Electron Transfer in Organized Assemblies of Biomolecules. Construction and Dynamics of Avidin/Biotin Co-immobilized Glucose Oxidase/Ferrocene Monolayer Carbon Electrodes

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There is a growing quest for the construction of supramolecular architectures associating several chemical functionalities in a spatially ordered manner. Directional electron transport is a key property when electrical activation of such molecular devices is requested. Knowing quantitatively how the system responds to electrical excitation is then an essential objective which opens up fundamental questions about the mechanism and dynamics of electron transport and of the electron-transfer activation of the chemical functionalities included in the structure. Immobilization of redox centers may be achieved by adsorptive or chemical attachment of monolayers on electrode surfaces or by entrapment in polymer or gel coatings in which other functionalities may also be included.[1] Although much has been learned from the construction and analysis of such systems, their spatial structure is generally not sufficiently defined or known to allow a full comprehension of the laws that govern their dynamics. An attractive alternative is to take advantage of the properties of biomolecules and derivatives in terms of molecular recognition for constructing spatially ordered systems. Among them, enzymes offer in addition a wealth of catalytic properties that may be triggered electrically provided that the system contains at least one redox enzyme. In most cases, direct electron transfer between the redox enzyme and the electrode is precluded by steric hindrance and/or denaturation. A mediator, serving as artificial cosubstrate, is therefore required to shuttle the electrons between the electrode and the enzyme. While several procedures have been proposed to immobilize redox enzymes and "wire"[2a] them to the electrode,[2] there have been very few attempts to analyze the dynamics of such systems and test the enzymatic activity once the attachment has been completed.[2d] In this respect, a strategy based on antigen−antibody interactions has been shown to fully preserve the activity of the enzyme in monolayer as well as in multilayer structures.[2d] These systems lend themselves to a quantitative cyclic voltammetric analysis of their dynamics. However, one drawback of these electrodes is that the mediator is not attached to the structure. There is a need of more integrated systems including the electron carrier which dynamics could be quantitatively analyzed.

We have found that making use of the biotin−avidin interaction in place of antigen−antibody interactions and of the presence of four noncooperative binding sites of avidin allows the construction, on glassy carbon (GC) electrodes, of the first example of a fully characterized monolayer where the enzyme and the electron carrier are simultaneously immobilized.[3,4] These electrodes are thus good models for investigating the factors governing the communication between electrical signal and biomolecules. Although this is not the primary objective of the present study, knowledge acquired along these lines on redox enzyme systems may be integrated in strategies for preparative-scale and biosensor applications.[5] A large variety of avidin and biotin derivatives are available thanks to their use in protein and enzyme assays,[6] thus leading to a facile generalization to other biomolecules and electron carriers.

The procedure we used for attaching both the enzyme and the cosubstrate, taking as example glucose oxidase and ferrocene, is sketched in Scheme 1. The long-chain biotin was first grafted, according to the recently described *N*-hydroxysuccinimide technology,[7] by reacting directly the GC surface with a chloroform solution of the commercially available *O*-2-[((*N*-hydroxysuccinimidyl)ethyloxy)carbonyl], *O′*-2-(*N*-biotinamidoethyl) poly(ethylene glycol) (NHS-CO_2-PEG-Biotin, average molecular weight 3500, average number of O-CH_2CH_2 units 69).The glucose oxidase conjugated avidin was then attached, thanks to its strong affinity for the biotin moieties.

Finally, the long-chain biotinylated ferrocene derivative, *O*-[(2-*N*-(β-ferrocenylethylamino)ethyloxy)carbonyl],*O′*-2-(*N*-biotinamidoethyl)poly(ethylene glycol) (Scheme 1) was bound to the remaining vacant sites of the monolayer of glucose oxidase conjugated avidin. This ferrocene derivative was obtained from the reaction of $FcCH_2CH_2NH_2$ with $NHSCO_2$-PEG-Biotin as depicted in the Supporting Information. The synthesis of a series of similar biotin derivatives has been recently reported.[8] Up to 10^{-10} mol/cm^2 may be deposited from a 10 mM chloroform solution of NHS-CO_2-PEG-Biotin.[7] It is tempting to attach the maximal amount of the biotin derivative on the surface in the

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(3) (a) The avidin−biotin technology has been previously used to bind horseradish peroxidase and glutamate dehydrogenase to a GC electrode after superficial oxidation aiming at the generation of carboxylic sites as already mentioned.[3b,c] Laser irradiation of "photobiotin"[3d] allows the attachment of the enzyme with less corrosion of the surface. In both cases, the cosubstrate was not included into the immobilized structure. (b) Pantano, P.; Morton, T. H.; Kuhr, W. G. *J. Am. Chem. Soc.* **1991**, *113*, 1832. (c) Pantano, P.; Kuhr, W. G. *Anal. Chem.* **1993**, *65*, 623. (d) Dontha, N.; Nowall, W. B.; Kuhr, W. G. *Anal. Chem.* **1997**, *69*, 2619.

(4) (a) Co-immobilization of the enzyme and the cosubstrate has previously been achieved by electrostatic or chemical binding with self-assembling thiols on gold.[4b−d] However, the dynamics of these electrodes have not been analyzed, and the percentage of active enzyme is not known. (b) Hodak, J.; Etchenique, R.; Calvo, E.; Singhal, K.; Bartlett, P. N. *Langmuir* **1997**, *13*, 2708. (c) Willner, I.; Heleg-Shabtai, V.; Blonder, R.; Katz, E.; Tao, G.; Bückmann, A. F.; Heller, A. *J. Am. Chem. Soc.* **1996**, *118*, 10321. (d) Bardea, A.; Katz, E.; Willner, I.; Bückmann, A. F. *J. Am. Chem. Soc.* **1997**, *119*, 9114. (e) Kajiya, Y.; Okamoto, T.; Yoneyama, H. *Chem. Lett.* **1993**, 2107. (f) Carbon gives much better electrochemical responses than gold in the oxidative potential range of interest thus allowing cyclic voltammetric analyses of the system dynamics. However, the attachment procedure should not be too corrosive as it is the case when it involves vigorous oxidation generating carboxylic anchoring groups.[4g,h] (g) Bourdillon, C.; Bourgeois, J. P.; Thomas, D. *J. Am. Chem. Soc.* **1980**, *102*, 4231. (h) Ianniello, R. M.; Yacynych, A. M. *Anal. Chem.* **1981**, *53*, 2090. (i) Arylation of carbon electrodes through electrochemical reduction of diazonium salts,[4j] followed by the same type of coupling with the enzyme minimizes corrosion,[4k] although extensive deactivation of the enzyme occurs.[4l] This observation and others[3] indicate that a substantial proportion of the enzyme may be denatured if it is immobilized too close to the electrode surface in an insufficiently hydrophilic environment. (j) Allongue, P.; Delamar, M.; Desbat, B.; Fagebaume, O.; Hitmi, R.; Pinson, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1997**, *119*, 201 and references therein. (k) Bourdillon, C.; Delamar, M.; Demaille, C.; Hitmi, R.; Moiroux, J.; Pinson, J. *J. Electroanal. Chem.* **1992**, *336*, 113. (l) Bourdillon, C.; Demaille, C.; Guéris, J.; Moiroux, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1993**, *115*, 12264.

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(7) (a) Anne, A.; Blanc, B.; Moiroux, J.; Savéant, J.-M. *Langmuir* **1998**, *14*, 2368. (b) This is also a noncorrosive derivatization technique.[4]

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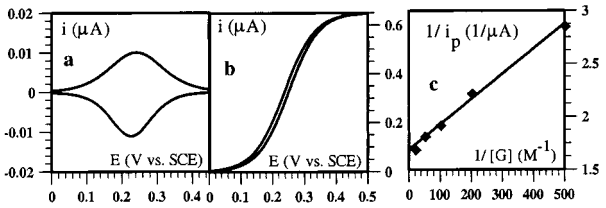
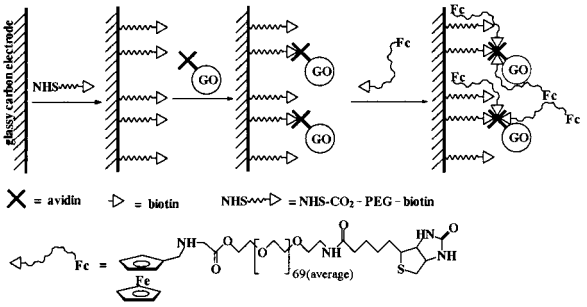


Figure 1. Cyclic voltammetry of the monolayer glucose oxidase−ferrocene electrode (see text) in the absence (a) and presence of 0.5 M glucose (b) phosphate buffer (pH = 8). Scan rate: 0.04 V/s. Temperature: 25 °C. (c) Variation of the plateau current with the glucose concentration.

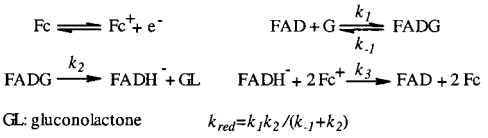
Scheme 1



hope of depositing a maximal amount of glucose oxidase on the electrode. However, successful co-immobilization leading to stable electrodes (less than 1.5% loss in the catalytic activity per day over 1 week) was obtained only by grafting not more than ca. 10^{-11} mol/cm^2. Electrodes obtained according to this optimized procedure should be carefully washed before use, including immersion in a phosphate buffer for periods as long as 20 h. With larger amounts of the biotin derivative deposited on the electrode, as for example with 10^{-10} mol/cm^2, the catalytic activity rapidly decreases with time (35% overnight). Under such conditions, the amount of biotin moieties on the surface is in large excess over the bound glucose oxidase conjugated avidins. Exposure of the electrode thus obtained to a solution of the biotinylated mediator does result in its incorporation in the enzyme layer. However, as soon as the electrode is washed and transferred into a test solution with no biotinylated mediator present, the attached biotinylated ferrocene is displaced by the excess biotins present on the electrode surface, owing to the reversibility of avidin−biotin complex formation (for more details see the Supporting Information).[6,9]

The cyclic voltammetry (Figure 1) of the optimized enzyme electrodes shows that the addition of glucose gives rise to a strong catalytic enhancement of the current[10] leading to S-shaped curves.[11] In the absence of glucose, integration of the surface reversible wave (Figure 1a) provides an estimate[12] of the surface concentration of the attached ferrocene mediator, $(6 \pm 3) \times 10^{-12}$ mol/cm^2. In the presence of glucose (Figure 1b), the plateau current, i_p, is expected to obey the following equation based on

Scheme 2



the mechanism depicted in Scheme 2,

$$\frac{1}{i_p} = \frac{1}{2FS\Gamma_E}\left(\frac{1}{k_3[\mathrm{Fc}]} + \frac{1}{k_2} + \frac{1}{k_{red}[\mathrm{G}]}\right)$$

S is the electrode surface area, Γ_E is the surface concentration of enzyme, [Fc] is the total where concentration of ferrocene and ferrocenium in the monolayer, and [G] is the glucose concentration. When ferrocene methanol is introduced in the solution, the catalytic current becomes at least 20 times larger. The effect of the attached mediator may thus be neglected as compared to the effect of the mediator in solution. Since k_3 (1.2×10^7 M^{-1} s^{-1}), k_2 (750 s^{-1}), and k_{red} (1.1×10^4 M^{-1} s^{-1}) for this solution mediator are known, the value of Γ_E, 3.5×10^{-12} mol/cm^2, ensues, indicating that, practically, all of the enzyme is active by comparison with antigen−antibody monolayers.[4l] It follows that there is a little less than 2 ferrocenes per glucose oxidase, in average, in the monolayer. When the $FcCH_2CH_2NHCO_2$-PEG-Biotin mediator is used in solution instead of ferrocene methanol, a smaller value of k_3, 6×10^5 M^{-1} s^{-1}, is found.

Coming back to the experiments where the enzyme electrode is introduced in a solution where no homogeneous mediator has been added, Figure 1c shows the variation of the plateau current with the concentration of glucose. Since k_3[Fc] is much smaller than k_2, its value, 13 ± 0.5 s^{-1}, is obtained from the intercept and k_{red}, $(9 \pm 4) \times 10^3$ M^{-1} s^{-1}, is obtained from the slope. The fact that this last value agrees with the value found in solution studies[13] confirms that functioning of the enzyme in the monolayer has not been significantly affected by the immobilization procedure. Assuming that the thickness of the layer within which the ferrocene moiety may move is twice the length of PEG 3500 (250 Å) leads to [Fc] $\approx 10^{-3}$ M and $k_3 \approx 10^4$ M^{-1} s^{-1}.

As discussed earlier,[14] there are three main steps in the reaction of the ferrocene mediators with glucose oxidase, namely, diffusion to the surface of the enzyme, adequate positioning at the bottom of the pocket connecting the prosthetic group to the enzyme surface, and electron transfer from the prosthetic group. The second step is rate determining with small ferrocenes.[14] This is also the case with the long-chain mediator when dispersed in the solution. The decrease in k_3 (by a factor of 17 as compared to ferrocene methanol) then results from the steric obstacle created by the long chain for reaching the bottom of the pocket. The further decrease in k_3 (by a factor of 60), observed when the long-chain mediator is immobilized, is likely to result from an increase of viscosity due to the entanglement of the various long chains present in the monolayer, making the diffusion to the enzyme surface rate determining.

This application of the avidin−biotin technology may be generalized to other enzymes and cosubstrates and to the step-by-step construction of ordered multilayer systems including the enzyme and a mediator attached by an arm of tailored length. It offers a friendly environment to the enzyme. Each step of the immobilization procedure can be carefully controlled leading to catalytic devices that are amenable to quantitative analysis through, e.g., cyclic voltammetry.

Supporting Information Available: Experimental details (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(10) (a) The curves in Figure 1 are corrected for a rather large background current. The raw curves are given in the Supporting Information. (b) In the absence of glucose, the reversible wave of the ferrocene derivative in the monolayer indicates a standard potential of 0.225 V vs SCE while a value of 0.165 is found when the ferrocene derivative is dispersed in the solution. Such a positive shift is also observed for soluble ferrocenes when going from water to PEG as the solvent,[10c] indicating that, in our case, the positive shift is most probably due to the large amount of $O\text{-}CH_2CH_2$ functionalities within the monolayer. (c) Ingram, R. S.; Murray, R. W. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 3941.

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