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A Sulfite Biosensor Fabricated by Immobilization of Sulfite Oxidase on Aluminum Electrode Modified with Electropolymerized Conducting Film (Polyaniline)

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The development of a sulfite biosensor based on the immobilization of sulfite oxidase in electropolymerized conducting film (polyaniline) is described. The method for the determination of sulfite is based on the use of aluminum electrode modified with films of polyaniline. Electropolymerization of polyaniline and simultaneous immobilization of sulfite oxidase on the aluminum were performed in an aqueous solution containing sulfite oxidase. The sulfite biosensor constructed by cycling the potential scan between +1.2 and -0.5 V *vs.* saturated calomel electrode (SCE) that showed a sensitive response to sulfite with a linear calibration graph in the concentration ranges of 0.006-5 mM sulfite and detection limit 0.002 mM sulfite (S/N = 3). The stability study of the resulted sulfite biosensor revealed that formation of a passive film on the aluminum surface causes improved stability of the electroactive films formed on the electrode surface. The bioelectrochemical response of the enzyme-modified electrode as a sulfite biosensor was investigated at different experimental conditions. The pH optimum of 8.5 was found when using phosphate buffer and the appropriate working temperature was accepted as 35 °C. The apparent Michaelis-Menten constant and the activation energy of the enzyme catalyzed reaction were also calculated.

Key Words: Sulfite biosensor, Aniline, Sulfite oxidase, Conducting polymer.

INTRODUCTION

Biosensors have recently attracted much interest as these bio-devices have been shown to have applications in clinical diagnostics, environmental monitoring, food freshness and bioprocess monitoring[1-8]. A number of methods have been developed for immobilization of enzymes, but electrochemical methods have been mainly used for the preparation of enzyme electrodes. Many studies have been done on immobilization of enzymes in various conducting polymers[9,10]. Due to the high conductivity properties and stability in air and aqueous solutions, conducting polymers

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are very useful materials for immobilization of enzymes. In this case, enzyme was immobilized directly into the conducting polymer film to form the enzyme electrode without using any agent. Polyaniline is one of the most attractive conducting polymers, which was used for various applications in modern electrochemistry. This is due to both the stability of polyaniline films produced electrochemically and their interesting electrochromic and conducting properties. Mu *et al.*[11,12] have studied suitability of polyaniline as polymeric film to immobilization of various enzymes. Determination of sulfite is very important for controlling its amount in food. According to food and drug administration (FDA), the safe amount of sulfide in foods should be less than 10 ppm. Also, due to the presence of sulfide in "acid rain", its determination in environmental samples is important. Conventional methods have been developed for the determination of sulfite in a variety of samples such as wines and preserved foods, but the application of these to natural waters is still unsatisfactory with regards to sensitivity, selectivity, analytical performance and simplicity[13].

Interest in developing biosensors for the determination of sulfide mainly in environmental samples has grown after the first report based on physical trapping of sulfite oxidase[14]. Sulfite concentration is determined by the measurements of the oxygen concentration decreases. This principle has been improved by coupling an oxygen sensor to a nylon membrane with the enzyme chemically immobilized on it[15]. Several types of transducers and enzyme immobilized procedures were previously reported: adsorption of sulfite oxidase on conducting salt (tetrathiofulvalene tetracyanoquinodimathane, TTF-TCNQ)[16], sulfite oxidase electroimmobilized into a polypyrrole film[17]. Enzyme immobilized on controlled pore glass was used for construction of an optical flow-through biosensor[18]. Insoluble hexacyanoferrates were used for preparation of sulfite biosensor based on sulfite oxidase[19]. Approaches to the use of biocatalysis other than sulfite oxidase were published. Sulfite ion sensor with use of immobilized organelles[20,21] and a microbial sensor of immobilized *Thiobacillus thiooxidase* cells[22] were described. Sulfite biosensor based on polytyramine has reported to be used in real samples as its application to wine analysis[23].

Sulfite biosensors take considerable interest for environmental and food analysis in the recent years. Sulfite determined in samples from river and seawater[15] and generally in spiked aqueous samples[18]. Among the several methods for the determination of sulfite, the FDA reference method is based on sulfite biosensor named Monnier-Williams method[24]. It shows the importance of sulfite biosensor and its study to development for various analyses.

This method is reliable and economic. In contrast, a gas chromatography method has been reported which is more accurate and has better sensitivity, but it requires expensive instrumentation and skilled operators. In a second class of methods, free or complex sulfite ion is determined directly using liquid chromatography[25], capillary electrophoresis[25], spectrophotometry[25] or electrochemistry[25], which has their own problems.

Electrochemical sulfite sensors are usually based on measuring the oxidation current of sulfite directly or that of hydrogen peroxide produced by the reaction with the enzyme sulfite oxidase. At the high oxidation potentials, other electroactive compounds in the sample are also oxidized at the working electrode and produce interfering currents. Various membranes, such as conducting polymers have been coated on the surfaces of electrodes to prevent interfering species from approaching the working electrode[26,27].

Stability of the electroactive film formed on the electrode surface is one of the most important problems in the preparation of modified electrodes. The aluminum substrate is very effective on the deposition of the electroactive and the film formed on the aluminum surface is very stable[25,28]. It was thought that this effect is corresponding to generation of passive film on the aluminum substrate. The role of passive surface on the stability of the electroactive film formed on the substrate electrode and its suitability for preparation of enzyme-modified electrodes has been discussed in the literature[25]. It was described that a lower charge is required for deposition of conducting polymer on a passive surface. This causes the enzyme incorporate into the polymeric film without change in its kinetic reaction (indicating by changes in value of Michaelis-Menten constant, K'm). The passive film formed on the electrode substrate is more suitable surface for deposition of the electroactive films, as it was reported for conducting polymers[25]. In addition of chemically modified electrodes with both insoluble hexacyanoferrates and conducting polymers, possibility of aluminum as a substrate electrode for immobilization of enzyme has also been reported.

In the present paper, an enzyme-modified electrode by the incorporation of sulfite oxidase into the electroactive film during the electropolymerization of aniline on the aluminum electrode has been reported. The bioelectrochemical response of the enzyme-modified electrode as a sulfite biosensor was investigated. The aim of this paper is to report a new sulfite biosensor based on conducting polymer and using aluminum as substrate electrode to improve the stability of the enzyme-modified electrode.

EXPERIMENTAL

Sulfite oxidase EC 1.8.3.1 from chicken liver was obtained from Sigma Company Inc. Other reagents used were of analytical-reagent grade (obtained from Merck Company Inc.). All solutions were prepared with doubly distilled water. The phosphate buffered potassium salt (0.05 M KH_2PO_4 + 0.05 M K_2HPO_4 + 0.1 M KNO_3) with pH of 8.5 was used as supporting electrolyte.

Construction of the enzyme electrode: The enzyme-modified electrode was prepared by electropolymerization of aniline from an aqueous solution containing sulfite oxidase. The enzyme was immobilized into the polyaniline film during the electrochemical polymerization of aniline in a solution of HCl-NaH_2PO_4 with pH of 8.5 containing 0.1 M aniline and 2.5 mg/mL of sulfite oxidase. The electropolymerization was done by cycling the potential scan between +1.2 and -0.5 V *vs.*

saturated calomel electrode (SCE). The polymerization time was about 0.5 h. Then the polyaniline-sulfite oxidase electrode was rinsed carefully with the corresponding buffer. The enzyme electrode was stored at 5 ºC in phosphate buffer (pH 8.5).

Sulfite measurements and apparatus: Determination and measurement of sulfite by the biosensor presented here is based on the enzyme-catalyzed reaction:

$$2SO_3^{2-} + 2H_3O^+ + 2O_2 \rightarrow 2SO_4^{2-} + H_2O_2$$

(in the presence of sulfite oxidase)

The determination of the response current is based on the formation of hydrogen peroxide during the enzyme-catalyzed reaction. The hydrogen peroxide is detected by the amperometric current method[29] during oxidation at the enzyme electrode:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

The electrochemical studies were carried out using a homemade potentiostat. The amperometric measurements were carried out using a Mulltimeter as the data were recorded by a computer. All potentials were referenced to saturated calomel electrode (SCE).

RESULTS AND DISCUSSION

The enzyme-modified electrode exhibits amperometric response towards sulfite. In study of the electrode response to sulfite, current increases to reaches it's maximum value and stays in equilibrium condition without any noticeable changes. The electrode reaches its steady state values of current after a relatively short time (< 50 s). The relationship of the biosensor sensitivity to the applied potential in the presence of 0.1 mM sulfite oxidase is shown in Fig. 1. Applied potential is not very effective on the electrode selectivity. However, high potential causes to oxidation of the other species such as ascorbic acid and oxygen, which can made positive error in real samples. Potential of 0.0 *vs.* SCE was chosen as operation potential due to many advantages of this potential such as ease of usage, stability of the electroactive film and low interferences. The response current of the enzyme-modified electrode is strongly dependence on pH of the solution. This is due to the activity and stability of the enzyme in various pHs and the optimum value of pH for sulfite oxidase is near neutral pHs[30].

The effect of pH on the electrode response was examined in the presence of 0.1 mM sulfite. Fig. 2 presents the effect of pH on the electrode response. The obtained results indicate that the optimum pH (8.5) is located in basic-neural range that this is due to the kinetic reaction of the enzyme. It is close to the optimum pH for free enzyme[30].

The enzyme-modified electrode displays a linear response to sulfite and acts as a sulfite biosensor. The relative standard deviation (RSD) for 0.1 mM sulfite was 3.5 % (for ten measurements) which indicates the electrode has a good reproducibility for sensing sulfite. The amperometric baseline did not exhibit any measurable drift in 0.1 mM sulfite and the noise level was less. Calibration plot for the determination

of sulfite is presented in Fig. 3. It indicates that the enzyme-modified electrode exhibits a linear range up to 0.5 mM sulfite with correlation coefficient of 0.994 at potential of 0.0 *vs.* SCE. It is seen that the dependence of current on sulfite concentration gives a straight line over the range of $6 \times 10^{-6} – 5 \times 10^{-3}$ M. The signal to noise characteristics (S/N = 3), indicates the detection limit of sulfide is 2×10^{-6} M.

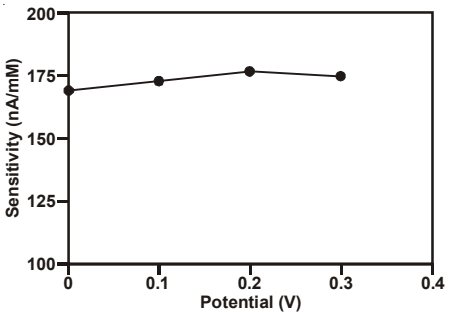

Fig. 1. Relationship between the response current and applied potentials of the enzyme-modified electrode

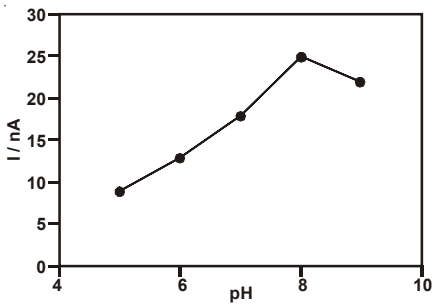

Fig. 2. The influence of solution pH on the response current of the enzyme-modified electrode in the presence of 0.1 mM sulfite

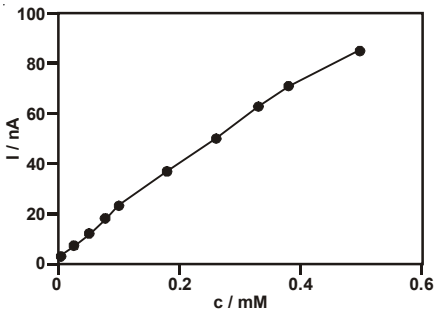

Fig. 3. Calibration plot of the electrode response towards sulfite

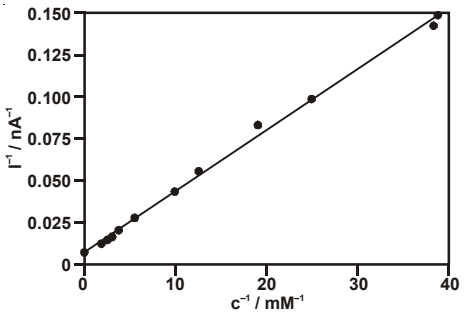

Fig. 4. Determination of the apparent Michaelis-Menten constant, K'm, for the enzyme-modified electrode

For the determination of the maximum current value and the apparent Michaelis-Menten constant, I-1 was plotted against ([sulfite])-1 which is shown in Fig. 4. The curve was obtained using the data presented in Fig. 3. The maximum current response was calculated from the intercept of the curve and was equal to 153.96 nA. The Michaelis-Menten, K'm, was calculated for the immobilized enzyme by an amperometric method as reported by Shu and Wilson[31]. The apparent Michaelis-Menten constant, K'm, was determined from slope of the curve which has value of 0.365 mM. This is very close to the magnitude of the Michaelis-Menten constant of free sulfite oxidase (0.39 mM) that shows the enzyme was not chemically modified and has its usual kinetic reaction.

The influence of temperature on the maximum response current of the enzyme-modified electrode was investigated in the presence of sulfite oxidase (Fig. 5). By increasing the temperature, current increases to reach its maximum value at 35 °C. It is indicated that the maximum value of the electrode response is 35 °C, which is optimum value for the sulfite biosensor.

Fig. 6 illustrates the curve obtained by plotting log I *vs.* 1/T from the data obtained in Fig. 5. By assumption of this fact that the electrode surface area, amount of enzyme substrate concentration are constant, the maximum response current of the enzyme modified electrode is dependence on the rate constant, k. By replacing log k with log I in Arrhenius equation, the slope of the curve (linear relationship) presents the activation energy, E_a. The activation energy of the enzyme-catalyzed reaction was calculated 23.2 kJ mol^{-1}.

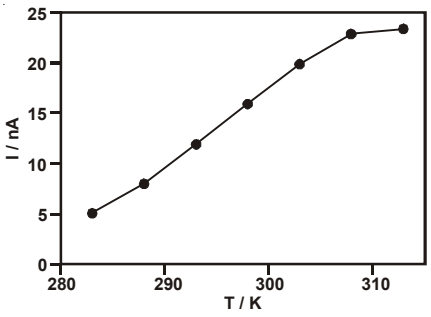

Fig. 5. Effect of temperature on the response current of the enzymemodified electrode

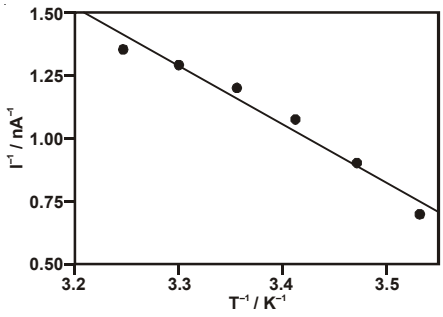

Fig. 6. Plot of log I *vs.* T^{-1} obtained from data of Fig. 5

The stability of the sulfite biosensor was examined during a long time of usage. Fig. 7. presents the changes in slope of the electrode response. As can be seen, the electrode is stable and has good selectivity in the first days of usage. After a certain time, a significant loss in current appears. This loss is due to the fact the enzyme is washed away from the film electrode, thus causing a sudden decrease in amperometric response. The enzyme formed on the electrode surface has a certain stability to remain on the polyaniline surface. Of course, this sudden decrease occurs during more than 10 d but it seems sudden due to the difference of two regions with high stability before and after of enzyme removing. However, after that the selectivity of the electrode approximately remains constant. Indeed, the sulfite biosensor has two different periods for useful application. However, the enzyme-modified electrode has a long useful lifetime due to the stability of the polyaniline film growth on the aluminum surface. After the first period of the electrode usage, sulfite oxidase immobilized on the surface of polyaniline film remove from the electrode surface and it causes a sudden decrease in amperometric response of the biosensor. But that part of enzyme incorporated into the conducting polymer film will remain until

breakdown of the conducting polymer. The obtained results show that the sulfite biosensor can be used for two different applications, for immediate usage and long term usage. It should be emphasized that another important parameter for decrease in stability of the enzyme-modified electrode is the activity of the enzyme. It is well-known behaviour of enzyme to gradual decrease of their activity. After long time of usage, activity of sulfite oxidase decreases as well as stability of the film formed on the electrode surface.

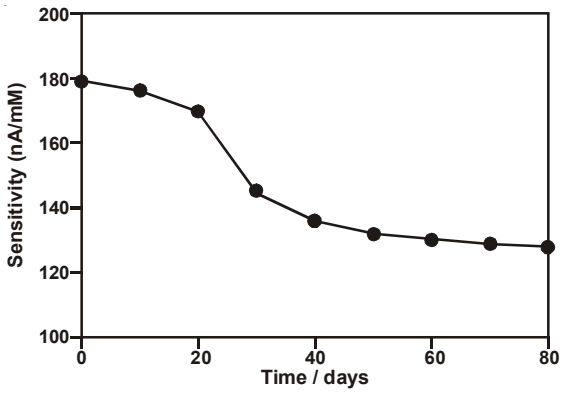

Fig. 7. Long-term behavior of the enzyme-modified electrode. The sensivity in the linear region of the calibration curves is given *vs.* the operation time

The thickness of the electroactive film is controlled by the amount of sulfite oxidase and aniline of the modifier solution, and by the charge passed through the electrochemical cell. The most difference of the mentioned electrode from the other enzyme-modified electrode is due to formation of the electoactive film on the electrode surface. During the electropolymerization process, experimental conditions are effective on the passivation of aluminum surface. As it was described, aluminum passivation causes to formation of more stable film on the electrode surface. The stable polyaniline film growth on the aluminum surface, keeps the incorporated enzyme for a long time. Moreover, aluminum passivation causes to difference in the nucleation and growth mechanism (NGM) of the conducting polymer that is effective on the electrochemical behaviour of the enzyme-modified electrode.

The error made by interferents is less for the determination of sulfite based on the sulfite biosensor, which is due to its high selectivity and low operation potential. The main interferences for the sulfite biosensor electrode is related to those compounds generate sulfite. Usually, these reactions occur at high pHs (higher than 9). Of course, according to the pH-dependence of the electrode presented at Fig. 2, lower pHs can be use. Although, the sulfite biosensor has a lower sensitivity at low values of pHs, but higher selectivity can be reached due to decrease of interfering effect.

Conclusion

The bioelectrochemical response of the enzyme-modified electrode based on electrochemical incorporation of sulfite oxidase into polyaniline aluminum modified electrode was investigated. The sulfite biosensor exhibits linear response to sulfite ion over a wide concentration range (3 decades) with low detection limit of 2×10^{-6} M. The biosensor has a good reproducibility and selectivity for sulfide. It was presented that aluminum electrode is a suitable substrate electrode for the preparation of enzymemodified electrodes and improves the stability of the film growth on it. Study of possibility of aluminum as substrate electrode for the preparation of enzyme modified electrodes with other conducting polymers and enzymes now is under investigation.

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