Bioelectrocatalysis for Synthetic Applications: Utilities and Challenges

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Abstract

The pairing of enzymes with electrochemistry to accomplish bioelectrocatalysis enables the inherent advantages of each to be harnessed toward efficient, green synthesis of fuels, feedstocks, agrochemicals and pharmaceuticals. This article seeks to describe the current advantages of bioelectrocatalysis for synthesis applications and recent research to further expand the scope of bioelectrocatalysis. We focus on recent progress in bioelectrocatalytic cofactor regeneration, selectivity, inert bond activation under aqueous conditions and how all three can be applied in enzymatic fuel cells. We also cover the current strategies to overcome the most prominent challenges in the field, with an emphasis on enzyme stability, cascades for increasing product complexity and directed evolution.

1. Introduction

The primary challenge in organic synthesis has always been selectivity, either stereo-, regio- or chemo-selectivity. Significant advancements in asymmetric synthesis have garnered Nobel Prizes[1], yet many important asymmetric transformations still necessitate toxic reagents, expensive transition metals and organic solvents under harsh conditions.[2] Electrochemical synthetic methods are an attractive green alternative, as they can utilize electricity directly from renewable sources. In addition, the tunability of the electrode potential allows for the targeting of one specific redox process and removes the need for stoichiometric chemical oxidants or reductants. While electrochemistry can have high chemoselectivity, it offers no intrinsic stereoselectivity.[3] In contrast, enzymes have evolved to be highly selective and function under mild, physiological conditions which makes them ideal catalysts to address many of these issues. On the other hand, biocatalysis often suffers from requiring expensive cofactors, but electrochemical based cofactor regeneration has proven to be quite effective.[4] The pairing of electrochemistry with biocatalysis mitigates many of the drawbacks for these orthogonal strategies.[5] Here, we demonstrate the advantages of bioelectrocatalysis for synthesis and highlight areas which need continued focus to advance its synthetic utility. Readers interested in an overview of the fundamental principles of bioelectrocatalysis are directed elsewhere.[6, 7]

2. Utility of Bioelectrocatalysis for Synthesis

Bioelectrocatalysis has been gaining more attention over recent years and has now advanced enough for the synthetic utility to have been demonstrated. The demonstrations most relevant to synthesis are in the areas of cofactor recycling, selectivity and activation of inert bonds under mild, typically aqueous, conditions. Enzymatic fuel cells further show how synthesis can be done in a more sustainable way and frequently incorporate the other advantages.

2.1 Cofactor Recycling

A growing number of synthetic routes in the pharmaceutical and agrochemical industries utilize enzymatic biocatalysis.[8] However, most enzymes require expensive, stoichiometric cofactors such as NADH, NADPH, FAD and ATP which can prevent economic viability.[9] Chemical and biological methods for recycling cofactors often introduces unwanted side products or lower enzyme activity.[4, 6] Bioelectrochemical regeneration can be accomplished for NAD+/NADH via the enzyme Diaphorase (DI) which can be mediated electrochemically without requiring additional substrates (generic example displayed in Figure 2.1).[6] Yuan et al.[10] improved this technique by immobilizing diaphorase within a cobaltocene poly(allylamine) redox polymer with pendant cobaltocene moieties capable of mediating electron transfer. This method had a high faradaic efficiency between 78%-99% with yields above 97% and circumvented common redox mediators which suffer from stability issues. The utility of this setup was demonstrated

by coupling it with an NADH dependent alcohol dehydrogenase. Similarly, the Armstrong group[11] reported a method for the regeneration of NADPH from $NADP^+$ via an indium tin oxide (ITO) electrode modified with ferredoxin-$NADP^+$ reductase (FNR). Electrophoretic deposition of ITO generated pores that enabled higher cofactor concentrations and led to improved catalytic activity when paired with a dehydrogenase. They separately coupled the regeneration scheme with a reductive aminase, imine reductase and malic enzyme. ATP is ubiquitous in vivo and often required for enzymatic synthesis. Sun *et al*[12]. used an FAD dependent acetate kinase to convert ADP to ATP in situ with a faradaic efficiency of 74% and 87% conversion. The required FAD was itself regenerated utilizing pyruvate oxidase (PO) and the substrates pyruvate and phosphate. Electrons from PO are shuttled to the anode using a ferrocenemethanol mediator. The method could also be applied to GTP recycling. While significant advances have been made to bioelectrocatytic cofactor regeneration, additional research is necessary.

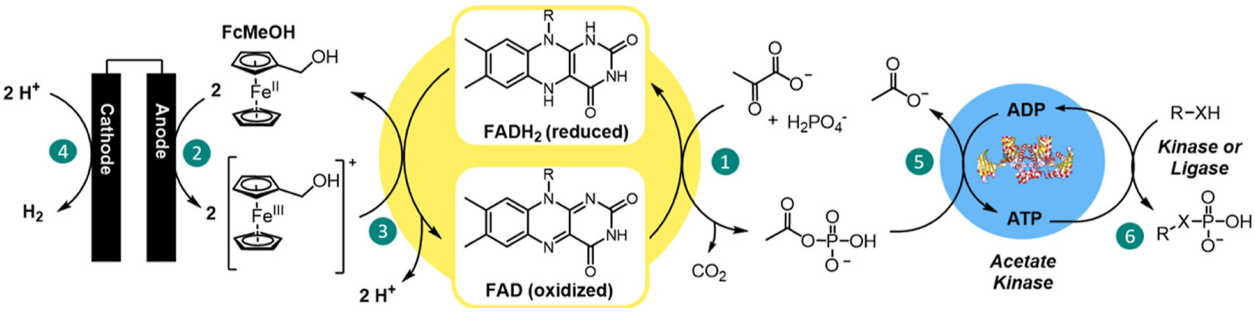

Figure 1. Bioelectrocatalytic ATP regeneration scheme. Reprinted with permission.[12]

2.2 Activation of inert bonds

Molecular nitrogen and carbon dioxide, while abundantly available, are the terminal oxidative products of many chemical processes and as such are thermodynamically inert. Bioelectrosynthetic techniques offer a way to incorporate these abundant but inert feedstocks into value-added products via inert bond activation (Figure 2.2). For example, ammonia and the other nitrogen containing compounds produced from the nitrogen reduction reaction (NRR) are critically important for agriculture and pharmaceuticals.[13] Nitrogenase enzymes are unique for their ability to activate molecular nitrogen for reduction under mild conditions.[14] The Minteer group[15] harnessed Nitrogenase in an enzymatic cascade to synthesize chiral amines. In this cascade, nitrogenase reduced molecular nitrogen into ammonia which enables pyruvate to be converted into alanine by an NADH dependent L-alanine dehydrogenase. ω-transaminase performs an enantioselective transamination between alanine and a ketone to form a chiral amine and regenerate pyruvate. Methyl viologen mediates electron transfer between the nitrogenase and diaphorase used to regenerate NADH. The Plumeré group[16], devised a bioelectrocatalytic carboxylation of crotonyl-CoA. Crotonyl-CoA carboxylase/reductase (Ccr) and ferredoxin $NADP^+$ reductase (FNR) were immobilized on a redox active hydrogel and then drop-casted onto a glassy carbon electrode. Ccr activates carbon dioxide for reduction and catalyzes the carboxylation of crotonyl-CoA. Ccr requires NADP and this was the first example of a redox active hydrogel mediating electron transport to facilitate regeneration of reduced cofactor NADPH. [16]Research into activating carbon dioxide toward the synthesis of fine chemicals has also been an active research area.[17, 18]

2.3 Selectivity

Asymmetric synthesis is one of the most important challenges in organic synthesis. Stereoisomers of pharmaceuticals often exhibit different biological activity. L-methamphetamine is a common nasal decongestant found in over the counter medications while the D-isomer is a highly addictive, illegal stimulant.[19] For this reason stereoselective reactions are always of interest and many biological catalysts are necessarily highly stereoselective. The carboxylation of crotonyl-CoA outlined in the previous section is also an example of this.[16] The carboxylation was both stereoselective, regioselective and resulted in one of the most complex products from fixing CO_2.

In a full biocatalytic route to produce the drug candidate islatravir, the first step involves the selective oxidation of a terminal alcohol to an aldehyde with the enzyme galactose oxidase(GOase).[20] The prochiral 2-ethynylglycerol is oxidized in a desymmetrization reaction to produce (*R*)-2-ethynylglyceraldehyde. Zhang, et al.[20] developed a bioelectrocatalytic approach in which GOase is activated through mediated electron transfer with the

anode (Figure 2.3). This allowed for direct turnover of the galactose oxidase without the need for adding expensive horseradish peroxidase. Other alcohol substrates could be selectively oxidized to the aldehyde. In another example, Guan and coworkers recently paired a direct electrochemical oxidation with a lipase-catalyzed stereoselective cross-coupling catalyzed of 2-substituted indoles and ketones. Numerous substrates showed a high degree of stereoselectivity while chemical oxidants drastically lowered the overall yield and selectivity.[20] The bioelectrocatalytic oxidation not only set a stereocenter, but selectively stops at the aldehyde. Traditional synthetic methods for partial oxidation often require expensive iodine complexes or toxic metal catalysts which leads industry to avoid them.[21]

2.4 Enzymatic Fuel Cells

Enzymatic fuel cells utilize bioelectrocatalysis to oxidize green fuels like hydrogen, ethanol and glucose. The electrons generated can be utilized to drive enzymatic electrosynthesis of value added products.[6] Research into electrochemical reductions often incorporate an EFC to highlight the ease with which bioelectrocatalysis slots into the green energy revolution and these examples often demonstrate all of the advantages discussed in the proceeding sections (displayed in figure 2.4) The Zhu lab[22] developed an EFC to cleanly drive the synthesis of the biologically relevant, L-DOPA. It relied on glucose oxidation and an electrochemical NAD^+ regeneration scheme in the anodic chamber to supply the driving force that enabled Tyrosinase to reduce L-tyrosine to L-DOPA. The setup had a high coulombic efficiency of 90% and was comparable to synthesis powered by a potentiostat. Additional examples of EFC's have been published recently, including the synthesis of chiral amines discussed previously.[15, 23]

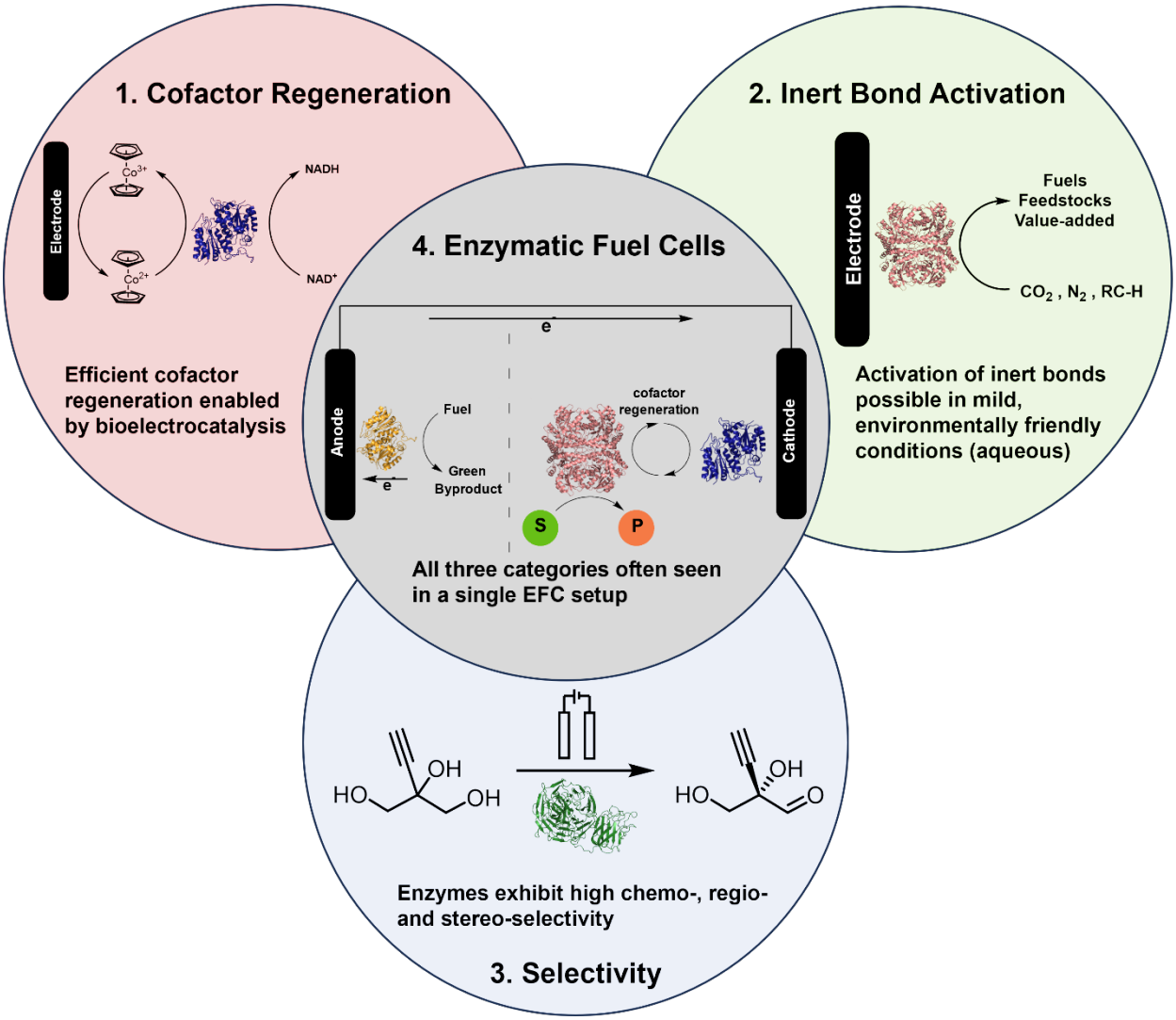

Figure 2. Summary of bioelectrocatalysis for synthesis. (1) adapted from another source.[10] (2) Transformations involving molecular nitrogen, carbon dioxide, C-H activation have been published.[16, 17, 23, 24] (3) Adapted from another source.[20] (4) EFC's which incorporate two or more of the above advantages are common.[15, 22, 23]

3. Challenges in Bioelectrosynthesis

As the use of enzymatic catalysts in organic electrosynthesis continues to expand, so must the ability to scale up bioelectrosynthetic reactions. When working toward a large-scale bioelectrosynthetic system, one must consider the stability of the enzyme(s), the efficiency of the reaction, as well as possible interactions with other compounds in the system. These are all areas in which bioelectrocatalytic systems still need to be improved. Enzymes only function under a narrow set of conditions without denaturing and, even when new systems or transformations are demonstrated, the stability over time can still be an issue. The size of enzymes can make efficient transport of electrons from the electrode surface difficult. While the inherent selectivity of biocatalysts can be highly beneficial, it can also be a downside by limiting substrate scope or the types of transformations that are possible. Less generalizability necessitates more system design is necessary meaning added time and costsThe remaining sections will discuss three areas of active research which hold the most promise for mitigating these challenges (displayed in Figure 4).

3.1 Enzyme Immobilization

The stability of enzymes in bioelectrosynthesis can be a significant challenge, especially when considering scalability of reactions. Immobilizing an enzyme on an electrode can lead to improved stability and catalytic activity, while also allowing them to be more readily incorporated into flow systems. Determining the most effective immobilization technique for a given enzyme requires substantial knowledge of the enzyme's structure and characteristics. Many types of materials have been used to immobilize enzymes, including polymers and metal organic frameworks.

A major challenge for immobilizing redox active enzymes is ensuring compatibility of the immobilizing matrix with the enzyme. To this end, Hickey *et al*.[25] used a novel pyrene-modified linear poly(ethylenimine) (LPEI) hydrogel to immobilize a variety of oxidoreductases on a carbon electrode. This method traps an enzyme in the hydrogel, allowing the active site of certain enzymes to get close enough to the electrode for electron transfer to occur without the use of a mediator. This method is not compatible with all enzymes, as some denature in the hydrogel and others see a significant decrease in enzyme activity.

In another example of a hydrogel successfully immobilizing an enzyme for electron transfer, Radomski *et al.*[26] used a ferrocene based redox hydrogel to immobilize glucose oxidase for the synthesis of gluconate from glucose (Figure 4b). This hydrogel traps the enzyme near the electrode surface, but the ferrocene moieties act as mediators for electron transfer to the active site. Their bioanodes displayed improved stability for glucose oxidase, lasting longer than 24 hours at a constant potential of 0.7 V vs. Ag/AgCl. Continued improvement in hydrogel stability will lead to possible industrial applications in the future.

Metal organic frameworks (MOFs) are becoming more popular as immobilization materials because they can be specifically designed and modified for a certain enzyme. For example, Yang *et al.*[27] immobilized pepsin on zeolitic imidazolate framework-8 (ZIF-8) using Ni^{2+} ions to anchor the enzyme on the material. Pepsin catalyzed the oxidation evolution reaction, requiring only a 127 mV overpotential at a current density of 10 mA cm^{-2} as compared to 690 mV without the Ni^{2+}. This method could be used to immobilize a variety of enzymes to achieve greater electrocatalytic efficiency.

To use enzymes industrially, continual advancement in enzyme immobilization techniques will be vital. Immobilization shows significant potential for improving bioelectrocatalytic systems. An important future direction will be utilizing multiple immobilized enzymes spatially separated on the same electrode or a "one-pot" multi-step synthesis on a single surface. Another promising avenue is tandem utilization of small molecule catalysts *and* immobilized enzymes operating without destabilizing each other.

3.2 Bioelectrochemical Cascades

Bioelectrosynthesis typically converts one substrate to one product, so several enzymes are often required for complex reactions. Bioelectrochemical cascades, in which a series of enzymes perform sequential reactions, have been found to selectively form complex products in "one-pot" experimental setups as well as in flow reactors (figure 4c). The Minteer Group[24] utilized three enzymes in a single pot to convert chemically inert hydrocarbons into imines.

The first step in the cascade used an alkane hydroxylase (alkB) to convert a hydrocarbon to a primary alcohol. An engineered choline oxidase oxidized the alcohol to an aldehyde, where a reductive aminase produced the imine. This selective pathway is not possible through standard organic synthesis. Taking this a step further, the Minteer Group[28] was able to utilize a modified version of the first two steps of their cascade, extracting the aldehyde into an organic solvent containing L-proline and diethyl azodicarboxylate, which catalyzed the production of an α-hydrazino aldehyde. This "three-stage" system combines bioelectrocatalysis, biocatalysis, and organocatalysis, offering new opportunities for C-H activation.

The reduction of CO_2 into value added products is one of the most important challenges facing the scientific community today. Jack *et al.*[29] designed a three chamber electrolyzer for the reduction of CO_2 into C6 pharmaceutical precursors, which can be used to synthesize statins. Their system used a copper-based gas diffusion electrode to convert CO_2 into ethanol, which was then sent to the enzymatic cascade. An alcohol dehydrogenase oxidized ethanol into acetaldehyde, which was converted to a 2,4-dideoxyhexose derivative, C6 lactol, using a 2-deoxyribose-5-phosphate aldolase. Converting CO_2 into pharmaceutical precursors is a unique route that can be achieved using enzymatic cascades, but efficiency continues to be a hindrance.

Cascade reactions often require non-enzymatic cofactor regeneration. To achieve this, El Housseini *et al.* [30] immobilized a rhodium catalyst for NADH regeneration along with L-lactate dehydrogenase (LDH) in a flow reactor to convert pyruvate to lactate. This method offers a way to use a small molecular catalyst and an enzyme in the same system. Their bioreactor displayed 79% faradaic efficiency along with a total turnover number for LDH of 180000.

The design of these cascade reactions is limited by enzyme compatibility and could be improved by the inclusion of organic or inorganic catalysts. However, enzymes and synthetic catalysts are rarely functional in the same conditions. More research is needed to improve the compatibility of enzymes with synthetic catalysts. For example, materials for immobilization, such as MOFs, could be used to improve the stability of an enzyme and a molecular catalyst in the same system. Other goals, such as newly designed flow and batch bioreactors, should continue to be worked toward to allow for more efficient cascades.

3.3 Directed Evolution for Improved Enzyme Stability

Directed evolution is an effective method of introducing desired characteristics to an enzyme by mimicking the process of Darwinian evolution.[31-33] This process, pioneered by Frances Arnold, is commonly used in biocatalytic systems to introduce new reactivity, widen an enzyme's substrate scope, improve the solvent tolerance and stability over the course of a reaction, and much more (figure 4a). To synthesize pharmaceuticals, specific functional groups are required in exact locations. This can be challenging with standard synthetic techniques, but enzymes offer unique reactivity and selectivity that can be improved upon with directed evolution. Rui *et al.*[34] engineered a (4-hydroxyphenyl)pyruvate dioxygenase via directed evolution to catalyze an abiological C(sp3)-H azidation reaction. The use of directed evolution improved the stereo-selectivity, displayed in Figure 3, as well as the chemo-selectivity and total turnover number of their system.

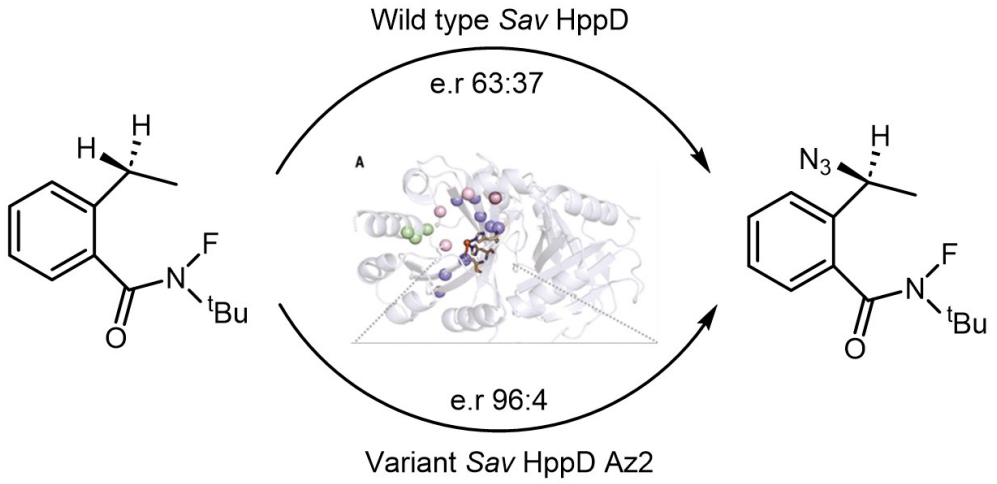

Figure 3. Directed evolution used to improve the enantiomeric ratio of azidation product. Adapted and reprinted with permission from Rui *et al.*[33]

In another example of the power of directed evolution, Athavale *et al.*[35] engineered heme-containing nitrene transferases to biocatalytically insert nitrogen into unactivated C(sp^3)-H bonds. These enzymes exhibited new reactivity with broad substrate scope for both amination and amidation. The scope of biocatalysis has grown significantly due to Arnold's work in directed evolution. This tool can be used in new ways to improve bioelectrocatalytic systems and introduce reactivity that organic electrochemistry cannot achieve. Directed evolution could be used to improve an enzyme's stability in a material used for immobilization, improve stability in the presence of commonly used co-solvents, or even improve the efficiency of electron transfer. However, directed evolution has rarely been applied with bioelectrosynthetic methods. Pairing directed evolution with electrosynthesis is an appealing path forward to increase the scope of bioelectrosynthesis and improve the scalability of bioelectrosynthetic reactions.

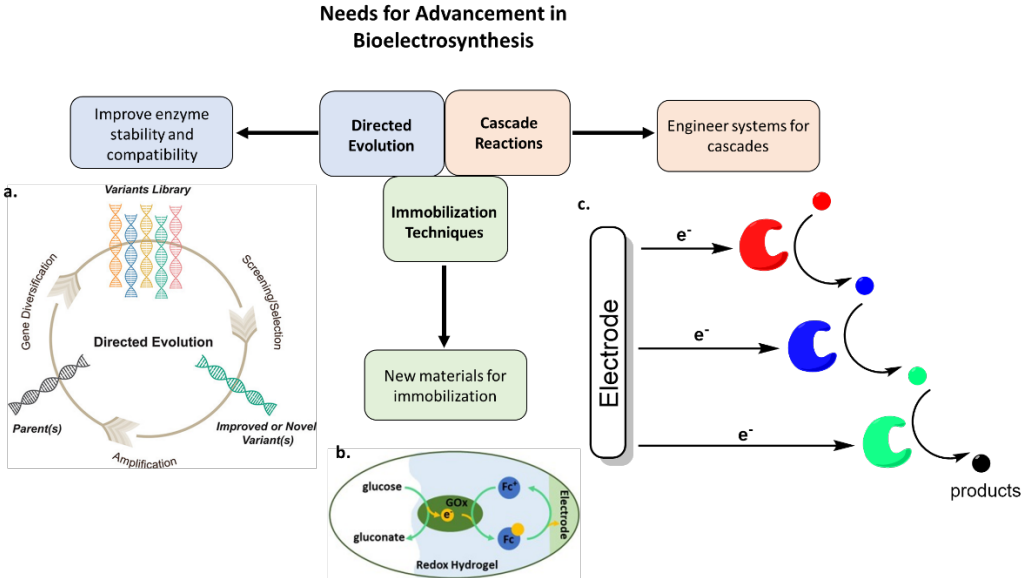

Figure 4. Future work in bioelectrocatalysis. **a.** Process of Directed Evolution. Reprinted with permission[32] **b.** Ferrocene redox hydrogel for gluconate synthesis. Reprinted with permission[25] **c.** Bioelectrochemical cascade for C-H functionalization. Reprinted with permission[27]

4. Conclusion

An ideal synthetic methodology for the 21st century exhibits high selectivity, wide substrate scope with great functional group tolerance, and is sustainable and environmentally friendly. Those attributes are some of the defining characteristics of bioelectrocatalysis. However, the high selectivity of enzymes often leads to a high substrate selectivity, inhibiting broader applicability. Directed evolution offers the ability to increase the substrate selectivity of a given enzyme, while also showing promise for another significant challenge in the field, enzyme stability. More focus in this area and improving enzyme immobilization techniques are needed. Research has begun in developing enzymatic cascades to address limitations in the complexity of products that can be synthesized. The inherent advantages of bioelectrocatalysis show enormous promise, but it is still a relatively small subfield that requires more attention.

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