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High Amplification Rates from the Association of Two Enzymes Confined within a Nanometric Layer Immobilized on an Electrode: Modeling and Illustrating Example

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Connecting an electrode with a soluble or an immobilized redox enzyme allows the transduction of specific chemical events taking place at its prosthetic group into easy-to-use electric signals. A route is thus opened to the gathering of mechanistic and kinetic information on the functioning of this class of enzymes on one hand and to biosensors applications on the other (substrate sensing or bioaffinity assays).[1] In all cases, establishing a model resulting in precise relationships linking the enzymatic kinetics and the electrochemical responses is an essential step for gaining meaningful kinetic data, which may additionally be used for rational sensor design and analytical performance optimization. So far such theoretical models have been derived for monoenzymatic systems, either involving direct electron transfer to the electrode[2] or mediated transfer by a redox cosubstrate with a soluble or an immobilized enzyme.[3-5]

For the reasons mentioned below, multienzymatic systems are gaining increasing attention, thus calling for an extension of theoretical treatments to schemes involving the coupling of two or more enzymes. Among multienzymatic systems, the coupling of several enzymes through substrate or cosubstrate regeneration allows the indirect investigation of redox-inactive enzymes and seems quite promising for amplifying the electrochemical responses in substrate detection[6] or bioaffinity assay[7] applications. However, in most previous examples, the coupled enzymes were entrapped in a thick membrane to the detriment of the biocomponents as well as the integrity and accessibility of the enzymes, resulting thus in rather modest amplification rates.

We have found on theoretical and experimental bases that confining two enzymes within one or within a small number of monolayers (Scheme 1) allows high amplification rates (higher than 1000), avoids membrane transport limitations, and lends itself to precise kinetic analyses that provide guidelines for optimization of the analytical sensitivity.

Scheme 1
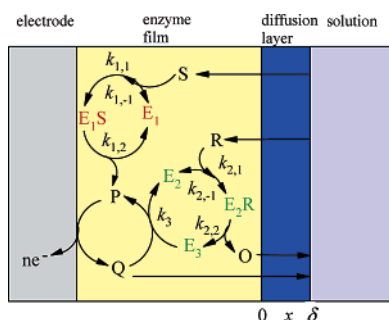
The first enzyme converts the substrate S into an electroactive product P, which is oxidized[8] at the electrode surface to give Q. Q serves as cosubstrate to the second enzyme in the conversion of the substrate R into the product O. We consider the case where detection is chronoamperometric with the electrode potential poised at a value positive enough for P to be immediately and entirely converted into Q. The first enzyme is assumed to follow a simple Michaelis−Menten kinetics (involving two forms, E_1 and E_1S) while the second, auxiliary, enzyme operates according to a ping-pong mechanism (involving the three forms E_2, E_2R, and E_3). In the absence of the amplifying enzyme, the current is obtained from eq 1.[9]

$$\frac{i_1}{nFS} = k_{1,2}\frac{[S]_{x=0}}{K_{1,M} + [S]_{x=0}}\Gamma_1^{\,0} \tag{1}$$

In the coupled enzyme system, the flux balances of P and Q write:

$$\frac{i}{nFS} = k_{1,2}\Gamma_{E_1S} + k_3[Q]_{x=0}\Gamma_{E_3} = k_3[Q]_{x=0}\Gamma_{E_3} - D_Q\left(\frac{d[Q]}{dx}\right)_{x=0} \tag{2}$$

respectively.[10] It follows that the amplification factor, $\mathscr{A}$ is expressed by:[9,10]

$$\mathscr{A} = \frac{i}{i_1} = 1 + \frac{k_3\Gamma_2^{\,0}\dfrac{\delta}{D_Q}}{1 + \dfrac{k_{1,2}\Gamma_1^{\,0}[S]_{x=0}}{K_{1,M}+[S]_{x=0}}\dfrac{k_3}{k_{2,2}}\dfrac{\delta}{D_Q}} = 1 + \frac{k_3\Gamma_2^{\,0}\dfrac{\delta}{D_Q}}{1 + \dfrac{i_1}{nFSk_{2,2}}\dfrac{k_3}{D_Q}\dfrac{\delta}{D_Q}} \tag{3}$$

When looking for the detection of either very small concentrations of the substrate S or for very small amounts of the affinity-deposited enzyme 1, the second term in the denominator vanishes, and the amplification factor becomes independent of either $[S]_{x=0}$ or Γ_1^0, as follows:

$$\mathscr{A} \rightarrow k_3\Gamma_2^{\,0}\frac{\delta}{D_Q} \tag{4}$$

The main factors for a good amplification are thus an auxiliary enzyme running fast toward Q and a large amount of this deposited onto the electrode.[11]

As an illustrative example, we have selected the β-galactosidase−diaphorase-coupled system. β-Galactosidase (β-Gal) was chosen as the primary enzyme label[12] because it is able to hydrolyze the p-aminophenyl-β-D-galactopyranoside (PAPG) substrate into an electroactive product, p-aminophenol (PAP). The PAP thus generated is next oxidized at the electrode into p-quinoneimine (PQI) according to a $-(2e^- + 2H^+)$ reaction. In the presence of the auxiliary enzyme, diaphorase (DI) from *Bacillus stearothermophilus*, PQI is reduced back to PAP, and the oxidized form of DI is finally regenerated in its reduced native state by its natural substrate, NADH. Selection of this bi-enzymatic system was guided by the following observations: (i) DI is very reactive toward PAP (bimolecular rate constant, k_3 larger than 10^8 M^{-1} s^{-1});[4] (ii) both enzymes have their optimal activity at approximately the same pH ($\sim 7.5-8.5$); (iii) the PAPG substrate is commercially available, essentially free from residual traces of PAP; (iv) both enzymes can be easily biotinylated. The step-by-step procedure for assembling
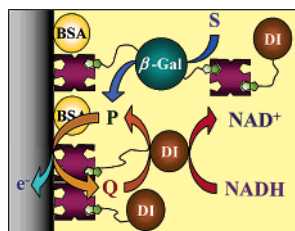
Figure 1. Avidin−biotin assemblage of β-galactosidase (β-Gal) and diaphorase from *Bacillus stearothermophilus* (DI). S: *p*-aminophenyl-β-D-galactopyranoside (PAPG), P: *p*-aminophenol (PAP), Q: *p*-quinoneimine (PQI). BSA: bovine serum albumin.
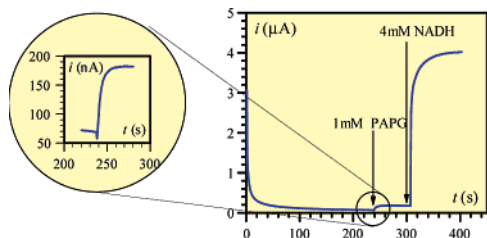

Figure 2. Amplification of the chronoamperometric response.[9] Successive injections of the first and second enzyme substrate, PAPG and NADH, respectively.

Table 1. Amplification of the Chronoamperometric Response

[β-Gal] (nM)[a]	Γ_2^0 (pmol cm^{-2})[16]	i_1 (nA)	i (μA)	$\mathscr{A}_{exp}$	$\mathscr{A}_{theo}$	Γ_1^0 (fmol cm^{-2})
100	0.47	105	4	38	41	650[b]
100	0.6	80	4.8	60	68	500[b]
1	0.75	8	3.15	394	548	50[b]
1	0.75	13	4.3	331	396	80[b]
0.1	0.9	<2	1.2	>1000	1720	8[c]
0.1	1.5	<2	1.3	>1000	2900	6[c]

[a] In the incubating solution. [b] From eq 1 in which $n = 2$, $[S]_{x=0}$ (1 mM) $\gg K_{1,M}$ (140 μM) with $k_{1,2}= 12$ s^{-1}.[9] [c] Estimated from eq 5.

the two biotinylated enzymes on the electrode surface is sketched out in Figure 1. We have selected the avidin−biotin immobilization strategy because it allows the assemblage of ordered protein multilayers[13] with a high degree of control and prevents denaturation of the deposited enzymes.[4,3d]

A saturated monolayer of neutravidin is first irreversibly adsorbed on the surface of the carbon electrode, followed by deposition of biotinylated β-Gal.[9,14] The electrode is next incubated in a neutravidin solution so as to fill the biotinylated sites born by the attached biotinylated β-Gal. A final incubation with biotinylated DI[9] leads to saturation of all neutravidin vacant sites.[15] A bi-enzyme layer of a few nanometers thickness is thus obtained, through which substrate and cosubstrate transport has no chance to interfere kinetically in the electrochemical response.

A typical chronoamperometric experiment is outlined in Figure 2. The potential is stepped from 0 to 0.3 V vs SCE so as to fulfill the condition that the concentration of P at the electrode surface is zero, still avoiding direct oxidation of added NADH or PAPG. After decrease and stabilization of the current, addition of PAPG to the solution results in a increase of the current up to a value that corresponds to the current previously defined as i_1. Successive additions of NADH (up to saturating concentrations), produced another, much larger, increase of the current that reaches a value that corresponds to the current previously defined as i. The results of similar experiments carried out for other values of the β-Gal concentration in the incubating solution, and therefore of its surface concentration, are gathered in Table 1.

Comparison of the experimental and predicted amplification factors (Table 1),[16] indicates a satisfactory agreement in all cases where the surface concentration of β-Gal was sufficient for the

current i_1 to be directly measurable (>2 nA). For the two lowest values of Γ_1^0, the amplification factor becomes independent of Γ_1^0 as expressed by eq 4.

In sum, we have demonstrated that very large amplification factors, as large as several thousands, can be reached experimentally, in agreement with appropriately derived theoretical predictions, thus opening the route to the rational design of high-performance substrate sensing or affinity assays applications.

Supporting Information Available: Experimental details and derivation of the theoretical relationships. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Transposition to reduction is straightforward.
(9) See Supporting Information.
(10) i_1 and i are currents, F the faraday, S, the electrode surface area. x is the distance to the electrode surface and δ the diffusion-convection layer thickness. D_Q is the diffusion coefficient of Q. The Γ s are the surface concentrations of the subscript enzyme. The rate constants are defined in Scheme 1 and through $K_{1,M} = (k_{1,-1} + k_{1,2})/k_{1,1}$, $k_1 = k_{1,1}$ $k_{1,2}/(k_{1,-1} + k_{1,2})$.
(11) Maximization of δ/D_Q is obtained with an immobile electrode, where the diffusion layer thickness is governed by natural convection.
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(14) Between these two steps, the electrode was exposed to a solution of bovine serum albumin (BSA) to fill the spaces remaining vacant after adsorption of neutravidin and thus to avoid nonspecific interactions during the next recognition steps.
(15) The penultimate step (neutravidin incubation) is important for relatively high coverages of β-Gal but may well be skipped for small coverages.
(16) For the reaction of native diaphorase with PAP in solution, $k_3 = 6 \times 10^8$ M^{-1} s^{-1}.[4] This value was divided by two to account for the decrease in reactivity observed, with ferrocenemethanol, upon biotinylation.[4] The other parameters were: $n = 2$, corresponding to the $-(2e^- + 2H^+)$ oxidation of PAP and $k_{2,2} = 700$ s^{-1}.[4] Γ_2^0 was derived from the recording of a cyclic voltammogram after addition of 20 μM ferrocenemethanol to the solution at the end of the above-described chronoamperometric experiments, using the preceding values for the two rate constants.[9] $\delta/D_Q = 6345$ cm^{-1} s was obtained from a chronoamperometric experiment where PAP was oxidized on an electrode covered with BSA.

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