**Enzyme Electrodes**

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# The Limiting Performance Characteristics in Bioelectrocatalysis of Hydrogenase Enzymes

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*Dedicated to Süd-Chemie on the occasion of its 150th anniversary*

The anticipated crisis in future energy production has stimulated a search for alternative energy sources. Molecular hydrogen is considered nowadays to be the most promising chemical fuel, providing the highest energy output relative to molecular weight and a combustion that is devoid of environmental pollutants, including carbon dioxide. Despite the automobile industry's orientation toward low-temperature platinum-based fuel cells, wide application of such systems in the future is highly doubtful because of 1) the high cost of platinum, which is growing exponentially,[1] and 2) the limited amount of this noble metal on our planet. The lowest Pt loading is about 2 g per kW of fuel cell, and any further decrease makes the fuel cell ineffective.[2] Thus, to support an annual production of 60000000 cars equipped with only 50 kW fuel cells, 6000 tons of platinum would be required. This figure is over 30 (!) times the present mining output. Applications of Pt are also limited by its susceptibility to poisoning by fuel impurities (CO, $H_2S$) and low selectivity.[3,4]

The only useful alternative to platinum-based electrocatalysis for $H_2$ oxidation is bioelectrocatalysis by hydrogenases,[3–7] first reported by our group more than 20 years ago.[7] Hydrogenases are responsible in nature for oxidation and/or evolution of molecular hydrogen. Hydrogenase-covered electrodes, in the absence of both dissolved enzyme and redox mediators, generate the equilibrium hydrogen potential under $H_2$ atmosphere[3,4] and even tolerate the presence of oxygen up to the explosion limit of $H_2$–$O_2$ mixtures.[8]

For use as a possible energy source, the catalytic system has to be optimized for maximum efficiency. We have found that bioelectrocatalysis by hydrogenases is facilitated by electropolymerized *N*-methyl-*N'*-(12-pyrrol-1-yldodecyl)-4,4'-bipyridine[5,9] (MPDB; see Experimental Section). This film seems to provide the optimal orientation of hydrogenase for effective electron transfer between the enzyme active site and the electrode.

As we reported earlier, the hydrogenase electrode, after immersion into hydrogen-saturated solution, reached the equilibrium hydrogen potential.[4,7,10] The claim that the observed zero current potential was indeed the hydrogen equilibrium potential was confirmed by its dependency on $H_2$ concentration and pH as predicted by Nernst's equation. At positive overvoltages, hydrogenase electrodes generate an anodic current in the presence of $H_2$ (Figure 1). The latter can only be attributed to hydrogen oxidation.[7] Cathodic current at negative overvoltages has been proven to occur as a result of $H_2$ evolution.[11]

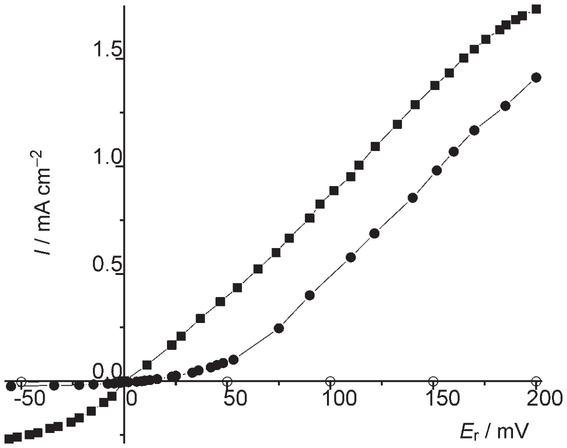

**Figure 1.** Current–voltage dependencies in $H_2$-saturated phosphate buffer solution (pH 7.0) of hydrogenase electrodes: *T. roseopersicina* (●); *D. baculatum* (■); no enzyme (○).

The total amount of hydrogenase on the electrode surfaces was estimated by recording the activity of the immobilized enzyme both in hydrogen oxidation by methyl viologen (monitored spectrophotometrically) and in $H_2$ evolution (controlled by means of chromatography). Because

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after immobilization enzyme activity is always less, this estimation should indicate the lower limit of the hydrogenase loading. For hydrogenase from *Thiocapsa roseopersicina*, it is $(45 \pm 10)$ pmol cm$^{-2}$ (Table 1), or $3 \times 10^{13}$ enzyme molecules per cm$^2$ of geometric surface.

***Table 1:*** Catalytic properties of hydrogenase electrodes.

| Hydrogenase source | Enzyme loading [pmol cm$^{-2}$] | $I_{200}$ [mA cm$^{-2}$] | $k_{e/c}$ [s$^{-1}$] | $k_{kin}$ [s$^{-1}$] |
|---|---|---|---|---|
| *T. roseopersicina* | $45 \pm 10$ | $1.4 \pm 0.2$ | $160 \pm 10$ | $120 \pm 10$ |
| *L. modestogalofillum* | $42 \pm 10$ | $1.2 \pm 0.2$ | $150 \pm 15$ | $100 \pm 10$ |
| *D. baculatum* | $40 \pm 10$ | $1.7 \pm 0.2$ | $220 \pm 10$ | $450 \pm 20$ |

The degree of surface occupation by the protein can be estimated by comparing its surface concentration with the lowest area required for one enzyme molecule. The latter found independently from Langmuir–Blodgett isotherms for hydrogenase from *T. roseopersicina* is 32 nm$^2$.[12] However, from loading this enzyme per geometric electrode area (see above), we obtain a value of 3.3 nm$^2$. Hence, the microscopic electrode area should be at least 10 times larger than its geometric area, which means that the surface roughness of the electrode support should not be less than 10. This figure coincides exactly with the lower limit of LSG roughness (see Experimental Section).

As proteins are of dielectric nature, the second and subsequent layers cannot be active towards hydrogen oxidation in the absence of freely diffusing redox mediators. Hence, direct bioelectrocatalysis is provided by the enzyme immobilized in a monolayer. From the current at 200 mV overvoltage ($I_{200}$), which is assumed to be at the level of maximum bioelectrocatalytic activity, we estimate the maximum rate of the hydrogenase reaction. This value, divided by the enzyme loading (Table 1), gives the enzyme catalytic constant in electrocatalysis ($k_{e/c}$).

It appears that the $k_{e/c}$ value for hydrogenases from *T. roseopersicina* and *Lamprobacter modestogalofillum* is similar to the corresponding catalytic constant estimated from homogeneous kinetics ($k_{kin}$). This fact strongly indicates that all detected enzyme molecules are immobilized in a monolayer. The slightly higher values of electrocatalytic constant point to an even larger amount of hydrogenase on the electrode surface compared to that estimated from the activity of the immobilized enzyme (which is expected). However, even the lower limit of enzyme loading in a monolayer requires surface roughness, which is the property of the electrode support used (see Experimental Section). Hence, we conclude that the electrode is almost completely covered with hydrogenase and nearly all enzyme molecules are properly oriented to achieve effective bioelectrocatalysis. In other words, the developed hydrogenase electrodes display nearly limiting performance characteristics in bioelectrocatalysis.

In contrast, for the most active hydrogenase from *Desulfomicrobium baculatum,* the electrocatalytic constant is half that of its homogeneous analogue (Table 1). Taking also into account that the $H_2$ oxidation current at the corresponding modified electrodes is the highest among all hydrogenases (Table 1, Figure 1), the most probable explanation for the observed difference in $k_{e/c}$ and $k_{kin}$ is that the operation of this particular enzyme electrode is in diffusion-limited conditions. Indeed, as seen in Figure 1, the current–voltage dependencies for enzyme electrodes are quite different. For hydrogenase from *T. roseopersicina*, the anodic current is exponentially dependent on a potential as predicted for charge-transfer-limiting reactions.[12] The electrode with immobilized enzyme from *D. baculatum* displays an overall sigmoid-type current–voltage dependence, known for diffusion-limited electrochemistry.[13] Moreover, at lower $H_2$ partial pressures the current–voltage dependence for *T. roseopersicina* hydrogenase is also changed to the diffusion-limited type confirming the above hypothesis.

From both fundamental and practical points of view, it is interesting to compare the behavior of the most active hydrogenase electrodes with Pt-based electrodes (platinum-vulcan, Pt loading 0.5 mg cm$^{-2}$) that are used in low-temperature fuel cells. Figure 2 shows that at low hydrogen concentrations (up to 30%) hydrogenase and Pt-based electrodes are similarly active. Only when the solution is saturated with $H_2$ does the activity of Pt fuel electrodes become slightly higher than that exhibited by the enzyme electrode. Even in sulfuric acid, the activity of the Pt-based fuel electrode is comparable to the hydrogenase electrode operated in a pH-neutral medium (Figure 2).

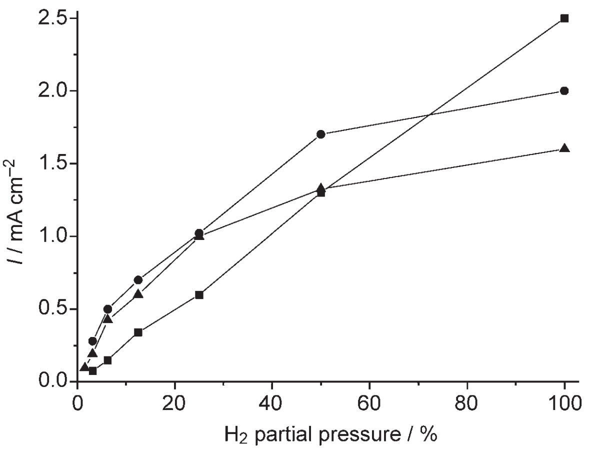

***Figure 2.*** Current of $H_2$ oxidation at 200 mV overvoltage as a function of its partial pressure in $H_2$–Ar: Pt-vulcan, pH 7.0 (●); Pt-vulcan, 0.05 $H_2SO_4$ (■); *D. baculatum* hydrogenase electrode, pH 7.0 (▲).

In summary, we have reported the limiting performance characteristics of hydrogenases in electrocatalysis. The activities of the most active hydrogenase electrodes in hydrogen oxidation from aqueous solution are quite similar to those of platinum-based fuel electrodes. It is thus expected that by hydrogenase entrapment into a conductive matrix (according to the technology presently used for Pt-based electrodes), it will be possible to elaborate highly active hydrogen enzyme electrodes with sufficient current characteristics for polyelectrolyte membrane based fuel cells.

## Experimental Section

Hydrogenases were isolated according to known procedures.[14] Carbon filament material LSG (with electrical resistivity of 50–70 m$\Omega$ cm and surface roughness of 10–30) was used as the electrode support. *N*-methyl-*N*′-(12-pyrrol-1-yldodecyl)-4,4′-bipyridine (MPDB), synthesized as in reference [15], was electropolymerized from aqueous solution to a surface coverage of $1.4\times10^{-7}$ mol cm$^{-2}$. Enzyme electrodes were prepared by overnight sorption of hydrogenase on LSG modified with poly(MPDB). After immobilization, the hydrogenase electrodes were thoroughly washed with aqueous buffer and tested in a solution that contained neither enzyme nor redox mediator.

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