ACS Energy LETTERS

Cite This: ACS Energy Lett. 2018, 3, 2736−2742

http://pubs.acs.org/journal/aelccp

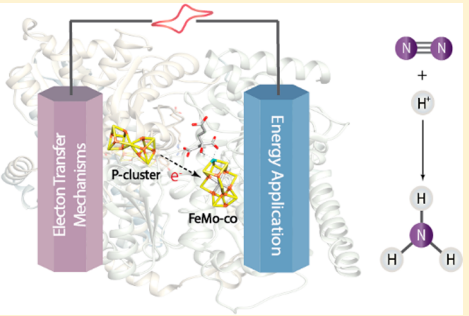
PERSPECTIVE

Nitrogenase Bioelectrocatalysis: From Understanding Electron-Transfer Mechanisms to Energy Applications

Rong Cai and Shelley D. Minteer*

Departments of Chemistry and Materials Science & Engineering, University of Utah, 315 South 1400 East Rm 2020, Salt Lake City, Utah 84010 United States

ABSTRACT: **Nitrogenase is the only enzyme known to reduce molecular nitrogen to ammonia, so it has been an oxidoreductase enzyme of great interest to the electrofuels and catalysis communities, as people consider the design of bioinspired nitrogen reduction catalysts for the production of ammonia. This Perspective details the use of nitrogenase as an electrocatalyst to learn mechanistic information about the electron-transfer mechanism as an inspiration for molecular and metal-based electrocatalysts and for a variety of energy applications, including energy-efficient ammonia production and biofuel cells. This Perspective also addresses the issues and challenges of nitrogenase bioelectrocatalysis that will need to be addressed in the coming years.**



Over the past decade, there was been a wealth of interest in electrofuels, where electricity is used to electrochemically generate a fuel (i.e., hydrogen). This has included a focus on nitrogen-based electrofuels produced from nitrogen reduction. There have been a wealth of molecular and heterogeneous electrocatalysts studied,[1−3] but it is very difficult to reduce nitrogen to ammonia. Therefore, researchers have turned to biological inspiration from the only enzyme known to reduce nitrogen to ammonia, which is called nitrogenase. Nitrogenases are complex oxidoreductase enzymes that are typically classified by their cofactor. The most studied nitrogenase is the MoFe nitrogenase that contains a FeMo-cofactor, but there are also VFe nitrogenases and FeFe nitrogenases with similar cofactors containing metal substitutions.[4] The different cofactors lead to different preferences for product selectivity (i.e., carbon dioxide reduction, proton reduction, nitrite reduction, and nitrogen reduction). These nitrogenase enzymes are actually a bienzyme cascade where the catalytic nitrogenase gains energy from a second protein called the Fe protein that delivers the energy from ATP to the nitrogenase,[5] as shown in Figure 1a. Therefore, it is difficult to understand electron-transfer mechanisms within the MoFe, VFe, or FeFe nitrogenase, because you may be limited by the electron transfer between the two proteins. Also, it is difficult to utilize this bienzyme cascade, because it requires the two proteins to dynamically interact (come together to transfer electrons then move apart to consume ATP and come together again to transfer electrons, etc.) and it is ATP-dependent enzyme, so ATP would need to be regenerated via an ATP regeneration scheme.[6−8] The ATP regeneration system complicates the electrochemical cell by adding an additional enzyme or enzymes to the catalytic cascade. In theory, the enzyme should be able to be controlled electrochemically. There is early work by Schultz and co-workers studying the electrochemistry of the FeMo-cofactor,[9] but there has not been an electrochemical study of the enzyme until the past few years, except traditional redox titrations to determine the redox potentials of the cofactors.

Bioelectrocatalysis is the field of research focused on utilizing oxidoreductase enzymes as electrocatalysts. There are two different classifications of bioelectrocatalysis in the literature: direct electron transfer and mediated electron transfer. Direct electron transfer means that the protein can directly communicate with the electrode through tunneling of electrons between the electrode and the cofactor[10] or other metal centers in the protein.[11] This allows for the bioelectrocatalysis to occur at the potential of the active site of the protein [or the additional metal centers in the protein (i.e., multiheme proteins will typically transfer electrons internally from the active site to the hemes and then the hemes to the electrode surface via an internal electron-transfer relay)].[11] The ability of bioelectrocatalysis to occur at the potential of the active site or other metal centers in the protein allows for accurate studies of enzyme kinetics and mechanisms. However, because of diffusional cofactors (i.e., NAD/NADH and NADP/NADPH) and buried active sites in oxidoreductase enzymes, it is not always possible to attach the protein to the

Received: August 31, 2018
Accepted: October 8, 2018
Published: October 8, 2018

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742

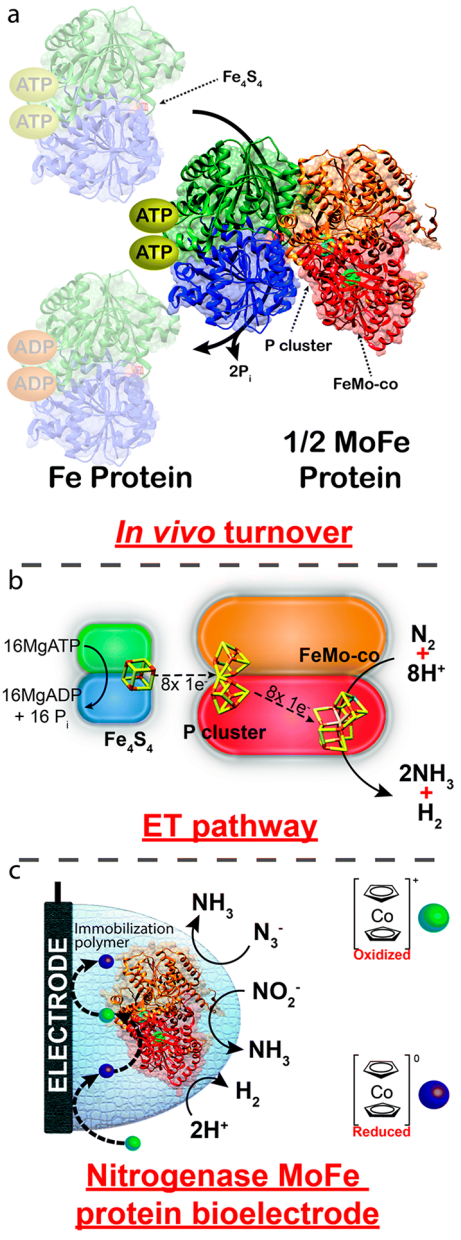


Figure 1. (a) Crystal structure of nitrogenase (PDB: 4WZA) from *Azotobacter vinelandii* illustrating the transient association of the Fe protein to the MoFe protein. (b) ET between the Fe_4S_4 cluster of the Fe protein to the P cluster and the FeMo-co of the MoFe protein. (c) Proposed route of heterogeneous ET of MoFe nitrogenase immobilized in poly(vinylamine) using cobaltocene as a diffusional electron mediator. Reprinted with permission from ref 15. Copyright 2016 Royal Society of Chemistry.

electrode in a configuration where the active site is within a reasonable tunneling distance to the electrode.[12,13]

When tunneling between the active site/cofactor and electrode is not possible or slow, mediated electron transfer can use a small molecule or polymer-based redox moiety to shuttle electrons to/from the electrode. This redox moiety should have a potential close to the potential of the active site and reversible electrochemistry.[14] Typically, mediated electron-transfer systems result in higher current densities than direct electron transfer, but they have potential inefficiencies. It is also important to note that mediators can do their own catalysis and are at potentials different than the active site, so they can result in studying kinetics and/or mechanisms that may or may not be the same as the protein in solution. In the field of bioelectrosynthesis for electrofuels, it is important to note that there are far fewer mediation schemes for reductive processes than oxidative processes (i.e., oxidation of the electrofuel).

Similar to other bioelectrocatalysis for electrofuels, the first reports of nitrogenase bioelectrocatalysis were with mediated electron transfer. It was found that cobaltocene/cobaltocenium dissolved in solution is an effective mediator for MoFe nitrogenase bioelectrocatalysis,[15] where the nitrogenase is immobilized in a nonconductive and nonmediating hydrogel (poly(vinylamine)), as shown in Figure 1b. The Vincent group also showed in 2017 that europium complexes in solution can be used as mediators for MoFe nitrogenase.[16] Nitrogenases are promiscuous enzymes, as shown in Figure 1c, so a variety of substrates have been studied for the bioelectrocatalysis of MoFe nitrogenases, including protons,[15] nitrite,[15] carbon dioxide,[17] and azide.[15] Some examples are shown in Figure 2. The cobaltocene-mediated system involves immobilizing the MoFe nitrogenase in a chemically cross-linked poly(vinylamine) hydrogel on carbon electrodes (i.e., glassy carbon or Toray carbon paper), where the hydrogel physically constrains the enzyme to the electrode, but the hydrogel is not conductive and does not contain immobilized redox moieties. The Seefeldt group has used this technique to investigate enzyme mechanism.[18] Specifically, they have studied the mechanism of hydrogen formation by metal-hydride protonation and electrochemically determined hydrogen/deuterium isotope effects.[18] Because nonelectrochemical assays require the Fe protein and electron transfer between the Fe protein and the MoFe protein is the rate-determining step of these assays, kinetic isotope effect measurements were not possible until this electrochemical assay was developed. This electrochemical assay showed that for the MoFe protein itself, a single hydrogen is involved in the rate-limiting step and that the rate-limiting step is indeed hydrogen gas formation. This work shows the utility of bioelectrocatalysis in studying enzyme mechanism.

One common strategy for immobilizing mediators is to produce redox polymers from the mediators and use the polymers for immobilizing the enzyme AND transferring the electrons from the electrode via self-exchange-based conduction. This strategy has been used for a variety of enzymes (hydrogenase, laccase, glucose oxidase, FAD-dependent glucose dehydrogenase, cellobiose dehydrogenase, and bilirubin oxidase),[19] but cobaltocene redox polymers do not mediate nitrogenase bioelectrocatalysis for any of the above reactions (i.e., proton reduction, nitrite reduction, azide reduction, carbon dioxide reduction, etc.), even though they mediate formate dehydrogenase bioelectrocatalysis of carbon dioxide reduction.[20] Future research will investigate other redox polymers to find redox polymers capable of promoting mediated bioelectrocatalysis, because combining the mediator and the immobilization polymer is typically a more efficient method of mediated bioelectrocatalysis.

It is important to note that no mediated bioelectrocatalysis of nitrogen reduction was observed with just the MoFe nitrogenase. Therefore, we explored combining the MoFe nitrogenase and the Fe protein into a biprotein cascade. Cobaltocene/cobaltocenium is not an effective mediator for the biprotein cascade of the MoFe nitrogenase and the Fe protein. We found that methyl viologen would act as a

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742

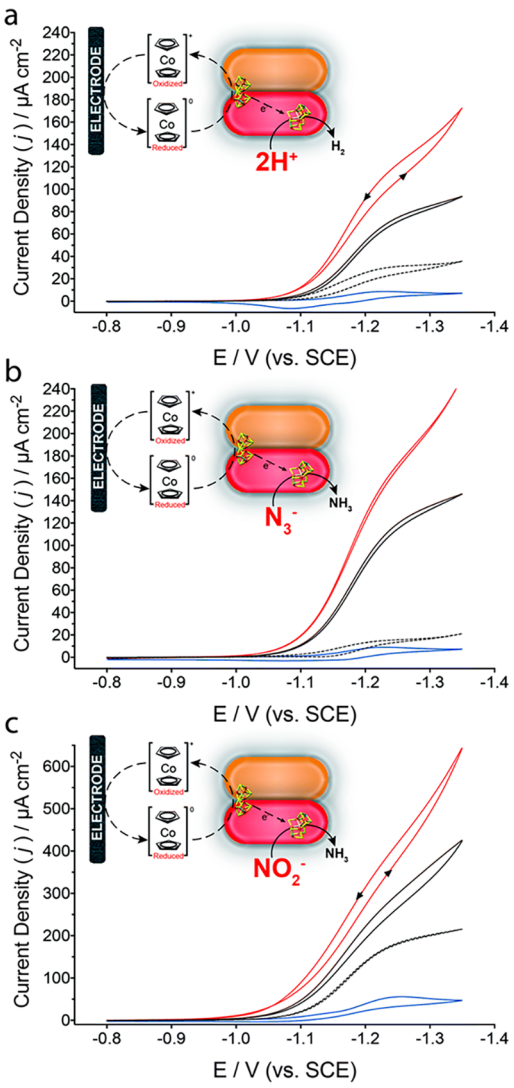


Figure 2. Cyclic voltammograms of MoFe protein bioelectrode in HEPES buffer (pH 7.4) containing 200 μM cobaltocene/cobaltocenium at a scan rate of 2 mV s^{-1} (solid black lines) in the (a) absence of any additional substrates or in the presence of (b) 50 mM azide or (c) 50 mM nitrite. MoFe protein bioelectrodes were also prepared with the β-98Tyr → His MoFe protein (red solid lines). Equivalent control bioelectrodes were prepared with either BSA (blue line) or apo-MoFe protein (black dashed line) in the presence of the respective substrates. Reprinted with permission from ref 15. Copyright 2016 Royal Society of Chemistry.

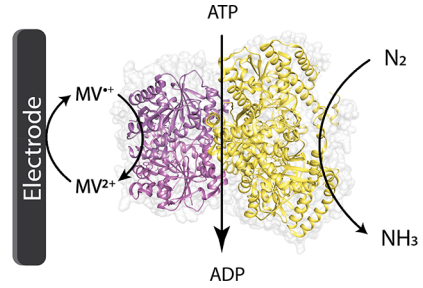


Figure 3. Schematic of the electrochemical Haber–Bosch process.

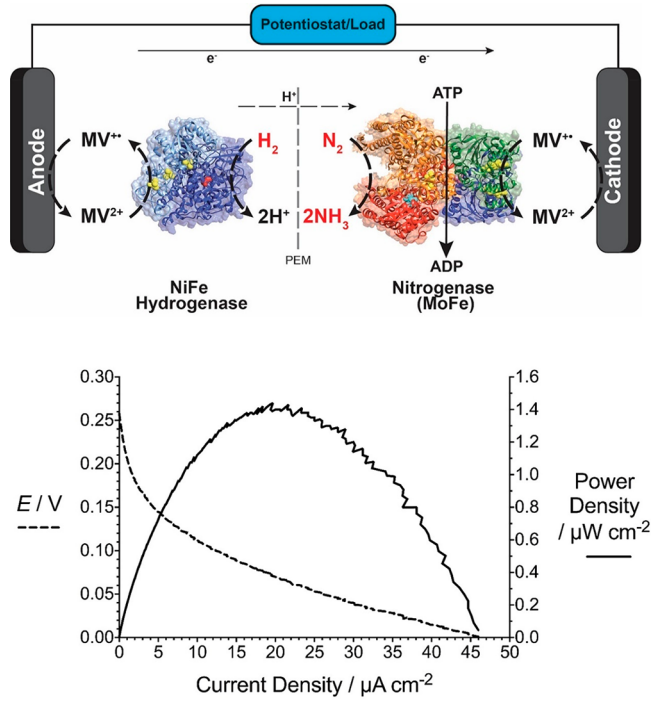


Figure 4. Hydrogen/nitrogen biofuel cell: (Top) Compartmentalization of hydrogenase and nitrogenase Fe/MoFe proteins by the use of a proton exchange membrane (PEM) leads to a fuel cell configuration that is able to utilize MV as the electron mediator in both chambers and simultaneously produce ammonia and electrical energy from hydrogen and nitrogen at room temperature and ambient pressure. (Bottom) Representative polarization (left y-axis) and power curves (right y-axis) of a hydrogen/nitrogen EFC. Reproduced with permission from ref 21. Copyright 2017 Wiley.

mediator for the biprotein cascade.[21] The MoFe nitrogenase and Fe protein cannot be immobilized at the electrode surface, because they require transient interactions for catalytic function, so we operated the biprotein cascade in solution with methyl viologen mediator in solution. This resulted in a biocathode that was capable of nitrogen reduction to ammonia, as shown in Figure 3. Coulombic efficiencies are relatively high (59 ± 6%) considering that the mediator and the enzymes are not immobilized.[21]

After showing nitrogen bioelectrocatalysis using the biprotein cascade, we combined this biocathode with a hydrogenase bioanode to produce a hydrogen/nitrogen biofuel cell. This hydrogen/nitrogen biofuel cell (Figure 4) resulted in an open-circuit potential of only 228 ± 28 mV,[21] because it is using the same mediator (methyl viologen) at both the anode and the cathode, but this system was designed to just show proof of concept that we could use a nitrogenase biocathode in a biofuel cell. It is also important to note that based on the polarization curve in Figure 4, the fill factor for this fuel cell is quite small, because the mediator is not immobilized at the cathode or the anode; there is no flow of reactants in this fuel cell; and the fuel cell configuration is an H-cell with a separation of a couple of centimeters between the anode and the cathode, which results in losses due to resistance. This biofuel cell had four main issues: (1) Nitrogenase is very oxygen sensitive, so the entire biofuel cell had to operate in a glovebag. (2) There is a substantial loss in potential due to the use of methyl viologen mediator at both the anode and cathode. (3) The biprotein cascade eliminates the possibility of immobilizing the enzyme, which is required for any

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736–2742

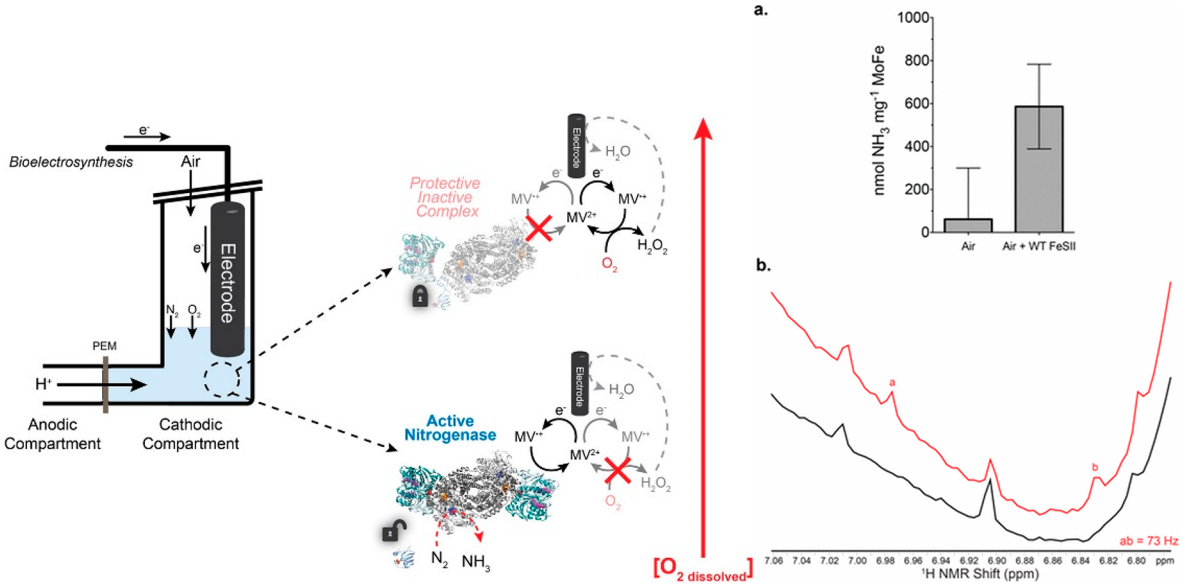


Figure 5. Shethna biocathodes: (Left) Schematic of the bioelectrochemical cell where N_2 was introduced to the cell by the injection of air over the headspace of the solution. (Right) (a) Fluorimetric quantification of NH_3 produced by nitrogenase bioelectrosynthetic N_2 reduction from air in the absence and presence of Shethna protein. (b) $^1H-^{15}N$ NMR (500 MHz) of $^{15}NH_4^+$ produced by the bioelectrosynthetic reduction of $^{15}N_2$ "air" (79% $^{15}N_2$/21% O_2) in the presence of FeSII, with (black) or without (red) ^{15}N decoupling. Reproduced from ref 22. Copyright 2017 American Chemical Society.

bioelectrochemical application to be feasible. (4) An ATP regeneration system is required to make this bioelectrode function, and that ATP regeneration system is expensive and energy intense.

In order to address the oxygen sensitivity of nitrogenases, we looked to nature for inspiration. In nature, there is a structural protein, named Shethna, that protects nitrogenase from oxygen. This protein is a conformational switch protein that locks the Fe protein and the MoFe protein into a super-complex that protects it from exposure to oxygen. When the oxygen concentration decreases, the protein switches and releases the supercomplex for bioelectrocatalytic use. The Shethna protein was combined with the Fe protein and the MoFe protein to produce a biocathode similar to the biocathode discussed above and shown in Figure 3, except for the addition of the Shethna protein. The Shethna protein does protect the Fe protein and the MoFe protein. The oxygen that is dissolved in the cell is consumed by the methyl viologen in the biocathode, so the system does function to produce ammonia directly from air,[22] as shown in Figure 5. The ammonia production was verified by fluorescent assay and NMR studies of bulk electrolysis with nitrogen-15 labeled nitrogen gas.[22] However, it is important to note that there are issues with both of these assays. The fluorescence assay shows positive response for other amines, so proper controls are always needed. The nitrogen-15 labeled nitrogen gas is produced from nitrogen-15 labeled ammonia, so there is always a small contamination; therefore, well-designed experimental protocols are also very important in this assay as well. This Shethna system addressed the issue of oxygen tolerance, but not the other issues with the nitrogen bioelectrocatalytic systems (i.e., ATP regeneration, potential losses due to mediators, etc.). It is also important to note that because the methyl viologen is consuming oxygen, then this strategy would dramatically decrease the Faradaic efficiency of the biocathode.

In order to eliminate the need for the Fe protein, direct electron-transfer systems are needed. Recently, it was reported that MoFe nitrogenase can be immobilized in a pyrene-modified linear poly(ethylenimine) (LPEI) hydrogel on a carbon electrode. This immobilization strategy showed bioelectrocatalysis for proton, nitrite, and nitrogen without the need for the Fe protein or ATP,[23] as shown in Figure 6. This solves the problem of immobilization, and this system is ATP-independent; more importantly, it allows for bioelectrocatalytic response at the potential of the electron relay in the protein. Although bioelectrocatalysis is usually established by cyclic voltammetric experiments in the presence and absence of substrate, square wave voltammetry is useful for determining cofactor potentials. Square wave voltammetry results of MoFe nitrogenase in the pyrene-modified LPEI show distinct redox peaks for both the P cluster and the FeMo-cofactor. This electrochemistry shows that both redox species can communicate with the electrode and allows for mechanistic studies of which mode of electron transfer occurs with each substrate of interest (proton reduction, nitrogen reduction, etc.). In the future, this direct bioelectrocatalysis system will be beneficial for studying kinetic isotope effects, inhibition, and the rates of internal electron transfer between the P cluster and the FeMo-cofactor. It will also help to better understand the role of ATP and the Fe protein in the catalytic process.

Although MoFe nitrogenase is the most studied nitrogenase, there are a wealth of other nitrogenases. Recently, the bioelectrochemistry of FeFe nitrogenase was explored for carbon dioxide reduction to formate and methane.[17] Also, the bioelectrochemistry of VFe nitrogenase was studied with cobaltocene mediators. VFe nitrogenase is an interesting nitrogenase, because it can catalyze carbon dioxide reduction to build carbon−carbon bonds. Although VFe nitrogenase can readily perform proton reduction like MoFe nitrogenase, this paper showed that it can also produce ethylene and propene from carbon dioxide, as shown in Figure 7.[24] This shows the utility of the nitrogenase active sites for (bio)electrocatalysis in

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742

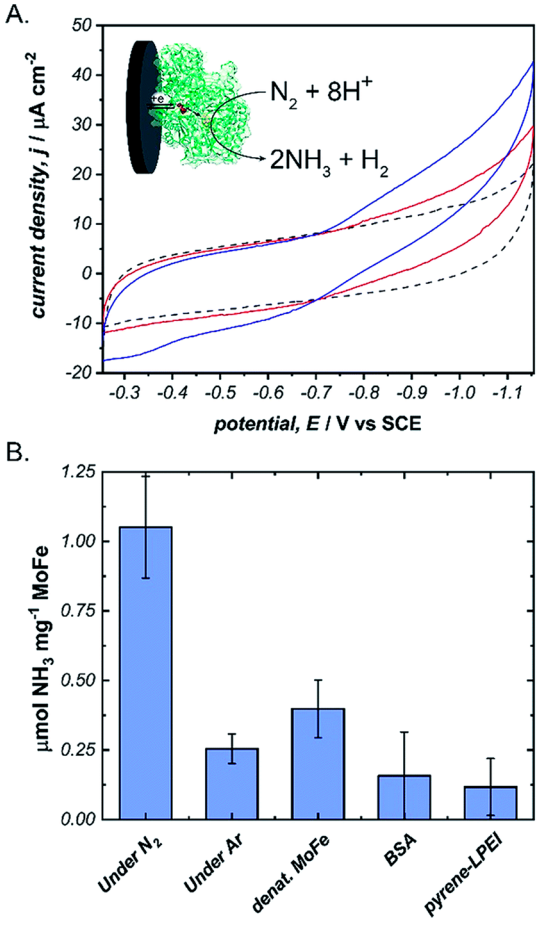


Figure 6. (A) Cyclic voltammograms of pyrene-modified LPEI films containing MoFe protein and 5 mg mL^{-1} MWCNT−COOH under Ar (black dashed trace) and after 5 min (red solid trace) or 10 min (blue solid trace) of bubbling of ultrahigh purity N_2. (B) Production of NH_3 using N_2 as the only substrate after constant potential bulk electrolysis of pyrene-LPEI/MoFe films for 8 h. Reproduced with permission from ref 23. Copyright 2018 Royal Society of Chemistry.

This MoFe nitrogenase direct electrochemistry shows that both redox species (FeMo-cofactor and the P cluster) can communicate with the electrode and allows for mechanistic studies of which mode of electron transfer occurs with each substrate of interest (proton reduction, nitrogen reduction, etc.).

general, whether hydrogen production, ammonia production, formate production, or hydrocarbon production. To date, all of these studies have been mediated electron transfer, but direct bioelectrocatalysis systems for FeFe nitrogenase and VFe nitrogenase will also be interesting for mechanistic studies of these enzyme systems as well.

Summary and Future Outlook. Over the last several years, there has been great interest in nitrogenase bioelectrocatalysis for a variety of applications. These applications include

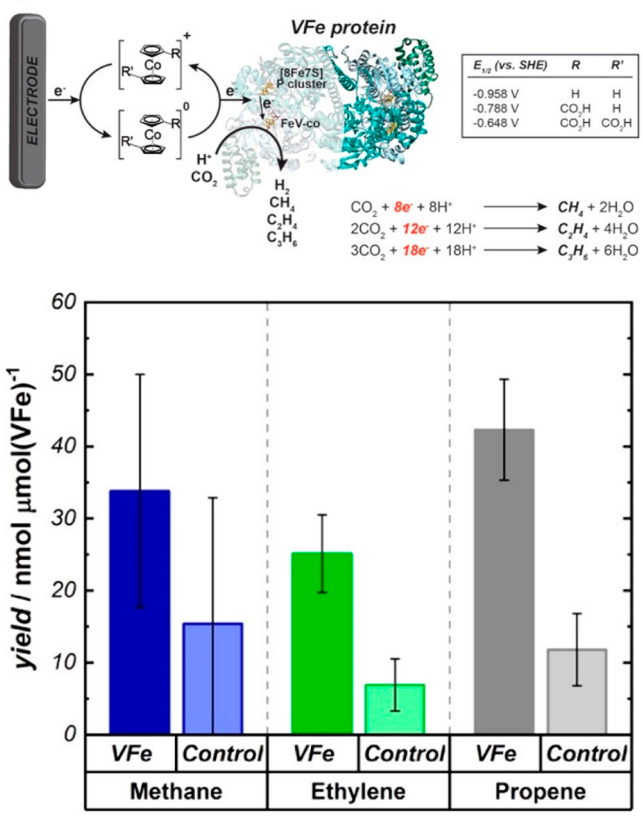


Figure 7. (Top) Bioelectrocatalytic turnover of VFe nitrogenase mediated by derivatives of cobaltocene/cobaltocenium. (Bottom) Product distribution of the bioelectrocatalytic carbon dioxide reduction reaction by VFe nitrogenase. Control experiments were performed with VFe that had been denatured by exposure to air for 30 min. Reproduced from ref 24. Copyright 2018 American Chemical Society.

utilizing electrochemistry to more directly understand enzyme mechanisms, because it allows for the ability to study the enzyme in the absence of other proteins (i.e., the Fe protein) and also with direct electron transfer without worrying about the effect of the redox mediator/dye on the chemistry and activity of the protein.[25] The information that will be learned from these fundamental studies will be of interest to the electrocatalysis community, who are using nitrogenase as their inspiration for molecular and materials-based electrocatalyst design. Other applications of nitrogenase bioelectrocatalysis include bioelectrosynthesis and biofuel cells. Bioelectrosynthesis is the use of nitrogenase in an electrolyzer for producing ammonia from nitrogen gas at atmospheric pressure and ambient temperature. The bioelectrosynthesis discussed above has high Faradaic efficiency but low stability (losing activity over the first few days of use). Recently, it has also been shown that nitrogenase bioelectrocatalysis can be used as the biocathode in a biofuel cell. Most biofuel cells utilize the oxygen reduction reaction catalyzed by a multicopper oxidase (i.e., laccase or bilirubin oxidase) as the biocathode and combine the biocathode with a glucose, glycerol, lactate, ethanol, hydrogen, or fructose bioanode.[26−28] This early work has showed that biocathodes can function with nitrogen to produce a value-added product (ammonia). This technology could also be applied to hybrid biosupercapacitors, which is a relatively new research area in biochemistry.

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742

Although the above applications are interesting and important to the energy community, there are many challenges that still need to be addressed, including increasing the current density of nitrogenase bioelectrodes and increasing the stability of nitrogenase bioelectrodes. In the world of bioelectrocatalysis, nanostructured materials have been designed and modified to improve current densities for a variety of enzyme electrocatalysis (i.e., laccase, fructose dehydrogenase, glucose oxidase, etc.),[29] but those materials engineering techniques have yet to be applied to nitrogenase. These include the use of gold nanoparticles, carbon nanotubes, graphene, and quantum dots. It is important to note that semiconductor quantum dots have been used for photocatalysis of nitrogen to ammonia with nitrogenase,[29] but not electrocatalysis. Although current density is technologically important, stability is probably the biggest issue with nitrogenase bioelectrodes today. Instability comes in several forms: temperature stability of the nitrogenase, oxygen stability of the nitrogenase, stability of the polymer/enzyme composite material, stability of the bioelectrodes to proteases, etc. This is a challenging issue for all bioelectrodes,[30] and there are several methods to address this issue.

There are many challenges that still need to be addressed in nitrogenase bioelectrocatalysis, including increasing the current density of nitrogenase bioelectrodes and increasing the stability of nitrogenase bioelectrodes.

One way to address stability issues is to focus on microbial bioelectrocatalysis versus enzymatic bioelectrocatalysis, because microbes grow, reproduce, and are continually producing new nitrogenase enzymes. There have been several systems utilizing algal and cyanobacteria bioelectrocatalysis for ammonia production recently. Leddy and Paschkewitz have electrochemically produced ammonia using a SA- mutant of *Anabaena variabilis* immobilized on a glassy carbon electrode with a hydrophobically modified Nafion film.[31,32] It has been shown that ferredoxin mediates nitrogenase bioelectrocatalysis in the cell when SA-1 mutant of *A. variabilis* is immobilized on indium tin oxide coated polyethylene,[33] so this would be an example of internally mediated bioelectrocatalysis, but the nitrogenase is being constantly reproduced by the cell and the cell is protecting the nitrogenase from oxygen through its natural mechanisms. Nocera and co-workers have also used nitrogenase with hydrogen-oxidizing bacteria *Zanthobacter autotrophicus* to produce ammonia from nitrogen, but here they are essentially producing hydrogen as the mediator for the bacteria.[34] These strategies address the stability issue, but because microorganism life must be sustained, they do require environmental control of temperature, pH, ionic strength, etc. They also typically result in lower volumetric catalytic activity because of the large volume of the microorganisms versus the individual proteins.

Another method to address stability issues is through materials strategies. For instance, hydrogenase is also a very oxygen-sensitive enzyme. Plumeré and co-workers designed a redox polymer system for immobilizing the enzyme that provides oxygen protection to the enzyme.[35] This system provides long-term oxygen tolerance of hydrogenase bioelectrodes, and similar strategies should work for nitrogenases. From a long-term stability perspective, many researchers have developed strategies for immobilizing and stabilizing proteins,[36] and many of these strategies could be applied to bioelectrodes. For instance, Kim and co-workers have designed immobilization strategies for protecting enzymes from proteases and providing temperature stability.[37−39] These techniques utilize cross-linking techniques, but there are also a wide variety of polymer-based stabilization techniques.[40−42] In the future, these materials strategies will need to be combined to improve the stability of nitrogenase bioelectrodes.

In conclusion, nitrogenase is an intriguing oxidoreductase enzyme that can be electrochemically evaluated through either mediated electron transfer or direct electron transfer. The results of electrochemical perturbation can be used to understand enzyme mechanism or for electrochemical energy applications (i.e., bioelectrosynthesis, biofuel cells, and biosupercapacitors), but there are still stability issues that need to be addressed for the use of nitrogenase in bioelectrochemical applications.

AUTHOR INFORMATION

Corresponding Author

*E-mail: minteer@chem.utah.edu.

ORCID

Shelley D. Minteer: 0000-0002-5788-2249

Notes

The authors declare no competing financial interest.

Biographies

Rong Cai is a Ph.D. student in the Department of Chemistry at the University of Utah under the supervision of Prof. Shelley Minteer. She received her M.S. degree from Chinese Academy of Science in 2015. She obtained her B.S. degree from Yunnan University, China in 2011.

Shelley Minteer received her Ph.D. in Chemistry at University of Iowa in 2000. She was a faculty member in the Department of Chemistry at Saint Louis University for 11 years before moving to University of Utah in 2011. She is a USTAR Professor of Chemistry and Materials Science & Engineering at the University of Utah. Her research interests are in the field of bioelectrocatalysis for energy, synthesis, and sensor applications. Her lab webpage can be found at https://chem.utah.edu/directory/minteer/index.php.

ACKNOWLEDGMENTS

The authors thank the Army Research Office MURI award (W911NF-14-1-0263).

REFERENCES

(1) Foster, S. L.; Bakovic, S. I. P.; Duda, R. D.; Maheshwari, S.; Milton, R. D.; Minteer, S. D.; Janik, M. J.; Renner, J. N.; Greenlee, L. F. Catalysts for nitrogen reduction to ammonia. *Nat. Catal.* **2018**, *1*, 490−500.

(2) Giddey, S.; Badwal, S. P. S.; Kulkarni, A. Review of electrochemical ammonia production technologies and materials. *Int. J. Hydrogen Energy* **2013**, *38*, 14576−14594.

(3) Kyriakou, V.; Garagounis, I.; Vasileiou, E.; Vourros, A.; Stoukides, M. Progress in the electrochemical synthesis of ammonia. *Catal. Today* **2017**, *286*, 2−13.

(4) Eady, R. R.; Leigh, G. J. Metals in the nitrogenases. *J. Chem. Soc., Dalton Trans.* **1994**, *19*, 2739−2747.

(5) Georgiadis, M.; Komiya, H.; Chakrabarti, P.; Woo, D.; Kornuc, J.; Rees, D. Crystallographic structure of the nitrogenase iron protein from Azotobacter vinelandii. *Science* **1992**, *257*, 1653−1659.

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742

(6) Kim, D. M.; Swartz, J. R. Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol. Bioeng.* **1999**, *66*, 180−188.
(7) Shih, Y.-S.; Whitesides, G. M. Large-scale ATP-requiring enzymic phosphorylation of creatine can be driven by enzymic ATP regeneration. *J. Org. Chem.* **1977**, *42*, 4165−4166.
(8) Andexer, J. N.; Richter, M. Emerging enzymes for ATP regeneration in biocatalytic processes. *ChemBioChem* **2015**, *16*, 380−386.
(9) Schultz, F. A.; Gheller, S. F.; Burgess, B. K.; Lough, S.; Newton, W. E. Electrochemical characterization of the iron-molybdenum cofactor from Azotobacter vinelandii nitrogenase. *J. Am. Chem. Soc.* **1985**, *107*, 5364−5368.
(10) Karyakin, A. A. Principles of direct (mediator free) bioelectrocatalysis. *Bioelectrochemistry* **2012**, *88*, 70−75.
(11) Ikeda, T.; Kano, K. Bioelectrocatalysis-based application of quinoproteins and quinoprotein-containing bacterial cells in biosensors and biofuel cells. *Biochim. Biophys. Acta, Proteins Proteomics* **2003**, *1647*, 121−126.
(12) Bartlett, P. N.; Al-Lolage, F. A. There is no evidence to support literature claims of direct electron transfer (DET) for native glucose oxidase (GOx) at carbon nanotubes or graphene. *J. Electroanal. Chem.* **2018**, *819*, 26−37.
(13) Milton, R. D.; Minteer, S. D. Direct enzymatic bioelectrocatalysis: differentiating between myth and reality. *J. R. Soc., Interface* **2017**, *14*, 20170253.
(14) Heller, A. Electrical wiring of redox enzymes. *Acc. Chem. Res.* **1990**, *23*, 128−134.
(15) Milton, R. D.; Abdellaoui, S.; Khadka, N.; Dean, D. R.; Leech, D.; Seefeldt, L. C.; Minteer, S. D. Nitrogenase bioelectrocatalysis: heterogeneous ammonia and hydrogen production by MoFe protein. *Energy Environ. Sci.* **2016**, *9*, 2550−2554.
(16) Paengnakorn, P.; Ash, P. A.; Shaw, S.; Danyal, K.; Chen, T.; Dean, D. R.; Seefeldt, L. C.; Vincent, K. A. Infrared spectroscopy of the nitrogenase MoFe protein under electrochemical control: potential-triggered CO binding. *Chem. Sci.* **2017**, *8*, 1500−1505.
(17) Hu, B.; Harris, D. F.; Dean, D. R.; Liu, T. L.; Yang, Z.-Y.; Seefeldt, L. C. Electrocatalytic CO_2 reduction catalyzed by nitrogenase MoFe and FeFe proteins. *Bioelectrochemistry* **2018**, *120*, 104−109.
(18) Khadka, N.; Milton, R. D.; Shaw, S.; Lukoyanov, D.; Dean, D. R.; Minteer, S. D.; Raugei, S.; Hoffman, B. M.; Seefeldt, L. C. Mechanism of nitrogenase H_2 formation by metal-hydride protonation probed by mediated electrocatalysis and H/D isotope effects. *J. Am. Chem. Soc.* **2017**, *139*, 13518−13524.
(19) Ruff, A. Redox polymers in bioelectrochemistry: Common playgrounds and novel concepts. *Curr. Opin. Electrochem.* **2017**, *5*, 66−73.
(20) Yuan, M.; Sahin, S.; Cai, R.; Abdellaoui, S.; Hickey, D. P.; Minteer, S. D.; Milton, R. D. Creating a low-potential redox polymer for efficient electroenzymatic CO_2 reduction. *Angew. Chem.* **2018**, *130*, 6692−6696.
(21) Milton, R. D.; Cai, R.; Abdellaoui, S.; Leech, D.; De Lacey, A. L.; Pita, M.; Minteer, S. D. Bioelectrochemical Haber−Bosch Process: An ammonia-producing H_2/N_2 fuel cell. *Angew. Chem., Int. Ed.* **2017**, *56*, 2680−2683.
(22) Milton, R. D.; Cai, R.; Sahin, S.; Abdellaoui, S.; Alkotaini, B.; Leech, D.; Minteer, S. D. The *in vivo* potential-regulated protective protein of nitrogenase in azotobacter vinelandii supports aerobic bioelectrochemical dinitrogen reduction *in vitro*. *J. Am. Chem. Soc.* **2017**, *139*, 9044−9052.
(23) Hickey, D. P.; Lim, K.; Cai, R.; Patterson, A. R.; Yuan, M.; Sahin, S.; Abdellaoui, S.; Minteer, S. D. Pyrene hydrogel for promoting direct bioelectrochemistry: ATP-independent electroenzymatic reduction of N_2. *Chem. Sci.* **2018**, *9*, 5172−5177.
(24) Cai, R.; Milton, R. D.; Abdellaoui, S.; Park, T.; Patel, J.; Alkotaini, B.; Minteer, S. D. Electroenzymatic C−C bond formation from CO_2. *J. Am. Chem. Soc.* **2018**, *140*, 5041−5044.
(25) Léger, C.; Elliott, S. J.; Hoke, K. R.; Jeuken, L. J.; Jones, A. K.; Armstrong, F. A. Enzyme electrokinetics: Using protein film voltammetry to investigate redox enzymes and their mechanisms. *Biochemistry* **2003**, *42*, 8653−8662.
(26) Calabrese Barton, S.; Gallaway, J.; Atanassov, P. Enzymatic biofuel cells for implantable and microscale devices. *Chem. Rev.* **2004**, *104*, 4867−4886.
(27) Zhou, M.; Wang, J. Biofuel cells for self-powered electrochemical biosensing and logic biosensing: A Review. *Electroanalysis* **2012**, *24*, 197−209.
(28) Davis, F.; Higson, S. P. Biofuel cells—recent advances and applications. *Biosens. Bioelectron.* **2007**, *22*, 1224−1235.
(29) Holzinger, M.; Le Goff, A.; Cosnier, S. Nanomaterials for biosensing applications: a review. *Front. Chem.* **2014**, *2*, 63.
(30) Kim, J.; Jia, H.; Wang, P. Challenges in biocatalysis for enzyme-based biofuel cells. *Biotechnol. Adv.* **2006**, *24*, 296−308.
(31) Leddy, J.; Paschkewitz, T. M. *Ammonia production using bioelectrocatalytical devices*. U.S. Patent US9506085B2, 2016.
(32) Paschkewitz, T. M. *Ammonia production at ambient temperature and pressure: An electrochemical and biological approach*. Ph.D. Thesis, University of Iowa, 2012.
(33) Knoche, K. L.; Aoyama, E.; Hasan, K.; Minteer, S. D. Role of nitrogenase and ferredoxin in the mechanism of bioelectrocatalytic nitrogen fixation by the Cyanobacteria anabaena variabilis SA-1 mutant immobilized on indium tin oxide (ITO) electrodes. *Electrochim. Acta* **2017**, *232*, 396−403.
(34) Liu, C.; Sakimoto, K. K.; Colón, B. C.; Silver, P. A.; Nocera, D. G. Ambient nitrogen reduction cycle using a hybrid inorganic−biological system. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 6450−6455.
(35) Plumeré, N.; Rüdiger, O.; Oughli, A. A.; Williams, R.; Vivekananthan, J.; Pöller, S.; Schuhmann, W.; Lubitz, W. A redox hydrogel protects hydrogenase from high-potential deactivation and oxygen damage. *Nat. Chem.* **2014**, *6*, 822−827.
(36) Balcão, V. M.; Vila, M. M. Structural and functional stabilization of protein entities: state-of-the-art. *Adv. Drug Delivery Rev.* **2015**, *93*, 25−41.
(37) Kim, M. I.; Kim, J.; Lee, J.; Jia, H.; Na, H. B.; Youn, J. K.; Kwak, J. H.; Dohnalkova, A.; Grate, J. W.; Wang, P.; Hyeon, T.; Park, H. G.; Chang, H. M. Crosslinked enzyme aggregates in hierarchically-ordered mesoporous silica: A simple and effective method for enzyme stabilization. *Biotechnol. Bioeng.* **2007**, *96*, 210−218.
(38) Kim, B. C.; Zhao, X.; Ahn, H.-K.; Kim, J. H.; Lee, H.-J.; Kim, K. W.; Nair, S.; Hsiao, E.; Jia, H.; Oh, M.-K.; et al. Highly stable enzyme precipitate coatings and their electrochemical applications. *Biosens. Bioelectron.* **2011**, *26*, 1980−1986.
(39) Kwon, K. Y.; Youn, J.; Kim, J. H.; Park, Y.; Jeon, C.; Kim, B. C.; Kwon, Y.; Zhao, X.; Wang, P.; Sang, B. I.; et al. Nanoscale enzyme reactors in mesoporous carbon for improved performance and lifetime of biosensors and biofuel cells. *Biosens. Bioelectron.* **2010**, *26*, 655−660.
(40) Chaniotakis, N. A. Review: Enzyme stabilization strategies based on electrolytes and polyelectrolytes for biosensor applications. *Anal. Bioanal. Chem.* **2004**, *378*, 89−95.
(41) O'Fagain, C. Enzyme stabilization-recent experimental progress. *Enzyme Microb. Technol.* **2003**, *33*, 137−149.
(42) Trubetskoy, V. S.; Burke, T. J. Engineered Polymeric Micelles: A novel tool for solubilization and stabilization of membrane protein targets for proteomics and drug discovery. *Am. Lab.* **2005**, *37*, 19−22.

DOI: 10.1021/acsenergylett.8b01637
ACS Energy Lett. 2018, 3, 2736−2742