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Supplementary Materials

Artificial, Photoinduced Activation of Nitrogenase Using Directed and Mediated Electron Transfer Processes

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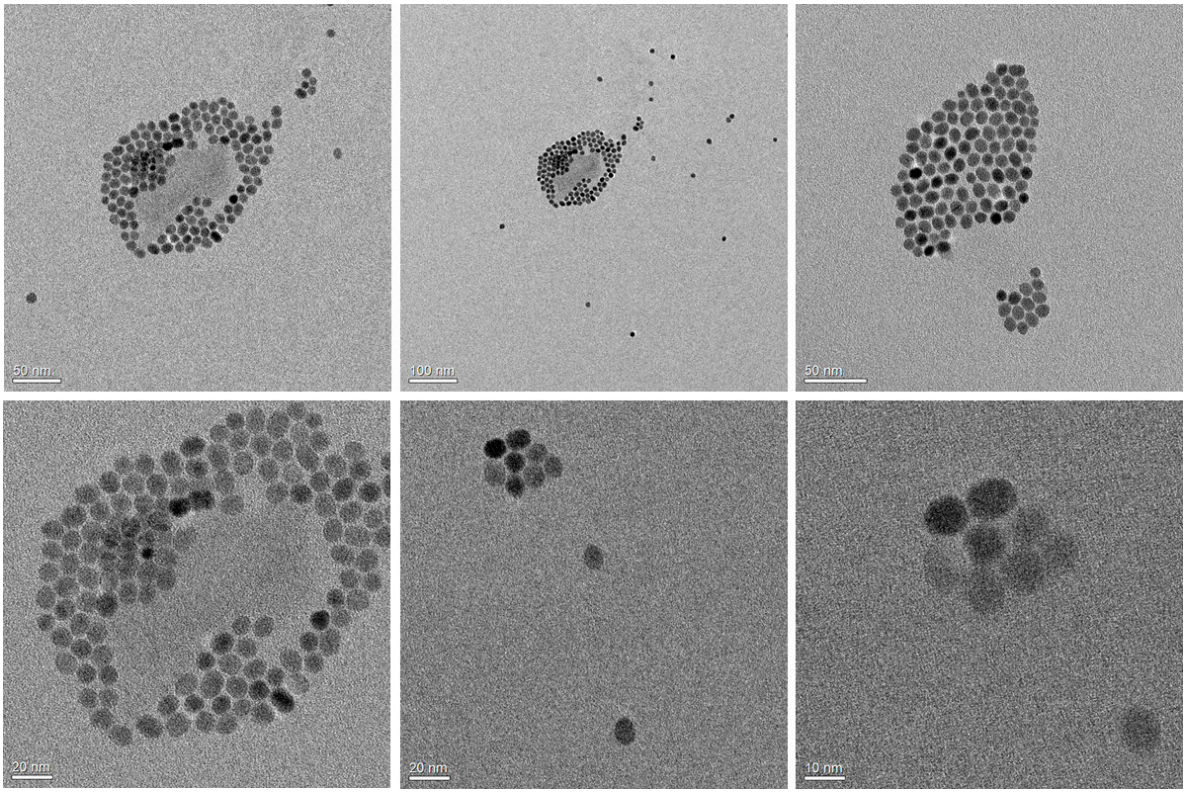

Figure S1. Additional TEM images of 10nm CdS-ME NPs.

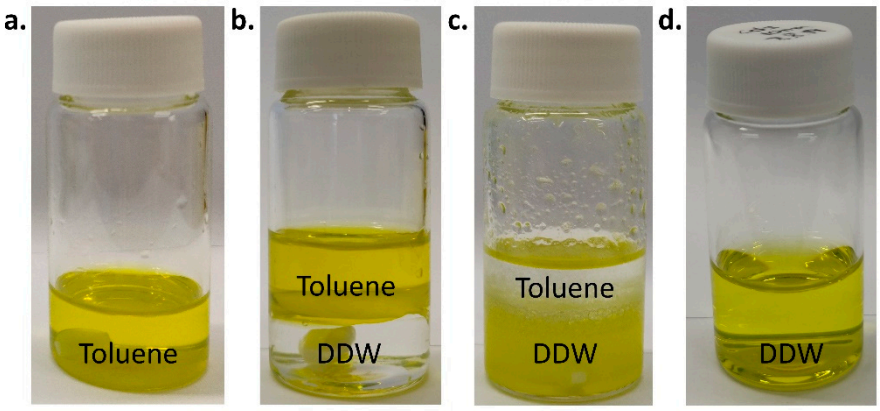

Figure S2. CdS Ligand Exchange to ME (a) CdS-OA NPs in Toluene, (b,c) Biphasic mixtures of Toluene and DDW before stirring (b) after stirring (c), d. CdS-ME NPs in DDW after filtration.

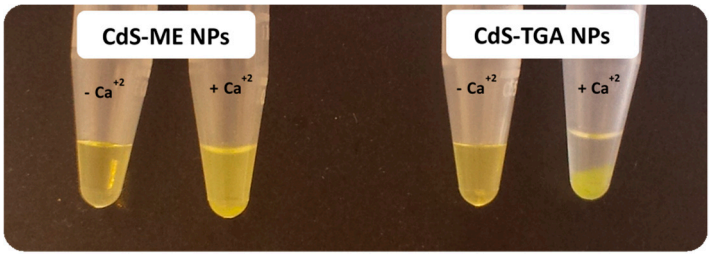

Figure S3. CdS-ME and Cd-TGA in the presence of positively charged divalent ions (Calcium ions), aggregation, and sedimentation were observed due to electrostatic crosslinking in the case of CdS-TGA NPs with the presence of calcium ions interaction.

CdS NPs Nanoparticle Concentration Determination

UV-Vis spectroscopy was used to determine the CdS NPs stock solution concentration and estimate band-gap (E_{bg}). 20 µL of the CdS stock was diluted in 180 µL of H_2O and the solution absorption between 300 to 700nm was measured. Using Beer's Law, Equation S1, the CdS NPs concentration were calculated:

Equation S1. $$C = \frac{A}{\varepsilon_{472\ nm} L}$$

where C is the molar concentration (mole·L^{-1}), A is the absorbance, L is the path length (cm), and ε_{472nm} is the molar extinction coefficient at 472 nm (2.56×10^6 M^{-1} cm^{-1}).
Equation S2 used for the determination of the E_g, when λ is the wavelength of the first peak in the UV-Vis absorbance spectrum, h represents Planck's constant (4.136×10^{-15} ev·sec) and c is the velocity of light (4.136×10^{17} nm/sec).

Equation S2. $E_g = hc/\lambda$ $E_g = 1240ev/\lambda$ (first peak)

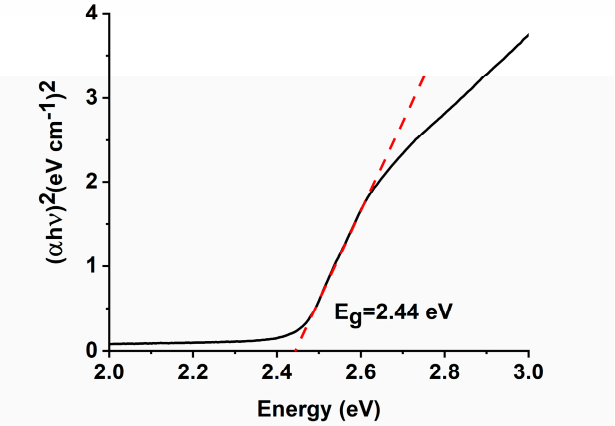

Figure S4. Tauc plot of the CdS NPs.

Agarose Gel Electrophoresis Characterization

In order to characterize the CdS NPs surface charges, a 0.6 wt% agarose (Lonza) gel in 1 X Tris-acetate-EDTA (TAE, Bio-Rad) was prepared. 0.6 g of agarose was dissolved in 100 mL of x1 TAE buffer. The solution was then microwaved until a clear, colorless solution was obtained. The mixture was then poured into the gel tray and allowed to solidify. CdS-ME / TGA NPs were then pipetted into the wells. A potential of 90 V is then applied for 30 minutes.

Zeta-potential Characterization

An additional method to determine the CdS NPs surface charges applied. Using Zetasizer Ultra of Malvern set up The CdS NPs were analyzed while dissolved in a 0.1 M phosphate buffer (PB) pH-7.2 and 51mM AA, CdS NPs concentration -120 nM.

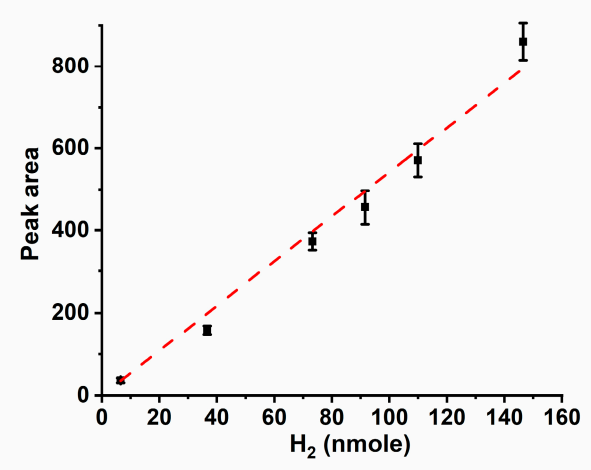

Figure S5. Calibration curve for H_2 using GC-TCD. (slope- 5.4±0.2, R-Square- 0.994)

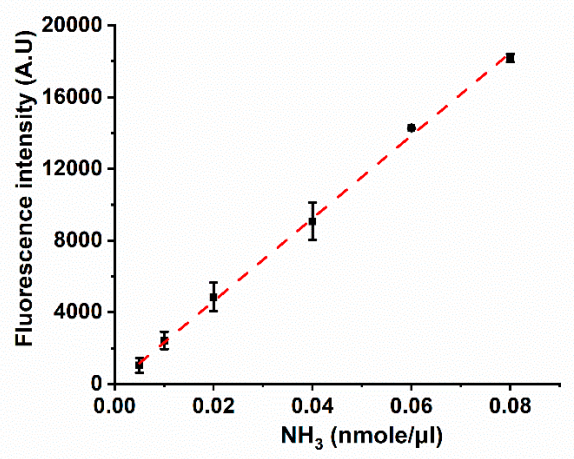

Figure S6. Calibration curve for NH_4Cl using OPA fluorescence assay after extraction to chloroform. (slope- 231000±2000, R-Square- 0.999)

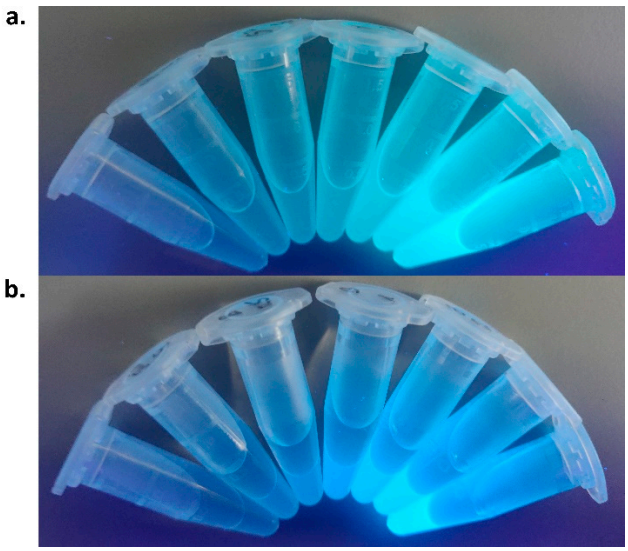

Figure S7. Fluorescence emission of calibration curve samples (NH_4Cl) after derivatization with OPA. (a) Before extraction to Chloroform, (b) After extraction to Chloroform.

Azotobacter Vinlandii Growth & Nitrogenase Purification [1,2]**:**

Azotobacter vinelandii strain DJ995 and DJ1194 which express his-tagged WT MoFe-P and αL158C MoFe-P respectively were grown using modified Burk media containing 2% sucrose, 0.8 mM $MgSO_4$, 0.6 mM $CaCl_2$-$2H_2O$, 10 μM Na_2MoO_4-$2H_2O$, 0.02 mM $FeSO_4$-$7H_2O$, 13 mM $C_2H_7NO_2$, 1.5 mM KH_2PO_4, and 4.6 mM K_2HPO_4. DJ995 & DJ1194 were seeded and grown on modified Burk agar plate (1.6% agarose) until sufficient colonies growth at 30 °C appeared. Then, the colonies were transferred to a 100 mL starter and incubated at 30 °C and 180 rpm. The optical density at 600 nm (OD_{600}) was monitored during the growth. Once the OD_{600} has reached ~1 (overnight incubation) 5 mL was transferred to a fresh 1L modified Burk media with 13 mM ammonium acetate at 30 °C and 180 rpm until OD_{600}~1.5 was reached (overnight incubation). Ammonium depletion was executed by centrifugation of the cells at 4 °C and 4,500 rpm for 25 min and resuspension in modified Burk's media without the ammonium acetate salts to induce the MoFe-P expression. The OD_{600} was monitored in order to ensure cell proliferation during MoFe-P expression ~4 hr (OD_{600}~1.5-2). The cells were then spun down (4 °C, 4,500 rpm, 25 min) and resuspended again in a minimal volume of modified Burk's media without ammonium acetate. The cells mixture was then divided into 50-mL conical centrifuge tubes, spun down again (4 °C, 8,000 rpm, 3 min) and the supernatant was removed before cell aliquots (~6 gr) were frozen in liquid nitrogen and stored in -80°C until MoFe-P purification.

Nitrogenase MoFe proteins, containing a 7xHis-tag on the α-subunit, were purified using metal affinity chromatography (1 mL, High-affinity Ni-NTA resin, GenScript, utilized in a glove box (O_2< 0.2 ppm). Cells aliquot (~6 gr) were dissolved in 20 mL of phosphate buffer containing NaCl and Sodium dithionite (0.1 M PB, 500 mM NaCl, 5 mM Sodium dithionite- DT, pH 7.2). The Precipitated cells were resuspended with an additional 20 mL of fresh PB containing NaCl and DT. Cells were lysed by Qsonica Q500- 500W sonicator installed in a glove box using a high voltage feedthrough connector (Lumitron), 15 sec on, 30 sec off cycles for 12 min total time on. (1/2" probe, 22% amplitude). The cell lysate was then centrifuged twice, a cycle of 30 min (10,000 rpm, 4°C) then the supernatant was transferred to a 50-mL conical centrifuge for a second 60 min cycle (10,000 rpm, 4°C). The reddish-black lysate was then loaded on an affinity Ni-charged resin. The column was washed with 18 column volumes of wash buffer (0.1 M PB, 500 mM NaCl, 50 mM imidazole, 1 mM DT, pH 7.2) to remove any loosely bonded proteins. Then, the MoFe-P was eluted using 3 column volumes of elution buffer (0.1 M PB, 500 mM NaCl, 500 mM imidazole, 1 mM DT, pH 7.2). In order to remove the excess of imidazole, the eluted MoFe-P solution was filtered by a 30 kDa centrifuge filter (Amicon Ultra-4, Mercury) at 4,200 rpm for 20 min and 4°C. The MoFe-P was resuspended in 0.1 M PB, 1 mM DT, pH 7.2. Finally, the purified MoFe-P was freezed in liquid nitrogen at a concentration of 4 μM further stored in -80°C in 100 μL aliquots to minimize freeze/thaw cycles.

Protein concentration was determined using UV-Vis spectroscopy with an estimated extinction coefficient of 313,240 $M^{-1} \cdot cm^{-1}$ at 280 nm and the purities were analyzed based on SDS-PAGE gels (SurePAGE, Bis-Tris 4-12%, GenScript).

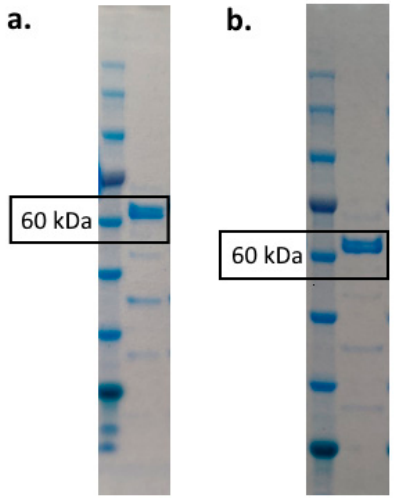

Figure S8. SDS/PAGE analysis of purified MoFe protein (a) WT (b) αL158C.

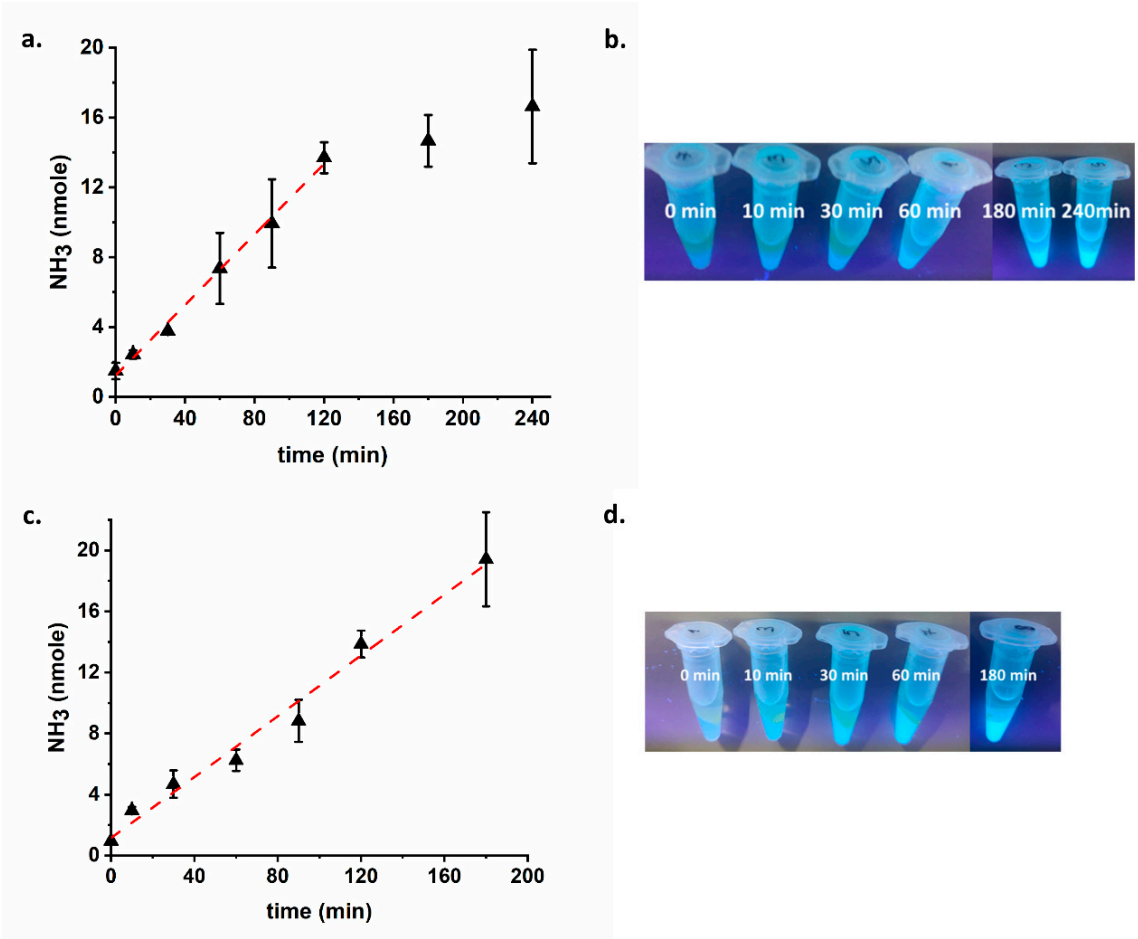

Figure S9. (a,c) kinetics of NH_3 production of mediated CdS-ME NPs: MoFe-P biohybrid systems, (a) WT MoFe-P and (c) αL158C MoFe-P. (b,d) NH3/OPA complex's fluorescence emission after extraction to Chloroform of different irradiation time points of mediated CdS-ME NPs: MoFe-P biohybrid systems, (b) WT MoFe-P and (d) αL158C MoFe-P.

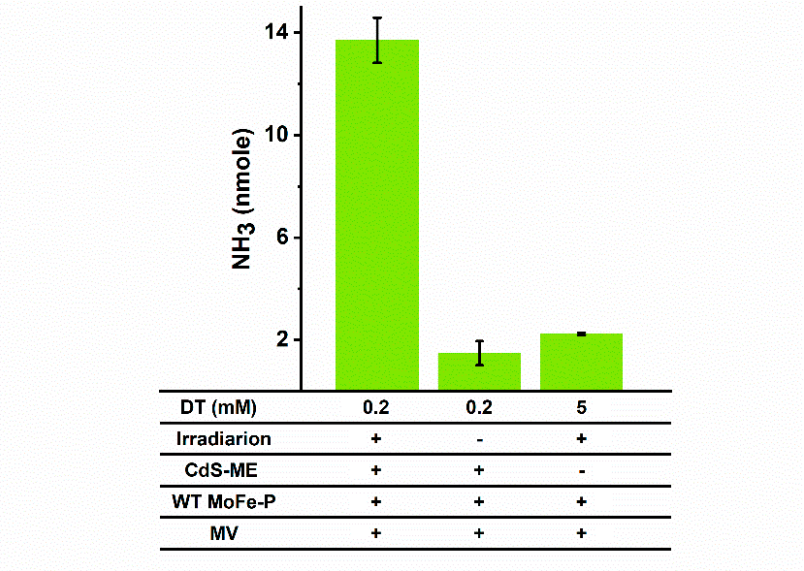

Figure S10. NH_3 production of WT MoFe-P activation by chemically reduced MV with Sodium dithionite (DT, with no CdS NPs) compared to CdS-ME NPs: WT MoFe-P biohybrid systems after 2 hr of irradiation and the same biohybrid system in dark condition as a control.

* Purified 100 µL aliquots of MoFe-P were stored in -80°C in 0.1 M PB, 1 mM DT, pH 7.2, when the MoFe-P was injected into the sealed glass vials the DT was diluted to 0.2 mM in the final reaction mixture volume of 560 µL.

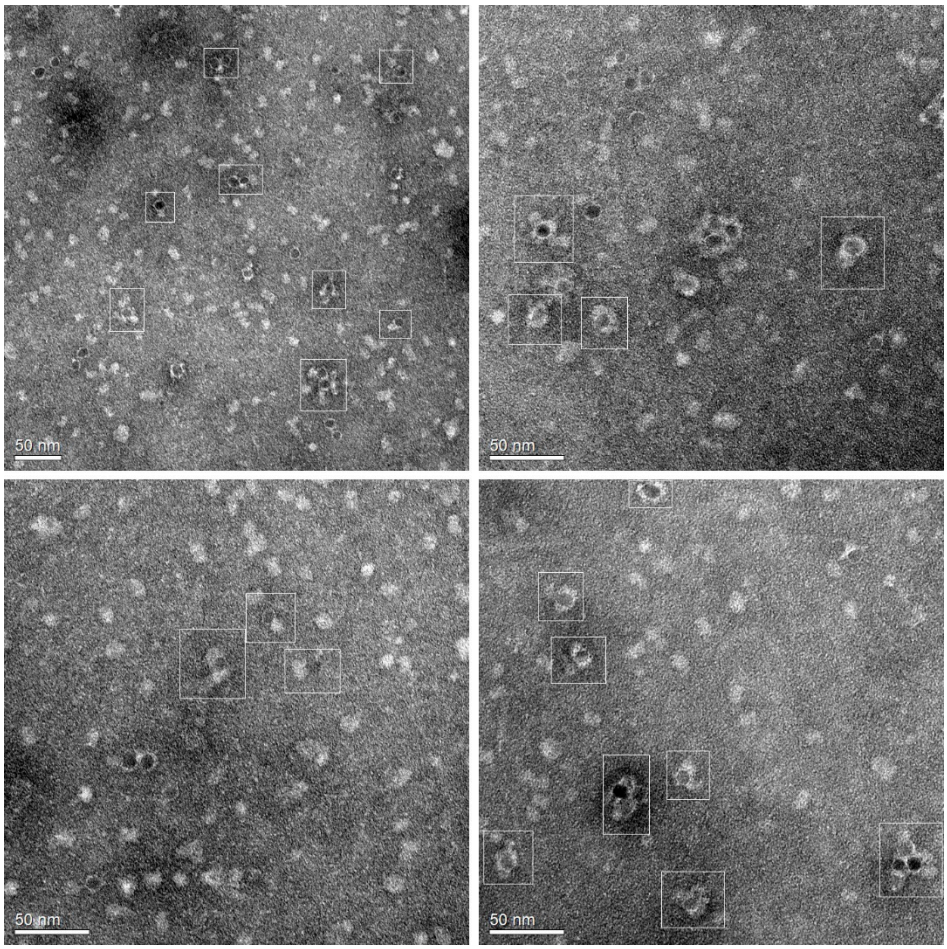

Figure S11. Additional TEM images of CdS-ME NPs: MoFe-P (αL158C) biohybrid systems, labeled by squares.

Table S1. specific activity of MoFe-P in mediated and non-mediated CdS-ME / TGA NPs : MoFe-P biohybrid systems.

Nitrogenase variant	CdS NPs-ligand	MV	S.A. (moleH_2/moleMoFe-P*min)	S.A. (moleNH_3/moleMoFe-P*min)
αL158C MoFe-P	CdS-ME	+	-	0.29 ± 0.02
	CdS-TGA		-	0.12 ± 0.01
	CdS-ME	-	41 ± 4	0.046 ± 0.004
	CdS-TGA		28 ± 2	0.061 ± 0.006
WT MoFe-P	CdS-ME	+	-	0.29 ± 0.04
	CdS-TGA		-	0.10 ± 0.01
	CdS-ME	-	15 ± 4	0.05 ± 0.01
	CdS-TGA		9 ± 3	0.050 ± 0.008
αL158C MoFe-P [b]	Fe-P [a,b]		21 ± 2	27 ± 7

[a] **N_2 reduction assay:** The reaction was done in 1.8 mL glass vials sealed with autosampler screw caps. Total reaction volume of 1.2 ml contained 2.5 mM $MgCl_26H_2O$ (Merck), 2.5 mM ATP (Sigma Aldrich), 10 mM sodium creatin phosphate hydrate (TCI, >98.0%) and 100 mM HEPES (Bio-Lab) as reaction buffer at pH 7.4. The solutions purged with N2 for 1 h prior to the injection of the MoFe-P, Fe-P, and 0.125 mg creatine phosphokinase (Sigma Aldrich) in an Ar-atmosphere glovebox (O_2< 0.2 ppm). The final concentrations of MoFe-P and Fe-P were 0.3 μM and 3 μM, respectively. The reactions were initiated by the addition of 90 μL of 100 mM DT and incubated for 2 hr at 30°C with gentle stirring.

[b] **Additional MoFe-P and Fe-P purification method:** MoFe-P and Fe-P which used for specific activity measurements were purified using ÄKTA go protein purification system (FPLC, General Electric Healthcare, GE). All chromatography was performed in an oxygen-free environment (O_2< 0.2 ppm) by using specially designed feedthrough connectors for the gove box. The feedthrough connector enables the loading of cell lysis or other fractions from the glove box onto different FPLC chromatography columns, and also enable the return of separated fractions back to the glove box in a closed cycle. All the buffers used for the separation were purged with Ar, stored in the glove box and contained 5 mM of DT. Firstly the cell lysate was separated into two main fractions using weak anion exchange chromatography. Filtered cell lysate (0.45μM filter) in a 50 mM PB and 100 mM NaCl solution buffered at pH 7.4 were loaded onto a GE HiPrep diethyl aminoethyl (DEAE) FF 16/10 Column, the column washed by 5 column volume (CV) with the same buffer. The separation was achieved by gradient elution between 100 to 500 mM NaCl in 50 mM PB, pH 7.4. The salt gradient was applied to the column at a flow rate of 2 ml/min over a total volume of 6 CV. The Fe-P fraction was further purified with size exclusion chromatography (SEC) on a GE Superdex 200 Increase 10/300 GL with a 50 mM pH 7.4 PB and 200 mM NaCl. His-tagged MoFe-P purified on a 1ml GE HisPrepTM FF affinity column using a step gradient. Nonspecific proteins were washed with 15CV of 50 mM PB and 500 mM NaCl and 10CV of 50 mM PB, 500 mM NaCl and 60 mM imidazole. His-tagged MoFe-P eluted with 50 mM PB, 500 mM NaCl and 350 mM imidazole elution buffer, pH 7.4. In order to remove excess imidazole, the eluted MoFe-P solution was filtered using a 30 kDa centrifuge filter (Amicon Ultra-4, Mercury) at 4,200 rpm for 20 min and 4°C, then MoFe-P was resuspended in 0.1 M PB, pH 7.4. Proteins concentration was determined using UV-Vis spectroscopy with an estimated extinction coefficient of 313,240 $M^{-1} \cdot cm^{-1}$ for MoFe-P and 27,570 $M^{-1} \cdot cm^{-1}$ for Fe-P at 280 nm and the purities were analyzed based on SDS-PAGE gels (SurePAGE, Bis-Tris 4-12%, GenScript).

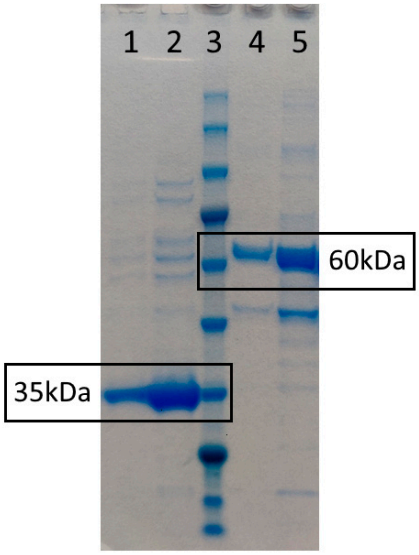

Figure S12. SDS/PAGE analysis of purified Fe-P (lane 1-2) and αL158C MoFe-P (lane 3-4) using FPLC system, molecular weight ladder (Lane 3).

References:

1. Burgess, B.K.; Jacobs, D.B.; Stiefel, E.I. Large-scale purification of high activity Azotobacter vinelandii nitrogenase. *Biochimica et Biophysica Acta (BBA) - Enzymology* **1980**, *614*, 196–209, doi:10.1016/0005-2744(80)90180-1.
2. Christiansen, J.; Goodwin, P.J.; Lanzilotta, W.N.; Seefeldt, L.C.; Dean, D.R. Catalytic and Biophysical Properties of a Nitrogenase Apo-MoFe Protein Produced by a *n ifB* -Deletion Mutant of *Azotobacter v inelandii* †. *Biochemistry* **1998**, *37*, 12611–12623, doi:10.1021/bi981165b.