Supporting Information (SI)

Direct Visible Light Activation of a Surface Cysteine-engineered [NiFe]-hydrogenase by Silver Nanoclusters

Liyun Zhang [a], Stephen E Beaton [a], Stephen B Carr [b, c] and Fraser A. Armstrong [a, *]

[a] Inorganic Chemistry Laboratory, Department of Chemistry, University of Oxford, South Parks Road, Oxford OX1 3QR, Oxfordshire, United Kingdom.

[b] Research Complex at Harwell, Rutherford Appleton Laboratory, Harwell Oxford, Didcot, United Kingdom.

[c] Department of Biochemistry, University of Oxford, Oxford OX1 3QU, Oxfordshire United Kingdom

[*] e-mail: fraser.armstrong@chem.ox.ac.uk

Table of content

Methods

Molecular biology

All electrochemistry and solution assays were performed with wild type and variant Hyd-2 enzymes produced using *E. coli* K-12 strain HJ001-hyp or HJ001-hyp-3S with the appropriate plasmid (Supplementary Table S2-S4). The Y'222C mutation was introduced in pO^c and the three cysteine-to-serine mutations (C197S, C432S, and C433S) were introduced into pMAK-hybC. All plasmid constructs were confirmed by DNA sequencing. The mutated hybC gene was transferred from pHybC-3S to the HJ001-hyp chromosome as previously described [1] and again, whole gene integrity was confirmed by DNA sequencing. Hyd-2 was then produced by expressing either the pO^c or pO^c Y'222C in HJ001-hyp or HJ001-hyp-3S depending on the variant.

The WT-Hyd-2, Y'222C-Hyd-2, 3S-Hyd-2 and 3S1C-Hyd-2 enzymes were prepared and crystallized as described previously.[2] X-ray diffraction data were collected at beamline i04-1 (Diamond Light Source, UK) to a maximum resolution of 1.7 Å using a Pilatus 6M-F detector and a wavelength of 0.92 Å. Data reduction was performed automatically with the autoPROC pipeline[3] running XDS[4] and Aimless[5]. Initial phase estimates were calculated by molecular replacement using Phaser[6] with wild type Hyd-2 (PDB ID **6EHQ**) as a search model. Variant amino acids were mutated and fitted manually using COOT[6] followed by restrained refinement in REFMAC5.[7] The resulting model showed excellent stereochemistry as assessed molprobity.[8]

Electrochemistry methods

All electrochemistry was performed in an anaerobic glove box containing a N_2 atmosphere (O_2 < 2 ppm. VAC box). Measurements were carried out using an Autolab potentiostat (PGSTAT128N) controlled by Nova software (EcoChemie). The three-electrode system comprised a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference. All experiments were carried out in 0.2 M MES buffer prepared by dissolving 2-(N-morpholino)ethanesulfonic acid (MES, Melford) in ultrapure water (Milli-Q, 18 MΩ cm).

Precise gas flow rates (BOC gases, high purity) were created using mass flow controllers (Sierra Instruments).

Fabrication of electrodes from TiO_2 particles

Indium tin oxide (ITO) slides (SPI Supplies, USA) of approximate dimensions 1 x 2 cm were cleaned by sonication for 15 min in ethanol, followed by acetone, and finally deionized H_2O. The slides were dried in the oven. Weighed amounts of TiO_2 powder (Degussa, P-25, 20 mg) and iodine (10 mg) were suspended in 20 mL acetone (Sigma-Aldrich). The suspension was sonicated for 30 min and then electrophoretic deposition was performed (10 V, 3 min) to create a film of TiO_2 on the surface of the ITO slides.[5, 9] The slides were converted into electrodes by making an ohmic contact with Cu wire on the uncoated area of the conducting glass and sealing with non-conducting epoxy.

Modification of TiO_2 electrodes by AgNCs-PMAA

A solution of AgNCs-PMAA was prepared as described previously.[10] The TiO_2 electrodes were modified by soaking in 10 mL of AgNCs-PMAA solution in the dark (24 hours at room temperature). The electrodes were thoroughly washed using distilled H_2O before testing.

Electrochemistry of hydrogenases attached to TiO_2 electrode surfaces

To study the electrochemistry of Hyd-2 and its variants on TiO_2 and AgNCs-PMAA/TiO_2 electrodes, aliquots of enzyme were drop-casted onto the electrode and left for a few minutes until partially dry. In a typical experiment, 10 µL of 10 µM enzyme was applied to an electrode with area 0.25 cm^2 (0.5 cm × 0.5 cm). All potentials were converted to the Standard Hydrogen Electrode (SHE) scale using the correction $E_{SHE} = E_{SCE} + 238$ mV at 30 °C.

Electrochemistry of hydrogenases attached to Au electrode surfaces

The gold working electrode consisted of a 2.5 mm length of gold wire (99.9985%, Alfa, UK; diameter 2 mm) attached to a brass rod using silver-load epoxy adhesive (RS components, UK).

The rod was glued into a Teflon casing using Araldite (3:1 CY1300:HY1300 (Ciba Geigy)), while the gold wire was embedded in an ethanol-resistant, epoxy resin (3:1 CY1300:HY932(Ciba Geigy)), which was cured by placing the electrode in a 80°C oven overnight then in a 140°C oven for a further 4 hours. Upon polishing, a flat, circular surface of gold becomes exposed to the solution. Prior to the voltammetry, the Au surface was cleaned mechanically by polishing successively with aqueous slurries of 1 μm, 0.3 μm and 0.05 μm Alumina; the Au surface was next cleaned electrochemically by cycling five times between 0.44 and 1.24 V. in 0.1M H_2SO_4 solution, at a scan rate of 0.1 V/s, starting and ending at 0.64 V. The upper limit was then increased to 1.59 V and a further fifteen cycles were performed before rinsing the electrode with water. After removing any excess water with tissue, the Hyd-2 enzyme was deposited by pipette (5 μL 2 mg/mL) and the electrode dried before recording cyclic voltammograms in 0.2M MES buffer pH 5.0 containing 0.1 M NaCl under 100% N_2, 25 ^{o}. All potentials were converted to the Standard Hydrogen Electrode (SHE) scale using the correction $E_{SHE} = E_{SCE} + 241$ mV at 25 °C.

Photocatalytic experiments and H_2 quantification

For photocatalytic experiments, the AgNCs-PMAA/TiO_2 nanoparticles were prepared as described previously.[11] All suspensions were prepared in a glovebox under a N_2 atmosphere before removal for measurements. Briefly, 5mg of AgNCs-PMAA/TiO_2 was dispersed and sealed in 5 mL of an aqueous solution of 0.1 M triethanolamine (TEOA), 0.1 M NaCl, pH 7.0 (all contained in a Pyrex pressure vessel, total volume = 22.5 mL). The suspension was sonicated for 15 minutes before purging with N_2 for 15 minutes. Subsequently, the Hyd-2 enzyme (20 μL of 12.5 μM solution) was added and the suspension stirred gently for 3 minutes to allow adsorption of the enzyme. For photocatalysis measurements, the stirred suspension was irradiated with visible light using a 300 W Arc Lamp, (Newport 67005) fitted with a 420 nm filter and held 5 cm from the vessel. The temperature was controlled by immersing the vessel in a water bath. Production of H_2 was monitored at regular intervals by removing small volumes (20 μL) of headspace gas for gas chromatography (GC) analysis. An Agilent 7890A series gas chromatograph (GC) with electronic pneumatic control optimized for trace gas analysis was used to monitor the

production of H_2 at regular intervals. Headspace gas samples of the reaction mixture were injected into the GC operating in pulsed splitless mode using a gas-tight Hamilton syringe. A 1.5 mm diameter split/splitless liner, a Restek ShinCarbon ST micropacked column, a micro thermal conductivity detector and high-purity N_2 carrier gas with a constant pressure of 25 psi were used.

H_2 production by other semiconductors/AgNCs-PMAA/3S1C-Hyd-2

Weighed amounts (100 mg) of pure anatase TiO_2, pure rutile TiO_2, ZnO, $SrTiO_3$ and ZrO_2 nanoparticles (<100 nm particle size, used as received from Sigma, CAS numbers 1317-70-0, 1317-80-2, 1314-13-2, 12060-59-2 and 1314-23-4, respectively) were suspended in 20 mL 5mg/mL AgNCs-PMAA aqueous solution by sonication for 15 min, then the suspension was protected by aluminum foil to avoid light and stirred at room temperature for 24 hours. Finally, the suspension was centrifuged at 3600 rpm/min for 20minutes. The powder was then washed by water and acetone. After drying, 5mg of AgNCs-PMAA/MO was dispersed and sealed in a 5 mL aqueous solution of 0.1 M triethanolamine (TEOA), 0.1 M NaCl, pH 7.0) contained in a Pyrex pressure vessel (total volume = 22.5 mL) by sonicating for 15 minutes. The solution was then purged with N_2 for 15 min. Subsequently, 3S1C- Hyd-2 (20 µL of 12.5 µM solution) was added and the suspension stirred gently for 3 minutes to allow adsorption of the enzyme. The stirred suspension was irradiated with visible light using a 300 W Arc Lamp, (Newport 67005) fitted with a 420 nm filter and held 5 cm from the vessel. The temperature was controlled by immersing the vessel in a water bath. Production of H_2 was monitored at regular intervals by removing small volumes (20 µL) of headspace gas for gas chromatography (GC) analysis.

Spectral measurements

Optical absorption spectra of the AgNC-PMAA solutions were acquired in the wavelength region from 200 nm to 800 nm using a Perkin Elmer Lambda 19 UV/Vis/NIR spectrophotometer. Emission spectra and photoluminescence titrations were recorded on a Fluorolog fluorescence spectrometer (HORIBA Scientific). All spectra were recorded with quartz cells of 10 mm path length.

Supplementary tables and figures

Table S1. The distances between the three surface-exposed cysteines (**S** atom) and Fe-S clusters (nearest **Fe** atom) or active site (**Ni** atom).

Cysteines	Cluster Name	Distance (Å)
C197	Distal	36
	Medial	25
	Proximal	25
	Active site	20
C432	Distal	36
	Medial	27
	Proximal	23
	Active site	17
C433	Distal	42
	Medial	33
	Proximal	30
	Active site	22

Table S2. Bacterial strains used in this study.

Strain	**Genotype**	**Purpose**
NEB DH5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	General cloning strain
HJ001-hyp	As HJ001, Δ*tatD::hypA1-X*	Used for production of native Hyd-2 and Y'222C
HJ001-hyp-3S	As HJ001-hyp, C197S, C432S, and C433S.	Used for production of 3S-Hyd-2 and 3S1C-Hyd-2.

Table S3. Oligonucleotide primers used, with their function and sequence.

Primer Name	**Primer Function**	**Primer Sequence**
HybO_Y'222C_F	Y'222C mutagenesis	5'- CCACCTCGGCTGTAAAGG-3'

	forwards primer	
HybO_Y'222C_R	Y'222C mutagenesis reverse primer	5'-TAAGTTTCTGGCCCTTTACAGCCGAGGTG GCACAGGCACCAGCCTTCG-3'
HybC_C433S_C432S_F	C433S_C432S mutagenesis forwards primer	5'-TCCACGCTGGGCCGTATTATTGGTCGTACC GTTCACAGTAGTGAATTGCAGGAT-3'
HybC_C433S_C432S_R	C433S_C432S mutagenesis reverse primer	5'-GTGAACGGTACGACCAATAATACGGCCCA GCGTGGA-3'
HybC_C197S_F	C197S mutagenesis forward primer	5'-TAGCGCACTACCTGCAAGCGTTGGAGAGCCA GCGTGACGC-3'
HybC_C197S_R	C197S mutagenesis reverse primer	5'-GCGTCACGCTGGCTCTCCAACGCTTGCAGGTA GTGCGCTA-3'

Table S4. The plasmid constructs used in this work

Plasmid	Identifier	Purpose	reference
pQE-80L hybO ΔTAT ΔTM C-terminal hexa-his tag	pO^C	Used for production of native Hyd-2 (in HJ001-Hyp) or 3S-Hyd-2 (in HJ001-hyp-3S)	2
pQE-80L hybO ΔTAT ΔTM,C-terminal hexa-his tag, Y'222C	pO^c Y'222C	Used for production of Y'222C (in HJ001-hyp) or 3S1C-Hyd-2 (in HJ001-hyp-3S)	This work
pMAK native *hybC*	pHybC	Used as parent plasmid for making the point mutations in *hybC*	This work
pMAK *hybC* C197S, C432S, and C433S	pHybC-3S	Contains three cysteines to serine mutations for *hybC*. Used to create HJ001-hyp-3S.	This work

Table S5. X-ray data collection and refinement statistics for the **3S1C-Hyd-2** variant.

Data collection	
PDB ID	6G7M
Space group	$P2_12_12_1$
Unit cell dimensions (Å)	a=100.24, b=100.98, c=169.42
Resolution (Å)	100.24-1.71 (1.74-1.71)
Total reflections	1387700 (67425)
Unique reflections	185232 (9153)
Completeness (%)	100 (100)
Multiplicity	7.5 (7.4)
$<I/\sigma>$	16.5 (2.2)
R_{merge} (%)	9.3 (83.8)
R_{pim} (%)	3.6 (33)
CC1/2	0.999 (0.819)
Refinement	
R_{work}/R_{free} (%)	15.7/19.0
No. of atoms	13668
Macromolecule	12729
Solvent	939
Average B-factors	
Macromolecule	18.8
Solvent	23.4
RMSD bond lengths (Å)	0.0159
RMSD angles (°)	1.63
Ramachandran plot (%) favoured / outliers	96.84/0.12
Clashscore	0.48

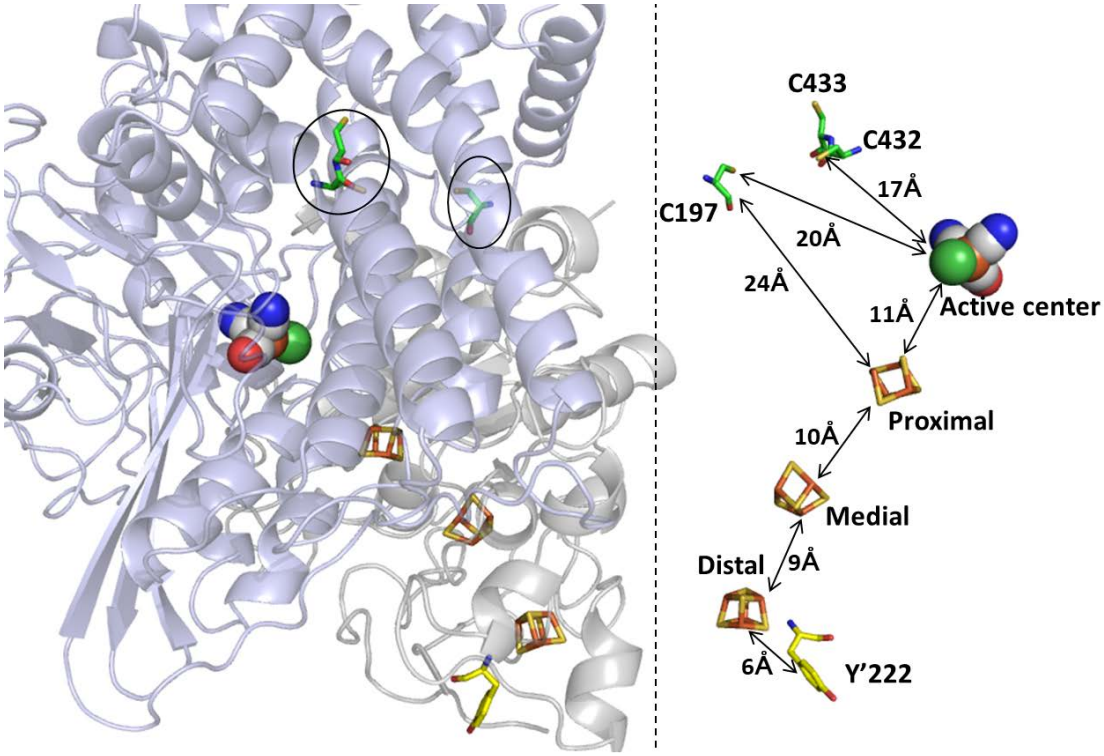

Figure S1. (Left) Location of surface-exposed cysteine residues in *E. coli* Hyd-2 (large subunit (α) blue, small subunit (β) grey). The three surface cysteines (C197, C432, C433) are circled and surface-exposed tyrosine 222 (Y'222) is shown in yellow. (Right) Distances between key groups, including the natural electron-transfer relay (using nearest S, Fe, atoms).

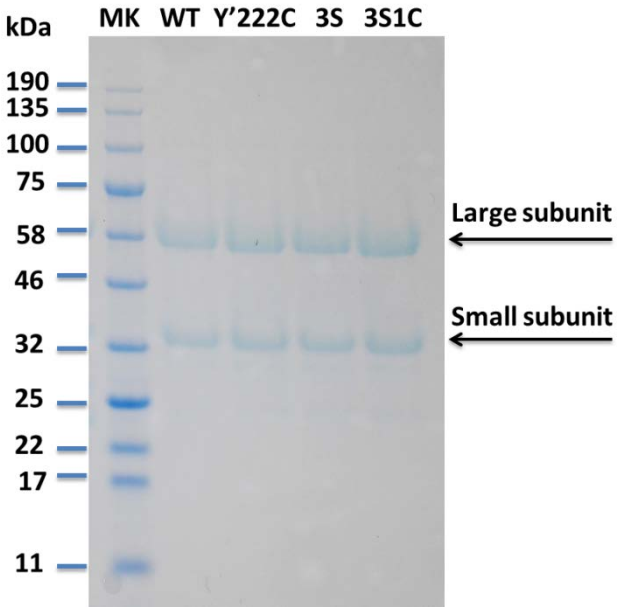

Figure S2. SDS-PAGE analysis of purified wt-Hyd-2, Y'222C-Hyd-2, 3S-Hyd-2 and 3S1C-Hyd-2.

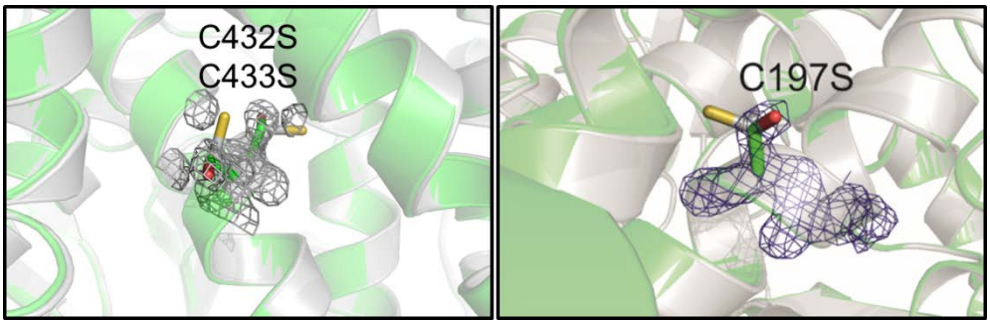

Figure S3. Electron density maps showing the positions of the variant amino acids. The more hydrophilic serine side chains point towards the solvent whereas the cysteine side chains pack against the surface of the protein. The 2mFo-Dfc map (blue) calculated for 3S1C-Hyd-2 is contoured at 1.5 r.m.s.d. (0.59 e/Å^3). Native residues are shown in grey and variant residues in green.

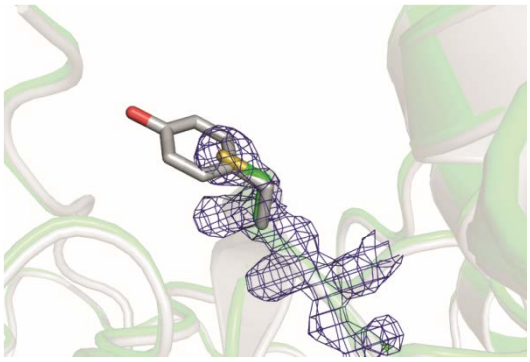

Figure S4. Structure comparison between Y'222 and C'222 in Hyd-2 and 3S1C-Hyd-2 dimers. The structure of hyd-2 is unperturbed by the introduction of the cysteine residue. The backbone atoms of the residues immediately surrounding C'222 can be superposed on those of the native enzyme with an r.m.s.d. of 0.16 Å. The positons of the side chains of these residues is also unaffected with the exception of H'223 which rotates to allow the imidazole ring to pack against the benzyl ring of nearby F'206, the cause of this rotation is unclear. The side chain of C'222 adopts the same rotamer as Y'222 in the native protein, but a small elongation in the electron density for the S-atom implies some rotational freedom about the Cα-Cβ bone. The electron density shown is a 2mFo-Dfc map calculated for 3S1C-Hyd-2 (contoured at 1.5 r.m.s.d. 0.59 e/Å3). Native residues are shown in grey and variant residues in green.

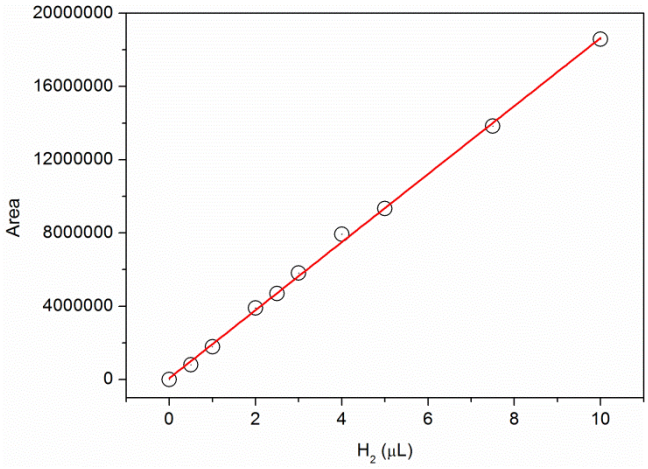

Figure S5. GC calibration for H_2 using pure H_2 at 1 atm, 25˚C. R^2 is 0.998. Carrier gas is N_2. A ShinCarbon ST Column was used with a flow rate of 12 mL/min.

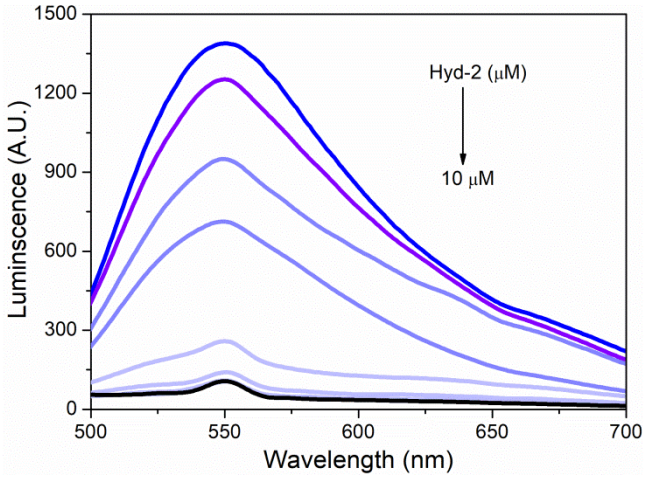

Figure S6. Photoluminescence spectra of AgNCs-PMAA (Ag atom concentration 20 µM by ICP-MS) showing effect of increasing **Hyd-2** concentration (0-10 µM) in 0.2 M MES buffer (500 µL, pH 6.0). λ_{ex} = 460 nm. Phtoluminescence decreases with addition of Hyd-2.

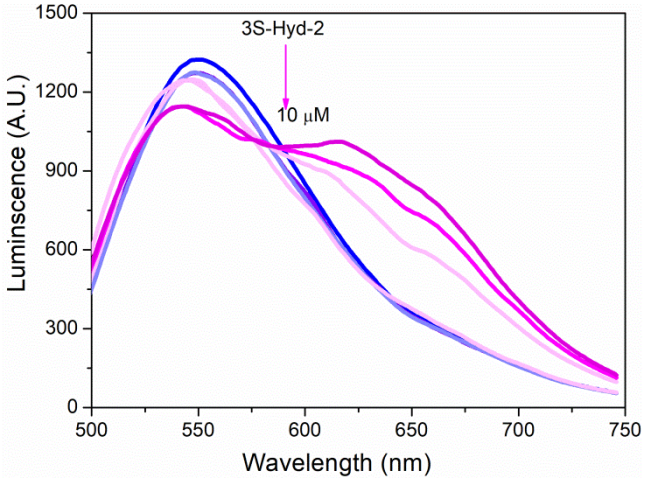

Figure S7. Photoluminescence spectra of AgNCs-PMAA (Ag atom concentration 20 µM by ICP-MS) showing effect of increasing **3S-Hyd-2** concentration (0-10 µM) in 0.2 M MES buffer (500 µL, pH 6.0). λ_{ex} = 460 nm.

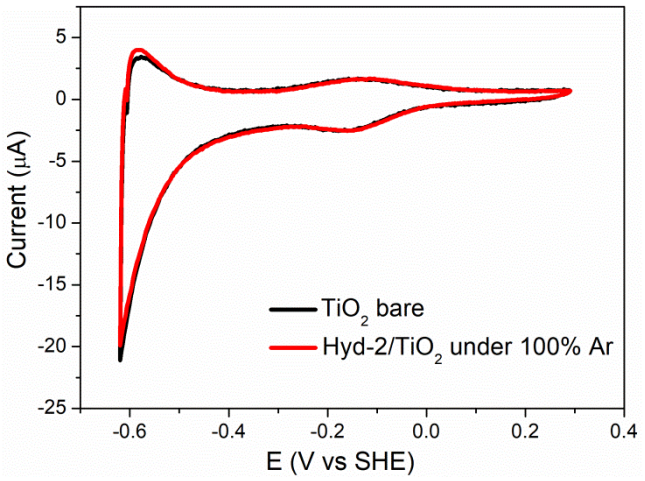

Figure S8. Cyclic voltammogram for Hyd-2 adsorbed on a TiO_2 electrode under an atmosphere of 100% Ar (red). The experiment (blank shown in black) was performed using a buffer containing 0.2 M MES and 0.1 M NaCl at pH 6.0, 30 °C. Scan rate = 5 mV^{-1}.

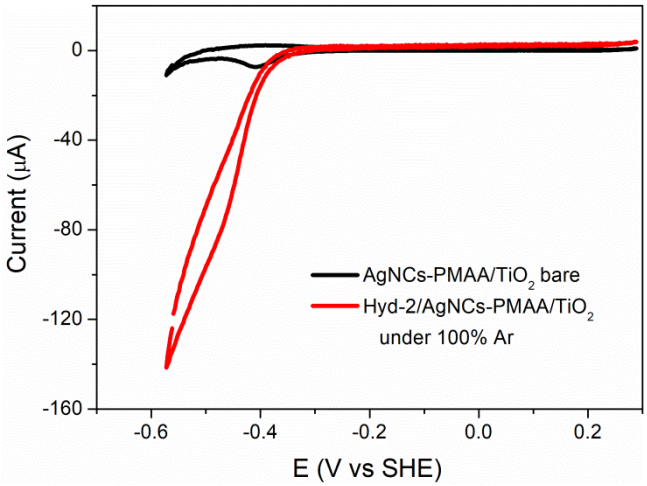

Figure 9. Cyclic voltammograms of Hyd-2 adsorbed on a AgNCs-TiO_2 electrode under an atmosphere of 100% Ar (red). The experiments were performed in 0.2 M MES (pH 6.0), 0.1 M NaCl, at 30 °C with a scan rate of 5 mV^{-1}.

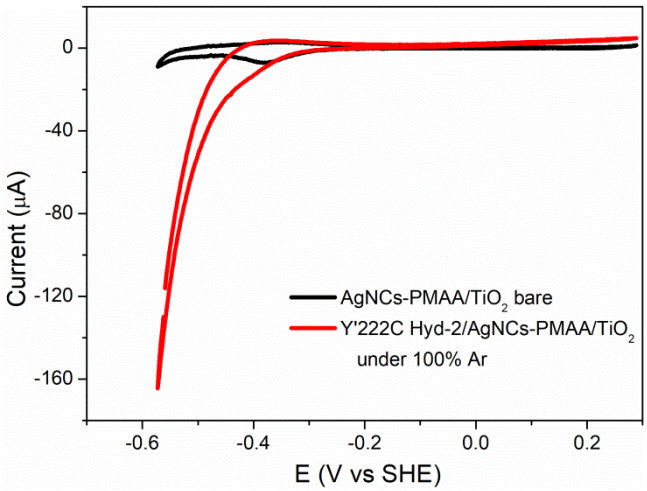

Figure S10. Cyclic voltammograms of Y'222C Hyd-2 adsorbed on a AgNCs-TiO_2 electrode under an atmosphere of 100% Ar (red). The experiments were performed in 0.2 M MES (pH 6.0), 0.1 M NaCl, at 30 °C with a scan rate of 5 mV^{-1}.

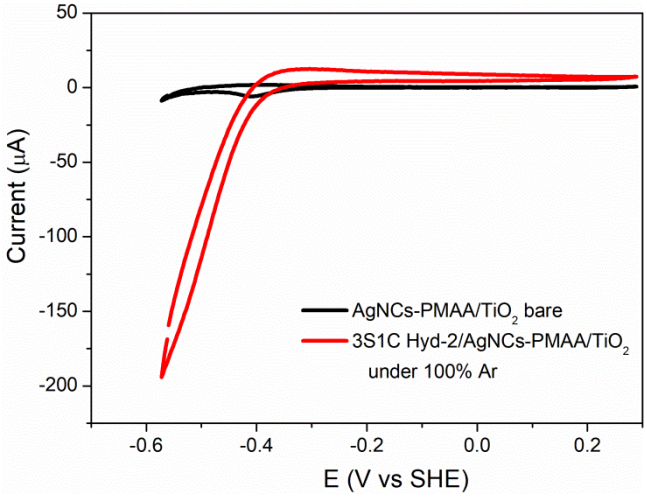

Figure S11. Cyclic voltammograms of 3S1C Hyd-2 adsorbed on a AgNCs-TiO_2 electrode under an atmosphere of 100% Ar (red). The experiments were performed in 0.2 M MES (pH 6.0), 0.1 M NaCl, at 30 °C with a scan rate of 5 mV^{-1}.

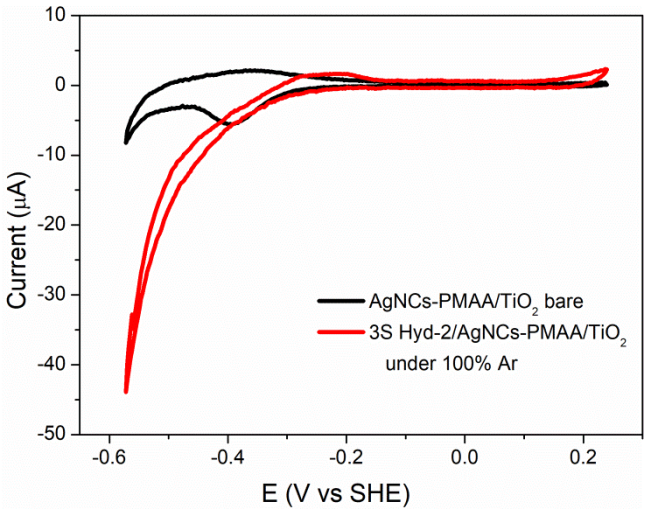

Figure S12. Cyclic voltammograms of 3S Hyd-2 adsorbed on a AgNCs-TiO_2 electrode under an atmosphere of 100% Ar (red). The experiments were performed in 0.2 M MES (pH 6.0), 0.1 M NaCl, at 30 °C with a scan rate of 5 mV^{-1}.

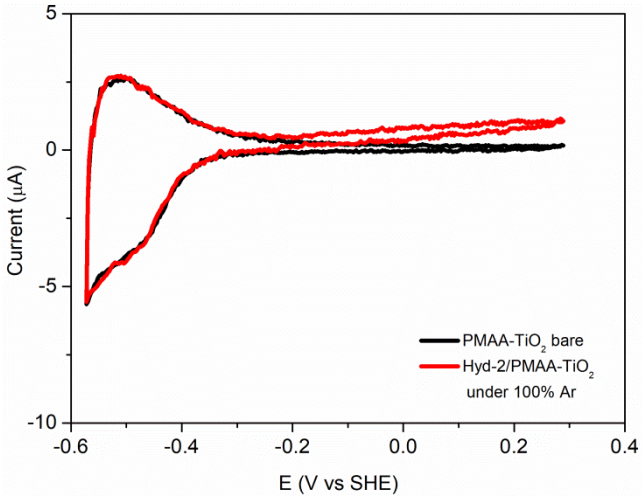

Figure S13. Cyclic voltammograms of Hyd-2 adsorbed on a PMAA modified TiO_2 electrode under an atmosphere of 100% Ar (red). The experiments were performed in 0.2 M MES (pH 6.0), 0.1 M NaCl, at 30 °C with a scan rate of 5 mV^{-1}.

References

1 R. M. Evans, E. J. Brooke, S. A. M. Wehlin, E. Nomerotskaia, F. Sargent, S. B. Carr, S. E. V. Phillips and F. A. Armstrong, *Nat. Chem. Biol.*, 2016, **12**, 46-50.

2 S. E. Beaton, R. M. Evans, A. J. Finney, C. M. Lamont, F. A. Armstrong, F. Sargent and S. B. Carr, *Biochem. J.*, 2018, **475**, 1353-1370.

3 S. Monaco, E. Gordon, M. W. Bowler, S. Delagenière, M. Guijarro, D. Spruce, O. Svensson, S. M. McSweeney, A. A. McCarthy, G. Leonard and M. H. Nanao, *J. Appl. Crystallogr.*, 2013, **46**, 804-810.

4 W. Kabsch, *Acta Crystallogr., Sect. D: Struct. Biol.*, 2010, **66**, 125-132.

5 A. J. McCoy, R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni and R. J. Read, *J. Appl. Crystallogr.*, 2007, **40**, 658-674.

6 P. Emsley, B. Lohkamp, W. G. Scott and K. Cowtan, *Acta Crystallogr., Sect. D: Struct. Biol.*, 2010, **66**, 486-501.

7 G. N. Murshudov, P. Skubak, A. A. Lebedev, N. S. Pannu, R. A. Steiner, R. A. Nicholls, M. D. Winn, F. Long and A. A. Vagin, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2011, **67**, 355-367.

8 V. B. Chen, W. B. Arendall, J. J. Headd, D. A. Keedy, R. M. Immormino, G. J. Kapral, L. W. Murray, J. S. Richardson and D. C. Richardson, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2010, **66**, 12-21.

9 H. G. Krüger, A. Knote, U. Schindler, H. Kern and A. R. Boccaccini, , *J. Mater. Sci.*, 2004, **39**, 839-844.

10 I. Diez, M. Pusa, S. Kulmala, H. Jiang, A. Walther, A. S. Goldmann, A. H. E. Muller, O. Ikkala and R. H. A. Ras, *Angew. Chem., Int. Ed.*, 2009, **48**, 2122-2125.

11 L. Zhang, M. Can, S. W. Ragsdale and F. A. Armstrong, *ACS Catal.*, 2018, **8**, 2789-2795.