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Electrical Contacting of Glucose Oxidase by Surface-Reconstitution of the Apo-Protein on a Relay-Boronic Acid-FAD Cofactor Monolayer

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Electrical contacting of redox-enzymes with electrodes is a major goal for developing amperometric biosensors,[1,2] biofuel cells,[3] and bioelectronic elements.[4] Integrated electrically contacted enzyme-electrodes were prepared by the tethering of redox-relay units to the enzyme associated with electrodes,[5] and by the immobilization of enzymes in redox-active polymers assembled on electrodes.[6] The effectiveness of electron transfer communication in these systems is, however, substantially lower than that of the enzymes with their native substrates.[7] This has been attributed to a nonoptimal modification of the redox-enzymes by the electroactive relay units, and to the random orientation of the enzymes in respect to the electrode supports.[2] We have demonstrated[8] that the reconstitution of apo-glucose oxidase (apo-GOx) on a relay-FAD (flavin adenine dinucleotide) monolayer associated with an electrode yields an aligned, electrically contacted, enzyme-electrode with an unprecedented effective electron-transfer communication that is similar to the electron transfer of the enzyme with its native substrate (oxygen). This efficient electrical communication between the surface-reconstituted bioelectrocatalyst and the electrode was utilized to develop enzyme-electrodes for a glucose sensor,[8] and for a biofuel cell.[3b] To generate the relay-FAD monolayer in these systems, the covalent coupling of a synthetic aminoethyl-FAD to the relay unit is a key step. The elaborate synthesis of this cofactor[9] turned the approach to be of limited practical utility. Here we wish to report on a new simple methodology for the surface-reconstitution of apo-flavoenzymes using a relay unit functionalized with a boronic acid ligand as the active component for the binding of native FAD. The reconstitution of apo-GOx on the relay-FAD monolayer yields an electrically contacted enzyme-electrode.

Boronic acid is an active ligand for the association of *cis*-diols, and particularly *cis*-diols which are a part of cyclic saccharides.[10] Accordingly, the relay-FAD monolayer is assembled on a Au-electrode as outlined in Scheme 1. Pyrroloquinoline quinone, PQQ (**1**), is covalently linked to a cystamine monolayer assembled on the electrode.[11] To the resulting monolayer, 3-aminophenylboronic acid (**2**), 1×10^{-3} M, is covalently linked, using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), 5×10^{-3} M, as a coupling reagent in 0.1 M HEPES-buffer, pH 7.3. The resulting electrode is treated with 1×10^{-3} M FAD (**3**) to yield the boronic acid-FAD complex on the monolayer assembly.

Figure 1A, curve a, shows the cyclic voltammogram of the resulting monolayer. The two redox-waves correspond to the quasireversible response of the FAD ($E° = -0.50$ V vs SCE) and the PQQ ($E° = -0.13$ V) units, pH 7.0, respectively. Coulometric assay of the redox waves of the electroactive units indicates that the surface coverage of the PQQ and FAD units is 1.8×10^{-10} and 1.6×10^{-10} mol·cm^{-2}, respectively (PQQ:FAD molar ratio is ca. 1:0.9). Treatment of the PQQ-FAD monolayer-functionalized

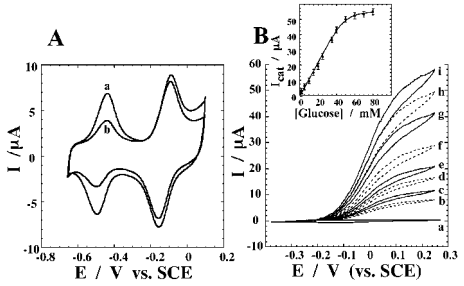
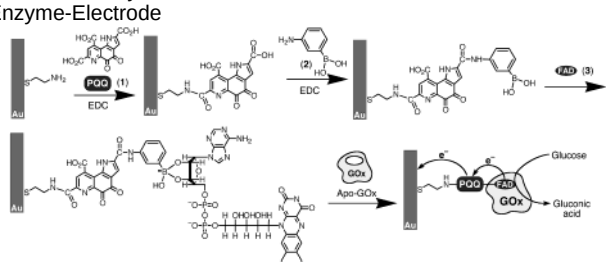


Figure 1. (A) Cyclic voltammograms of the PQQ-FAD-functionalized Au-electrode: (a) before reconstitution, (b) after reconstitution with apo-GOx; potential scan rate, 200 mV·s^{-1}. (B) Cyclic voltammograms of the GOx reconstituted on the PQQ-FAD-functionalized Au-electrode (geometrical area 0.3 cm^2, roughness factor ca. 1.3) in the presence of different concentrations of glucose: (a) 0, (b) 5, (c) 10, (d) 15, (e) 20, (f) 25, (g) 35, (h) 40, (i) 50 mM; potential scan rate, 2 mV·s^{-1}. Data were recorded in 0.1 M phosphate buffer, pH 7.0, under Ar. Inset: Calibration plot of the electrocatalytic currents ($E = 0.2$ V) at variable glucose concentrations.

Scheme 1. Assembly of the Reconstituted GOx-Electrode and the Bioelectrocatalytic Oxidation of Glucose with the Enzyme-Electrode



electrode with apo-GOx results in the surface-reconstitution of the protein on the functionalized electrode, Figure 1A, curve b.

Microgravimetric quartz-crystal-microbalance measurements following the reconstitution of apo-GOx on a Au/quartz piezoelectric crystal (AT-cut, 9 MHz) modified with the PQQ-FAD monolayer indicate a surface coverage of the enzyme that corresponds to 2×10^{-12} mol·cm^{-2}. Figure 1B shows the cyclic voltammograms of the resulting surface-reconstituted enzyme-electrode in the presence of variable concentrations of glucose. An electrocatalytic anodic current is observed in the presence of glucose implying that the surface-reconstituted enzyme is electrically contacted with the electrode, and that the enzyme is bioelectrocatalytically active toward the oxidation of glucose. The electrocatalytic anodic current is observed at the redox potential of the PQQ units indicating that PQQ mediates the oxidation of the $FADH_2$ formed upon the oxidation of glucose.

In a control experiment outlined in Scheme 2, route a, 3-aminophenylboronic acid (**2**) was directly linked to a cysteic acid monolayer assembled on the Au-electrode. The cofactor FAD was then linked to the boronic acid ligand, and apo-GOx was recon-

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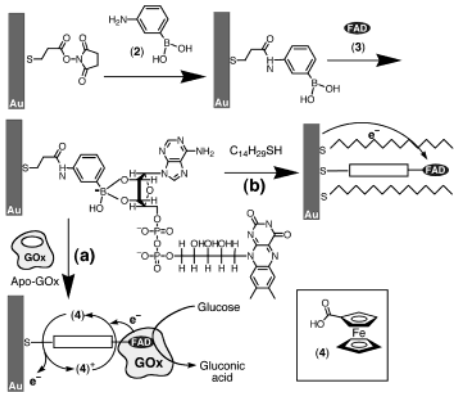
Scheme 2. Functionalization of a Au-Electrode with the FAD Cofactor[a]



[a] (a) Reconstitution of a non-rigidified FAD-monolayer with GOx and the biocatalytic oxidation of glucose by the enzyme-electrode in the presence of **4** as a diffusional mediator. (b) Assembly of a regidified FAD-monolayer and its chronoamperometric reduction.

stituted onto the monolayer. The resulting surface-reconstituted enzyme-electrode lacks direct electrical communication with the electrode, although the enzyme is reconstituted in a biologically active configuration that is evident by the bioelectrocatalyzed oxidation of glucose in the presence of ferrocene carboxylic acid (**4**) as a diffusional electron mediator. This control experiment clearly reveals that the PQQ units mediate the electron transport between the FAD redox-site and the electrode surface in the integrated system (Scheme 1).

Figure 1B, inset, shows the derived calibration curve corresponding to the currents transduced by the enzyme-electrode at different concentrations of glucose. The current response saturates at glucose concentrations higher than 60 mM. The saturated current value corresponds to the highest turnover rate of the biocatalyst. From the known surface coverage of the enzyme, and knowing the saturation value of the current density ($i_{max} = 140\ \mu A \cdot cm^{-2}$), we estimate the electron-transfer turnover rate to be ca. 700 s^{-1} at 25 °C. This value is similar[12] to the electron-transfer turnover rate of glucose oxidase with O_2, its native substrate. The efficient electron-transfer turnover rate of the reconstituted enzyme has important consequences on the properties of the enzyme electrode. The amperometric response of the enzyme-electrode in the presence of glucose is not interfered by oxygen. Similarly, the amperometric responses of the electrode ($E = 0.0$ V) in the presence of glucose are unaffected by 20 mM of ascorbic acid or 20 mM of uric acid, common interferants to glucose sensing electrodes. That is, the nonspecific oxidation of the interferants has a small effect (<5%) on the currents originating from the glucose oxidation.

A final aspect that should be considered relates to the mode of association of the FAD-cofactor to the boronic acid ligand. The FAD cofactor includes the diol functionalities of the ribose unit and of the linear glycerol unit. Previous studies[13] indicated that the association constant of the saccharide unit to the boronic acid ligand is substantially higher than that of the linear polyol, and hence we suggest that the binding of ribose to the boronate is the binding mode in the monolayer structure. A single binding mode of the FAD-cofactor to the boronic acid ligand has been confirmed by chronoamperometric experiments. The 3-aminophenylboronic acid component was covalently linked to the thiolated cysteic acid

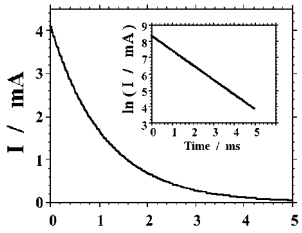


Figure 2. Chronoamperometric current transient corresponding to the reduction of the rigidified FAD-monolayer upon the application of potential step from −0.4 to −0.6 V. Inset: Semilogarithmic plot of the chronoamperometric transient. The data were recorded in 0.1 M phosphate buffer, pH 7.0, under Ar.

monolayer associated with the Au-electrode, and the monolayer was interacted with FAD to yield the boronate complex. The resulting monolayer was rigidified with $C_{14}H_{29}SH$, in ethanol solution (1 mM, 2 h), Scheme 2, route b. It was demonstrated[14] that the interfacial electron-transfer rate constants to electroactive units in monolayer configurations are sensitive to their spatial separation from the electrode and to the mode of binding. The association of FAD to the boronic acid ligand by the two possible modes would yield a chronoamperometric transient with a biexponential kinetics that correspond to the electron-transfer rate constants to the two modes of binding of the FAD units. Figure 2 shows the chronoamperometric transient corresponding to the reduction of the FAD unit. The current transient follows a single-exponential decay, Figure 2 (inset), suggesting a single mode of association of the FAD unit.

In conclusion, we have demonstrated a new method for the surface-reconstitution of apo-flavoenzymes by the assembly of a PQQ-boronate-FAD monolayer on the electrode. The resulting reconstituted enzyme reveals effective electrical contact with the electrode, and the enzyme-electrode is insensitive to interferants.

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