Covalent linkage of glucose oxidase to modified basal plane pyrolytic graphite electrodes and the use in the ferrocene-mediated amperometric measurement of glucose

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Abstract

A simple, fast and effective method for the immobilization of glucose oxidase onto a modified basal plane pyrolytic graphite electrode is described. To enhance the rate of electron exchange with the electrode, a ferrocene derivative was used as a mediator. The stability of the enzyme electrode was evaluated using direct current cyclic voltammetry.

Introduction

The use of sensitive electrochemical techniques in biosensor design is a rapidly developing area. Amperometric enzyme electrodes combine the advantages of the specificity of the enzyme for the recognition of particular molecules with the direct transduction of the rate of the reaction into current. In these devices, the redox enzymes are attached to the electrodes where electron transfer proceeds, either through a direct transfer between the active site and the electrode, or through a soluble charge carrier. In practice, the replacement of the natural redox partner of the enzyme by a low molecular weight redox mediator proved to be more effective and convenient in achieving fast electron transfer between electrodes and enzymes. Since the potential at which the detection is carried out is determined by the redox potential of the mediator, interference from other electroactive species can be avoided by careful selection of the mediator. The synthesis of derivatives of a particular redox mediator is a powerful tool, and critical for the manipulation of a given system. On the other hand, the type of immobilization technique used to confine enzymes onto electrode surfaces has a marked effect on the usefulness of the amperometric enzyme electrode. The most stable immobilized enzymes are those prepared through chemical bridges to a surface [1, 2], but there are many results with physically entrapped enzymes as well [3–5].

In recent years we have been involved in the improvement of immobilization techniques for redox enzymes and the search for suitable redox mediators. The success with the glucose sensor [6], discovered in our laboratory, promoted further research on devices based on the glucose oxidase (GOD) electrode [7–9]. In these sensor configurations, a substituted ferrocene (bis(η^5-cyclopentadienyl)iron, $Fecp_2$) is used to shuttle electrons between enzyme and electrode. The formal potential of the ferrocene derivative depends upon the substituents on either or both of the cyclopentadienyl rings, but the electron transfer reaction retains their advantageous quality of rapidity and reversibility. The system is not dependent on oxygen; the ferricinium ion replaces oxygen as a cofactor for GOD.

$$\text{Glucose} + \text{GOD}_{(ox)} \longrightarrow \text{Gluconolactone} + \text{GOD}_{(red)}$$

$$\text{GOD}_{(red)} + 2Fecp_2R^+ \longrightarrow \text{GOD}_{(ox)} + 2Fecp_2R + 2H^+$$

$$2Fecp_2R \longrightarrow 2Fecp_2R^+ + 2e^-$$

A linear current response, proportional to glucose concentration, is observed over the diagnostic range and is free from interference.

We report here a simple, yet very effective method, of immobilizing GOD onto a modified basal plane pyrolytic graphite (BPG) electrode, the synthesis of the competent redox mediator: 1,1′-dimethyl-3-(1-hydroxy-2-aminoethyl)ferrocene (DMFEA) and the results with this system using direct current cyclic voltammetry.

Experimental

Materials

GOD (EC. 1.1.3.4.) from *Aspergillus niger* was provided by MediSense Inc.; D-glucose (AnalaR) was from BDH;

0020-1693/92/$5.00

dimethylferrocene was purchased from Strem Chem. Co.; $POCl_3$, nitromethane, EDC, laurylamine, *N*-methyl formanilide and 10% Pd/C were supplied by Aldrich Chem. Co.; BPG was from Union Carbide. Silica gel 60 (Merck) was used for chromatographic separations. All solvents were AnalaR grade and all solutions were prepared from AristaR grade reagents (BDH) in ultra pure water (Millipore). The supporting electrolyte was 20 mM K_2HPO_4, pH 6.9; the latter was adjusted with 0.1 M $HClO_4$. Glucose solutions were prepared 24 h prior to use to allow equilibration of α- and β-anomers.

Apparatus

D.c. cyclic voltammetry experiments were performed at room temperature with a two-compartment cell that had a working volume of 0.5 ml. The working compartment accommodated a 1 cm^2 platinum gauze counter electrode in addition to the 5 mm diameter BPG working electrode. A saturated calomel electrode (SCE) was used as a reference in a sidearm which connected to the working compartment via a luggin capillary. All potentials are referred to the SCE. An Ursar Scientific potentiostat was used with a Gould series 60 000 A3 chart recorder to control the potential of the working electrode.

Synthesis

Figure 1 shows the reaction sequence used to prepare DMFEA.

1,1'-Dimethylferrocene carboxaldehyde (I)

13 g (0.1 mol) *N*-methylformanilide and 14.6 g (0.1 mol) $POCl_3$ were dissolved in 10 ml of dry dichloromethane and stirred at room temperature for 45 min. To this mixture was dropwise added 11.3 g (0.05 mol) dimethylferrocene in 70 ml of dry dichloromethane under nitrogen. The reaction mixture was stirred at room temperature for 3 days in the dark. Then it was poured onto 200 ml ice-cold saturated Na acetate. The organic phase was washed with 10% $NaHCO_3$, dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification was carried out on a silica gel column using dichloromethane as eluent. Yield 11 g (86%). IR: ν(C=O) 1672 cm^{-1}.

1,1'-Dimethyl-3-(1-hydroxy-2-nitroethyl)ferrocene (II)

A Schlenk tube containing **I** (5 g, 21 mmol), nitromethane (2.2 ml, 42 mmol) and 200 ml absolute alcohol was fitted with a stirrer and cooled to 0 °C. Sodium metal (1 g, 42 mmol) was dissolved in 30 ml dry methanol at 0 °C and stirred for 3 h. After the addition of base, the sodium salt precipitated out as a solid. The reaction mixture was cooled to −78 °C; the resulting solids washed with dry petroleum ether. To liberate the free nitro compound from the salt form, the solids were

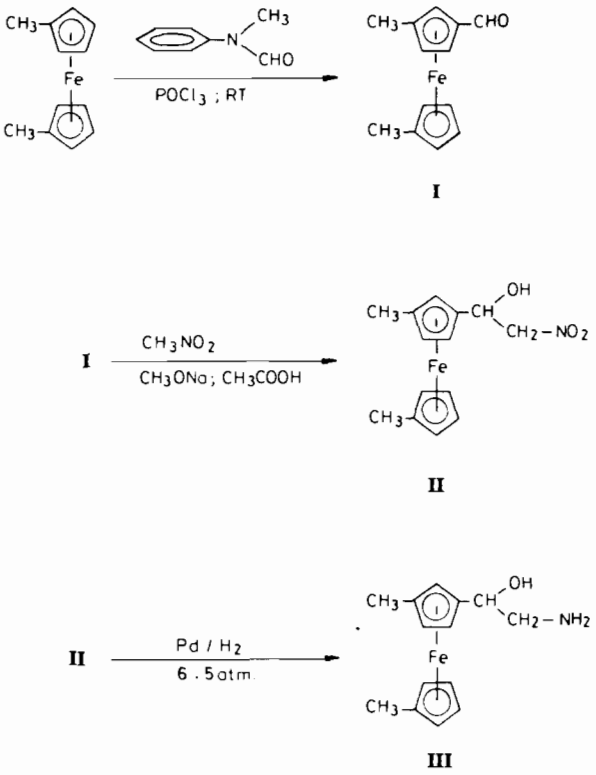

Fig. 1. Synthesis route for the preparation of DMFEA.

suspended in dry ether; 1.1 ml freshly distilled glacial acetic acid was added under N_2 and stirred at room temperature overnight. Crushed ice was added to the dark red solution and the precipitate was washed with 5% sodium bicarbonate solution then dried. The solvent was removed *in vacuo* to give a dark red oil (4 g, 66%).

1,1'-Dimethyl-3-(1-hydroxy-2-aminoethyl)ferrocene (III)

A glass pressure vessel containing compound **II** (4 g) in 200 ml dry methanol and 500 mg Pd/C catalyst was filled with H_2 gas (6.5 atm) and the mixture was stirred for 15 h. Thereafter the reaction mixture was filtered and the solvent removed to give 2.5 g (69%) yellow solids. IR: 3354 and 3273 cm^{-1}, two sharp peaks N–C stretching. *Anal.* Calc. for $C_{14}H_{19}NOFe$: C, 61.54; H, 6.96; N, 5.13. Found: C, 60.97; H, 6.80; N, 5.15%.

Construction of GOD electrode

Figure 2 illustrates the preparation of the enzyme electrode. A freshly cut basal plane pyrolytic graphite electrode was dipped into a saturated methanolic solution of laurylamine for 5 min, then sonicated for 5 min. To 100 μl 0.1 mM aqueous solution of GOD, 100 μl 10% aqueous solution of 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added at 5 °C and the previously modified BPG electrode

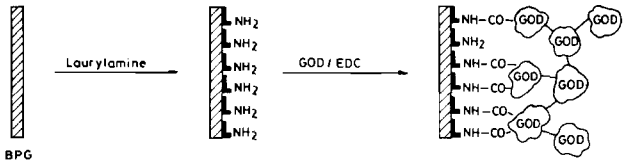

Fig. 2. Schematic illustration of electrode modification.

was immersed in it for 20 min. After several washings with Millipore water the electrode was stored in 20 mM phosphate buffer at 4 °C.

GOD electrode pretreatment

Prior to experiments, the electrode response was stabilized by continuous operation of the electrode under potentiostatic control at 250 mV in 7 mM glucose over a 10 h period. Thereafter the electrodes were found to give more stable responses.

Results and discussion

We have investigated a number of immobilization procedures for GOD. Figure 2 outlines our most successful approach. This consists basically of two different modification steps for the BPG electrode: adsorption and chemical coupling. The long alkyl chain of laurylamine is strongly adsorbed at the very apolar BPG electrode surface, effectively covering the electrode area with amino groups; further reactions can be undertaken in order to covalently couple the GOD onto the electrode. A water soluble carbodiimide (EDC) was chosen to perform the coupling. The carbodiimide activates the surface carboxylic groups of GOD which would then react with any amino group available to form amide bounds. Since the enzyme itself also contains surface amino groups, the GOD will not only couple to the electrode, but to other molecules of GOD as well. The enzyme immobilization described here results in an electrode having not just a monolayer of GOD, but a cluster of them are attached to its surface, where the highly soluble mediator (ferricinium cation) can easily diffuse to facilitate electron transfer between electrode and enzyme.

The choice of the mediator used was based on our previous investigations [10] that ferrocene and its analogues act as effective mediators to a wide range of oxidoreductases including GOD. These ferricinium forms are rapid oxidants for the enzyme GOD. The results obtained with a large number of ferrocene analogues showed the second-order rate constant to be highly dependent on the charge on the mediator. Positively charged ferrocenes are superior to negatively charged ones. Highly soluble compounds with low redox potentials are favored. The DMFEA synthesized by us proved to be the most effective redox mediator for GOD so far.

Figure 3 shows the current–voltage curves of the BPG electrode before and after modification with GOD. Comparing the data obtained by cyclic voltammetry between A and B we can conclude that the GOD is immobilized onto the electrode (case B) which is manifested by the decrease of capacitance of the electrode. In the presence of 0.2 mM DMFEA (C) the direct current cyclic voltammogram obtained with the GOD electrode exhibits a reversible, one electron redox couple with $E_{1/2}$ at 140 mV versus SCE characteristic of the ferrocene analogue. On the addition of D-glucose (50 mM), a large catalytic current is observed at oxidizing potentials (D), indicating, that the enzyme catalyzed oxidation of glucose is electrochemically coupled via DMFEA. The catalytic current (i_{cat}) is 30-fold larger than that of the non-coupled signal (C).

The performance of this GOD electrode was evaluated daily for over a month (Fig. 4). The electrode

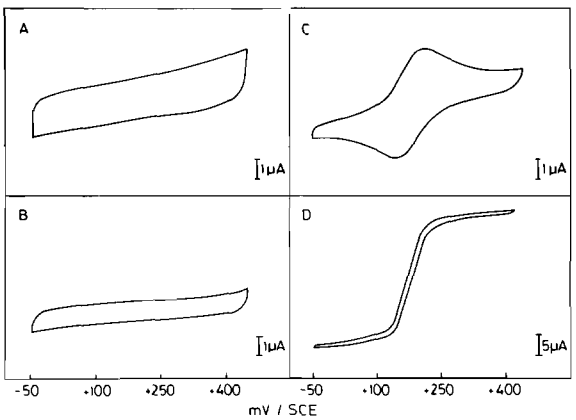

Fig. 3. D.c. cyclic voltammograms recorded in 20 mM phosphate buffer pH 6.9 at a scan rate of 10 mV/s between −50 and +450 mV vs. SCE. A, Freshly cut BPG electrode; B, the above electrode modified with laurylamine and GOD immobilized onto it (see text); C, in 0.2 mM DMFEA containing solution using the GOD electrode; D, as C in 50 mM glucose solution.

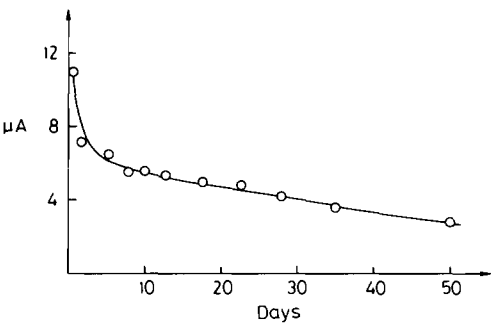

Fig. 4. Steady-state current response of the GOD electrode with time using 50 mM glucose and 0.2 mM DMFEA at an applied potential of 250 mV vs. SCE in 20 mM phosphate buffer pH 6.9.

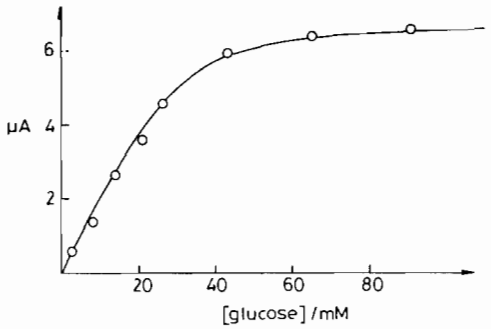

Fig. 5. Glucose concentration dependence of steady-state current of a 10 day old GOD electrode in the presence of 0.2 mM DMFEA in 20 mM phosphate buffer. Applied potential is 250 mV vs. SCE.

was operated in the steady state at a potential where the enhanced cathodic current reached a plateau. The initial high loss in response was circumvented by preconditioning the electrode prior to operation. In spite of the preconditioning procedure, the response decreased over the first couple of days, but afterwards gave a rather steady response for 30 days and only slowly decayed thereafter. It seems that the GOD in this immobilized state is very stable and remains active much longer than in other immobilized forms [11, 12]. This kind of attachment allows the enzyme to be used for many more analyses without losing its catalytic activity.

A typical steady-state current calibration curve for a 10 day old GOD electrode is shown in Fig. 5. Since a glucose dependent current is only realized when the electrode is poised sufficiently positive to generate the ferricinium ion, the potentiostat was operated at 250 mV versus SCE (60 mV positive of $E_{1/2}$ for DMFEA). The background current at the electrode in the absence of glucose was measured, $i_0 = 1.2$ μA and substracted from the data in Fig. 5. The electrode gave a linear current response in the range of 1–30 mM glucose and finally became insensitive to increasing glucose concentrations.

Conclusions

We have described a fast and highly effective method for the immobilization of GOD onto a BPG electrode and have shown its use in the DMFEA-mediated amperometric measurement of glucose. The stability of the enzyme electrode and its steady response for over a month are features that make it fit for clinical or biotechnological monitoring. Given further development, this routine could be extended to other enzymes too.

Acknowledgement

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