Journal of Electroanalytical Chemistry 823 (2018) 588–592

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Development of amperometric biosensors using screen-printed carbon electrodes modified with conducting polymer and nanomaterials for the analysis of ethanol, methanol and their mixtures

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ARTICLE INFO

Keywords:
Biosensor
Screen printed carbon electrodes
Polyneutral red
Alcohol oxidase
Alcohol dehydrogenase
Ethanol-methanol mixture

ABSTRACT

Amperometric biosensors based on alcohol dehydrogenase (ADH) and alcohol oxidase (AOx) were developed for analysis of ethanol, methanol and their mixtures. The first biosensor based on ADH responds only to ethanol, the second biosensor based on AOx responds to both methanol and ethanol. Screen printed carbon electrodes (SPCEs) were used as the base electrodes. They were first modified with nanoparticles (multiwalled carbon nanotubes and gold nanoparticles) and polyneutral red film. Then the enzyme ADH was immobilized on them to prepare ADH based biosensor for ethanol analysis or the enzyme AOx was immobilized on them to prepare AOx based biosensor for methanol analysis. The analytical characterization parameters sensitivity, linear range, limits of detection and quantification were found as 0.432 μA·mM^{-1}, 178.5–1000 μM, 53.5 μM and 178.5 μM, respectively, for ethanol analysis with AOx based biosensor, as 0.509 μA·mM^{-1}, 335.9–1000 μM, 100.8 μM and 335.9 μM, respectively, for methanol analysis with AOx based biosensor and 0.490 μA·mM^{-1}, 320–1000 μM, 96.1 μM and 320.2 μM, respectively for ethanol analysis with ADH based biosensor. The same parameters were also determined for the analysis of different mixtures with AOx based biosensor. The developed biosensors have been tested on a commercial alcoholic drink for the analysis of its ethanol and methanol contents.

1. Introduction

Ethanol and methanol are used in many areas as solvent, antifreeze, raw material for chemical synthesis, disinfectant and main ingredient of alcoholic beverages [1]. Ethanol used in alcoholic beverages is obtained by fermentation of sugars during which methanol is formed as a natural product [2]. The amount of methanol in falsified drinks is higher than the amount of methanol in fermented alcohol. Methanol is known to be toxic and harmful to human health. Intake of methanol to the body may cause blindness or even death [3]. Therefore, the analysis of methanol in the presence of ethanol is very important. Identification and analysis of alcohols at high sensitivity, selectivity and accuracy are necessary not only in alcoholic beverages but also in food industry and in paper industry as well as in agricultural, environmental, clinical and judicial applications. Many analytical methods based on gas and high performance liquid chromatography, electrophoresis and spectroscopy have been developed for the analysis of ethanol, methanol and other aliphatic alcohols [4–9]. Although these methods are precise and reliable, they have disadvantages such as being complex and time consuming, requiring expensive equipment and pre-processing such as distillation. Amperometric methods with biosensors can overcome these disadvantages. Alcohol dehydrogenase (ADH) or Alcohol oxidase (AOx) based biosensors are used for the analysis of samples containing single alcohol compound, in particular ethanol. ADH is an oxidoreductase enzyme. It catalyzes the oxidation of primary aliphatic and aromatic alcohols, except methanol, to aldehydes by cooperating with NAD^+ coenzyme which is reduced to NADH. It should be noted that there are a few works reporting response of ADH to methanol as well. For example, Jimenez et al [10] reported greater response for methanol than ethanol with the use of ADH in a biosensor including polydiallylmethylsilane and Pt nanoparticles in its formulation. Other works for ethanol analysis using ADH based biosensors include the recent ones by Samphao et al. [11,12]. On the other hand, AOx is an enzyme that catalyzes the oxidation of short chain, low molecular weight aliphatic alcohols to their corresponding aldehydes. It uses oxygen as an acceptor. The flavin adenine dinucleotide (FAD), the cofactor of the AOx enzyme, is reduced to $FADH_2$ in the reaction and then oxidized back to FAD by O_2 in the medium. The conventional ways to follow reactions catalyzed by

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https://doi.org/10.1016/j.jelechem.2018.07.009
Received 31 January 2018; Received in revised form 8 June 2018; Accepted 4 July 2018
Available online 05 July 2018
1572-6657/

oxidases are to measure the decrease in oxygen concentration in the solution or the increase in concentration of hydrogen peroxide formed in oxidation of $FADH_2$ to FAD by O_2 [13]. Since enzymes usually have the ability to catalyse the oxidation of one alcohol, the studies on selective analysis of alcohol mixtures using biosensors are limited. In one study for ethanol analysis in the presence of methanol, a complex chemiluminometric system with three enzymes consisting of catalase, formaldehyde dehydrogenase and AOx was used [14]. In another study, an AOx biosensor was used for the analysis of 5 alcohols [15]. The only work that we could find in literature about the analysis of ethanol-methanol mixture using biosensor is the one reported by Bucur et al. [16]. Screen printed electrodes were used as the base electrodes in this work. They were modified by meldola blue mediator coupled with ADH enzyme or by cobalt phthalocyanine mediator coupled with AOx enzyme. Chronoamperometric measurements were carried out at −10 mV with the ADH based biosensor and at +600 mV with the AOx based biosensor [16]. In the work of Bucur et al. [16], working pH was not optimized. Measurements were made at an average pH of 8 which was based on optimum pH values of enzymes given by the producers. This pH is rather high for the stability of NAD^+ coenzyme of ADH based biosensors and AOx enzyme [17]. On the other hand the rather high working potential of +0.6 V to follow oxidation of H_2O_2 with AOx based biosensor may bring the interference problem into consideration.

PNR has been used as a redox mediator in several works involving oxidase based biosensors. Pauliukaite and Brett [18] developed a glucose biosensor by modifying a carbon film electrode with PNR and GOx. In their work, glucose detection was achieved amperometrically at −0.25 V. It was stated that PNR behaved as mediator and catalyzed the oxidation of $FADH_2$. Qu et al. [19] succeeded the determination of glucose using glassy carbon electrode modified with MWCNT, PNR and GOx at −0.2 V without any interference. Barsan et al. [20] developed an alcohol biosensor by modifying a carbon film electrode with PNR, AOx, GA and bovine serum albumin. They reported that PNR served as a redox mediator for AOx enzyme in this biosensor. In another study [21], bismuth film electrode was modified with GOx and NR monomer was used as a redox mediator in the solution for the determination of glucose.

The purpose of the present study is to develop enzyme biosensors based on ADH and AOx for the analysis of samples containing ethanol, methanol and their mixtures by amperometric method at low potentials. Screen printed carbon electrodes (SPCEs) are selected as base electrodes for the biosensor due to their low cost and practical use. They are modified with multiwalled carbon nanotubes (MWCNTs) and gold nanoparticles (AuNPs) to improve electronic conductivity, and also with polyneutral red (PNR) conductive polymer to act as a mediator. Then ADH or AOx is immobilized onto the modified electrodes.

2. Experimental

2.1. Chemicals and reagents

Alcohol oxidase (AOx, from E.C. *Hansenula sp.*, 1.1.3.13., 0.6 unit·mg^{-1} solid), alcohol dehydrogenase (ADH, from *Saccharomyces cerevisiae*, E.C. 1.1.1.1., 340 units·mg^{-1} solid), sodium salt of β-nicotinamide adenine dinucleotide (NAD^+, *from Saccharomyces cerevisiae*), solution of glutaraldehyde (GA, 25 wt% in H_2O), AuNPs (10 nm stabilized suspension in 0.1 mM phosphate buffer solutions, PBSs), gelatin (G) were purchased from Sigma-Aldrich. Ethanol, methanol, KH_2PO_4, K_2HPO_4, KCl, $K_3Fe(CN)_6$, neutral red (NR), DMF, NaOH and HCl were purchased from Merck. MWCNTs were from DropSens. They were carboxyl functionalized and synthesized by chemical vapor deposition. Their diameter is 10 nm, length is 1.5 μm, C content is > 95%, mass fraction purity is 0.99. They were dried under vacuum and used without any further purification. Water taken from Millipore Milli-Q Direct Q-3 ultrapure water system was used in experiments. K_2HPO_4, KH_2PO_4 and KCl were used in PBS preparations and AOx, ADH, NAD^+, GA and G solutions were prepared in these PBSs (in 0.1 M KCl).

2.2. Electrochemical measurements

Disposable screen-printed carbon electrodes (SPCEs) were supplied by DropSens (Oviedo, Spain, ref. DRP110). They have a combined three electrode system; first a carbon working electrode (WE), then a silver reference electrode (RE) and finally a carbon counter electrode (CE). All these electrodes were deposited on a ceramic support by printing technology. The dimensions of the electrode system are 3.4 × 1.0 × 0.05 cm with WE of 4 mm in diameter. These electrodes are inexpensive and can be utilized with samples in micro volumes. SPCE was connected to a potentiostat (PalmSens, produced by Palm Instrument, Netherland). All electrochemical measurements were performed with this potentiostat which was controlled by PalmSens PS Trace 4.8 software. The cells (10 mL) used in electrochemical measurements were also provided by Dropsens. SPCEs were pretreated electrochemically before their use by applying a potential of +1.4 V for 300 s in a 50 mM buffer solution at pH = 7.0. A Mettler Toledo pH-meter was used for pH measurements. Amperometric measurements were carried out under controlled magnetic stirring. A new electrode was used for each assay in electrochemical experiments which were performed at room temperature.

2.3. Preparation and application of biosensors

SPCEs were modified with MWCNTs, AuNPs and PNR as described in our earlier works [22,23]. They were then further modified with ADH or AOx to act as a biosensor toward ethanol and methanol in samples containing the two; alone or in mixtures. In order to prepare the biosensor sensing to ethanol alone, 5 μL of an ADH solution (prepared by dissolving 1 mg ADH in 10 μL 50 mM PBS of pH 7.0) was carefully spread on the WE of SPCE which was already modified with MWCNT, PNR and AuNP. In order to prepare the biosensor sensing to both methanol and ethanol, a solution was prepared by dissolving 1.7 mg AOx and 1 mg gelatin (G) in 10 μL of 50 mM PBS of pH 7.0. This solution was kept in water bath at 38 °C for 15 min and then carefully spread over the WE of SPCE which was already modified with MWCNT, PNR and AuNP. Both of these modified electrodes were dried for 1 h at 4 °C and then 1 μL 1% glutaraldehyde (GA) solution (prepared in 50 mM PBS of pH 7.0) was dropped on them to cross-link ADH or AOx. Further drying of electrodes for another half an hour at the same temperature is followed by rinsing with ultrapure water to remove any excess GA. Electrodes prepared in this way were assigned by notation of SPCE/MWCNT/PNR/AuNP/ADH/GA for ADH based biosensor and by the notation of SPCE/MWCNT/PNR/AuNP/AOx/G/GA for AOx based biosensor.

The developed biosensors were tested in determining ethanol and methanol contents of a model mixture and a commercial alcoholic drink by chronoamperometric method at a working potential of +0.20 V for ADH based biosensor and −0.30 V for AOx based biosensor.

3. Results and discussion

3.1. Factors affecting the biosensor performance

3.1.1. Optimization of pH

SPCE/AOx/G/GA biosensors were prepared to examine the effect of pH on the response of AOx based biosensors to methanol. They were used for current measurements at a potential of −0.45 V in solutions containing 100 μM methanol prepared in 50 mM PBS having different pH. A current vs pH plot is shown in Fig. 1. It is clearly seen that the optimum pH is 7.0 at which maximum current is attained. Thus, subsequent measurements were carried out at this pH for AOx based biosensor. The optimum pH for ADH based biosensors (SPCE/MWCNT/PNR/ANP/ADH/GA) was already determined as 7.75 in our previous study [22].