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A Redox Hydrogel Protects the O_2-Sensitive [FeFe]-Hydrogenase from *Chlamydomonas reinhardtii* from Oxidative Damage**

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Abstract: *The integration of sensitive catalysts in redox matrices opens up the possibility for their protection from deactivating molecules such as O_2. [FeFe]-hydrogenases are enzymes catalyzing H_2 oxidation/production which are irreversibly deactivated by O_2. Therefore, their use under aerobic conditions has never been achieved. Integration of such hydrogenases in viologen-modified hydrogel films allows the enzyme to maintain catalytic current for H_2 oxidation in the presence of O_2, demonstrating a protection mechanism independent of reactivation processes. Within the hydrogel, electrons from the hydrogenase-catalyzed H_2 oxidation are shuttled to the hydrogel–solution interface for O_2 reduction. Hence, the harmful O_2 molecules do not reach the hydrogenase. We illustrate the potential applications of this protection concept with a biofuel cell under H_2/O_2 mixed feed.*

The prospect of replacing noble metals with earth-abundant elements such as Co, Ni and Fe in catalysts for H_2 oxidation[1] and H_2 evolution[2] opens up the perspective for cost-efficient fuel-cell anodes and electrolyzer cathodes. However, the most active Fe/Ni-based catalysts for H_2 oxidation and H_2 evolution typically suffer from deactivation even by traces of O_2,[3] which implies that technological applications would require high-purity H_2 feed and strict anaerobic conditions.

Hydrogenases are among the most active catalysts for the H_2/H^+ interconversion.[4] The search for O_2-tolerant hydrogenases and the elucidation of the deactivation/protection mechanism[5] are among the most promising research strategies for the design of robust enzymes[3b,6] and biomimetic synthetic catalysts.[3a] However, the catalytic performance, both in terms of turnover frequency and overpotential, typically becomes worse with increasing O_2 tolerance.[4b,7]

We recently proposed an alternative strategy for the technological application of highly active catalysts without changing their intrinsic O_2 sensitivity.[8] We integrated a [NiFe]-hydrogenase as a model H_2-oxidizing catalyst in viologen-based redox hydrogels which resulted in high catalytic current densities for H_2 oxidation at low overpotential even in the presence of O_2 (5% partial pressure in the H_2 feed). The proposed protection mechanism is based on the hydrogenase-catalyzed H_2 oxidation reaction within the film that provides electrons for the viologen-catalyzed O_2 reduction reaction at the outer layers of the film near the hydrogel–electrolyte interface (Model A, Figure 1A). Hence, the inhibitor O_2 is quenched before it reaches the layer of active hydrogenase.

Other concepts based on thin viologen films,[11] quinone-containing membranes,[12] and porous electrodes[1e] were used to enhance the O_2 tolerance of various O_2-sensitive [NiFe]-hydrogenases. However, the protection from O_2 was attributed to reactivation of the hydrogenase by the reductive force of the viologen radical cation (Model B, Figure 1B) or to restricted diffusion of O_2 into the porous electrode matrix.[1e] The former mechanism may also contribute to the protection of the reversibly inhibited [NiFe]-hydrogenase integrated in the viologen-based redox hydrogel film. However, a mechanism that requires reactivation would imply that only reversibly inhibited catalysts can be protected and a mechanism based on restricted O_2 diffusion would delay but not prevent inactivation as demonstrated for porous graphite materials.[1e] Hence, based on these protection mechanisms, large families of irreversibly inhibited catalysts, such as [FeFe]-hydrogenases[13] and most synthetic catalysts,[14]

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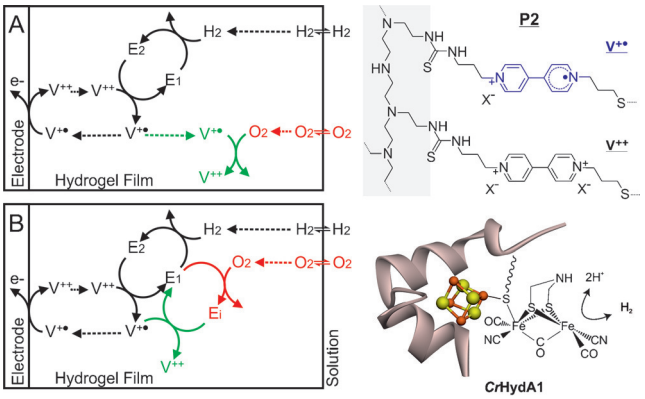

Figure 1. Left: Proposed models for the O_2 protection mechanism in redox hydrogel films. A) O_2 is reduced in a thin layer close to the hydrogel–solution interface preventing O_2-induced deactivation of the hydrogenase in the inner layers of the film.[8] B) O_2 deactivates the enzymes within the film and the reductive force of the redox hydrogel reactivates the enzyme, whereby the relative rates of the reaction (solid arrows) and diffusion (dashed arrows) processes define the overall catalytic performance.[9] Inhibitor and deactivation pathways are represented in red, reactivation/protection processes in green. Top right: Schematic representation of the viologen-modified polymer (P2) with the reduced viologen moiety in blue. Bottom right: Schematic representation of the *Cr*HydA1 active site (PDB entry 3LX4).[10]

would still be excluded from potential technological applications.

Simulations based on kinetic and mass transport parameters, which were used for the analysis of the chemical reactions and the associated time-dependent concentration profiles within redox hydrogel films containing a [NiFe]-hydrogenase, suggest that the reactivation step is not required for catalyst protection.[9] Here, we apply the hydrogel protection concept to the extremely O_2-sensitive [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* (*Cr*HydA1). Unlike [NiFe]-hydrogenases, the active site of [FeFe]-hydrogenases is irreversibly destroyed by O_2.[5b,15] If the model based on O_2 reduction which we proposed in our previous work (Figure 1 A) is correct, the redox hydrogel should also protect the [FeFe]-hydrogenase *Cr*HydA1.

The redox polymer (P1) previously used for the [NiFe]-hydrogenase was designed to enable 1) the formation of stable hydrogel films, 2) the generation of high catalytic current densities for H_2 oxidation, and 3) the protection from oxidative damage.[8] The viologen moieties were selected as the functionalities responsible for electron transfer and protection processes in the polymer while a polyethylenimine backbone was chosen to facilitate electrostatic interactions with the partially negatively charged [NiFe]-hydrogenase for hydrogel formation. For the integration of the [FeFe]-hydrogenase, the polymer structure was adjusted to account for the specific properties of the protein such as charge distribution and size (see the Supporting Information). Nevertheless, the substitution at the viologen moieties is kept unaltered to ensure that the redox-buffering and oxygen-reducing properties of the resulting polymer (P2, Figure 1) are equivalent to the one previously used for the [NiFe]-hydrogenase.

P2 viologen moieties have a redox potential of $E_{V^{++}/V^{+\bullet}} = -260$ mV (vs. standard hydrogen electrode (SHE)), almost identical to that of P1[8] (Figure 2 A, blue trace). Upon addition of H_2, a film of P2 on a glassy carbon electrode does not result in any catalytic current (Figure 2 A and B, blue traces). When P2 is immobilized on the electrode together with *Cr*HydA1, in the presence of H_2, the resulting redox hydrogel displays a catalytic oxidation current with an onset potential corresponding to that of the viologen groups from the hydrogel (Figure 2 A), proving that the viologen moieties are responsible for the electron transfer from the enzyme to the electrode. Catalytic currents were stable (Figure S2), with current densities of $(444 \pm 55)\ \mu A\,cm^{-2}$ (five individually prepared electrodes). Although the redox hydrogel film is deposited on flat electrodes, the measured current densities are similar to those obtained for nanostructured electrodes modified with viologen polymer and a [NiFe]-hydrogenase.[16] The significant driving force imposed by the redox potential

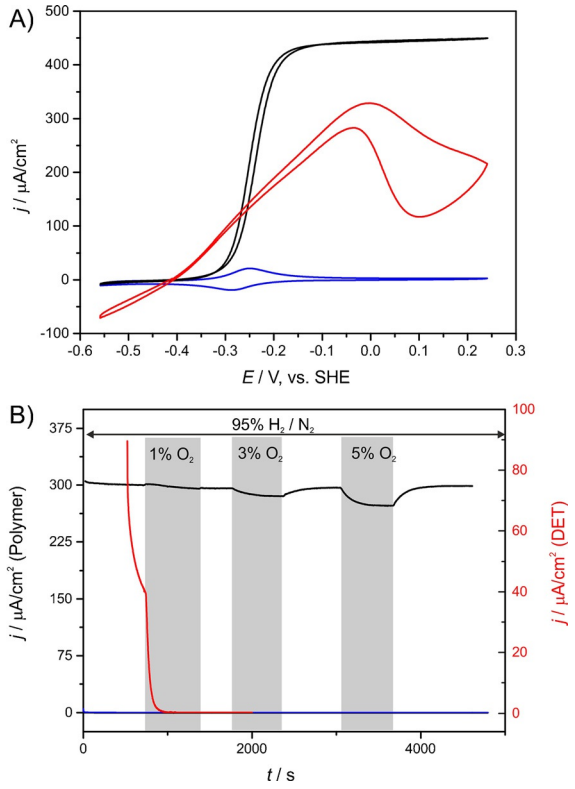

Figure 2. A) Comparison of cyclic voltammograms (CV) of a P2 film without a hydrogenase electrode (blue), with a *Cr*HydA1 [FeFe]-hydrogenase/polymer electrode (black) and with a covalently modified pyrolytic graphite electrode in which the hydrogenase is in direct electron transfer (DET) configuration (red). Experimental conditions: electrode rotation rate 2000 rpm, pH 7.0, 25 °C, 20 $mV\,s^{-1}$ and 1 bar of H_2 (black and red traces) and 20 $mV\,s^{-1}$ and 1 bar of H_2 (blue trace) B) Chronoamperometry (CA) for evaluation of oxygen tolerance. Black trace: glassy carbon electrode (GCE) drop-coated with *Cr*HydA1 [FeFe]-hydrogenase/polymer P2, blue trace: GCE drop-coated with the polymer P2 only, red trace: pyrolytic graphite electrode with the same enzyme in DET regime. The catalytic current is decreasing prior to O_2 exposure due to fast potential inactivation at the applied potential. During O_2 exposure the concentration of H_2 was kept constant at 95 % and N_2 was used as an inert gas to complete the mixture. CA recorded at +141 mV vs. SHE, pH 7.0, 25 °C, 2000 rpm and gas flow of 2000 sccm.

of the viologen moiety favors fast electron transfer from the hydrogenase to the viologen while the reverse process is impeded. Therefore, no significant catalytic current is observed for H_2 evolution with the [FeFe]-hydrogenase embedded in P2 (Figure 2 A).

It has been described that [FeFe]-hydrogenases inactivate both reversibly and irreversibly at high potentials in the presence of H_2.[17] This process is clearly visible as a decay of the catalytic H_2 oxidation current at potentials above −100 mV vs. SHE when the enzyme is adsorbed on a highly oriented pyrolytic graphite electrode under direct electron transfer conditions (Figure 2 A and B, red traces). All hydrogenases suffer from high potential deactivation regardless of whether flat or highly porous electrodes[1e,18] are used, which limits their applications. This is not observed in the CV of the electrode with the hydrogenase in the viologen-modified hydrogel. Under mediated ET conditions the enzyme only experiences the formal potential of the viologen redox couple independently of the applied potential at the electrode. Since $E_{V^{++/V+\cdot}}$ is lower than the inactivation potential of the hydrogenase, the oxidized viologen moieties in the redox polymer cannot induce hydrogenase inactivation, showing the protection towards high potential inactivation as found for P1 with the [NiFe]-hydrogenase.[8]

The major challenge was to protect the enzyme towards O_2 inactivation. When the [FeFe]-hydrogenase is adsorbed onto the electrode under direct electron transfer conditions (Figure 2 B, red trace), the catalytic current quickly vanishes and does not recover upon switching back to H_2 100 %. When the enzyme is embedded in the redox hydrogel matrix, it can sustain H_2 oxidation activity even in the presence of 5 % O_2 in the H_2 gas feed for up to 5 min (Figure 2 B, black). In the presence of the H_2/O_2 mixed atmosphere, the cathodic current for O_2 reduction expected at potential negative relative to $E_{V^{++/V+\cdot}}$ is mostly absent, demonstrating that H_2 serves as the reducing agent (Figure S4). When the O_2/H_2 mixed atmosphere is changed back to 100 % H_2, the catalytic current is fully recovered. These results are strong evidence supporting model A (Figure 1) in which protection from O_2 inside the polymer film relies on the reduction of O_2 at the outer layers. In the absence of O_2, the hydrogenase oxidizes H_2, transfering the electrons to a nearby oxidized viologen moiety. Then electron transfer takes place through the viologens to the electrode surface, where the viologen is oxidized back. When O_2 is added to the gas mixture, it is consumed on the surface of the hydrogel film by the reduced viologen moieties, leaving the interior of the hydrogel film anaerobic. The catalytic current drop observed during the chronoamperometric experiment in the presence of O_2 results from the diversion of electrons produced from the hydrogenase-catalyzed H_2 oxidation to the hydrogel/electrolyte interface where these electrons are sacrificed to reduce O_2. The recovery of the catalytic current upon O_2 removal from the gas feed provides clear evidence for the protection mechanism. In the case of the [NiFe]-hydrogenase, it could be speculated that this current drop and recovery is related to the deactivation and reactivation of the enzyme (model B, Figure 1).[11] This is not the case for the [FeFe]-hydrogenase where inactivation is completely irreversible even in the presence of small amounts of O_2; in this case no recovery should be observed after returning to pure H_2, as illustrated by the measurement in the direct electron transfer regime. Therefore, the protection mechanism relies on the electron diversion to the surface of the hydrogel film to reduce O_2 (model A), and it is not a result of enzyme reactivation (model B).

Alternative concepts based on porous carbon materials that restrict O_2 diffusion were previously proposed to protect [NiFe]-hydrogenases.[1e] However, such a mechanism is not expected to contribute to the protection achieved with the redox hydrogel since the diffusion of small molecules in this highly solvated polymer matrix is not significantly impeded.[19] Moreover, the application of porous carbon materials for the protection of [FeFe]-hydrogenases still remains to be demonstrated.

Despite being the most active hydrogenases,[20] [FeFe]-hydrogenases have never been used in fuel cells because of their extreme O_2 sensitivity. Since our experiments demonstrate that the hydrogenase from *Cr*HydA1 can sustain H_2 oxidation in the presence of O_2 and it is not inactivated when exposed to high potentials, we tested our electrode in a single-compartment biofuel cell operating under anode-limiting conditions. Here, the enzyme is exposed to the harshest conditions possible in a fuel cell, namely the presence of O_2 and high potentials. These conditions lead to poor performances of fuel cells in which the hydrogenase is in a direct electron transfer configuration, even when O_2-tolerant hydrogenases are employed.[18,21] To ensure anode-limiting conditions we used an oversized O_2-reducing cathode. The cathode was based on bilirubin oxidase (BOD) covalently immobilized on a carbon cloth surface.[22] Individual measurements of the two half-cells under a 95 % H_2/5 % O_2 feed demonstrate that the fuel cell is limited by the current on the hydrogenase-viologen-hydrogel electrode (Figure S3). The fuel cell was tested by increasing the load between cathode and anode while recording the current (Figure 3). It displayed an open-circuit voltage of 1080 mV, a short-circuit current of 280 $\mu A\,cm^{-2}$, and a maximum power density of 225 $\mu W\,cm^{-2}$ (based on the anode area).

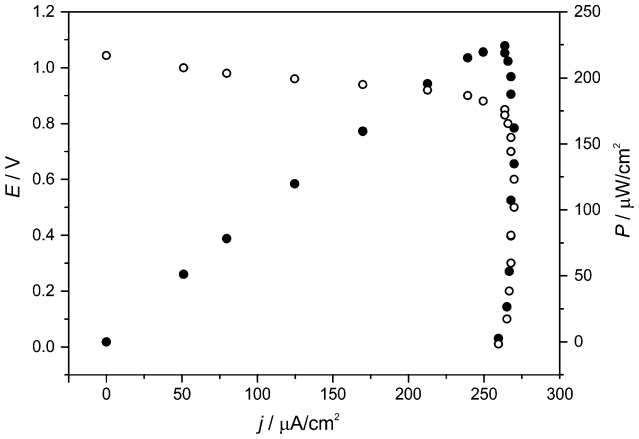

Figure 3. Cell voltage (*E*, open circles) and power density (*P*, solid circles) vs current density at a single-compartment fuel cell under H_2/O_2 mixed feed with the redox hydrogel/*Cr*HydA1 [FeFe]-hydrogenase anode and a BOD cathode.

Much effort has been expended to understand the O_2 inactivation mechanism of this class of hydrogenases.[5b,15,23] The main strategies to improve their O_2 tolerance to date relied either on the design of specific mutant proteins,[24] or on the generation and screening of large random mutagenesis libraries,[25] both up to now with rather limited success. These results show how our chemical approach of using a viologen-modified hydrogel effectively protects an extremely O_2-sensitive hydrogenase to such an extent that its utilization in a fuel cell with a mixed H_2/O_2 gas feed becomes possible. The fuel cell performance was comparable to that of earlier reported non-nanostructured fuel cells based on O_2-tolerant hydrogenases.[26] Moreover, these results with a hydrogenase that is irreversibly inactivated by O_2 confirm our model for the O_2 protection of catalysts inside the viologen hydrogel. The enzyme remains active at O_2 levels as high as 5% in H_2 which is far above the level of O_2 impurities present in the gas feeds typically used in technological applications. This opens up new possibilities to exploit extremely active but highly O_2-sensitive and irreversibly deactivated catalysts in fuel cells and in photocatalytic H_2-evolving systems.[27] Future development of the redox hydrogel will focus on increasing the kinetics for O_2 reduction to water[8,28] to prevent the partially reduced reaction intermediates such as superoxide and hydrogen peroxide from degrading the viologen[29] and possibly the enzyme upon extended exposure.

Keywords: biocatalysis · biofuel cells · hydrogenases · O_2 protection · viologen hydrogel

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