Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Hydrogen Bioelectrogeneration with pH-Resilient and Oxygen-Tolerant Cobalt Apoenzyme-Saccharide

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Summary

S1.0 Experimental section

S1.1 Reagents

Agarose heteropolysaccharide, sulfuric acid (98%), hydroxocobalamin acetate (CoP), Sephadex G-25, apo-protein from myoglobin equine skeletal muscle was purchased from Sigma-Aldrich.

S1.2 Apo-reconstituted protein: Apo-Myo with CoP (CoMyo) and SacCoMyo

The apo-reconstituted protein with CoP was obtained according to previous reported and adapted methodology[1]. A stock solution of CoP 1 μmol L^{-1} was previous prepared in purified water and added to a solution of apo-protein (0.1 - 0.5 mM) at room temperature (25 °C). The final solution was gently stirred and incubated for 20 – 30 minutes before purification in a chromatographic column containing Sephadex G-25. The stock concentration of apo-reconstituted CoMyo after purification was determined by the Soret band at 418 nm (136000 mol^{-1} L^{-1} cm^{-1}).

SacCoMyo was prepared by dispersing the previous prepared solution (500 μL of the stock solution of CoMyo 1.4 μmol L^{-1}) of the protein into agarose aqueous solution (0.4 mg mL^{-1}). The incorporated agarose per mol of artificial protein relationship was the following: $1.43x10^{-6}$ mols (protein solution) / $1.2x10^{-3}$ mols (agarose solution) ~ $1,19x10^{-3}$. We performed calculations based on the concentration of the previously purified artificial CoMyo protein 1.43 μmol L^{-1}.

S1.3 Bioelectrodes Preparation

The artificial protein CoMyo was immobilized by dispersing a previously prepared solution (500 μL of the stock solution of CoMyo at 1.4 μmol L^{-1}) into an agarose aqueous solution (0.4 mg mL^{-1}). This method was employed to prevent protein leaching from the electrode surface. The identical procedure was utilized to retain CoP on the electrode surface or to produce (SacCoP). The resulting bioelectrode configurations were carbon paper/SacCoP and carbon paper/SacCoMyo.

S1.4 Electrochemistry experiments

All electrochemical measurements were performed using a μ-Autolab Type III (Metrohm Autolab, Utrecht, The Netherlands) with software Nova 2.1.5. The carbon paper-modified electrodes with apo-protein and apo-reconstituted proteins with CoP were used as working electrodes, $Ag/AgCl_{sat}$ as the reference electrode, and Pt wire as the auxiliary electrode. The measured potentials vs. Ag/AgCl were converted

to the reversible hydrogen electrode (RHE) scale according to the following equation (1)[2].

$$E_{\mathrm{RHE}} = E_{\mathrm{Ag/AgCl}} + 0{,}059\mathrm{pH} + E^{0}_{\mathrm{Ag/AgCl}} \quad (1)$$

For the realization of the cyclic voltammetry and chronoamperometry experiments, an electrochemical cell with 100 mL volume was used. The supporting electrolyte was H_2SO_4 0.5 mol L^{-1} for pH 1, phosphate buffer solution 0.1 mol L^{-1} for pH 7 and acetate buffer solution 0.1 mol L^{-1} for pH 3.

S1.5 Electronic spectroscopy

The electronic spectroscopy experiments in the ultraviolet-visible (UV-vis) range (300 nm – 700 nm) were conducted using a Jasco V-760 spectrophotometer with a quartz cell having a 1 mL capacity. The concentration of the apo-reconstituted CoMyo was determined following the protein's purification through Sephadex G-25.

S1.6 Vibrational spectromicroscopy (micro-FTIR)

Vibrational spectroscopy (micro-FTIR) was performed using a Bruker Vertex 70V spectrometer with a Hyperion 3000 microscope attached (Bruker Gmbh, Ettlingen, Germany). Micro-FTIR spectra were obtained in the transmittance mode. The agarose, Apo-Myo, CoMyo and SacCoMyo were placed on the CaF_2 window with a cooled mercury telluride and cadmium detector (MCT). The spectra were recorded from an average of 128 accumulations with a spectral resolution of 4 cm^{-1} and a spectral window of 3800–730 cm^{-1} using an objective lens with 36x magnification.

S1.7 DEMS setup

The working electrodes for the DEMS experiments were prepared on one side of a porous carbon disc sheet (Toray carbon, 10%) that was pressed with a gas-diffusible PTFE (Polytetrafluoroethylene; 0.02 μm; Gore-Tex) membrane layer behind using a hydraulic press (1 ton weight for 1 min), and the biocatalyst materials were coated by drop-casting on the surface of the electrode. The prepared working electrodes were inserted into the cap of a homemade PEEK (polyether ether ketone) holder and added 2 more porous PTFE layers behind with a porous polymer frit support to avoid electrolyte leakage. It was gently fastened to the end of the stainless-steel capillary tube setup that connects the cell to the mass spectrometer (Pfeiffer Vacuum QMA 200). The execution of the DEMS experiments was performed according to the previous procedure adapted[3]. The ionic current signal (m/z = 2) data is continuously monitored and detected by the specific

software (Quadera) system for the mass spectrometry to evaluate the production of hydrogen gas by water splitting. In this work, the geometric area of the working electrode exposed to the electrolyte was considered to be 0.38 cm^2 (7 mm of diameter), and electrical contact was made by inserting the edge of a thin titanium strip wrapped in a Teflon tape that connects the edge of the carbon disc inserted in the PEEK holder.

S1.8 Long-term stability and pH-dependence

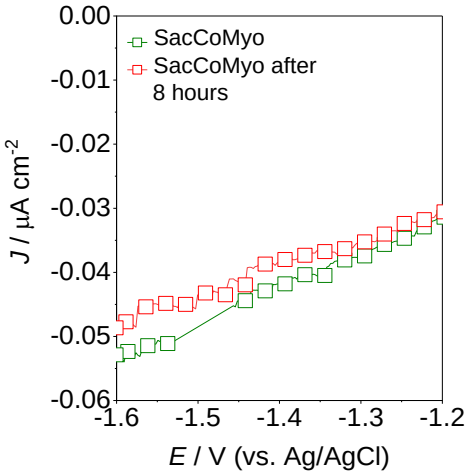


Figure S1. Long-term stability of oxygen-tolerant and pH-resilient SacCoMyo for hydrogen bioelectrogeneration. Turnover frequency (TOF) for the SacCoMyo as a function of overpotential (green) and after 8 hours of experiments. Supporting electrolyte H_2SO_4 0.5 mol L^{-1}, pH 1, under saturated oxygen conditions.

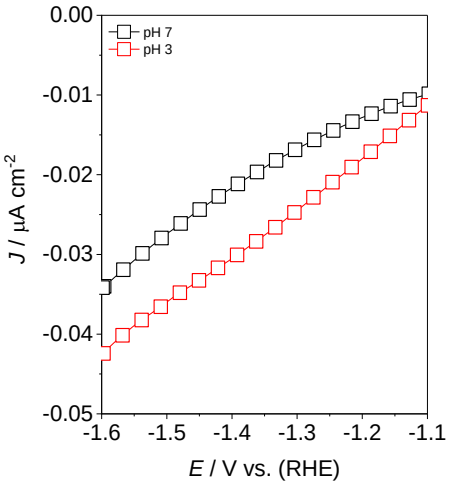


Figure S2. Cyclic Voltammograms of SacCoMyo. The effect of pH on the electrochemical behaviour of SacCoMyo for pH 7 (black) and pH 3 (red). Supporting electrolyte pH 7: Phosphate buffer solution 0.1 mol L^{-1}. Supporting electrolyte pH 3: Acetate buffer solution 0.1 mol L^{-1}.

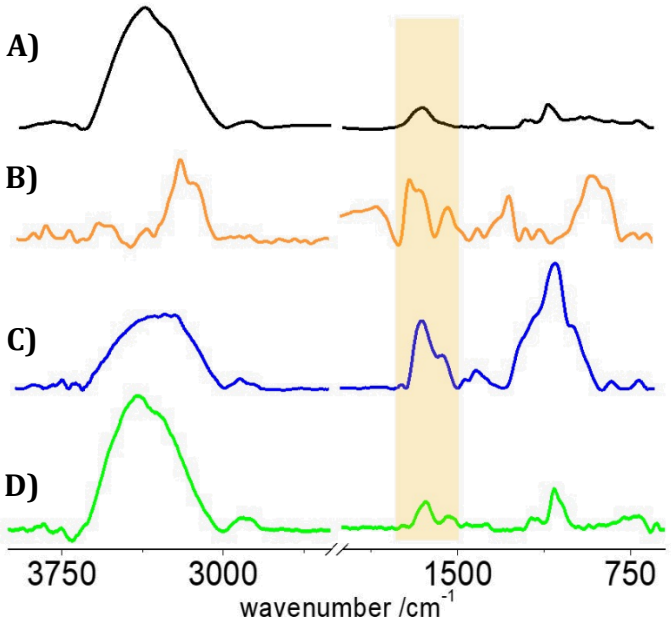
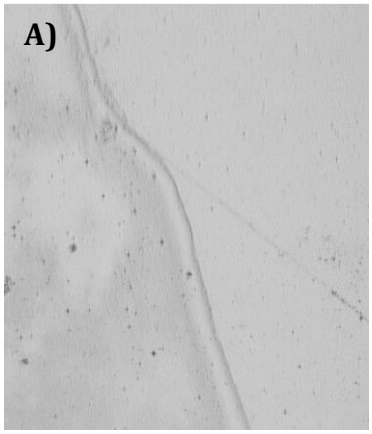
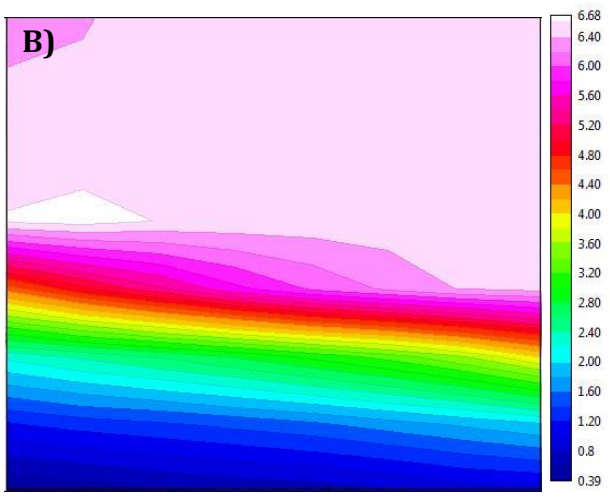


Figure S3. micro-FTIR spectra. **A)** Agarose. **B)** Apo-Myo. **C)** CoMyo. **D)** SacCoMyo. The IR vibrational spectra were collected in the spectral range from 3800 – 730 cm^{-1} at a spectral resolution of 8 cm^{-1} with accumulation of 64 scans. The highlighted square shows the amide I region from 1800 cm^{-1} to 1600 cm^{-1} used for the integration and obtention of the 2D and 3D chemical mapping for B), C) and D) (Figures S4 – S7).

Table S1. Vibrational stretching bands obtained from micro-FTIR for agarose, Apo-Myo, CoMyo and SacCoMyo.

Sample	Wavenumber	Attribution
Agarose	3401 cm^{-1}	-OH stretching
	2897 cm^{-1}	-CH stretching
	1644 cm^{-1}	C=O stretching
	1080 cm^{-1}	Glycosidic bonding
Apo-Myo	3207 cm^{-1}	Amide A
	1847 cm^{-1}	C=O stretching
	1640 cm^{-1}	Amide I
	1510 cm^{-1}	Amide II
	1245 cm^{-1}	Amide III
	879 cm^{-1}	Myoblobin
CoMyo	3300 cm^{-1}	Amide A and -OH stretching
	2927 cm^{-1}	-CH stretching
	1641 cm^{-1}	Amide I
	1542 cm^{-1}	Amide II
	1400 cm^{-1}	C-O-C stretching
	1053 cm^{-1}	P-O stretching
SacCoMyo	3400 cm^{-1}	OH stretching
	2900 cm^{-1}	-CH stretching
	1640 cm^{-1}	Amide I
	1524 cm^{-1}	Amide II
	1184 cm^{-1}	Amide III
	1080 cm^{-1}	Glycosidic bonding
	769 cm^{-1}	Myoblobin





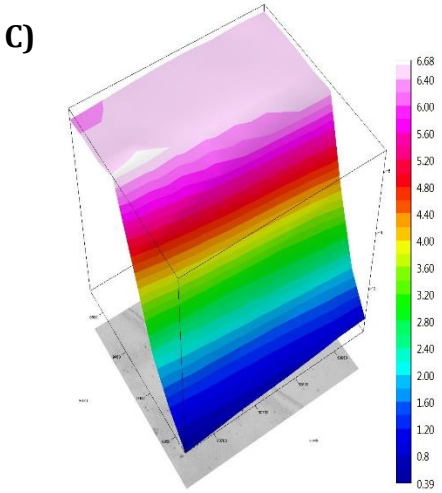
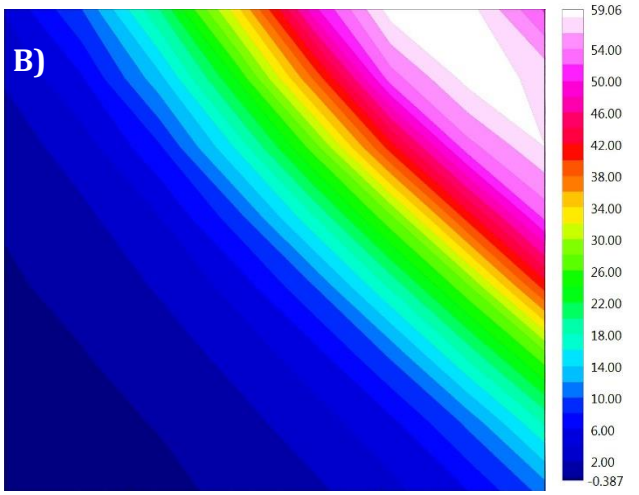


Figure S4. micro-FTIR spectra of Agarose. **A)** Optical image (x15) of Agarose dry-spotted on polycrystalline gold surface. **B)** 2D chemical map showing the distribution of the spectral bands of Agarose. **C)** 3D chemical map obtained from Bruker Vertex 70V micro-FTIR spectrometer. The scale bar is 20 µm. The IR vibrational spectra were collected in the spectral range from 3800 – 730 cm^{-1} at a spectral resolution of 8 cm^{-1} with accumulation of 64 scans.



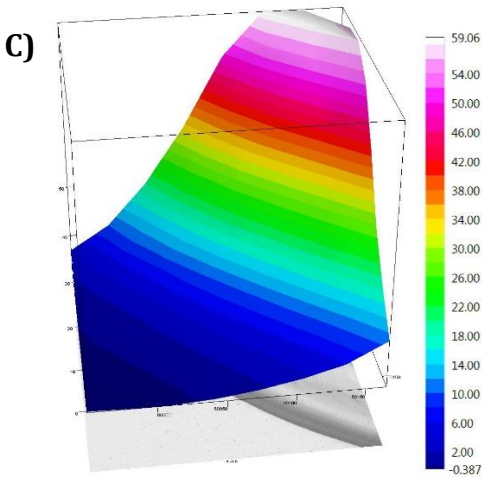


Figure S5. micro-FTIR spectra of Apo-Myo. **A)** Optical image (x15) of Apo-Myo dry-spotted on polycrystalline gold surface. **B)** 2D chemical map showing the distribution of the spectral bands of Agarose. **C)** 3D chemical map obtained from Bruker Vertex 70V micro-FTIR spectrometer. The scale bar is 20 µm. The IR vibrational spectra were collected in the spectral range from 3800 – 730 cm^{-1} at a spectral resolution of 8 cm^{-1} with accumulation of 64 scans. The integration of the infrared signal was realized in the amide I region from 1800 cm^{-1} to 1600 cm^{-1}.

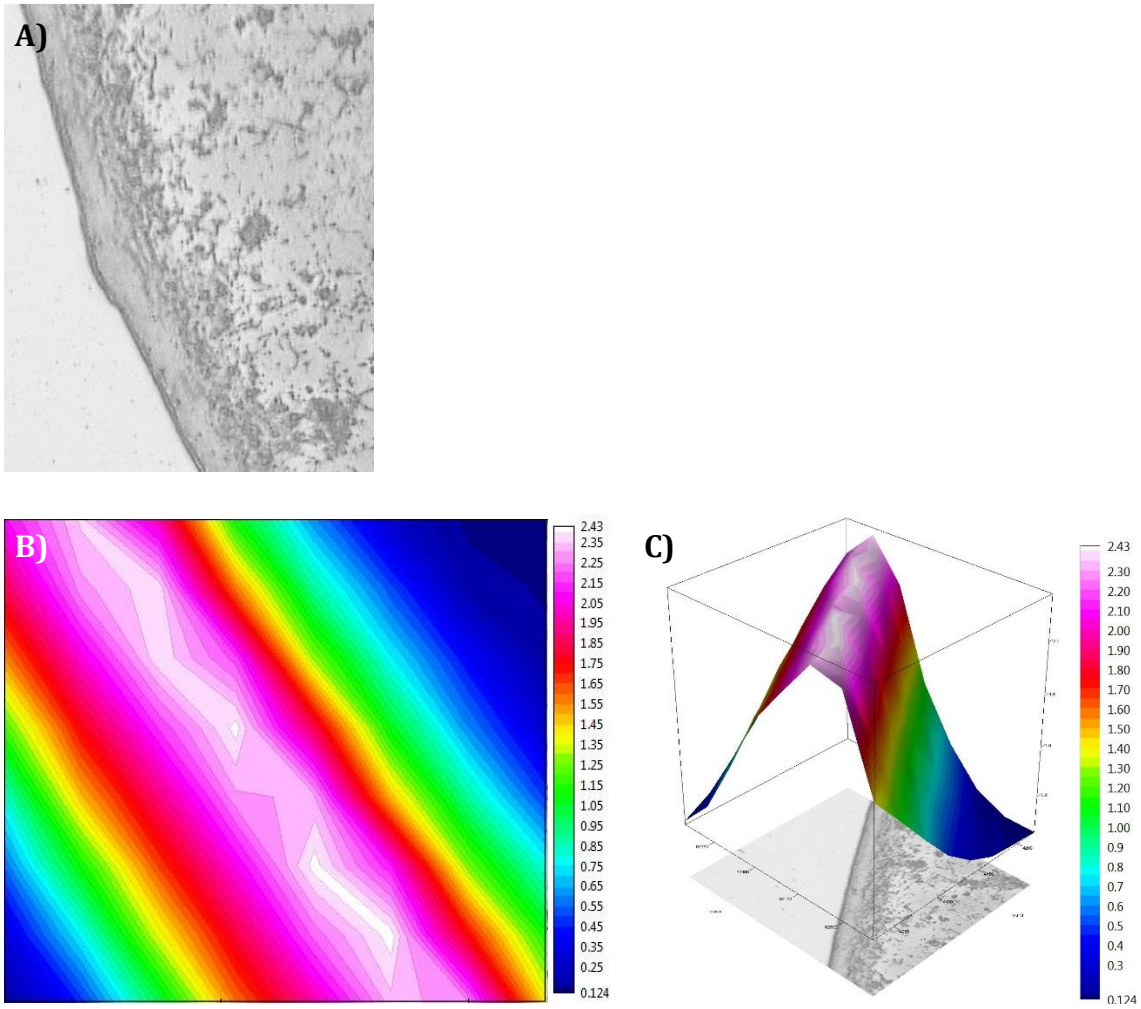


Figure S6. micro-FTIR spectra of CoMyo. **A)** Optical image (x15) of Apo-Myo dry-spotted on polycrystalline gold surface. **B)** 2D chemical map showing the distribution of the spectral bands of Agarose. **C)** 3D chemical map obtained from Bruker Vertex 70V micro-FTIR spectrometer. The scale bar is 20 μm. The IR vibrational spectra were collected in the spectral range from 3800 – 730 cm^{-1} at a spectral resolution of 8 cm^{-1} with accumulation of 64 scans. The integration of the infrared signal was realized in the amide I region from 1800 cm^{-1} to 1600 cm^{-1}.

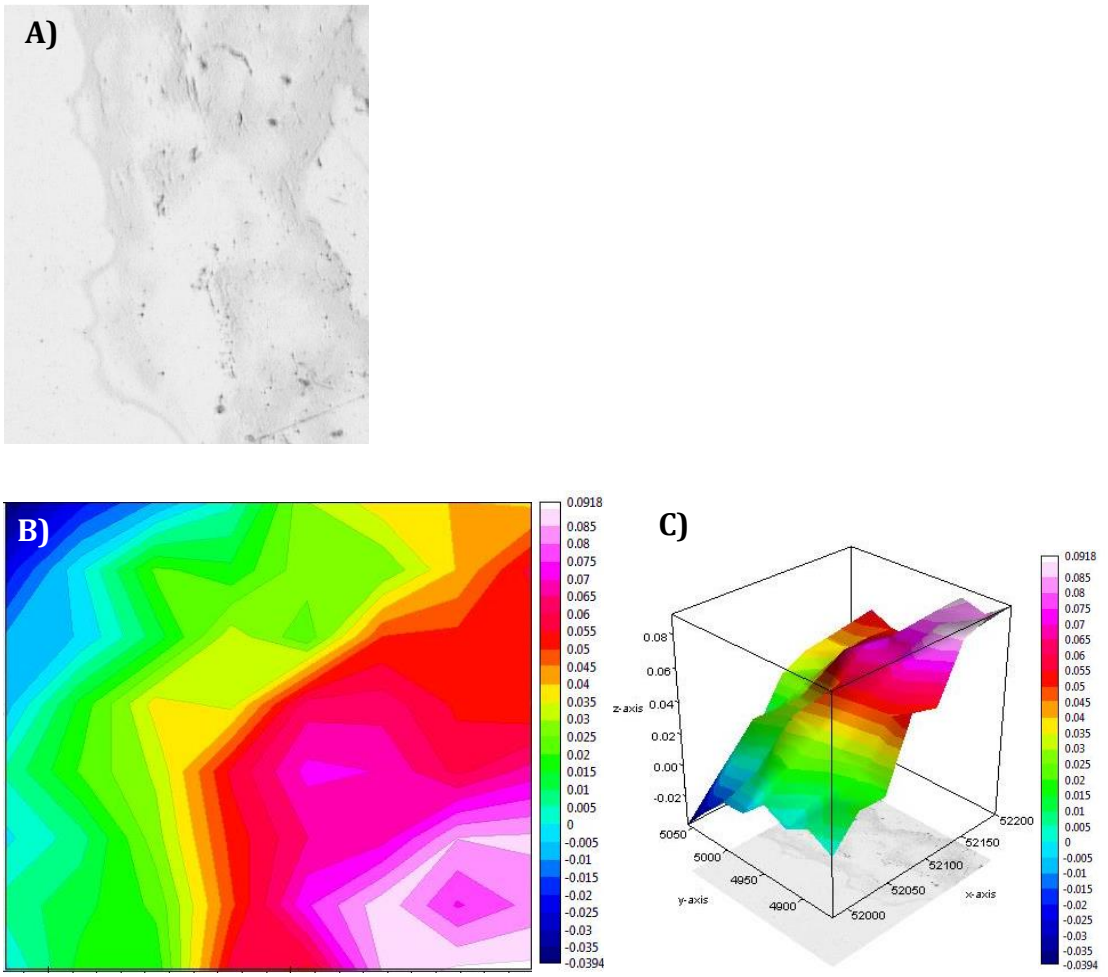


Figure S7. micro-FTIR spectra of SacCoMyo. **A)** Optical image (x15) of Apo-Myo dry-spotted on polycrystalline gold surface. **B)** 2D chemical map showing the distribution of the spectral bands of Agarose. **C)** 3D chemical map obtained from Bruker Vertex 70V micro-FTIR spectrometer. The scale bar is 20 µm. The IR vibrational spectra were collected in the spectral range from 3800 – 730 cm^{-1} at a spectral resolution of 8 cm^{-1} with accumulation of 64 scans. The integration of the infrared signal was realized in the amide I region from 1800 cm^{-1} to 1600 cm^{-1}.

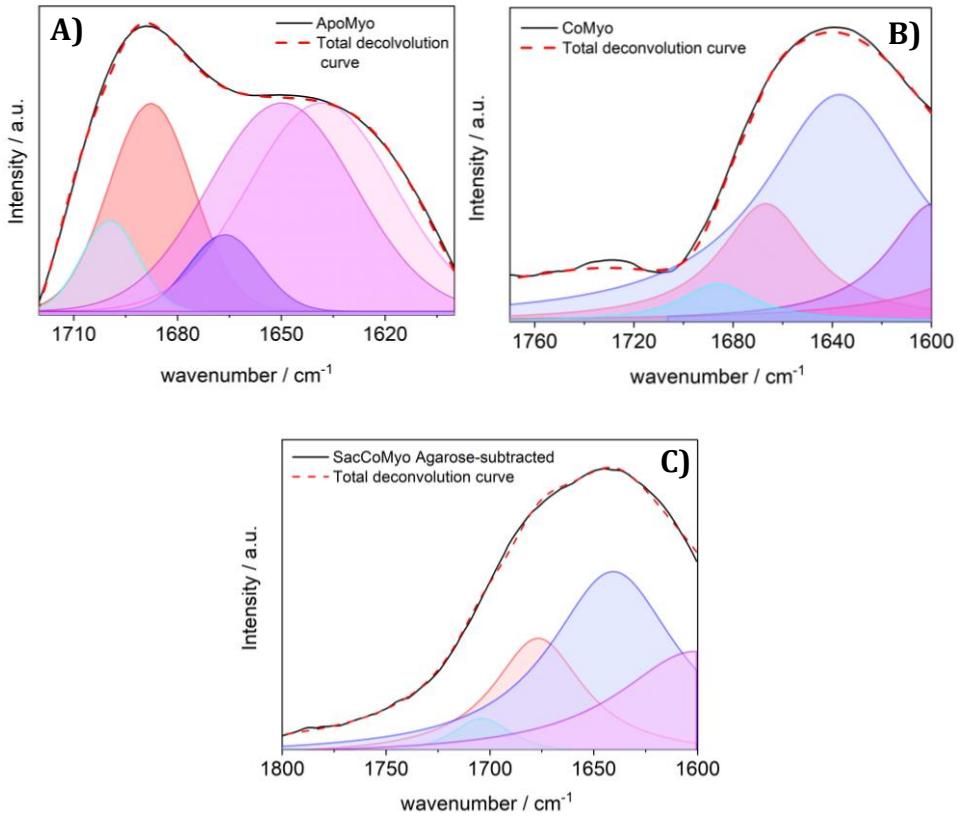


Figure S8. Amide I band deconvolution and secondary structure percentages at the border of Apo-Myo (A), CoMyo (B) and SacCoMyo (C) deposited on to gold polycrystalline surface, while the different types of secondary structure (α-helices, β-sheets + β-turns, and random coils) are colored in cyan, pink, blue, purple and red, respectively.

S2.0 References

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(2) Venkatkarthick, R.; Davidson, D. J.; Ravichandran, S.; Vengatesan, S.; Sozhan, G.; Vasudevan, S. Eco-Friendly and Facilely Prepared Silica Modified Amorphous Titania (TiO2–SiO2) Electrocatalyst for the O2 and H2 Evolution Reactions. *Catal Sci Technol* **2015**, *5* (11), 5016–5022. https://doi.org/10.1039/C5CY00805K.

(3) De Souza, J. C. P.; Silva, W. O.; Lima, F. H. B.; Crespilho, F. N. Enzyme Activity Evaluation by Differential Electrochemical Mass Spectrometry. *Chemical Communications* **2017**, *53* (60), 8400–8402. https://doi.org/10.1039/C7CC03963H.