Electron Transfer in Organized Assemblies of Biomolecules. Step-by-Step Avidin/Biotin Construction and Dynamic Characteristics of a Spatially Ordered Multilayer Enzyme Electrode

Nathalie Anicet,[1a] Christian Bourdillon,[1b] Jacques Moiroux,*[,1a] and Jean-Michel Savéant*[,1a]

Laboratoire d'Electrochimie Moléculaire, Unité Mixte de Recherche Université, CNRS No. 7591, Université de Paris 7, Denis Diderot, 2 place Jussieu, 75251 Paris Cedex 05, France, and the Laboratoire de Technologie Enzymatique, UPRES-A No. 6022, Université de Technologie de Compiègne, BP 20529, 60205 Compiègne Cedex, France

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The avidin−biotin technology may be used successfully to build stable and spatially ordered assemblies of monomolecular enzyme layers on surfaces as illustrated with the example of glucose oxidase and glassy carbon electrodes. With ferrocene methanol as the cosubstrate, cyclic voltammetry allows a detailed analysis of the catalytic responses, thus providing a demonstration of the spatial order of the multilayer structure and an estimate of the average distance between monomolecular layers. The respective advantages of the avidin−biotin and antigen−antibody technologies are discussed.

There is a continuous interest for associating specific chemical functionalities with electrodes so as to trigger them electrically or to translate chemical signals into easily measurable electrical signals.[2] A well-documented way of achieving these objectives consists in entrapping the chemical functionalities together with redox centers in polymer or gel coatings.[3] As far as chemical specificity is concerned, enzymes offer remarkable properties and ways of "wiring"[4a] them to electrode surfaces by means of redox gels or polymers have been investigated.[4] Imitation of natural systems has inspired a quest for more precisely organized systems based on the docking properties of proteins.[5] As far as the connection between such molecular architectures and electrodes is concerned, two modes of linking have been reported. One involves electrostatic interactions[6] and the other antigen−antibody binding.[7] Applying the latter approach to the example of glucose oxidase, the step-by-step deposition of up to 12 successive fully active monomolecular layers was achieved. The cyclic voltammetry of these electrodes allowed the dissection of their catalytic responses, revealing the respective roles of the enzymatic kinetics and of the diffusion of the cosubstrate through the assembly of monomolecular layers. The detailed analysis of the latter factor provided evidence that these multilayers are indeed spatially ordered and an estimate of the distance between the successive monomolecular layers was obtained.

One drawback of the antigen−antibody technique is the scarce availability of the pertinent biomolecules, namely the enzyme conjugated antibody and the noninhibitory monoclonal antibody to the enzyme. Since biotinylated molecules are more readily accessible, we have attempted to develop a technique allowing the construction of similar assemblies of monomolecular layers based on the strong avidin−biotin affinity.[8] Few examples of the use of the avidin−biotin binding to derivatize electrode surfaces have been reported. One consisted in the covalent derivatization of a highly oxidized carbon electrode by avidin, followed by the grafting of a monolayer of a biotinylated enzyme.[9] Strong oxidation of the carbon surface leading to large background-currents was subsequently avoided by use of a *N*-hydroxysuccinimide function[10a] to bind a poly(ethylene glycol) long-chain biotin derivative onto the surface. A monomolecular layer of glucose oxidase conjugated avidin was then bound on top of the biotin layer.[10b] The versatility offered by the avidin−biotin technology allowed, in this case, not only the attachment of a monomolecular layer of glucose oxidase onto the electrode surface but also the immobilization of the cosubstrate (a ferrocene) within the same structure. Successive layers of biotinylated enzymes were deposited on a platinum electrode after irreversible adsorption of avidin.[11] In view of the deposition technique, it is unlikely that the successive layers are monomolecular layers and the procedure does not seem quite reproducible.[11]

We performed the step-by-step construction of an assembly of monomolecular layers leading to the structure sketched in Scheme 1, up to 12 monomolecular layers. The enzyme coatings thus obtained are reasonably stable, the decrease in enzymatic activity being less than 10% over a period of 40 days. The dynamics of the system was analyzed by means of cyclic voltammetry after addition of glucose and of a ferrocene mediator to the solution. Diffusion of the latter through the structure was used to sense its spatial order and to estimate the distance between the successive monomolecular layers.

Results and Discussion

Construction of the Monomolecular Layer Assembly. A key initial step is the deposition of an appropriate anchoring layer onto the electrode surface that would allow further binding of glucose oxidase units. Several strategies may be envisaged for this purpose. Covalent attachment of biotin onto glassy carbon surfaces has been previously described.[9] It involves vigorous oxidation of the surface thus leading to a large background current[12] which would prevent precise kinetic measurements. Avidin may be adsorbed on platinum,[11] but platinum electrodes also exhibit exceedingly large background currents in the pH and potential ranges of interest. Moreover, our attempts to use this procedure did not lead to stable coatings.

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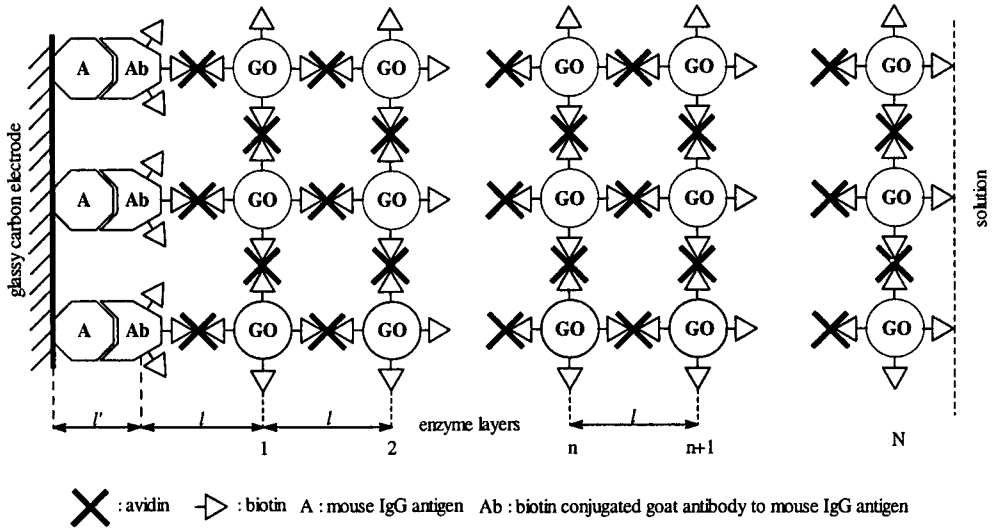
SCHEME 1



SCHEME 2

$$P \rightleftharpoons Q + e^- \qquad (0)$$

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

$$FADG \xrightarrow{k_2} FADH^- + GL \qquad (2)$$

$$FADH^- + 2Q \xrightarrow{k_3} FAD + 2P \qquad (3)$$

As another possible strategy, we attempted to adsorb avidin on a glassy carbon surface by prolonged dipping into an avidin solution. After simple rinsing, the electrode was then allowed to react with a solution containing biotinylated glucose oxidase. The amount of deposited active enzyme was estimated from the cyclic voltammetric response of the electrode after addition of glucose and of the mediator, ferrocene methanol, in the solution. When a significant amount of active enzyme is deposited on the electrode, a catalytic S-shaped wave is observed and the surface concentration, Γ_E, of active enzyme may be derived from the plateau current, i_p, according to eq 1 in the framework of Scheme 2, using for the rate constants previously determined values.[7]

$$i_{p,corr} = i_p - i_d = \frac{2FSk_3\Gamma_E C_P^0}{1 + k_3 C_P^0\left(\frac{1}{k_2} + \frac{1}{k_{red} C_G^0}\right)} \qquad (1)$$

where F is the faraday, S the electrode surface area, C_P^0 the bulk mediator concentration, C_G^0 the bulk glucose concentration, $k_{red} = k_1k_2/(k_{-1} + k_2)$, i_d is the diffusion current in the absence of glucose.

In the present case, no catalytic activity was detected, implying that neither avidin nor biotinylated glucose oxidase has been adsorbed on the surface. This is not very surprising in view of the previous observation that glucose oxidase does not adsorb on glassy carbon in most circumstances or, when it does, it rapidly loses its activity.[12b] We have found, however, that avidin-labeled glucose oxidase adsorbs on glassy carbon and retains appreciable catalytic activity, up to a surface concentration of 5×10^{-13} mol/cm^2 in catalytically active enzyme. However, half of the deposited enzyme was washed away upon standing overnight in the classical PBS buffer (see Experimental Section), indicating that adsorption of avidin-labeled glucose oxidase is not a viable technique for attaching the anchoring layer to the surface.

We therefore resort to another strategy, namely adsorption of a mouse IgG antigen to which a biotin-conjugated goat antibody to mouse IgG is successively bound. Avidin and biotinylated glucose oxidase were then immobilized successively (Scheme 1). To operate the binding between the biotinylated electrode and avidin so as to obtain a reproducible and persistent system, nonspecific protein interactions should be avoided. We made a preliminary study of this problem by binding avidin-labeled glucose oxidase, rather than the avidin + biotinylated glucose oxidase, to the biotinylated goat antibody to mouse IgG. Using the cyclic voltammetric method described earlier, it was found that up to 36×10^{-13} mol/cm^2 of active enzyme was deposited after 3 days of immersion in a solution of avidin-labeled glucose oxidase. Such a large value of the surface concentration of active enzyme (concentrations obtained with the previously described antigen−antibody technique are of the order of 25×10^{-13} mol/cm^2) pointed to the interference of nonspecific interactions. Indeed, ca. 8×10^{-13} mol/cm^2 could be washed away by prolonged immersion of the electrode in the PBS buffer. It was also found that nonspecific binding could be prevented by treatment of the immobilized layers of mouse IgG and biotinylated goat antibody to mouse IgG with gelatin and addition of gelatin to the solution of avidin labeled enzyme. Thus, coming back to the successive binding avidin + biotinylated glucose oxidase, gelatin was systematically added, as described in the Experimental Section, to avoid the interference of any type of nonspecific binding. Successful attachment of biotinylated glucose oxidase to the first layer of deposited avidin required an accurate selection of the concentration and of the time of immersion in the solution of biotinylated glucose oxidase. We indeed found that, at a given concentration, Γ_E does not reach a limiting value upon increasing the immersion time which would indicate the formation of a saturated monomolecular layer. Instead, Γ_E goes through a maximum as shown in Figure 1. This observation indicates that the avidin in the layer is the object of a competition between binding to the biotinylated surface and binding with the biotin heads of the biotinylated glucose oxidase in the solution, implying that the avidin−biotin reaction is reversible in spite of its strongly favorable equilibrium constant.[13] An increase in concentration shortens the time required to reach the maximum and increases the rate at which the avidin layer vanishes into the solution

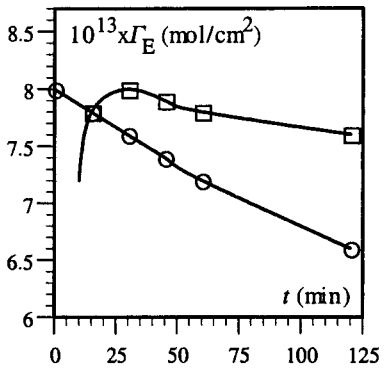


Figure 1. Exposure of a GC electrode covered with a layer of A, a layer of Ab, and a layer of avidin (see Scheme 1) to a PBS buffer solution of biotinylated glucose oxidase containing 0.1 mg/mL gelatin. Variation of the surface concentration of active enzyme with the time of exposure for two concentrations of biotinylated glucose oxidase, 10 μg/mL (□) and 80 μg/mL (○).

(Figure 1). The optimal concentration and time of exposure were as depicted in the Experimental Section.

The same procedure was repeated for the deposition of the successive monomolecular layers. Up to 12 monolayers were deposited on the electrode in this manner. When not in use, the electrodes were stored in the PBS buffer. They exhibit a good stability losing less than 10% activity over a period of 40 days. As with the antigen−antibody construction technique,[7c] any number of deposited active enzyme layers may be deactivated by iodoacetate, and successive active layers may be grafted on top of them following the same procedure. One advantage of the avidin−biotin technique over the antigen−antibody technique is that a much shorter time is required for the deposition of one monomolecular layer, 45 min instead of 17 h.

Cyclic Voltammetric Analysis of the Catalytic Response. Diffusion of the Mediator. Structure of the Monomolecular Layer Assembly. Immersion of the enzyme electrode into a pH = 8 phosphate buffer containing 0.5 M glucose results in an S-shaped cyclic voltammogram, independent of the scan rate, which increases as the number of glucose oxidase monomolecular, *N*, increases (Figure 2a). The variation of the plateau current, i_p, with *N* is shown in Figure 2b for a series of concentrations of mediator, C_P^0, ranging from 0.4 to 0.005 mM. When the mass transport of the mediator is fast enough for not interfering kinetically in the catalytic response, the plateau current obeys eq 2, a slightly modified version of eq 1 in which

$$i_{p,corr} = i_p - i_d = \frac{2FSk_3\Gamma_E\kappa_Q C_P^0}{1 + k_3\kappa_Q C_P^0\left(\frac{1}{k_2} + \frac{1}{k_{red}\kappa_G C_G^0}\right)} \qquad (2)$$

the partition coefficients, between solution and multilayer coating, have been introduced, κ_Q for ferrocenium and κ_G for glucose. Γ_E now stands for the total surface concentration of the glucose oxidase contained in the whole assembly of monomolecular layers. The rate constants k_2, k_3, and k_{red} are known:[7,14,15] $k_2 = 700\ s^{-1}$, $k_3 = 1.2 \times 10^7\ M^{-1}\ s^{-1}$, $k_{red} = 1.1 \times 10^4\ M^{-1}\ s^{-1}$. An alternative useful form of eq 2 is as follows.

$$\frac{2FS}{i_{p,corr}}\Gamma_E = \frac{1}{k_2}\left(1 + \frac{K_{M,Q}}{\kappa_Q C_P^0} + \frac{K_{M,G}}{\kappa_G C_G^0}\right) \qquad (3)$$

where the mediator and glucose Michealis constants, $K_{M,Q} = k_2/k_3 = 5.8 \times 10^{-5}$ M and $K_{M,G} = k_2/k_{red} = 6.4 \times 10^{-2}$ M,

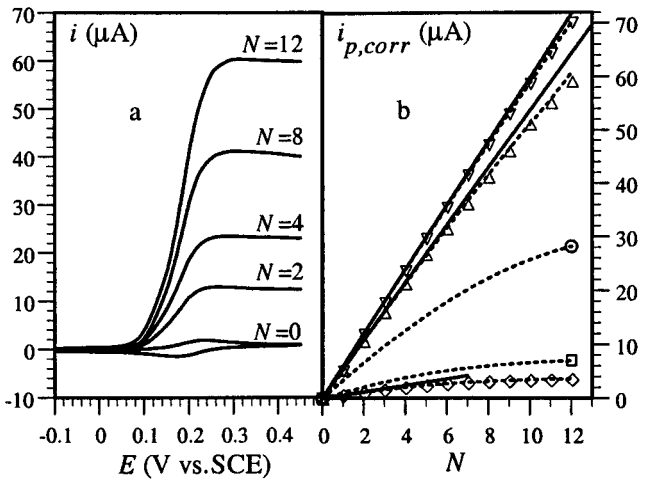


Figure 2. Cyclic voltammetry of glucose oxidase electrodes in a pH = 8 phosphate buffer as a function of the number of monomolecular glucose oxidase layers, *N*. Scan rate: 0.04 V/s. Temperature: 25 °C. Mediator: ferrocene methanol. Glucose concentration: 0.5 M. (a) Cyclic voltammograms at ferrocene concentration $C_P^0 = 0.2$ mM. (b) Variation of the plateau current, i_p, with the number of monomolecular layers for $C_P^0 = 0.4$ (▽), 0.2 (△), 0.05 (○), 0.01 (□), 0.005 mM (◇). The full lines show the predicted variation in the absence of mediator mass transport limitation. The dotted lines represent the simulation of the interference of the mediator mass transport for $l = 550$ Å (see text).

respectively, have been introduced. If the same amount of active enzyme is present in each of the monomolecular layers, $\Gamma_E = N\Gamma_E^0$, where Γ_E^0 is the surface concentration of enzyme per monomolecular layer. Thus, if this is indeed the case and if the mass transport of the mediator does not interfere, the plateau current is predicted to be proportional to the number of monomolecular layers. Figure 2b shows that this behavior is reached asymptotically when N → 0. It also shows that the deviation from proportionality is the more significant the smaller the concentration of mediator.

A large concentration of glucose, 0.5 M, was present in the solution in all experiments summarized in Figure 2. Under these conditions, the diffusion of glucose throughout the multilayer coating does not interfere in the kinetics of the catalytic process. Its concentration remains constant, equal to $\kappa_G C_G^0$ inside the coating and to C_G^0 in the solution. Unless κ_G is much smaller than unity, the value of the glucose Michealis constant is such that for = 0.5 M, the glucose kinetics is close to saturation. The third term within the parentheses in eq 3 is equal to 0.128 assuming $\kappa_G = 1$. Thus, if κ_G is not much different from 1, its influence on the global kinetics may be neglected. The validity of this assumption will be confirmed further on.

At the highest mediator concentration, the second term within the parentheses in eq 3 is equal to only 0.145 assuming $\kappa_Q = 1$. It follows that the influence of κ_Q on the catalytic current is small and thus that the surface concentration of enzyme per monomolecular layer, Γ_E^0, may be derived with a good approximation from eq 4, obtained from eq 3 by setting $\kappa_G = 1$, $\kappa_Q = 1$ and, $\Gamma_E = N\Gamma_E^0$.

$$\Gamma_E^0 = \frac{i_{p,corr}}{N}\frac{1}{2FSk_2}\left(1 + \frac{K_{M,Q}}{C_P^0} + \frac{K_{M,G}}{C_G^0}\right) \qquad (4)$$

From the linear behavior observed up to $N = 7$ in the $C_P^0 = 0.4$ mM experiment we thus obtain $\Gamma_E = (8.05 \pm 0.05) \times 10^{-13}$ mol/cm^2. Since the biotinylated glucose oxidase we used is 66% active (see Experimental Section), the total amount of

enzyme in each monomolecular layers is 12×10^{-13} mol/cm^2. The same approximation applies to the $C_P^0 = 0.2$ mM experiment up to $N = 5$. At the lowest mediator concentration, the influence of κ_Q on the catalytic current becomes significant (with $\kappa_Q = 1$, the second term within the parentheses in eq 3 is equal to 11.6). From the initial slope of the $i_{p,corr}/N$ plot at this concentration (Figure 2b) we conclude that κ_Q is very close to 1 ($\kappa_Q = 1.00 \pm 0.03$).

The increasing deviation of the $i_{p,corr}/N$ plot from proportionality as C_P^0 decreases (Figure 2b) suggests that the diffusion of the mediator interferes in the kinetics. The rate of mediator diffusion decreases proportionally to C_P^0, whereas the contribution of the rate of the enzymatic reaction decreases less rapidly (eq 3). Diffusion thus tends to control the overall kinetics more and more as C_P^0 decreases.[16] In order to investigate the effect of mediator diffusion quantitatively, we need to know three additional parameters, namely the partition coefficient of the reduced form of the mediator, κ_P, and the diffusion coefficients of the reduced and oxidized forms of the mediator within the coating, D_P and D_Q, respectively. In the solution, the two diffusion coefficients are practically the same, i.e., $D = 6.7 \times 10^{-6}$ cm^2/s in the buffer.[17a] The increase in viscosity resulting from the addition of 0.5 M glucose lowers its value to $D = 5.5 \times 10^{-6}$ cm^2/s.[17b] At a scan rate of 0.1 V/s, the diffusion layer thickness is ca. 10^5 Å, i.e., more than 10 times the thickness of a coating composed of 12 monomolecular layers as will appear from the estimate of a monomolecular layer below. In the absence of glucose, a reversible ferrocene/ferrocenium wave is observed at any scan rate. In such experiments carried out at 0.1 V/s with a 12 monomolecular layer electrode, the diffusion coefficients D_P and D_Q should therefore not interfere in the apparent standard potential $E_{P/Q}^0$, obtained as the midpoint between the anodic and cathodic peak potentials, which is simply expressed as $E_{P/Q}^0{}' = E_{P/Q}^0 + (RT/F) \ln(\kappa_P/\kappa_Q)$. Since we observed that the voltammograms are the same in the presence and absence of coating, it follows that $\kappa_P = \kappa_Q = 1.00 \pm 0.03$. At a scan rate of 20 V/s, the diffusion layer is about equal to the thickness of a 12 monomolecular layer coating. Since, at 20 V/s, both the height and the location of the wave are the same with and without coating, we may conclude that the diffusion coefficients of P and Q are the same as in the solution in the absence of glucose, and presumably in the presence of glucose too. Thus, $D_P = D_Q = 5.5 \times 10^{-6}$ cm^2/s in the presence of 0.5 M glucose seems a reasonable approximation. The lack of variation of the diffusion coefficients from the solution to the coating and the fact that the partition coefficients are close to unity suggest that the monomolecular assembly is quite an open structure. This observation confirms the assumption made earlier that the partition coefficient of glucose is also close to unity.

Knowing the value of these various parameters, we may now model the effect of mediator diffusion on the catalytic current as a function of the distance l between each glucose oxidase monomolecular layer using the same finite difference approach as depicted previously in the case of antigen−antibody coatings.[7c,18] An excellent fit of the whole set of experimental data (Figure 2b) is obtained for $l = 550$ Å. Figure 3 shows in a more detailed manner the results of the simulation for $C_P^0 = 0.005$ mM, where the effect of mediator diffusion is the largest. The average error on the plateau current is ± 0.1 μA. The resulting error on l is shown in Figure 3. As expected, it rapidly increases as the number of monomolecular layers decreases because the interference of the mediator diffusion diminishes

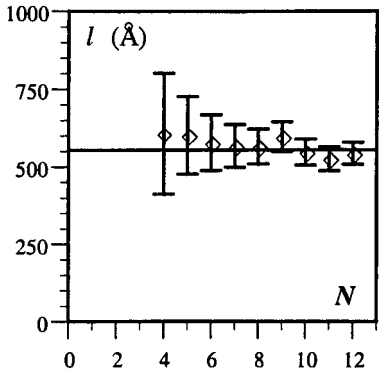


Figure 3. Cyclic voltammetry of glucose oxidase electrodes in a pH = 8 phosphate buffer as a function of the number of the number of monomolecular glucose oxidase layers, *N*. Scan rate: 0.04 V/s. Temperature: 25 °C. Mediator: ferrocene methanol, = 0.005 mM. Glucose concentration: 0.5 M. Estimation of *l* and of the standard deviation for $N = 4-12$ from the simulation of the effect of mediator diffusion.

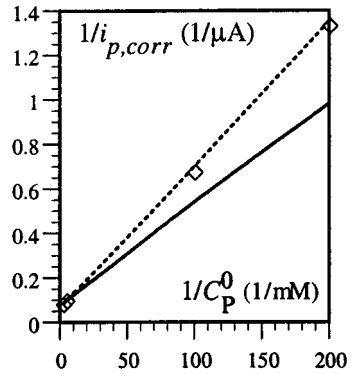


Figure 4. Cyclic voltammetry of a glucose oxidase electrode coated with 12 inactive monomolecular layers and 13th and 14th active monomolecular layers in a pH = 8 phosphate buffer. Scan rate: 0.04 V/s. Temperature: 25 °C. Mediator: ferrocene methanol. Glucose concentration: 0.5 M. Variation of the catalytic plateau current with the mediator concentration,. Solid line: behavior expected in the absence of mediator diffusion. Dotted line: simulation of the effect of mediator diffusion with $l = 550$ Å.

accordingly. The weighted average value of l is thus found to be $l = 554 \pm 17$ Å.[19]

The effect of mediator diffusion may also be illustrated by experiments in which the monomolecular enzyme layers initially deposited onto the electrode surface are deactivated before additional active monomolecular layers are deposited on top of them. The experiments summarized in Figure 4 were carried out with 12 inactivated monomolecular layers, on top of which 13th and 14th active layers were deposited according to the same procedure as described earlier. At a large mediator concentration ($C_P^0 = 0.4$ mM), the mediator diffusion does not interfere and catalytic current obeys eq 4. We thus found that $\Gamma_{E,13}^0 = 6.1 \times 10^{-13}$ mol/cm^2 and $\Gamma_{E,14}^0 = 7.9 \times 10^{-13}$ mol/cm^2. As previously observed with antigen−antibody multilayers,[7c] the amount of active enzyme deposited in the first active layer on top of the set of inactive layers is somewhat less than the optimum coverage before inactivation. However, the optimum coverage is restored in the next active layer. As shown in Figure 4, the variation of the catalytic current with the mediator concentration is far from the variation that is expected in the absence of a mediator diffusion effect. In contrast, the simulation of the mediator diffusion effect with the same value of l, 550 Å, as already used in the preceding simulations is in excellent agreement with the experimental data.

If glucose oxidase and avidin were to be regarded as spherical, the distance between two successive monomolecular layers would be of the order of 200 Å (100 Å for glucose oxidase,[20a−c] 2 × 15 Å for the biotin "arms",[20d] 80 Å for avidin[20e]). The fact that a significantly larger distance is found experimentally is presumably related to the inclusion into the structure of the bovine serum albumin (not shown in Scheme 1) required to eliminate the protein nonspecific binding. Since the avidin and biotin moieties are firmly attached together, it is anticipated that the presence of the bovine serum albumin forced the proteins to adopt a drawn-out structure. It should be recalled in this connection that the biotin arms are grafted onto the particularly soft carbohydrate layer surrounding the glucose oxidase proteic core.

Conclusions

Provided concentrations and exposure times are carefully selected, the avidin−biotin technology allows the construction of stable assemblies of monomolecular layers of glucose oxidase on glassy carbon. Up to 14 monomolecular layers have been deposited this way, and there is no reason that this number cannot be augmented. In each monomolecular layer, the surface enzyme activity is the same as in solution. The stability of the multilayer coating (less than 10% loss in activity after 40 days) is also critically dependent on the nature of its anchoring onto the electrode surface. The antigen−antibody technology used in the present work for biotinylating the electrode surface is quite satisfactory in this respect. The dynamics of their catalytic activity toward the oxidation of glucose in the presence of a reversible one-electron reversible couple is conveniently analyzed by means of cyclic voltammetry. Evidence that a spatially ordered structure, templating the electrode surface as sketched in Scheme 1, is thus constructed derives from two observations. One is that the catalytic plateau current is proportional to the number of monomolecular layers at high concentrations of the mediator. The second derives from the analysis of the mediator diffusion across the structures which effect appears as the mediator concentration is decreased and/or the number of monomolecular layers increased. The good agreement between the experimental data and simulations based on this structure provides evidence of its reality. Another outcome of the analysis is the average distance between successive monomolecular layers which is found equal to 550 Å. Comparison of this value with the value obtained with antigen−antibody constructions, 475 Å, as well as the values of the surface concentration of enzyme (active and inactive), 12×10^{-13} mol/cm^2 (vs 12×10^{-13} mol/cm^2), points to a lower longitudinal and lateral compactness of the avidin−biotin structure. Related to this low compactness is the observation that the diffusion coefficients of each member of the ferrocene/ferrocenium mediator couple are closely the same as in the solution. Their partition coefficients are practically equal to 1. This is also true for glucose.

One general advantage of the avidin−biotin technology over the use of antigen−antibody interactions is the more extended availability of the corresponding biomolecules which may prove of interest for future extension to pluri-enzymatic systems. Although the deposition of each monomolecular layer needs a more careful selection of the experimental conditions (because of the reversibility of the avidin/biotin binding), it requires less time with the avidin−biotin technology than with the antigen−antibody method, typically, 45 min instead of 17 h.

Experimental Section

Chemicals. The mouse IgG (whole molecule) and the biotin conjugated goat antibody to mouse IgG (whole molecule) were from Organon Teknika Cappel. Avidin was purchased from Pierce. Glucose oxidase from *A. Niger* was from Boehringer Manheim (grade I). Biotinamidocaproyl hydrazide (BLCH) and the Sephadex G25, PD-10 column for gel filtration were from Sigma and Pharmacia Biotech, respectively. Between each run, the column was equilibrated with a PBS pH 7.4 buffer. Centricon-30 was from Amicon. All other chemicals were purchased from Aldrich. They were used as received. The stock solutions of glucose were allowed to mutarotate overnight before use. The PBS buffer was made of 0.01 M KH_2PO_4, 0.15 M NaCl, and 0.01% sodium azide, and pH was adjusted to 7.4 with a 1 M NaOH solution.

Biotinylated Glucose Oxidase. After repeated failures in our attempts at immobilizing commercially available biotinylated glucose oxidases of various origins, we chose to prepare this compound ourselves from the very beginning. The long-chain biotin reagent BLCH was anchored to the shell of carbohydrates surrounding glucose oxidase according to the following procedure.[21a] Periodate oxidation (200 μL of a 0.088 M $NaIO_4$ aqueous solution) of the carbohydrates of glucose oxidase (2 mL of a 5 mg/mL solution in pH 5.5 acetic acid/acetate buffer) was allowed to proceed for 20 min in the dark. The reaction was stopped by gel filtration on the PD-10 column, the eluent being the PBS buffer (3.5 mL). The first 2 mL containing 2.86 mg/mL of periodate oxidized glucose oxidase was collected and added to a BLCH solution (3.33 mg in 0.5 mL). The resulting reaction mixture was kept in the dark for $2^1/_2$ h at 0 °C. The hydrazone was reduced by $NaCNBH_3$ (20 μL of a 5 M solution in 1 M NaOH) at 0 °C in the dark during 40 min. In order to avoid the formation of polymers, the remaining free aldehyde groups were blocked by reaction with ethanolamine (100 μL of a 1 M solution adjusted at pH 9.6 with concentrated HCl) for 30 min. The mixture was then gel filtered twice. In order to remove whatever remained of the biotinylating reagent, the filtered solution was centrifuged twice through a Centricon-30 microconcentrator. The catalytic activity of the biotinylated enzyme in solution was assayed by means of cyclic voltammetry,[14] and the protein[22] and the biotin contents[8b,21b,23] were assayed according to the detailed procedures given in the literature. Before centrifugation through the Centricon-30 microconcentrator, the catalytic activity of the reagent related to the protein content was 100% and the biotin content was 17.4 biotin per glucose oxidase. When centrifuged twice, the activity and the biotin/glucose oxidase ratio became 66% and 5.6, respectively. When centrifuged five times, the activity and the biotin/glucose oxidase ratio became 50% and 5.7, respectively. Thus we decided to use the twice centrifugated reagent. The final solution was aliquoted and stored below −20 °C.

Successive Immobilization of Enzyme Monolayers. Adsorption of mouse IgG resulted from a 2 h exposure of the electrode surface to a 1 mg/mL solution of the IgG in the PBS buffer. The electrode was then thoroughly rinsed, washed with the buffer, dipped into a 0.1 mg/mL solution of gelatin in PBS for 15 min, and rinsed again. The biotinylated antimouse IgG (20 μg/mL in PBS) was then used to recognize the adsorbed mouse IgG for 6 h. Direct adsorption of the biotinylated antimouse IgG did not give well reproducible results. The first avidin−biotin reaction was allowed to proceed during the immersion of the electrode, for 20 h, in a 0.1 mg/mL gelatin plus 20 μg/mL avidin solution in PBS buffer. After another thorough washing, the electrode was dipped, for 30 min at 4

°C, in a 0.1 mg/mL gelatin plus 10 μg/mL biotinylated glucose oxidase PBS buffered solution and washed. For the immobilization of the next monolayer on top of the first or of any preceding layer we proceeded as follows. The electrode was first immersed for 15 min in a 0.1 mg/mL gelatin plus 20 μg/mL avidin PBS buffered solution, washed, and immersed for another 30 min in the 0.1 mg/mL gelatin plus 10 μg/mL biotinylated glucose oxidase PBS buffered solution at 4 °C. When not in use, the electrode was stored at 4 °C in the PBS buffer.

Cyclic Voltammetry Instrumentation. The glassy carbon electrode (0.07 cm^2 surface area) and the instrument were the same as previously described.[7a] Unless otherwise specified, the temperature in all experiments was 25 °C. The pH 8 phosphate buffer then used was made of 0.0365 M KH_2PO_4 plus 0.01% sodium azide and pH adjusted with a 1 M NaOH solution, leading to an ionic strength of 0.1 M. All solutions were purged from dioxygen before each run.

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(1) (a) Université de Paris 7. (b) Université de Compiègne.

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