects, determinations were performed using the standard additions method. The results obtained with the biosensors showed a good correlation with those certified by the suppliers despite the potential interferences, which are normally found in real samples, and allowed us to ascertain the practical applicability of the proposed biosensor.

Table 1. Results obtained for ethanol determination in beverage samples using the Pt/PPy-PVS/MB/ADH-NAD^+ based biosensor.

Beverage samples	Label indicated alcohol content (% v/v)	Determined value[a] (% v/v)	Relative Deviation (%)
Beer	5.00	5.17 ± 0.03	3.40
Vodka	37.50	38.5 ± 0.41	2.67
White wine	12.00	11.89 ± 0.06	0.92

[a]Average of 3 measurements

In the present study, a strategy was employed to develop a new ADH and MB based amperometric biosensor for the determination of ethanol. An amperometric ethanol biosensor with immobilization of ADH and NAD^+ on PPy-PVS film was accomplished. MB was used as the mediator and the oxidation potential of

NADH, in the presence of MB, decreases from 0.5 V to –0.072 V. The results demonstrated that the polypyrrole–polyvinylsulfonate film is an efficient support for MB adsorption that provided an excellent environment for NADH oxidation as well for ADH immobilization. Therefore, this material was very useful for a simple and effective way to develop amperometric biosensors highly sensitive to ethanol. An important advantage of the constructed biosensor is the low applied potential of its working electrode, –0.072 V (vs. Ag/AgCl). In our study there was no interference effect of electroactive substances such as ascorbic acid or uric acid. However, an interference effect of other alcohols, mostly methanol, was observed. This can be minimized by increasing the selectivity of the biosensor in the following studies. The most promising features of our device are its high cost-effectiveness, simple sensor design, ease of operation, low limit of detection, wide linear ranges, good repeatability, and long-term storage stability. A comparison of the analytical characteristics of ADH based ethanol biosensors is given in Table 2. The electrocatalytic effect of MB and an appropriate environment of polypyrrole mixed with polyvinylsulfonate resulted in an ethanol biosensor with high sensitivity for NADH and low potential ethanol detection. All these characteristics make the Pt/PPy-PVS/MB/ADH-NAD^+ ethanol biosensor an interesting alternative to other determination methods for ethanol in clinical, industrial, food, and environmental analysis.

Table 2. Analytical characteristics of ADH based ethanol biosensors.

Working electrode	Detection potential (V)	Linear range (mM)	Limit of detection (LOD) (μM)	References
ADH/titania sol-gel/Meldola's blue/MWCNT/Nafion	–0.05	0.05–1.1	25	5
CNTB/ADH/MB	+0.05	0.0001–0.004	0.1	30
SPE/MB/Nafion/ADH-NAD^+	–0.17	1–5	11	36
CA-TBO/ADH/GCE	–0.4–+0	0.01–0.4	5	7
NiHCF/ADH/Au	+0.55	Up to 5	0.5	41
QH-ADH / PEGDGE /graphite	+0.30	0.01–0.2	10	4
Pt/PPy-PVS/MB/ADH-NAD^+	–0.072	0.001–0.01 0.01–0.1	0.1	This work

MWCNT; multiwall carbon nanotube, CNTP; carbon nanotube paste, SPE; screen printed electrode, CA; cellulose acetate, TBO; toluidine blue O, GCE; glassy carbon electrode, NiHCF; Nickel hexacyanoferrate, PEGDGE; poly(ethylene glycol) diglycidyl ether

3. Experimental

3.1. Equipment and reagents

Electrochemical studies were carried out by using a CHI 1230B Instrument using a 3-electrode cell. The working electrode was a platinum (Pt) plate (0.5 cm^2). The auxiliary and reference electrodes were Pt wire and Ag/AgCl electrodes (3 M KCl), respectively. pH values of the buffer solutions were measured with an ORION Model 720A pH/ion meter. Temperature control was achieved with a Grant GD 120 thermostat. SEM was performed using a JEOL JEM 100 CX II scanning electron microscope (JEOL, Peabody, MA, USA) equipped with a Link analytical system.

Alcohol dehydrogenase (EC 1.1.1.1, purified from the Saccharomyces Cerevisiae, activity of $\geq$300 unit/mg) was purchased from Sigma. Ethanol (absolute) was purchased from Merck. β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), and β-nicotinamide adenine dinucleotide sodium salt (NAD^+)

were purchased from Sigma. Meldola's blue (MB: 8-Dimethylamino-2,3-benzophenoxazine hemi(zinc chloride) salt, $C_{18}H_{15}ClN_2O$-$0.5Cl_2Zn$) was obtained from Sigma. Pyrrole and sodium polyvinylsulfonate (PVS) were supplied by Fluka and Aldrich, respectively. All other chemicals were obtained from Sigma. All the solutions were prepared with the use of distilled water.

3.2. Preparation of Pt/PPy-PVS film electrode

The surface of the cleaned Pt plate electrode[42] was covered with PPy-PVS film by the electropolymerization of pyrrole in medium with sodium polyvinylsulfonate.[43] The Pt plate electrode was immersed in 10 mL of solution of 0.1 M pyrrole and 2.5 mL (25%) of sodium polyvinylsulfonate. The solution was purged with argon in order to remove the oxygen. Pyrrole electropolymerization was carried out on the Pt electrode surface by cyclic voltammetric scans between –1.0 and 2.0 V at a scan rate of 50 mV/s (vs. Ag/AgCl electrode (3 M KCl)) with 2 cycles. After electropolymerization, the PPy-PVS film was rinsed with deionized water to remove the unreacted pyrrole monomer.

3.3. Preparation of the ethanol biosensor

Firstly, MB (5 mM) was dropped upon the PPy-PVS film and the electrode was dried at room temperature. A solution consisting of 270 U of ADH enzyme, 1 mg of bovine serum albumin, 5 mM NAD^+, 0.1 M pH 7.0 phosphate buffer, and 15 μL of 2.5% glutaraldehyde was dropped upon the PPy-PVS/MB film. The electrode was dried at room temperature and washed with 0.1 M phosphate buffer solution (pH 7.0) several times in order to remove the excess nonimmobilized enzyme and glutaraldehyde. The immobilized enzyme electrode was kept in a refrigerator at 4 °C in phosphate buffer when not in use.

3.4. Amperometric measurements

Determination of ethanol was carried out by using controlled potential electrolysis (CPE). Determination of the oxidation potential of MB was carried out using differential pulse voltammetry (DPV). Determination of ethanol was performed by the oxidation of MB (red.) at –0.072 V (vs. Ag/AgCl electrode (3 M KCl)). The enzyme electrode was immersed into 0.1 M phosphate buffer (pH 7.0). The solution contained 0.1 M potassium chloride as a supporting electrolyte. The electrode was brought to equilibrium by keeping it at –0.072 V (vs. Ag/AgCl electrode (3 M KCl)). Steady-state current (i_a) was recorded. Ethanol was added to the cell from stock solution and the system was stirred. The currents (i_b) obtained at –0.072 V were recorded. The current values ($\Delta i = i_b - i_a$) were plotted against the ethanol concentration in order to determine the working range of the electrode. Reactions on the electrode surface occurred according to the mechanism shown in Figure 13.

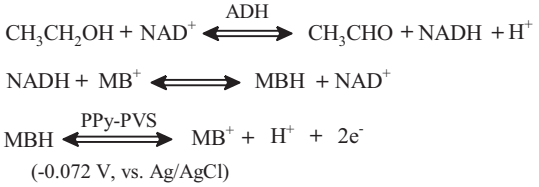

Figure 13. Reactions on the electrode surface for ethanol determination.

Acknowledgments

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