

Electronic Supporting Information (ESI)

Catalytic Electrochemistry of a [NiFeSe]-Hydrogenase on TiO₂ and Demonstration of its Suitability for Solar H₂ Production

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Contents

Experimental Section	page 2
Results and Discussion	page 6
References	page 8
Supporting Figures S1-S5	page 9

Experimental Section.

General Considerations. All starting reagents were obtained from commercial suppliers and were of the highest available purity: they were used as received unless otherwise noted. The [NiFeSe]-hydrogenase was purified from French pressed *Desulfomicrobium baculatum* cells using a previously published method.¹ The final homogeneous enzyme was assessed by mass spectrometry, with measurement of 55 kDa and 31 kDa for the respective masses of the large and small subunits. A mixed buffer system was used for the PFV experiments: this solution consisted of 15 mM in each of sodium acetate, MES (2-[N'-morpholino]ethanesulfonic acid), HEPES (N'-[2-hydroxyethyl]-piperazine-N'-2-ethanesulfonic acid), TAPS (N'-tris[hydroxymethyl]-methyl-3-amino-propanesulfonic acid), and CHES (2-[N'-cyclohexylamino]ethane-sulfonic acid), with 0.10 M NaCl as supporting electrolyte. For the photocurrent and photocatalysis experiments a pH 7.0 buffer consisting of 25 mM Tris (tris(hydroxymethyl)aminomethane) and 25 mM EDTA (ethylenediaminetetraacetic acid tetrasodium salt) was used. All solutions were titrated with dilute NaOH or HCl to the desired pH at the experimental temperature. Purified water (Millipore: 18 M Ω cm) was used for all measurements and the [NiFeSe]-H₂ase was handled in a VAC glovebox under an anaerobic N₂ atmosphere (O₂ less than 2 ppm). TiO₂(F2) nanoparticles (60 nm particle size; pure anatase) from Shova Denko were provided by Prof. M. Grätzel and Dr. K. Sivula from the Ecole Polytechnique Fédérale de Lausanne, Switzerland, and TiO₂(P25) particles (21 nm particle size; 80% anatase, 20% rutile; BET: 50±15 m² g⁻¹) from Evonik (former Degussa).

Preparation of Sensitizers and Attachment to TiO₂ Nanoparticles. [Ru(bipy)₂(4,4'-(PO₃H₂)₂bipy)]Br₂² (RuP; bipy = 2,2'-bipyridine), [Ru(bipy)₂(4,4'-(CO₂H)₂bipy)]Cl₂³ (RuC), and [Ru(NCS)₂(4,4'-(CO₂H)₂bipy)₂]⁴ (N3) were prepared as reported previously and their chemical structures are depicted in the insert of Figure S3. Complexes RuC and RuP were isolated upon replacement of the chlorido ligands of [Ru^{II}Cl₂(bipy)₂] by 4,4'-dicarboxy-2,2'-bipyridine or diethyl 4,4'-diphosphonato-2,2'-bipyridine, respectively. The phosphonated esters in the precursor form of RuP were hydrolyzed with Me₃SiBr in MeOH. Photosensitizer N3 was prepared from commercially available Ru^{III}Cl₃·3H₂O *via* [Ru^{II}Cl₂(4,4'-

(CO₂H)₂bipy)₂] and replacement of the chlorido ligands by thiocyanate. Multinuclear-NMR, ESI-MS, IR and UV-vis spectroscopy confirmed the composition and purity of the prepared compounds. Although RuP was used for all photochemical experiments in the main text, the photocatalytic properties of RuP-TiO₂ at buffered pH 7.0 have also been compared with RuC and N3 attached to TiO₂. These results justified the suitability of RuP for TiO₂ sensitization for the H₂ production experiments (see below).

The RuP dye-sensitized TiO₂ nanoparticles (20 μmol sensitizer per g TiO₂) were prepared by the following procedure: RuP (3.6 mg, 4.0 μmol) dissolved in water (1 mL) was added dropwise to an aqueous slurry of TiO₂ nanoparticles (200 mg) in water (19 mL) under vigorous stirring. The resulting mixture was stirred at room temperature overnight under protection of light by aluminum foil resulting in quantitative adsorption of RuP. No absorption peaks in the visible region of the UV-visible spectrum (GBC Cintra 10 UV-visible spectrometer) were observable in the supernatant upon centrifugation. Quantitative attachment was also achieved with RuC and N3 at pH 3.0 (no significant UV-visible absorbance due to Ru complexes was detected upon filtration), but the carboxylate-linked chromophores RuC and N3 desorbed readily and almost quantitatively upon increasing the pH to 7.0 in EDTA/Tris buffered solutions. A detailed discussion of adsorption of RuC and RuP on TiO₂ can be found in the literature.⁵

Electrochemical Measurements. The TiO₂-ITO (ITO = indium-doped tin oxide) working electrodes (0.25 cm² TiO₂ surface area) were prepared as described in the literature.⁶ A viscous suspension of TiO₂ nanoparticles in 0.10 M nitric acid (25–50 mg/75 μL) was sonicated for 10 min and then spread onto ITO-coated glass using a glass slide and an adhesive tape spacer to protect the rest of the conducting glass substrate from the suspension. The thin TiO₂ film was dried in air at 80 °C for several hours, annealed at 450 °C for 0.5 h and used within 1-2 days. The pyrolytic graphite edge (PGE) electrodes (0.2 mm diameter) were polished with an aqueous slurry of 1.0 μm α-alumina-powder and sonicated for several seconds prior to use. Each film of [NiFeSe]-H₂ase was prepared by dipping the working electrode (either TiO₂ or PGE) in a dilute solution of the enzyme (*ca.* 2 μM hydrogenase in a mixed buffer solution at pH 7.0 at room

temperature under N₂ atmosphere) containing a co-adsorbate, polymyxin B sulfate (0.20 mg mL⁻¹), for 30 s and then immersing the electrodes immediately into the electrochemical cell solution. However, the co-adsorbate was later found to be not essential for a good film growth on TiO₂-ITO electrodes.

Protein film voltammetry (PFV) was performed using an Autolab PGSTAT 20 electrochemical analyzer controlled by GPES software (Eco Chemie, The Netherlands), and equipped with a digital staircase scan generator. A two-compartment three-electrode glass electrochemical cell was used with a platinum auxiliary electrode and a saturated calomel reference electrode (SCE) held in a sidearm separated from the main cell compartment and linked by a Luggin capillary. The cell was thermostated by a water jacket connected to a water flow thermostat at 20 °C, and the reference electrode sidearm was maintained at room temperature. Blank cyclic voltammograms with the TiO₂-ITO and PGE working electrodes without enzyme on the electrode, or the ITO electrodes (no TiO₂) immersed into the solution of [NiFeSe]-H₂ase, showed no current due to H₂ oxidation or proton reduction over the range of at least 0.0 to -0.8 V *vs.* SCE. The gas saturation in the cell solutions was maintained with either H₂ (Premier Grade, Air Products) or N₂ (Oxygen Free, BOC) by bubbling through the cell solutions during measurements. The potentials are quoted versus SCE, and can be converted to the standard hydrogen electrode (SHE) by adding +245 mV at 20 °C.⁷ In several cases the voltammetric response of protein-free as well as protein-loaded TiO₂ films showed charging/discharging currents with variable extent at *ca.* -0.8 to -0.6 V *vs.* SCE, which are characteristic for electron injection into sub-bandgap states of the metal oxide films. The term film loss refers to a decrease in current due to enzyme desorption, enzyme denaturation or unfavorable conformational rearrangement on the electrode surface.

The photocurrent experiments were performed with an applied voltage of 0.5 V *vs.* SCE using a pH 7.0 buffer (25 mM EDTA / 25 mM Tris) in air. The freshly annealed TiO₂-ITO electrodes were immersed into a 10⁻⁴ M solution of the sensitizer (pH 3.0 ± 0.2) overnight. For the less soluble sensitizer N3, a saturated solution was used. The dye-sensitized electrodes were then removed, carefully rinsed with water and put into the cell solution 15 min prior to measurements to allow for equilibration in the pH 7.0 buffer solutions. Measurements were

performed at 20 °C, with the first 5 min being a dark period followed by visible light illumination (UV cut-off filter; $\lambda > 400$ nm) with a focused 250 W tungsten halogen lamp. After 65 min a dark period (100 s) was applied and followed again by illumination. The lamp was switched on at least five minutes prior illumination to allow for equilibration of light intensity and the focused light beam was covered with an Al foil during the dark periods.

Photocatalytic Experiments and H₂ Quantification. The samples for photocatalytic H₂ production were prepared in a glovebox under a N₂ atmosphere. The RuP-sensitized TiO₂ particles (5 mg; 0.1 μ mol RuP, see above) were suspended in EDTA (25 mM)/Tris (25 mM) buffer (4.5 mL, pH 7.0) in a Pyrex pressure reaction vessel and subjected to ultrasound treatment at room temperature to form a colloidal slurry. Then [NiFeSe]-H₂ase (10 μ L of 5 μ M solution) was added under vigorous stirring. The reaction vessel was sealed with a septum and taken out of the glovebox. Then Ar was bubbled through the reaction mixture to remove N₂ and traces of H₂, whereupon a fixed amount of an internal standard (CH₄) was injected into the headspace of the reaction vessel. The photocatalytic experiments were performed by visible light illumination (UV cut-off filter; $\lambda > 420$ nm) with a focused 250 W tungsten halogen lamp of a stirred colloidal RuP-TiO₂-H₂ase particle reaction mixture thermostated at 25 °C with a water-jacket. The light intensity (45 mW cm⁻²) was measured with a Melles Griot Broadband Power/Energy Meter 13PEM001. The pH of the reaction mixture was measured after 8 h of illumination and was in all cases between 7.0 and 7.2. Blank experiments in the absence of RuP, TiO₂ or [NiFeSe]-H₂ase under the same experimental conditions showed no detectable amounts of H₂ or only tiny traces of H₂. The amount of H₂ produced was quantified using a Unicam Pro-GC gas chromatograph with a 5 Å molecular sieve column, thermal conductivity detector and Ar carrier gas with a flow rate of 2 mL min⁻¹. The column oven was held isothermally at 40 °C during measurements. Headspace gas samples were injected into the spectrometer with a gas-tight Hamilton syringe. The response factor for H₂/CH₄ ($rf(\text{H}_2/\text{CH}_4) = 3.87 \pm 0.03$) under experimental conditions was established by calibration with known amounts of H₂ and CH₄, and was determined before and after a series of measurements.

Results and Discussion.

PFV Studies with [NiFeSe]-H₂ase: TiO₂(P25) vs. TiO₂(F2) vs. PGE. Two different types of TiO₂ nanoparticles, Evonik's (formerly Degussa) P25, TiO₂(P25), and Shova Denko's F2, TiO₂(F2), with 21 and 60 nm medium particle sizes, respectively, were used for electrochemical and photochemical experiments. Both TiO₂-ITO electrodes showed increased [NiFeSe]-H₂ase film stability compared to PGE, and their time-dependent voltammetric response with stationary working electrodes is depicted in Figures 2a, S1 and S2.⁸

Furthermore, TiO₂-ITO electrodes with adsorbed enzymes show high current densities [up to three times higher for TiO₂(F2)-ITO than PGE]. The surface topologies of PGE and nanocrystalline TiO₂ electrodes have been studied in detail by scanning electron microscopy.⁹ Although a PGE surface is very rough, the mesoscopic TiO₂-electrodes are likely to have, in addition, many pores and channels for the enzyme to become entrapped; a property that could explain the stability and high substrate turnover density of H₂ase on TiO₂-ITO. These properties make H₂ases adsorbed on TiO₂-ITO not only suitable for photocatalytic application, but also for future fundamental electrochemical studies of redox-proteins, electrodes in enzyme fuel-cell devices, solid-state electron relays for electron-transfer between adsorbed pairs of proteins, and spectro-electrochemical applications.

Selection of RuP Sensitizer: RuP vs. RuC vs. N3. The stable attachment of a ruthenium sensitizer to TiO₂ under the employed experimental conditions (buffered pH 7 solution) was essential prior attaching Ru-TiO₂ to [NiFeSe]-H₂ase for solar H₂ production. We compared three ruthenium dyes for attachment to TiO₂: (i) [Ru^{II}(NCS)₂(4,4'-(CO₂H)₂bipy)₂] (N3), which is a benchmark sensitizer for photo-voltaic Grätzel type solar cells, (ii) [Ru^{II}(bipy)₂(4,4'-(CO₂H)₂bipy)]Cl₂ (RuC) with three bipyridyl ligands, and (iii) its phosphonic acid analogue [Ru^{II}(bipy)₂(4,4'-(PO₃H)₂bipy)]Cl₂ (RuP). The photocurrent generation profiles of the ruthenium-dye sensitized TiO₂(P25)-ITO electrodes with an applied voltage of 0.5 V vs. SCE in air at pH 7.0 are shown in Figure S3. The larger the photocurrent the more electrons are injected from the adsorbed sensitizers to the

conduction band of the TiO₂ electrode. The minor photocurrents of N3 and RuC are due to almost quantitative detachment of the carboxylate-linked sensitizers from the TiO₂-ITO electrodes in the buffered pH 7.0 solution (confirmed by UV-vis spectroscopy), whereas the strong attachment of the phosphonic groups in RuP to TiO₂ allowed for quantitative attachment and large photocurrents upon visible light irradiation ($\lambda > 400$ nm).^{2,5} The photocurrent response of visible light illuminated ($\lambda > 400$ nm) RuP-TiO₂(F2)-ITO electrodes is depicted in Figure S4.

Photo-hydrogen Production Experiments: TiO₂(P25) vs. TiO₂(F2). The observed fast interfacial electron transfer, excellent electrocatalytic H₂ production activity and stable attachment of [NiFeSe]-H₂ase on TiO₂ with the enzyme ability to catalyse H₂ production without rigorous anaerobic conditions allow us to use this hybrid system for photocatalytic H₂ production. Both RuP-TiO₂(P25) and RuP-TiO₂(F2) were employed in the photo-hydrogen production experiments. Sensitization of the TiO₂-H₂ase conjugates resulted in a large increase in H₂ production for both nanoparticles upon visible light irradiation: GC analysis showed *ca.* 1.9×10^5 turnovers (mol H₂ per mol H₂ase in the H₂ production assembly) for RuP-TiO₂(P25)-H₂ase (Figure 2c) and *ca.* 1.0×10^5 for RuP-TiO₂(F2)-H₂ase (Figure S5) after 8 h of illumination. Control experiments (in the absence of RuP, TiO₂, H₂ase or light) showed only tiny traces of H₂ production, with the largest amount being formed in the TiO₂(F2)-H₂ase (no RuP) system (TON = 6×10^3) which may arise from trace contamination of the TiO₂ particles. The excellent photocatalytic properties of TiO₂(P25) nanoparticles have been widely recognized.¹⁰ The higher H₂ production rates using TiO₂(P25) than TiO₂(F2) confirm the unique properties of TiO₂(P25) and make it a bench-mark solid-state electron-relay/photocatalyst for our future enzyme-catalyzed H₂ production experiments.

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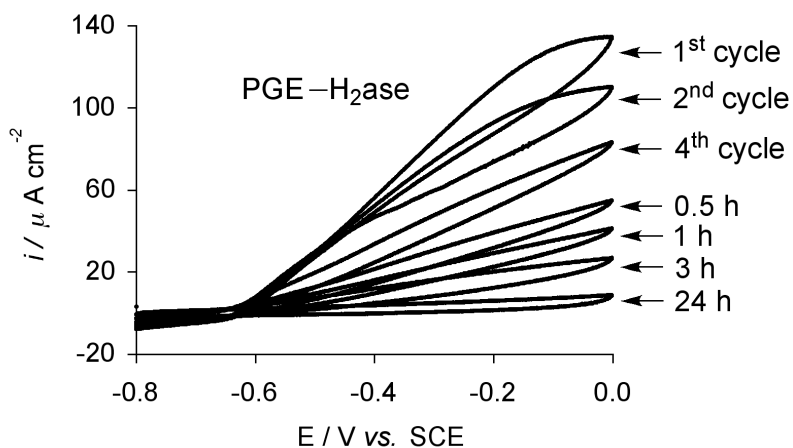


Figure S1. Protein film voltammograms of [NiFeSe]-H₂ase on a pyrolytic graphite edge working electrode under 1 bar H₂ at 20 °C, pH 7.0, at a scan rate of 10 mV s⁻¹ showing consecutive scans (cycles indicated) and measurements after 0.5 h, 1 h, 3 h and 24 h.

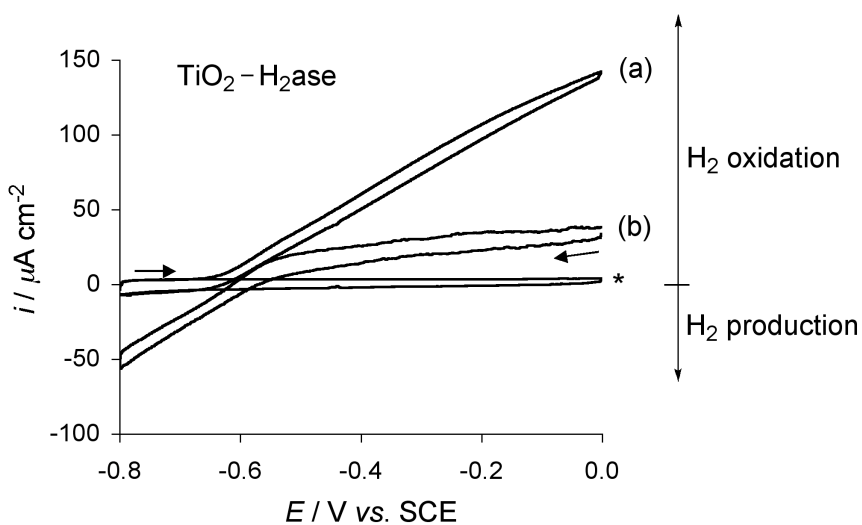


Figure S2. Protein film voltammograms of [NiFeSe]-H₂ase adsorbed to a TiO₂(P25)-ITO working electrode at pH 7.0 at 20 °C at a scan rate of 10 mV s⁻¹ under (a) 1 bar H₂ and (b) 1 bar N₂ (in the presence of small amounts of H₂ that were present in the glovebox atmosphere; an open electrochemical cell was used). Control scan in the absence of H₂ase is marked with an asterisk. The protein film voltammogram of H₂ase-TiO₂(F2)-ITO is shown in Figure 2a, main text.

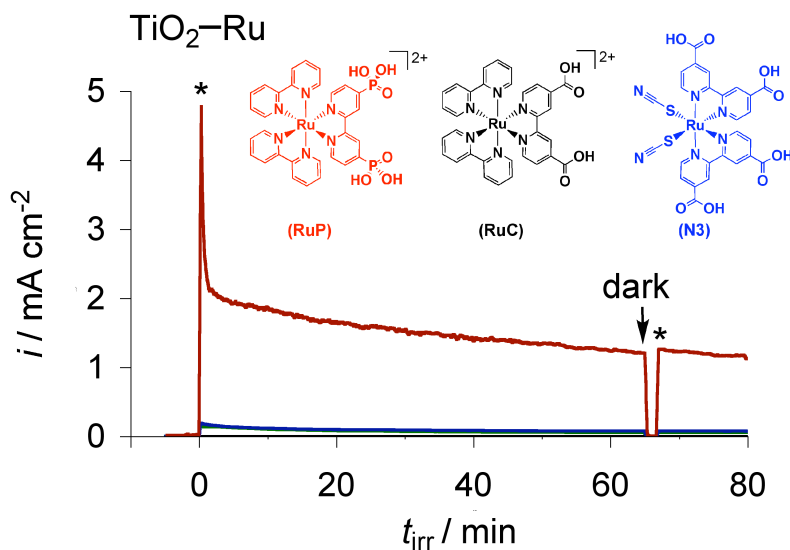


Figure S3. Visible light-induced ($\lambda > 400 \text{ nm}$) photocurrent obtained with ruthenium dye-sensitized $\text{TiO}_2(\text{P25})\text{-ITO}$ electrodes at pH 7.0 in EDTA/Tris buffer with an applied voltage of 0.5 V *vs.* SCE in air. The response of a freshly prepared RuP- $\text{TiO}_2\text{-ITO}$ with chromophore RuP (phosphonate linkers) is shown in red and for RuC and N3 (carboxylate linkers) in green and blue, respectively. The insert displays the chemical structures of RuP, RuC and N3. An asterisk indicates illumination start and a short dark period after 65 min is also shown.

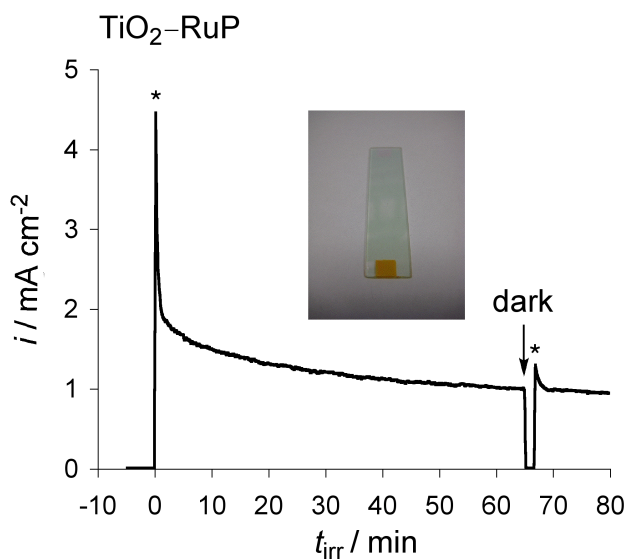


Figure S4. Visible light-induced ($\lambda > 400 \text{ nm}$) photocurrent obtained with a dye-sensitized RuP- $\text{TiO}_2(\text{F2})\text{-ITO}$ electrode at pH 7.0 in EDTA/Tris buffer with an applied voltage of 0.5 V *vs.* SCE in air. The photoanode has been sensitized at pH 3.0 overnight and was immersed into the pH 7.0 buffer 15 min prior measurement. An asterisk indicates the beginning of illumination periods and a dark period is also shown. The photo-current response for RuP- $\text{TiO}_2(\text{P25})\text{-ITO}$ is shown in Figures 2b (main text) and S3. The insert shows a RuP- $\text{TiO}_2\text{-ITO}$ electrode.

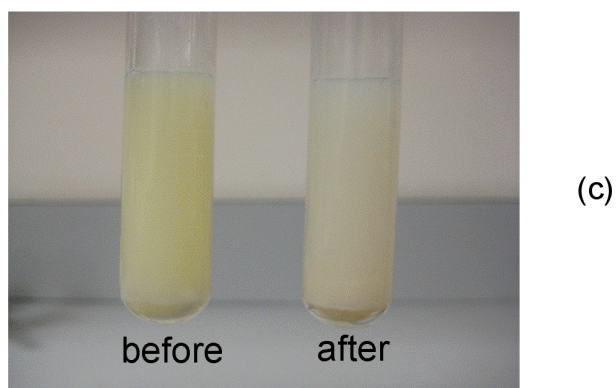
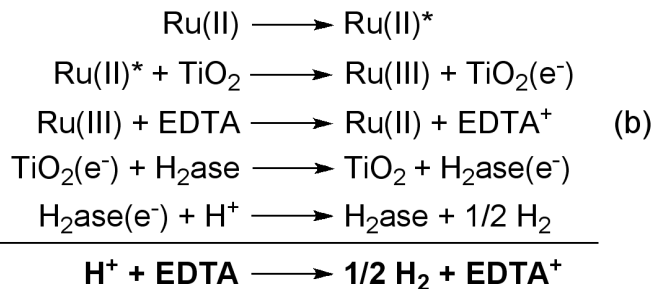
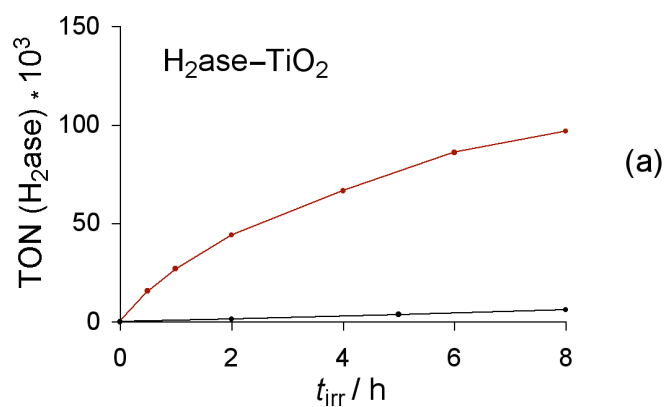


Figure S5. (a) Turnover numbers (in thousands) for H₂ production with respect to H₂ase from photocatalytic hydrogen production experiments ($\lambda > 420$ nm) containing RuP-TiO₂(F2)-H₂ase particles. Control scan (no dye) shown in black. The photocatalytic response for RuP-TiO₂(P25)-H₂ase is shown in Figure 2c, main text. (b) Overall reaction scheme of sacrificial photo-H₂ production. (c) A colloidal RuP-TiO₂-H₂ase assembly in sacrificial electron donor buffer before (left) and after (right) irradiation for 8 h.