ARTICLE

Supplementary Information for

Pyrene-Modified Hydrogel for Direct Bioelectrocatalysis: ATP-Independent Electroenzymatic Reduction of N_2

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Chemicals and Instruments

Purified laccase was purchased from Amano Enzyme Inc. Ethylene glycol diglycidyl ether (EGDGE) crosslinker was purchased from Polysciences, Inc; carboxylic acid functionalized Multi-wall carbon nanotubes (MWCNT-COOH) were purchased from Cheap Tubes. All other chemicals used were purchased from Sigma Aldrich and used as received without further purification. Water used was filtered with Ultrapure MilliQ system. A gene of *Azotobacter vinelandii* encoding flavodoxin (nifF) and *Synechococcus sp.* encoding the [2Fe-2S] ferredoxin (petF) were purchased as a synthetic gene from Integrated DNA Technologies, Inc and a plasmid pBTR(hCc) was purchased from Addgene. A prelinearized pET28a expression vector (Novagen) from Gibson assembly® and a QuikChange Lightning kit from Agilent Genomics were purchased. Primers were designed and produced by the DNA/peptide core facility at the University of Utah.

Experimental Procedures

1. Enzyme expression and purifications

1.1 Recombinant horse cytochrome c.

The method for the expression and purification of recombinant horse cytochrome c was from a previously published procedure.[1] The purchased plasmid pBTR(hCc) was transferred into *E.coli* strain BL21(DE3) and was cultured by shaking at 37℃ in 5 mL rich medium overnight with ampicillin. This culture was used to inoculate 2 L of the same medium and was incubated while shaking at 37℃ for 30 hours. Cells were collected by 8000 rpm centrifugation.

The purification starts with adding 3 mL of lysis buffer per gram of cell collected and was stirred at room temperature for 1 hour and overnight at 4℃. This solution was pushed through a french press, then centrifugated for 20 minutes at 8000 rpm with an addition of ammonium sulfate to a final concentration of 300 g L^{-1}. After the centrifugation, the supernatant was dialyzed twice overnight in 10 L water and 2 L of low salt buffer, respectively. The dialysate was purified using S-Sepharose column via FPLC using a linear gradient of salt concentration. The purified cytochrome c was eluted at a high salt concentration.

1.2 Recombinant flavodoxin (Fld)

Flavodoxin with a histidine tag (6x) at the C terminus was expressed by the nifF gene of *A. vinelandii*, which was cloned into a prelinearized pET28a expression vector. Mutant Cys69Ala flavodoxin protein (Fld) was generated using the QuikChange Lightning kit and primers were designed by the use of the associated software. Sequencing of the final plasmids confirmed the successful incorporation of the desired mutations. *E. coli* C43 cells were transformed with the corresponding vectors.

Mutant Fld was produced in *E. coli* C43 cells. A 20 mL starter was in Luria-Bertani (LB) medium at 37℃ at 200 rpm. This starter was used to culture 2 L of the same LB with 50 mg L^{-1} kanamycin A. The cells were grown to the optical density at 600 nm ($OD_{600\ nm}$) of ~0.6-0.8 at 37 °C where protein expression was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 0.2 mM and flavin mononuleotide (FMN) to a final concentration of 10 mg mL^{-1}. After 12 h of induction shaken at 150 rpm at 30 °C, the cells were harvested by centrifugation at 8000 rpm for 10 minutes, washed in 50 mM Tris, 200 mM NaCl buffer (pH 8). The collected cells were green with a final yield of 13 grams from a 2 L culture.

All purification steps were conducted anaerobically under an argon atmosphere. The cells were resuspended in Buffer A (50 mM Tris, 200 mM NaCl, 2 mM sodium dithionite (DT), 1 mM dithiothreitol (DTT) pH 8) with an addition of 50 µg DNAse and 1 mg mL^{-1} lysozyme and the cell solution were left to react for 1 hour at 4℃ followed by sonication. The cell lysate was centrifuged at 26,000xg for 1 h at 4 °C. Fld in the supernatant was purified by FPLC with immobilized metal affinity chromatography (IMAC) over HisTrap column (GE Healthcare, 5 mL) pre-equilibrated with buffer A, and then washed with buffer A containing 50 mM imidazole. Mutant Fld was eluted with buffer A containing 250 mM imidazole. In order to remove the imidazole, the eluted mutant Fld was loaded onto a HiPrep 26/10 desalting column pre-equilibrated with 50 mM Tris buffer pH 8.

1.3 Recombinant ferredoxin

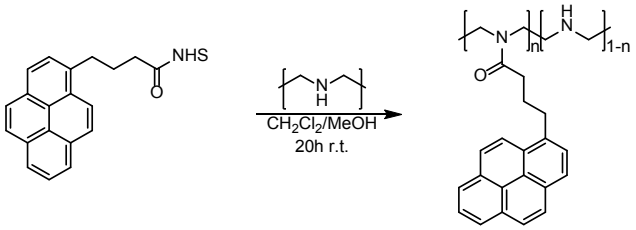
The growth and the purification of ferrodoxin was followed from a previous published publication[2] [2Fe-2S] ferredoxin (petF from Synechococcus sp.) was produced by *E. coli* C43 grown in LB medium containing 50 μg mL^{-1} kanamycin A. The protein expression was induced once the OD_{600} had reached ~0.6 by the addition of 0.2 mM IPTG and 50 μM $FeCl_3$. This cell culture was incubated for 6 hours at 30℃ and the cells were harvest in 50 mM MOPS, 300 mM NaCl buffer (pH 7) via centrifugation, washing and lysing the cells by the use of microfluidizer (3× passages @ ~18,000 psi). The collected cells were centrifuged again to collect the red supernatant that was purified with HiTrap columns (GE Healthcare, 5 mL) and HiPrep 26/10 desalting column pre-equilibrated with 50 mM MOPS buffer (pH 7). The final ferredoxin concentration was determined via the μ-bradford assay.

1.4 Nitrogenase

A mutant strain of *A. vinelandii* were grown and the N-terminal poly(histidine-tagged MoFe that has been purified according to a previous published publication[2]. Briefly, *A. vinelandii* was cultured in 18 L of Burk medium with 10 mM NH_4^+. Once the optical density of the culture was around 1.5 at 600 nm, the medium was switched to a NH_4^+ free Burk medium to encourage nitrogenase production.

Purification were performed in an anoxic tent (O_2 < 0.5 ppm). Cells were lysed via osmotic shock. His-tagged MoFe was purified by IMAC over HisTrap HP columns (GE Healthcare), before being purified further by anion exchange chromatography (Q-Sepharose, GE Healthcare) over a linear gradient of NaCl (200−650 mM). Fractions containing nitrogenase were pooled and the protein was desalted using a Hitrap desalting column (5 mL GE Healthcare) equilibrated using 100 mM O_2-free MOPS, pH 7.0 and subsequently flash frozen and stored at -80 °C. The final nitrogenase concentration was determined to be 27 mg mL^{-1} via Biuret method. It is important to note that Tris buffer should be avoided in the final stages of nitrogenase purification as it may lead to false positives while detecting ammonia production via the fluorescence assay described herein.

2. Pyrene-LPEI synthesis



Pyrene-LPEI was prepared by first dissolving 1-pyrenebutyric acid N-hydroxysuccinimide ester (pyrene-NHS, 0.269 g, 0.7 mmol) in a 1:1 DMSO/MeOH mixture (5 mL total volume) and then adding the pyrene-NHS solution into a stirring solution of linear poly(ethylenimine) (LPEI) (0.05 g, 1.2 mmol) in MeOH (5 mL). To ensure solubility throughout the reaction, CH_2Cl_2 (10 mL) was added to the reaction solution, which was then stirred for 24 hours at 35-40 °C in a closed flask to prevent evaporation. The reaction mixture was allowed to cool to room temperature, and MeOH and CH_2Cl_2 were evaporated under reduced pressure. The crude product mixture in DMSO was added dropwise to a stirring solution of toluene (150 mL), causing the polymer to precipitate rapidly. Toluene was decanted and the solid polymer was collected by vacuum filtration. The solid polymer was washed with THF (30 mL x3) to remove unreacted pyrene-NHS. Any remaining THF was evaporated under reduced pressure resulting in a pale yellow solid (0.18 g). The extent of LPEI substitution was determined by ^{1}H-NMR ($CDCl_3$) and is described in ESI Section 6.3 below.

3. Bioelectrode Fabrication

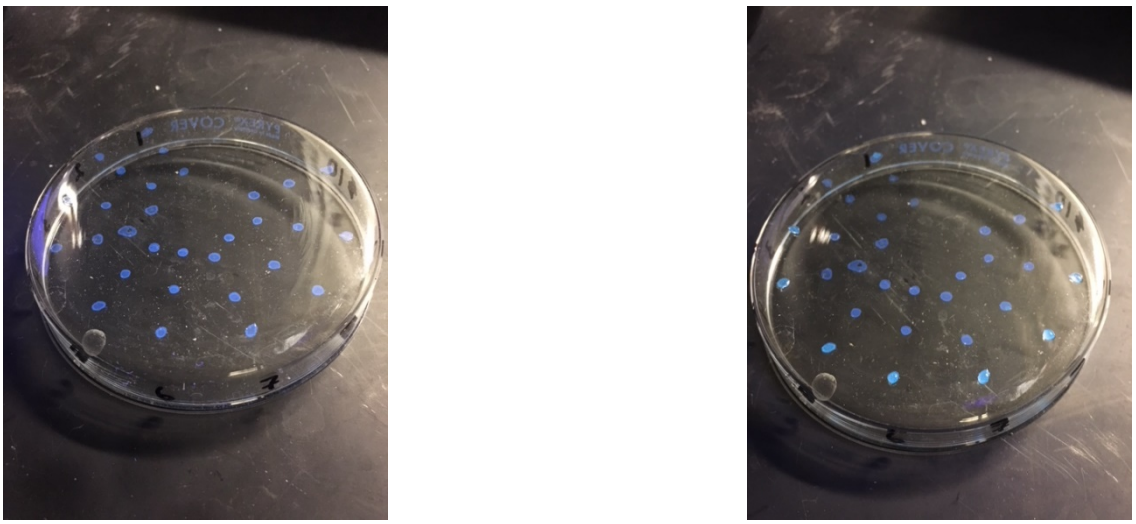
3.1 Preparation

Stock solutions of 10 mg mL^{-1} enzyme (nitrogenase, laccase, flavodoxin, ferredoxin, and horse cytochrome c), 10 mg mL^{-1} pyrene-LPEI, and 13 vol% ethyleneglycol diglycidyl ether (EGDGE, 6 μL in 45 μL H_2O) were prepared in water. Pyrene-LPEI/protein film coating solutions were prepared by first combining 70 μL of stock pyrene-LPEI solution with 30 μL of stock enzyme solution and vortexing briefly

(~10 sec). To the pyrene-LPEI/protein solution was then added 3.75 µL of stock EGDGE solution, followed by additional vortexing (~10 sec). Pyrene-LPEI/protein/EGDGE solution (30 µL) was drop-coated onto a 0.25 cm^2 Toray electrode (0.5 cm x 0.5 cm) in 10 µL increment, allowing 5-10 minutes to dry between each deposition. Modified electrodes were allowed to cure overnight and used without further purification. For films containing CNTs, the stock pyrene-LPEI solution was added to various amounts of carboxylated short multiwalled carbon nanotubes (MWCNT-COOH); this mixture was vortexed briefly (~30 sec) followed by the addition of protein and EGDGE solutions as described above.

3.2 Optimization of pyrne-LPEI hydrogel using laccase

The bioelectrode solution was optimized using laccase by varying the final concentration of the EGDGE solution and the amount of MWCNT-COOH. The EGDGE solution was varied from 2.2 to 29% volume. The final bioelectrode solutions without MWCNT-COOH were deposited onto a glass surface and dried overnight. The deposited hydrogels were washed with water and any altercation to the shape of the hydrogel was monitored under UV-light. At 13% by volume or 6 µL of EGDGE in 39 µL of water showed the least shape change of the deposited hydrogels as shown in picture S1. The amount of MWCNT-COOH was varied to make a final concentration of 1 mg mL^{-1}, 5 mg mL^{-1} and 10 mg mL^{-1} solution. Any enhancement of electrochemical response was monitored by cyclic voltammetry. At 5 mg mL^{-1} MWCNT-COOH, the electrochemical response was clearly observed without increasing the capacitance as shown in figure S1.



Picture 1. (a, left) 22% Pyrene-LPEI under UV light, and (b, right) 22% Pyrene-LPEI under UV light after washing with water.

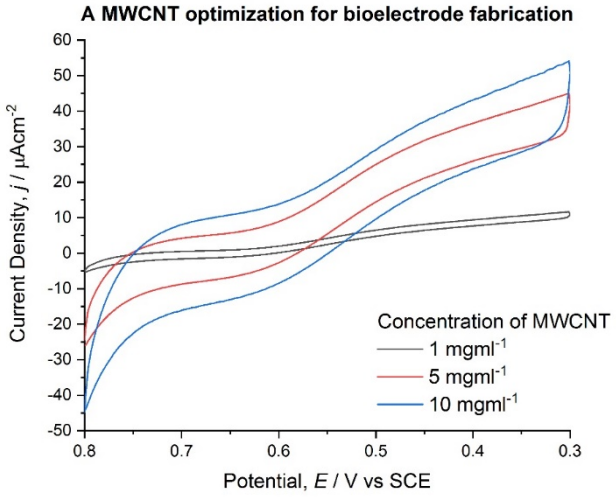


Figure S1. Representative bioelectrocatalytic CVs of various MWCNT loadings using laccase solution.

4 Electrochemical Experiments

4.1 Instruments and techniques

All bioelectrodes made using nitrogenase, laccase, flavodoxin, and ferredoxin was tested using cyclic voltammetry (CV) with a scan rate of 5 mV sec^{-1} and square-wave voltammetry (SWV) with a CH Instruments 1033 potentiostat. A three-electrode set up was used with a SCE reference electrode, a platinum mesh counter electrode and Toray carbon paper electrodes.

4.2 Nitrogenase bioelectrocatalysis

CVs of nitrogenase were performed in an inert atmosphere (Ar) glove box using 4 mL of a degassed 100mM MOPS buffer of pH 7.0. As a substrate, a final concentration of 100 mM sodium nitrite was added. A three-electrode setup in a sealed round bottom glass was prepared with nitrogenase biocathode in 100 mM MOPS. N_2 (g) was bubbled into the apparatus between each CV performed.

4.3 Laccase bioelectrocatalysis

CV of laccase was performed in air using 10 mL of 100 mM citric buffer of pH 4.5 from 0.8 V to 0.3 V at 5 mV s^{-1}. The buffer was bubbled with N_2 (g) for 5 minutes prior to performing the first CV. Afterwards, O_2 was bubbled for 5 minutes before the next CV was performed. A control CV was performed with bioelectrodes using unmodified LPEI instead of pyrene-LPEI as described in Section 5.4.

4.4 Determination of protein redox potentials

Square wave voltammetry (SWV) was performed for flavodoxin and ferredoxin electrodes. Then, a CV potential range was determined to be $\pm$ 0.4 V from the SWV peak potentials.

5 Laccase Activity Assay Using ABTS

5.1 Laccase in solution

A stock solutions of 0.54 mM ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), 0.1 mg mL^{-1} laccase solution and 100 mM citric/phosphate buffer of pH 4.5 were prepared in water. All absorbance measurements were triplicated using Thermo 50 Scientific© Evolution 260 Bio UV-Visible Spectrophotometer.

The procedure for determining laccase activity using ABTS was adopted from a previous publish paper[3]. A stock solution of laccase was diluted to a range of concentrations in the buffer (0.063 mg mL^{-1}, 0.031 mg mL^{-1}, 0.016 mg mL^{-1}, 0.0078 mg mL^{-1}, 0.0039 mg mL^{-1}). 1.125 mL of 100 mM citric/phosphate buffer at pH 4.5, 0.125 mL of the ABTS stock solution, and 0.125 mL of a laccase solution at a desired concentration were added to a cuvette. The solution was mixed by inversions before monitoring the absorbance. Figure S2 shows a linear region of laccase activity and the specific activity was calculated to be 8.1 ± 0.3 U mg^{-1} where U is defined as the amount of laccase that oxidized 1 μmol of ABTS per minute using the ABTS activity assay.

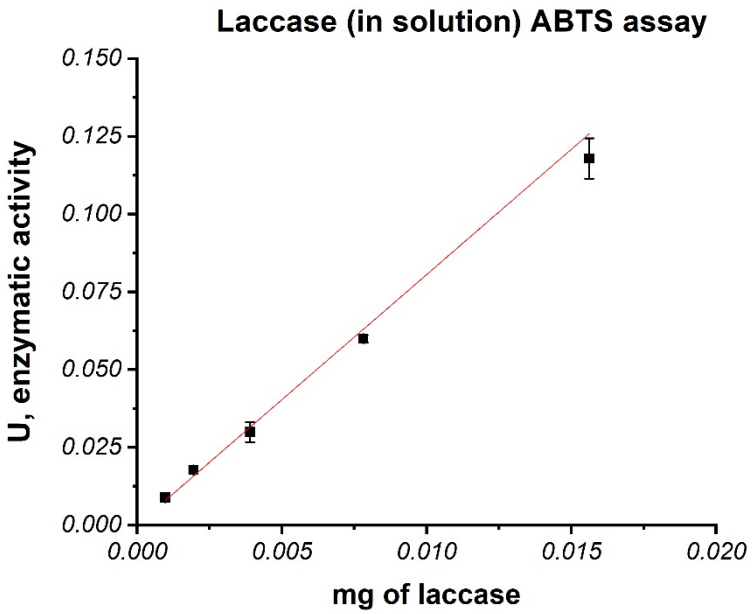


Figure S2. Plot used to calculate specific activity for homogeneously dissolved laccase. Activity was determined using 100 mM phosphate/citrate buffer, pH 4.0, 25 °C.

5.2 Laccase in pyrene-LPEI hydrogel

Using the same diluted laccase solution used in 3.1, bioelectrode solution were prepared according to 1 chemicals and instruments for electrochemical experiments without depositing onto Toray electrodes. Using 3 µL of this solution, a pyrene-LPEI hydrogel was deposited onto a cuvette avoiding the incident light but still in contact with the assay solution. The cuvettes were left to dry overnight before the absorbance was measured. Figure S3 (left) shows a linear curve fitting of laccase activities in a pyrene-LPEI hydrogel and the specific activity of a laccase in a pyrene-LPEI hydrogel is calculated to be 1.2 ± 0.1 U mg^{-1}.

5.3 Laccase with anthracene modified carbon nanotubes

A bioelectrode solution was prepared using a previously published procedure.[4] Using 150 µL of the 100 mM citrate/phosphate buffer, 3 mg laccase was dissolved and was mixed with 15 mg of anthracene modified carbon nanotubes (An-MWCNT). The mixture was sonicated for 10 minutes, briefly vortexed and 50 µL of TBAB-modified Nafion was lastly added. The final mixture was briefly vortexed and sonicated. Onto each cuvette, 1 µL, 3 µL, 10 µL, and 30 µL of the final mixture was deposited. For the ABTS activity assay, 0.318 mL of the stock ABTS solution and 3.182 mL of the 100 mM citrate/phosphate buffer was added to the cuvette prior to the experiment. A stir bar was placed in the cuvette for a uniform mixture through the experiment. Figure S3 (right) shows a linear curve fitting of a laccase activity in the An-MWCNT/TBAB-Nafion paste and the specific activity was calculated to be 0.027 U mg^{-1}.

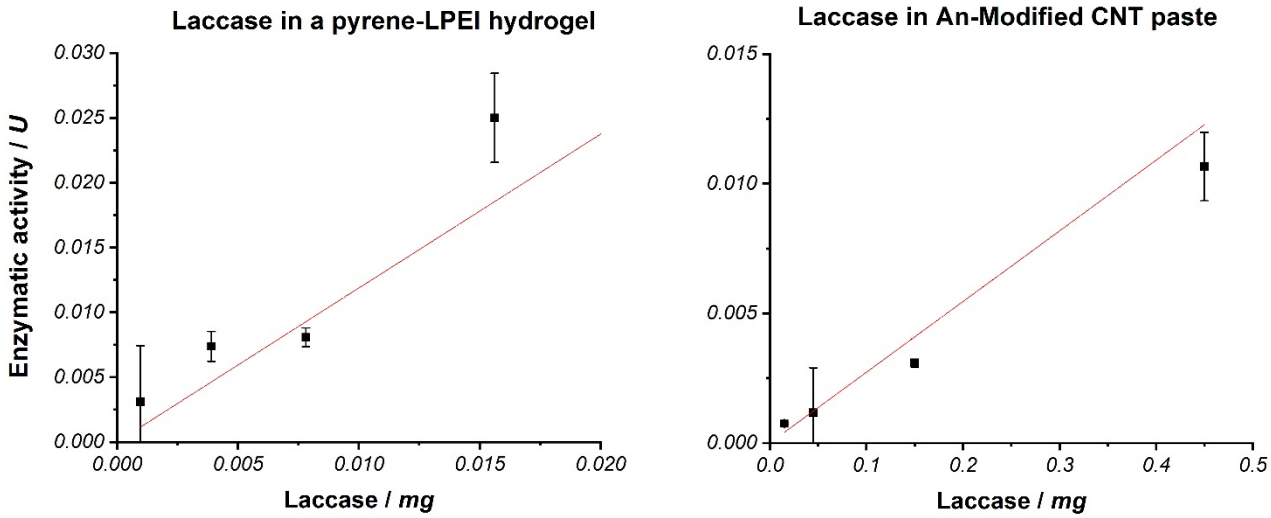


Figure S3. Plots used to calculate specific activity for laccase in either a pyrene-LPEI hydrogel (left) or An-MWCNT/TBAB-Nafion hydrogel (right). Activity was determined using 100 mM phosphate/citrate buffer, pH 4.0, 25 °C.

5.4 Control experiments using unsubstituted LPEI with laccase and MoFe

Cross-linked films of LPEI have previously been utilized to facilitate direct electron transfer with redox proteins by enabling favorable electrostatic interactions between the polymer, enzyme, and electrode surface.[6] To determine the extent that electrostatics of this type alone play in enabling DET for the pyrene-LPEI films, we performed a control experiment, in which laccase was immobilized in a similar manner using unmodified LPEI.

Stock solutions of 10 mg mL^{-1} laccase and 8 vol% ethyleneglycol diglycidyl ether (EGDGE, 4 µL in 45 µL H_2O) were prepared in water. In addition, a stock solution of unmodified LPEI (10 mg mL^{-1}) was prepared by dissolving LPEI in 0.1 M aqueous HCl. It should be noted that dilute aqueous acid was necessary to dissolve the unsubstituted LPEI. Film coating solutions of LPEI/laccase were prepared by first adding 70 µL of stock LPEI solution to 0.5 mg of carboxylated MWCNTs and vortexing briefly (~10 sec). Subsequently, 30 µL of stock laccase solution was added to the LPEI/CNT mixture followed by vortexing briefly (~10 sec). To the LPEI/CNT/protein solution was then added 3.75 µL of stock EGDGE solution, followed by additional vortexing (~10 sec). Aliquots of the combined LPEI/CNT/laccase/EGDGE solution (30 µL) were drop-coated onto a 0.25 cm^2 Toray electrode (0.5 cm x 0.5 cm) in 10 µL increment, allowing 5-10 minutes to dry between each deposition. Modified electrodes were allowed to cure overnight and used without further purification.

Cyclic voltammograms of the resulting films (Figure S4, left) in the presence of either N_2 or O_2 were performed to quantify their capacity for electrocatalytic O_2 reduction. The resulting electrocatalytic current density of LPEI/CNT/laccase films at 0.3 V vs SCE, 22 ± 13 µA cm^{-2}, was substantially lower than that of

the corresponding film containing pyrene-modified LPEI (Figure S4, right), which exhibited catalytic current density of 107 ± 24 μA cm^{-2}.

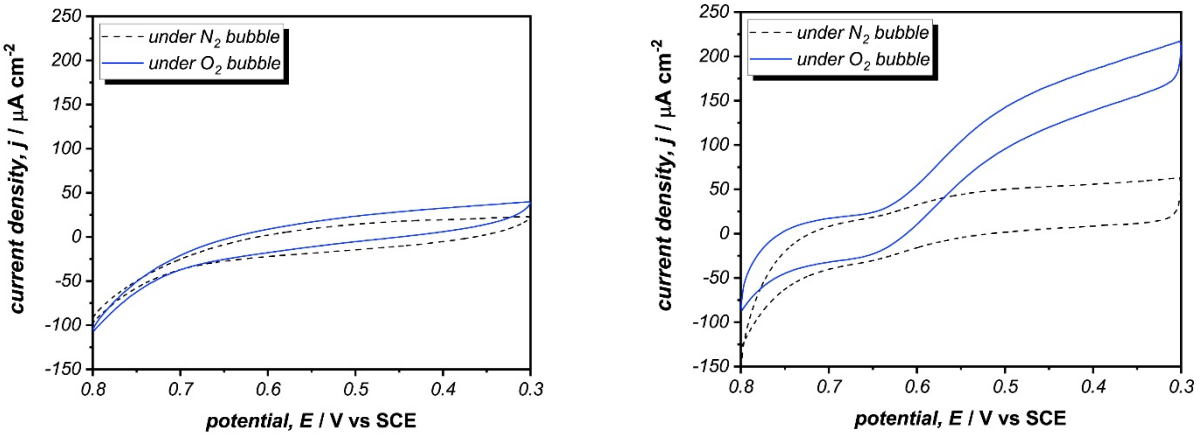


Figure S4. Representative CVs of laccase immobilized in unmodified LPEI (left) and pyrene-modified LPEI (right) in the absence (---) or presence of O_2 (—). Experiments were performed using 100 mM citrate, pH 4.5, at 25 °C and 5 mV sec^{-1}.

Similar experiments were performed using unmodified LPEI films with the MoFe protein of nitrogenase, in which CVs were performed in the absence and presence of N_2 (Figure S5A). A small increase in the reductive current density can be observed in response to bubbling N_2 through the solution for 10 min; however, this current increase is negligible compared to bioelectrocatalytic N_2 response exhibited by pyrene-LPEI/MoFe films (Figure 4A). In addition, square wave voltammetry of films prepared using unmodified LPEI with MoFe protein exhibit no apparent features in the potential range of the p-cluster (Figure S5B). This is in stark contrast to the peak observed when pyrene-LPEI is employed, further suggesting that electrostatic interactions alone cannot account for the electrochemical responses observed.

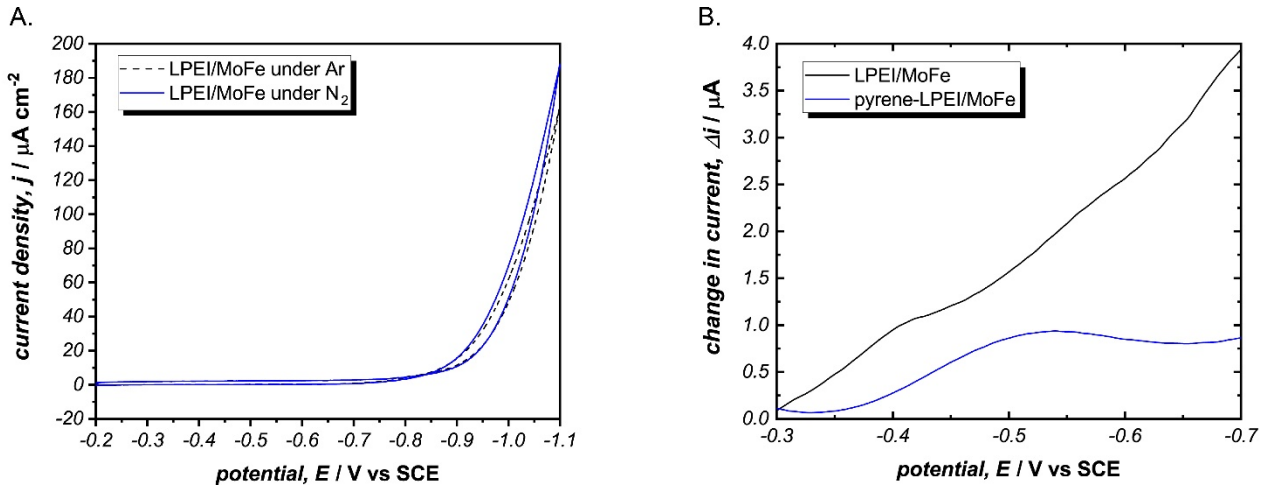


Figure S5. (A) Representative CVs of unmodified LPEI hydrogels containing 5 mg/mL MWCNT-COOH and MoFe protein under Ar (---) and after 10 minutes of bubbling N_2 (—). (B) Representative SWVs comparing MoFe hydrogels prepared using unmodified LPEI (—) and pyrene-LPEI (—). Experiments were performed using 100 mM MOPS buffer, pH 7.0 at 25 °C. CVs were performed at 5 mV sec^{-1}, while SWVs were performed at 10 Hz.

6 Pyrene-LPEI characterization

6.1 UV-VIS characterization

A stock solution of pyrene-LPEI was made by dissolving 2 mg of pyrene-LPEI in 5.04 mL of water. The stock solution was diluted to a concentration range from 60 μg mL^{-1} to 0.94 μg mL^{-1}. Using UV-visible Spectrophotometer, the absorbance of each pyrene-LPEI solution was monitored from 220 nm to 600 nm.

Figure S6 shows a full spectrum of pyrene-LPEI. Figure S7 shows a calibration curve of pyrene-LPEI and Table S1 shows the calculated molar absorptivity at each maximum wavelength.

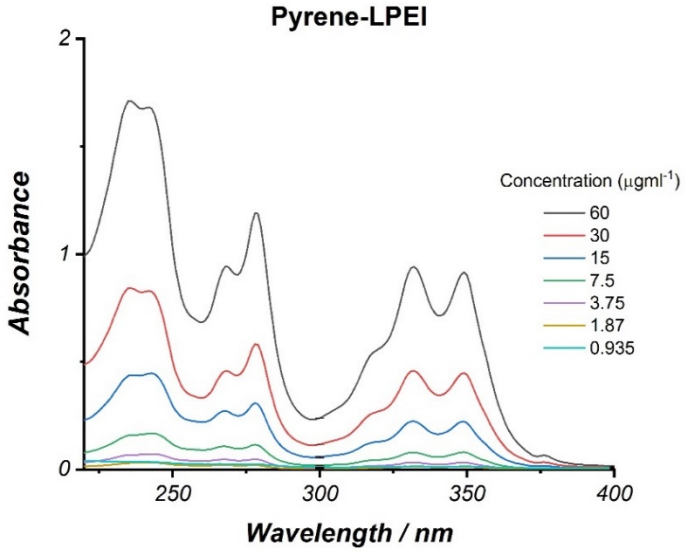


Figure S6. Absorbance spectrum of pyrene-LPEI.

λ_{max}	*Molar absorptivity*	*error*	*R-squared*
350 nm	0.0148	0.0003	0.99
332 nm	0.0155	0.0003	0.99
275 nm	0.0165	0.0002	0.99
235 nm	0.0283	0.0004	0.99

Table S1. The calculated molar absorptivity of pyrene-LPEI

6.2 Fluorescence characterization

On a 3 cm long and 0.6 cm wide Toray paper, 300 µL of the MWCNT -COOH solution prepared by dispersing 5 mg of MWCNT in N-methyl-2-pyrrolidone (NMP) was deposited and dried completely prior to an experiment. A same sized Toray electrode was prepared without a MWCNT-COOH deposition. All florescence measurements were excited at 345 nm and the florescence intensity was monitored from 350 nm to 600 nm using the Hitachi Fluorescence Spectrophotometer F-7000. A quartz cuvette used in the experiment was cleaned using a pipe cleaner with water, ethanol and 1M sodium bicarbonate between each measurement.

A 3.75 µg mL^{-1} pyrene-LPEI solution was used to measure the fluorescence intensity. A Toray paper was diagonally placed in a cuvette and the fluorescence intensity was measured. This step was repeated with the previously prepared MWCNT-Toray using a fresh 3.75 µg mL^{-1} pyrene-LPEI solution. For a control experiment, water was used instead of the pyrene solution. At 375 nm, non-complexed pyrene was observed and at 475 nm, pyrene in an excimer formation (pi-stacked) was observed. At 390 nm, a scattering light is observed. Regardless of the placement of a Toray and MWCNT-Toray, excimer formation of pyrene-LPEI was observed as shown in Figure S8.

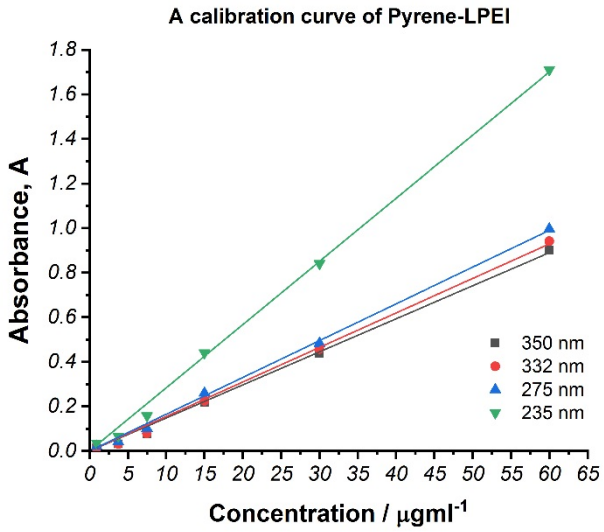


Figure S7. Calibration curves of pyrene LPEI at the λ_{max}.

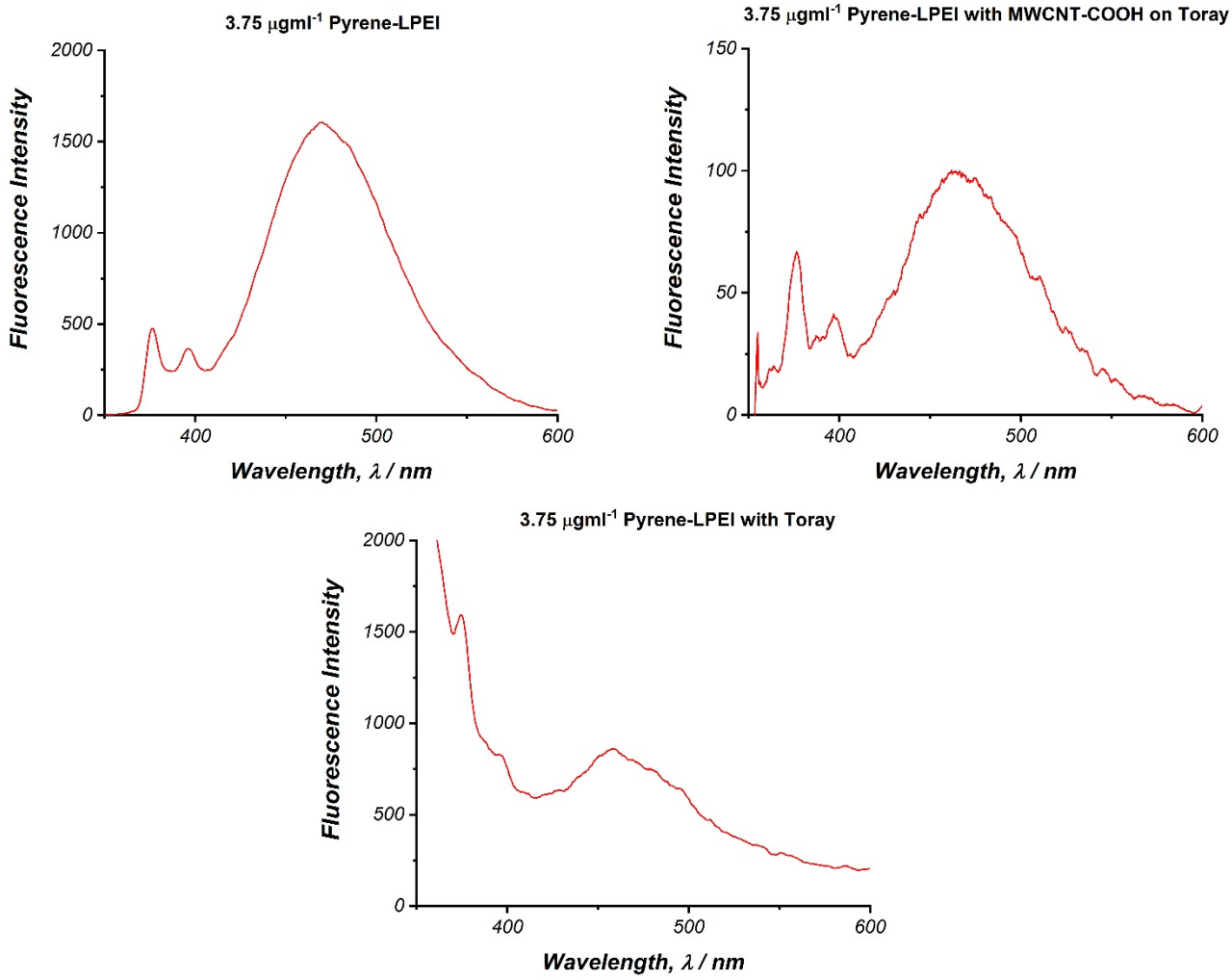


Figure S8. (a, top left) A representative fluorescence of pyrene-LPEI. A higher fluorescence intensity at 475 nm indicates a higher concentration of pyrene excimers than a non-complexed pyrene in the hydrogel solution. (b, top right) A representative fluorescence of pyrene-LPEI with MWCNT-Toray in a cuvette. The background fluorescence of MWCNT-Toray in water was subtracted. (c, bottom) A representative fluorescence of pyrene-LPEI with Toray paper in a cuvette. The background fluorescence of Toray in water was subtracted.

6.3 ^{1}H-NMR

^{1}H-NMR was performed with the synthesized pyrene-LPEI in deuterated chloroform using 400 MHz NMR. The NMR spectra of pyrene-LPEI and 1-pyrenebutyric acid N-hydroxysuccinimide ester (pyrene-NHS) are shown in Figure S9a and S9b, respectively. Pyrene substitution was calculated to be 22% using the equation described previously.[5]

Pyrene percent substitution = 4 / (backbone hydrogen integration -2) *100

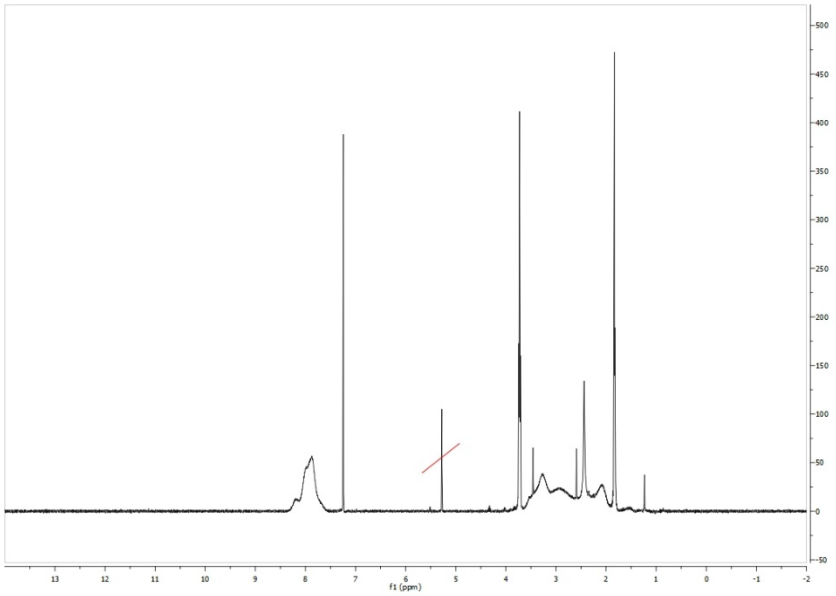


Figure S9a. A ^{1}H NMR spectra of pyrene-LPEI in $CDCl_3$.

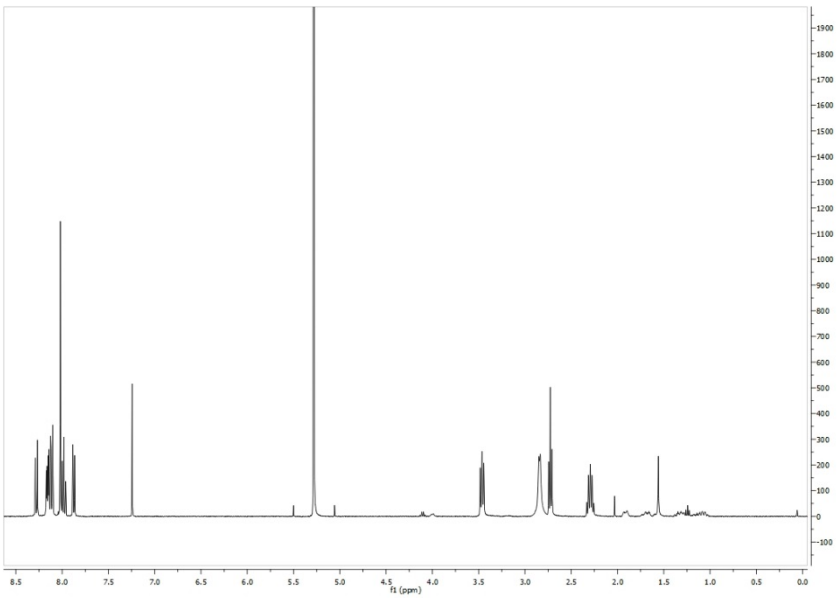


Figure S9b. A ^{1}H NMR spectra of pyrene-NHS in $CDCl_3$.

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