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Hydrogen production at high Faradaic efficiency by a bio-electrode based on TiO₂ adsorption of the new [FeFe]-hydrogenase from *Clostridium perfringens*

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Keywords

15 Bio-hydrogen; [FeFe]-hydrogenase; hybrid device; titanium dioxide.

Abstract

The [FeFe]-hydrogenase CpHydA from *Clostridium perfringens* was immobilised by adsorption on anatase TiO₂ electrodes for clean hydrogen production. The immobilized enzyme proved to perform direct electron transfer to and from the electrode surface and catalyses both
20 H₂ oxidation (H₂ uptake) and H₂ production (H₂ evolution) with a current density for H₂ evolution of about 2 mA cm⁻¹. The TiO₂/CpHydA bioelectrode remained active for several days upon storage and when a reducing potential was set, H₂ evolution occurred with a mean Faradaic efficiency of 98%. The high turnover frequency of H₂ production and the tight coupling of electron transfer, resulting in a Faradaic efficiency close to 100%, support the
25 exploitation of the novel TiO₂/CpHydA electrode as a powerful device for H₂ production.

Introduction

Research of new energy sources in place of fossil fuels is of paramount importance and hydrogen is considered as a promising alternative because it is a renewable, clean and low cost energy carrier. For this purpose, hydrogen producing devices are being developed, including bio-hybrid devices in which hydrogenases can be used as efficient catalysts, in place of noble metals such as platinum [1-4].

To this end both [NiFe]- and [FeFe]-hydrogenases (H_2 ases) have been used on a wide variety of solid electrodes, such as pyrolytic graphite edge (PGE), glassy carbon and carbon felt electrodes, gold electrodes, TiO_2 electrodes but also carbon nanotubes and nanowires, CdTe nanocrystals and CdS nanorods. Electrochemical, photoluminescence and Raman spectroscopy studies demonstrate that the hydrogenases remain catalytically active and therefore can be exploited as efficient catalysts [5-10]. Several electrochemical setups have been proposed exploiting the catalytic properties of both [NiFe]- and [FeFe]-hydrogenases from various bacterial and some algal species [6,11-12].

Many types of carbon-based electrodes have been reported in the literature because of their non-specific interaction with hydrogenases and, most importantly, these biohybrid systems displayed high current densities. Moreover, carbon materials are less expensive compared to platinum electrodes and they have high electroactive surface area on which hydrogenases can be adsorbed, especially the carbon felt electrode [6].

In previous years, semiconductor-based electrodes have attracted a great deal of interest both for hydrogenase-coupling in the perspective for the possible photoactivation by direct UV/Vis illumination or by mean of antenna systems coupled to the semiconductors.

Titanium dioxide was used because of its photochemical properties alone or coupled to sensitising dyes. In fact, the ability of RuP- TiO_2 nanoparticles to absorb both UV and visible light has been exploited for light-driven H_2 production by *D. baculatum* [NiFeSe]- H_2 ase absorbed on it [7]. Not only titanium oxide, but also other materials like CdTe and CdS show intrinsic photochemical properties so the photochemical H_2 production by CdS nanorod and CdTe nanocrystal [FeFe]- H_2 ase complexes has been well investigated [8,13].

To obtain a reliable electrochemical response, independently from the electrode material on which hydrogenases are immobilized, a very good electrical contact is fundamental to allow an efficient electron transfer between the enzyme and the solid electrode surface. This is generally reflected in high efficiency in the electrocatalysis, with minimal uncoupling or wastage of electrons supplied by the electrode for molecular hydrogen production. In some cases, immobilization by simple absorption is sufficient and can be stabilized by hydrogen bonds, Van der Waals force, charge-charge and polar interactions between the side chain of superficial residues of the protein and the functional groups of the solid surface. This is the case of glassy carbon, carbon felt, PGE and also TiO_2 electrodes. The advantage is the very simple and low cost procedure and also the minimal interference with protein structure and stability, which might instead be negatively affected by the chemical covalent coupling procedures. In other cases, a covalent immobilization by means of flexible molecules that expose reactive functional groups is required to stably bind the protein in a correct orientation on the solid surface [14-18]. In a recent work [FeFe]-hydrogenases from *C. acetobutylicum* and *C. reinhardtii* were covalently immobilized onto pyrolytic graphite modified with a carboxybenzenediazonium [19]. The efficacy of a covalent immobilization *versus* a simple adsorption is usually measured in terms of good coupling and long-term bioelectrode stability. The direct electron transfer (ET) is influenced not only by interfacial electron transfer, which is overcome by means of a good electron coupling, but also by mass transport of the substrates from the bulk solution to the interface and of the products through the

opposite direction. Since hydrogenases generally possess high turnover frequencies and low K_M values, the mass transport could result in a limitation for catalysis. To overcome or minimize this problem, a rotating disc electrode (RDE) is often used [6,19,20]. RDEs are very efficient for measuring rate constants and give a detailed fundamental characterization of the system but the applicative outcomes are limited. Conversely, stationary electrodes are easier to use and can be exploited for
5 working devices.

Concerning the protein catalyst, although both [NiFe]- and [FeFe]-hydrogenases are of interest for the development of biodevices, [FeFe]-hydrogenases have remarkably higher k_{cat} (or turnover frequency, *i.e.* TOF), up to 10^4 sec^{-1} , approximately one to two orders of magnitude higher than [NiFe]-enzymes. The isolation and characterisation of novel [FeFe]-hydrogenases grants the availability of more stable and efficient catalysts, widening the possible choice for
10 applicative purposes.

In this work, a novel [FeFe]-hydrogenase CpHydA (encoded by CPF_2655 gene) from a *Clostridium perfringens* strain newly isolated in a pilot plant with high hydrogen productivity [21,22] was immobilized by adsorption on stationary TiO₂ electrodes. The heterologous expression in *E. coli* was achieved thanks to a previously established system [23] and it allowed to obtain a pure and active protein in good yields. Anatase TiO₂ was used because of its ability to bind
15 hydrogenases [9] with a favourable orientation for electron transfer by simple adsorption; this is possibly due to a major influence of electrostatic interactions between the partially negative electrode surface and positively charged aminoacids (mainly lysines and arginines) surroundings the stem region of this kind of hydrogenase near the [2Fe2S] cluster and the distal [4Fe4S] cluster, where the electrons are suggested to be delivered by ferredoxin in the natural system. The choice of TiO₂ was dictated also by the availability, low cost and safety of use of the material [24,25] compared to other used
20 semiconductors. The major limiting characteristic of TiO₂ for further exploitation of the biohybrid devices in photochemistry, *i.e.* the prevalent absorption in the UV region, is currently being overcome by the availability of doped variants with extended absorption band in the visible spectrum [26-29], still maintaining the surface determinants crucial for a good interaction with the protein.

The resulting hydrogen producing bioelectrode was validated by chronoamperometric experiments and tested for
25 production of hydrogen gas by quantitative measurement in gas chromatography. Efficiency and stability were studied and results are discussed comparing the present system to others reported in the literature. The high efficiency and good stability of this biohybrid system provides a benchmark for alternative future devices for biohydrogen production.

2. Materials and Methods

2.1 Recombinant expression and purification

30 Recombinant expression of the hydrogenase CypHydA from *Clostridium perfringens* SM09 [22] was performed in *E. coli* Rosetta2(DE3) as previously described [30] upon transformation of the competent cells with plasmid pECPF2655, obtained by ligating the entire coding sequence of CPF_2655 into the empty expression vector pECr1 [23] between NdeI and XhoI sites. After the induction, cells were incubated over night under pure argon flow to maintain anaerobic conditions in a water bath at 30°C. To prevent oxygen inactivation of the active CpHydA all the following manipulations were performed into a
35 glove box (Plas Labs) under an anaerobic hydrogen/nitrogen atmosphere; before use, all solutions were vacuumed, equilibrated with the anaerobic atmosphere and supplemented with 2-20 mM sodium dithionite.

Purification of CpHydA was carried out under strict anaerobic conditions by affinity chromatography using Strep-Tactin

Superflow high capacity cartridges (IBA) and following manufacturer's instructions. The enzyme was eluted and stored in 100 mM Tris·HCl, 150 mM NaCl pH 8.0 supplemented with 2 mM sodium dithionite and 2.5 mM desthiobiotin.

Coomassie-stained SDS-PAGE was used to determine the purity and the molecular weight of the purified enzyme (64.7 kDa). Protein concentration was assayed with Bradford assay using bovine serum albumin as standard.

5 2.2 Hydrogenase activity assay

Hydrogenase activity (hydrogen evolution) of the purified enzyme in solution was tested as previously described [31]. Briefly, 10 mM dithionite-reduced methyl viologen was used as artificial electron donor at 37°C in 100 mM Tris·HCl, 150 mM NaCl pH 8.0. The evolution of H₂ both from the soluble enzyme with methyl viologen as electron donor and from the bio-electrodes poised at reducing potentials was quantified by gas chromatography using an Agilent Technologies 7890A
10 instrument equipped with purged packed inlet, Molesieve 5A column (30 m, ID 0.53 mm, film 25 mm) and thermal conductivity detector; argon was used as carrier gas. The typical activity of the purified soluble enzyme used for the immobilisation experiments was 1500 μmol H₂/min/mg of protein.

2.3 Enzyme immobilization

All experiments in this section were performed in 50 mM N-(2-Acetamido)-2-aminoethanesulfonic acid (Aces) pH 6.8
15 containing 100mM NaCl as additional supporting electrolyte.

The anatase electrodes were manufactured by Solaronix (Switzerland). They are screen printed and supported on fluorine-doped tin oxide (FTO); the anatase layer is a disk of 6 mm diameter and 250 μm thickness composed of 20 nm nanoparticles (Ti-Nanoxide T/SP).

Enzyme immobilization was achieved as previously described for another [FeFe]-hydrogenase [9] in an anaerobic glove
20 box (Belle Technology) under a pure nitrogen atmosphere. The enzyme solution was incubated on top of the TiO₂ electrode in a wet chamber at room temperature for approximately 2 hours. Control experiments were routinely performed using enzyme-free buffer. Before use electrodes were rinsed with anaerobic buffer.

2.4 ATR-FTIR spectroscopy

25 The spectra were acquired at room temperature using a Bruker Tensor 27 FT-IR spectrometer (Bruker Instruments). The sample chamber was purged with 99.9999% pure nitrogen gas.

For the characterisation of CpHydA immobilized on anatase electrodes, the attenuated total reflectance (ATR) tool (Harrick Scientific Products) was used.

Spectra were acquired with a resolution of 2 cm⁻¹ accumulating 256 scans. The baseline correction was obtained using
30 the Opus 6.0 software (Bruker Instruments) by the concave rubberband algorithm.

2.5 Electrochemical characterisation

Cyclic voltammetry experiments were carried out in the anaerobic glove box. The reference electrode was Ag/AgCl 3M NaCl (BASi); the counter electrode was made of a platinum wire and the stationary working electrode was the TiO₂
35 electrode. All potentials have been corrected by +0.209 V to be referred to the Standard Hydrogen Electrode (SHE).

Cyclic voltammetry was performed with Autolab PGSTAT12 potentiostat (EcoChemie) controlled by "General Purpose

Electrochemical System” (GPES) software at a scan rate of 20 mV s⁻¹. All scans were initiated at the most negative potential. Current density was calculated by correcting for the electrode geometric area.

Stability test of the electrodes with CpHydA was performed by collecting cyclic voltammograms at 20°C over time, using the same electrodes (4 replicas) for several days (up to 8 days). Electrodes were stored at 4°C in anaerobic conditions.

Chronoamperometry was performed poisoning the working electrode at a potential of -741 mV vs SHE. The electrochemical cell (BASi) was filled with 700 uL buffer solution and sealed with a rubber stopper. The gas phase was sampled with a SampleLock Gastight syringe (Hamilton) and analyzed by gas chromatography.

Turnover frequency (TOF) was calculated as the ratio between the molar of H₂ produced at the end of the experiment and the moles of enzyme immobilized, assuming that all the enzyme was adsorbed and electro-active.

The Faradaic efficiency was calculated as the ratio between the moles of H₂ produced by the electrode and the equivalent of electrons passed through the electrode. The equivalents of electrons were measured by the mathematical integration of the current traces obtained during chronoamperometry experiments.

3. Results and discussion

3.1 Immobilization on anatase electrodes of the *Clostridium perfringens* [FeFe] hydrogenase

The novel [FeFe] hydrogenase CpHydA from *Clostridium perfringens* SM09 studied in this paper was newly isolated from a highly efficient hydrogen producing pilot plant, and was employed as biocatalyst to modify a TiO₂ nanostructured electrode. The active soluble enzyme was immobilised by a simple and low-cost adsorption technique and subsequently these bioelectrodes were characterised for their features and performances as hydrogen producing devices.

Adsorption of CpHydA on anatase electrodes was firstly probed for structural integrity of the enzyme by ATR-FTIR spectroscopy. Figure 1A spectrum shows the typical amide I, II and III bands. The amide I band (in the range 1600-1700 cm⁻¹) corresponds mainly to the stretching vibration of the C=O and C-N groups in the peptide bonds. The amide II band (in the range 1500-1600 cm⁻¹) is due to the out-of-phase combination of the N-H in plane bend and the C-N stretching vibration. These bands result from a group of overlapped signals related to the secondary structure of the enzyme. The amide III band (in the range 1200-1400 cm⁻¹) is associated to the in-phase combination of the N-H bending with C-N stretching vibration and is also influenced by side chains structure [32]. The ATR-FTIR spectrum indicates that the enzyme is bound to the surface and that the secondary structure is conserved

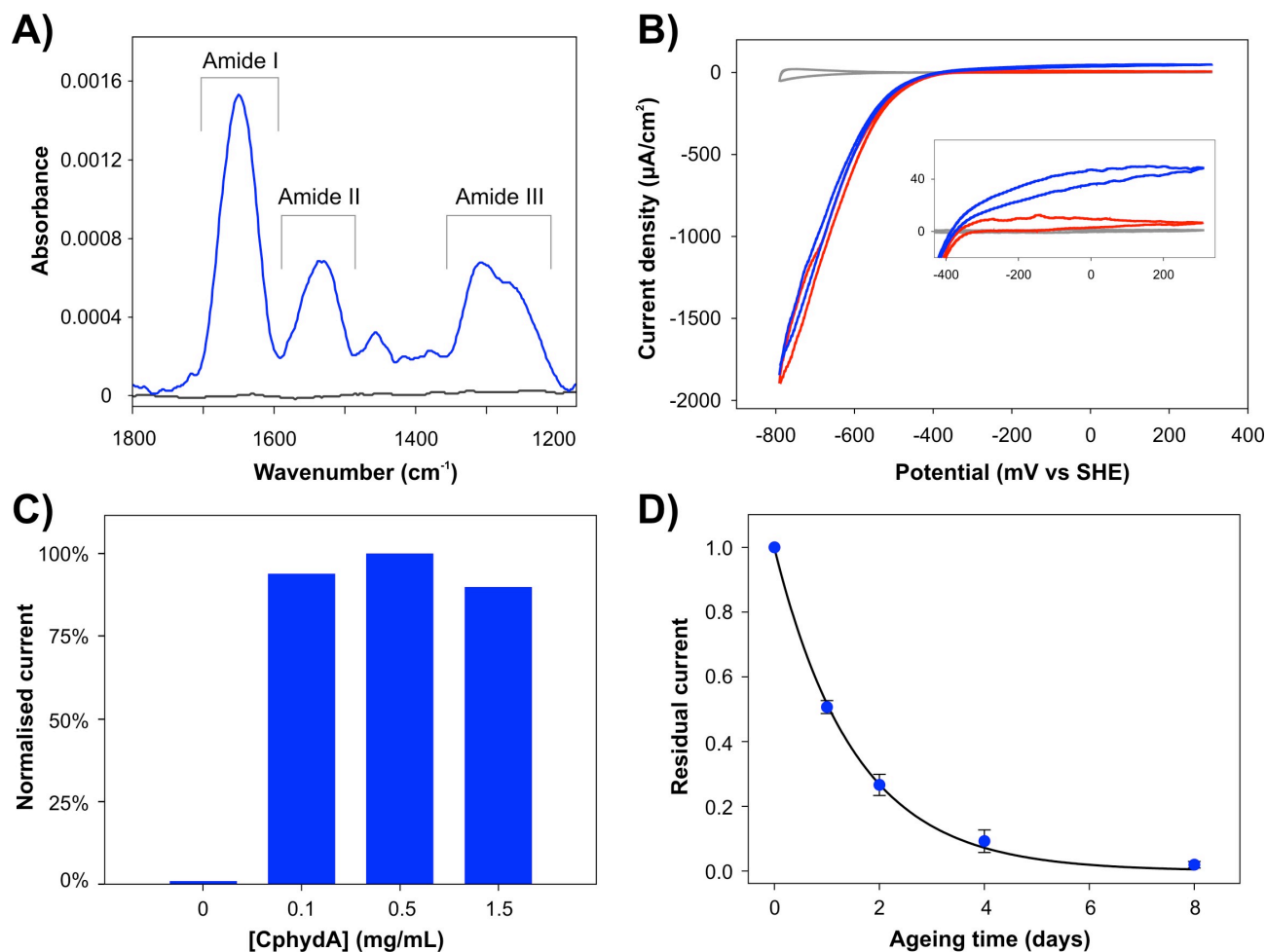
The functionality of the immobilized enzyme was investigated by cyclic voltammetry (fig. 1B). The electrodes with immobilized CpHydA have a reductive catalytic current due to the H⁺ reduction to H₂ (namely H₂ evolution) that has an onset potential of about -400 mV vs SHE. In the presence of exogenously added H₂, also an oxidative catalytic current due to H₂ oxidation (namely H₂ uptake) can be detected. It is also possible to observe that the addition of H₂ does not cause a large current drop in the reductive region, demonstrating that the immobilized CpHydA does not suffer from product inhibition under these conditions, as previously observed for similar enzymes [33]. The overall behaviour is coherent to that observed before for other [FeFe]-hydrogenases immobilized on TiO₂ electrodes [9].

Cyclic voltammetry demonstrates that CpHydA is immobilized in an active form and that it can perform direct electron transfer with the electrode. Also, the current density observed in H₂ evolution is about 2 mA cm⁻¹, which is significantly higher than previously reported on static electrodes [6,9].

The effect of protein concentration was investigated (fig. 1C) to determine the optimal amount of CpHydA to be used,

that was found to be the lowest tested concentration: 0.1 mg/mL (*i.e.* 1.5 μ M). This concentration gives the highest possible current density and increasing concentrations do not result in an improvement of the catalytic current.

The stability of the electrodes with CpHydA was tested over time (fig. 1D). Cyclic voltammograms were collected using the same electrodes for several days and the current decay was plotted and fitted with an exponential equation. A significant current can be detected up to 4 days with a calculated half-life of 1 day; these values are similar to previous reports where both adsorption and covalent immobilization on carbon electrodes were used [19].



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Fig. 1 Immobilization of CpHydA on anatase electrodes. A) ATR-FTIR spectrum of CpHydA adsorbed on the anatase electrode. B) Cyclic voltammograms of the immobilized enzyme obtained in the absence (red line) or in the presence of H_2 (blue line); grey line represents the bare TiO_2 electrode only treated with buffer as control. C) Effect of different CpHydA concentrations on the maximum reductive current in cyclic voltammograms. D) Stability of the $\text{TiO}_2/\text{CpHydA}$ electrode; the electrodes were anaerobically stored and tested by cyclic voltammetry at different time-points; the residual reductive current was plotted.

3.2 Hydrogen evolution from the electrodes

To test the ability of the TiO₂/CpHydA electrode to produce hydrogen, chronoamperometry experiments were performed and the gas accumulated in the head-space of the reaction vial was analysed by gas chromatography (fig. 2).

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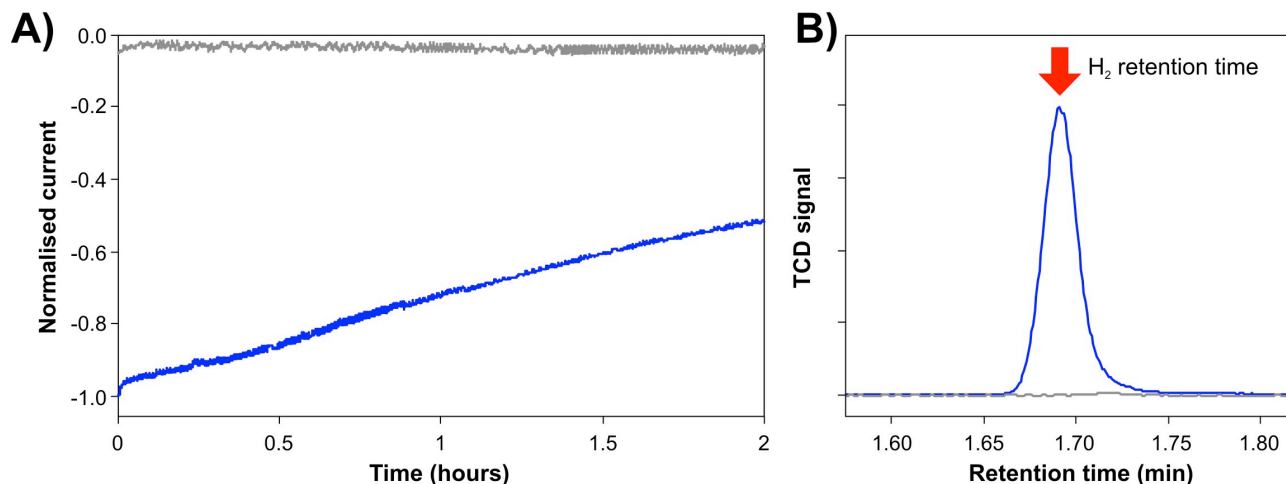


Fig. 2 Chronoamperometry and H₂ evolution. A) The potential of the working electrode was set to -741 mV vs SHE and the current was recorded for 2 hours. B) At the end of the experiment, the gas phase was analysed by gas chromatography. Blue line represents the TiO₂/CpHydA bioelectrode; grey line represents a control of the bare TiO₂ electrode.

10

The current traces showed a current drop that is typical of immobilized redox enzymes and that has been attributed to both inactivation and desorption [9,19,34,35]. Nevertheless, in this case the current drop after 30 minutes is much smaller when compared to another [FeFe]-hydrogenase adsorbed on TiO₂ [9]; moreover, when the current drop is compared on the time-scale of hours, it is similar to that observed with [FeFe]-hydrogenases covalently immobilized on carbon electrodes [19].

15

Gas chromatographic analysis of the gas phase showed the presence of H₂ only when CpHydA was immobilized. The Faradaic efficiency of the process, calculated as the ratio between the produced H₂ and the electrons used, is very high: 98 ± 1% (Table 1). The turnover frequency (TOF) calculated for this system is ≥ 4 s⁻¹, assuming that all the protein loaded onto the electrode is actually electroactive (Table 1).

20

Table 1. H₂ evolution from the electrode. The Faradaic efficiency was calculated as the ratio between the total amount of H₂ evolved and the number of electron equivalents used, The turnover frequency (TOF) was calculated as the ratio between the total H₂, the amount of protein and the time.

Total H ₂	Total charge	Protein amount	Duration	Efficiency	TOF
874±14 nmol	173±4 mC	≤30 pmol	120 min	98±1%	≥ 4.0 s ⁻¹

25

Since cyclic voltammograms of hydrogenases always display catalytic currents, due to the presence of protons as substrate, the quantitation of the adsorbed protein in electroactive form is very difficult. The estimate amount of immobilised protein on surfaces by ATR-FTIR has been proposed in some systems on the basis of the recorded intensity of the amide I, II and III signals [36,37] but the quantitation suffers from several uncertainties due to the thickness of the system during cell assembly. In this case the measurements were performed on the protein adsorbed on the TiO₂ electrode layered on the FTO glass, a thick and fragile support, and the protein-TiO₂ surface system was then placed in contact with the Germanium crystal and tightened. The recorded amide II band intensities were below 1 mAU (Fig 1A). A very approximate comparison with a published study of amide II intensity of known concentration of standard proteins [36] suggests that such intensities are measured for maximum protein concentration of 0.05 µg/cm², i.e. in our system 0.0145 µg of total immobilised protein. The turnover number of 4 s⁻¹ was calculated on a total protein loading of 2 µg (20 µl of a 0.1 mg/ml protein solution loaded onto the electrode, which means 30 pmol), i.e. a total biocatalyst amount overestimated by two order of magnitude. The turnover number of ≥4 s⁻¹ is therefore a largely underestimated value, and it is more likely in the range of hundreds s⁻¹, given the estimate proposed above and considering that the amount of protein loaded onto the electrode for adsorption is for sure in excess and that a thorough electrode rinsing was performed before testing the behaviour in chronoamperometry. The range of reported TOF for biohybrid systems involving [FeFe] hydrogenases and surfaces or electron donors is between 0.14 [38] and 900 s⁻¹[13], with other calculated TOF of about 20-100 s⁻¹[8,39].

4. Conclusions

The adsorption of a novel hydrogenase catalyst onto TiO₂ granted the development of a bioelectrode with extremely high Faradaic efficiency, close to 100%, and with a good stability over time, comparable with other adsorption-based systems.¹⁹ The preparation is quite simple and straightforward and the cost of the starting material is kept low due to the large availability of titanium and to the very low production cost of TiO₂ nanoparticles. The advantage of TiO₂ being a non-toxic and stable semiconductor strengthen the results reported here. This also paves the way for photoactivation trials, especially given the high coupling of electron transfer efficiency to the biocatalyst. Other very interesting semiconductors, which similarly have been coupled to hydrogenases for electro- and photo-production of hydrogen [8,10], although their higher turnover frequencies, suffer from some drawbacks given by high toxicity. This has to be taken into account when comparing to TiO₂ based systems: they might counterbalance a lower turnover number with a higher long-term sustainability. The excellent coupling properties of this hydrogenase with the static bioelectrode suggest a possible applicative outcome for hydrogen production, with hydrogenase replacing rare-metal platinum catalyst. This has been proposed and supported in several reviews as a valuable perspective for future trends in hydrogen production [39,40]. In fact hydrogenase catalyst works on a Fe based chemistry, therefore does not require highly expensive and rare metals.

The approach here presented, *i.e.* the exploitation of a novel hydrogenase recently isolated from a highly productive hydrogen pilot-plant fed with waste material [22], is strengthening the efforts towards a systematic identification of ideal biocatalysts to grant sustainable energy production.

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References

- [1] P. M. Vignais, B. Billoud, Occurrence, classification, and biological function of hydrogenases: an overview, *Chem. Rev.* 107 (2007) 4206-4272.
- [2] J. A. Cracknell, K. A. Vincent, F. A. Armstrong, Enzymes as working or inspirational electrocatalysts for fuel cells and electrolysis, *Chem. Rev.* 108 (2008) 2439-2461.
- [3] T. W. Woolerton, S. Sheard, Y. S. Chaudhary, F. A. Armstrong, Enzymes and bio-inspired electrocatalysts in solar fuel devices, *Energy Environ. Sci.* 5 (2012) 7470-7490.
- [4] P. W. King, Designing interfaces of hydrogenase-nanomaterial hybrids for efficient solar conversion, *Biochim. Biophys. Acta* 1827 (2013) 949-957.
- [5] T. J. McDonald, D. Svedruzic, Y.-H. Kim, J. L. Blackburn, S. B. Zhang, P. W. King, M. J. Heben, Wiring-up hydrogenase with single-walled carbon nanotubes, *NanoLetters* 7 (2007) 3528-3534.
- [6] M. Hambourger, M. Gervaldo, D. Svedruzic, P. W. King, D. Gust, M. Ghirardi, A. L. Moore, T. A. Moore, [FeFe]-hydrogenase-catalyzed H₂ production in a photoelectrochemical biofuel cell, *J. Am. Chem. Soc.* 130 (2008) 2015-2022.
- [7] E. Reisner, D. J. Powell, C. Cavazza, J. C. Fontecilla-Camps, F. A. Armstrong, Visible light-driven H(2) production by hydrogenases attached to dye-sensitized TiO(2) nanoparticles, *J. Am. Chem. Soc.* 131 (2009) 18457-18466.
- [8] K. A. Brown, S. Dayal, X. Ai, G. Rumbles, P. W. King, Controlled assembly of hydrogenase-CdTe nanocrystal hybrids for solar hydrogen production, *J. Am. Chem. Soc.* 132 (2010) 9672-9680.
- [9] S. Morra, F. Valetti, S. J. Sadeghi, P. W. King, T. Meyer, G. Gilardi, Direct electrochemistry of an [FeFe]-hydrogenase on a TiO₂ electrode, *Chem. Commun.* 47 (2011) 10566-10568.
- [10] M. B. Wilker, K. E. Shinopoulos, K. A. Brown, D. W. Mulder, P. W. King, G. Dukovic, Electron transfer kinetics in CdS nanorod-[FeFe]-hydrogenase complexes and implications for photochemical H₂ generation, *J. Am. Chem. Soc.* 136 (2014) 4316-4324.
- [11] G. Goldet, C. Brandmayr, S. T. Stripp, T. Happe, C. Cavazza, J. C. Fontecilla-Camps, F. A. Armstrong, Electrochemical kinetic investigations of the reactions of [FeFe]-hydrogenases with carbon monoxide and oxygen: comparing the importance of gas tunnels and active-site electronic/redox effects, *J. Am. Chem. Soc.* 131 (2009) 14979-14989.
- [12] D. Svedruzic, J. L. Blackburn, R. C. Tenent, J. D. R. Rocha, T. B. Vinzant, M. J. Heben, P. W. King, High-performance hydrogen production and oxidation electrodes with hydrogenase supported on metallic single-wall carbon nanotube networks, *J. Am. Chem. Soc.* 133 (2011) 4299-4306.
- [13] K. A. Brown, M. B. Wilker, M. Boehm, G. Dukovic, P. W. King, Characterization of photochemical processes for H₂ production by CdS nanorod-[FeFe] hydrogenase complexes, *J. Am. Chem. Soc.* 134 (2012) 5627-5636.
- [14] F. J. M. Hoeben, I. Heller, S. P. J. Albracht, C. Dekker, S. G. Lemay, H. A. Heering, Polymyxin-coated Au and carbon nanotube electrodes for stable [NiFe]-hydrogenase film voltammetry, *Langmuir* 24 (2008) 5925-2931.
- [15] E. Lojou, M. Brugna, N. Candoni, S. Dementin, M. T. Giudici-Ortoni, Biocatalysts for fuel cells: efficient hydrogenase orientation for H₂ oxidation at electrodes modified with carbon nanotubes, *J. Biol. Inorg. Chem.* 13 (2008) 1157-1167.
- [16] H. Krassen, S. Stripp, G. von Abendorth, K. Ataka, T. Happe, J. Heberle, Immobilization of the [FeFe]-hydrogenase CrHydA1 on a gold electrode: design of a catalytic surface for the production of molecular hydrogen, *J. Biotechnol.* 142 (2009) 3-9.
- [17] H. Krassen, S. T. Stripp, N. Böhm, A. Berkessel, T. Happe, K. Ataka, J. Heberle, Tailor-Made Modification of a Gold Surface for the Chemical Binding of a High-Activity [FeFe] Hydrogenase, *Eur. J. Inorg. Chem.* (2011) 1138-1146.
- [18] C. Madden, M. D. Vaughn, I. Diez-Pérez, K. A. Brown, P. W. King, D. Gust, A. L. Moore, T. A. Moore, Catalytic turnover of [FeFe]-hydrogenase based on single-molecule imaging, *J. Am. Chem. Soc.* 134 (2012) 1577-1582.
- [19] C. Baffert, K. Sybirna, P. Ezanno, T. Lautier, V. Hajj, I. Meynial-Salles, P. Soucaille, H. Bottin, C. Lèger, Covalent attachment of FeFe hydrogenases to carbon electrodes for direct electron transfer, *Anal. Chem.* 84 (2012) 7999-8005.

- [20] K. A. Vincent, A. Parkin, F. A. Armstrong, Investigating and exploiting the electrocatalytic properties of hydrogenases, *Chem. Rev.* 107 (2007) 4366-4413.
- [21] B. La Licata, F. Sagnelli, A. Boulanger, A. Lanzini, P. Leone, P. Zitella, M. Santarelli, Investigating and exploiting the electrocatalytic properties of hydrogenases, *Int. J. Hydrogen Energy* 36 (2011) 7861-7865.
- [22] S. Morra, M. Arizzi, P. Allegra, B. La Licata, F. Sagnelli, P. Zitella, G. Gilardi, F. Valetti, Expression of different types of [FeFe]-hydrogenase genes in bacteria isolated from a population of a bio-hydrogen pilot-scale plant, *Int. J. Hydrogen Energy* 39 (2014) 9018-9027.
- [23] S. Morra, B. Mongili, S. Maurelli, G. Gilardi, F. Valetti, Isolation and Characterization of a New [FeFe]-Hydrogenase from *Clostridium perfringens*, *Biotechnol. Appl. Biochem.* (2015) in press doi: 10.1002/bab.1382.
- [23] P. W. King, M. C. Posewitz, M. L. Ghirardi, M. Seibert, Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system, *J. Bacteriol.* 188 (2006) 2163-2172.
- [24] J. Zhang, Q. Xu, Z. Feng, M. Li, C. Li, Importance of the relationship between surface phases and photocatalytic activity of TiO₂, *Angew. Chem. Int. Ed.*, 2008, 47, 1766-1769.
- [25] W. J. Foo, C. Zhang, G. W. Ho, Non-noble metal Cu-loaded TiO₂ for enhanced photocatalytic H₂ production, *Nanoscale* 5 (2013) 759-764.
- [26] M. Ni, M. K. H. Leung, D. Y. C. Leung and K. Sumathy, A review and recent developments in photocatalytic water-splitting using TiO₂ for hydrogen production, *Renewable Sustainable Energy Rev.* 11 (2007) 401-425.
- [27] S. Livraghi, M. C. Paganini, E. Giamello, A. Selloni, C. Di Valentin, G. Pacchioni, Origin of photoactivity of nitrogen-doped titanium dioxide under visible light, *J. Am. Chem. Soc.*, 2006, 128, 15666-15671.
- [28] G. Barolo, S. Livraghi, M. Chiesa, M. C. Paganini, E. Giamello, Mechanism of the Photoactivity under Visible Light of N-Doped Titanium Dioxide. Charge Carriers Migration in Irradiated N-TiO₂ Investigated by Electron Paramagnetic Resonance, *J. Phys. Chem. C* 116 (2012) 20887-20894.
- [29] J. Huo, Y. Hu, H. Jiang, C. Li, In situ surface hydrogenation synthesis of Ti³⁺ self-doped TiO₂ with enhanced visible light photoactivity, *Nanoscale* 6 (2014) 9078-9084.
- [30] I. Yacoby, L. T. Tegler, S. Pochekeilov, S. Zhang, P. W. King, Optimized expression and purification for high-activity preparations of algal [FeFe]-hydrogenase, *PLoS ONE* 7 (2012) e35886.
- [31] S. Morra, A. Giraud, G. Di Nardo, P. W. King, G. Gilardi, F. Valetti, Site saturation mutagenesis demonstrates a central role for cysteine 298 as proton donor to the catalytic site in CaHydA [FeFe]-hydrogenase, *PLoS ONE* 7 (2012) e48400.
- [32] A. Barth, Infrared spectroscopy of proteins, *Biochim. Biophys. Acta* 1767 (2007) 1073-1101.
- [33] V. Fourmond, C. Baffert, K. Sybirna, S. Dementin, A. Abou-Hamdan, I. Meynial-Salles, P. Soucaille, H. Bottin, C. Léger, The mechanism of inhibition by H₂ of H₂-evolution by hydrogenases, *Chem. Commun.* 49 (2013) 6840-6842.
- [34] V. Fourmond, T. Lautier, C. Baffert, F. Leroux, P.-P. Liebgott, S. Dementin, M. Rousset, P. Arnoux, D. Pignol, I. Meynial-Salles, P. Soucaille, P. Bertrand, C. Léger, Correcting for electrocatalyst desorption and inactivation in chronoamperometry experiments, *Anal. Chem.* 81 (2009) 2962-2968.
- [35] V. Hajj, C. Baffert, K. Sybirna, I. Meynial-Salles, P. Soucaille, H. Bottin, V. Fourmond, C. Léger, Reductive inactivation of FeFe hydrogenase and implication for catalysis, *Energy Environ. Sci.* 7 (2014) 715-719.
- [36] K. K. Chittur, FTIR/ATR for protein adsorption to biomaterial surfaces, *Biomaterial*, 19 (1998) 357-69.
- [37] M. Müller, B. Kessler, N. Houbenov, K. Bohatá, Z. Pientka, E. Brynda, pH dependence and protein selectivity of poly(ethyleneimine)/poly(acrylic acid) multilayers studied by in situ ATR-FTIR spectroscopy, *Biomacromolecules* 7 (2006) 1285-1294.
- [38] C. E. Lubner, R. Grimme, D. A. Bryant, J. H. Golbeck, Wiring photosystem I for direct solar hydrogen production, *Biochemistry* 49 (2010) 404-414.
- [39] C. E. Lubner, A. M. Applegate, P. Knörzer, A. Ganago, D. A. Bryant, T. Happe, J. H. Golbeck, Solar hydrogen-producing bionanodevice outperforms natural photosynthesis, *Proc. Natl. Acad. Sci.* 108 (2011) 20988-20991.
- [40] M. G. Walter, E. L. Warren, J. R. McKone, S. W. Boettcher, Q. Mi, E. A. Santori, N. S. Lewis, Solar water splitting cells, *Chem. Rev.* 110 (2010) 6446-6473.