Redox-Polymer-Wired [NiFeSe] Hydrogenase Variants with Enhanced O₂ Stability for Triple-Protected High-Current-Density H₂-Oxidation Bioanodes

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Dedicated to Prof. Bernd Speiser on the occasion of his retirement.

Introduction

Bioelectrocatalysis has gained huge importance in the past decades and is considered a promising research field for the development of novel sustainable energy conversion and storage systems[1] as well as for the green production of value-added chemicals and (solar) fuels.[2] In particular, the use of biocatalysts to produce sustainable H₂/O₂-powered biofuel cells with high power output has become a major research area in this context. Although the biocathodes used for such devices are mainly based on rather robust, stable, and easy-to-wire multi-copper oxidases such as bilirubin oxidase or laccase,[3] the biocatalyst at the bioanode, that is, highly active H₂-oxidizing Ni/Fe-based hydrogenases, typically suffers from pronounced O₂ sensitivity and fast inactivation at high potentials especially under anode-limiting conditions.[4] Hence, the preparation of hydrogenase-based bioanodes requires the implementation of specific protection systems, for example, based on O₂-reducing low-potential redox polymers, or the use of O₂-tolerant but usually less active hydrogenases, such as, for instance, the hydrogenases from Escherichia coli,[5] Ralstonia eutropha,[6] or Aquifex aeolicus.[7] The introduction of O₂-reducing redox polymers for electrical wiring and protection of air-sensitive hydrogenases was successfully demonstrated for various hydrogenases including [NiFe] hydrogenase from D. vulgaris Miyazaki F,[8] [FeFe] hydrogenase from Chromydomonas reinhardtii,[9] and [NiFeSe] hydrogenase from D. vulgaris Hildenborough.[10] Moreover, effective protection was even observed for thin films[11] and polymer/enzyme-modified gas-diffusion electrodes (GDEs), which could be incorporated in membrane-free biofuel cells that exhibited benchmark power densities for polymer-based systems.[12] In addition, the low-potential redox polymer...
not only acts as an O₂-quenching matrix but also as a Nernst buffer system for the biocatalyst, preventing inactivation at high potentials. Hence, low-potential redox polymers provide a double protection system for such sensitive materials, which was even transposable to synthetic catalysts. Moreover, hydrogenases deactivated under aerobic conditions may be reactivated by the low-potential polymer matrix, as was shown previously for [NiFe] and [NiFeSe] hydrogenases.

Although O₂-tolerant hydrogenases derived from the above-mentioned microorganisms can be operated in the presence of distinct levels of O₂ inactivation at high potentials still remains an issue. Furthermore, the overpotential for H₂-oxidation is often at more positive values than those of O₂-sensitive [NiFe] and [FeFe] hydrogenases. Evidently, this will limit the power output of a related biofuel cell because this value will directly affect the maximum open-circuit voltage (OCV). Hence, a combination of redox polymers (double protection shield) and hydrogenase variants with enhanced O₂ tolerance would allow the development of triple-protected bioanodes. We want to emphasize that although the redox polymer requires a slightly more positive redox potential than the hydrogenase itself to ensure successful electron exchange at oxidative conditions, the open-circuit potential of the corresponding bioanode and, consequently, the OCV of the cell will not be limited by the mid-point potential of the redox polymer owing to the pseudo-capacitive properties of the polymer matrix. This effect was shown for glucose oxidase/bilirubin oxidase as well as hydrogenase/bilirubin oxidase-based biofuel cells in which the anodic catalyst (glucose oxidase, hydrogenase) was electrically wired by means of a redox polymer.

The properties of enzymes can be modulated by enzyme engineering. For instance, glucose oxidase that uses O₂ as a natural electron acceptor could be turned into an O₂-insensitive enzyme by site-directed mutagenesis and cofactor redesign. Artificial maturation of [FeFe] hydrogenase allows for the fine tuning of the properties of the active center of the enzyme. The synthetic nature of the active site allows for the incorporation of specific ligands or the alteration of the overall ligand sphere to adjust the properties of the whole enzyme. Following this approach, the stability of [FeFe] hydrogenases could be enhanced, and the overall activity of the enzyme can be controlled. Although the maturation process occurs spontaneously without any helper proteins or additional cofactors, the preparation of the active site of a [FeFe] hydrogenase requires distinct synthetic efforts and the maturation process is an additional step in the preparation of the active enzyme. In contrast, once developed, variants of an enzyme showing altered properties are produced directly from the living organisms. The additional maturation step and complex synthesis is thus not required.

Recently, it was shown that variants (G491A and G491S) of the [NiFeSe] hydrogenase from D. vulgaris Hildenborough with modification in a specific amino acid close to the active site led to enhanced stability of the biocatalyst in the presence of O₂ while retaining a high activity for H₂-oxidation. For [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27). In the wild-type [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27). In the wild-type [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27). In the wild-type [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27). In the wild-type [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27). In the wild-type [NiFeSe] hydrogenase, oxygen reaches the active center of the enzyme (1), whereas in the variants G491A and G491S, the pathway to the active center is partially blocked by altered amino acid residues, which hamper the access of O₂ (2). For a detailed structural and mechanistic description, see Ref. (27).
was evidenced by protein film electrochemistry conducted in a direct electron transfer (DET) regime and in the presence of O₂.[27] However, the DET mode does not provide any protection against high-potential inactivation or against high O₂ concentrations and is hence impractical for potential applications. Nevertheless, it shows that variants of this type of hydrogenase can be prepared with enhanced O₂ stability.

In this contribution, we combined the advantages of the enhanced O₂-stable variants of [NiFeSe] hydrogenase from *D. vulgaris* Hildenborough and the O₂ quenching and Nernst buffer properties of low-potential viologen-modified redox polymers to fabricate high-current-density bioanodes, which could be successfully incorporated into H₂/O₂-powered biofuel cells exhibiting benchmark power densities at moderate catalyst loadings.

Results and Discussion

Electrical wiring

The redox polymer P(N₃MA-BA-GMA)-vio (poly(3-azido-propyl methacrylate-co-butyl acrylate-co-glycidyl methacrylate)-vio, with vio = 1-(5-hexyn-1-yl)-1’-methyl-4,4’-bipyridinium; see Figure S1 in the Supporting Information) was used previously for productive electrical wiring and protection of wild-type [NiFeSe] hydrogenase from *D. vulgaris* Hildenborough (wt-[NiFeSe]).[10] Benchmark H₂-oxidation currents of approximately 1.7 mA cm⁻² at optimized conditions were found for P(N₃MA-BA-GMA)-vio/wt-[NiFeSe]-modified glassy carbon electrodes.[10] Stimulated by our previous findings, we exploited the possibility to electrically wire two O₂-tolerant [NiFeSe] variants, namely G491A and G491S, to the same electrode material.

Indeed, cyclic voltammograms of drop-cast P(N₃MA-BA-GMA)-vio/G419A and P(N₃MA-BA-GMA)-vio/G419S films measured under turnover conditions, that is, under H₂ atmosphere (Figure 2a, b, red curves), showed pronounced catalytic H₂-oxidation waves with half-wave potentials (a and b: approximately −0.32 V vs. SHE), which matches closely the mid-point potential of the polymer-bound viologen units (≏0.34 V vs. SHE, black curves). The behavior is in line with the results measured for the wild type (Figure S2 in the Supporting Information). We hence conclude that the [NiFeSe] variants can also be productively wired through the redox polymer P(N₃MA-BA-GMA)-vio in a mediated electron-transfer regime. Moreover, long-term chronoamperometric measurements over 7 h under continuous turnover conditions showed similar operational stability for the wild type and the two variants (Figure S3 in the Supporting Information).

The maximum current densities *J*ₘₐₓ measured for the individual, freshly prepared electrodes were calculated as average values from three electrodes as (450 ± 84) μA·cm⁻² for G491A and (476 ± 172) μA·cm⁻² for G491S. The wild type shows a *J*ₘₐₓ value of (752 ± 259) μA·cm⁻² (note that for the wild type and the variants, storage of the modified electrodes leads to a decrease in overall electrode activity). The results are in line with the measured activities of the used enzyme batches [G491A: (1918 ± 119) s⁻¹ and G491S: (2416 ± 387) s⁻¹; note that the standard deviations are overlapping], which are below the activity of the wild-type enzyme (4850 ± 260 s⁻¹)[27] and below the maximum values reported previously for the variants (see values above).[27]

The steady-state current observed at high potentials (> −0.2 V vs. SHE, Figure 2, red lines) under turnover conditions indicates that the variants can also be effectively protected against high-potential inactivation in contrast to the operation under DET conditions for this type of hydrogenases.[29, 30]

**Figure 2.** Cyclic voltammograms recorded at a scan rate of 10 mV s⁻¹ of (a) P(N₃MA-BA-GMA)-vio/G491A and (b) P(N₃MA-BA-GMA)-vio/G491S films immobilized on glassy carbon disk electrodes (3 mm) in the absence (black lines, 100 % Ar, purged through solution) and presence of H₂ (red lines, 100 % H₂, purged through solution). Working electrolyte: phosphate buffer, 0.1 M, pH 7.3, room temperature.

Oxygen tolerance

Because the low-potential redox polymer acts as an O₂-quenching matrix and thus protects the enzyme from O₂ damage,[10] the stability of the variants and the wild type against oxygen in the immobilized state was measured in the absence of H₂ in chronoamperometric experiments. Under these conditions, electrons from H₂-oxidation are absent and cannot be used by the polymer matrix to reduce incoming O₂.[8, 31]

Figure 3 shows chronoamperometric experiments at an applied potential (*E*ₚₛₛ) of +160 mV (vs. SHE) under alternating gas-mixture atmospheres. The O₂ content in the gas feed was stepwise increased after each H₂ cycle. To ensure that all H₂ had been removed before the O₂ was added to the gas feed, the cell was purged with argon. After the background current
was reached, the film was exposed to an O\textsubscript{2}/Ar mixture with varying O\textsubscript{2} content (5, 10, and 15\%, gray shaded areas in Figure 3). The wild type shows a steady decrease of the H\textsubscript{2}-oxidation activity over all O\textsubscript{2}/Ar cycles. After exposure to 15\% O\textsubscript{2}, the electrode remains inactive when switching the gas feed back to H\textsubscript{2}. This is consistent with a fast in-diffusion of O\textsubscript{2} to the active center of the enzyme (Figure 1a). In contrast, both variants show a rather constant current output after the 5 and 10\% O\textsubscript{2} cycle. Moreover, even after exposure to 15\% O\textsubscript{2}, both electrodes still show a remarkable activity towards H\textsubscript{2}-oxidation (G491A: \(\approx 20\%\) of the initial H\textsubscript{2}-oxidation current; G491S: \(\approx 35\%\)). The results demonstrate that the variants indeed exhibit an increased O\textsubscript{2} tolerance compared with the wild-type enzyme owing to a partial blocking of molecular oxygen (hampered access) based on the altered amino acid residues in the variants (Figure 1a) and, by this, that the variants provide an additional protection for the proposed H\textsubscript{2}-oxidation bioanodes. The electrochemical results obtained with the polymer/enzyme films are in line with the results reported for operating the same hydrogenases in the DET regime.\(^{[27]}\) However, strong variations in the residual currents were observed after exposure to O\textsubscript{2}, which is attributed to variations in film thickness and inhomogeneities of the catalytic layers, leading to different diffusion profiles of O\textsubscript{2}. However, in all experiments the variants showed a higher stability towards O\textsubscript{2}.

**Reactivation**

For the wild-type [NiFeSe] hydrogenase, which was deactivated under aerobic conditions, reactivation is known to occur quickly at rather negative potentials.\(^{[29,30]}\) Moreover, the reduced low-potential polymer matrix P(N\textsubscript{3}MA-BA-GMA)-vio is also able to reactivate the inactive [NiFeSe] hydrogenase.\(^{[10]}\)

To evaluate a possible reactivation behavior of the two [NiFeSe] hydrogenase variants, glassy carbon electrodes modified with P(N\textsubscript{3}MA-BA-GMA)-vio/G491A and P(N\textsubscript{3}MA-BA-GMA)-vio/G491S films were exposed to O\textsubscript{2} until complete inactivation occurred, as evidenced by the current dropping back to background values. Application of a negative potential of \(-440\;\text{mV}\) (vs. SHE; polymer is fully reduced, inactive mediator form) for 500 s leads to reactivation of the enzyme (Figure 4) as indicated by the oxidative currents, which were observed again when the potential was stepped back to \(+160\;\text{mV}\) (vs. SHE; mediator is oxidized, active form). Both potentials were applied under a 90\% H\textsubscript{2}/10\% Ar gas feed. The wild type shows the same behavior (see Figure S4 in the Supporting Information and Ref. [10]).

For practical applications, this effect is highly desirable because a potentially necessary exchange of a deactivated electrode in a device can be prevented. Instead, a short cathodic potential pulse might reactivate the electrode, and operation can be resumed.

**Membrane-free H\textsubscript{2}/O\textsubscript{2} biofuel cells**

Oxygen-tolerant hydrogenases typically display higher redox potentials, which will decrease the maximum OCV of a corresponding biofuel cell compared with their O\textsubscript{2}-sensitive analogues. However, because the O\textsubscript{2}-tolerant variants G491A and G491S show similar H\textsubscript{2}-oxidation potentials as the wild type,\(^{[27]}\) the electrical wiring is possible with the same polymer (Figure 2). Hence, we expect similar OCV values for related biofuel cells as for those based on the wild-type hydrogenase. To evaluate the performance of the bioanodes in a biofuel cell, polymer/hydrogenase-modified glassy carbon electrodes were combined with a gas-diffusion O\textsubscript{2}-reducing bilirubin oxidase-based biocathode. The use of a gas-diffusion system at the
**Bacillus pumilus**

Hydrogenases were achieved. To demonstrate the relevance of the O$_2$-tolerant [NiFe-Se] variants, carbon cloth-based gas-diffusion layers were first modified with P(GMA-BA-PEGMA)-vio [poly(glycidyl methacrylate-co-butyl acrylate-co-poly(ethylene glycol)methacrylate)]-vio; for the structure and synthesis of this polymer, see Figure S1 in the Supporting Information and Ref. [12], respectively. Films followed by the immobilization of an active P(N$_2$,MA-BA-GMA)-vio/G491S layer (Figure 1b; for a detailed description of the preparation process see the Experimental Section; electrodes are denoted as P(GMA-BA-PENMA)-vio//P(N$_2$,MA-BA-GMA)-vio/G491S; owing to the limited amount of enzyme, only the variant G491S was used for the preparation of a H$_2$-oxidation gas-diffusion layer).

The less hydrophilic viologen-modified polymer P(GMA-BA-PENMA)-vio acts as an adhesion layer between the hydrophilic active layer and the hydrophobic carbon cloth surface. More-over, the underlying redox polymer layer prevents contribution from DET between the enzyme and the porous electrode surface and excludes high-potential inactivation.

Under gas-diffusion conditions, the bioanode showed absolute H$_2$-oxidation currents of approximately 0.8 mA (Figure 5a). After switching the potential to +160 mV vs. SHE (t > 500 s), H$_2$-oxidation currents indicate successful reactivation. Working conditions: phosphate buffer, 0.1 M, pH 7.3, room temperature, electrodes were deactivated by extensive exposure to O$_2$ until any H$_2$-oxidation current was absent.

**Figure 4.** Chronoamperometric experiments with aerobically deactivated (a) P(N$_2$,MA-BA-GMA)-vio/G491A and (b) P(N$_2$,MA-BA-GMA)-vio/G491S films immobilized on glassy carbon disk electrodes (3 mm $^2$). First, a potential of -440 mV vs. SHE was applied for 500 s to fully reduce the viologen-modified polymer (the enzyme is reactivated during reduction via the polymer). After switching the potential to +160 mV vs. SHE (t > 500 s), H$_2$-oxidation currents indicate successful reactivation. Working conditions: phosphate buffer, 0.1 M, pH 7.3, room temperature, electrodes were deactivated by extensive exposure to O$_2$ until any H$_2$-oxidation current was absent.

Membrane-free biofuel cells prepared with the gas-diffusion $Bp$-BOD-based biocathode in combination with P(N$_2$,MA-BA-GMA)-vio//wt-[NiFeSe] (Figure S6a in the Supporting Information), P(N$_2$,MA-BA-GMA)-vio/G491A (b), and P(N$_2$,MA-BA-GMA)-vio/G491S (c) bioanodes showed OCV values of 1.06, 1.05, and 1.06 V, respectively. As expected, all biofuel cell assemblies show similar or even identical OCV values (note that all electrodes show very similar enzyme and polymer loadings), indicating again the benefits of the two variants compared with typically used O$_2$-tolerant hydrogenases with higher redox potentials. The maximum power density with respect to the electrode surface area of the bioanode was observed at 0.8 V for all fuel cells and was estimated to be 340 $\mu$W cm$^{-2}$ (wt-[NiFeSe]), 325 $\mu$W cm$^{-2}$ (G491A), and 271 $\mu$W cm$^{-2}$ (G491S). The values are similar to other polymer-based biofuel cells using [NiFe]$^{[8]}$ and [FeFe]$^{[9]}$ hydrogenases.

Recently, we showed that the use of gas-diffusion layers modified with polymer/wt-[NiFeSe] and polymer/[NiFe] films displayed enhanced power output owing to an enhanced mass transport of the gaseous substrate H$_2$ towards the bioanode.$^{[12]}$ Current densities for the bioanode of close to 8 mA cm$^{-2}$ and power densities of 3.8 mW cm$^{-2}$ for biofuel cells with a bilirubin oxidase-modified gas-diffusion biocathode were observed.$^{[12]}$ To demonstrate the relevance of the O$_2$-tolerant [NiFeSe] variants, carbon cloth-based gas-diffusion layers were first modified with P(GMA-BA-PENMA)-vio [poly(glycidyl methacrylate-co-butyl acrylate-co-poly(ethylene glycol)methacrylate)]-vio; for the structure and synthesis of this polymer, see Figure S1 in the Supporting Information and Ref. [12], respectively. Films followed by the immobilization of an active P(N$_2$,MA-BA-GMA)-vio/G491S layer (Figure 1b; for a detailed description of the preparation process see the Experimental Section; electrodes are denoted as P(GMA-BA-PENMA)-vio//P(N$_2$,MA-BA-GMA)-vio/G491S; owing to the limited amount of enzyme, only the variant G491S was used for the preparation of a H$_2$-oxidation gas-diffusion layer).

The less hydrophilic viologen-modified polymer P(GMA-BA-PENMA)-vio acts as an adhesion layer between the hydrophilic active layer and the hydrophobic carbon cloth surface.$^{[12]}$ Moreover, the underlying redox polymer layer prevents contribution from DET between the enzyme and the porous electrode surface and excludes high-potential inactivation.$^{[12]}$

Under gas-diffusion conditions, the bioanode showed absolute H$_2$-oxidation currents of approximately 0.8 mA (Figure 5a). The modified surface area of the carbon cloth-based bioanode has a diameter of approximately 4 mm, which results in a surface area of the active layer of approximately 0.126 cm$^2$, and thus maximum current densities of 6.3 mA cm$^{-2}$ were achieved. The values are similar to previously reported polymer-based gas-diffusion systems equipped with wt-[NiFeSe] and [NiFe] hydrogenases (Table S1 in the Supporting Information). However, care must be taken when comparing current densities measured with porous electrodes. Because of the 3D structure of the electrodes, the real surface is often unknown. Hence, the catalyst loading is a better value for comparison. For the
G4915-based electrodes, the catalyst loading is 8.4 nmol cm$^{-2}$/1.06 nmolelectrode$^{-1}$. Interestingly, for the wt-[NiFeSe] hydrogenase, current densities of only 5.3 mA cm$^{-2}$ were observed with a substantially higher catalyst loading of 27.0 nmol cm$^{-2}$/3.4 nmolelectrode$^{-1}$ as reported in our previous work (see Ref. [12] and Table S1 in the Supporting Information), which largely exceeds the values of the G4915 variant at almost identical overall polymer loading [wt-[NiFeSe]: 230 µg electrode$^{-1}$ (previous work, Ref. [12]); G4915: 260 µg electrode$^{-1}$]. At a lower catalyst loading of 12.1 nmol cm$^{-2}$/1.53 nmolelectrode$^{-1}$ (polymer loading 230 µg electrode$^{-1}$), which is only slightly higher than the loading of the G4915 enzyme, the wt-[NiFeSe] shows a $J_{\text{max}}$ value of only 3.6 mA cm$^{-2}$ (see Ref. [12]). This effect might be related to an improved incorporation of the G9415 variant in the polymer film when immobilized on the rather hydrophobic carbon cloth-based electrodes. A stronger interaction prevents leaching of the enzyme and thus ensures a higher local concentration of the biocatalyst during the experiment. In addition, a loss of activity in the immobilized state for the wild type may also contribute to a reduced electrode activity. An effect of different polymer-to-enzyme ratios can be ruled out because almost identical polymer loadings were used for all experiments. However, the effect seems to be specific for the porous, hydrophobic carbon cloth electrodes because the wild type shows a higher activity on flat glassy carbon electrodes (see Figure 2 and Figure S2 in the Supporting Information).

To evaluate the performance of the gas-diffusion P(GMA-BA-PEGMA)-vio/P(N,MA-BA-GMA)-vio/G4915 electrode in an all-gas-diffusion membrane-free $\text{H}_2/\text{O}_2$ biofuel cell, the bioanode was combined with an $\text{O}_2$-reducing biocathode modified with bilirubin oxidase from Myrothecium verrucaria (Mv-BOD), for comparison purposes because it was used in our previously reported experiments$^{[34]}$. For the immobilization of Mv-BOD, the carbon cloth was first modified with 2-ABA (2-amino benzoic acid) to ensure a proper orientation of the enzyme on the electrode surface. The modifier was anchored in an electrochemical grafting process by applying an oxidative potential pulse.$^{[38]}$

The Mv-BOD was then immobilized by means of a conventional drop-casting process and was operated in the DET regime.$^{[12, 33]}$ A high catalyst loading was used to ensure anode-limiting conditions (nominal enzyme loading: 1.2 mg electrode$^{-1}$). Absolute currents under gas-diffusion conditions ($100\% \text{O}_2$) reached approximately 2 mA (Figure S7 in the Supporting Information), which largely outperforms the bioanode ($\approx 0.8$ mA, Figure 5a). The fully assembled $\text{H}_2/\text{O}_2$ biofuel cell (Figure 5b) showed an OCV of 1.14 V, which is slightly higher than the values obtained on glassy carbon electrodes (1.05–1.06 V); this might be attributed to the slightly lower overpotential for $\text{O}_2$ reduction of Mv-BOD compared with $\text{Bp-BOD}^{[34]}$. The maximum power density was reached at 0.7 V and was estimated to be 4.4 mW cm$^{-2}$. This value even outperforms our previously reported value for the [NiFe]-based biofuel cell (3.6 mW cm$^{-2})^{[12]}$ and—to the best of our knowledge—sets a new benchmark for a biofuel cell using redox-polymer-based bioanodes (Table S1 in the Supporting Information). Moreover, the catalyst loading is significantly lower than the [NiFe] system (31.8 nmol cm$^{-2}$) reported previously (Table S1 in the Supporting Information).

Cyclic voltammograms (Figure S8 in the Supporting Information) measured before and after biofuel cell operation showed similar values for the bioanode, with the slightly higher currents after the biofuel cell test most likely as a result of changed diffusion properties inside the polymer/enzyme layer, for example, owing to swelling and/or slightly changed local pH values, which will affect the overall activity of the enzyme. In contrast, the current of the biocathode was slightly decreased after the biofuel evaluation (Figure S7 in the Supporting Information). This again highlights the high stability of the bioanode in a membrane-free biofuel cell under anode-limiting conditions. The operational stability of the biofuel cell was tested at a constant load of 0.7 V (Figure 5c). After 10 h of continuous operation, 75% of the initial current density remained. Cyclic voltammograms measured after the long-term experiment showed significantly lower currents for the bioanode (Figure S8 in the Supporting Information) and the biocathode.
(Figure S7 in the Supporting Information). We want to emphasize that the size of the polymer signals (Figure S7 in the Supporting Information, dashed black curve) were also decreased compared with the voltammograms measured with the freshly prepared electrode. Thus, not only does deactivation/decomposition of the enzyme contribute to the decreased activity after long-term operation, but the loss of immobilization matrix may also have an effect. Nevertheless, the bioanode shows an outstanding performance and demonstrates the potential applicability of G941S (enhanced O₂ tolerance) as a highly active and stable catalyst in a membrane-free biofuel cell device. Moreover, the proposed H₂-oxidation bioanodes combine the advantages of the protection matrix (O₂ quenching; no high-potential deactivation) and the enhanced enzyme stability of the hydrogenase variants (blocks of O₂ access) in accordance with the mechanism depicted in Figure 1b and thus demonstrate a triple-protection system for the high-current-density H₂-oxidation bioanodes.

Conclusions

The two [NiFeSe] variants show a higher O₂ tolerance than the wild type in the immobilized state, which is in line with results reported for the enzymes operated in a direct electron transfer regime. In combination with the redox polymer-based protection matrix, the proposed bioanodes reveal a triple-protection system that ensures stable operation. Moreover, we could demonstrate that the two [NiFeSe] variants show similar performance as the wild type and as [NiFe] hydrogenases when incorporated into a conventional redox-polymer-based biofuel cell. In addition, the use of gas-diffusion layers ensured high substrate transport towards the active polymer/enzyme layer, allowing H₂-oxidation currents of approximately 6.3 mA cm⁻² for the G491S variant at comparative-ly low catalyst loadings. Combination of the gas-diffusion bioanode with a gas-diffusion O₂-reducing biocathode allowed for the fabrication of a H₂/O₂-powered biofuel cell with benchmark performance in a membrane-free configuration. We conclude that the novel O₂-tolerant [NiFeSe] variants are promising candidates for biofuel cell applications, demonstrating that enzyme engineering is indeed a powerful tool, which may be used to not only overcome sensitivity issues but also to further enhance the activity and stability of biocatalysts.

Experimental Section

Chemicals and materials

All chemicals and materials were purchased from Sigma–Aldrich, Alfa-Aesar, VWR, Acros-Organsics, or Fisher Scientific and were used as received (reagent or analytical grade) except where otherwise noted. For the preparation of all aqueous solutions, deionized water from a Millipore water-purification system was used. The synthesis and characterization of the redox polymer poly(3-azidopropyl methacrylate-co-butyl acrylate-co-glycidyl methacrylate)-vio [P(IN,MA-BA-GMA)-vio, with vio = (1-(5-hexyn-1-yl)-1'-methyl-4,4'-bipyridinium)] was described previously in Ref. [10]. It was used as an aqueous solution with a concentration of 7.5 mg mL⁻¹. The synthesis and characterization of the less hydrophilic redox polymer poly(3-glycidyl methacrylate-co-butyl acrylate-co-poly(ethylene glycol) methacrylate)-vio [P(GMA-BA-PEGMA)-vio] was described previously in Ref. [12]. It was used as an aqueous solution with a concentration of 7.5 mg mL⁻¹.

Enzymes

The isolation and purification of the wild-type [NiFeSe] hydrogenase from D. vulgaris Hildenborough (wt-[NiFeSe]) was described previously in Ref. [28]. The activity for H₂ uptake was measured as (4850 ± 260) s⁻¹. The enzyme was stored in Tris-HCl buffer, 20 mm, pH 7.6 at −80 °C at a concentration of 170 μM. The preparation of the [NiFeSe] variants G491A and G491S is described in Ref. [27]. Their H₂ uptake was measured to be (191±11) s⁻¹ (G491A) and (2416±387) s⁻¹ (G491S). The [NiFeSe] variants were stored in Tris-HCl, 20 mm, pH 7.6 at −80 °C (G491A: 82.96 μM; G491S: 53.20 μM).

Bilirubin oxidase from Bacillus pumilus (Bp-BOD) was isolated and purified according to protocols reported in Ref. [32]. The protein was stored in 50 mM borate buffer, pH 9, at −80 °C; concentration = 54.75 mg mL⁻¹; activity = 713 Um⁻¹. Bilirubin oxidase from Myrothecium verrucaria (Mv-BOD, lyophilized powder, 15–65 Um⁻¹ protein) was obtained from Sigma–Aldrich and stored at −20 °C as a powder. For electrode modification, the enzyme was dissolved in phosphate buffer, 0.1 M, pH 7.3, at a concentration of 15 mg mL⁻¹.

Electrochemical experiments

All electrochemical experiments were conducted under the corresponding atmosphere (argon, hydrogen, oxygen, and their mixtures) and at room temperature by using a Gamry Reference 600 potentiostat in a three-electrode configuration with an Ag/AgCl/3 M KCl reference electrode. All potentials were rescaled to the standard hydrogen electrode (SHE) according to the equation

\[ E_{\text{SHE}} = E_{\text{Ag/AgCl/KCl}} + 210 \text{ mV} \]

Phosphatase buffer (0.1 M, pH 7.3) was used as electrolyte for all experiments. For cyclic voltammetric and chronoamperometric experiments, a Pt counter electrode and modified glassy carbon disk working electrodes (3 mm) were used. The latter were polished by using, first, diamond particles (3 μm) followed by Al₂O₃ powder (1 μm, then 0.3 μm) according to protocols. Measurements under gas-diffusion conditions were performed in a homemade glass cell with carbon cloth-based gas-diffusion electrodes (MFT, carbon foam sheet, porous C, 0.454 mm thick, ≈10 μm cm⁻²), porosity ≈31 μm coated on one side with a conductive Nafion/Teflon-based microporous film (50 μm), carbon content 5 mg cm⁻², EQ-bcgd1-14005-LD). Thermal mass flow controllers (GFC17, Aalborg Instruments and Controls) were used to adjust the desired atmosphere and gas mixtures with predefined compositions (for compositions of the gas feed, see the main text and figures). The back of the gas-diffusion electrode was exposed to the corresponding gas atmosphere (bioanode) or to air/O₂ (biocathode). During the experiments in gas-breathing mode, the electrochemical cell/electrolyte was continuously purged with an argon stream to prevent permeation of O₂ into the bulk electrolyte. For characterization of the biofuel cells, power curves were measured by stepped potential chronoamperometric experiments to minimize contributions from capacitive charging currents. After each potential step, steady-state currents were used to calculate the corresponding power values.
Modification of glassy carbon electrodes with hydrogenase/polymer films

All films were prepared by means of a standard drop-casting process. For this, stock solutions of the polymer P(N,MA-BA-GMA)-vio (7.3 mg mL⁻¹) were mixed with 3 μL of phosphate buffer (0.1 M, pH 7.3), and the corresponding hydrogenase was added (wt-NiFeSe): 0.5 μL, 170 μm in Tris-HCl buffer, 20 mm, pH 7.6; G491A: 1 μL, 82.96 μm in Tris-HCl buffer; G491S: 1.56 μL, 53 μm in Tris-HCl buffer). For electrode modification, 1.3 μL of the stock solution was drop-cast onto the 3 mm glassy carbon disk electrode. The modified electrodes were incubated overnight at 4 °C and air dried for 1 h prior to use. In a typical experiment, three electrodes were modified from the same stock solution.

Modification of carbon cloth electrodes with hydrogenase/polymer films

First, 20 μL of the polymer P(GMA-BA-PEGMA)-vio (7.5 mg mL⁻¹ in water) was drop-cast on the microporous side of the carbon cloth electrode and dried overnight at room temperature. Subsequently, 20 μL of G491S (53 μm in Tris-HCl, 20 μm, pH 7.6) was mixed with 15 μL of P(N,MA-BA-GMA)-vio (7.3 mg mL⁻¹ in water) and drop-cast onto the already existing polymer spot (diameter of ≈ 4 mm). The electrode was dried overnight at 4 °C.

Modification of carbon cloth electrodes with Bp-BOD

The bare carbon cloth was pre-wetted with ethanol on both sides and rinsed with water. Then, the microporous side of the gas-diffusion layer was modified with 20 μL of the 8-BpBox solution (54.75 mg mL⁻¹ in borate buffer, 50 mm, pH 9). The electrode was dried overnight at 4 °C.

Modification of carbon cloth electrodes with Mv-BOD

For the preparation of the Mv-BOD-based cathode, the microporous side of the ethanol-treated carbon cloth gas-diffusion electrode was first modified with 2-aminobenzoic acid (2-ABA) in an electrochemical grafting process in 0.1 M KCl/5 mm 2-ABA/water by applying a potential pulse of ~0.8 V vs. Ag/AgCl/3 M KCl for 60 s according to procedures reported in Refs. [12,33]. The modified electrode was rinsed with water and further modified with 120 μL of an aqueous Mv-BOD solution (10 mg mL⁻¹, nominal enzyme loading: 1.2 mg electrode⁻¹) and dried at 4 °C overnight.

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Conflict of interest

The authors declare no conflict of interest.

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