Step-by-Step Immunological Construction of a Fully Active Multilayer Enzyme Electrode

Christian Bourdillon,[1a] Christophe Demaille,[1b] Jacques Moiroux,[1b] and Jean-Michel Savéant[1b]

Laboratoire de Technologie Enzymatique
Unité Associée au CNRS No. 1442
Université de Technologie de Compiègne
B.P. 649, 60206 Compiègne Cedex, France
Laboratoire d'Electrochimie Moléculaire
de l'Université Denis Diderot (Paris 7)
Unité Associée au CNRS No. 438
2 place Jussieu, 75251 Paris Cedex 05, France

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In a previous article,[2] we described an antibody–antigen method for attaching a monolayer of enzyme onto an electrode surface. The method was illustrated with the deposition of a monolayer of glucose oxidase on a glassy carbon electrode. Full activity of the enzyme monolayer thus immobilized was demonstrated by determination of the amount of enzyme by radioactive $^{125}I^*$ labeling and analysis of the cyclic voltammetric responses recorded in the presence of glucose, using ferrocene methanol as mediator. Immunological methods have been used earlier to deposit enzymes on electrode surfaces,[3] but the techniques used did not allow precise determination of the amount of deposited active enzymes. Chemical attachment and entrapment in polymers are methods that have also been used to immobilize enzymes on electrode surfaces.[4]

We now report an antibody–antigen method allowing the step-by-step synthesis of a set of successive glucose oxidase monolayers onto a glassy carbon electrode surface. The essential step for attaching a second monolayer to the first glucose oxidase monolayer immobilized on the electrode surface by the technique described in ref 2 involves using monoclonal antibodies to glucose oxidase (C), as sketched in Figure 1. At one end, C binds to the glucose oxidase moiety contained in the glucose oxidase conjugate, B. At the other end, it serves as an antigen for the glucose oxidase conjugate B, playing then the same role as the sacrificial antigen A, directly adsorbed at the electrode surface. Since the only monoclonal antibodies to glucose oxidase that are available are those produced in mouse, we replaced the rabbit antigen A and the anti-rabbit glucose oxidase conjugate B used in reference 2 by their mouse and anti-mouse analogs, respectively. In this manner, it was possible to attach a second monolayer to the first, a third to the second, and so on.[14]

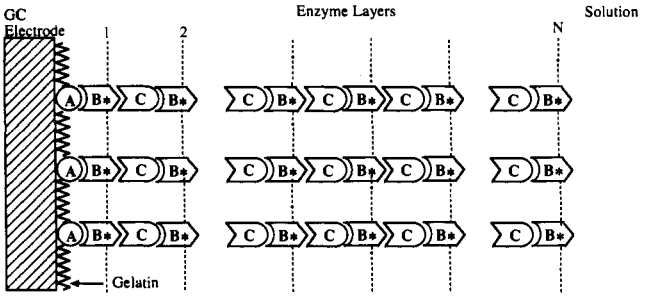

Figure 1. Sketch of the construction of N monolayers of glucose oxidase on a glassy carbon electrode surface. (A) Adsorbed mouse IgG (antigen); (B) anti-mouse IgG glucose oxidase conjugate (antibody); (C) monoclonal antibody to glucose oxidase produced in mouse. An asterisk indicates the approximate location of a glucose oxidase moiety.

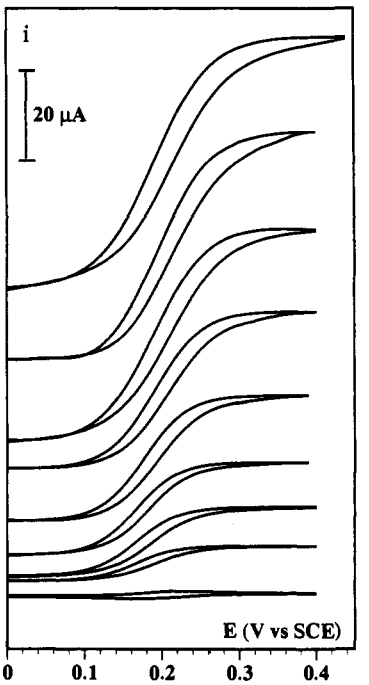

Figure 2. Cyclic voltammetry of the catalysis of glucose oxidation by the glucose oxidase electrode as a function of the number of monolayers (N) in the presence of glucose (0.1 M) with ferrocene methanol (0.1 mM) as mediator in a pH 8 phosphate buffer (ionic strength, 0.1 M). From bottom to top: N = 0, 1, 2, 3, 4, 5, 6, 7, and 8 (zero current in each voltammogram is defined by the horizontal line at the foot of the wave). Scan rate, 0.04 V/s. Temperature, 25 °C.

Typical voltammograms of methanol ferrocene obtained in the presence of glucose with electrodes with 1–8 successively attached monolayers are shown in Figure 2. The same reversible wave of ferrocene methanol was obtained at a bare electrode or at any of the electrodes coated with glucose oxidase in the absence

(1) (a) Université Technologique de Compiègne. (b) Université Denis Diderot.
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of glucose in the solution. In the presence of glucose, the increasingly large catalytic responses observed as the number of monolayers increases may be analyzed as follows.

With a single monolayer, the cyclic voltammetric current produced by the enzymatic oxidation of glucose by means of a mediator couple P/Q may be decomposed, as shown earlier,[2] in two contributions. One is the P/Q current response obtained in the absence of glucose and the other, the catalytic contribution, i_{cat}, obeys the following equation:

$$i_{cat} = \frac{2FSk_3\Gamma^0_{E,1}[Q]_0}{1 + k_3[Q]_0\left(\frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1k_2[G]}\right)} \quad (1)$$

where $\Gamma^0_{E,1}$ is the surface concentration of enzyme present in the monolayer, S is the electrode surface area, $[Q]_0$ is the concentration of the active form of the mediator, Q, at the electrode surface, and [G] is the concentration of glucose at the enzymatic sites (the bulk concentrations of the mediator and of glucose are denoted C^0_P and C^0_G, respectively). The various rate constants in eq 1 are those defined in the following reaction scheme:

$$P \rightleftarrows Q + e^- \quad (0)$$

$$FAD + G \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} FADG \quad (1)$$

$$FADG \xrightarrow{k_2} FADH_2 + GL \quad (2)$$

$$FADH_2 + 2Q \xrightarrow{k_3} FAD + 2P \quad (3)$$

where FAD and $FADH_2$ are the oxidized and reduced forms of glucose oxidase, respectively, FADG is the enzyme–substrate complex, G represents β-D-glucose, and GL represents gluconoδ-lactone.

(14) Solutions of gelatin (0.1 mg/mL), glucose oxidase conjugated antibody (10 μg/mL), and monoclonal antibody to glucose oxidase (40 μg/mL) were all prepared in a buffer composed of 0.01 M KH_2PO_4 and 0.15 M NaCl. The pH was adjusted at 7.4 with a 1 M NaOH solution. In the two latter cases, 0.1 mg/mL of sodium azide was also added to the solution to prevent the formation of bacterial colonies. Adsorption of the antigen resulted from a 2 h exposure of the electrode surface to a low concentration (*ca.* 3 μM) solution in order to minimize aggregation. The electrode was then thoroughly washed with the buffer and dipped for 10 min in the solution of gelatin. After another thorough washing, the antigen–antibody reaction took place after overnight immersion of the electrode in the solution of glucose oxidase conjugated antibody. This procedure ensures that the first monolayer is saturated with catalytically active glucose oxidase (over 2 h exposure to the antigen, the catalytic activity remains steady). The next monolayers were immobilized on top of the first or any preceding layer. We proceeded as follows. The electrode was first immersed in a mixture of two mouse IgG monoclonal antibodies to glucose oxidase of different origins for 5 h, thus allowing recognition and binding to the glucose oxidase units of the preceding layer. After thorough washing, the electrode thus obtained was left overnight in contact with the glucose oxidase conjugated antimouse IgG. Assays of the total enzymatic activity, as described in the text, showed that no increase of the amount of glucose oxidase thus immobilized resulted from increased immersion times and/or antibody concentrations in each of the two steps of the procedure. When not in use, the electrode was stored in the buffer solution containing 0.1 mg/mL NaN_3.

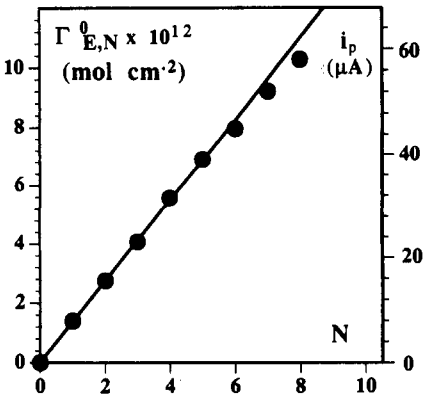

Figure 3. Plateau currents (i_p) and ensuing values (from eq 2) of the amount of glucose oxidase deposited on the electrode surface, $\Gamma^0_{E,N}$, as a function of the number of monolayers, N.

At the plateau of the wave, $[Q]_0 = C^0_P$. Thus eq 2 can be used to determine the amount of active glucose oxidase deposited on

$$i_{cat} = \frac{2FSk_3\Gamma^0_{E,1}C^0_P}{1 + k_3C^0_P\left(\frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1k_2C^0_G}\right)} \quad (2)$$

the electrode surface, $\Gamma^0_{E,1}$ since in the experiments represented in Figure 2, glucose is in large excess and therefore [G] = C^0_G. The rate constants to be used in the application of eq 2 have been determined previously:[15] $k_3 = 1.2 \times 10^7$ M^{-1} s^{-1}, $k_2 = 700$ s^{-1}, and $k_{red} = k_1k_2/(k_{-1} + k_2) = 10^4$ M^{-1} s^{-1}. With a single monolayer immobilized by means of the mouse/anti-mouse procedure, it was found that $\Gamma^0_{E,1} = 1.4 \times 10^{-12}$ mol cm^{-2}, somewhat less than with the rabbit/anti-rabbit procedure ($\Gamma^0_{E,1} = 2.6 \times 10^{-12}$ mol cm^{-2}).[16]

If diffusion of the mediator through the multilayer film (N layers) is sufficiently rapid, eq 2 also applies replacing $\Gamma^0_{E,1}$ by $\Gamma^0_{E,N}$. The plateau current should then be proportional to N. As seen from Figure 3, this is indeed what is observed experimentally. In other words, $\Gamma^0_{E,N}$ derived from the application of eq 2 to the plateau currents is proportional to N, showing that the same surface concentration of glucose oxidase, Γ^0_E, is present in each of the successively deposited monolayers.[16]

Simulation of the plateau currents, taking account of the diffusion of the mediator through the enzyme film with a diffusion coefficient equal to the value in the solution, reproduced satisfactorily the slight downward deviation from linearity that can be observed in Figure 3. We may thus conclude that the enzyme film is a remarkably open structure not obstructing the mediator diffusion to any significant extent.

It was also observed that the huge catalytic activities thus obtained are stable in time: the decrease of the enzymatic activity for electrodes in use or stored[14] was less than 10% for a period of 20 days.

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(16) In 45% of B (Figure 1), glucose oxidase, although enzymatically inactive, is still present and recognized by C.