Iron-sulfur-based Single Molecular Wires for Enhancing Charge Transport in Enzyme-based Bioelectronic Systems

Aishwarya Mahadevan^a, Teshan Fernando^a and Sandun Fernando^{a*}

^a Department of Biological and Agricultural Engineering, 303C Scoates Hall, Texas A&M University, College Station, TX 77843, USA

*Corresponding Author's Email address: sfernando@tamu.edu (S. Fernando)

†Phone: (979) 845 9793, Fax: (979) 862 3442

Abstract

When redox enzymes are wired to electrodes outside a living cell (*ex vivo*), their ability to produce a sufficiently powerful electrical current diminishes significantly due to the thermodynamic and kinetic limitations associated with the wiring systems. Therefore, we are yet to harness the full potential of redox enzymes for the development of self-powering bioelectronics devices (such as sensors and fuel cells). Interestingly, nature uses iron-sulfur complexes ([Fe-S]), to circumvent these issues *in vivo*. Yet, we have not been able to utilize [Fe-S]-based chains *ex vivo*, primarily due to their instability in aqueous media. Here, a simple technique to attach iron (II) sulfide (FeS) to a gold surface in ethanol media and then complete the attachment of the enzyme in aqueous media is reported. Cyclic voltammetry and spectroscopy techniques confirmed the concatenation of FeS and glycerol-dehydrogenase/nicotinamide-adenine-dinucleotide (GIDH-NAD⁺) apoenzyme-coenzyme molecular wiring system on the base gold electrode. The resultant FeS-based enzyme electrode reached an open circuit voltage closer to its standard potential under a wide range of glycerol concentrations (0.001-1 M). When probed under constant potential conditions, the FeS-based electrode was able to amplify current by over 10 fold as compared to electrodes fabricated with the conventional pyrroloquinoline quinone-based composite molecular wiring system. These improvements in current/voltage responses open up a wide range of possibilities for fabricating self-powering, bio-electronic devices.

Keywords

Bioanode, Enzyme immobilization, Molecular wire, Redox enzymes, Self-assembled monolayers, Bioelectronics

1. Introduction

Redox enzymes are naturally occurring bioelectronic entities that can act as power plants or self-powering sensors due to their ability to generate an electron stream in the presence of an enzyme-specific analyte. However, when these enzymes are wired to electrodes (Heller 1990) outside a living cell (i.e., *ex vivo*), their ability to produce a sufficiently powerful electrical current diminishes significantly due to the thermodynamic limitations associated with the coenzymes.

Common coenzymes (also known as cofactors) are typically resistant to cyclic oxidation and reduction *ex vivo*. Furthermore, they require special molecules, known as electron mediators, to help extract electrons from the coenzyme and transfer them to the final target. The molecular wires that consist of mediators and other interfacing molecules that tether the enzyme to the base electrode (Katz et al. 1997), although help mitigate the thermodynamic issues, create issues associated with electron transport kinetics due to the increased resistance that results from the increased length. This lengthy wiring scheme results due to known electron mediators not having the correct combination of prosthetic groups to anchor the enzyme system (i.e., the apoenzyme-coenzyme complex) from one end and the base electrode from the other. Linker molecules with appropriate functional groups are introduced into the wiring scheme to fulfill the anchoring function. Fig. 1A shows a commonly used, conventional wiring scheme that comprises cystamine, pyrroloquinoline quinone (PQQ), and phenyl boronic acid for tethering the nicotinamide adenine dinucleotide (NAD^+) coenzyme-dependent apoenzyme onto a metal electrode for sensing and biofuel cell applications.

Fig. 1: Schematic of wiring systems. (a) Complex conventional wiring of the coenzyme NAD^+ to a gold electrode using a series of molecules including a cystamine linker, a PQQ mediator, and a phenyl boronic acid linker; (b) Simplified wiring of NAD^+ coenzyme directly onto the gold electrode by the multi-functional mediator, FeS

The outcome of such a long wiring scheme is high ohmic resistance, resulting in constrained electron transport (Johnston et al. 2007; Park et al. 1999; Reed et al. 1997) and producing fuel cells with low power density and sensors with low sensitivity (Minteer et al. 2007; Reed et al. 1997; Riklin et al. 1995). The lack of an effective molecular wiring system that can allow unimpeded charge transport is a significant problem that hinders the ability to harness the full potential of redox-enzymes-based, self-powering bioelectronic devices.

Interestingly, nature uses an array of unique molecules, i.e., the iron-sulfur complexes ($[\text{Fe-S}]$), to circumvent the thermodynamic and kinetic electron transport issues associated with redox enzymes. Iron-sulfur compounds are the key molecules that link enzyme (apoenzyme and coenzyme) complexes to the supporting surfaces in mitochondrial and photosynthetic electron transport chains (Berk et al. 2000). Iron-sulfur groups are ideal linker molecules because they: 1) can mediate electron transport; 2) have correct

prosthetic groups to anchor the enzyme complex at one end and provide metal support at the other (sulfur coordinates with metal centers (Graham and Dingman 2006; Love et al. 2005; Mrksich et al. 1996; Tour et al. 1995; Zhong and Porter 1994) and onto proteins with cysteinyl residues (Meyer 2008), while iron coordinates with the heterocyclic nitrogen atoms (Cline et al. 1985; Lavrenova et al. 1986; Marlin et al. 1999) or amine linkages (Bedford et al. 2005; Liu et al. 2013; Zuo et al. 2013) that are present in the coenzymes and cellular peptides); and, quite importantly, 3) are short enough to alleviate charge transport limitations. Even so, to date, we have been unable to duplicate [Fe-S]-based electron transport chains *ex vivo*, primarily due to the instability of iron-sulfur compounds in aqueous media.

The aim of this work was to determine whether iron-sulfur molecules can be used effectively as linker molecules to attach the redox enzyme complexes to supporting surfaces *ex vivo*, and, if so, to investigate their performance in the electrical transport function. As a working model, a glycerol-sensitive gold bioanode is described based on direct attachment of the glycerol-dehydrogenase (GIDH)-NAD⁺ apoenzyme-coenzyme complex onto the supporting gold surface using iron (II) sulfide (FeS) mediation. A conventional PQQ-based electrode, described previously (Mahadevan et al. 2014), was used as the control. The performances of the two electrode systems were compared using amperometric and potentiometric studies.

2. Materials and Methods

2.1 Reagents and apparatus

Iron (II) sulfide (FeS), NAD⁺-dependent glycerol dehydrogenase from *Cellulomonas* sp. (EC.1.1.1.6), β -nicotinamide adenine dinucleotide (NAD⁺), glycerol $\geq 99\%$, and glutaraldehyde were purchased from Sigma-Aldrich, USA (www.sigmaaldrich.com). 50 mM KOH solution made in 35 wt% H₂O₂ was used to clean the surface of the gold. FeS was suspended in $\geq 99.5\%$ ethanol, and β -NAD⁺, GIDH, and glutaraldehyde solutions were prepared in a 0.1 M phosphate buffer (pH = 7). Molecular-biology-grade water obtained from Sigma-Aldrich was used to prepare all the aqueous-based solutions and for rinsing and cleaning purposes throughout this study. Two-millimeter gold-disk working electrodes, Ag/AgCl reference electrodes, and Pt auxiliary electrodes were purchased from CH Instruments, Inc., Austin, TX.

The potentiometric and amperometric tests were performed using a CHI8003D Potentiostat from CH Instruments, Inc. Water containing the enzyme stimulants (Mahadevan et al. 2014) viz. $(\text{NH}_4)_2\text{SO}_4$ and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ was used as the carrier electrolyte throughout the study.

2.2 Fabrication of the bioanode

Layer-by-layer self-assembly via dip-coating was used to fabricate the FeS-based enzyme electrodes. First, the gold (Au) electrodes were prepared for modification by following the three-step rigorous cleaning procedure as described earlier (Mahadevan et al. 2014). The clean Au electrodes were immersed in a 0.3 M FeS solution suspended in ethanol for 2 h. Then the FeS-modified electrodes were immersed in 1 mM of $\beta\text{-NAD}^+$ for 2 h followed by 1 mg mL⁻¹ of GIDH for 2 h for formation of NAD⁺ and GIDH monolayers respectively. The FeS-NAD⁺-GIDH functionalized electrodes were lastly treated with 10% (v/v) glutaraldehyde for 20 min to crosslink and secure the molecular layers. Every step in the dip-coating procedure was followed by rinsing the electrodes with water to eliminate physisorbed molecules, if any. Fig. 1B shows the FeS-based wiring scheme.

Attachment of FeS to the Au surface was verified via a combination of techniques involving cyclic voltammetry (described below), and X-ray photoelectron spectroscopy (XPS) and Fourier Transform Infra-red (FTIR) spectroscopy (described in the Supplementary Material section).

2.3 Ferricyanide-voltammetry to confirm SAM formation

Electrochemical experiments were performed via the conventional three-electrode system (i.e., enzymatic working electrode, Pt counter electrode, and Ag/AgCl reference electrode) placed in an electrochemical cell containing 5 mL of potassium ferricyanide. A single potential sweep was individually performed for each successive layer of the multi-layer SAMs attached to the Au electrode (with constant surface area 0.031 cm²). A sweep rate of 50 mV/s was used for all the cyclic voltammetric measurements. A bare Au electrode was used as an ancillary control (or standard) electrode. All experiments were performed at room temperature.

2.4 Potentiometric analysis

Instantaneous open circuit voltage (OCV) measurements of the enzyme electrodes were taken using a potentiostat via the conventional three-electrode system in 5 mL total volume of the carrier electrolyte containing 0.001-1 M glycerol.

2.5 Amperometric analysis

An array of amperometric tests were conducted for the enzyme electrodes via the typical three-electrode system in glycerol (0.001-1 M). First, cyclic voltammetry was conducted in a total volume of 5 mL of carrier electrolyte containing glycerol. Current responses for potential sweeps between 0 - 1.5 V and 0.665 - 1.3 V, at a scan rate of 50 mV/s were recorded. The effect of current with respect to time at a constant potential, $E = 1.3$ V, was analyzed using constant potential amperometry for 60 s. To evaluate the diffusion coefficient(s) of glycerol, chronoamperometry was conducted at initial $E = 0.665$ V and final $E = 1.3$ V.

3. Results and Discussion

3.1 Surface characterization of the bioanode

Fig. 2 shows the ferricyanide cyclic voltammograms (CVs) for the electrode(s) obtained after each dip-coating. The current response after each immersion step was used to verify the formation of a self-assembled-monolayer (SAM) on the Au electrode. Change in current and peak splitting of the $\text{Fe}(\text{CN})_6^{3-/4-}$ voltammetry have been attributed to change in electron-transfer kinetics caused by the blocking behavior of the densely packed assemblies on the surface of the electrode (Dowdy and Leopold 2010). Deposition of a SAM layer across the conductive pathway increases the length of the conductor, which in turn, increases the ohmic resistance at a given potential, resulting in the reduction of peak current. Hence, the decrease in current and increase in peak shift after each dip-coating step seen in Fig. 2 can be attributed to the formation of successive SAM layers on the Au surface.

Fig. 2: Surface characterization of anode using cyclic voltammetry. Cyclic voltammograms of the bare Au electrode (yellow) and the modified Au electrode after formation of each SAM layer, conducted in 0.01 M potassium ferricyanide with 0.1 M KNO_3 as the supporting electrolyte at a potential scan rate of 50 mV/s. A constant surface area of the Au electrode, i.e., 0.031 cm^2 , was used throughout the study

3.2 Potentiometric analysis of the bioanode

It was observed that the instantaneous OCVs produced by the FeS-based electrodes were significantly greater than those of the PQQ-based electrode (Fig. 3). It was inciting to observe that the FeS-based electrode reached the standard potential E^0 (half-wave potential) for all glycerol concentrations while the PQQ-based electrode clearly failed to reach the rated maximum for all substrate concentrations. In fact, the OCV values deteriorated significantly in the conventional electrode with increasing glycerol concentrations, which was likely due to increased resistance resulting from the conventional and much lengthier wiring scheme.

Fig. 3: Potentiometric analysis of bioanode. Instantaneous OCV obtained by FeS-based and PQQ-based enzyme electrodes in aqueous glycerol concentrations (0.001 – 1 M) containing enzyme stimulants

3.3 Amperometric analysis of the bioanode

CVs showing the amperometric response of the FeS-based enzyme electrode in glycerol concentrations ranging from 0 – 1 M are shown in Fig. 4A. In the CVs, the anodic and cathodic currents that correspond to the catalytic oxidation and reduction of glycerol/dihydroxyacetone by GIDH further validates the presence of immobilized enzymes and the retention of their activity. It was apparent that the FeS-based system showed comparable increases in the anodic peak currents ($|I_a|$) and cathodic peak currents ($|I_c|$) at 1.3 and 0.665 V, respectively, for increasing glycerol concentrations. A correlation plot between the concentration of glycerol and the peak currents was developed to compare $|I_a|$ and $|I_c|$ of the Fe-based and PQQ-based electrodes (Fig. 4B). Apart from having a strong logarithmic correlation with the concentration of glycerol (between 0.001 and 1 M), the FeS-based system showed a consistent increase in anodic currents $|I_a|$ for all glycerol concentrations when compared with those of the PQQ-based system.

Fig. 4: Amperometric analysis of bioanode. (a) Cyclic voltammograms for the FeS-based enzyme electrode for increasing concentrations of glycerol (a = 0 M, b = 0.001 M, c = 0.01 M, d = 0.1 M, and e = 1 M) at a scan rate of 50 mV/s; (b) Peak current (I_p) at 1.3 V and 0.665 V vs. glycerol concentrations measured by enzymatic electrodes (FeS-based and PQQ-based) derived from CV scans between 0 and 1.5 V at a scan rate of 50 mV/s. Fitting equations for FeS-based @ 1.3 V are $y = 1.4969\ln(x) + 28.573$, $R^2 = 0.9883$ and for PQQ-based, they are $1.3337\ln(x) + 25.092$, $R^2 = 0.9595$; FeS-based @ 0.665 V are $y = -1.139\ln(x) - 16.924$, $R^2 = 0.9397$ and for PQQ-based are $-1.01\ln(x) - 16.734$, $R^2 = 0.9578$; (c) Equilibrated (60 s) constant potential amperometric measurements of anodic currents for FeS-based and PQQ-based enzyme electrodes in glycerol concentrations in the range of 0.001 – 1 M at $E = 1.3$ V; (The analysis portrays how the two electrode systems will behave in a practical bioelectronics application situation, such as biosensing.); (d) Comparison of peak current increase at 1.3 V for FeS-based and PQQ-based electrode systems using cyclic voltammetry and constant potential amperometry

To compare the performance of the electrode systems in a practical bioelectronics application, such as biosensing, a constant potential amperometry study was performed at 1.3 V pertaining to anodic oxidation. The results of this study indicated that the FeS-based electrodes had peak current amplifications ranging from 200% to 1100% compared to the conventional electrode, depending on the concentration of the substrate that was used (Figs. 4C and 4D). The limit of detection (LOD) for the FeS electrode was found to be 1.97 μ A. It is likely that this remarkable increase in the performance of the FeS-based electrode was as a result of replacement of the longer cystamine, PQQ, and boronic acid wire segments with the shorter FeS segments. The current-voltage analysis alludes to the ability of FeS to

mediate the electron transfer between the enzyme system and the electrode surface and the ability of the FeS molecules to transport these charges more effectively - likely due to the shortened path.

Furthermore, diffusion coefficients of glycerol obtained by the conventional PQQ-based and the novel FeS-based electrodes were compared. Diffusion coefficients reflect the nature of the transport phenomena that occur between the substrate and an electroactive system. This comparison was necessary to confirm that the current-voltage responses that were observed were actually due to differences in the wiring and not due to variations in the diffusion of the substrate onto the surfaces of the electrodes with different morphologies. Chronoamperometry technique was used to evaluate the diffusion coefficient(s) of glycerol (Fig. 5A).

Fig. 5: Evaluating diffusion coefficient of glycerol using chronoamperometry. (a) Chronoamperograms for FeS-based enzyme electrode in 0 – 1 M glycerol; (b) Cottrell plot (I vs $t^{-1/2}$) obtained from chronoamperograms of FeS-based enzyme electrode in 0.001 – 1 M glycerol; (c) Correlation of bulk concentration and diffusion coefficients (Initial $E = 0.665$ V and Final $E = 1.3$ V over the FeS-based and PQQ-based electrodes.)

Oxidative and reductive chronoamperograms for the FeS-based electrode appear symmetrical and identical for all glycerol concentrations (Fig. 5A), indicating that the rate of charge transfer did not differ as

the glycerol concentrations varied (within the given voltage range). This shows that the system was not affected by the limitations of charge/mass transport. To further verify this, diffusion coefficients were derived for the two forms of electrodes. Cottrell plot (I vs $t^{-1/2}$, Fig. 5B) and the associated equation, $I = \frac{nFA\sqrt{DC}}{\sqrt{\pi t}}$, (where, D is the diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$), C is the bulk concentration (mol cm^{-3}), and n , F , A , and t are electron number, Faraday number, surface area of the electrode, and time, respectively) were used to evaluate the diffusion coefficients (D) (Bard and Faulkner 1980). The D values obtained for the PQQ-based electrode correlated well with that of the FeS-based electrode, as shown in Fig. 5C. An average diffusion coefficient of $1.24 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$ for glycerol concentrations of $0.001 - 1 \text{ M}$ was obtained with the FeS-based electrode, which was close to that obtained for the conventional PQQ-based electrode ($1.68 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$). The analysis indicated that the diffusion of the solute to the surfaces was equal for the two electrodes, irrespective of their surface morphologies.

4. Conclusions

It was possible to tether glycerol-dehydrogenase/nicotinamide-adenine-dinucleotide (GIDH-NAD⁺) apoenzyme-coenzyme complex onto a supporting gold electrode using inorganic iron (II) sulfide-based (FeS-based) single-molecular-wire. To avoid dissociation in aqueous media, FeS was anchored to the gold base electrode in anhydrous ethanol medium. Attachment of the apoenzyme-coenzyme complex was conducted subsequently in aqueous media. Successful concatenation of the molecular layers was verified using cyclic voltammetry and spectroscopy. Open circuit voltage of the glycerol-consuming cell developed with conventional PQQ-based electrodes was consistently lower than the standard potential, while that of the FeS-based electrodes consistently reached the standard potential. When the two electrode systems were probed with applied potential viz. cyclic voltammetry at glycerol concentrations between $0.001 - 1 \text{ M}$, the FeS-based electrodes consistently generated higher peak currents than the conventional PQQ-based electrode. Constant potential amperometry which emulates the use of such electrodes for real-world applications indicated over a 1000% increase in the peak currents when the FeS-based enzyme electrode was used with lower glycerol concentrations. On an average, the FeS-based electrode obtained 780% increase in the peak currents over the entire range of substrate

concentrations that were tested (i.e., 0.001 – 1 M glycerol) when compared with the conventional PQQ-based electrode. This remarkable enhancement of the electrical charge transport properties was attributed to: 1) the reduced internal resistance of the enzymatic electrode resulting from the shorter FeS single-molecular-wire as opposed to the conventional PQQ-based wiring system that requires a series of biomolecules to foster enzyme attachment; and 2) the ability of FeS to be a single-molecular anchoring agent to mediate electron shuttling between NAD^+ and the supporting electrode. Although we are just now getting around to duplicating this phenomenon on the laboratory bench, nature has long been able to transport electrons quite efficiently since eukaryotes came into existence. And, iron-sulfur has been the key first link to anchor the enzyme complex with the rest of the chain in the mitochondrial and photosynthetic electron transport chains. Based on the findings, we have come one step closer to utilizing this knowledge outside a living cell.

Acknowledgments

This article is based on work supported by the National Science Foundation under Grant CBET-1243311. We are grateful to Dr. R. Karthikeyan for allowing access to his laboratory to carry out a significant portion of the studies (during the time at which the Principle Investigator, Fernando's laboratory was under renovation).

References

- Bard, A.J., Faulkner, L.R., 1980. *Electrochemical methods: fundamentals and applications*. Wiley New York.
- Bedford, R.B., Bruce, D.W., Frost, R.M., Hird, M., 2005. Simple iron-amine catalysts for the cross-coupling of aryl Grignards with alkyl halides bearing [small beta]-hydrogens. *Chemical Communications*(33), 4161-4163.
- Berk, A., Zipursky, S., Lodish, H., 2000. *Molecular Cell Biology* 4th edition.
- Cline, J.F., Hoffman, B.M., Mims, W.B., LaHaie, E., Ballou, D.P., Fee, J.A., 1985. Evidence for N coordination to Fe in the [2Fe-2S] clusters of Thermus Rieske protein and phthalate dioxygenase from Pseudomonas. *Journal of Biological Chemistry* 260(6), 3251-3254.
- Dowdy, C.E., Leopold, M.C., 2010. Enhanced electrochemistry of nanoparticle-embedded polyelectrolyte films: Interfacial electronic coupling and distance dependence. *Thin Solid Films* 519(2), 790-796.
- Graham, D., Dingman, S., 2006. *Material Matters*. Sigma Aldrich.
- Heller, A., 1990. Electrical wiring of redox enzymes. *Accounts of Chemical Research* 23(5), 128-134.

Johnston, D.E., Strachan, D.R., Johnson, A.T.C., 2007. Parallel fabrication of nanogap electrodes. *Nano letters* 7, 2474-2477.

Katz, E., Heleg-Shabtai, V., Willner, B., Willner, I., Bückmann, A.F., 1997. Electrical contact of redox enzymes with electrodes: novel approaches for amperometric biosensors. *Bioelectrochemistry and Bioenergetics* 42(1), 95-104.

Lavrenova, L.G., Ikorskii, V.N., Varnek, V.A., Oglezneva, I.M., Larionov, S.V., 1986. High-temperature spin transition in coordination compounds of iron(II) with triazoles. *Journal Name: Sov. J. Coordinat. Chem.; (United States); Journal Volume: 12:2; Other Information: Translated from Koordinatsionnaya Khimiya, 12: No. 2, 207-215(Feb 1986), Medium: X; Size: Pages: 119-127.*

Liu, T., DuBois, D.L., Bullock, R.M., 2013. An iron complex with pendent amines as a molecular electrocatalyst for oxidation of hydrogen. *Nat Chem* 5(3), 228-233.

Love, J.C., Estroff, L.A., Kriebel, J.K., Nuzzo, R.G., Whitesides, G.M., 2005. Self-Assembled Monolayers of Thiolates on Metals as a Form of Nanotechnology. *Chemical Reviews* 105(4), 1103-1170.

Mahadevan, A., Gunawardena, D.A., Karthikeyan, R., Fernando, S., 2014. Potentiometric vs amperometric sensing of glycerol using glycerol dehydrogenase immobilized via layer-by-layer self-assembly. *Microchimica Acta*, 1-9.

Marlin, D.S., Olmstead, M.M., Mascharak, P.K., 1999. Carboxamido Nitrogens Are Good Donors for Fe(III): Syntheses, Structures, and Properties of Two Low-Spin Nonmacrocyclic Iron(III) Complexes with Tetracarboxamido-N Coordination. *Inorganic chemistry* 38(13), 3258-3260.

Meyer, J., 2008. Iron–sulfur protein folds, iron–sulfur chemistry, and evolution. *J Biol Inorg Chem* 13(2), 157-170.

Minteer, S.D., Liaw, B.Y., Cooney, M.J., 2007. Enzyme-based biofuel cells. *Current Opinion in Biotechnology* 18(3), 228-234

Mrksich, M., Chen, C.S., Xia, Y., Dike, L.E., Ingber, D.E., Whitesides, G.M., 1996. Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proceedings of the National Academy of Sciences* 93(20), 10775-10778.

Park, H., Lim, A.K.L., Alivisatos, A.P., Park, J., McEuen, P.L., 1999. Fabrication of metallic electrodes with nanometer separation by electromigration. *Appl. Phys. Lett.* 75, 303-304.

Reed, M.A., Zhou, C., Muller, C.J., Burgin, T.P., Tour, J.M., 1997. Conductance of a molecular junction. *Science* 278, 252-254.

Riklin, A., Katz, E., Willner, I., Stocker, A., Bückmann, A.F., 1995. Improving enzyme-electrode contact redox modifications cofactors. *Nature* (376,), 672-675.

Tour, J.M., Jones, L., Pearson, D.L., Lamba, J.J.S., Burgin, T.P., Whitesides, G.M., Allara, D.L., Parikh, A.N., Atre, S., 1995. Self-Assembled Monolayers and Multilayers of Conjugated Thiols, .alpha.,.omega.-Dithiols, and Thioacetyl-Containing Adsorbates. Understanding Attachments between Potential Molecular Wires and Gold Surfaces. *Journal of the American Chemical Society* 117(37), 9529-9534.

Zhong, C.-J., Porter, M.D., 1994. Evidence for Carbon-Sulfur Bond Cleavage in Spontaneously Adsorbed Organosulfide-Based Monolayers at Gold. *Journal of the American Chemical Society* 116(25), 11616-11617.

Zuo, W., Lough, A.J., Li, Y.F., Morris, R.H., 2013. Amine(imine)diphosphine Iron Catalysts for Asymmetric Transfer Hydrogenation of Ketones and Imines. *Science* 342(6162), 1080-1083.

Appendices

Equations

Equation (1):
$$I = \frac{nFA\sqrt{DC}}{\sqrt{\pi t}}$$

Figures

Fig. 1. Schematic of wiring systems. (a) Complex conventional wiring of the coenzyme NAD⁺ to a gold electrode using a series of molecules including a cystamine linker, a PQQ mediator, and a phenyl boronic acid linker; (b) Simplified wiring of NAD⁺ coenzyme directly onto the gold electrode by the multi-functional mediator, FeS

Fig. 2: Surface characterization of anode using cyclic voltammetry. Cyclic voltammograms of the bare Au electrode (yellow) and the modified Au electrode after formation of each SAM layer, conducted in 0.01 M potassium ferricyanide with 0.1 M KNO₃ as the supporting electrolyte at a potential scan rate of 50 mV/s. A constant surface area of the Au electrode, i.e., 0.031 cm², was used throughout the study

Fig. 3: Potentiometric analysis of bioanode. Instantaneous OCV obtained by FeS-based and PQQ-based enzyme electrodes in aqueous glycerol concentrations (0.001 – 1 M) containing enzyme stimulants

Fig. 4: Amperometric analysis of bioanode. (a) Cyclic voltammograms for the FeS-based enzyme electrode for increasing concentrations of glycerol (a = 0 M, b = 0.001 M, c = 0.01 M, d = 0.1 M, and e = 1 M) at a scan rate of 50 mV/s; (b) Peak current (I_p) at 1.3 V and 0.665 V vs. glycerol concentrations measured by enzymatic electrodes (FeS-based and PQQ-based) derived from CV scans between 0 and 1.5 V at a scan rate of 50 mV/s. Fitting equations for FeS-based @ 1.3 V are $y = 1.4969\ln(x) + 28.573$, $R^2 = 0.9883$ and for PQQ-based, they are $1.3337\ln(x) + 25.092$, $R^2 = 0.9595$; FeS-based @ 0.665 V are $y = -1.139\ln(x) - 16.924$, $R^2 = 0.9397$ and for PQQ-based are $-1.01\ln(x) - 16.734$, $R^2 = 0.9578$; (c) Equilibrated (60 s) constant potential amperometric measurements of anodic currents for FeS-based and PQQ-based enzyme electrodes in glycerol concentrations in the range of 0.001 – 1 M at E = 1.3 V; (The analysis portrays how the two electrode systems will behave in a practical bioelectronics application situation, such as biosensing.) ; (d) Comparison of peak current increase at 1.3 V for FeS-based and PQQ-based electrode systems using cyclic voltammetry and constant potential amperometry

Fig. 5: Evaluating diffusion coefficient of glycerol using chronoamperometry. (a) Chronoamperograms for FeS-based enzyme electrode in 0 – 1 M glycerol; (b) Cottrell plot (I vs $t^{-1/2}$) obtained from chronoamperograms of FeS-based enzyme electrode in 0.001 – 1 M glycerol; (c) Correlation of bulk concentration and diffusion coefficients (Initial E = 0.665 V and Final E = 1.3 V over the FeS-based and PQQ-based electrodes.)