J|A|C|S
COMMUNICATIONS

Published on Web 02/23/2002

Molecular "Wiring" Enzymes in Organized Nanostructures

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Received December 18, 2001

We report on the "*molecular wiring*" efficiency of glucose oxidase in organized self-assembled nanostructures comprised of enzyme layers alternating with layers of an osmium derivatized poly(allylamine) cationic polyelectrolyte, acting as redox relays. Varying the relative position of the active enzyme layer in nanostructures alternating active enzyme and inactive apoenzyme we have investigated the mechanisms of electrical signal generation from biomolecular recognition. The specific rate of bimolecular $FADH_2$ oxidation ("wiring efficiency") is limited by the diffusion-like electron hopping mechanism in the multilayers.

Organized multilayers formed by alternated electrostatic adsorption of anionic and cationic polyelectrolytes provide a simple way to fabricate ultrathin functional films on solid surfaces with nanometer resolution.[1] Films including enzymes and redox polymer "wires" in their structure allow high selectivity toward molecular recognition and electrical communication in sensing devices.[2,3] By using organized enzyme nanostructures we report here how the molecular "wiring" efficiency depends on the relative position of the active enzyme layer in the multilayer structure. This is relevant to the rational design of miniature intelligent devices where biomolecule recognition can be combined to microelectronics to achieve fast monitoring of biochemicals. Molecular recognition with enzymes and with enzyme-labeled immuno and genomic electrodes based respectively on the antigen−antibody interaction and single strand DNA (ss-DNA) hybridization[4,5] with self-immobilized redox relays to generate an electrical signal can be integrated in circuits leading to intelligent biochips of the future.[6]

Enzymes deposited in ordered monolayers and multilayer systems have been described by using different assembling techniques for enzyme immobilization such as Langmuir−Blodgett,[7] self-assembled monolayers,[8−10] step-by-step electrostatic adsorption of alternate multilayers,[2,11−13] antigen−antibody interaction,[14,15] avidin−biotin interaction,[16] surfactants films,[17] electrostatic adsorption of hyperbranched polyelectrolytes,[18] etc. Electrically "wired" enzymes have attracted much attention recently because of their potential applications in reagentless biosensors and molecular devices.[19] In large proteins such as glucose oxidase (GOx) (186 000 g mol^{-1}), direct electron transfer from $FADH_2$ buried inside the protein structure, to the electrode surface from the prosthetic group is hindered. Heller and co-workers have demonstrated that the electrical communication between the $FADH_2$ in glucose oxidase and electrodes can be facilitated by electrostatic complexing of the negatively charged enzyme in a solution of pH above the isoelectric point (4.2) with a cationic quaternized poly(vinylpiridine) and poly(vinylpiridine) $Os(bpy)_2Cl$ redox mediator copolymer.[20] Furthermore, based on this concept Heller introduced a two-component epoxy technique combining GOx and other oxidases with the polycationic redox mediator cross-linked with a bifunctional reagent to yield a hydrogel.[21]

Scheme 1

$$\mathrm{PAH\text{-}Os(II)} \longrightarrow \mathrm{PAH-Os(III)} + e^-$$

$$2\mathrm{PAH\text{-}Os(III)} + \mathrm{GOx(FADH_2)} \xrightarrow{k} \mathrm{GOx(FAD)} + 2\mathrm{PAH\text{-}Os(II)}$$

$$\mathrm{GOx(FAD)} + S \underset{}{\overset{k_m}{\longleftrightarrow}} \mathrm{GOx(FAD-S)} \xrightarrow{k_{cat}} \mathrm{GOx(FADH_2)}$$

It is worthwhile noticing that in the hydrogel there is a random distribution of components with little control over the molecular orientation while spatially ordered enzyme assemblies built with the same active components of the hydrogels offer several advantages over random polymers.[2,15] The step-by-step electrostatic adsorption between a charged surface and oppositely charged molecules in solution introduced by Decher with molecular-level control[22−24] offers the possibility of regulating the adsorption and restricting the deposition to a monolayer.

In this communication we analyze how the relative position of the active enzyme layer in the supramolecular structure affects the efficiency of the redox polyelectrolyte layers acting as "molecular wires".

For this purpose, we first consider the double redox−enzyme catalytic cycle for the oxidation of β-D-glucose (S) catalyzed by glucose oxidase (GOx) and mediated by the self-contained molecular "wire polymer" PAH-Os in the organized self-assembled multilayer as depicted in Scheme 1. In the absence of glucose mass transport limitations in the ultrathin enzyme film, the expression for the current density is given by[2]

$$i_{cat} = \frac{2Fk_{cat}\Gamma_E}{1 + \dfrac{k_{cat}}{k[\mathrm{Os(III)}]} + \dfrac{K_{MS}}{[S]}} \qquad (1)$$

The Michaelis constant for β-D-glucose is $K_{MS} = (k_{-1} + k_{cat})/k_1$; k_{cat} and k are the enzyme turnover (glucose oxidation) and the bimolecular $FADH_2$ reoxidation rate constant, respectively, and Γ_E is the surface concentration of the total active enzyme wired by the Os polymer while [S] is the concentration of β-D-glucose. Figure 1 shows typical plots of the electrochemical response in β-D-glucose solution and a nonlinear fit to eq 1 from which the quantities k[Os] and Γ_E have been derived assuming $k_{cat} = 700\ s^{-1}$ and $K_{MS} = 25$ mM.[2]

The relevance of the relative position for a single active enzyme layer in the multilayer has been investigated by stepwise electrostatic adsorption of one active GOx layer and three inactive FAD-free apoenzymes sandwiched by PAH-Os layers. If the bimolecular collisional rate were isotropic one would expect the same "electrical wiring" efficiency no matter what the relative enzyme to osmium polymer position in the nanostructure was. However, Figure 1a

10.1021/ja017802u CCC: $22.00

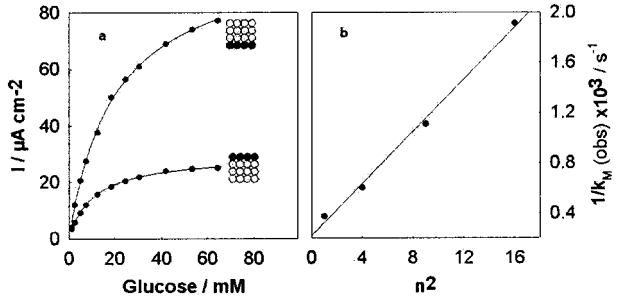

Figure 1. (a) Typical catalytic current response to β-D-glucose concentration for self-assembled nanostructured thin films of PAH-Os/GOx/(PAH-Os/$(ApoGOx)_3$ and (PAH-Os/$ApoGOx)_3$/PAH-Os/GOx and best fit lines to eq 1. ApoGOx: FAD-free glucose oxidase. (b) Plot of the observed reciprocal reoxidation rate constant $1/k_M$(obs) versus n^2 ($n = \Delta x/d_{layer}$ is the distance from the electrode surface expressed in terms of the number of successive self-assembled layers).

shows that the catalytic response for the two limiting cases where the active GOx layer is progressively moved from the bottom layer adjacent to the thiolated gold electrode toward the top layer in contact with the electrolyte is quite different. We have found a progressive decrease in the value of k[Os] from 2665 s^{-1} for the bottom layer adjacent to the underlying electrode to 523 s^{-1} for the top layer in contact with the external electroyte. Therefore we demonstrate that the relative position of the active GOx in the multilayer determines the "wiring efficiency", although the redox charge is approximately the same in all four experiments.

Cyclic voltammetry experiments have shown that all redox sites are equally accessible for electron transfer by hopping between adjacent osmium sites in the time scale of the experiment, ca. $\nu F/RT \approx 20$ to 40 s^{-1} (ν is the electrode potential scan rate). However the steady-state oxidation of a GOx at a given distance from the electrode surface demands a much higher flux of electrons (i.e. depending on the rate-determining step either $k_{cat} \approx 700$ s^{-1} or $k \leq 5000$ s^{-1}).

The generation of an electrical signal from the molecular recognition by GOx involves the following sequence of events: $FADH_2$ reoxidation, $k_M = k$[Os], and propagation of electrons from the active layer to the underlying electrode through the multilayer. Therefore, the observed rate of $FADH_2$ reoxidation, k_M(obs), can be expressed as the sum of the reciprocals of the reoxidation rate k_M in the absence of electron propagation limitations and the electron hopping diffusion constant k_D:

$$\frac{1}{k_M(\text{obs})} = \frac{1}{k_M} + \frac{1}{k_D} \qquad (2)$$

We can express k_D as the reciprocal of the characteristic diffusion time given by the Einstein equation $\tau = \Delta x^2/2D_e$ with $\Delta x = nd_{layer}$, where n is the layer number and d_{layer} the interlayer average distance and D_e is the electron-hopping diffusion coefficient.

The linear dependence of $1/k_M$(obs) vs n^2 as predicted by eq 2 is shown in Figure 1b.

From the intercept we calculate the maximum reoxidation constant $k_M = 4500$ s^{-1}, and from the slope and $d_{layer} \approx 5$ nm, $D_e = 1.2 \times 10^{-9}$ cm^2 s^{-1} in excellent agreement with the electron hopping diffusion constant measured in random hydrogels of the same composition by ultramicroelectrode chronoamperometry. The reoxidation efficency, $k = 4.5 \times 10^4$ M^{-1} s^{-1}, has been obtained from k_M and [Os] ≈ 0.1 M in the films.

We therefore conclude that the flux of electrons that can reoxidize the enzymatic $FADH_2$ in GOx layers farther away from the underlying electrode surface is limited by the propagation of electronic charge in the self-assembled multilayers.

In the present report, using well organized self-assembled multilayers, we have been able to show for an enzyme that can achieve fast reoxidation rates by the self-contained molecular wire, that the flux of electrons available is limited by the diffusion-like propagation of charge in the multilayer.

Acknowledgment. The authors acknowledge financial support from University of Buenos Aires (UBA), Argentine Science Research Council (CONICET), and Motorola Semiconductor Sector (Arizona, U.S.A.). E.J.C. acknowledges a Guggenheim Fellowship 2000. E.J.C. and C.D. are Research Fellows of CONICET (Argentina) and A.W. holds a Research Grant from CONICET (Argentina).

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JA017802U