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A miniaturized glucose biosensor based on the coimmobilization of glucose oxidase and ferrocene perchlorate in nafion at a microdisk platinum electrode

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Abstract

A miniaturized glucose biosensor based on the coimmobilization of Fc^+ (ferrocene perchlorate)/GOD (glucose oxidase) in nafion film at the surface of a microdisk platinum electrode was fabricated and successfully used for the amperometric determination of glucose. The influences of various experimental conditions, including the relative amounts of glucose oxidase in diluted nafion aqueous solution, the concentration of ferrocene perchlorate and oxygen etc., were investigated in this paper. Ferrocene perchlorate as a redox mediator could catalyze the oxidation of the generated H_2O_2 based on the enzymatic reaction of glucose in the presence of glucose oxidase and oxygen at a favorable lower working potential (ca. 0.25 V vs. SCE). Moreover, it could also oxidize the reduced flavin adenine dinucleotide ($FADH_2$) of glucose oxidase directly in anaerobic environment. The response time and the detection limit under an optimal parameters were 2 min and 1×10^{-5} M, respectively. The interferences of ascorbic acid and uric acid could be obviously reduced because of the ion-selective characteristics of nafion film and a favorable lower working potential. From the Michealis–Menten analysis, the apparent Michaelis constants for glucose and the maximum limiting currents determined were 10.7 mM and 5.1 nA for the incorporation of Fc^+ in 1.00 mM Fc^+ solution, 7.06 mM and 5.85 nA in 2.00 mM Fc^+, respectively. Moreover, using water instead of organic solvents for nafion dilution made this enzyme electrode exhibit a good stability and reproducibility for a long-term use. © 1997 Elsevier Science S.A.

Keywords: Miniaturized biosensor; Glucose oxidase; Microelectrode; Ferrocene perchlorate; Nafion

1. Introduction

The electrochemical biosensors, with special emphasis on the development of small and portable devices, have been extensively investigated. Such devices are very useful in neurological and physiological studies by improving biocompatibility, operating in a very small volume and fabricating multi-probes and arrays. The fabrication is commonly accompanied with microelectronic technologies such as screen-printing or photolithographic schemes, which are suitable for large-scale production [1,2].

Enzyme immobilization is a key step in fabrication of the sensitive and stable biosensors. Usually, it was carried out by different approaches including covalent-binding, cross-linking and entrapment in a gel or polymer [3–6]. The latter has caused extensive interest because of its simple one-step making. Nafion, as a polymer, has been widely used for enzyme immobilization, moreover, the nafion modified electrode could exclude the effects of some interference such as ascorbic acid and uric acid etc. [7,8].

The third-generation biosensors based on the coimmobilization of redox mediators with enzyme at the electrode surface have been established [9,10]. In general, the direct regeneration of the electroactivity of reduced enzymes species is a kinetically hindered process. Oxygen has been served as a mediator for the regeneration of reduced enzyme under aerobic conditions [11]. But, its working potential is higher. A suitable mediator could be used to reduce the working potential of biosensors based on its catalytic characteristics for H_2O_2. Gulce et al. [12] studied the use of the redox polymer PVF for the immobilization of glucose oxidase and the determination of glucose under aerobic

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conditions. Ferricinium centers which are present in the polymeric material act as mediators for the oxidation of hydrogen peroxide which is produced during the enzymatic reaction. Such redox mediators include quinones, dyes, ferrocene and their derivatives etc. They could be employed to catalyze the oxidation of biomaterials, and the corresponding amperometric biosensors were fabricated [13–16].

Enzyme modified microelectrodes have been increasingly interesting because of their prospect for in vivo. Most of them are dependent of the determination of hydrogen peroxide [17]. Thus it is necessary for them to be improved by performing at a lower working potential based on the mediator catalytic characteristics for hydrogen peroxide. Here, the chemically modified microelectrode by the incorporation of ferrocene perchlorate depending on the cation-exchange characteristics of nafion was reported. A new amperometric microbiosensor of glucose was successfully achieved which relied on the coimmobilization of glucose oxidase and ferrocene perchlorate in nafion at the surface of a microdisk platinum electrode. The results would be significant for the development of miniaturization of biosensors.

2. Experimental

2.1. Reagents

All solutions were prepared with double-distilled water and all reagents were analytical reagent grade. The glucose oxidase and β-D-glucose were purchased from Sigma, nafion (5% v/v) ethanol solution was obtained from Aldrich.

The dilution of nafion was performed by mixing water and nafion (5%) with the ratio of 9:1 (v/v). Ferrocene perchlorate was obtained from the Coordination Institute of Nanjing University, China. Ascorbic acid and uric acid were purchased from Shanghai Institute of Biochemistry, Chinese Academy of Sciences.

β-D-glucose stock solution was prepared prior to use for at least 24 h for mutarotation. The determination of glucose was by adding aliquot glucose stock solution in cell.

2.2. Apparatus

A microdisk platinum electrode ($\Phi = 20$ μm) with outer diameter of 3.0 mm and a cylinder platinum electrode were used as working and counter electrodes, respectively. The reference electrode was a saturated calomel electrode (SCE).

All experiments were performed on a three-electrode with a BAS 100B system. The experimental temperature was controlled at 25 ± 0.1°C.

2.3. Preparation of the chemically modified electrode

Prior to modification, the microdisk platinum electrode was polished with 400, 800 mesh sandpaper and 0.05 μm aluminum slurry and then cleaned by sonicating in double-distilled water for 10 min. The electrode was pretreated by a continuous cyclic sweep in the range of 0.0– +1.5 V at 50 mV s^{-1} in buffer until a stable background was observed.

Nafion/GOD (0.05 μl) solution was spread on the whole electrode surface and let evaporate in air at room temperature for 30 min to form the immobilized film. Then, these nafion modified electrodes were stored in buffer at 4°C.

The ferrocene perchlorate mediator was incorporated into GOD/nafion modified film by a cyclic sweep in phosphate buffer solution containing ferrocene perchlorate in the range of −0.1– +0.6 V until a constant voltammogram occurs.

3. Results and discussion

3.1. Incorporation of ferrocene perchlorate into nafion/GOD film and its electrochemical characteristics

Fig. 1 showed the incorporation process of Fc^+ into nafion/GOD film covered on the electrode surface. With the increase of scan numbers, the anodic and cathodic peak currents increased gradually. It is obvious that ferrocene perchlorate diffused into the nafion/GOD film and this mediator was stored in the film. The voltammetric peaks would reach constant when the scan time was up to 4 min. This value is far quicker than that of Ref. [7] in which it was performed at a conventional electrode.

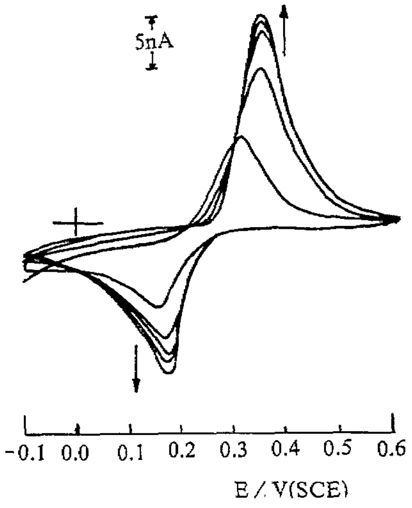

Fig. 1. Cyclic voltammograms showing the incorporation of ferricinium ion into nafion/GOD film from a 2.0 mM Fc^+ solution at 40 mV s^{-1} for 20 cycles.

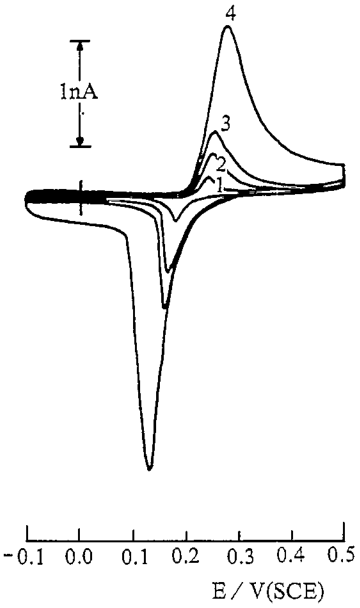

Fig. 2. Cyclic voltammograms of Fc^+/nafion film in 2.0 mM Fc^+ at scan rate of (a): (1) 1, (2) 5, (3) 10, and (4) 40 mV s^{-1}, respectively.

For the anodic peaks, they were higher than the corresponding cathodic peaks, consistent with the oxidation of an adsorbed deposit. Ferrocene is water insoluble, so, it is difficult to diffuse into solution. In contrast, the Fc^+ is slightly water soluble, and it is litter easily released from the film into bulk solution than ferrocene. Fc^+ could be stored in nafion at a higher concentration according to its distribution coefficient for cation between the nafion and solution.

It was found that the film thickness would influence the incorporation process of Fc^+. For a thin film, Fc^+ can diffuse more quickly into the film than that for a thicker film, meanwhile, the voltammograms changed more slowly with the increase of sweep numbers, moreover, only less amounts of Fc^+ could be incorporated into the film.

Fig. 2 showed the dependence of cyclic voltammograms on the scan rate on a nafion/Fc^+ electrode when Fc^+ was incorporated in nafion in a 200 mM Fc^+ phosphate buffer solution. With the increasing scan rate, the cathodic peak potential shifted toward a more negative direction, and the anodic peak potential would shift in a more positive value. Meanwhile, for the scan rate shown, the peak current correlated linearly with the square root of the scan rate. This resemblance to a semi-infinite diffusion process was consistent with the slow charge transport in nafion [19].

3.2. Hydrogen peroxide response on Fc^+/nafion coated electrode

The overpotential for the oxidation of hydrogen peroxide is higher at bare electrode, and the detection potential is usually beyond over + 0.6 V (vs. SCE) [18]. However, hydrogen peroxide could be detected at this modified electrode only at + 0.25 V which was reduced for 350 mV than that mentioned above. It also showed an amplified current response. Fig. 3 showed the catalytic oxidation of hydrogen peroxide at this modified electrode and the dependence of the current response on H_2O_2 concentration. Under the addition of H_2O_2, the anodic peak current increased quickly, and its cathodic peak current almost disappeared. The ferricinium sites apparently acted as a mediator for the oxidation of H_2O_2. It could be expressed as follows:

$$H_2O_2 + Fc^+ \rightarrow O_2 + Fc + 2H^+$$

The catalytic current resulted from the reoxidation of ferrocene sites:

$$Fc \rightarrow Fc^+ + e$$

The response currents were linear with the concentration of H_2O_2 in the range of 1×10^{-5}–5×10^{-3} M.

3.3. Catalytic oxidation of glucose

Ferrocene perchlorate is mobile at the interface of nafion and solution due to its slight water solubility and smaller molecular size, but an addition of a suitable amount of Fc^+ into the electrolyte would ensure that a constant concentration Fc^+ might be kept in the nafion film, which can provide a reservoir of cation, and a low diffusion of mediator from nafion film could be observed [19]. Therefore, a pseudo-third generation microdevice was obtained by incorporating Fc^+ mediator into nafion film after GOD immobilization. However, when no Fc^+ in solution was provided for equilibrium

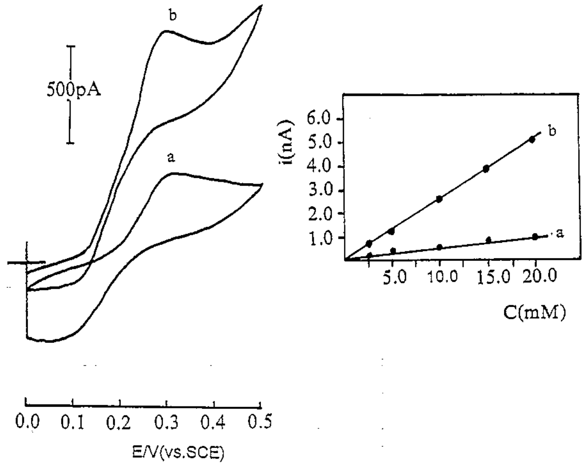

Fig. 3. Cyclic voltammograms of Fc^+/nafion film at 40 mV s^{-1} in (a) 2.0 mM Fc^+, (b) (a) + 5.0 mMH_2O_2. Inset: the response curve of H_2O_2 at (a) Fc^+/nafion modified Pt electrode; (b) bare pt electrode.

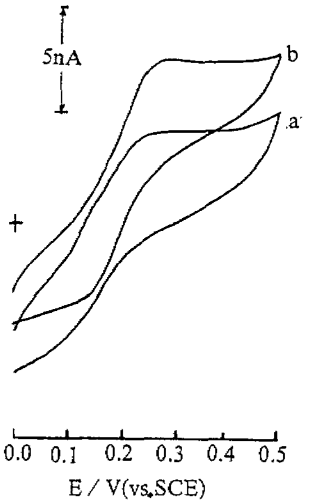

Fig. 4. Cyclic voltammograms of nafion/GOD film in 2.0 mM Fc^+ at 25 mV s^{-1}. (a) After equilibration of Fc^+ at the interface of nafion and solution. (b) With 7.5 mM β-D-glucose in solution.

of the film, the electrode would easily lose its electrochemical activity.

Fc^+ is slightly instable and began to degrade after 2–3 days if left in the concentrated form, but it is more stable in diluted form (up to 4 months) [20]. In our experiments, it was also observed that Fc^+ precipitation would appear when the Fc^+ concentration was beyond 10.0 mM. However, the Fc^+ precipitation was difficult to be observed for a long time when the concentration of Fc^+ was lower than 4.0 mM.

Fig. 4 showed the cyclic voltammogram which was obtained in the absence and presence of glucose. The catalytic current resulted from the added glucose which diffuses into the film and reacts with oxygen in the presence of glucose oxidase. The generated hydrogen peroxide was catalyzed by Fc^+. Meanwhile, the increasing Fc was rexoidized again. It could be expressed as follows:

$$Fc \rightarrow Fc^+ + e$$

$$Fc^+ + H_2O_2 \rightarrow O_2 + Fc + 2H^+$$

$$O_2 + \text{glucose} \xrightarrow{GOD} H_2O_2 + \text{gluconic acid}$$

The observed catalytic peak potential was at + 0.25 V. Such lower working potential could reduce the noise of current response, therefore, a glucose biosensor relatively high sensitivity could be obtained.

The dependence of peak currents on concentration of glucose was shown in Fig. 6(a). The response current increased linearly to 7.5 mM then deviates above 7.5 mM. A calibration curve was shown in Fig. 6(b). The apparent Michealis constant and the maximum limiting current, calculated from the Michealis–Menten analysis, were 7.06 mM and 5.85 nA, respectively.

3.4. Effects of relative amounts of GOD and nafion, the concentration of Fc^+ and oxygen on the amperometric determination of glucose

Fig. 5 showed the effects of the relative amounts of glucose oxidase and nafion in film on the response current of glucose. The response current almost increased linearly with the increase of the amount of glucose oxidase up to 5 mg ml^{-1}. No obvious response current was observed for lower amounts of glucose oxidase. However, it would also be reduced under a higher ratio of GOD/nafion due to the bad adhesion of the film to the surface of electrode.

It was observed that high concentration of Fc^+ incorporated into the GOD/nafion modified film would delay the response time for the catalytic oxidation of glucose. Meanwhile, it would also take a longer time for the establishment of mediator equilibrium at the interface between film and solution.

The response time for reaching maximum current was ca. 2 min for 1.0 mM Fc^+ and 4 min for 2.0 mM Fc^+. But it was quicker than that for conventional biosensors. The response curves of glucose on different concentration of Fc^+ were shown in Fig. 6(b)Fig. 7(a).

The calibration curves of glucose were obtained in the presence of oxygen using linear sweep voltammetry (Fig. 6). Obviously, the linear response region for 1.0 mM Fc^+ was wider than that for 2.0 mM Fc^+.

The response current was reduced when the test solution was deaerated with nitrogen (Fig. 7(b)). It showed that oxygen plays an important role in the catalytic process. Meanwhile, the electroactivity of Fc^+ reduced the working potential due to the electrocatalysis for the oxidation of the generated hydrogen peroxide. Moreover, it was found that the Fc^+ could also oxidize the reduced flavin adenine dinucleotide of glucose and the maximum limiting currents were 10.71 mM and 5.1 nA under aerobic condition, 6.23 mM and 1.98 nA under anaerobic condition, respectively. This is

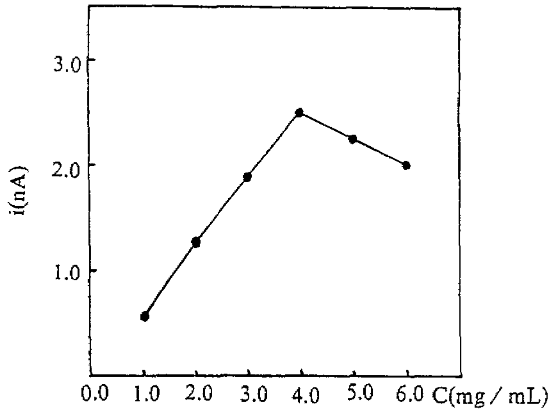

Fig. 5. The effects of the ratio of GOD to nafion on response at + 0.25 V after adding 5.0 mM β-D-glucose.

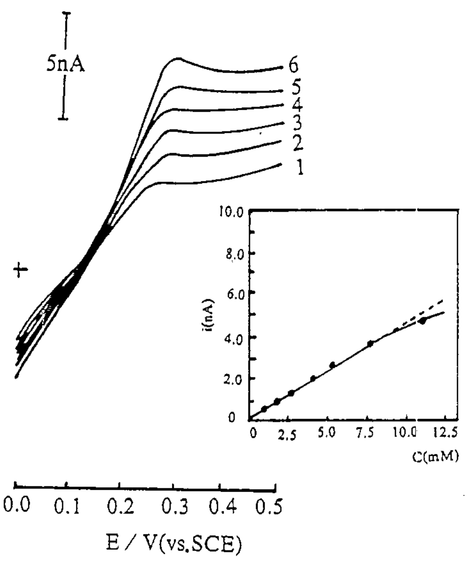

Fig. 6. (A) The linear sweep voltammograms by adding various concentration of β-D-glucose in 2.0 mM Fc^+. (1) 0.0 mM, (2) 2.5 mM, (3) 5.0 mM, (4) 7.5 mM, (5) 11.0 mM, (6) 20.0 mM. (B) The inset figure was the calibration curve of glucose. Scan rate: 25 mV s^{-1}.

related to the characteristic of nafion film, because nafion film usually possesses the ability of adsorbing oxygen [21].

3.5. Interference of ascorbic acid, uric acid and the stability of modified electrode

The determination of biomaterials are usually interfered by ascorbic acid and uric acid, etc. Many attempts have been carried out to exclude the effects of these interferences, which included the pretreatment of electrode, modification of electrode, and reduction of the working potential, etc.

The response currents for ascorbic acid and uric acid at the bare platinum microelectrode were compared with that at the nafion covered one, in which the working potential was chosen at +0.25 V. The response currents of 1.0 mM ascorbic acid were 10 and 2.0 nA at bare and the nafion covered microelectrode, respectively. No response currents were observed for 1.0 mM uric acid at this modified electrode because of its lower working potential. So, this nafion modified microelectrode exhibited an excellent feature for the reduction of interferences.

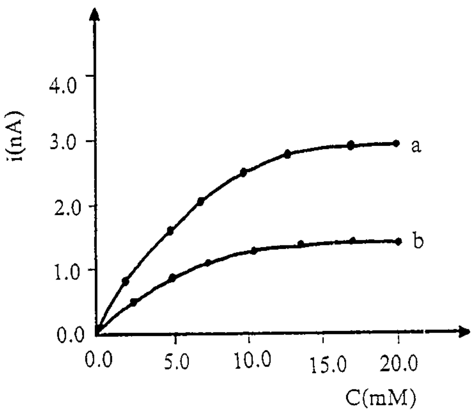

Fig. 7. The calibration curve of β-D-glucose containing 1.0 mM Fc^+ (a) with oxygen gas, (b) deaerated.

The enzyme's inactivity usually made the enzyme electrode instable and little reproducible. Nafion coating, formed by using nafion solution diluted by organic solvents, could cause denaturation of enzyme. Here, nafion was diluted by using water instead of alcohol or buffer previously used. This is very important for reserving the enzyme activity. The results showed that this modified microelectrode had a good stability and reproducibility when it was stored in phosphate buffer (pH 5.9) at 4°C for 1 month, and the response current reduced only about 20%. But, it would reduce more than 50% after it was placed for 60 days.

Acknowledgements

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