Electronic Supplementary Material (ESI) for Chemical Communications.

Converting a Cysteine-Rich Natively Noncatalytic Protein to an Artificial Hydrogenase

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Supplementary Information

Experimental section

General procedures

All chemicals and reagents are of analytical grade and used as received without further purification. All glassware and plasticware are soaked in a 10 mM ethylenediaminetetraacetic acid (EDTA) bath overnight, followed by overnight soaking in 10% and 1% nitric acid baths, respectively. These are then thoroughly washed with Milli-Q (Millipore Sigma) water. All buffers are made using chelexed (Sigma-Aldrich) deionized water, which is again chelexed overnight, followed by pH adjustment and filtration.

Peptide synthesis and purification

The α-MT and its mutants are synthesized, purified, and identified using previously reported procedures.[1] Peptide concentration is calculated using an extinction coefficient ε_{280nm} of 5500 $M^{-1}cm^{-1}$ for one Trp.

Preparation of peptide samples

For all sample preparations of αMT and its mutants, lyophilized peptides are dissolved in 10 mM tris pH 7.5 buffer, reduced with 100-fold excess and a 50-fold excess of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), respectively for 10 min to reduce any disulfide bonds that may be present. Excess TCEP is later removed by a PD10 column equilibrated with a low pH buffer (pH 2.5) and collected in septa-capped vials. Elution of the sample using a low-pH buffer removes any metal contamination.[2] The eluted peptides are adjusted to the required pH and degassed for 10 min before each experiment. Metallation was performed with 1-4 equiv of $NiSO_4$ addition to αMT or 1 equiv addition to the mutants. For Ni_4- αMT preparation, Ni^{II} was added in small aliquots with ~10 min equilibration time. Because the activity assays were performed with stoichiometric amounts of Ni^{II}, in general, no additional steps were taken to purify the metallated samples after metal addition.

ESI-MS

All samples were analyzed on Thermo Scientific Orbitrap Exploris 240 (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an Ultimate 3000 Nano UHPLC system (Dionex, Sunnyvale, CA, USA) using a 150 × 0.075 mm PepMap 100 C18, 2 μm particle size, analytical column (Thermo Fisher Scientific) in trapping mode with a C18 trap cartridge. The peptides were separated using mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid) at a flow rate of 0.3 μL/min. The peptide separation gradient consisted of 2−30% solvent B over 4 min, ramped to 95% solvent B over 2 min, held for 4 min, and then returned to 2% solvent B over 1 min and held for 6 min. The peptides were eluted directly into the nanospray source of an Orbitrap Exploris instrument controlled with Xcalibur version 3.1 (Thermo Fisher, San Jose, CA) using a conductive nanospray emitter. The spray voltage was set to 2400 V, and the ion transfer tube was set to 300 °C. The MS1 data were collected at a resolution of 60,000 with a scan range of 250−2000 m/z.

CD spectroscopy

The apo αMT sample is prepared as mentioned previously. The Ni^{II}-bound αMT are prepared anaerobically by adding Ni^{II} in aliquots to apo αMT and equilibrated for 10-min between each addition. The CD data are recorded on a JASCO J-1500 spectrometer using a 1 mm path-length quartz cuvette. Data processing was performed using Origin.

UV/Vis spectroscopy

Metal titration of αMT and its mutants are performed by anaerobic addition of Ni^{II} solution in a 1 cm path length septa-capped cuvette (Starna Cells) and the spectral changes are monitored using a Cary 5000 UV/Vis/NIR spectrophotometer (Agilent). Ni^{II} is added using a gastight syringe (Hamilton) followed by 10 min incubation to reach equilibrium after each Ni^{II} addition before measurements. The absorbance of apo αMT is subtracted from Ni^{II}-bound spectra to obtain the differential absorbance (ΔA). Appropriate dilution correction is done as necessary.

pH titrations

Ni_4-αMT is prepared by adding 4 eq of Ni^{II} to αMT in unbuffered solutions. The pH of the solution is adjusted by adding small aliquots of concentrated KOH followed by 10 min stirring. After each addition, UV-vis spectra are recorded. The spectral changes are plotted against pH.

Photocatalysis

Photocatalysis experiments are performed with 2 mL solutions of anaerobically prepared 30μM catalyst, 1 mM of respective photosensitizer (RuPS, FL, ErB, and EY) and the corresponding amount of the sacrificial electron donor (TEOA, TEA or AA) in 10 mM tris buffer as tabulated in Table S2. This solution is adjusted to the required pH before transferring to septa-capped pyrex tubes. The white light source (λ>400 nm; Thor Laboratories) of 180 mW power is used in these experiments. The low buffer concentration is used to provide a sufficient buffering capacity while minimizing the effect of ionic strength on photocatalytic H_2 production, as the latter effect can be a contributing factor in such assays. 250 μL of headspace gas is sampled in 30 min intervals using gas-tight syringes (VICI) and injected into a GC-2014 instrument (Shimadzu) operating with N_2 as the carrier gas. H_2 is detected using a thermal conductivity detector (TCD) and the area under the peak is converted to ppm using calibration standards. TONs are calculated from the ratio of the moles of H_2 produced per mole of the catalyst. Experiments of αMT mutants are performed under optimized conditions with 2 mL solutions of anaerobically prepared 30 μM mutants, 1 mM RuPS, and 100 mM AA.

Emission assays of photosensitizers

A 2.5 mL solution of 1 mM of RuPS in 10 mM Tris buffer is prepared in a quartz cuvette and adjusted to a final pH of 5.6. The quenching experiments have been performed by the addition of aliquots of AA up to 130 mM. The quenching with Ni_4-αMT is studied in a concentration range up to 50 μM. The emission intensity is recorded using a PTI fluorimeter with the respective excitation and emission wavelengths (λ_{ex} = 510 nm, λ_{em} = 600 nm). Data is analyzed using Origin and fit to the Stern-Volmer equation:

$F_0/F = 1 + K_q\tau_0[Q]$

F_0 and F are the initial and final intensity, respectively; [Q] is quencher concentration, τ_0 is the lifetime of fluorophore in the absence of quencher (0.63 μs in water), and K_q is the quenching rate constant.

Electrochemical studies

The composites are prepared by following the previously reported literature procedures.[3] First, 0.2% single-walled carbon nanotubes (SWNTs) were dissolved in a 4:1 ratio of water: ethanol mixture followed by the addition of 0.1% (∼17 μL) nafion in ethanol. The suspension was then sonicated for 1h at 37 °C. To this, aliquots of the hetero-bifunctional linker, 1-pyrenebutyric acid N-hydroxy succinimide ester (4 μL of 4 mg/mL) (PBSE) in DMF was added and the mixture was incubated for 1h at RT. The final samples were prepared by mixing the SWNT/PBSE composite and Ni_4-αMT in a 1:1 ratio and incubated for 12−16 h at 4 °C. Finally, 10 μL of the assay composite was dropped on the glassy carbon electrode (GCE) and dried under a gentle stream of N_2.

All electrochemistry experiments are performed using a Pine Wave Driver 20 bipotentiostat using GC as the working electrode, coiled Pt wire as the counter electrode, and Ag/AgCl (in saturated KCl) as the reference electrode. The experiments are performed by dropping 10 μL of 164 μM Ni_4-αMT on GCE, followed by drying under a gentle flow of nitrogen. All experiments are performed using a 20 mM mixed buffer solution of sodium acetate, 2-ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), and Tris in 0.1 M KCl as the supporting electrolyte. A scan rate of 100 mV/s is used.

The CPE experiments are performed under inert atmosphere with 7 mL of 40 μM Ni_4-αMT solutions prepared in 10 mM acetate buffer pH 5.5 containing 0.1 M KCl in a 50 mL three-necked pear-shaped flask with stirring, using a 2 mm glassy carbon rod used as the working electrode polarized at −1.25 V vs Ag/AgCl. The counter electrode is separated from the main chamber using a fritted tube. To measure H_2 production, 250 μL of headspace gas was syringed out after 1 h of CPE and quantified similar to the photocatalysis experiments. CPE experiments of αMT mutants are performed with 7 mL of 40 μM of mutants bound to 1 eq Ni^{II}.

Table S1. Physical parameters of αMT and its variants.

Protein	λ_{max} (nm)	$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)	Ni^{II} eq.[a]	K_d (μM)[b]
αMT	290	42,049	2.0	29
	322	29,909	2.0	73
	430	13,167	2.8	310
	600	1,210	3.8	
αMT-1	289	20,750	1	ND
	322	6,788		
	474	469		
	601	197		
αMT-2	280	17,669	1	ND
	288	16,823		
	320	12,274		
	620	318		
αMT-3	283	17,531	1	ND
	288	17,539		
	322	5,225		
	466	523		
	606	185		
αMT-4	282	19,349	1	ND
	288	19,210		
	322	5,826		
	479	465		
	591	199		

[a]Extracted by fitting the Hill equation; [b]extracted by fitting the K_d equation.[4] ND – not determined.

Table S2. Photocatalytic conditions used for reaction optimization are shown below.

PS (concentration)	SED (concentration)	Acidity	TON	Initial TOF (s^{-1})
RuPS (1mM)	AA (100mM)	pH 5.6	260	0.0967
RuPS (1mM)	TEA (10%)	pH 12	83	0.0027
RuPS (1mM)	TEOA (15%)	pH 8.5	0	
ErB (1mM)	AA (100mM)	pH 5.6	0	
ErB (1mM)	TEA (10%)	pH 12	0	
ErB (1mM)	TEOA (5%)	pH 8.5	20	
ErB (1mM)	TEOA (10%)	pH 8.5	26	
ErB (1mM)	TEOA (15%)	pH 8.5	125	0.0076
ErB (1mM)	TEOA (25%)	pH 8.5	11	
FL (1mM)	AA (100mM)	pH 5.6	0	
FL (1mM)	TEA (10%)	pH 12	0	
FL (1mM)	TEOA (15%)	pH 8.5	6	
EY (1mM)	AA (100mM)	pH 5.6	0	
EY (1mM)	TEA (10%)	pH 12	9	
EY (1mM)	TEOA (5%)	pH 7.1	9	
EY (1mM)	TEOA (15%)	pH 7.1	11	

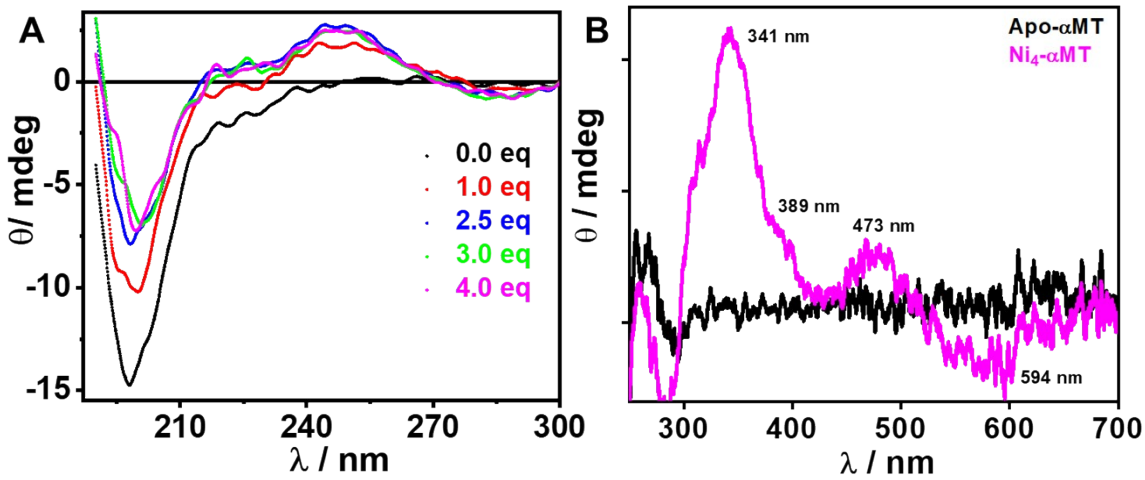


Fig. S1. A) CD spectra of 30 μM αMT in the presence of various Ni^{II} equiv. B) Visible CD spectrum of 2 mM apo αMT and in the presence of 4 equiv Ni^{II}. Data collected in 10 mM Tris pH 7.5.

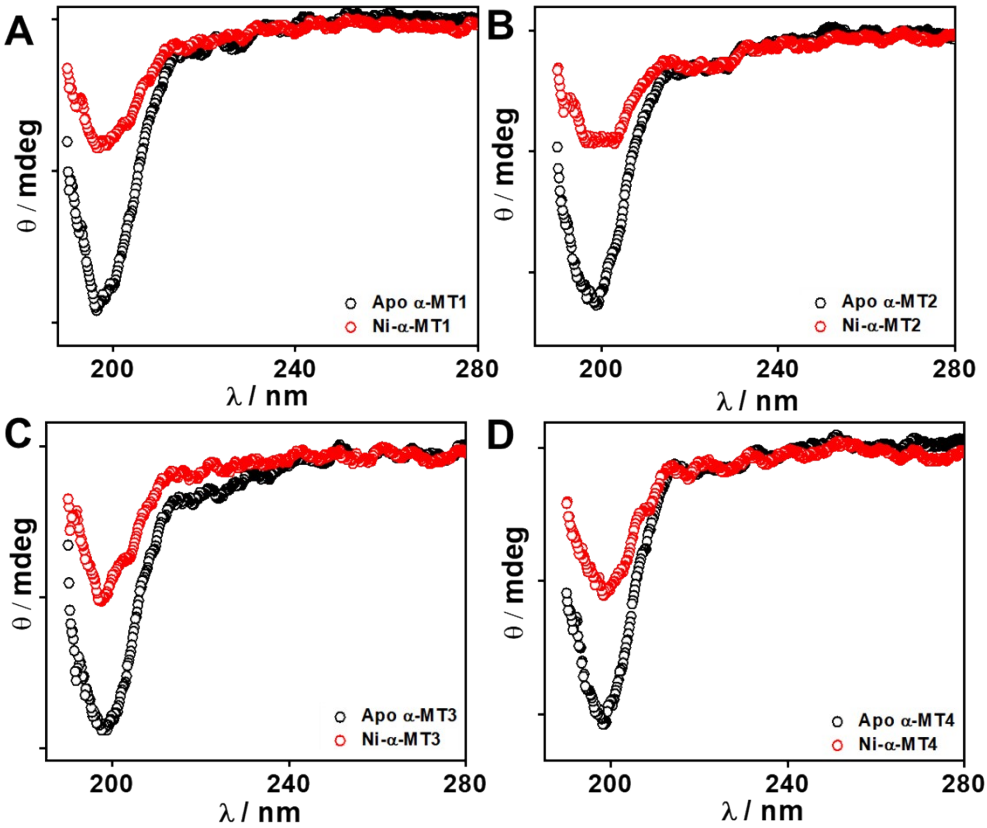


Fig. S2. CD spectra of 30 μM αMT1-4 in the absence and presence of 1 equiv Ni^{II}, collected in 10 mM Tris pH 7.5.

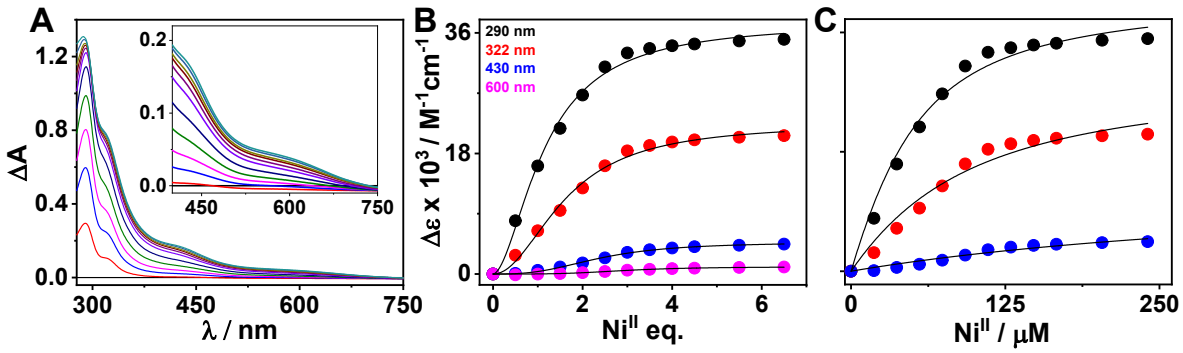


Fig. S3. (A) UV/Vis titration spectra of 30 μM αMT with Ni at pH 7.5. (B) Plots of differential absorptivity vs. eq of Ni^{II}. The solid black lines represent fits to the data to extract the number of Ni^{II} eq at which each band saturates.(C) Plots of differential absorptivity vs. concentration of Ni^{II}. The solid black lines represent fits[4] to the data to extract K_d (Table S1). The Y axis is the same as in B.

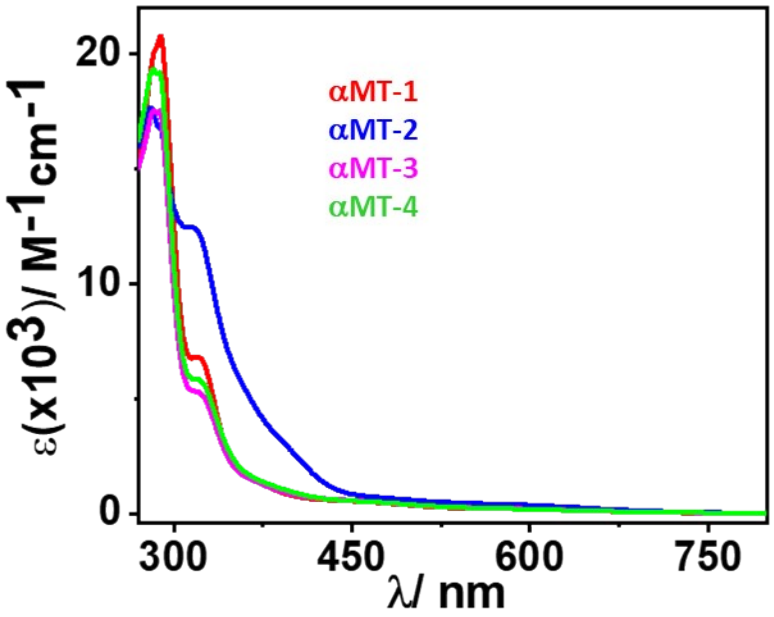


Fig. S4. UV/Vis spectra of 30 µM Ni^{II}-αMT mutants at pH 7.5.

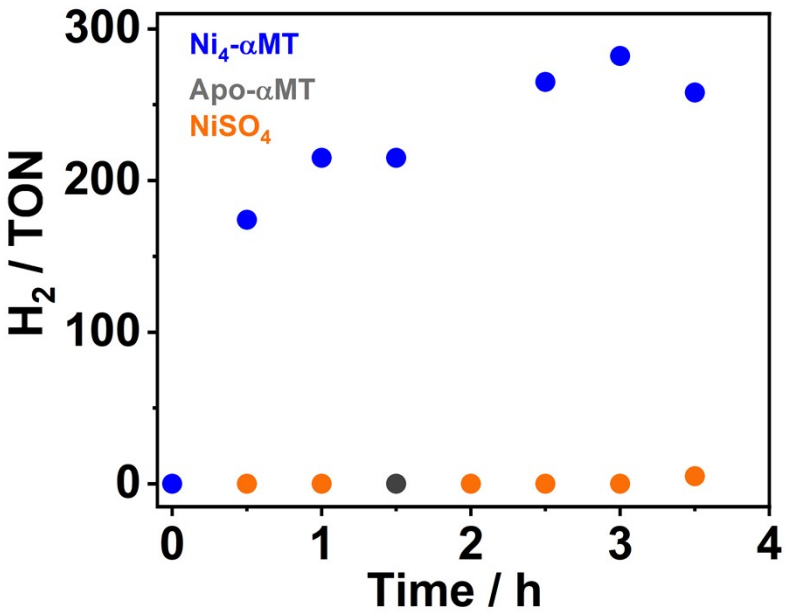


Fig. S5. Plots of time-dependent H_2 production at pH 5.6 by Ni_4-αMT (blue), apo αMT (grey), and $NiSO_4$ (orange). Data were collected at 30 μM concentration of protein using RuPS/AA. The apo protein and $NiSO_4$ were also used at 30 μM.

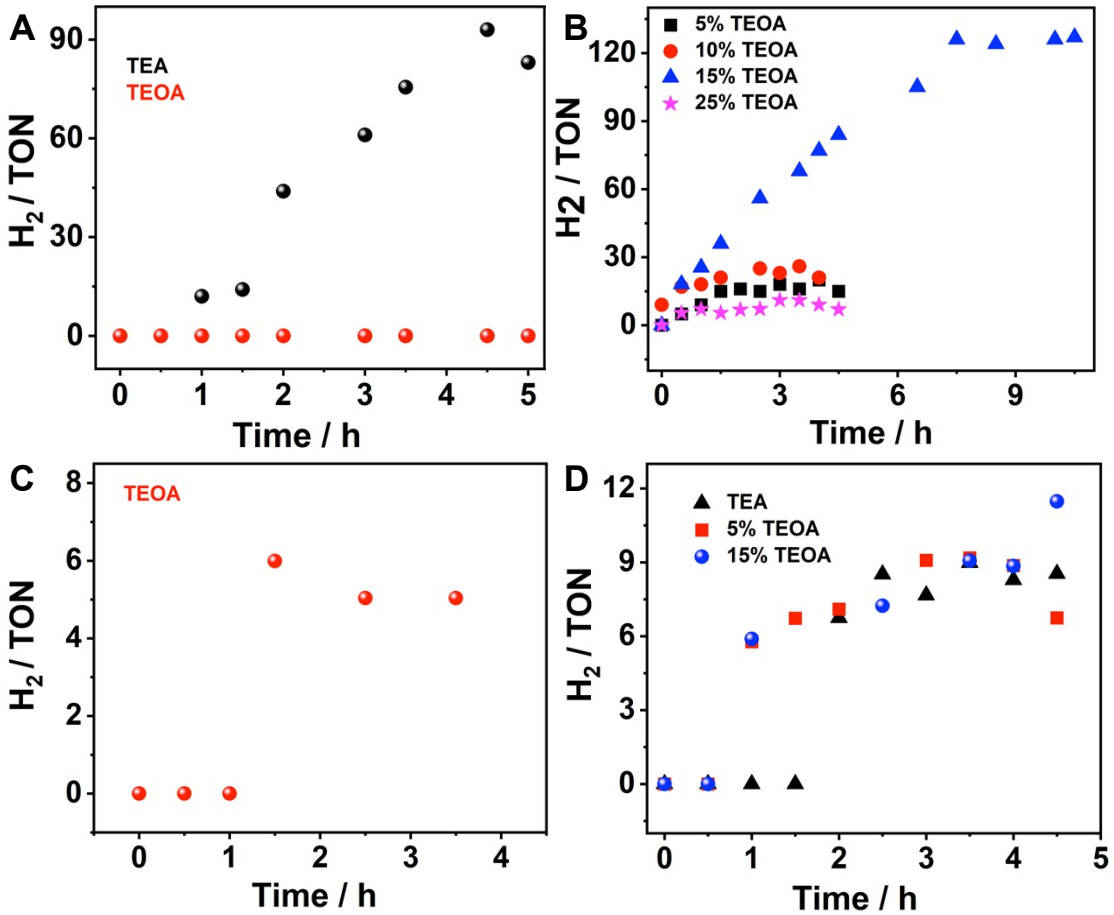


Fig. S6. Plots of time-dependent H_2 production by 30 μM Ni_4-αMT using (A) RuPS, (B) ErB, (C) FL, and (D) EY using different electron donors. Reaction conditions for each experiment are shown in Table S2.

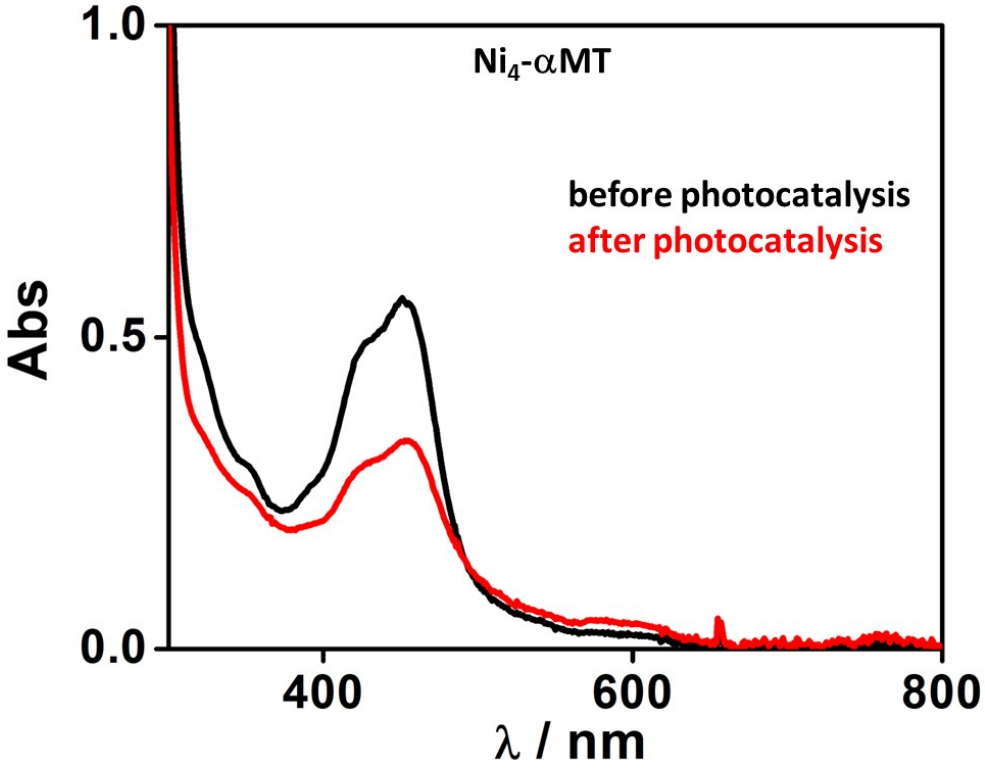


Fig. S7. UV-vis spectra of Ni_4-αMT photosample before irradiation (black) and after the photocatalysis experiment (red). A 10-fold dilution of the samples are done prior to measurement.

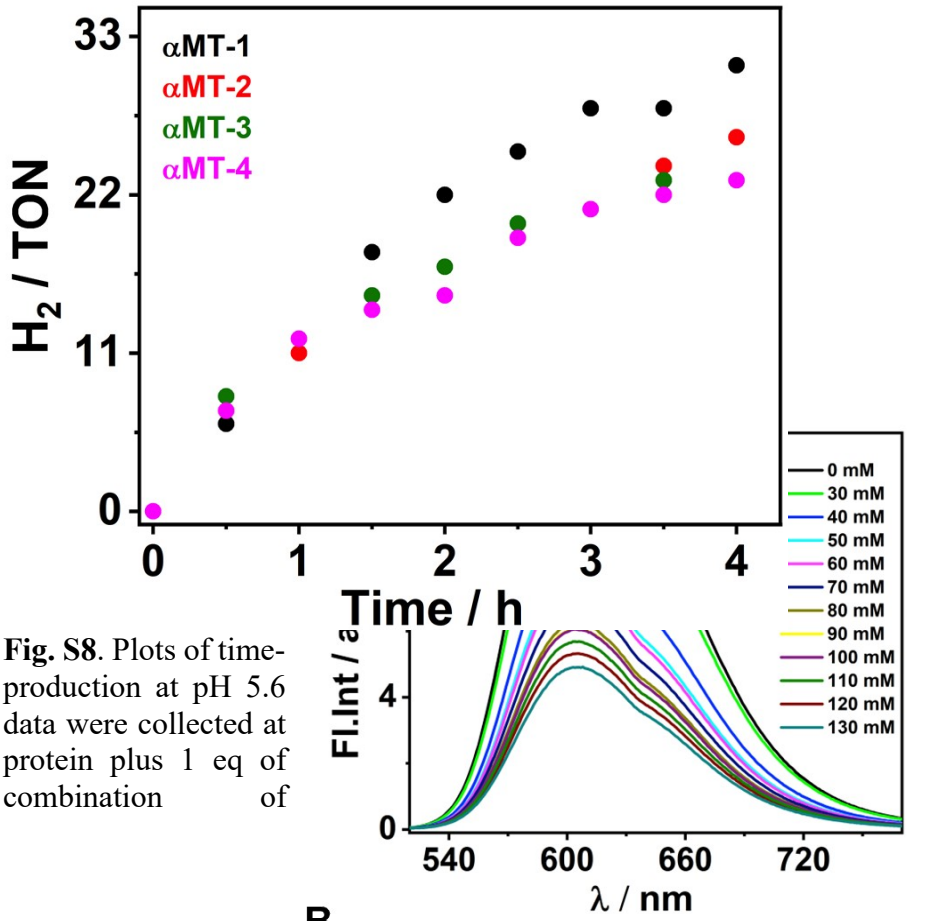
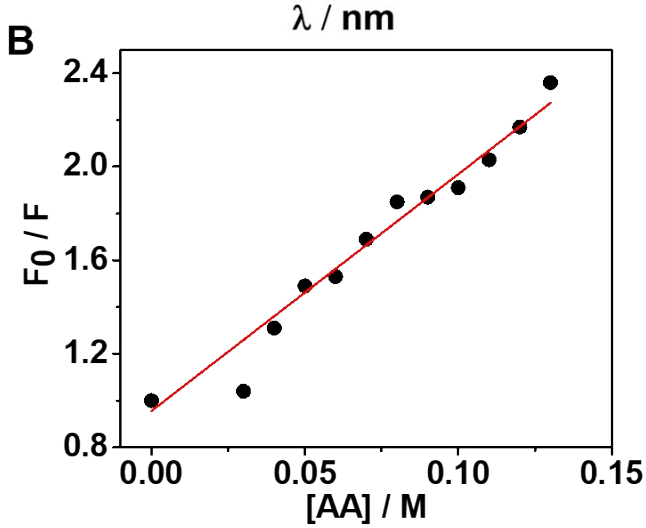


Fig. S8. Plots of time-dependent H_2 production at pH 5.6 by αMT variants. All data were collected at 30 μM concentration of Ni^{II} protein plus 1 eq of RuPS/AA. combination of



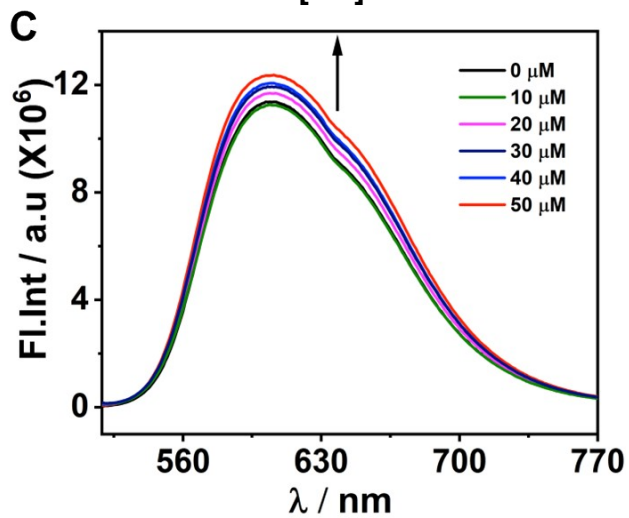


Fig. S9. Quenching of 1 mM RuPS in 10 mM tris buffer pH 5.6 (λ_{ex}= 510 nm) (A) as a function of AA concentration. (B) The corresponding Stern-Volmer plot. (C) RuPS emission as a function of Ni_4-αMT concentration showing no quenching.

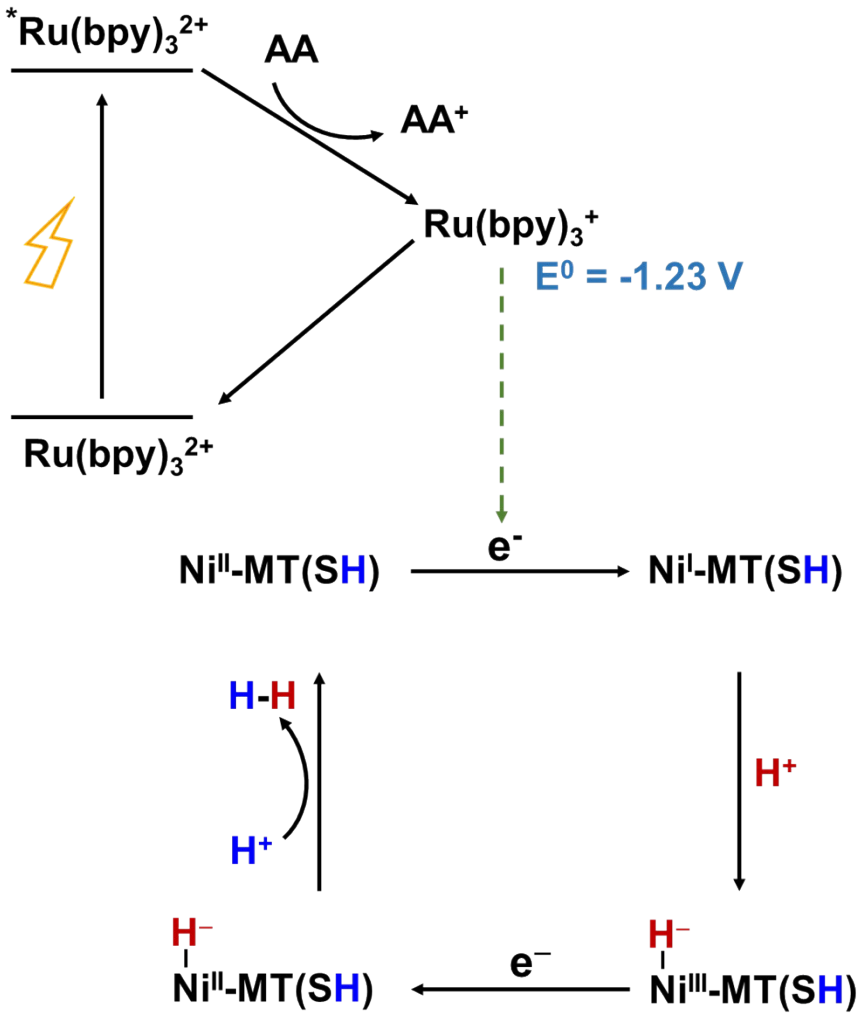


Fig. S10. Reductive quenching pathway of HER using RuPS/AA combination.

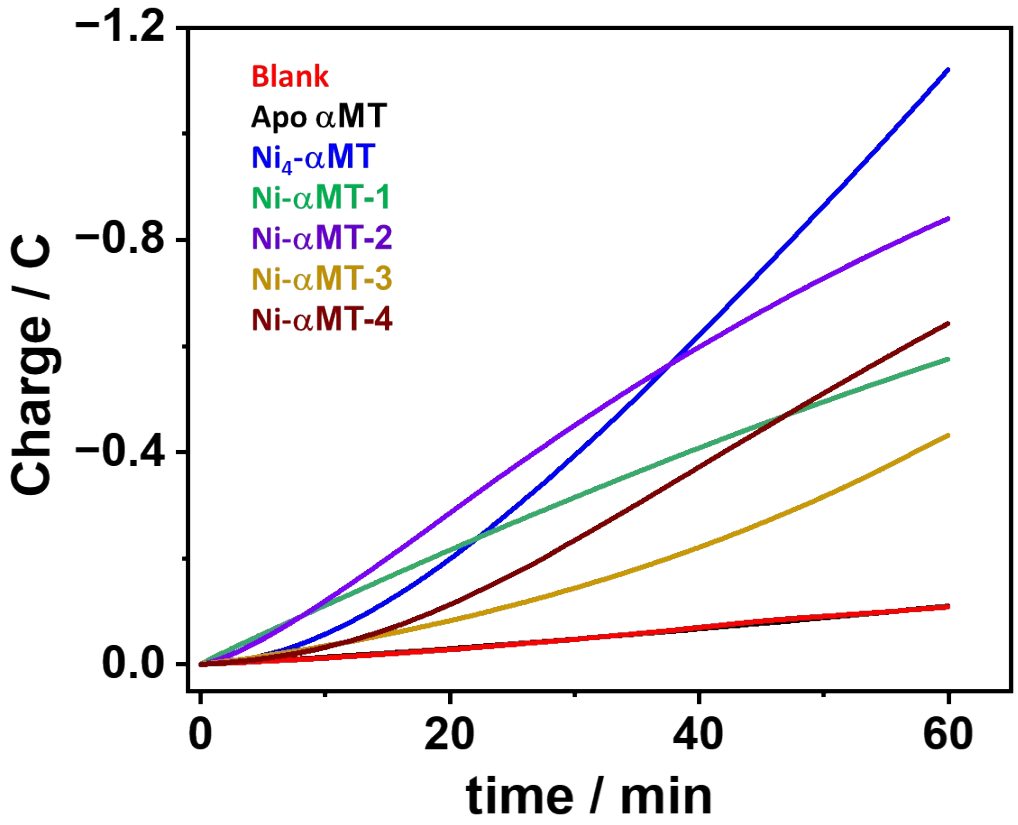


Fig. S11. Charge passed in Coulombs during 1 h of CPE of 7 mL 40 μM solutions of Ni_4-αMT (blue), Ni-αMT-1 (green), Ni-αMT-2 (violet), Ni-αMT-3 (dark yellow), Ni-αMT-4 (wine), apo-αMT (black), and blank (red) using a 3 mm glassy carbon electrode polarized at −1.25 V vs Ag/AgCl. pH = 5.5. No detectable H_2 was produced in the control samples.

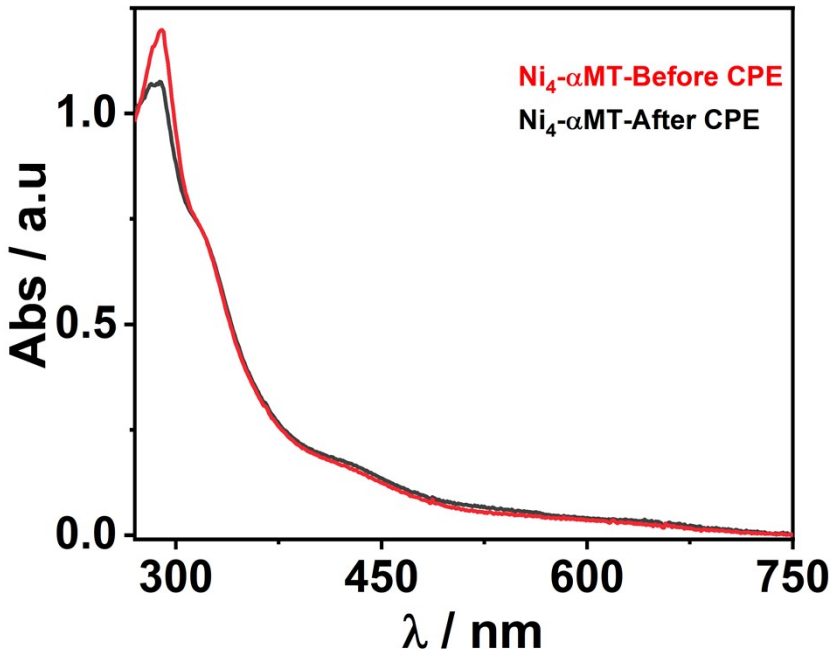


Fig. S12. UV-vis spectra of Ni_4-αMT samples before and after CPE.

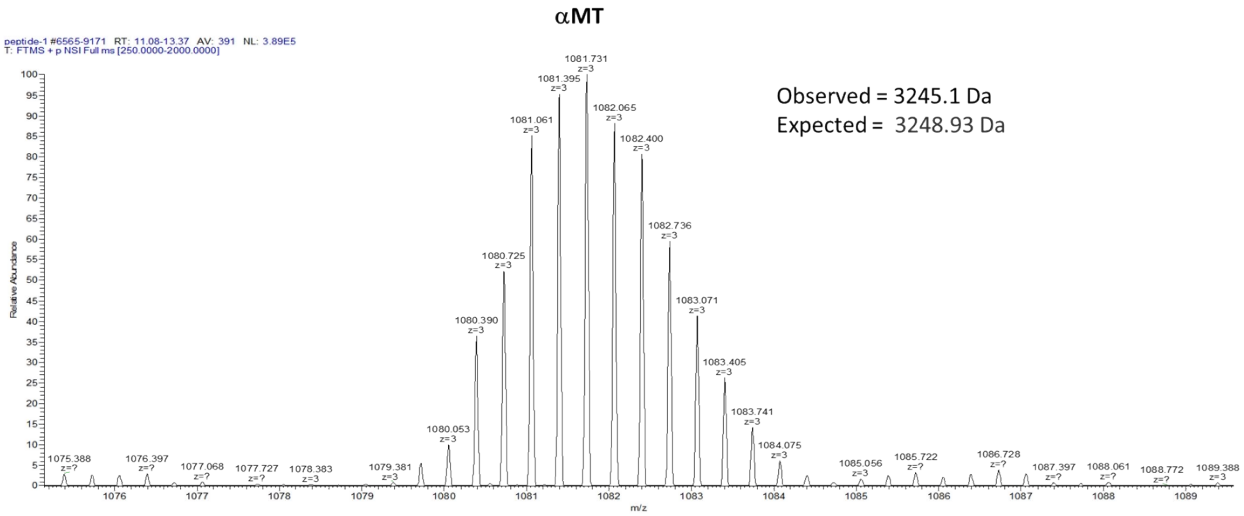


Fig. S13. ESI-MS data for apo αMT.

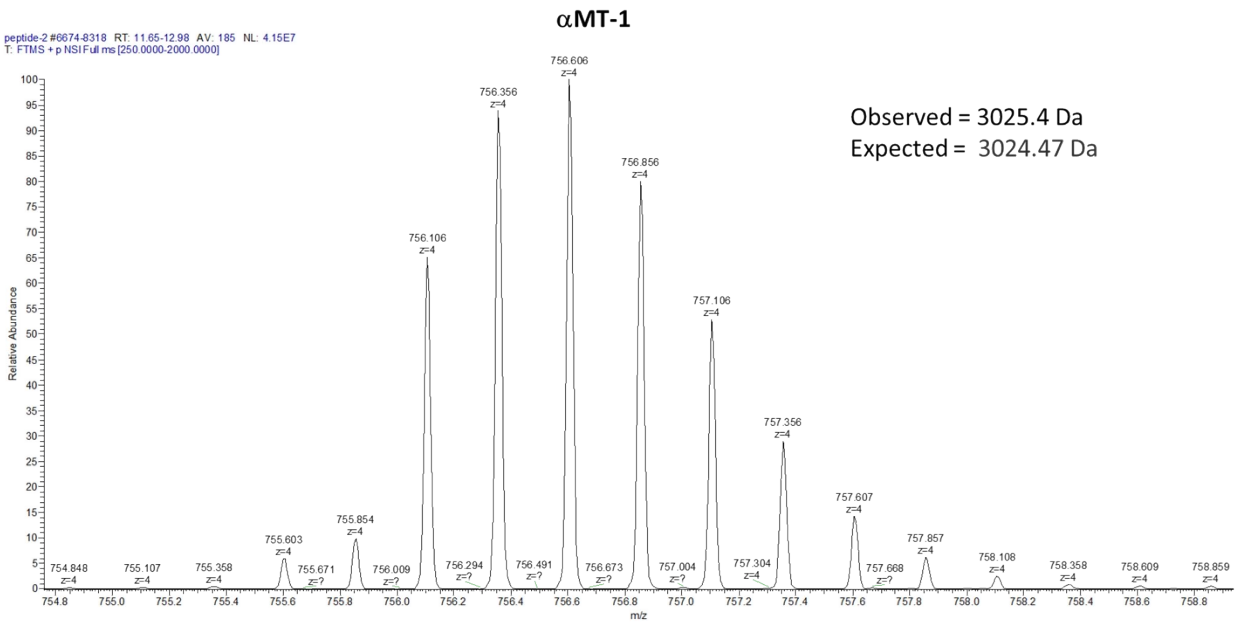


Fig. S14. ESI-MS data for apo αMT-1.

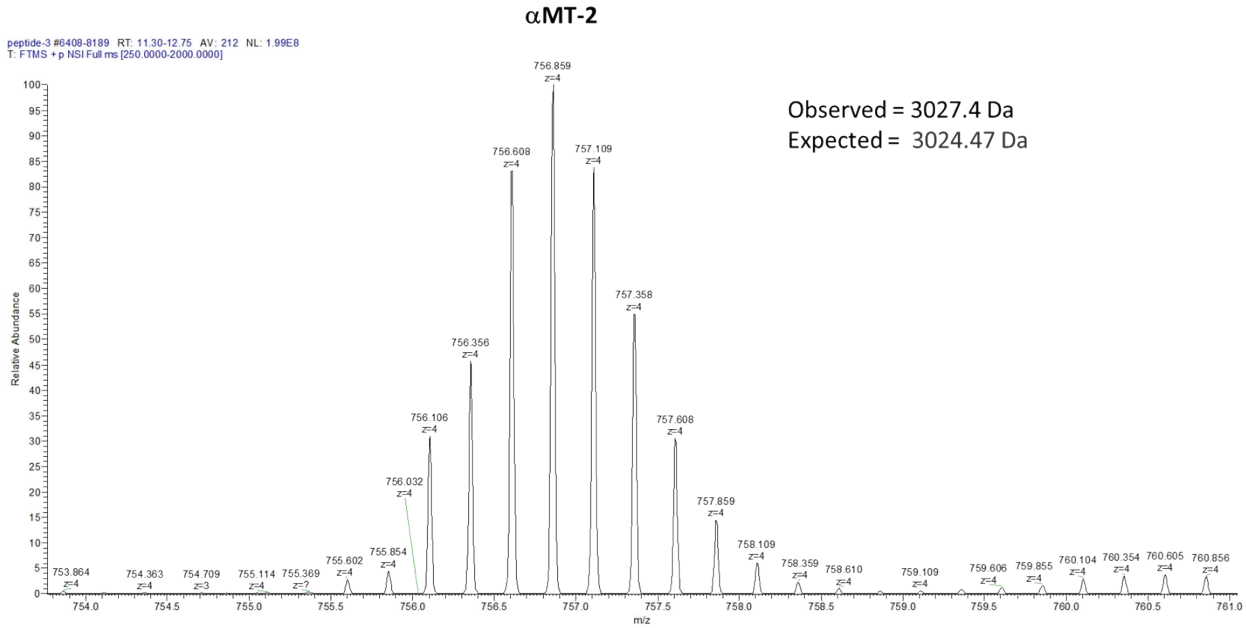


Fig. S15. ESI-MS data for apo αMT-2.

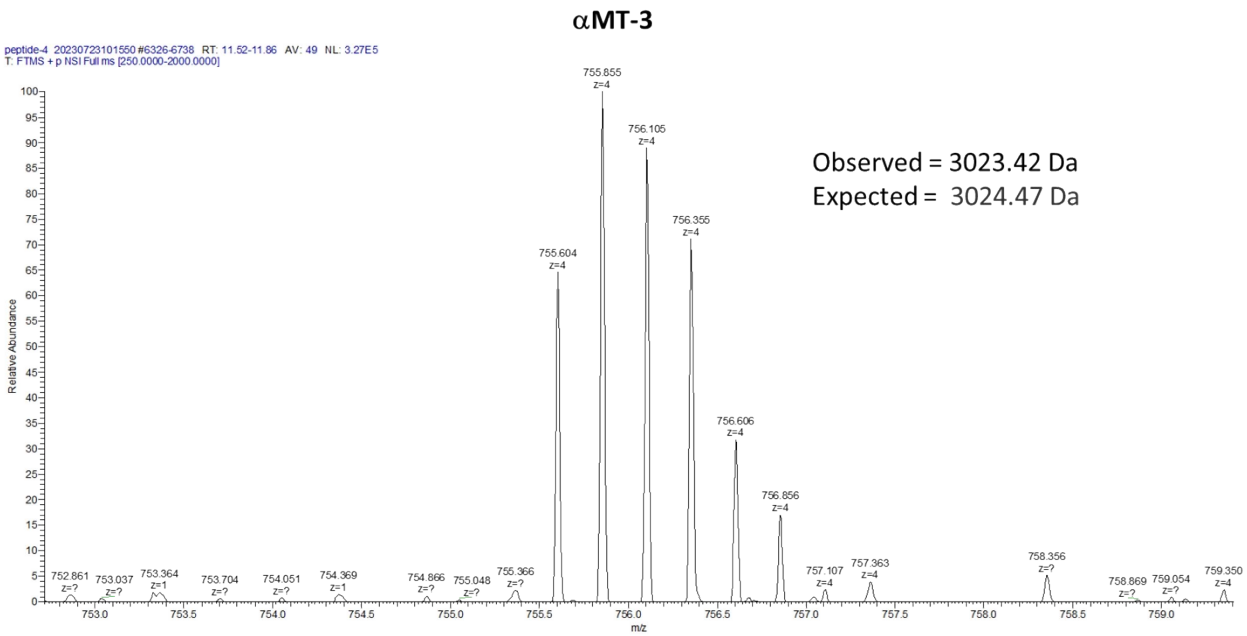


Fig. S16. ESI-MS data for apo αMT-3.

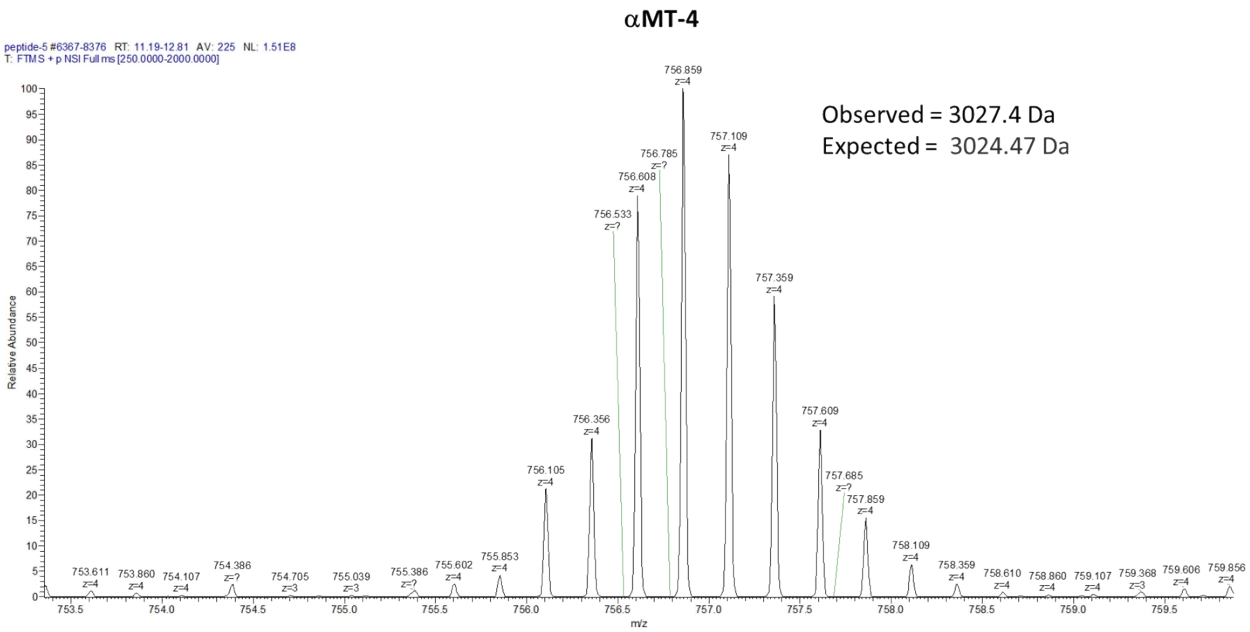


Fig. S17. ESI-MS data for apo αMT-4.

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[3] a) S. Chakraborty, S. Babanova, R. C. Rocha, A. Desireddy, K. Artyushkova, A. E. Boncella, P. Atanassov, J. S. Martinez, *J. Am. Chem. Soc.* **2015**, *137*, 11678-11687; b) Z. Nazemi, P. Prasad, S. Chakraborty, *ChemElectroChem* **2020**, *7*, 1029-1037; c) D. Prakash, S. Mitra, M. Murphy, S. Chakraborty, *ACS Catal.* **2022**, *12*, 8341-8351.
[4]S. Chakraborty, D. S. Touw, A. F. A. Peacock, J. Stuckey, V. L. Pecoraro, *J. Am. Chem. Soc.* **2010**, *132*, 13240-13250.