Supplementary information for

Site-specific attachment of a H_2-evolving artificial metalloenzyme onto carbon nanotubes via electrografting of a protected thiophenolate diazonium salt

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Materials and Methods

Chemicals and general procedures

Multiwalled carbon nanotubes (MWCNT, 10 nm diameter, purity > 99%) and all the other reagents were purchased from Sigma-Aldrich and were used as received without any purification. All solvents were of analytical grade. Ferrocenemethanethiol, 4-ethynylbenzenediazonium tetrafluoroborate[1] and 8-mercapto-N-(1,10-phenanthrolin-5-yl) octanamide[2] were prepared as previously described. Distilled water was passed through a Milli-Q water purification system. The electrochemical experiments were carried out in a three-electrode electrochemical cell using a Autolab PGSTAT100 Potentiostat and a Palmsens 4 Potentiostat. The MWCNT electrodes were used as working electrodes. Pt wire was used as counter electrode and the reference electrode was based on the $Ag/AgNO_3$ 10^{-2} M reference electrode in MeCN and Saturated Calomel Electrode (SCE) in water. All potentials are given versus Fc/Fc^+ in MeCN and the SCE in water. All current densities are normalized towards the geometrical surface area of the MWCNT/glassy carbon electrode (0.071 cm^2). Experiments were repeated on at least three electrodes.

XPS analysis was performed using a Thermoelectron ESCALAB 250 device (ICGM, France). The X-ray excitation was provided by a monochromatic Al-Kα (hυ=1486.6 eV) source. The analyzed area was ~0.15 mm^2. The background signal was removed using the Shirley[3] method. The surface atomic concentrations were determined from photoelectron peak areas using the atomic sensitivity factors reported by Scofield[4]. Binding energies (BE) of all core levels were referred to the C=C of C1s carbon at 284.4 eV.

Synthesis of 4-diazophenyl disulfide bis(tetrafluoroborate)

A solution of 292 mg (1.2 mmol) 4-Aminophenyl disulfide in 20 mL of MeCN was cooled to -40 °C under a stream of argon. Then, 270 mg (2.4 mmol) of $NOBF_4$ was gradually added. After 1 hour of stirring, the insoluble fraction was filtrated, washed with cold MeCN and Et_2O. The product is obtained a white powder. Yield: 320 mg (60 %).

^1H NMR (400 MHz, CD_3CN): δ=8.38 (d, J=7.2 Hz, 4 H), 8.00 (d, J=7.2 Hz, 4 H), MS (ESI) : $[M-BF4)]^+$ = 359.06.

MWCNT-based Electrode preparation

Glassy carbon electrodes (GCE) were cleaned prior to used using a polishing plate for 1 min and 1 µm diamond paste. The electrodes were then sonicated in a 1:1 v ratio of Ethanol:acetone solution for 30 min. 5 mg of MWCNT were sonicated in 1 ml of NMP for 4 h. 20 µL of the dispersion were drop-coated on a glassy carbon electrode and dried during 1h under vacuum. The edges of the CNT film were reduced, affording a 0.07 cm^2 electrode surface.

4-Diazophenyl disulfide bis(tetrafluoroborate) electrografting and chemical cleavage

5 mg of 4-diazophenyl disulfide bis(tetrafluoroborate) are dissolved in 8 mL of 0.1 M Tetrabutylammonium Perchlorate (TBAP)/Acetonitrile (MeCN) electrolyte solution. The MWCNT working electrode is then placed inside the electrochemical cell, with an $Ag/AgNO_3$ (10 mM TBAP in MeCN) and platinum wire as reference and counter electrodes respectively. Electrografting was performed in a glovebox by cyclic voltammetry (CV) at 20 mV s^{-1} using a PalmSens4 potentiostat. After electrografting, the working electrode is thoroughly rinsed with acetonitrile and stored in the glovebox. Chemical cleavage of the disulfide bridges was then made by soaking the functionalized electrodes in a DMF solution containing 17 mM of (TCEP) for 1 h and then rinsed 3 times with DMF and 3 times with MeCN.

Functionalization with ferrocene and Rubredoxin

Electrodes were soaked in 5 mM of Ethynylferrocene or Ferrocene Hexanethiol, in dichloromethane (CH_2Cl_2) for 1h. The electrodes were finally washed in dichloromethane or DMF three times and MeCN three times.

Rubredoxine-based electrodes were elaborated by soaking the functionalized electrode in a 2.5mg mL^{-1} of protein solution in 50 mM HEPES pH 7.6 for 1 h. The electrodes were further rinsed in the HEPES buffer solution at pH 7.6. Electrochemical measurements were made just after these last rinsing steps.

Cloning, production and purification

The synthetic genes of rubredoxin (Rd) WT and N14C mutant from *Clostridium pasteunarium* (UniprotKB accession code: P00268) were obtained from Genscript in pET15b as codon-optimized genes for expression in *E. coli*.

Amino acid sequences:

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                10         20         30         40         50
WT   MKKYTCTVCG YIYNPEDGDP DNGVNPGTDF KDIPDDWVCP LCGVGKDQFE EVEE
N14C MKKYTCTVCG YIYCPEDGDP DNGVNPGTDF KDIPDDWVCP LCGVGKDQFE EVEE
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E. coli BL21 (DE3) cells harboring wild-type *Cp*Rd-WT or *Cp*Rd-N14C were cultured in 2 L of lysogeny broth (LB) medium, supplemented with 100 mg per L of Ampicillin, under stirring at 37 °C. The expression was induced using 0.5 mM of IPTG once the culture reached an OD_{600} of 0.5-0.6. The cultures were then left at 20 °C and 180 rpm overnight before harvesting by centrifugation. The pellets were then frozen in liquid nitrogen and stored at -80 °C.

Bacterial pellets from 2 L cultures were re-suspended in 100 mL of 50 mM HEPES pH 7.6 (Buffer A) containing a protease inhibitor tablet “complete” by Roche and lysed by sonication. The cell debris were removed by centrifugation at 25 000 rpm for 25 min at 4°C (Rotor JA 30.50). Each clear lysate (WT, N14C or D32C) was loaded at 1.5 mL min^{-1} onto a 15 mL Q Sepharose column equilibrated in buffer A. After washing with 100 mL of buffer A, the protein was eluted

with 200 mL of a 0-50 % linear gradient of buffer B (Buffer A + 1 M NaCl). The fractions were analyzed by SDS-PAGE, and those containing *Cp*Rd were pooled and concentrated (cut-off 5 kDa) before loading onto a Superdex 75 16/600 size exclusion column (GE Healthcare) equilibrated in buffer C (Buffer A + 150 mM NaCl). The fractions were collected, analyzed by SDS-PAGE, and those containing pure *Cp*Rd were dialyzed overnight at 4 °C in buffer A. Pure *Cp*Rd WT and *Cp*Rd N14C were concentrated to 2 mg mL^{-1} and 6.6 mg mL^{-1} before fast freezing and storage at -80 °C, respectively.

Metal substitution

The protocol was adapted from references[5,6].All metal substitution steps were carried out in a nitrogen atmosphere glove box (O_2 < 2.0 ppm). The protein was incubated in the presence of 0.15 M DTT for 10 minutes. After incubation, it was precipitated at 4 °C with 5% trichloroacetic acid until a white precipitate was formed. The colorless precipitate was collected by centrifugation at 5000×g for 10 minutes and the supernatant was discarded. The pellet was resuspended in a 0.5 M Tris-HCl buffer at pH 8.5, and the TCA precipitation step was repeated to ensure that all originally bound metals were removed. After repeating DTT incubation, TCA precipitation, and centrifugation, the pellet was again resuspended in 0.5 M Tris-HCl buffer. A 2.5-fold molar excess of $NiSO_4$ was added to the solution, and the sample was incubated at 4 °C for 16 hours to allow for complete metal incorporation. The solution was then centrifuged at 17,900×g to pellet any metal adduct precipitate that had formed. The protein-containing supernatant was finally exchanged and concentrated in 50 mM HEPES buffer pH 7.6 using a 3 kDa Centricon (two times) to remove excess of metal salts and DTT-Ni adducts. The nickel-substituted rubredoxin is yellow and was checked by UV-Vis and quantified accordingly.

ESI-MS

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC/ESI-MS) was performed on a 6210 LC/ESI-TOF mass spectrometer interfaced with an HPLC binary pump system (Agilent Technologies). The mass spectrometer was calibrated with standard calibrants (ESI-L, Low concentration tuning mix, Agilent Technologies) before measurements and mass spectra were recorded in the 20-4000 *m/z* range in the positive ion mode. All solvents used were HPLC grade (Chromasolv, Sigma-Aldrich), trifluoroacetic acid (TFA) was from Acros Organics (puriss., p.a.). Solvent A was 0.03% TFA in water, solvent B was 95% acetonitrile-5% water-0.03% TFA. Just before analysis the protein samples were diluted in acidic denaturing conditions to a final concentration of 5 µM with solution A (0.03% TFA in water). Samples were thermostated at 10°C in the autosampler and the analysis was run by injecting 4 µL of each sample. They were first trapped and desalted on a reverse phase-C8 cartridge (Zorbax 300SB-C8, 5µm, 0.3mm IDx5mm, Agilent Technologies) for 3 minutes at a flow rate of 50 ul/min with 100% solvent A and then eluted with 70% solvent B at flow rate of 50 ul/min for MS detection. The RP-C8 cartridge was then re-equilibrated for 4 min with 100% solvent A at a flow rate of 50 ul/min. MS spectra were acquired and the data processed with MassHunter

workstation software (v. B.02.00, Agilent Technologies) and with GPMAW software (v. 7.00b2, Lighthouse Data, Denmark).

SEC-MALLS

The samples were injected manually into a SEC-MALLS system (Wyatt Dawn HELEOS-II 18-angle light scattering detector and Wyatt Optilab rEX refractive index monitor linked to a Shimadzu HPLC system comprising a LC-20AD pump and SPD20A UV/Vis detector) using a Superdex 200 10/300 Increase size exclusion column eluted with buffer containing 50 mM HEPES at pH 7.2, 300 mM NaCl, and 1 mM TCEP. The data were analyzed using the ASTRA software (version 6) and the molecular masses were calculated for each sample. Protein concentration in all samples was determined by integration of the differential refractive index (dRI) peak after injection of 20 µL protein.

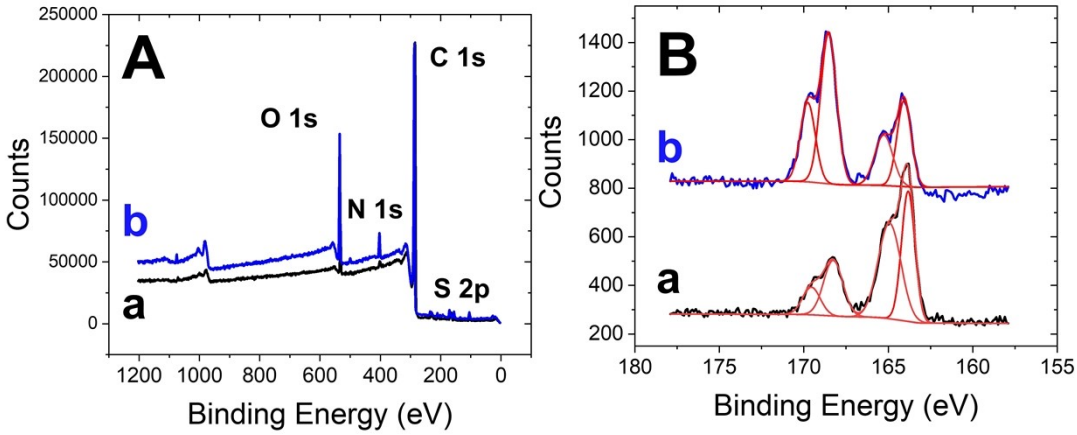
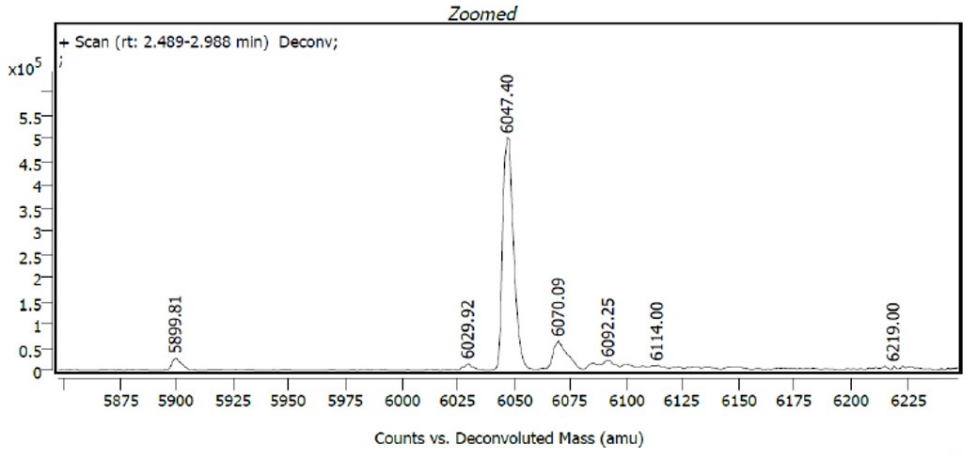


Figure S1. (A) XPS survey spectra and associated deconvolution (red) and (B) XPS spectra at S 2p core levels for (a, black) MWCNT electrode modified with DPDS and (b, blue) MWCNT electrode modified with *Cp*Rd-N14C.

_Cp_Rd-WT

Expected mass: 6047.63 Da



_Cp_Rd-N14C

Expected mass: 6036.67 Da

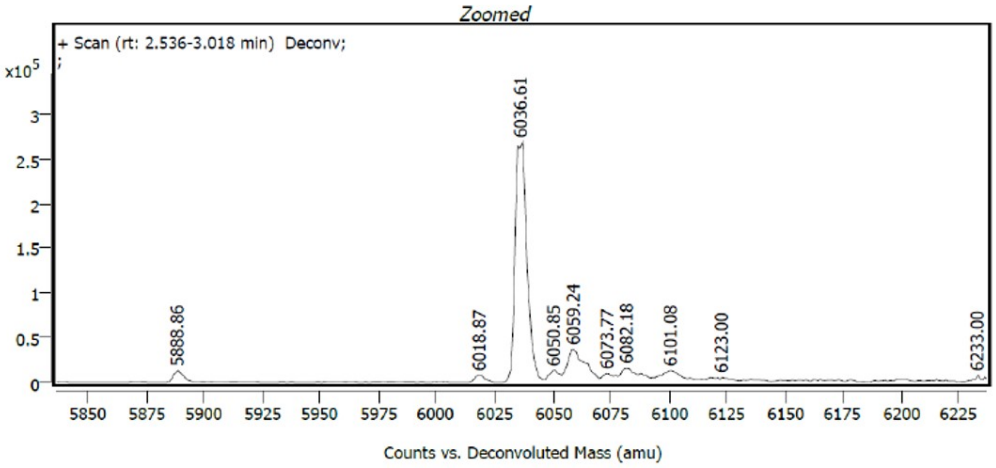
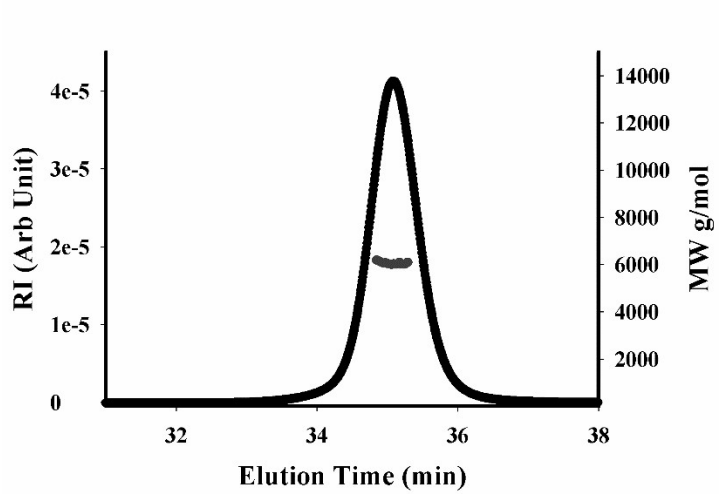


Figure S2. ESI-MS spectra of *Cp*Rd WT and *Cp*Rd N14C.

WT



N14C

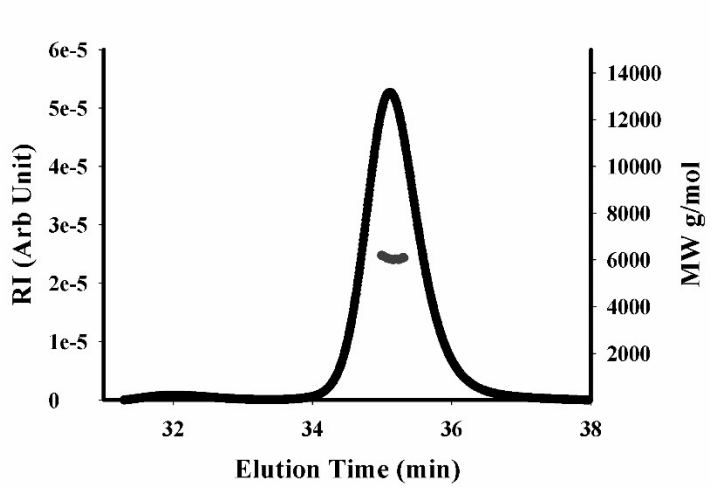


Figure S3. SEC MALLS analysis of protein samples. Top: *Cp*Rd WT concentration 2 mg mL^{-1}, MW 6 +/- 0.1 kDa. Bottom: *Cp*Rd-N14C Concentration 2.4 mg mL-1, MW 6 +/- 0.1 kDa.

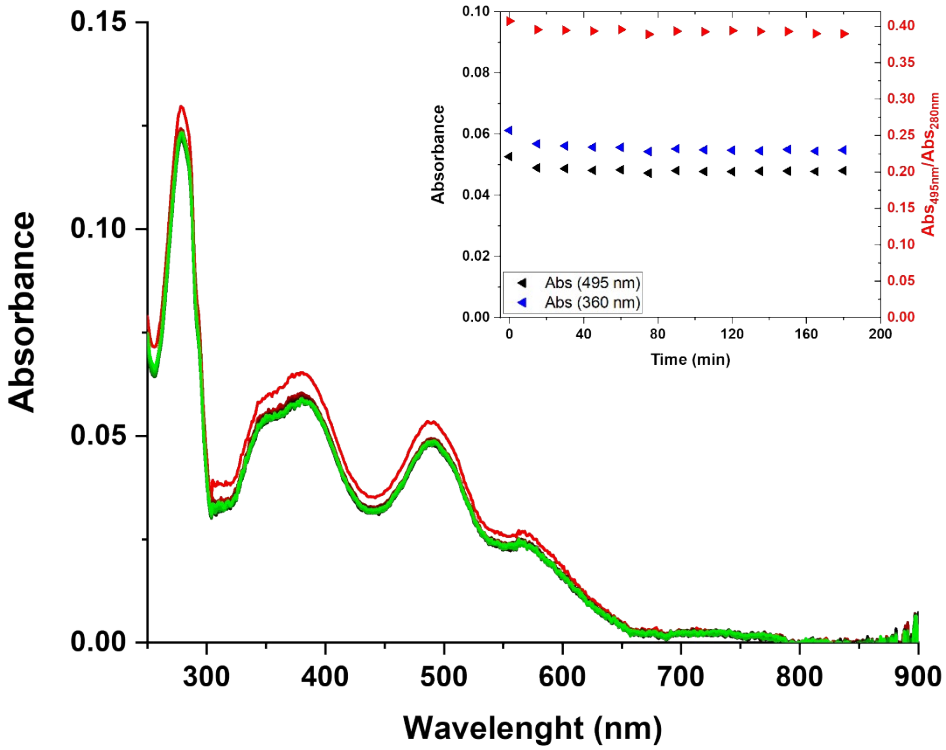


Figure S4. UV/Vis spectra of *Cp*Rd WT recorded over time. 10 µM *Cp*Rd WT in 50 mM HEPES buffer pH 7.6 recorded every 15 minutes. Inset, Absorbance at 495 nm (black triangles, left axis), 360 nm (blue triangles, left axis), and the absorbance ratio A_{495}/A_{280} (red triangles, right axis) were monitored over 200 minutes at 25 °C time-dependent monitoring under continuous stirring (100 rpm).

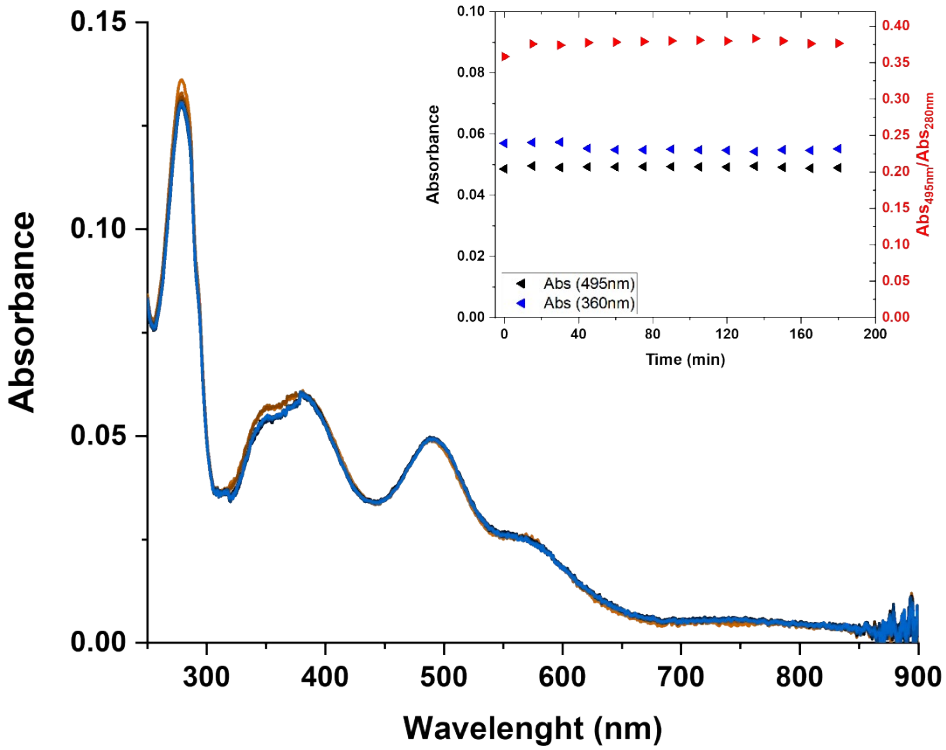


Figure S5. UV/Vis spectra of *Cp*Rd N14C recorded over time. 10 µM *Cp*Rd N14C in 50 mM HEPES buffer pH 7.6 recorded every 15 minutes. Inset, Absorbance at 495 nm (black triangles, left axis), 360 nm (blue triangles, left axis), and the absorbance ratio A_{495}/A_{280} (red triangles, right axis) were monitored over 200 minutes at 25 °C time-dependent monitoring under continuous stirring (100 rpm).

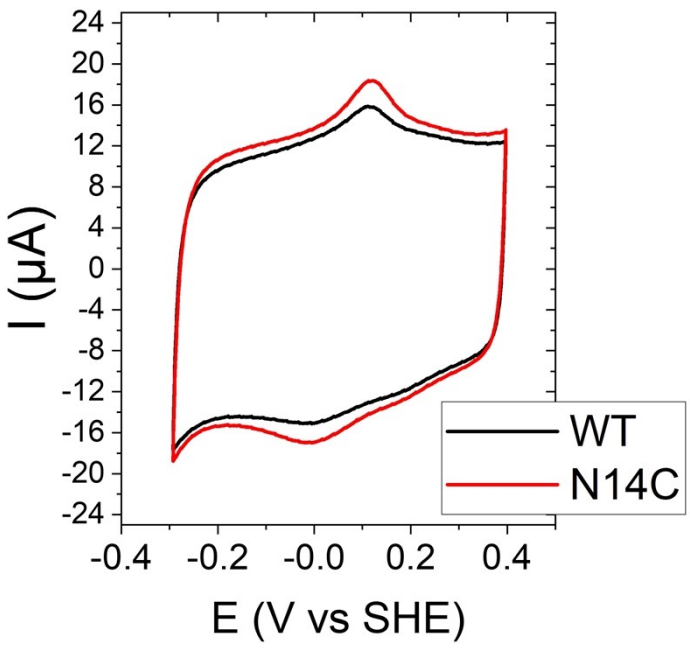


Figure S6. CV of MWCNT electrodes modified with *Cp*Rd WT and CpRd N14C in 50 mM HEPES (pH 7.6, v = 10 mV s^{-1}).

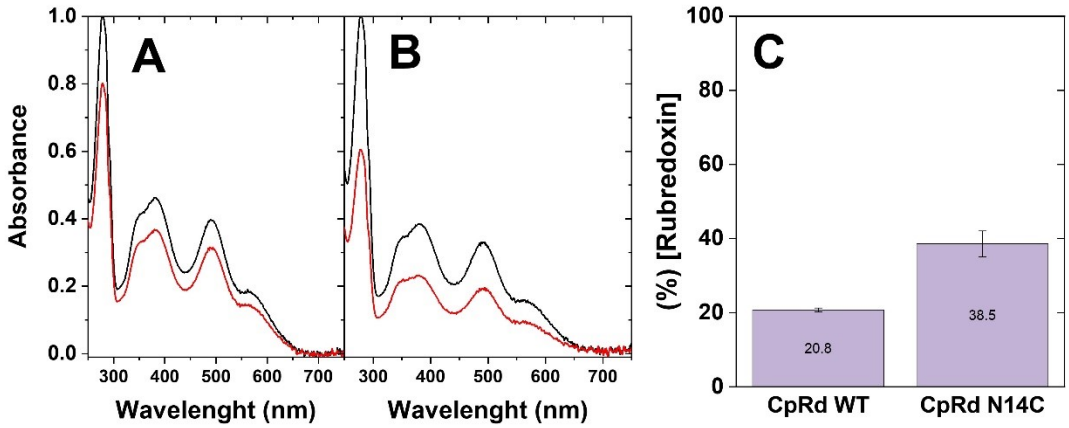


Figure S7. UV/Vis spectra for (A) Fe-CpRd WT and (B) Fe-CpRd N14C in 50 mM HEPES buffer pH 7.6 before (black) and after soaking a 1 mg mL^{-1} solution on thiolate-modified MWCNT electrode ; (C) Percentage of the amount of retained rubredoxin during the soaking step

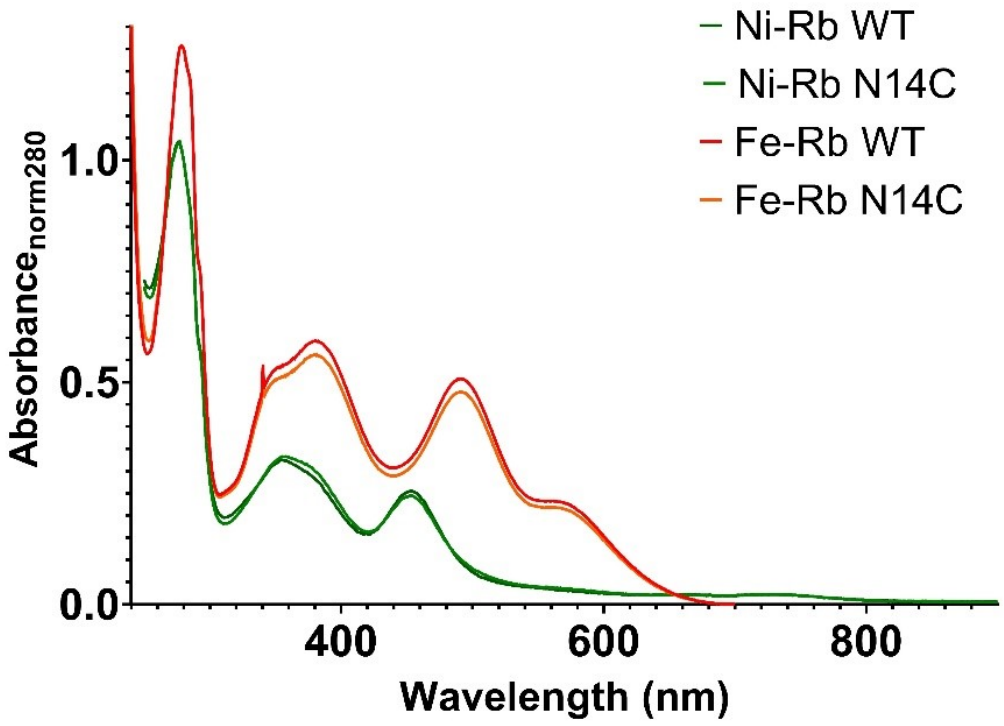


Figure S8. UV/Vis spectra. In red and orange are the Fe-*Cp*Rd WT and Fe-*Cp*Rd N14C, respectively, in 50 mM HEPES buffer pH 7.6. In green and light green are the Ni-*Cp*Rd WT and Ni-*Cp*Rd N14C, respectively, in 50 mM HEPES buffer pH 7.6.

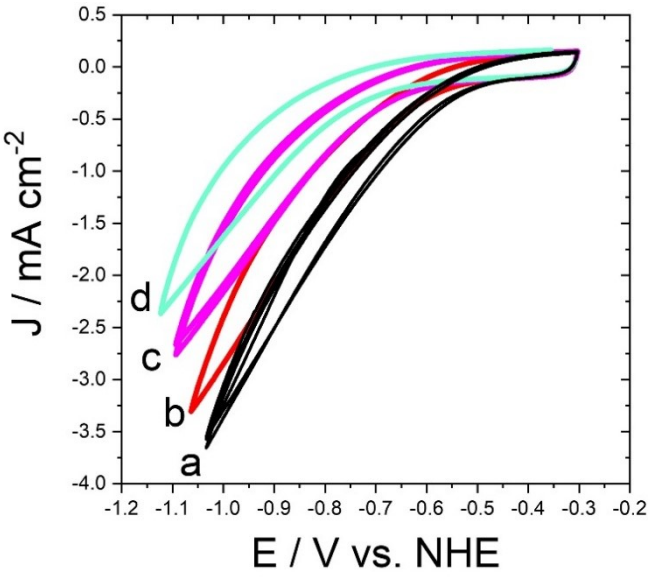


Figure S9. CV of MWCNT electrode modified with Ni-*Cp*Rd N14C in 50 mM of BRB buffer at pH (a) 3.5, (b) 4, (c) 4.5 and (d) 5 (3 scans, v = 10 mV s^{-1}).

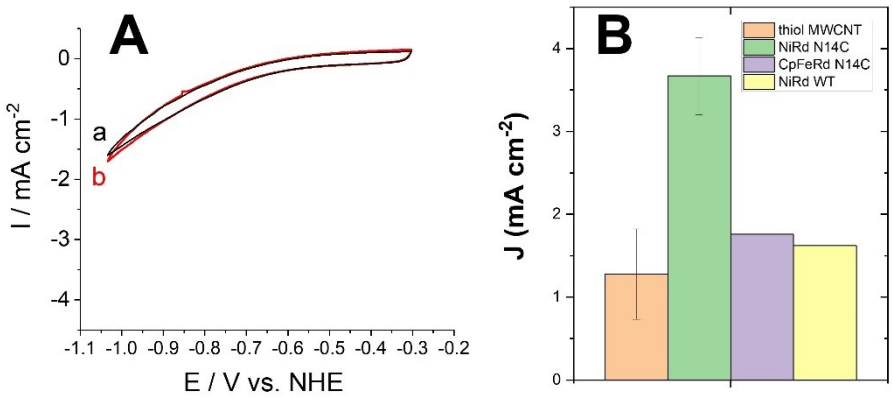


Figure S10. (A) CV of thiolate-modified MWCNT electrode (a) without and (b) with Fe-*Cp*Rd N14C in 50 mM of BRB buffer at pH 3.5 (v = 10 mV s^{-1}) ; (B) Current density measured at -1 V for thiolate-modified MWCNT electrode, thiolate-modified MWCNT electrode with Ni-*Cp*Rd N14C, Fe-*Cp*Rd N14C and Ni-*Cp*Rd WT in 50 mM of BRB buffer at pH 3.5 (v = 10 mV s^{-1})

Table S1. HER performances of nanostructured electrodes modified with artificial Ni metalloenzymes, bio-inpired Ni complex and hydrogenases.

Supported Catalyst	**Electrode**	**Γmax (nmol cm^{-2})**	**Current density (mA cm^{-2})**	**TOF (s^{-1})**	**Ref**
Ni-Rd	Modified MWCNT (site-specific/covalent)	9.9	4 at -1 V vs SHE (pH 3.5)	2	This work

Ni-Rd	Modified MWCNT (covalent)	0.34	0.32 at -1 V vs SHE (pH 3.5)	34	[7]
Bisdiphosphine Ni(II) complex	Modified MWCNT (covalent)	1.5	4 at –0.3 V vs SHE (0.5 M H2SO4)	14	[8]
[NiFeSe] Hydrogenase	Modified MWCNT (non-covalent)	0.011	0.2 at -0.6 V vs. SHE (pH 7.6)	94	[9]
CpI FeFe hydrogenase	nanoITO (non-covalent)	9	-8 at -0.8 V vs SHE (pH 7)	41	[10]

References

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