A Fully Active Monolayer Enzyme Electrode Derivatized by Antigen–Antibody Attachment

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Abstract: The immobilization of monolayers and submonolayers of glucose oxidase on carbon electrodes by adsorption of rabbit IgG (antigen) and reaction with a glucose oxidase conjugated antibody, the antirabbit IgG produced in goat, is described. As revealed by radioactive ^{125}I labeling and by cyclic voltammetry, using ferrocene methanol as mediator, the enzyme monolayers thus immobilized are fully active and persistent. The fact that the mediator couple remains reversible in the presence of the enzyme film allows a particularly simple and quick derivation of primary and secondary plots characterizing the enzyme kinetics from the cyclic voltammetric responses. Comparison with chemically derivatized electrodes investigated in the same manner under similar conditions shows the superiority of the immunological attachment technique.

Immobilization of enzymes onto electrode surfaces as applied to the catalysis of electrochemical reactions has attracted active attention in recent years in relation to the design and optimization of biosensors[2] and of biotechnological processes.[3] In most cases, the presence of a mediator (cosubstrate) that shuttles electrons between the electrode and the enzyme prosthetic group is required. The mediator may be dispersed in the solution that contains the substrate. In systems where the enzyme is entrapped in a polymer matrix, the mediator may also be attached to the polymer or to the enzyme itself thus providing a "wiring" of the enzyme to the electrode surface.[4]

Chemical immobilization seems preferable to direct adsorption on the electrode surface which appears to result in denaturation of the enzyme.[5] Among the various chemical techniques employed to immobilize a monolayer of enzyme on an electrode surface, the most popular method consists, using a carbon electrode, of oxidizing vigorously the surface so as to create a large number of carboxylic acid sites which are reacted successively first with carbodimide and then with the enzyme.[6,7] One drawback of the method is that base-line currents arising from the oxidation of the carbon surface may hamper a precise electrochemical kinetic characterization of systems constructed along these lines. The exact amount of active enzyme attached to the surface is not easily quantitized since the oxidative corrosion of the carbon surface may have changed its area by a significant but hardly measurable factor.[8]

An interesting extension of the surface oxidation–carbodiimide technique has been recently proposed.[9] A biotin unit is attached on the electrode surface, and the enzyme is itself biotinilated, the two biotin units being then connected by an avidin unit taking advantage of the strong biotin–avidin affinity.

It is also possible to employ a reductive attachment technique whereby the carbon surface is first grafted with *p*-carboxyphenyl radicals generated from the electrochemical reduction of the corresponding diazonium salts and then coupled with the enzyme through the carbodiimide method.[8b] The base-line oxidation currents thus obtained are less than with the oxidative method. However the amount of active enzyme thus grafted has not been determined with precision.

The deposition of multilayers of enzymes on electrode surfaces has been carried out by entrapment within polymer matrixes.[4,10] Another method leading to a reticulated multilayer film consists in oxidizing the surface and then reacting the carboxylic acid groups simultaneously with carbodiimide and the enzyme.[11] With such systems, the presence of several layers of enzyme is expected to enhance the catalytic current. This effect may however be

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counteracted by the slowness of the diffusion of the substrate through the film and/or the slowness of the shuttling of electrons through physical diffusion of the mediator or electron hopping between mediator sites when these are irreversibly bound to the film structure.[12]

Although immunological techniques have been previously used to deposit enzymes on surfaces,[13] they have not so far been applied to the attachment of enzyme monolayers on electrode surfaces in the aim of obtaining a fully active and persistent catalytic system.[14]

We describe in the following sections such an antigen–antibody immobilization procedure taking glucose oxidase as an example of attached enzyme. The combined use of cyclic voltammetry of the electrode thus modified and radioactive ^{125}I labeling allowed a precise determination of the amount of active enzyme in the deposited monolayer and the full characterization of the catalysis kinetics. Particular attention was paid to the obtention of small background currents and to the preservation of the electrochemical reversibility of the electron-shuttling mediator. We also compared the activity of the enzyme in this system with that obtained with the chemical reductive method evoked earlier.[8b]

The excellent results obtained with the antigen–antibody technique in terms of catalyst activity and stability allow us to envision the use of the same technique for a future step-by-step synthesis of systems containing successive enzyme layers enabling a precise control of their activity and spatial arrangement.

Results and Discussion

Ferrocene methanol was selected as mediator. The kinetics of the catalysis of the oxidation of glucose by dissolved glucose oxidase by means of this particular mediator has been fully characterized in an earlier work.[15] In the absence of the substrate, the mediator gives rise to a one-electron (chemically and electrochemically) reversible cyclic voltammetric wave. It increases in height and progressively loses its chemical reversibility upon addition of glucose. The increase of the peak height is thus a measure of the kinetics of the overall catalytic reaction from which the pertinent rate constants could be derived. We used the same approach in the present case where the enzyme is attached to the electrode surface. It is important to examine whether or not the film of immobilized enzyme affects the electrochemical reversibility of the mediator oxidation at the electrode. Optimal efficiency of the catalysis is reached when the mediator couple remains electrochemically reversible (continues to obey the Nernst law) in the potential scan rate range of interest. The fullfilment of these conditions also allows a simpler derivation of the catalysis kinetics from the experimental data. All these cyclic voltammetric experiments were carried out in a phosphate buffer at pH 8 (0.1 M ionic strength), a pH where the reaction between ferrocenium methanol, the active form of the mediator, and the reduced flavin of the enzyme is the fastest.[15] The electrode material was glassy carbon, not oxidized, carefully polished, and rinsed in all cases.

We first attempted to simply adsorb glucose oxidase onto the carbon electrode. The resulting catalytic increase of the cyclic voltammetric peak current was very weak, less than 10% of the original peak height at 0.02 V/s. Either the adsorption of glucose oxidase on the surface is too weak, or adsorption causes denaturation of the enzyme as already observed by means of ellipsometry on platinum.[5] Simple adsorption is therefore not a suitable means for preparing an efficient enzyme electrode.

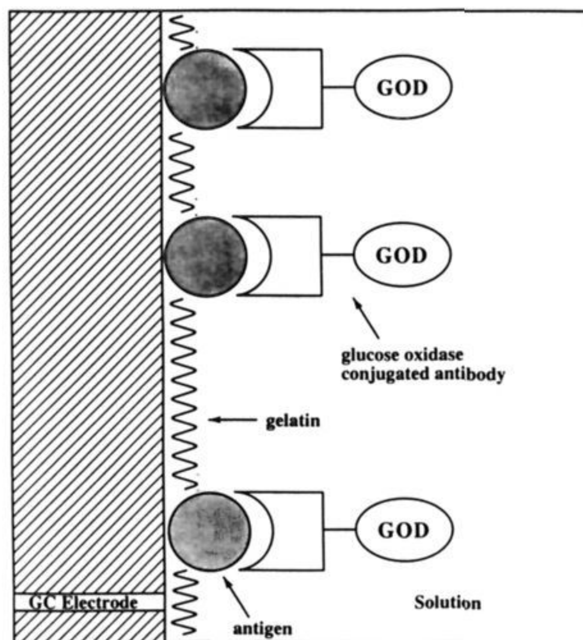

Figure 1. Sketch of the monolayer glucose oxidase electrode obtained by absorption of rabbit IgG (antigen) and reaction with a glucose oxidase conjugated antibody, the antirabbit IgG (whole molecule) produced in goat.

The structure of the electrode prepared by the antigen–antibody technique is sketched in Figure 1. Adsorption of rabbit IgG, used as a sacrificial antigen, was followed by adsorption of gelatin and specific binding of a glucose oxidase conjugated antibody (with a fully active glucose oxidase moiety) as described in the Experimental Section. Direct adsorption of the glucose oxidase conjugated antibody on the glassy carbon electrode did not lead to efficient catalysis most probably because of inactivation of a large amount of the enzyme. It is likely that the rabbit IgG used as sacrificial antigen also undergoes some denaturation upon adsorption. It is nevertheless still recognized by the glucose oxidase conjugated antibody whose enzyme remains full activity as will be shown latter on. The presence of gelatin also prevents self-inhibiting adsorption of ferrocene methanol on the electrode surface. In the absence of glucose, the anodic peak current, i_p^0, indeed remains proportional to the square root of the scan rate (v) up to 10 V/s as expected for a fast electron-transfer reaction[16] whereas the ratio $i_p^0/v^{1/2}$ starts to decrease above 1 V/s in the absence of gelatin. Likewise, the anodic-to-cathodic peak potential separation remains equal to 60 mV[16] up to 10 V/s in the presence of gelatin, whereas it becomes larger and larger above 1 V/s in the absence of gelatin.

The thickness of the gelatin layer is most probably small, as represented in Figure 1, since it does not hamper the binding of the glucose oxidase conjugated antibody with the adsorbed antigen as revealed by the determinations of the catalytic efficiencies described below.

A typical cyclic voltammogram showing the occurrence of the catalytic reaction is shown in Figure 2. The characteristic rate constants of the catalytic process may be derived from the cyclic

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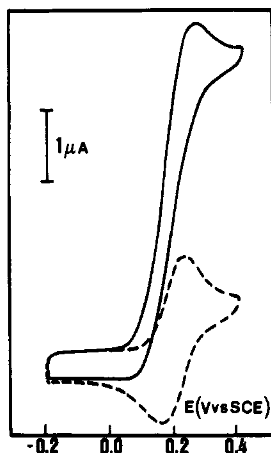
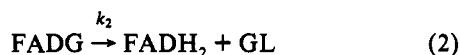


Figure 2. Cyclic voltammetry of the catalysis of glucose oxidation by the glucose oxidase electrode prepared by the antigen–antibody technique with ferrocene methanol (0.1 mM) as mediator in a pH 8 phosphate buffer (0.1 M ionic strength). The dashed and solid lines represent the cyclic voltammogram (0.04 V/s) in the absence and presence of glucose (0.1 M) respectively. Temperature: 25 °C.

voltammograms as follows. As discussed earlier (ref 15 and references cited therein) the catalytic process consists of the following sequence of reactions

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

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



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

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

(P, Q, reduced and oxidized forms of the mediator respectively; FAD, $FADH_2$, oxidized and reduced form of the prosthetic groups of glucose oxidase respectively; FADG, enzyme–substrate complex; G, β-D-glucose; GL, glucono-δ-lactone).

P diffuses from the bulk of the solution, where its concentration is C_P^0 to the electrode surface obeying the second Fick's law for linear diffusion. *Q* diffuses back from the electrode to the solution where its concentration is zero also obeying the second Fick's law:

$$\frac{\partial[Q]}{\partial t} = D\frac{\partial^2[Q]}{\partial x^2}$$

(*t*, time; *x*, distance from the electrode surface, *D*, diffusion coefficient, assumed to be the same for *Q* and *P*). At time $t = 0$, the concentrations of *P* and *Q* are equal to C_P^0 and 0, respectively, throughout the solution. At the electrode surface, the *P*/*Q* couple obeys the Nernst law as discussed earlier

$$\frac{[P]_0}{[Q]_0} = \exp\left[-\frac{F}{RT}(E - E^0)\right]$$

(E^0 is the standard potential of the mediator couple).

The current *i* flowing through the electrode is

$$i = F\left\{-DS\left(\frac{\partial[Q]}{\partial x}\right)_0 + 2k_3[Q]_0\Gamma_{\mathrm{FADH_2}}\right\}$$

(anodic currents are counted as positive) where *S* is the electrode surface area and Γ_{FADH_2}, the amount of $FADH_2$ at the surface. Assuming that FAD, FADG, and $FADH_2$ obey the steady-state approximation within the surface film and taking account of the fact that glucose is in large excess

$$i = -FSD\left(\frac{\partial[Q]}{\partial x}\right)_0 + \frac{2Fk_3\Gamma_E^0[Q]_0}{1 + k_3[Q]_0\left(\frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1k_2[G]}\right)}$$

where Γ_E^0 is the total amount of enzyme present at the electrode surface.

It follows from the above equations that the first term is equal to the current observed in the absence of glucose and that

$$[Q]_0 = \frac{C_P^0}{1 + \exp\left[-\frac{F}{RT}(E - E^0)\right]}$$

The second term in the expression of the current which represents the increase due to the catalytic reaction

$$i_{\mathrm{cat}} = \frac{2Fk_3\Gamma_E^0C_P^0}{1 + \exp\left[-\frac{F}{RT}(E - E^0)\right] + k_3C_P^0\left(\frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1k_2[G]}\right)} \quad (1)$$

may thus be derived from experimental data by simply subtracting the curve obtained in the absence of glucose to that obtained with glucose present in the solution, as represented in Figure 2.

We checked, for all experiments carried out at various glucose concentrations and amounts of enzyme on the electrode surface, that the magnitude of i_{cat} was independent of the scan rate as predicted by eq 1.

The stability of the electrode response, which can be taken as a measure of the persistency of the activity of the attached enzyme, was found to be remarkably good, of the order of 0.3% decay per day (more precisely, 15% after 45 days with 2–3 h functioning per day).

The magnitude of Γ_E^0, the total amount of enzyme present at the electrode surface, was varied by changing, in the last step of the electrode preparation, the concentration of enzyme in the solution where the electrode was dipped and/or the duration of its exposure to the solution. It was observed that, upon increasing one or the other of these factors (see Experimental Section), the magnitude of i_{cat} reached a limiting value indicating that the electrode surface then became saturated in enzyme.

The maximal value of Γ_E^0 thus reached was determined by radioactive ^{125}I labeling of the antirabbit IgG (see Experimental Section). We found that the total amount of enzyme at saturation was (1.8 ± 0.1) 10^{-13} mol with the 3-mm diameter GC electrodes we used throughout the work, i.e., (2.6 ± 0.2) 10^{-12} mol·cm^{-2} per unit geometric area. The molecule of glucose oxidase is a compact ellipsoid[5,18] whose size is *ca.* 8 nm × 6 nm × 5 nm.[18b] Each molecule may be attached in various orientations; therefore, the Stokes radius, 4.3 nm,[5] is a reasonable estimate for its projection area, i.e., 58 nm^2. Since the attachment proceeds randomly *ca.* 60% of the electrode area can be covered.[19] Thus each molecule of glucose oxidase occupies an area of *ca.* 100 nm^2, and the superficial concentration of enzyme which should correspond to the saturation of a monolayer can be estimated as 1.7 × 10^{-12} mol·cm^{-2}. Comparing the latter figure with the coverage determined by radioactive ^{125}I labeling would imply that the actual area of the electrode is 1.5 times its geometrical area, a quite reasonable value in view of the procedure used to polish the electrode surface (see Experimental Section). Although the above size and surface area estimates are certainly approximate, we

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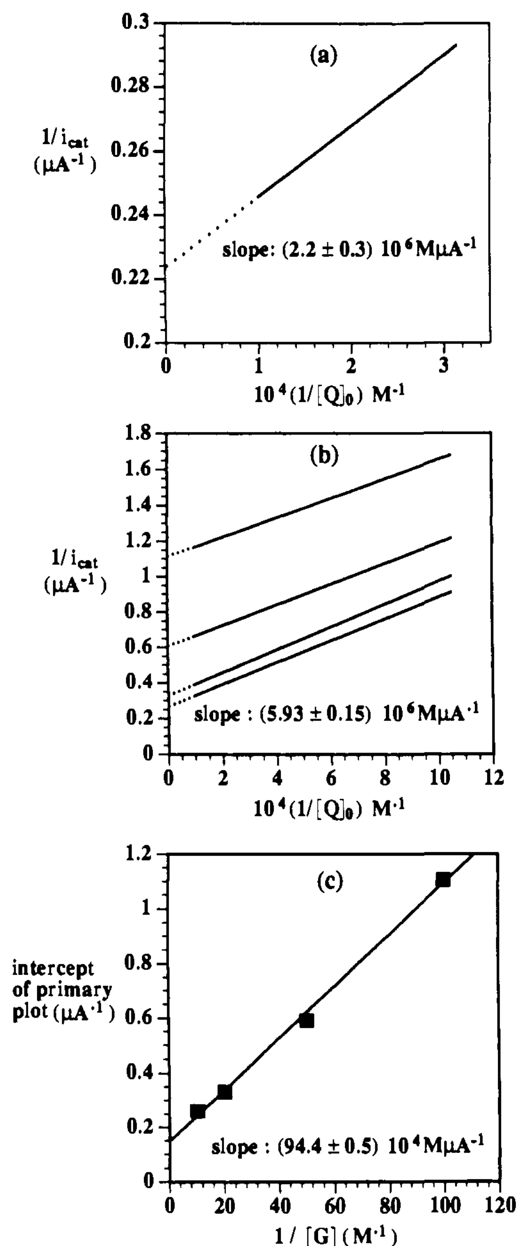

Figure 3. Antigen–antibody immobilized glucose oxidase GC electrode. (a) Primary plot obtained at saturation coverage of the enzyme with a 0.01 M glucose concentration. (b) Primary plots obtained with a partial (37%) enzyme coverage for (from top to bottom) 0.01, 0.2, 0.05, and 0.1 M glucose. (c) Secondary plot derived from the intercepts of the primary plots in (b).

can conclude that the electrode surface is covered by a monolayer of close-packed enzyme units.

Once the amount of enzyme present on the electrode surface is known, the rate constants of the key steps of the catalytic process may be derived from the cyclic voltammograms using the expression of i_{cat} given in eq 1. This is more conveniently performed after recasting eq 1 in the following form

$$\frac{1}{i_{cat}} = \frac{1}{2Fk_3\Gamma_E^0}\frac{1}{[Q]_0} + \frac{1}{2F\Gamma_E^0}\left(\frac{1}{k_2} + \frac{1}{k_{red}[G]}\right) \quad (2)$$

($k_{red} = k_1k_2/(k_{-1} + k_2)$ according to the usage in enzyme kinetics).

Thus, at each glucose concentration, a straight line should be obtained upon plotting $1/i_{cat}$ against $1/[Q]_0 = 1 + \exp[-(F/RT)(E - E^0)]$. A characteristic "primary plot" may thus be derived from each cyclic voltammogram in a straightforward manner. Unlike commonly used procedures, there is no need in the present case to carry out a series of experiments in which the mediator concentration would be systematically varied. The simplicity of the present procedure derives from the coincidence of the enzymatic site and that of the nernstian production of the oxidized form of the mediator.

Table I. Characteristic Rate Constants of Glucose Oxidation by Glucose Oxidase Immobilized on the Glassy Carbon Electrode by Antibody–Antigen Attachment

enzyme	immobilized monolayer[a]	in solution	
mediator	ferrocene methanol	ferrocene methanol[b]	O_2[c]
k_3, $M^{-1}\,s^{-1}$	$(1.3 \pm 0.3) \times 10^7$	$(1.2 \pm 0.1) \times 10^7$	
k_2, s^{-1}	600 ± 200	680 ± 100	750
k_{red}, $M^{-1}\,s^{-1}$	$(0.9 \pm 0.3) \times 10^4$	$(1.1 \pm 0.2) \times 10^4$	1.1×10^4

[a] Present work. [b] From ref 15. [c] From ref 17.

The primary plot obtained with a saturated enzyme electrode at a 10^{-2} M concentration of glucose is shown in Figure 3a. According to eq 2, its slope is $^1/_2Fk_3\Gamma_E^0$. The value of k_3 reported in Table I was derived from the value of this slope, knowing Γ_E^0 from the radioactive labeling experiments. The value of k_3 thus obtained is with an excellent precision the same as that measured in homogeneous solution with the same mediator.[15]

We may thus conclude that the glucose oxidase monolayer attached to the electrode by the antigen–antibody technique is fully active.

Primary plots obtained at several concentrations of glucose with a smaller enzyme coverage are shown in Figure 3b. They are satisfactorily linear and parallel as predicted from eq 2. From the common slope of these plots and the slope in Figure 3a, it is found that the enzyme coverage is 37% the saturation coverage in these experiments.

The "secondary plot" may then be derived by plotting the intercepts of the primary plots against $1/[G]$ (Figure 3c). The slope and intercept of the secondary plot then allow (eq 2) the derivation of k_{red} and k_2, respectively. The characteristic rate constants of the immobilized enzyme system are summarized in Table I. We see that their values are all very close to those determined in homogeneous solution with the same mediator and, for k_{red} and k_2, with dioxygen as the cosubstrate.

For comparison, we investigated, in a similar manner, with the same mediator, glucose oxidase modified glassy carbon electrodes prepared by the reductive covalent attachment method described in ref 8b. Using radioactive ^{125}I labeling of the glucose oxidase, we found a coverage of $(3.3 \pm 0.3)\ 10^{-12}$ mol·cm^{-2} per unit geometric area.

The proportion of these enzyme units that are effectively active was then determined from the catalytic current observed in the presence of glucose. However, as illustrated in Figure 4, the simple method used in the preceding case was no longer applicable since the mediator wave in the absence of glucose is no longer reversible. The primary plot was thus derived from the catalytic plateau currents obtained with several concentrations of the mediator. Indeed, when the catalytic current has reached, beyond the peak, a limiting value, the reversibility of the mediator does not matter any longer since the concentration of the oxidized form of the mediator at the electrode surface $[Q]_0$ then equals the bulk concentration of its reduced form, C_P^0. We found that the ratio between the intercept and the slope of the linear plot thus obtained (Figure 4), namely, $(k_3/k_2) + (k_3/k_{red}[G])$ is the same as in the case of the antigen–antibody attachment and the same as is homogeneous solution. This observation allows one to conclude that the enzyme units that are responsible for the catalysis are each fully active. The proportion of active enzymes over the total of the enzymes present at the electrode surface may then be derived from the slope of the primary plot, $^1/_2Fk_3\Gamma_E^0$. It is thus found that only 4% of the covalently immobilized glucose oxidase units are enzymatically active. This considerable loss of activity, as compared to the case of antigen–antibody attachment, presumably results from a denaturation of the enzyme in the

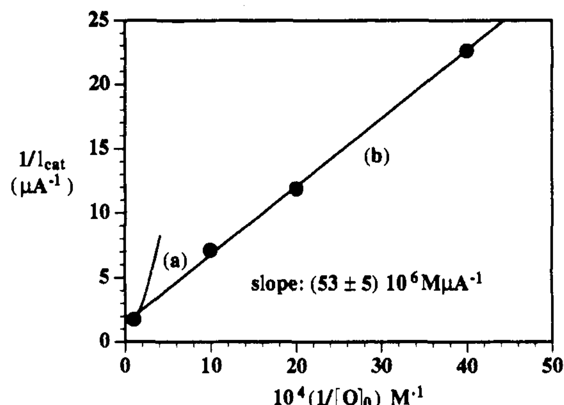

Figure 4. Glucose oxidase derivatized GC electrode prepared by the reductive covalent attachment method.[8b] Primary plots at a 0.5 M glucose concentration obtained upon plotting the inverse of the catalytic contribution to the current in a single cyclic voltammogram against $1/[Q]_0 = 1 + \exp[-(F/RT)(E - E^0)]$ (a) and the inverse of the catalytic plateau current for various values of C_P^0 against $1/[Q]_0 = 1/C_P^0$ (b).

hydrophobic environment created by the substituted phenyl groups linked to the electrode surface.

Conclusions

The use of radioactive ^{125}I labeling and of cyclic voltammetry, using ferrocene methanol as mediator, has allowed the precise demonstration that the immunological immobilization technique described above is able to produce remarkably efficient catalytic electrodes in terms of activity and stability. The monolayers or submonolayers of enzyme that may thus be constructed in a controlled manner are fully active. The presence of the enzyme film does not alter significantly the electrochemical reversibility of the mediator. It follows that primary plots characterizing the enzyme kinetics, and from them secondary plots, may be derived in a straightforward manner from each single cyclic voltammogram.

The immunological immobilization technique appears as clearly superior to chemical covalent attachment methods particularly to the reductive method involving the grafting of substituted phenyl groups to the electrode surface.

The technique may be generalized to other enzymes and constitutes a starting point for the step-by-step construction of multilayer systems with precise control of activity and spatial arrangement.

Experimental Section

Materials. Glucose oxidase from *Aspergillus niger* was from Boehringer Mannheim (grade I). ChromPure rabbit IgG (whole molecule) was from Jackson ImmunoResearch Laboratories. The affinity purified glucose oxidase conjugated antirabbit IgG (whole molecule) produced in goat from Organon Teknika Cappel. The full enzymatic activity of the glucose oxidase labeled antibody was ascertained as follows. The concentration of active glucose oxidase labeled antibody was measured electrochemically, as previously described,[15] in an aliquot of the commercial solution dissolved in a pH 8, 0.1 M ionic strength phosphate buffer solution with 0.1 mM and 0.5 M ferrocenemethanol and glucose concentrations, respectively. In these experiments the glassy carbon electrode was covered with a gelatin layer in order to avoid adsorption of the glucose oxidase antibody onto the electrode surface (see below). The concentration of enzymatically active FAD was thus found to be 1.4×10^{-5} M. The total FAD concentration in the glucose oxidase labeled antibody was spectrochemically determined at 452 nm by reference to genuine glucose oxidase in exactly the same background solution. It was found to be 1.3×10^{-5} M showing that the glucose oxidase moiety in the glucose oxidase labeled antibody is fully active. ^{125}I sodium iodide and ^{125}I labeled antirabbit IgG (whole molecule) raised in donkey were from Amersham. IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was obtained from Pierce. D-(+)-glucose and the CAF 4 elastomer were from Prolabo. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate (CMCT) and *n*-tetrabutylammonium tetrafluoroborate (n-Bu_4BF_4) were from Sigma and Fluka, respectively. Ferrocenemethanol and all other chemicals were purchased from Aldrich. All materials were used as received. The stock solutions of glucose were allowed to mutarotate overnight before use. All solutions were purged of oxygen before each electrochemical measurement.

Instrumentation. The cyclic voltammetry working electrode was a 3-mm diameter glassy carbon disk from Tokai Corp. sealed in epoxy resin or laterally covered with the CAF 4 elastomer. The electrode surface was polished to mirror-finish with diamond pastes (down to 1 μm) and ultrasonically rinsed in dichloromethane before use. The electrochemical equipment was as described previously.[15]

Immobilization of Glucose Oxidase. Immunological Immobilization of Glucose Oxidase. Immobilization of glucose oxidase by means of the antigen/glucose oxidase conjugated antibody reaction was performed as follows. The background solution for each reactant was made of 0.15 M NaCl plus 10^{-2} M KH_2PO_4 adjusted to pH 7.4 with sodium hydroxide. The glassy carbon surface was first saturated with adsorbed rabbit IgG by leaving the electrode, for 2 h in a water-saturated atmosphere, in contact with a 0.5 mg/mL solution of rabbit IgG dropped on top of the carbon disk with a sterilized microsyringe. The electrode was thoroughly washed with the background solution and dipped for 10 min in a 0.1 mg/mL solution of gelatin. After a new thorough washing, the antigen/antibody reaction took place during the immersion of the electrode into a solution of the glucose oxidase conjugated antirabbit IgG. The concentration of the latter and the immersion duration were varied so as to control the surface concentration of immobilized glucose oxidase. For example the results used in Figures 2 and 3 were obtained with an electrode which had been immersed for 30 min in a solution containing 5 μg/mL of the glucose oxidase conjugate. Electrochemical measurements showed that the catalytic efficiency increased, with increasing conjugate concentration and/or immersion time, up to a plateau value indicating that the monolayer was saturated with catalytically active glucose oxidase. A systematic study showed that such a saturation was reached after immersing the electrode 3 h into a 10 μg/mL solution of conjugate. Finally the enzyme electrode was rinsed thoroughly with the background solution and stored at 5 °C in the background solution.

Immobilization of the ^{125}I Labeled Antirabbit IgG. The procedure was as described above, the ^{125}I labeled antirabbit IgG being used in place of the glucose oxidase conjugated antirabbit IgG. In order to ensure the saturation of the monolayer the immersion time was 4 h in the commercial solution of ^{125}I antirabbit IgG (concentration 10 μg/mL, radioactivity 10 μCi/μg). The glassy carbon rod was laterally painted with the CAF 4 elastomer so as to leave only the disk tip of the carbon material in contact with the reactants. The elastomer cover was scratched before introduction of the rinsed electrode into the scintillation counter. The measured radioactivity showed that $(1.8 \pm 0.1) \times 10^{-13}$ mol of ^{125}I labeled antibody was attached, the nonspecific binding being negligible since only 3.2×10^{-15} mol adsorbed onto the same (gelatin pretreated) glassy carbon disk. The geometric area of the disk being 0.07 cm^2, the superficial concentration of the saturated monolayer of ^{125}I labeled antibody per unit of geometric area was $(2.6 \pm 0.2) \times 10^{-12}$ mol·cm^{-2}. Radioactive measurements also showed that the ^{125}I labeled glucose oxidase and antirabbit IgG do not adsorb onto the gelatin layer.

Reductive Covalent Immobilization of Glucose Oxidase. As already reported[8b] the maximum coverage of the electrode with catalytically active glucose oxidase covalently linked to the carbon surface within the framework of this method can be obtained as follows. *p*-Phenylacetic diazonium tetrafluoroborate[8b,20] dissolved (5 mM) in acetonitrile plus 0.1 M n-Bu_4BF_4 is reduced electrochemically at the glassy carbon cathode. The potential of the electrolysis and its duration are −0.9 V *vs* SCE and 4 min, respectively. The electrode is ultrasonically rinsed in acetone for 15 min and immersed in a 0.1 M solution of CMCT in 0.02 M acetate buffer at pH 4.6 for 30 min. Then the electrode is rinsed in 0.1 M phosphate buffer (pH 7.2) and dipped overnight at 5 °C in a solution of glucose oxidase (1 mg/mL) in the 0.01 M phosphate buffer (pH 7.2). After being thoroughly rinsed in the buffer, the modified electrode is stored at 5 °C in the 0.01 M phosphate buffer (pH 7.2).

Preparation of ^{125}I Labeled Glucose Oxidase.[21] IODO-GEN (100 μg) dissolved in dichloromethane (1 mL) gave a solid film adhering onto the inner bottom of a test tube after exhaustive evaporation of the solvent under a gentle stream of dry nitrogen. Phosphate buffer (80 μL, 0.5 M, pH 7.25) plus 40 μL of a 1 mg/mL solution of a 1 mg/mL solution of glucose oxidase in the same buffer and 8 mCi of the commercial solution of ^{125}I sodium iodide were introduced into the tube and allowed to react

in the presence of IODO-GEN for 5 min under vortexed agitation. Phosphate buffer (0.1 M, pH 7.25) was added then to the reaction mixture up to a total volume of 1 mL. The resulting solution was desalted by centrifugation at 2000 × g during 10 min through the membrane of a Centricon 30 vial (30 000 MW cutoff). The same operation was repeated twice and then twice again with 0.01 M phosphate buffer (pH 7.25). The duration of the last centrifugation was 1 h so as to concentrate glucose oxidase, the final volume being *ca.* 40 μL. A more precise value of this volume was deduced from its weight. A 5-μL aliquot was removed for γ scintillation counting. The measurement gave a radioactivity of 90 mCi/mL. After making allowance for the radioactivity of the filtrate (less than 0.13 mCi/mL) the iodination efficiency was found to be 5.6 mol ^{125}I/mol of glucose oxidase. This efficiency is *ca.* 30 times greater than that obtained with an iodination procedure mediated by lactoperoxidase and hydrogen peroxide instead of IODO-GEN and reported earlier in the literature.[11a] Another aliquot of 5 μL was removed for the kinetic characterization of the catalytic activity of the ^{125}I labeled glucose oxidase in solution by means of cyclic voltammetry as detailed in a previous paper.[15] No loss in enzyme activity was detected upon iodination. The remaining ^{125}I labeled glucose oxidase was covalently linked, as described above, to glassy carbon electrodes laterally painted with the CAF 4 elastomer. The lateral cover of elastomer was scratched before introduction into the scintillation counter.

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