

Electronic Supplementary Information for:

Bioelectrocatalytic NAD⁺/NADH Inter-Conversion: Transformation of an Enzymatic Fuel Cell into an Enzymatic Redox Flow Battery

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Experimental

Materials

NAD-dependent glucose dehydrogenase (NAD-GDH, E.C. 1.1.1.47) was purchased from Toyobo USA Inc. (*Bacillus sp.*, recombinantly-expressed in *E. coli*) and used without any additional purification. Diaphorase (DH, EC 1.6.5.2) was purchased from Sekisui Diagnostics (*Bacillus megaterium*) and used without any additional purification. Laccase M120 (EC 1.10.3.2) was purchased from Amano Enzymes and was used without any additional purification. Bilirubin Oxidase (BOx, EC 1.3.3.5) was purchased from Amano Enzymes (*Myrothecium species*) and was used without any additional purification. Alcohol dehydrogenase (ADH, EC 1.1.1.1) was purchased from Sigma-Aldrich (*Saccharomyces cerevisiae*) and was used without any additional purification. β -Nicotinamide adenine dinucleotide reduced form disodium salt trihydrate (NADH) and β -nicotinamide adenine dinucleotide (NAD⁺) were purchased from Research Products International. Ferrocene methanol, glutaraldehyde, Nafion[®] 212, benzyl bromide, 4,4'-dipyridyl, 1,3-dibromopropane and glucose were purchased from Sigma Aldrich. Sodium phosphate dibasic anhydrous and citric acid monohydrate were purchased from Fisher Scientific. Ethylene glycol diglycidyl ether (EGDGE) was purchased from Polysciences, Inc. Toray carbon paper (TGP-H-060, non-wet-proofed) was purchased from Fuel Cell Earth. Glassy carbon electrodes (3 mm in diameter) and saturated calomel reference electrodes (SCE) were purchased from CH Instruments. Solutions were prepared using deionized (DI) water taken from a Millipore Type 1 (Ultrapure) Milli-Q system (18.2 M Ω cm⁻¹).

Synthesis of BPV-LPEI

Benzylpropylviologen-linear poly(ethylenimine) (N-benzyl-N'-propyl-4,4'-bipyridinium, BPV-LPEI) was synthesized by the modification of a previously-reported procedure.¹ LPEI was synthesized as previously reported.² Initially, 4,4'-dipyridyl (1.1x mol. eq., 6 g) was dissolved in 100 mL of dry acetone (under N₂) and heated to 60 °C. Benzyl bromide (1 mol. eq., 5.97 g) was added in a drop-wise fashion over 30 minutes and the resulting mixture was stirred at 60 °C for 7 hours, after which a pale-turquoise precipitate formed. The reaction mixture was cooled, filtered and washed with excess acetone to yield monobenzylviologen (77 % mol. yield).

Next, monobenzylviologen (2.55 g, 1 mol. eq.) and 1,3-dibromopropane (10.8 g, 7x mol. eq.) were added to 9:1 acetonitrile/methanol under N₂ and refluxed for 48 hours. The resulting mixture containing a yellow precipitate was reduced to 25 % volume *in vacuo*, cooled, and the precipitate was collected by vacuum filtration. The yellow solid was washed with an equivalent

volume of acetonitrile and further washed with excess acetone until the yellow solid was shiny in appearance. The product was collected with methanol and dried to yield 2.3 g (57 % mol. yield) of benzylbromopropylviologen (BBPV, Figures S1-S5).

Finally, BBPV (530 mg, 1 mol. eq.) was added to a solution of linear poly(ethylenimine) (215 mg, 5 mol. eq.) dissolved in 8:2 acetonitrile:methanol (50 mL) and left to reflux overnight. The solvent mixture was removed *in vacuo* and washed with excess dichloromethane to yield a dark blue polymer, benzylpropylviologen (BPV-LPEI, Figure S6).

Preparation of bioelectrodes

DH/BPV-LPEI bioelectrodes were prepared by drop casting 3 μL of a mixture containing 21 μL BPV-LPEI (2.5 mg/mL), 9 μL DH (10 mg/mL), and 1.125 μL EGDGE (10% v/v). These electrodes were dried under positive airflow at room temperature overnight. GDH/C₈-LPEI/DH/BPV-LPEI bioanodes were prepared by drop casting an additional 3 μL of a NAD-GDH-containing mixture onto the DH/BPV-LPEI bioanode. The additional mixture contained 21 μL C₈-LPEI (10 mg/mL), 9 μL GDH (10 mg/mL), and 0.316 μL glutaraldehyde (62.5 mM). These electrodes were then dried under positive airflow at room temperature for two hours.

The Laccase biocathodes were prepared by painting a mixture onto Toray carbon paper, adapted from a previously-reported procedure.³ The mixture was prepared by first mixing 7.5 mg Anthracene-modified MWCNTs (Ac-MWCNTs) with 25 μL of tetrabutylammonium bromide-modified (TBAB) Nafion (20 mg/mL) and mixing and sonicating this mixture multiple times. While the Ac-MWCNTs and TBAB modified Nafion mixture was vortexed and sonicated 1.5 mg of laccase was added to 75 μL of 100 mM citrate phosphate buffer (7.0 pH) and vortexed. The two mixtures were then added together, vortexed and sonicated. The resulting mixture was painted onto 6 Toray electrodes with geometric surface areas of 0.25 cm² and left to dry for two hours at room temperature under positive airflow.

Electrochemical methods

All electrochemical measurements were conducted on a CH Instrument Inc. model 660e potentiostat at room temperature, with the exception of charging and discharging experiments. Characterization of the anode was conducted in 100 mM phosphate/citrate buffer (pH 7.0) using a three-electrode setup that included a glassy carbon electrode as the working electrode, a saturated calomel reference electrode (SCE) and a platinum mesh counter electrode. EFCs were characterized by recording the open circuit potential for 10 minutes, followed by a linear sweep voltammetry from the OCP to 0.001 V at 1 mV s⁻¹. Cycling tests were conducted on a VSP Biologic potentiostat/galvanostat and were characterized by a galvanotactic charge/discharge of 704 nA cm⁻².

Enzymatic activity assays and denatured enzyme controls

Enzymatic activity assays were performed to evaluate the activity of NAD-GDH and DH before immobilization. Specific activities were performed in 100 mM phosphate/citrate buffer (7.0). NAD-GDH was evaluated by following the production of NADH at 340 nm in the presence of

10 mM NAD⁺ and 25 mM of glucose, yielding a specific activity of 119 ± 17.3 U mg⁻¹ solid. DH was evaluated using the same buffer where NADH (2mM) was the electron donor and dichlorophenolindophenol (0.04 mM) was the electron acceptor. The specific activity of DH was determined to be 18.8 ± 1.23 U mg⁻¹ solid. Denatured enzyme control samples (NAD-GDH and DH) were prepared by heating aliquots of the enzymes to 100 °C for 3 hrs.

REFERENCES

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2. S. A. Merchant, T. O. Tran, M. T. Meredith, T. C. Cline, D. T. Glatzhofer and D. W. Schmidtke, *Langmuir*, 2009, **25**, 7736-7742.
3. M. T. Meredith, M. Minson, D. Hickey, K. Artyushkova, D. T. Glatzhofer and S. D. Minteer, *ACS Catal.*, 2011, **1**, 1683-1690.

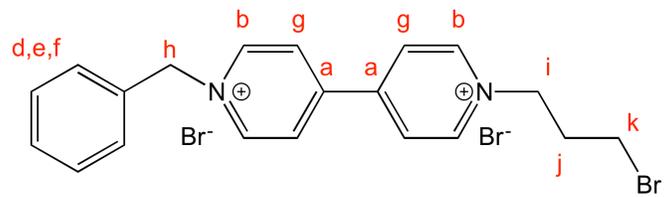


Figure S1. Annotated structure of MBBPV. H and C assignments were made using the following ^1H and ^{13}C 1D and 2D NMR spectra.

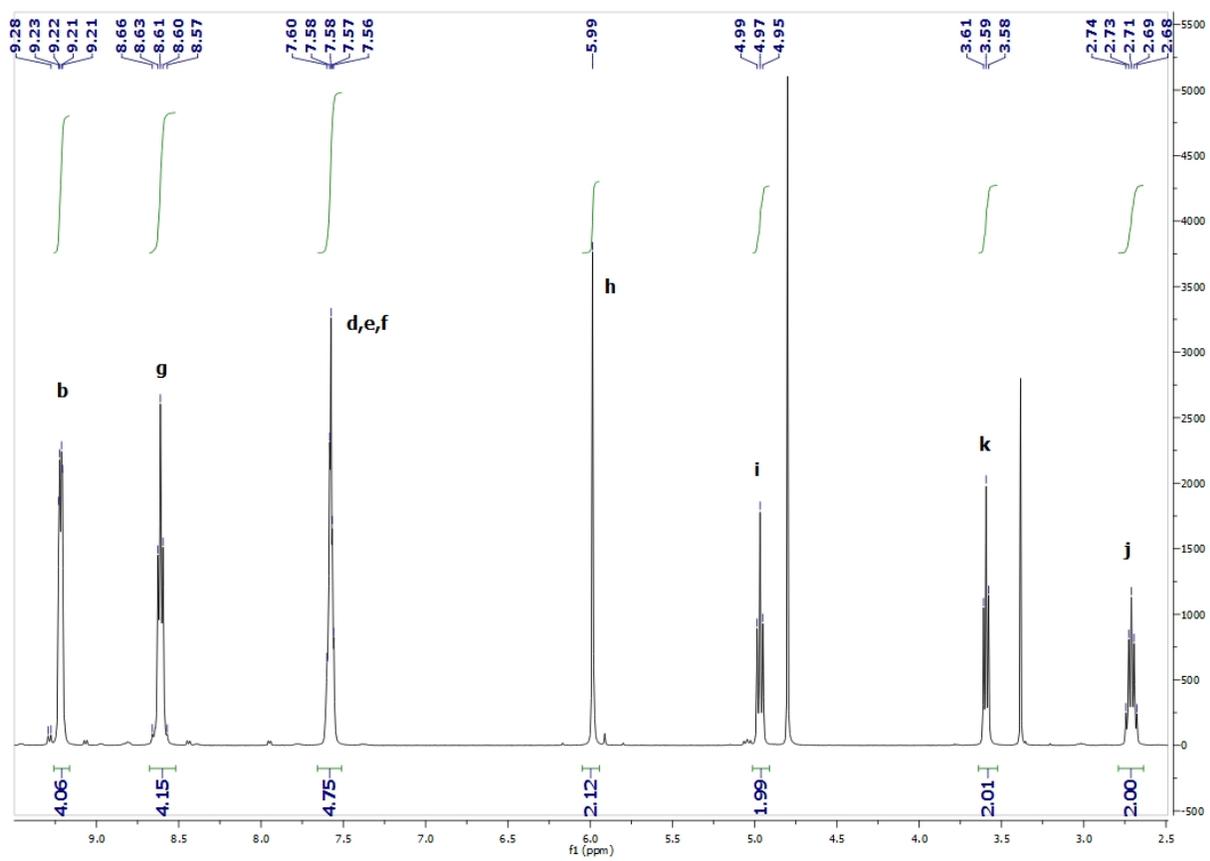


Figure S2. ^1H (400 MHz, D_2O) spectra of MBBPV. ^1H NMR (400 MHz, D_2O) δ 9.22 (dd, $J = 6.8, 2.3$ Hz, 1H), 8.61 (t, $J = 6.1$ Hz, 1H), 7.62 – 7.55 (m, 1H), 4.97 (t, $J = 7.1$ Hz, 1H), 3.59 (t, $J = 6.1$ Hz, 1H), 2.71 (p, $J = 6.7$ Hz, 1H).

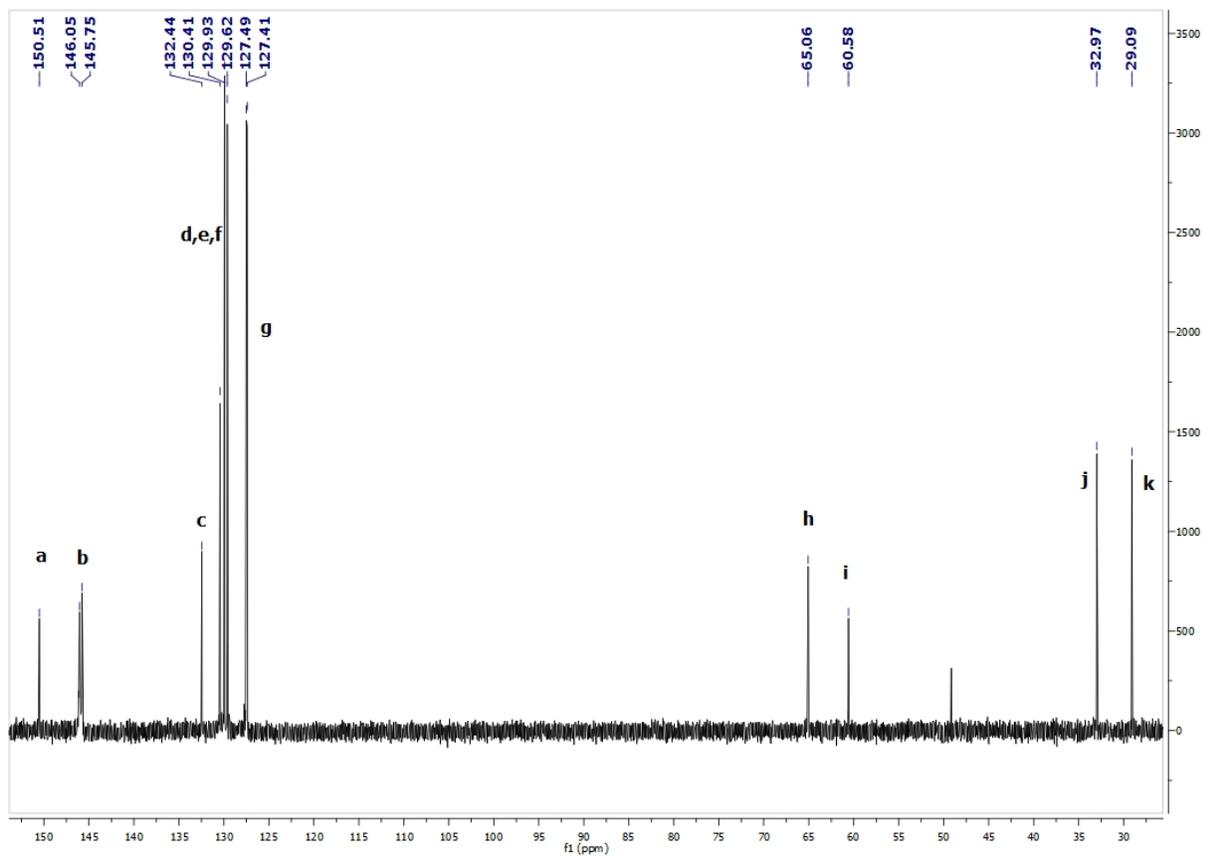


Figure S3. ^{13}C (100 MHz, D_2O) spectra of MBBPV. ^{13}C NMR (101 MHz, D_2O) δ 150.51, 146.05, 145.75, 132.44, 130.41, 129.93, 129.62, 127.49, 127.41, 65.06, 60.58, 32.97, 29.09.

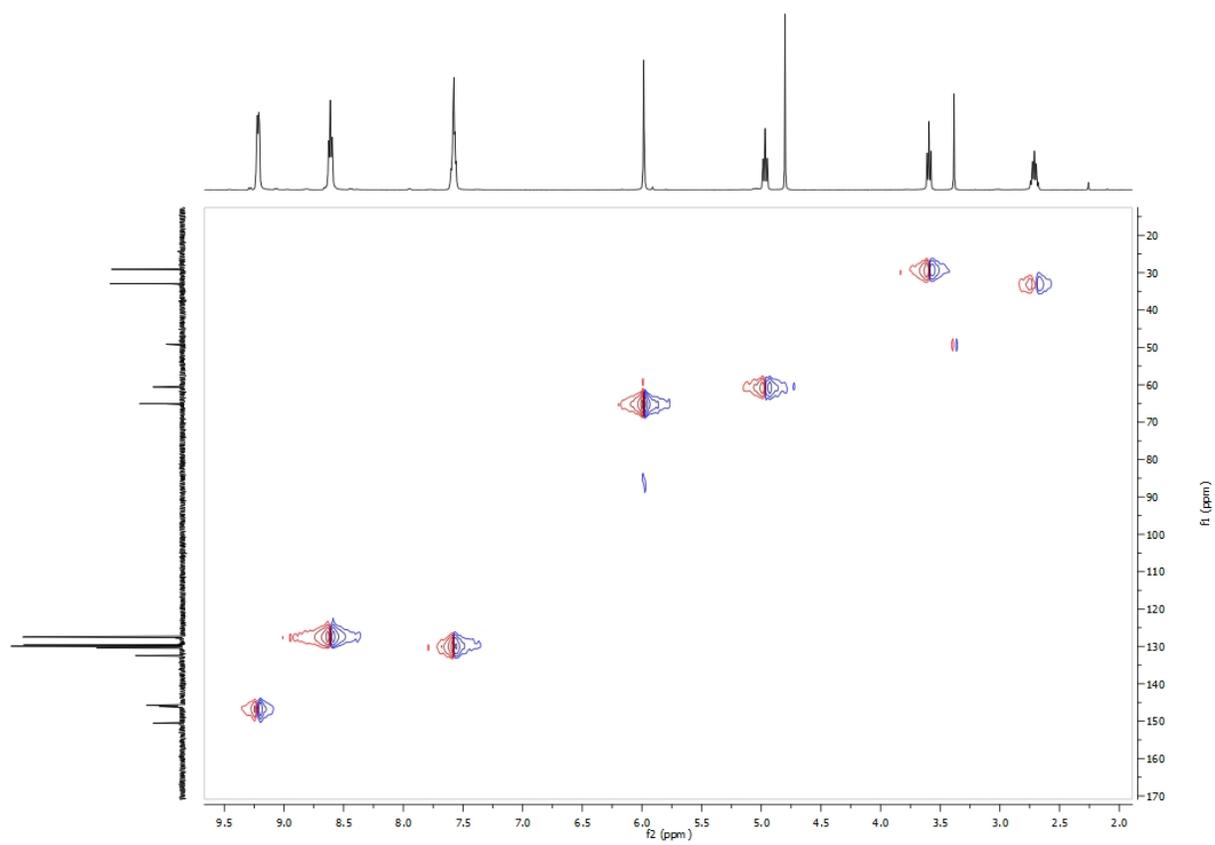


Figure S4. HMQC spectra of MBBPV.

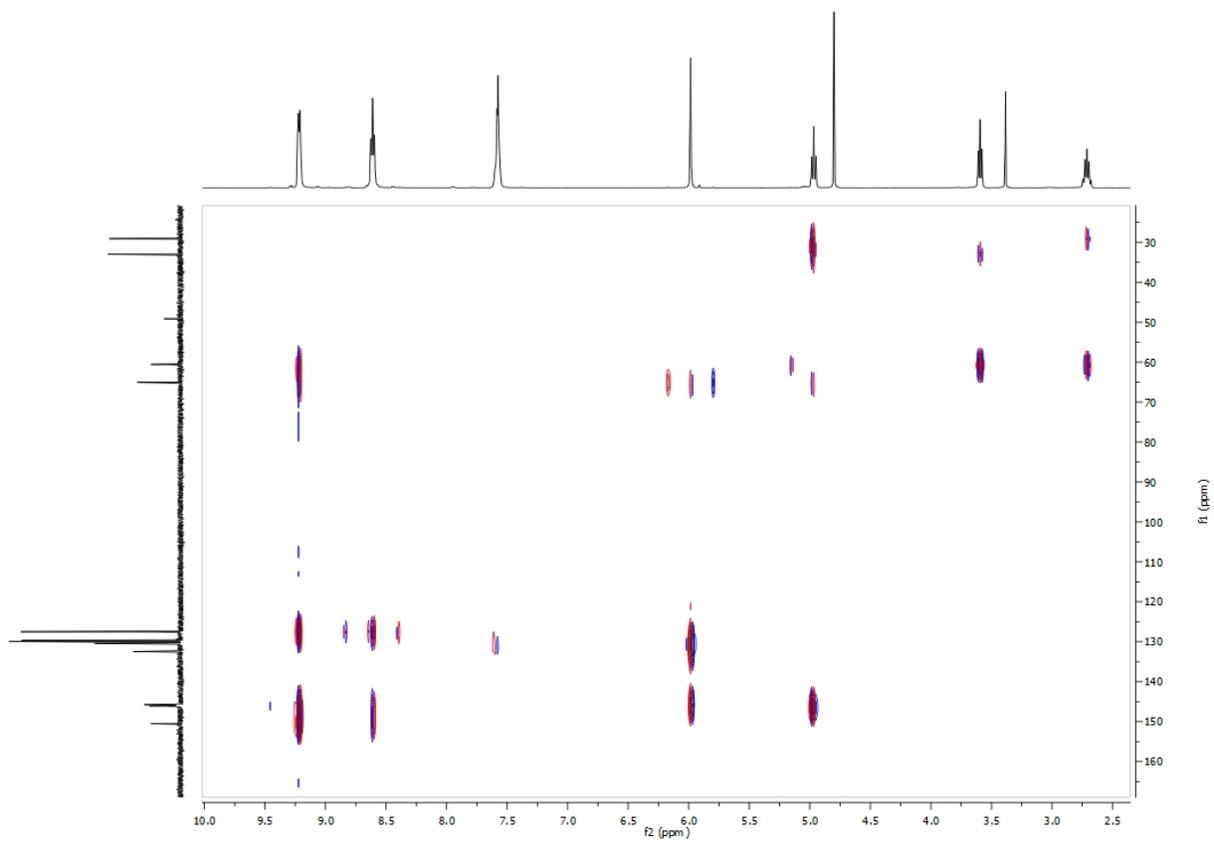


Figure S5. HMBC spectra of MBBPV.

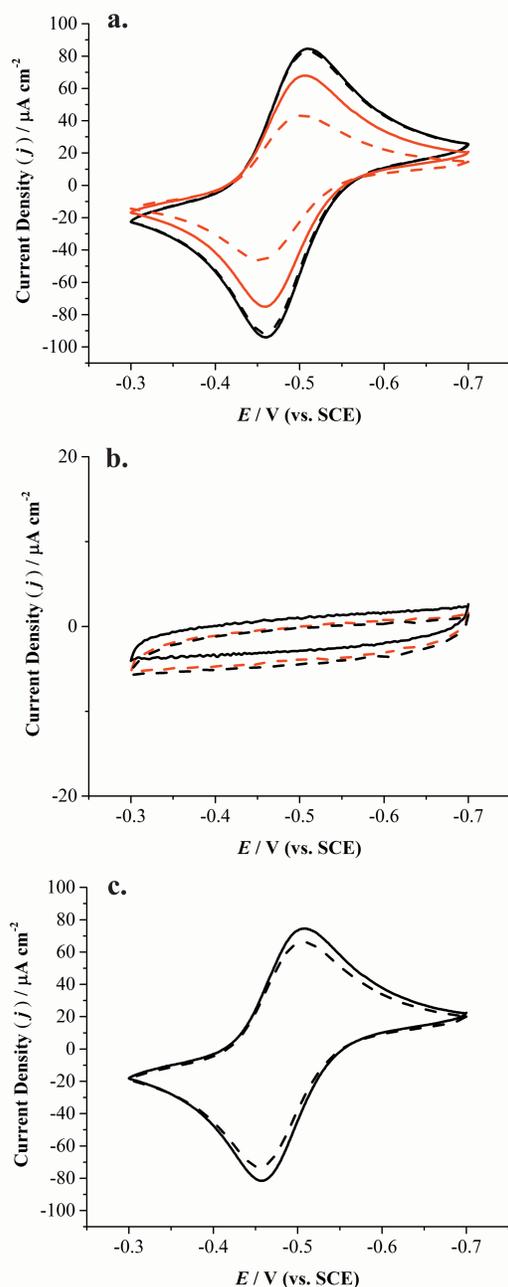


Figure S6. (a) Control experiments performed using diaphorase (DH) that was denatured by heating. Cyclic voltammetry was performed at 5 mV s^{-1} in a stirred, N_2 -purged phosphate-citrate buffer solution (pH 7.0, 0.1 M) in the absence (black solid line) or presence of 5 mM NAD^+ (black dashed line) in the absence (red solid line) or in the presence of 5 mM NADH (red dashed line). (b) Control experiments performed using redox-silent $\text{C}_8\text{-LPEI}$ in the place of BPV-LPEI. Cyclic voltammetry was performed at 5 mV s^{-1} for DH/ $\text{C}_8\text{-LPEI}$ in a stirred, N_2 -purged phosphate-citrate buffer solution (pH 7.0, 0.1 M) in the absence (black solid line) or presence of 5 mM NAD^+ (black dashed line) or in the presence of 5 mM NADH (red dashed line). (c) Control experiments performed with heat-denatured NAD-GDH. Cyclic voltammetry was performed at 5 mV s^{-1} in a stirred, N_2 -purged phosphate-citrate buffer solution (pH 7.0, 0.1 M) containing 5 mM NAD^+ in the absence (black solid line) or presence of 100 mM glucose .

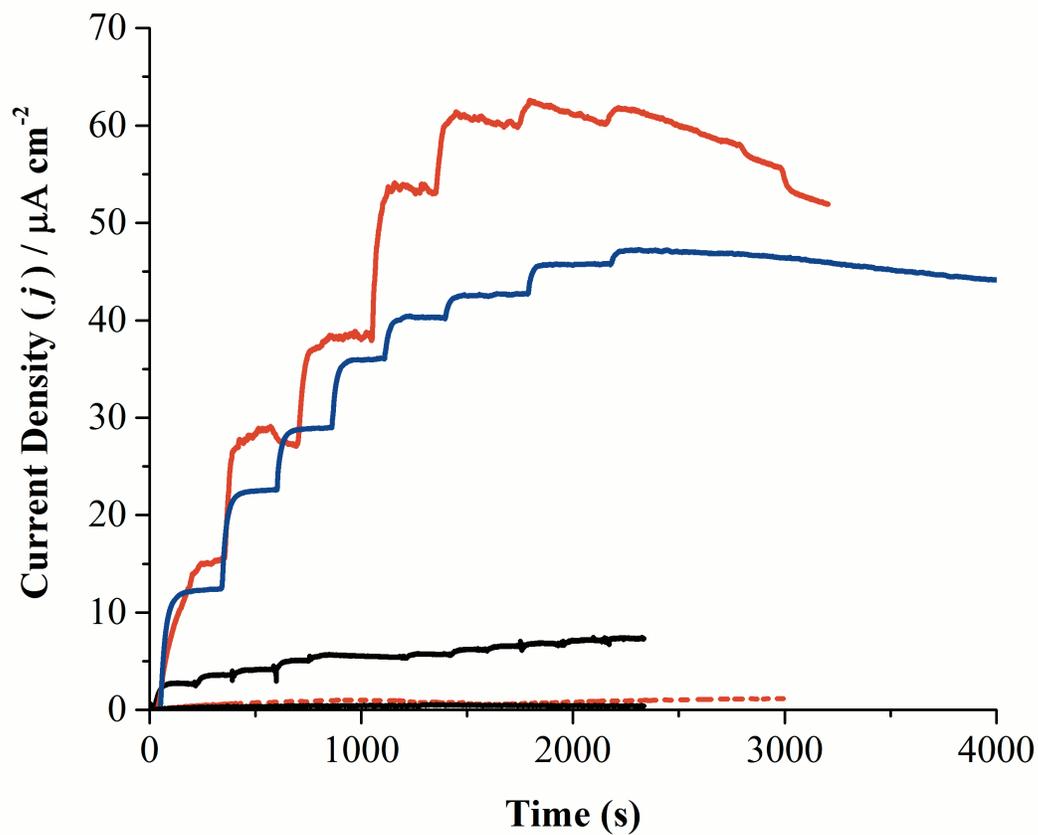


Figure S7. Amperometric steady-state i - t curves for the injection of glucose (solid blue), NAD^+ (solid black), NADH (solid red). Control experiments were also performed with denatured DH with NAD^+ (dashed black), and NADH (dashed red). Oxidative electrochemistry was performed at -0.38 V vs. SCE, whereas reductive electrochemistry was performed at -0.63 V vs. SCE.

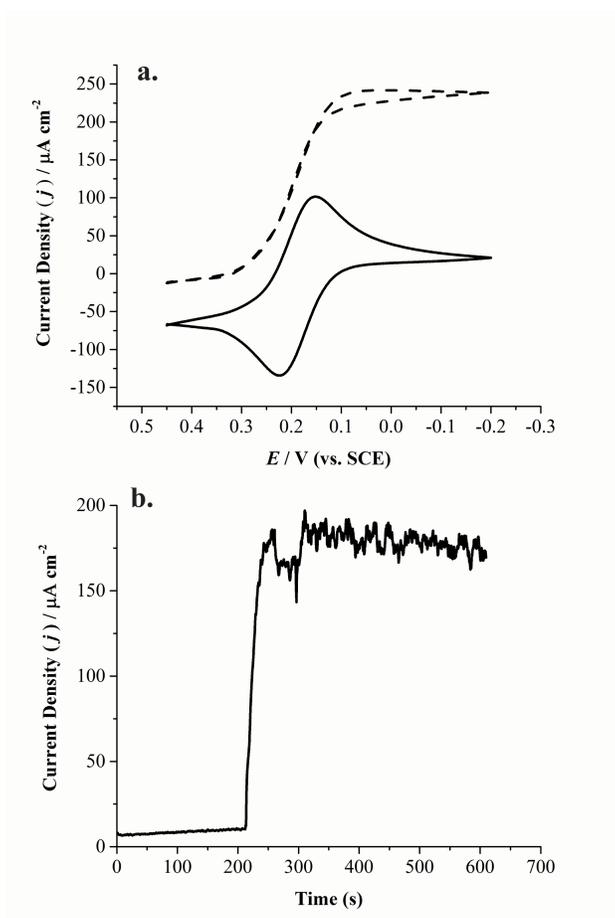


Figure S8. (a) Cyclic voltammetry and amperometric $i-t$ analysis of Fc-mediated O_2 reduction by diffusive bilirubin oxidase (BOx). Cyclic voltammetry was performed at 5 mV s^{-1} in a stationary, phosphate-citrate buffer solution (pH 7.0, 0.1 M) containing 1 mM ferrocene methanol⁺ in the absence (black solid line) or presence of BOx. Raw Amperometric $i-t$ curves of the injection of BOx in 1 mM ferrocene methanol in stirred phosphate-citrate buffer (pH 7.0, 0.1 M); the electrode potential was poised at 0 V vs. SCE.

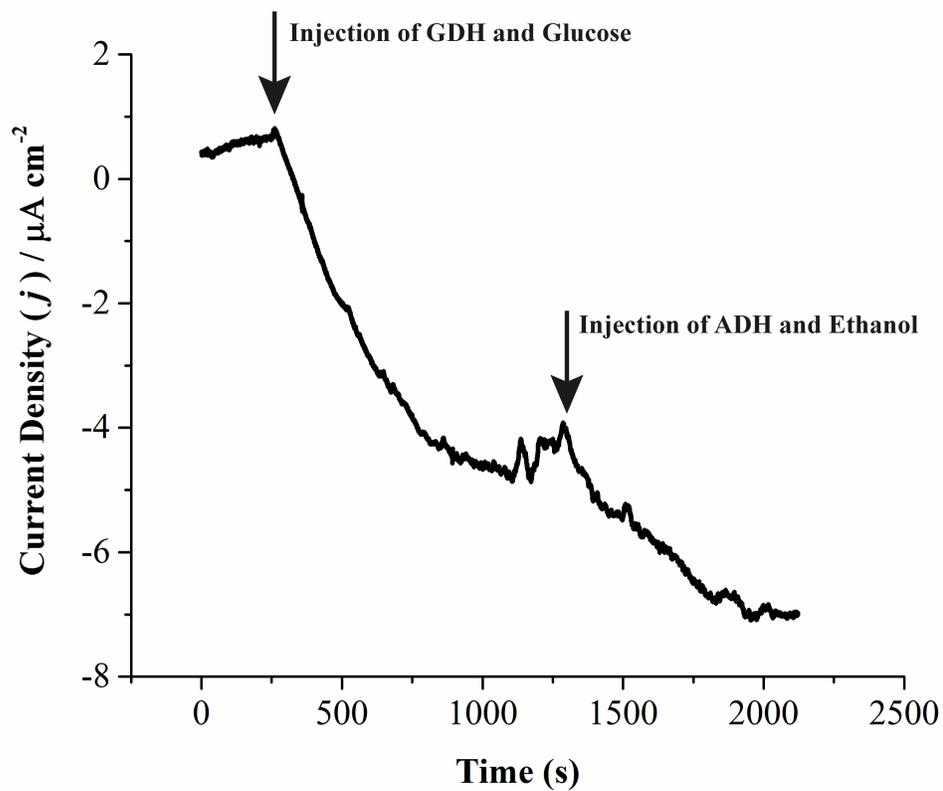


Figure S9. Amperometric i - t curves of the injection of GDH and glucose followed by an injection of ADH and ethanol in stirred citrate phosphate buffer (100 mM, 7.0 pH, N_2 -purged) containing 5 mM NAD^+ , performed in a 3-electrode configuration with an applied potential of -0.38 V vs. SCE.