Fabrication of a Sulfite Biosensor by the Use of Coducting Polymer

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ABSTRACT: *In this research, an enzyme modified electrode has been produced during the electropolymerization of aniline through incorporation of Sulfite oxidase into a conducting polymer. Then the bioelectrochemical response of resulted sulfite biosensor was investigated at different experimental conditions. Study of the stability 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 optimum pH and temperature were 8.5 and 35 ℃, respectively. The apparent Michaelis-Menten constant and the activation energy of the enzyme catalyzed reaction were calculated.*

KEY WORDS: *Sulfite biosensor, Aniline, Sulfite oxidase, Conducting polymer.*

INTRODUCTION

There is a considerable interest in biosensors based on electrocatalysis systems of enzymes since *Clark* and *Lyons* made the first report in 1960s [1-4]. 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 [5-8].

Due to the high conductivity properties and stability in air and aqueous solutions, conducting polymers 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.* [9-11] 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 is less than 10 ppm

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(10 mg/kg). 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 [12].

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 [13]. 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 [14]. Several types of transducers and enzyme immobilized procedures were previously reported: adsorption of sulfite oxidase on conducting salt (tetrathio-fulvalene tetracyanoquinodimathane, TTF-TCNQ) [15], sulfite oxidase electroimmobilized into a polypyrrole film [16]. Enzyme immobilized on controlled pore glass (CPG) was used for construction of an optical flow-through biosensor [17]. Insoluble hexacyanoferrates were used for preparation of sulfite biosensor based on sulfite oxidase [18]. Approaches to the use of biocatalysis other than sulfite oxidase were previously published. Sulfite ion sensor with use of immobilized organelles [19,20] and a microbial sensor of immobilized *Thiobacillus thiooxidase* cells [21] were described. Sulfite biosensor based on polytyramine has been reported to be used in real samples as its application to wine analysis [22].

Sulfite biosensors have taken considerable interest for environmental and food analysis in the recent years. Sulfite is determined in real environmental samples such as samples from river and seawater [14] and generally in spiked aqueous samples [17]. Among the several methods for the determination of sulfite, the FDA reference method is based on sulfite biosensor named *Monnier-Williams* method [23]. It shows the importance of sulfite biosensor and the study of its development for a variety of applications and analyzes.

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 [24], capillary electrophoresis [25], spectrophotometry [26] or electrochemistry [27], which have 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 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 [28,29].

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 film and the film formed on the aluminum surface is very stable [30-34]. 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 [34,35]. 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 [35]. In addition to chemically modified electrodes with both insoluble hexacyanoferrates and conducting polymers, the possibility of aluminum as a substrate electrode for immobilization of enzyme has also been reported.

In the present paper, we wish to report a novel enzyme-modified electrode by the incorporation of sulfite oxidase into the electroactive film during the electropolymerization of aniline on the aluminum electrode. The bio-electrochemical 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 was obtained from Sigma. Other reagents used were of analytical-reagent grade (obtained from Merck). 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. 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).

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. SCE. The polymerization time was about 30 min. Then the polyaniline-sulfite oxidase electrode was rinsed carefully with the corresponding buffer. The enzyme electrode was stored at 5 °C in phosphate buffer (pH 8.5).

The sulfite oxidase electrode reaction is as follows:

$$2SO_3^{2-} + 2H_3O^+ + 2O_2 \rightarrow 2SO_4^{2-} + H_2O_2 \quad (1)$$

(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 [36] during oxidation at the enzyme electrode:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

RESULTS AND DISCUSSION

The enzyme-modified electrode exhibits amperometric response towards sulfite. In study of the electrode response to sulfite, current increases to reach it's maximum value and stays in equilibrium condition without any noticeable change. 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 oxidation of the other species such as ascorbic acid and oxygen, which can make positive error in real samples. Potential of 0.0 vs. SCE was chosen as operation potential due to the 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 dependent 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 [37,38].

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-neutral range that this is due to the kinetic reaction of the enzyme. It is close to the optimum pH for free enzyme [37,38].

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 low. 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 6×10^{-6} - 5×10^{-3} M. The signal to noise characteristics (S/N = 3), indicates the detection limit of sulfide is 2×10^{-6} M.

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* constant, K'm, was calculated for the immobilized enzyme by an amperometric method as reported by *Shu* and *Wilson* [39]. 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

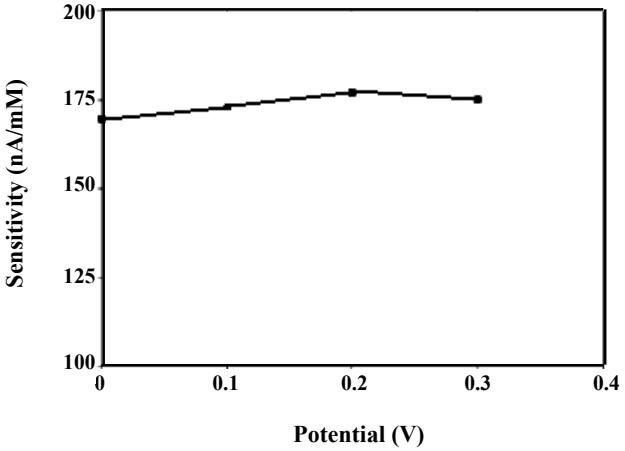

Fig. 1: Relationship between the response current and applied potentials of the enzyme-modified electrode.

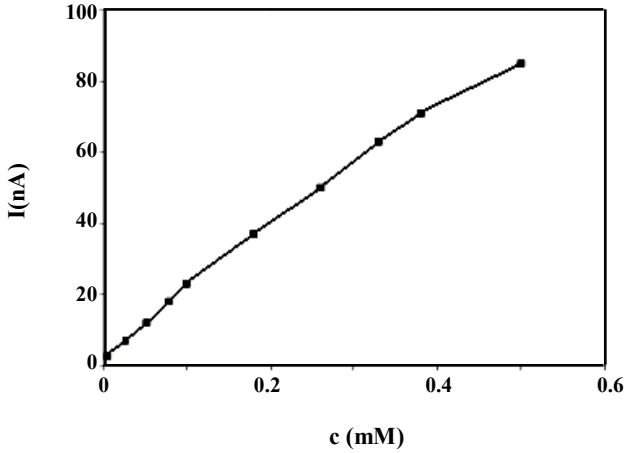

Fig. 3: Calibration plot of the electrode response towards sulfite.

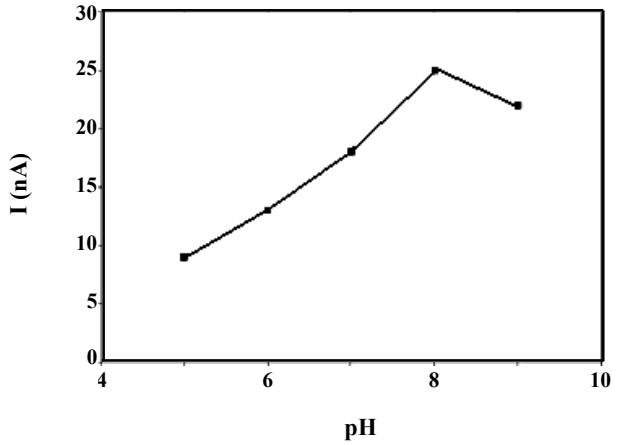

Fig. 2: The influence of solution pH on the response current of the enzyme-modified electrode in the presence of 0.1 mM sulfite.

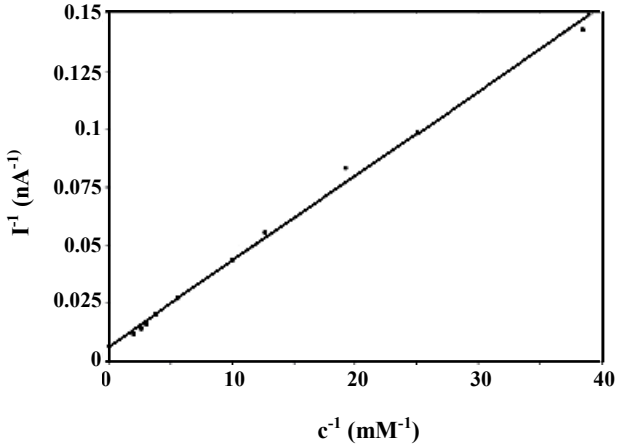

Fig. 4: Determination of the apparent Michaelis-Menten constant, K'm, for the enzyme-modified electrode.

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 versus 1/T from the data obtained in Fig. 5. By assumption of the fact that the electrode surface area and the amount of enzyme substrate concentration are constant, the maximum response current of the enzyme modified electrode depends 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, Ea. The activation energy of the enzyme-catalyzed reaction was calculated 23.2 kJ mol^{-1}.

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.

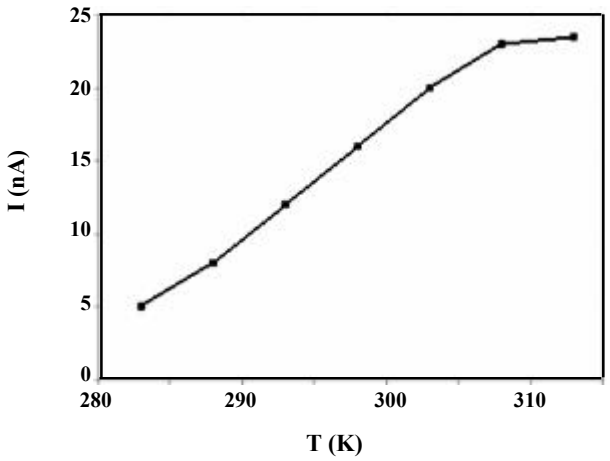

Fig. 5: Effect of temperature on the response current of the enzyme-modified electrode.

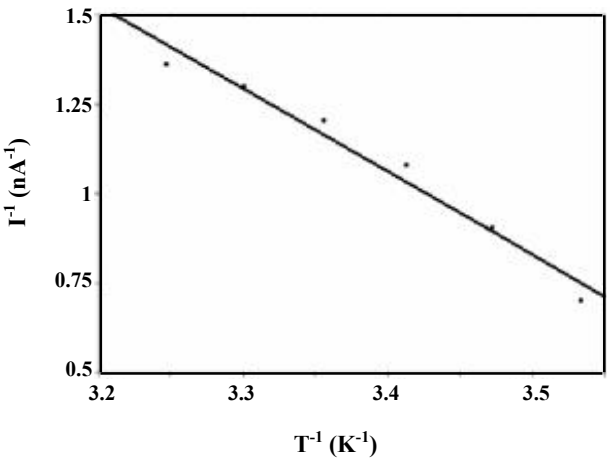

Fig. 6: Plot of log I versus T-1 obtained from data of Fig. 5.

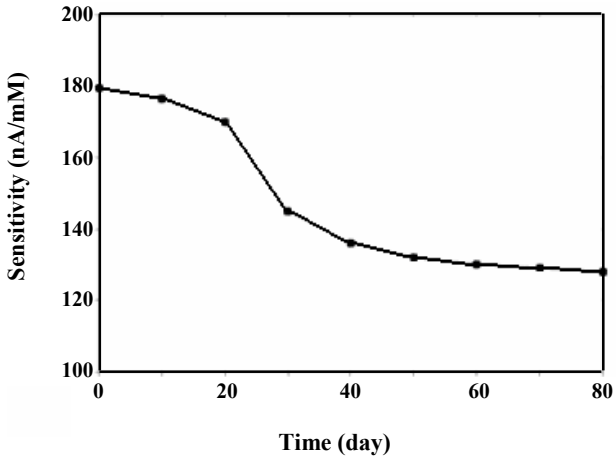

Fig. 7: Long-term behavior of the enzyme-modified electrode. The sensivity in the linear region of the calibration curves is given vs. the operation time.

The 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 days but it seems sudden due to the difference of two regions with high stability before and after enzyme removal.

However, after that the selectivity of the electrode approximately remains constant. Indeed, the sulfite biosensor has two different periods for useful application. 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 is removed from the electrode surface and this causes a sudden decrease in amperometric response of the biosensor. But that part of the 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 activity of the enzyme. Gradual decrease of activity is a well-known behavior of enzymes. After long time of usage both the activity of sulfite oxidase as well as the stability of the film formed on the electrode surface decrease.

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 main difference of the mentioned electrode from the other enzyme-modified electrode is the 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 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 a difference in the nucleation and growth mechanism (NGM) of the conducting polymer that is effective on the electrochemical behavior 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 that 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.

CONCLUSIONS

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 enzyme-modified 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 is now under investigation.

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