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[NiFe]Hydrogenase from *Citrobacter* sp. S-77 Surpasses Platinum as an Electrode for H_2 Oxidation Reaction**

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In memory of Saori Ogo

Abstract: *Reported herein is an electrode for dihydrogen (H_2) oxidation, and it is based on [NiFe]Hydrogenase from Citrobacter sp. S-77 ([NiFe]$_{S77}$). It has a 637 times higher mass activity than Pt (calculated based on 1 mg of [NiFe]$_{S77}$ or Pt) at 50 mV in a hydrogen half-cell. The [NiFe]$_{S77}$ electrode is also stable in air and, unlike Pt, can be recovered 100 % after poisoning by carbon monoxide. Following characterization of the [NiFe]$_{S77}$ electrode, a fuel cell comprising a [NiFe]$_{S77}$ anode and Pt cathode was constructed and shown to have a a higher power density than that achievable by Pt.*

Though platinum (Pt) is famously rare and expensive, it is used as an electrode in many electrochemical applications because it is chemically inert and has excellent electrical properties. Platinum is particularly useful as an anode in the hydrogen half-cell because it also acts as a catalyst which homolytically cleaves dihydrogen (H_2) to hydrogen radicals ($H^{\cdot}$) with a very small overpotential.[1] Thus, platinum forms a crucial part of the standard hydrogen electrode (SHE)—the defining electrode that all electrochemical potentials are measured against.[2] In fact, platinum is such a good electrode for the hydrogen half-cell, it is only its expense and CO poisoning problems that give chemists a reason to look for alternative materials.[3]

One class of alternative electrodes is enzyme electrodes, where an enzyme is used either in solution or as part of a solid matrix to catalyze the electrochemical reaction of interest. Enzyme electrodes, however, are usually inefficient and primarily of interest for their selectivity. In the case of the electrode in the hydrogen half-cell, [NiFe]Hydrogenases ([NiFe]H_2ases) have been considered because they are excellent catalysts for hydrogen oxidation reactions (HORs).[4–7] These enzymes are, however, either too sensitive to dioxygen (O_2) or too inactive for successful application in a hydrogen half-cell.

Armstrong and co-workers have used a purified, membrane-bound [NiFe]H_2ase from *Allochromatium vinosum* as an anode catalyst for an H_2–O_2 fuel cell in a solution system.[6] The [NiFe]H_2ase anode has H_2 oxidation ability equal to or better than a platinum anode. However, to date, H_2ases have not been used an electrode of a polymer electrolyte fuel cell (PEFC).[6–11]

We have recently reported the discovery of a unique form of [NiFe]H_2ase from *Citrobacter* sp. S-77 ([NiFe]$_{S77}$).[12] This membrane-bound [NiFe]H_2ase is not only O_2 stable but also highly active. Herein, we describe its employment as an electrode in the hydrogen half-cell and demonstrate that it outperforms [NiFe]H_2ase from *Desulfovibrio vulgaris* Miyazaki F ([NiFe]$_{DvMF}$) and Pt/C in both mass activity and reaction resistance. We further demonstrate the construction of an enzymatic PEFC that outperforms a standard platinum fuel cell.

An H_2 anode employing [NiFe]$_{S77}$ was prepared as follows. Carbon black was added to an aqueous solution (10 mM MOPS/KOH buffer, 150 mM NaCl, pH 7.0, MOPS = 3-(*N*-morpholino)propanesulfonic acid) of [NiFe]$_{S77}$ and the suspension was loaded onto carbon cloth ([NiFe]$_{S77}$: 0.19 $\mu g_{[NiFe]S77}\,cm^{-2}$, 2.1 $pmol\,cm^{-2}$). A membrane electrode assembly (MEA) was assembled with the H_2ase anode and a Pt/C cathode using a Nafion membrane. [NiFe]$_{S77}$ is stable enough to handle under air and no Nafion solution was required. The amino-acid sequence of [NiFe]$_{S77}$ is almost identical to that of Hyd-2 from *Escherichia coli*. Pairwise alignment scores of large and small subunits are 93 and 95, respectively. The Hyd-2 is classified into O_2-sensitive H_2ase and the enzyme can be reactivated after inactivation by O_2. A [NiFe]$_{DvMF}$ anode was also prepared by the same procedure as

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that used for the [NiFe]$_{S77}$ anode, except its handling without O_2.

The properties of [NiFe]$_{S77}$ as an anode for HORs were investigated and revealed an extraordinarily high mass activity (Figure 1 and Figure S1 in the Supporting Information). The [NiFe]$_{DvMF}$ also acted as an anode with much lower mass activity compared to that of the [NiFe]$_{S77}$ anode. The anode potentials of [NiFe]$_{S77}$ and [NiFe]$_{DvMF}$ under open circuit conditions were almost 0 V versus the reversible hydrogen electrode (RHE), as seen with platinum. The mass activity at an iR-free overpotential of 50 mV in the hydrogen half-cell is 33 times higher than that of [NiFe]$_{DvMF}$ and an extraordinary 637 times higher than that of platinum (Figure 1), as calculated based on 1 mg of [NiFe]$_{S77}$, [NiFe]$_{DvMF}$, or platinum (506,000 times calculated based on 1 mg of Ni and Fe atoms of the active center in [NiFe]$_{S77}$ in Figure S2 or Pt). The value for the previous record holder, a Pt/cobalt/manganese (PtCoMn) alloy, was reported at an iR-free overpotential of 1 mV and here we found a mass activity, based on 1 mg of Ni and Fe atoms in the enzyme active center (see Figure S2), of nearly 200 times that of the value for the PtCoMn alloy and over 1000 times that of Pt/C ([NiFe]$_{S77}$: 7830 A mg$_{NiFe}^{-1}$, PtCoMn: approximately 40 A mg$_{Pt}^{-1}$ at 1 mV and Pt/C: 7.5 A mg$_{Pt}^{-1}$ at 1 mV).[13,14] The enzymatic properties are also impressive when compared to other known H_2ases. [NiFe]$_{S77}$ achieved a turnover frequency [TOF = (mol of H_2/mol of [NiFe]$_{S77}$) per second] of 893 000 s^{-1} at plateau current density using a H_2/N_2 (50%/50%) gas mixture (Figure S3). The TOF for the [NiFe]$_{S77}$ anode is 63 times the TOF for the [NiFe]$_{DvMF}$ anode (14100 s^{-1}; Figure S3), 99 to 595 times the reported TOFs for [NiFe]H_2ase from *Allochromatium vinosum* (1500–9000 s^{-1}),[15] and much higher than the TOF for platinum (1.2 s^{-1}; Figure S3).[7] The loss of anode performance in the PEFC was about 9% after 120 cycles and 3 hours in which voltage cycled between 0 to 200 mV (Figure S4), while 77% of the activity was lost after 3 hours as determined by a spectrophotometric assay with benzyl viologen as an electron acceptor (Figure S5), and suggests that [NiFe]$_{S77}$ could be stabilized in the PEFC.

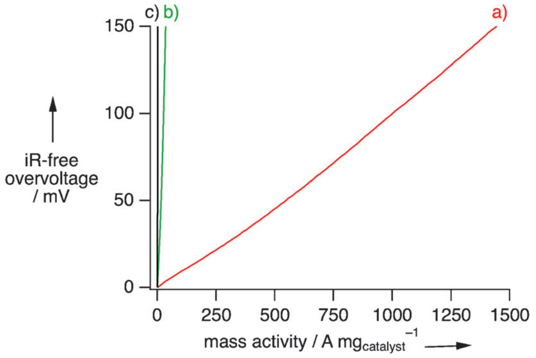

Figure 1. iR-free overvoltages as a function of mass activity for HORs for a) [NiFe]$_{S77}$ (sweep rate: 10 mV s^{-1}), b) [NiFe]$_{DvMF}$ (sweep rate: 1 mV s^{-1}), and c) Pt/C (sweep rate: 10 mV s^{-1}) anodes under H_2-O_2 fuel cell conditions. Pt and Pt/C were used as the reference and counter electrodes, respectively, for all the tested fuel cells. MEA area: 5 cm^2. Anode: [NiFe]$_{S77}$ (0.19 μg$_{[NiFe]S77}$ cm^{-2}), [NiFe]$_{DvMF}$ (0.20 μg$_{[NiFe]DvMF}$ cm^{-2}), or Pt/C (0.10 mg$_{Pt}$ cm^{-2}) prepared without a Nafion solution. Cathode: Pt/C (1.0 mg$_{Pt}$ cm^{-2}) prepared with a Nafion solution. Flow rate of water-saturated H_2 and O_2 gases: 200 mL min^{-1}. Temperature: 60 °C. Humidity: 100%. The control experiment without the enzyme is shown in Figure S1. The other control experiments are shown in Table S3.

It has been reported that the TOFs for HORs with [NiFe]H_2ases, determined by electrochemical analysis, are higher than the TOFs determined by spectrophotometric assay with benzyl or methyl viologen as an electron acceptor (entries 1–3 in Table S1).[8,10] The same tendency is observed for [NiFe]$_{S77}$ and [NiFe]$_{DvMF}$ (entries 4 and 5 in Table S1). In consideration of the TOFs obtained from PEFCs (entries 4 and 5 in Table S1) and solution-type fuel cells (entries 1–3 in Table S1), there is a possibility that PEFC is more suitable for [NiFe]H_2ases to act as highly active catalysts.

The impedance characteristics were also analyzed, thus revealing remarkably low impedance (0.13 Ω), even lower than those of [NiFe]$_{DvMF}$ (2.6 Ω) and platinum (0.34 Ω). Figure 2 shows that the Ohmic resistance (0.019 Ω) is much smaller than the reaction resistance in the anode reaction, and indicates the reaction rate is dominated by the oxidation of H_2 rather than proton transfer in the Nafion membrane.

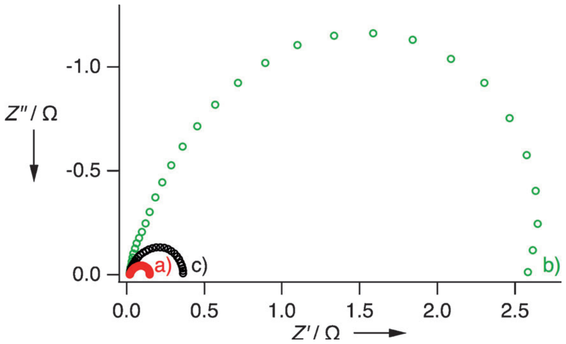

Figure 2. Impedance spectra for HORs for a) [NiFe]$_{S77}$, b) [NiFe]$_{DvMF}$, and c) Pt/C anodes in H_2-O_2 fuel cell conditions. Pt and Pt/C were used as the reference and counter electrodes, respectively, for all the tested fuel cells. The spectra were measured under open circuit conditions by changing the frequency from 0.1 to 10^5 Hz. MEA area and the other conditions are the same as those described in the caption of Figure 1.

Exchange current density was higher and activation energy was lower for [NiFe]$_{S77}$, compared to those values of [NiFe]$_{DvMF}$ and platinum electrodes. Tafel plots of [NiFe]$_{S77}$, [NiFe]$_{DvMF}$, and platinum reveal the exchange current densities at 60 °C as 0.211, 0.0466, and 0.120 A cm^{-2}, respectively (see Figures S6–S8). The activation energies ([NiFe]$_{S77}$: 10.1 kJ mol^{-1}, [NiFe]$_{DvMF}$: 14.5 kJ mol^{-1}, Pt/C: 13.5 kJ mol^{-1}) were calculated from Arrhenius plots using the exchange current densities (see Figure S9).

An obvious, and necessary, application for such an extraordinary electrode is to serve as an anode of a hydrogen fuel cell (Figure 3). The [NiFe]$_{S77}$ fuel cell affords an open circuit voltage of 0.95 V and a maximum power density of 180 mW cm^{-2}, which is 44 times higher than that of

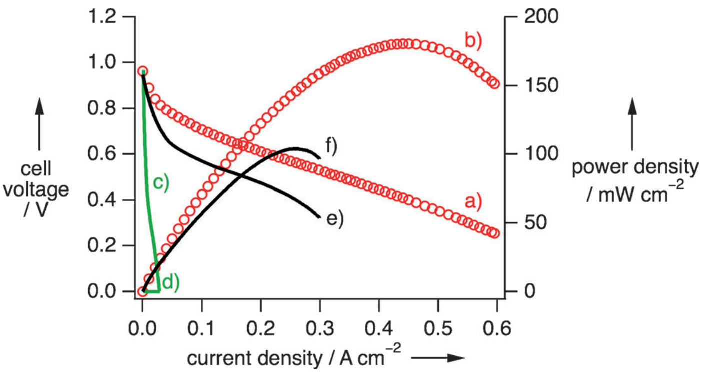

Figure 3. Polarization curves for a) $[NiFe]_{S77}$ (sweep rate: 50 mV s^{-1}), c) $[NiFe]_{DvMF}$ (sweep rate: 1 mV s^{-1}), and e) Pt/C (sweep rate: 10 mV s^{-1}) anodes in H_2-O_2 fuel cell conditions. Power density curves for b) $[NiFe]_{S77}$ (sweep rate: 50 mV s^{-1}), d) $[NiFe]_{DvMF}$ (sweep rate: 1 mV s^{-1}), and f) Pt/C (sweep rate: 10 mV s^{-1}) anodes in H_2-O_2 fuel cell conditions. Pt/C was used as the counter electrode for all the tested fuel cells. MEA area and the other conditions are the same as those described in the caption of Figure 1. The control experiment without enzyme is shown in Figure S10. The other control experiments are shown in Table S3.

4.1 mW cm^{-2} produced from the $[NiFe]_{DvMF}$ fuel cell. This cell is the first construction of a $[NiFe]H_2$ase-based PEFC.[6–11] The PEFC performance for $[NiFe]_{S77}$ and $[NiFe]_{DvMF}$ was optimized by conducting various control experiments (see Table S3). The large difference in the value of the maximum power densities produced from PEFC, 180 mW cm^{-2} (entry 1 in Table S2), and solution-type fuel cells, 1.67 and 0.3 mW cm^{-2} (entries 2 and 3 in Table S2),[8a,9] could be caused by the different fuel cell system and different cathode catalyst between Pt/C for our PEFC and bilirubin oxidase for solution-type fuel cells.

We found that $[NiFe]_{S77}$ can recover its activity after carbon monoxide (CO) poisoning (see Figures S11 and S12). The H_2 feed to the fuel cell was switched to an H_2/CO (CO 103.2 ppm) gas mixture, which resulted in a loss of performance to 12 % of the original power density (Figure S12 b). Reinstating the H_2 feed allowed recovery to 100 % of the original power density. The CO-inhibition/recovery behavior is similar to that for the $[NiFe]H_2$ase from *Allochromatium vinosum*,[6] but different from that for a Pt/C which showed only a partial recovery of platinum sites.[16] As described above, $[NiFe]_{S77}$ is almost identical to Hyd-2 from *Escherichia coli* in terms of the amino-acid sequence. The Hyd-2 is known as CO-sensitive H_2ase that can be reactivated by H_2 after CO-inhibition, and is similar to $[NiFe]_{S77}$ when observed in the PEFC.

To answer the question of how H_2ase enzymes can be so much more efficient than platinum at hydrogen splitting, we note that the two materials use completely different mechanisms to separate the H_2. H_2ases cleave H_2 heterolytically [Eq. (1)][4–6,17,18] while platinum cleaves it homolytically [Eq. (2)].[1] Although homolytic cleavage is preferable in the gas phase ($\Delta G^0_{hetero} = 1642$ kJ mol^{-1}, $\Delta G^0_{homo} = 407$ kJ mol^{-1}),[19,20] heterolytic cleavage is favorable ($\Delta G^0_{hetero} = 176$ kJ mol^{-1}, $\Delta G^0_{homo} = 442$ kJ mol^{-1}) in the aqueous phase.[20,21] Since water-saturated H_2 gas covers even the solid supported electrodes in our experiments, it is the aqueous phase values that are of most importance.

$$H_2 \rightarrow H^+ + H^- \rightarrow 2\,H^+ + 2\,e^- \qquad (1)$$

$$H_2 \rightarrow 2\,H^\bullet \qquad \rightarrow 2\,H^+ + 2\,e^- \qquad (2)$$

In conclusion, we have created a new electrode for the oxidation of H_2 and it outperforms platinum in terms of mass activity, reaction resistance, and activation energy. We are convinced that $[NiFe]_{S77}$ will provide the basis of a new standard for H_2 electrodes.

Experimental Section

Preparation of $[NiFe]_{S77}$ anode: An aqueous solution of $[NiFe]_{S77}$ (9.5 µg mL^{-1}, 10 mM MOPS/KOH buffer, 150 mM NaCl, pH 7.0) was prepared by 5 times dilution of the original aqueous solution of $[NiFe]_{S77}$ (47.7 µg mL^{-1}). Carbon black (5.0 mg) was added into 100 µL of the diluted solution (10 mM MOPS/KOH buffer, 150 mM NaCl, pH 7.0) containing $[NiFe]_{S77}$ (0.95 µg) and the resulting suspension was loaded on a waterproof carbon cloth (5 cm^2) to make a gas diffusion electrode by drying under air at room temperature. The loading of $[NiFe]_{S77}$ is 0.19 µg cm^{-2}.

Preparation of $[NiFe]_{DvMF}$ anode: An aqueous solution of $[NiFe]_{DvMF}$ (9.8 µg mL^{-1}, 10 mM MOPS/KOH buffer, 150 mM NaCl, pH 7.0) was prepared by 5 times dilution of the original aqueous solution of $[NiFe]_{DvMF}$ (49.1 µg mL^{-1}). Carbon black (5.0 mg) was added into 100 µL of the diluted solution (10 mM MOPS/KOH buffer, 150 mM NaCl, pH 7.0) containing $[NiFe]_{S77}$ (0.98 µg) and the resulting suspension was loaded on a waterproof carbon cloth (5 cm^2) to make a gas diffusion electrode by drying under an N_2 atmosphere at room temperature. The loading of $[NiFe]_{DvMF}$ is 0.20 µg cm^{-2}.

Preparation of Pt/C anode: Carbon black (4.4 mg) and Pt/C (1.1 mg) were loaded on a waterproof carbon cloth (5 cm^2) to make a gas diffusion electrode. The loading of Pt is 0.10 mg cm^{-2}. No Nafion solution was used to make the Pt/C anode for comparison with the $[NiFe]_{S77}$ anode prepared without a Nafion solution.

Preparation of Pt/C cathode: To a Nafion solution (59 mg) was added Pt/C (11 mg), in which a Nafion-to-carbon ratio is 2, and the resulting suspension was sonicated for 1 h. The suspension was loaded on a waterproof carbon cloth (5 cm^2) to make a gas diffusion electrode. The loading of Pt is 1.0 mg cm^{-2}.

Preparation of anode and cathode for control experiments: The anode and cathode were prepared according to the same methods described above except amounts of $[NiFe]_{S77}$, $[NiFe]_{DvMF}$, Pt, and carbon black (see Table S3).

MEA and fuel cell assembly: The Nafion membrane 212 was boiled in 3 % H_2O_2 aqueous solution for 1 h, deionized water for 1 h, 1 M H_2SO_4 aqueous solution for 1 h, and deionized water for 1 h. A piece of the pretreated Nafion 212 was sandwiched between two gas diffusion electrodes which are prepared by the above procedure to obtain MEA and the MEA was assembled in 5 cm^2 fuel cell hardware (Figure S20). A 5 cm^2 single cell with one reference electrode of Pt (EFC-05-02-REF, TOYO Corporation) is used for measurement of anode polarization curves and impedance spectra. The reference electrode of Pt is a reversible hydrogen electrode (RHE) that is positioned inside the single cell on anode side.

Polarization measurement: Polarization curves were measured on 2- or 3-electrode setup. Anode polarization curves were measured on the 3-electrode setup by connecting RE, WE, and CE to the reference electrode, anode, and cathode, respectively. Fuel cell polarization curves were measured on the 2-electrode setup by connecting RE1 and CE to anode and connecting RE2 and WE to cathode, where the current and voltage values were recorded using linear sweep voltammetry at a scan rate of 1–50 $\mathrm{mV\,s^{-1}}$. iR-free overvoltage of anode was obtained from the following equation: $\eta(\text{anode}) = V(\text{anode}) - iR$, where η is overvoltage, V is voltage, i is current density, and R is Ohmic resistance determined by each impedance measurement for $[\mathrm{NiFe}]_{\mathrm{S77}}$, $[\mathrm{NiFe}]_{\mathrm{DvMF}}$, and Pt/C anodes.

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