Fundamentals, Applications, and Future Directions of Bioelectrocatalysis

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ABSTRACT

Bioelectrocatalysis is an interdisciplinary research field combining biocatalysis and electrocatalysis via the utilization of materials derived from biological systems as catalysts to catalyze the redox reactions occurring at an electrode. Bioelectrocatalysis synergistically couples the merits of both biocatalysis and electrocatalysis. The advantages of biocatalysis include high activity, high selectivity, wide substrate scope, and mild reaction conditions. The advantages of electrocatalysis include the possible utilization of renewable electricity as an electron source and high energy conversion efficiency. These properties are integrated to achieve selective biosensing, efficient energy conversion, and the production of diverse products. This review seeks to systematically and comprehensively detail the fundamentals, analyze the existing problems, summarize the development status and applications, and look toward to the future development directions of bioelectrocatalysis. First, the structure, function, and modification of bioelectrocatalysts are discussed. Second, the essentials of bioelectrocatalytic systems, including electron transfer mechanisms, electrode materials, and reaction medium, are described. Third, the application of bioelectrocatalysis in the fields of biosensors, fuel cells, solar cells, catalytic mechanism studies, and bioelectrosyntheses of high-value chemicals are systematically summarized. Finally, future developments and a perspective on bioelectrocatalysis are suggested.

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1. INTRODUCTION

1.1. Bioelectrocatalysis and Bioelectrocatalysts

Bioelectrocatalysis is the utilization of materials derived from biological systems as catalysts to catalyze the redox reactions occurring at the electrode.[1] Bioelectrocatalysis is an interdisciplinary research field of biocatalysis and electrocatalysis. The traditional biocatalysis has the advantages of high activity, high selectivity, mild reaction conditions, and diverse catalytic functions.[2, 3] Electrocatalysis achieves the flexible conversion between chemical energy and electrical energy. Bioelectrocatalysis synergistically combines the advantages of biocatalysis and electrocatalysis, allowing it to provide an excellent opportunity for sustainable green chemistry.[4] As the executor of the catalytic functions, the bioelectrocatalyst serves as the fundamental working component of the bioelectrocatalytic system. Oxidoreductases represent a large class of enzymes that account for nearly 25% of all known proteins. They can catalyze reduction-oxidation reactions and transport an electron(s) between the two substrates with an enzyme cofactor.[5, 6] In recent decades, an increasing number of studies indicate that the oxidoreductase-catalyzed reactions can be coupled with the electrode. The electrode can substitute one of the enzyme substrates and act as either an electron donor or an electron acceptor to support the oxidation or reduction of the second substrate.[4, 5] Accordingly, the isolated oxidoreductases are the most basic and commonly used bioelectrocatalyst. In addition to isolated oxidoreductases, organelles (especially mitochondria and chloroplasts), the subcellular microcompartmentalization structures, can also be employed as bioelectrocatalysts to catalyze electrochemical reactions. These organelles contain a series of oxidoreductases to form electron transfer chains, which have electrochemically active species, such as

ubiquinone or cytochrome *c*, that can communicate with the electrode.[7-11] Some living microbial cells, which are called electroactive microbial cells, are another type of bioelectrocatalyst. These electroactive microbes have evolved unique functional structures, including electrically conductive pili (e-pili) and conductive membrane structures, that mediate the electrical communication with an electrode and finally accomplish long-distance electron transfer mechanisms between electrodes and intracellular oxidoreductases.[12-16]

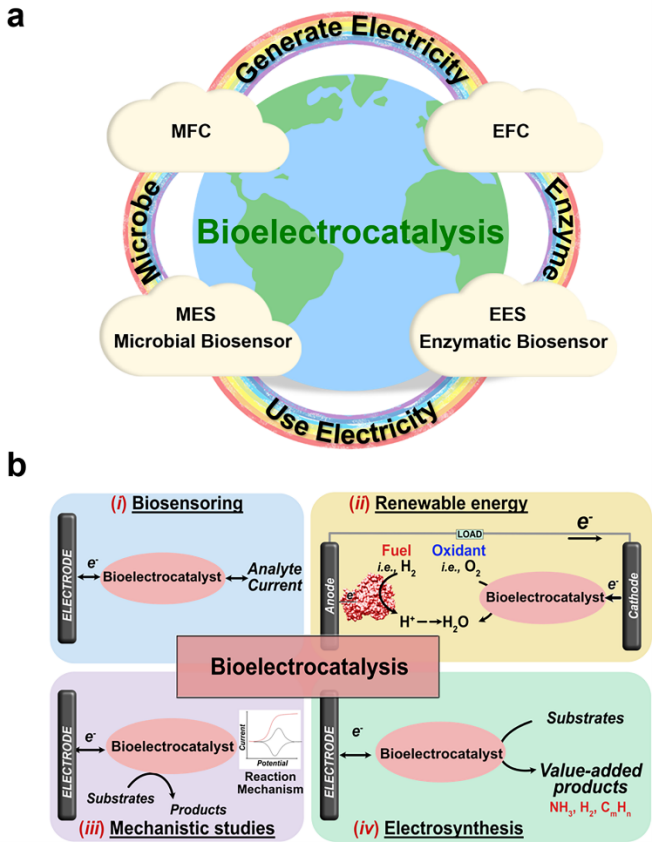

Figure 1. (a) Schematic representation of the types of bioelectrocatalytic systems. MFC, microbial fuel cell; EFC, enzymatic fuel cell; MES, microbial electrosynthesis; EES, enzymatic electrosynthesis. Reprinted with permission from ref. 17 with modification. Copyright 2020 Elsevier. (b) Schematic overview of different applications of bioelectrocatalytic systems. Reprinted with permission from ref. 5 with modification. Copyright 2020 Wiley.

1.2. The Types and Applications of Bioelectrocatalytic Systems

Figure 1a shows that the energy transition of bioelectrocatalytic systems can be (1) the conversion of chemical energy into electrical energy to generate electricity, or (2) the utilization of electrical energy to achieve a specific chemical reaction and achieve the conversion of electrical energy into chemical energy. This energy conversion can be catalyzed by either isolated oxidoreductases or electroactive microbial cells. The combination of different energy conversion processes with specific bioelectrocatalysts yields distinct types of bioelectrocatalytic systems. Specifically, the use of oxidoreductases or electroactive microbial cells to catalyze the conversion of chemical energy into electrical energy is the working principle of both enzymatic fuel cells and microbial fuel cells, respectively. Additionally, the utilization of electrical energy catalyzed by oxidoreductases and electroactive microbial cells is the foundational basis of electrochemical enzymatic biosensors, electrochemical microbial biosensors, enzymatic electrosyntheses, and microbial electrosyntheses.[17] Depending on the different types of bioelectrocatalysis systems mentioned above, the mutual transformation between chemical energy and electrical energy can be effectively realized and finally implemented for specific functions. To date, bioelectrocatalysis systems have gained significant attention in four major areas (**Figure 1b**): (1) biosensing, (2) renewable bioelectricity production, (3) functional mechanism studies, and (4) bioelectrosynthesis of valuable chemicals.[5, 18, 19]

Bioelectrocatalysis has been extensively applied in the design and development of bioelectrochemical sensing technologies. Electrochemical biosensors are characteristically defined as platforms where a transducer (electrode) contains a bioelectrocatalyst that acts as the biorecognition element.[20] The transducer is typically covered with a chemically

selective layer. In certain cases, biosensors can act as a kind of fuel cell with a target analyte as the fuel (self-powered biosensors). Through specific chemical interactions between the bioelectrocatalyst and the target analyte of interest, related information about the type and concentration of the target analyte can be obtained by transforming the response into an electrically detectable signal.[21] Both oxidoreductase and electroactive microbial cell biocatalysts can be used in the construction of biosensors for specific analyte detection and sensing applications in the fields of medical diagnostics and health monitoring,[22, 23] chemical testing,[24-26] environmental monitoring,[27, 28] as well as food and drink analyses.[29, 30] Future work in the development of biocatalyst-based biosensing systems needs to focus on the optimization of biosensor architectures, specifically in terms of stability, sensitivity, and reproducibility. Additionally, trends in designing miniature, paper-based, and wearable biosensing platforms are essential for low-cost mass production, improved analytical performance, and capabilities for multi-analyte detection. In the case of bioelectrocatalysis for renewable electricity production, the target fuels are oxidized at the anode, which results in the generation of an electrical current. Subsequently, the generated current is utilized to power an external load. Since biofuel cells are critical technologies for the production of clean and sustainable energy, significant research efforts have been devoted to design, develop, and enhance these bioelectrochemical platforms, namely with regard to fundamental knowledge of bioelectrocatalysis and electron transfer mechanisms, selection of electrode materials, and optimized system designs.[31] In studies concerned with the catalytic mechanisms of oxidoreductases and the electron transfer mechanism of electroactive microbial cells, initial research studies mainly focused on the use of amperometry, protein films, and cyclic voltammetry, to investigate the kinetics, inhibition,

thermodynamics, and transport parameters of electrochemically-active enzymes.[5, 32, 33] On a more in-depth, fundamental level, the bioelectrocatalytic measurements can be used to study the electron transfer mechanism occurring with each substrate of interest as well as the intramolecular electron transfer pathways of oxidoreductases via the electrochemical communications between oxidoreductases and electrodes.[34-37] The preparation of value-added chemicals, clean biofuels, and degradable materials is the promising application of bioelectrocatalysis. Currently, bioelectrocatalysis has gained interest in the synthesis of fine chemicals, desired biofuels, and materials,[38] especially, the production of redox co-factor-dependent CO_2 reduction,[39, 40] N_2 fixation,[35, 41, 42] as well as the biosynthesis of chiral products.[43-45] The electrochemical system could use cheap and clean electricity as the electron source to supply enough reducing equivalents to effectively support the preparation reaction catalyzed by different types of bioelectrocatalysts. Combined with the benefits of high activity and high selectivity of bioelectrocatalysts, the area of bioelectrocatalysis becomes an indispensable approach to modern biomanufacturing.[4, 46]

1.3. The Principal Issue of Bioelectrocatalysis

The four applications mentioned above can be further grouped into two categories: (1) analysis-oriented applications (biosensors and mechanism study systems) and (2) product-oriented applications (renewable bioelectricity systems and bioelectrosynthetic systems). The core issue of both analysis-oriented and product-oriented systems is the electron transfer efficiency, which plays a fundamental role in the performance of bioelectrocatalytic system. An efficient electron transfer process is favorable to reduce the detection limit and improve the sensitivity of biosensors. In the research of catalytic mechanisms, efficient electron transfer processes are helpful to sensitively capture the

current responses during catalysis, thereby more accurately exploring the catalytic mechanism. For the product-oriented applications, an efficient electron transfer process is necessary for the generation of high current and power density in bioelectricity systems and high space-time yield of bioelectrosynthetic systems. However, the active sites of most oxidoreductases are buried deep within the protein, which obstructs the electrochemical contact between the redox enzyme and the electrode surface.[47] For electroactive microbes, the extracellular electron transfer rates are typically slow as they are limited by the insulating characteristics of cell membrane layers.[48] Although specific bacteria have developed redox membrane proteins as functional motifs with electron transfer capabilities, these proteins are relatively embedded in the cell membrane.[49] Current research studies to improve electron transfer efficiency in bioelectrocatalytic systems adopt relative approaches, including (1) the modification of bioelectrocatalysts via either protein engineering of oxidoreductases or metabolic engineering of electroactive microbial cells, (2) the development of novel electrode materials and electrode modification methods, and (3) the design and application of new reaction media.

This review article starts by presenting the structural features of bioelectrocatalysts, namely, oxidoreductases and electroactive bacterial cells, that promote electron transfer and the bioelectrocatalyst modifications that further enhance the electron transfer. Next, we introduce a discussion on electron transfer mechanisms. We then provide a detailed overview of the technical points in the construction of bioelectrocatalytic systems from the view of the electrode and reaction medium. Finally, the applications of bioelectrocatalysis for biosensing purposes, renewable bioelectricity production, mechanistic studies, and bioelectrosynthesis of valuable chemicals are assessed. By summarizing the current

research progress herein, this review article projects an outlook of the development and future directions of bioelectrocatalysis based on the different application areas. We expect this review article to provide engaged readers with relatively general knowledge of bioelectrocatalysis and a useful reference for future research efforts.

2. THE TYPE AND MODIFICATION OF BIOELECTROCATALYSTS

The bioelectrocatalyst is the functional component in bioelectrocatalytic systems. The isolated oxidoreductases and electroactive microbial cells are the two most common and widely used types of bioelectrocatalysts. With the continuous progress of protein engineering, metabolic engineering, and synthetic biology, the catalytic properties of oxidoreductases and electroactive bacteria can be effectively regulated and enhanced to make them more adaptable for practical use and applications in bioelectrocatalysis systems.

2.1. Oxidoreductases

Oxidoreductases are biological redox proteins that catalyze electron transfer reactions by reduction or oxidation of substrates.[50] In contrast to the conventional redox molecular catalysts, oxidoreductases are large molecules composed of an insulating protein shell and small redox cofactor motifs. The structure of the protein shell serves as the biological recognition element for substrates, which endows the selectivity and specificity of the enzyme. The redox cofactor motifs are made of metal prosthetics, including heme centers (Fe), iron-sulfur clusters (Fe-S), copper centers (Cu), and molybdenum centers (Moco), as well as non-metal prosthetics, including FAD or FMN and pyrroloquinoline quinone (PQQ). These redox cofactor motifs are the functional core unit of oxidoreductases, which

have the capability of achieving electron transfer with electrode surfaces and often use electron mediators. Their delicate coordinate sphere is usually buried deep within the protein to exclude the outside solvent. After electron transfer, the redox equivalents can be immediately stored into these prosthetic groups.

2.1.1. Heme-containing Oxidoreductases. Heme is a molecule that contains the porphyrin complex of iron (II)-heme-or iron (III)-hemin as a prosthetic group. It is capable of forming several reduced and oxidized states. Over a wide potential range, the heme electrochemical properties (e.g., the formal potential ($E^{0'}$) for its redox conversion between Fe^{2+} and Fe^{3+}) can change depending on the protein environment; for example, the formal potential changes from –0.27 V *vs* SHE for horseradish peroxidase to 0.26 V *vs* SHE for cytochrome *c*.[51] These heme-containing enzymes have different functions. Namely, they (1) are capable of either reversibly combining oxygen for transport (hemoglobin) or storing it in a combined form (myoglobin),[52] (2) participate in electron transfer processes (cytochrome *b* and *c*),[53] (3) catalyze the reduction of oxygen to water (cytochrome *c* oxidase),[54] (4) oxidize different functional groups and realize the activation of C-H bond by molecular oxygen (monooxygenase P450),[55] and (5) catalyze the decomposition of peroxides (catalase and peroxidase).[56]

2.1.2. Iron-sulfur (Fe-S) Cluster- and Multi-metal Center-containing Oxidoreductases. Fe-S cluster-containing oxidoreductases are those in which iron atoms are bound with sulfur-containing ligands. The simplest chemical Fe-S clusters are the rhombic [2Fe-2S] and the cubane [4Fe-4S] types, which contain iron ($Fe^{2+/3+}$) and sulfide (S^{2-}). Fe-S clusters are normally bound with proteins via the coordination of iron ions by histidine or cysteine residues.[57, 58] The typical Fe-S cluster-containing proteins are

ferredoxin, hydrogenase, and nitrogenase. Ferredoxin is an electron shuttle that transports electrons between electron donor and electron acceptor proteins (e.g., putidaredoxin mediated electron transfer between P450cam and putidaredoxin reductase).[59] A Fe-S cluster is present in the active site of ferredoxin. The ferredoxin redox reaction is represented as $Fd(Fe^{3+}) + e^- \rightleftharpoons Fd(Fe^{2+})$.[60] Cyclic voltammetric current-potential responses of ferredoxin isolated from a variety of bacteria have been reported, with E^0 ranging from –0.15 to –0.7 V *vs* SCE, depending on the electrode material.[61, 62] In hydrogenase, the principal function of the Fe-S cluster is to secure the metabolic processes through hydrogen oxidation. Under certain conditions, hydrogenase is also capable of splitting water to produce hydrogen.[63, 64] In both NiFe and Fe-only hydrogenases, the active sites are deeply buried under the protein surface. Transport of H^+/H_2 to/from the active sites almost certainly takes place through specific channels in the protein matrix, and the sites are wired to the surface for electron exchange with their partner redox proteins by a conduit of Fe-S cluster.[65] Fe-only hydrogenases have one hydrogen-binding cluster and one or multiple [4Fe-4S] clusters per molecule, whereas NiFe hydrogenase usually have one Ni, one [3Fe-4S], and one or more [4Fe-4S] clusters per molecule.[66] Nitrogenase is an enzyme that is capable of reducing nitrogen to ammonia and is typically classified by the cofactor. The most studied nitrogenase is the MoFe nitrogenase, which contains an iron-molybdenum cofactor (FeMo-cofactor). In addition, there is also iron-vanadium cofactor (VFe) nitrogenases and iron-iron cofactor (FeFe) nitrogenases. The nitrogenase enzymes are comprised of two component metalloproteins, a catalytic component (MoFe, VFe, or FeFe protein) and an electron-transferring ATP-hydrolyzing iron-protein (Fe-protein).[35, 67] The nitrogenase Fe-protein has one [4Fe-4S] cluster bridged between the two subunits. The

[4Fe-4S] cluster can be stabilized in three core oxidation states, particularly 2+, 1+, and 0, and hence can act as a two-electron donor.[68] The $\alpha_2\beta_2$-tetrameric MoFe-protein contains two unique clusters per $\alpha\beta$-subunit pair: (1) the [8Fe-7S] P-cluster located at the $\alpha\beta$-subunit interface, and (2) the [Mo-7Fe-9S-X-homocitrate] FeMo-cofactor positioned within the α-subunit. Nitrogenase catalysis involves a chain of multifaceted formation and dissociation processes between the MoFe-protein and Fe-protein. In this process, electrons are sequentially transferred from the [4Fe-4S] cluster of the Fe protein, through the P-cluster, finally to the FeMo-cofactor of the MoFe protein, where the N_2 reaction and ammonia production eventually occurs.[67]

2.1.3. Copper-containing Oxidases. Copper is a critical cofactor that is involved in biological oxidation-reduction reactions and oxygen transport.[69] The essential role of copper-containing proteins is associated with the transfer of electrons and oxygen to catalyze oxidative reactions.[70] Based on their spectroscopic features, copper sites can be divided into three categories to reflect the electronic and geometric structure of the active site: type 1 (T1) or blue copper, type 2 (T2) or normal copper, and type 3 (T3) or coupled binuclear copper centers.[71] A prominent feature of copper proteins is that they function almost exclusively in the metabolism of O_2 or NO_x compounds. Also, copper proteins usually correlate with oxidizing organic/inorganic radicals.[72] The $E^{o'}$ of the Cu^{2+}/Cu^{+} redox couple can be modulated by ligand type and coordination geometry and by the extended amino acid environment compared to the $E^{o'}$ value of the Cu^{2+}/Cu^{+} redox couple in water (150 mV *vs* NHE).[73] Laccase is another typical copper-containing oxidase that catalyzes the oxidation of phenols and n-diphenylenediamines. The terminal acceptor of the electrons is oxygen. The redox potential of the multi-copper active site is intricately linked to the

protein substrate specificity and its ability to oxidize phenolic substrates, which is thermodynamically driven by the concomitant reduction of molecular oxygen.[74]

2.1.4. Flavoproteins. The flavin enzymes have a flavin cofactor (e.g., flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD)). They perform the role of electron carriers from the substrate to either other carriers or oxygen. For its molecular oxygen reactivity, a flavin cofactor is in its reduced form. This electron-rich reduced flavin can use molecular oxygen as an electron acceptor. Upon one-electron transfer from a reduced flavin to oxygen, a complex, consisting of a superoxide and the flavin radical, is formed.[75] Oxygen activation in these flavin oxidases typically involves the formation of a (transiently) stable flavin C_{4a}-oxygen adduct. Depending on the protonation state, this peroxy species reacts with nucleophilic or electrophilic substrates, thereby splitting the oxygen-oxygen bond.[76] Flavin oxidases catalyze several oxygenation reactions, including hydroxylation, epoxidations, Baeyer-Villiger oxidations, and sulfoxidation with high regio- and/or enantio-selectivity.[75]

2.1.5. PQQ-containing Enzymes. All PQQ-dependent oxidoreductases contain the bound cofactor PQQ along with or without heme moieties. The cofactor PQQ is coordinated with the apo-enzyme via Ca^{2+} ions, and electrons are transferred from the substrate via PQQ to the heme groups and finally to the electron acceptor.[77] Moreover, some of the PQQ-dependent enzymes can transfer electrons directly to solid surfaces[78] or conducting polymers.[79] There are two structural categories of PQQ-containing oxidoreductases. The first category, referred to as quinoproteins, contains only PQQ in the active site. This category includes glycerol, aldose, and glucose dehydrogenase.[80] The second category containing PQQ and one or more heme groups are known as

quino(hemo)proteins (e.g., fructose dehydrogenase and alcohol dehydrogenase).[81] In this case, electrons from substrates are transferred via PQQ to the heme group and then to the natural electron acceptor. PQQ is reduced by two electrons at a noticeably higher redox potential (+90 mV) in comparison with NAD^+ (–320 mV) or FAD (–45 mV).[77] A vital feature of this PQQ structure is the ortho quinone at the C4 and C5 position of the quinolone ring, which becomes reduced to the quinol during catalysis. The C5 carbonyl in the oxidized form is highly reactive towards nucleophiles, such as alcohols, ammonia, amines, cyanide, and amino acids. At pH 7, the midpoint redox potential of the isolated PQQ is approximately +90 mV, but this value likely changes with environmental conditions in the PQQ-dependent enzymes.[82]

2.2. Electroactive Microbial Cells

For the first time in 1911, Potter demonstrated that bacterial microorganisms have current producing capabilities, thus introducing the idea of using whole cells as bioelectrocatalysts.[83] Consequently, electroactive microbial cells, capable of donating or accepting electrons, have been employed as bioelectrocatalysts in different microbial bioelectrochemical systems,[19, 84-87] such as biosensors for analytical applications,[88, 89] microbial fuel cells (MFCs) for biomass conversion,[38, 90-94] harvesting electricity schemes,[95-97] platforms for remediation of pollutants,[98-100] as well as electrosynthesis for H_2 production,[101, 102] O_2, and CO_2 reduction.[103, 104] Due to various advantages, including good efficiency, high stability, and persistent growth, electroactive microbial cells have been applied as new-generation biological catalysts. In addition to the aforementioned oxidoreductases as enzymatic biocatalysts, electroactive microbial cells can act as alternative bioelectrocatalysts. In principle, bioelectrocatalytic systems utilizing

electroactive microbial cells can be considered a "bag of enzymes."[21, 105] In contrast to oxidoreductases, microorganisms can catalyze a broad range of reactions in which the electroactive microbes act as self-duplicating bioreactors of miniature sizes. Microbial biocatalysts contain complex metabolic networks; therefore, they catalyze reactions in a less specific manner relative to isolated oxidoreductases. The use of whole microbial cells as bioelectrocatalysts offers several benefits over enzyme-based biocatalytic systems. Namely, microbial cells do not require enzyme purification steps and provide enhanced stability for biocatalytic platforms. However, these microbial biocatalysts have limitations in comparison to oxidoreductases, including (1) requirement for a continual supply of nutrients and energy to support the living cells, (2) lack of specificity, and (3) slower rates of signal generation. While numerous microorganisms are known to be electrochemically active in nature, certain microbial cells have developed distinct electron transfer mechanisms to establish electrical communication with electrode surfaces. Herein, we present an overview of two well-studied electroactive microorganisms and their unique electron transfer mechanisms. Additionally, we provide a summary of other electrochemical microbes with putative conductive filaments, as well as electrode-microorganism interactions.

2.2.1. Two Representative Electroactive Microbial Cells *Geobacter sulfurreducens* and *Shewanella oneidensis*. The two most intensely studied model electroactive bacteria are Gram-negative mesophilic *Shewanella oneidensis* and *Geobacter sulfurreducens*. These exoelectrogens achieve direct electron transfer mechanisms through *c*–type cytochromes, which are located on the outer cell membranes.[106-110] This electron transfer occurs via direct physical contact where bacterial layers form on the electrode surface.

While this electron transfer type has low extracellular potential losses, its rates are limited due to the nanometer scale of the electron transfer range, and the limited number of microorganisms that make direct electrochemical contact with the electrode.[111] Additionally, *G. sulfurreducens* and *S. oneidensis* can enable long-distance extracellular electron transfers via conjugating *c*–type cytochromes into conductive nanowires and/or pili.[112-116] Several studies have proposed distinct mechanisms for these processes; however, some remain extremely controversial.[117-121]

In the case of metal-reducing strain *S. oneidensis* MR-1, analyses have identified a porin-cytochrome complex MtrCAB consisting of (1) periplasmic decaheme *c*–type cytochrome (MtrA), (2) an outer membrane ß-barrel porin (MtrB), and (3) an outer membrane decaheme *c*–type cytochrome (MtrC), as the major proteins in the respiratory metal reduction mechanisms. Located on the outer cell membrane, MtrC protein can donate electrons over a wide potential range. Electron transport from the periplasm to MtrC occurs via a transmembrane electron transfer module containing MtrA protein and the incorporated sheath MtrB protein (**Figure 2a**). Thus, these proteins form a complex to perform extracellular electron transfer to metal oxides. The protein arrangement in complex MtrCAB, which spans approximately 40 Å of the outer cell membrane, allows electron transfer from one side of the lipid bilayer to the other via a 20-hemes chain between the two cytochromes. While other proteins, including the membrane decaheme *c*–type cytochrome OmcA, are also hypothesized to be involved in the metal reduction process, their roles appear to be minor. A few mechanisms have been suggested to elucidate the extracellular electron transfer in *S. oneidensis*, including direct contact of microorganisms with metal oxides, use of electron shuttles (e.g., flavins, quinones), and

use of conductive nanowires. Indirect electron transfer mechanisms for *S. oneidensis* have been proposed as these bacteria species secrete small electroactive molecules, including flavins, acting as either (1) diffusing mediators between electrodes and cytochromes on the outer membrane or (2) bound co-factors for the cytochromes,[122-127] that mediate long-range extracellular electron transfer. Conductive pili-based nanowires have also been associated with facilitating electron transfer at a long distance in *S. oneidensis* strains.[92, 116, 128, 129] Yet, additional studies have shown that mutant *S. oneidensis* strains that lack these conductive pili are capable of reducing metals comparably to the wild-type strains.[122, 130, 131] A research study by Reguera and co-workers examining *S. oneidensis* pili concluded that the pili are non-conductive via conducting tip atomic force microscopy.[112, 132] However, a later study argued that *S. oneidensis* have conductive pili.[128] More recent studies have demonstrated that the supposedly conductive *S. oneidensis* pili were dried extensions of the outer cell membrane.[114] Therefore, electron transfer mechanisms in respiratory metal reduction by *S. oneidensis* appear to result mostly from direct contact between cytochromes on the outer cell membrane and the solid metal oxides.[133]

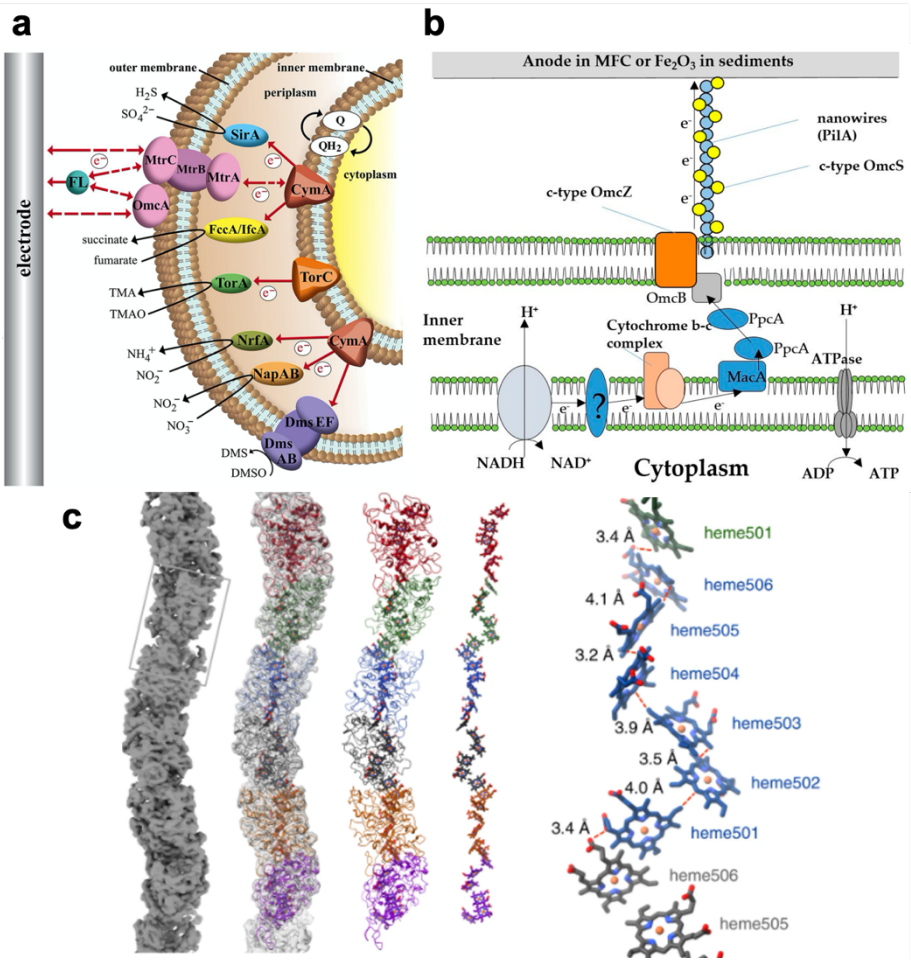

Figure 2. Structural features of electron transfer in model electroactive microbial cells and their interactions with electrode surfaces in bioelectrochemical schemes. (a) The electron transfer mechanism and conductive membrane structure of *S. oneidensis*, showing unique Mtr-pathway and terminal reductases. Quinones (Q) transfer electrons to CymA or TorC, which pass electrons to MtrCAB or terminal reductases. MtrCAB complex interacts with electrode surface either via direct contact or flavin molecules. The dashed arrows in indicate theoretical electron flow direction, whereas the solid arrows indicate experimentally determined electron flow path. Reprinted with permission from ref. 227. Copyright 2015 Frontiers. (b) The electron transfer mechanism branched, outer membrane cytochromes (OMCs) system, and conductive pili structure in *G. sulfurreducens*. Electrons are transferred between inner membranes, periplasm and outer membrane, and an electrode through a cytochrome chain and menaquinones (MQ). Reprinted with permission from ref. 135. Copyright 2019 MDPI. (c) The structure of *G. sulfurreducens* nanowires with closely arranged hemes in filamentous OmcS and labeled hemes. The interatomic contact distances (to the right in (c)) between adjacent porphyrins are 4.1 Å or less. Reprinted with permission from ref. 138. Copyright 2019 Springer Nature.

G. sulfurreducens can also engage in direct extracellular electron transfers via self-assembly of the *c*–type cytochromes into conductive pili structures. In *G. sulfurreducens*, the electrically conductive microbial nanowires, typically referred to as e-pili, are type IV pili, which consist of PilA protein. These e-pili connect the inner membrane with an outer electron acceptor, facilitating direct interspecies electron transfer (**Figure 2b**).[14, 115, 134, 135] Various other proteins, such as OMCs, might be involved in transporting electrons to an electron acceptor via type IV pili structures,[136] which are critical for efficient extracellular electron transfer of biofilms.[112] However, the fundamental mechanism of electron transport is a debated topic (**Figure 2c**).[119] Malvankar and co-workers have proposed a "metallic-like model," suggesting that electron transport occurs through stacked π-orbitals of aromatic amino acids.[116, 137] The overlapping π-π aromatics, which are packed 3–4 Å,[138] are a structural motif of the conductive type IV pili that facilitate long-range electron transport.[113, 116, 137] However, Wang *et al.* demonstrated that the *G. sulfurreducens* conductive filaments, composed of a micrometer-long polymerized chain of hexaheme cytochrome OmcS, are responsible for long-distance electron transport instead of PilA e-pili.[139] PilA protein facilitates the secretion of OmcS outside of the cells. The inter-subunit coordination, along with parallel stacking of heme OmcS pairs, promote stability of the protein-protein interface. Additionally, the study by Wang *et al.* showed closely stacked (<4–6 Å) hemes to continuously promote electron transfer between OmcS monomers. However, this conclusion was challenged by reports from Lovley and co-workers,[12, 13] claiming the perspective that PilA e-pili are the structural motif responsible for long-range electron transport because of several reasons. First, the long-range electron transport mechanism requires the formation of a thick electroconductive biofilm. A research study

demonstrated that the removal of the *omcS* gene had no apparent impact on the current production from the biofilm.[140] Second, the expression of pilin genes in *G. sulfurreducens* resulted in strains with low-conductivity pili, but more outer-surface OmcS.[14] Expression of mutant pilin genes led to the formation of less conductive *G. sulfurreducens* biofilms, thus indicating that OmcS filaments do not participate in long-range electron transport.[141] Additionally, *G. sulfurreducens* mutant strains have been designed to express lower OmcS amounts and higher PilA levels in comparison to wild-type *G. sulfurreducens*, resulting in higher current generation and formation of more conductive biofilms.[145] Moreover, studies have shown that there is no correlation between PilA expression and OmcS secretion.[115] Finally, the cell culturing conditions used by Wang and co-workers are inadequate for e-pili expression as PilA was barely detectable during their filament preparation step, thus calling into question the relevance of referenced results. Future work is necessary to provide a deeper understanding of the fundamental electron transport mechanism in *G. sulfurreducens*. *G. sulfurreducens* has also been shown to excrete flavin-based electron shuttles, yet these are not mobile and mainly operate when bound to cytochromes.[142, 143]

Due to their direct electron transfer capabilities, specifically long-range pili-based electron transfer, these two well-studied Gram-negative microbial species represent highly efficient and desirable exoelectrogens as bioelectrocatalysts for practical applications. The pivotal features of pili-based conductivities require further investigations for their use in the development of novel sustainable bioelectronic materials.[144] Consequently, these two electroactive bacteria have been employed in a various biotechnological applications, including microbial fuel cells and bioelectrosynthesis (e.g., maintenance of redox balance during fermentation and bioremediation). In microbial fuel cells, *S. oneidensis* and *G.*

sulfurreducens oxidize organic substrates and transfer electrons to the anode to convert chemical energy to electrical energy.[145-147] *G. sulfurreducens* form well-structured biofilms on anodes that generate high power outputs in microbial fuel cells.[147] Microbial fuel cells with bacterial co-cultures containing *G. sulfurreducens* have displayed improved current generation compared to pure *G. sulfurreducens* cultures. For example, a bioelectrochemical system in which *G. sulfurreducens* was co-cultured with non-electroactive *Escherichia coli* generated higher currents compared to the monomicrobial culture of *G. sulfurreducens*, which is due to O_2 reduction by *E. coli*.[148] In contrast to donating electrons to anodes, these electroactive microorganisms can also accept electrons from cathodes. Under anoxic conditions, *S. oneidensis* MR-1 directly accepts electrons from cathode surfaces. The Mtr extracellular electron transfer pathway then transfers electrons to the quinone/quinol pool in the cytoplasmic membrane where the electrons are utilized in fumarate reduction.[16] Although bioelectrosynthetic systems using *S. oneidensis* and *G. sulfurreducens* are still in their initial stages, they show a promise as platforms for bioelectrocatalyic applications. Future research studies should focus on the development and characterization of bioelectrosynthesis systems to produce carbon-neutral and advanced biofuels, as well as high-value chemicals, using these two electroactive microorganisms as model species.

2.2.2. Other Electroactive Microbial Cells. To date, three mechanisms of extracellular electron transfer have been suggested to elucidate the respiratory activity of substrates in electroactive bacterial microorganisms. Specifically, the three electron transfer strategies occur via direct contact, nanowires, and/or electron shuttles (**Figure 3**).[149] The most studied model bacteria systems are aforementioned *S. oneidensis* and *G. sulfurreducens*, both of

which use multiheme *c*-type cytochromes (Section 2.2.1). Both organisms are also proposed to form conductive microbial nanowires. As a solution for long-range electron transport, bacterial microorganisms in nature have developed mechanisms to produce putative microbial protein filaments, which are microbial nanowires[116] with conductive properties *in vivo* under physiological conditions. These microbial nanowires offer opportunities for increased microbe-electrode interactions. Additionally, they are critical players in electron exchange between species and electronic communication between microbes and electron acceptors in their environmental surroundings. As discussed previously, the microorganism *G. sulfurreducens* has been found to produce electrically conductive nanowires during electrode reduction. Following this discovery, putative conductive filaments have also been observed in several other electroactive microorganisms, including *S. oneidensis* MR-1.[150] *Geobacter* species produce type IV pilin proteins mainly composed of subunit protein PilA. In contrast, *S. oneidensis* microbial nanowires are outer membrane extensions with porin-cytochrome complexes that are responsible for extracellular electron transfer.

Direct electron transfer between interspecies facilitated by conductive filaments was initially observed in co-cultures of *G. sulfurreducens* and *Geobacter metallireducens*,[132, 151] which adapted a means to share electrons, promoting a mutual metabolism. Summers and co-workers demonstrated that *G. metallireducens* used ethanol as the electron donor in the growth medium, while *G. sulfurreducens* used the provided fumarate as the electron acceptor.[132, 151] Various research findings have confirmed the importance of conductive pili for interspecies electron transfer in both *Geobacter* microorganisms.[151, 152] Research studies have observed microbial nanowires in the iron-reducing *Rhodopseudomonas*

palustris RP2 strain[153] and the sulfate-reducing *Desulfovibrio desulfuricans*.[154] Additional reports have detected microbial nanowires to form in the iron-oxidizing *Acidithiobacillus ferrooxidans*.[155, 156] Photosynthetic microbes, such as unicellular *Synechocystis* species, can also develop microbial nanowires under electron acceptor-limiting and high light intensity conditions.[128] Furthermore, researchers have identified other photosynthetic bacteria, such as *Microcystis aeruginosa* and *Nostoc punctiforme*, to develop putative conductive filaments when exposed to high light intensities.[157, 158]

The conductive microbial nanowires in *G. sulfurreducens*, *Aci. ferrooxidans,* and *Synechocystic* sp. are type IV pili, which are the most common pili type in microorganisms.[128, 155-157, 159, 160] These microbial nanowires forming in distinct microorganisms vary in (1) width because type IV pili have abilities to create pili bundles, resulting in different widths, (2) pili length due to cell culture preparation techniques and microbe ages that can lead to fracture of long, delicate pili motifs, and (3) molecular masses of their subunits.[128, 155-157, 159, 160] In addition to their unique functional characteristics (e.g., twitching motility, DNA uptake),[128, 157, 159-161] type IV pili are considered significant multifunctional extracellular structures as they play critical roles in electron transfer. The microbial nanowires in *G. sulfurreducens* are PilA subunit polymers, in *Synechocystic* sp. they are made of PilA1, and in *Aci. ferrooxidans* they are suspected to be composed of PilV and PilW proteins.[128, 155-157, 159, 160] Further studies are necessary to identify the potential roles of the later in electron transfer processes. Pili-like structures have been identified in several other microorganisms, including *Aeromonas hydrophila, R. palustris, D. desulfuricans*, *Mi. aeruginosa*, and *No. punctioforme*, however, more elaborate studies are necessary to confirm the protein identities and structures.[119-121, 153, 154, 162] *Pelotomaculum*

thermopropionicum is also known to form flagellum-based appendages, which are electrically conductive,[128, 162] but the physiological roles and protein components are not yet known.

Fundamental knowledge about extracellular electron transfer or electroactive activities in Gram-positive bacteria is limited,[163, 164] as this group of bacterial species was thought to be unable to transfer electrons across their non-conductive peptidoglycan cell wall without using external electron mediators.[165] However, Marshall and co-workers reported electrochemical evidence of direct electrode reduction by *Thermincola ferriacetica*.[166] Additional studies examining the physiology, electrochemistry, and genetics of *Thermincola potens* species reported the electron transfer mechanism to depend on *c*-type cytochromes linked to the cell wall,[167, 168] but the generated current was low. In addition, Light and co-workers demonstrated that food-borne pathogen *Listeria monocytogenes* employs a unique flavin-based extracellular electron transfer mechanism to carry electrons to iron or electrode surfaces.[169] By completing a genetic screening to identify *L. monocytogenes* mutants with reduced extracellular ferric iron reductase activity, the researchers identified an eight-gene locus responsible for extracellular electron transfer. This gene locus encodes a specific NADH dehydrogenase that separates extracellular electron transfer from aerobic cellular respiration processes by shuttling electrons to a distinct membrane-based quinone pool. The study also demonstrates the activity of an extracellular flavoprotein, in combination with flavin molecule shuttles, facilitates electron transfer to extracellular acceptors. In another study by Light *et al.*, an enzyme family of putative extracellular reductases.[170] The research group showed that in flavination modifications of the fumarate reductase sub-family enables the enzyme to receive electrons

from the extracellular electron transfer chain and support the growth of *L. monocytogenes*. These primary experimental findings point to a simple electron conduct that is compatible with the membrane structures of Gram-positive bacteria, thus providing important evidence of extracellular electron transfer activities of other electrogenic microbes. Therefore, future studies also need to investigate and define extracellular electron transfer mechanisms in Gram-positive microbes.

In addition to electroactive bacteria that produce putative conductive filaments, several microbial species are also capable of self-producing redox-active metabolites that can serve as extracellular electron shuttles (mediators).[171] The electrochemically-active metabolites leave the cell in their reduced states to transfer electrons to long-distance extracellular oxidants, return inside the cell in the oxidized state, after which these molecules get re-reduced. Thus, this redox cycling of extracellular electron shuttles allows certain microbes to facilitate electron transfer within bioelectrocatalytic systems. The best-characterized microorganisms with extracellular electron shuttle-producing abilities are *Pseudomonas aeruginosa* and *S. oneidensis*. Namely, *P. aeruginosa* secretes redox-active, nitrogen-containing heterocyclic metabolites known as phenazines, whereas *S. oneidensis* self-produces yellow-pigmented flavin molecules.[122, 149, 171-175] In the case of *P. aeruginosa*, phenazines facilitate electron transfer across cell membranes, transferring content from inside the cell to the extracellular environments.[172, 173] As part of the phenazine biosynthetic pathway, *P. aeruginosa* strains are known to produce at least five distinct phenazine derivatives.[172] While *P. aeruginosa* phenazines are mostly studied, in the context of quorum sensing, as metabolites that allow the microbial cells to communicate with neighboring *P. aeruginosa* cells in defense to other competitive microbes, phenazines also

serve as redox electron shuttles for mediated electron transfer.[176] For instance, Rabaey and co-workers have demonstrated the use *of P. aeruginosa* phenazine production for enhancement of electron transfer rates in microbial fuel cells.[177] Similarly, *S. oneidensis* cell cultures accumulate flavin species, namely riboflavin (B2) and flavin mononucleotide, which can act as electron shuttles to facilitate the reduction of substrates, such as several forms of Fe(III) oxide.[149] The secreted flavins by *S. oneidensis* MR-1 are reduced in the Mtr respiratory route.[149] Research findings, characterizing the crystal structures of the outer membrane-associated cytochrome MtrC, demonstrated the flavin mononucleotide binding domains to be near two-solvent exposed heme groups,[178] therefore, postulating biochemical-based insight into how flavin electron shuttles enable respiration. Several other microorganisms, including *Lactococcus lactis*, *Klebsiella pneumonia*, and *Sphingomonas xenophaga*, have been reported to produce cyclic quinones as extracellular electron shuttles.[18, 179-181]

Apart from only a few model microorganisms, the mechanisms of extracellular electron transfer in microbial cells that are designated as electroactive are not yet investigated. Consequently, it is challenging to comparatively evaluate the electroactivities of diverse bacterial species. Thus, experimental setups are required to characterize microbial electroactivities in pure cell cultures, particularly about the functional link between current and microbial metabolism, to elucidate the extracellular electron transfer processes.

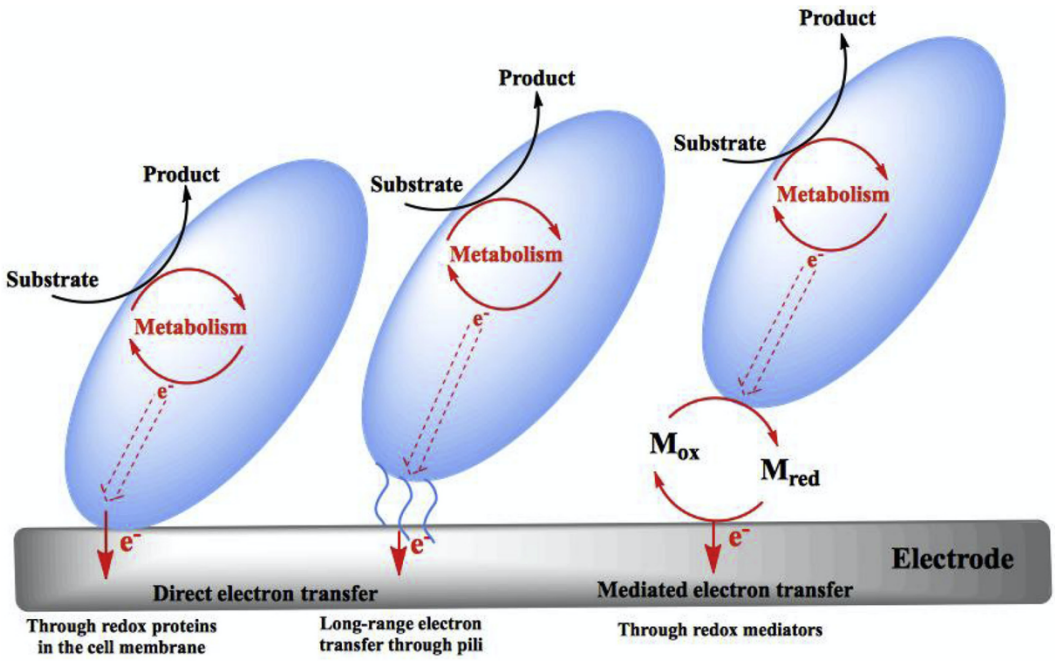

Figure 3. The three mechanism of electron transfer in microbial cells. To the left is (1) direct contact via cell membrane bound *c*-type cytochromes. To the right is (2) mediated electron transfer via extracellular redox-active electron shuttles (mediators). In the middle is (3) long-range electron transfer though pili in a bacterial microorganism. Reprinted with permission from ref. 164. Copyright 2019 Elsevier.

2.3. Modification of Oxidoreductases via Protein Engineering

By natural design, oxidoreductases are not optimized for applications in bioelectrocatalysis systems. In most cases, the redox-active motifs are deeply buried in an insulating protein shell, and sophisticated control mechanisms regulate electron transfer mechanisms with oxidoreductases to prevent random electron transfer, radical formation, and futile use of energy.[182] To overcome the physiological constraints and improve the kinetic properties and electrochemical performance, oxidoreductases need to be modified, making them more adaptable for use in bioelectrocatalysis systems. For example, engineered enzymes with a stronger affinity towards specific analytes, better stability, faster electron transfer rates, and residues able to provide oriented immobilization, result in enhanced biosensor performance.[183] Deglycosylation is favorable for the electron transfer between

oxidoreductase and electrode.[184] Protein engineering is an effective approach to break through the natural physiological constraints. The common protein engineering methods involve mainly rational design and directed evolution.[6]

2.3.1. Rational Design of Proteins. Rational protein design requires accurate information of the protein structure or at least a reliable computational model. The site-directed mutagenesis guided by structural information is the method employed in the rational design for the modification of protein structures and the improvement of catalytic properties.[185] To rationally modify oxidoreductase for bioelectrocatalytic applications, several strategies have been investigated, including trimming oxidoreductases, surface modifications, amino acid substitutions at/around the active-sites, and protein modifications for oriented immobilization (**Figure 4**).[186]

2.3.1.1. Trim of Oxidoreductases. Truncating an oxidoreductase at the C-terminal, the N-terminal, or a loop structure can shorten the original electron transfer pathway and open up a redox-active site to make it close enough to the conducting support. FAD-dependent glucose dehydrogenase contains a FAD cofactor in α subunit (catalytic subunit) and an electron transfer subunit (β subunit). The β subunit is a cytochrome *c*-like molecule containing three heme c. The electron transfer pathway of FAD-dependent glucose dehydrogenase is proposed to proceed in sequence from FAD, through heme 3, to heme 2, then to heme 1, and finally to the electron acceptors in solution. To establish a direct electron transfer process between the FAD-dependent glucose dehydrogenase and an electrode, a truncated β subunit composed of only heme 3 was designed and constructed based on the 3D homology model. This modified FAD-dependent glucose dehydrogenase had a simplified electron transfer pathway. Heme 3 was exposed in proximity to the

electrode. The result showed that the truncated β subunit could accept electrons from the FAD cofactor and exhibited directed electron transfer with the electrode (**Figure 4a**).[187] D-fructose dehydrogenases have a similar structure and electron transfer pathway. Some engineered D-fructose dehydrogenases with truncated electron transfer pathways have been constructed as well. These engineered D-fructose dehydrogenases also exhibited improved electron communication ability with the electrode surfaces.[188, 189]

2.3.1.2. Surface Modifications. Protein surface modifications can facilitate electrochemical modification between oxidoreductases and electrodes. Deglycosylation is a representative and an effective surface modification method performed to enhance the electron transfer between the prosthetic group of oxidoreductases and the electrode. Glycosylation is regarded as one of the most important posttranslational modifications after protein synthesis, which is an effective way of generating a diversity of proteins and modulating the protein function.[190] However, the glycosylation of the oxidoreductase surface blocks the electron transfer between the prosthetic group and the electrode. Consequently, the removal of the glycosyl from the surface of oxidoreductase is conducive to a closer contact of the prosthetic group and the electrode due to the downsizing of dimensions of oxidoreductases on the electrode surface. Research work by Ortiz and co-workers presented the effect of deglycosylation on the electrochemical properties of cellobiose dehydrogenase (**Figure 4b**).[184] After the deglycosylation treatment, the graphite electrodes modified by cellobiose dehydrogenase exhibited a 40-65% higher catalytic current (I_{max}) value in the presence of the substrate than the electrode modified with glycosylated cellobiose dehydrogenase. This increase can be attributed to the downsizing of cellobiose dehydrogenase and enhanced directed electron transfer due to

deglycosylation. Gorton and co-workers studied the effect of deglycosylation and surface-exposed cysteine residues on the direct electron transfer (DET) properties of horseradish peroxidase (HRP). The non-glycosylated HRP was heterogeneously expressed by *E. coli* without glycosylation modification. The lack of the glycosyl barrier significantly reduced the distance between the active site of HRP and the electrode. The non-glycosylated HRP was adsorbed on a pre-oxidized gold electrode and generated more than a 30-fold increase in electron transfer rate compared with the native HRP. In addition to cellobiose dehydrogenase and HRP, the deglycosylation strategy has also been used on glucose oxidase (GOx) to enhance the electron transfer properties.[191, 192]

2.3.1.3. Amino Acid Substitutions at the Active Site. Site-directed mutagenesis is a common approach used to improve the catalytic properties of an enzyme, such as specific activity, stability, and/or kinetic parameters. For oxidoreductases, the improved properties can make it more suitable for application in electrochemical systems. In the case of methylamine dehydrogenase, a Phe55 residue with large steric hindrance, which located at the substrate access channel, was substituted to alanine (an amino acid with much smaller steric hindrance). After the modification, mutant methylamine dehydrogenase exhibited approximately a 400-fold lower K_m value towards histamine relative to that of a wild-type methylamine dehydrogenase. The mutant methylamine dehydrogenase was immobilized on an electrode to design a histamine biosensor, showing Michaelis-Menten behavior in response to varying histamine concentrations and a 3-fold lower K_m than the biosensor with immobilized native methylamine dehydrogenase. The limit of detection for the histamine biosensor based on the immobilized mutant methylamine dehydrogenase was 5 μM, which is four times lower compared to the 20 μM detection limit of biosensor based on the native

methylamine dehydrogenase.[193] In addition to an improvement of the catalytic properties, the rational design of proteins can also be used to change the substrate preference of enzymes. Specifically, for oxidoreductases, the coenzyme or the electron mediator preference can be changed. The utilization of a more stable, more efficient, and cheaper electron mediator is of great significance for enzymatic fuel cells. Chen *et al*. developed a rational design strategy to change the coenzyme specificity of 6-phosphogluconate dehydrogenase (6PGDH) from its $NADP^+$ to NAD^+ (**Figure 4c**). Through the amino acid-sequence alignment of $NADP^+$ and NAD^+ preferred 6PGDH enzymes and computer-aided substrate-docking, four residues involved in coenzyme binding were identified, and the mutant N32E/R33I/T34I was constructed. The mutant 6PGDH was applied in a biobattery. The maximum power density and current density of the biobattery catalyzed by the mutant were 0.136 mW cm^{-2} and 0.255 mA cm^{-2}, which are ~25% higher than those obtained from wild-type 6PGDH.[194]

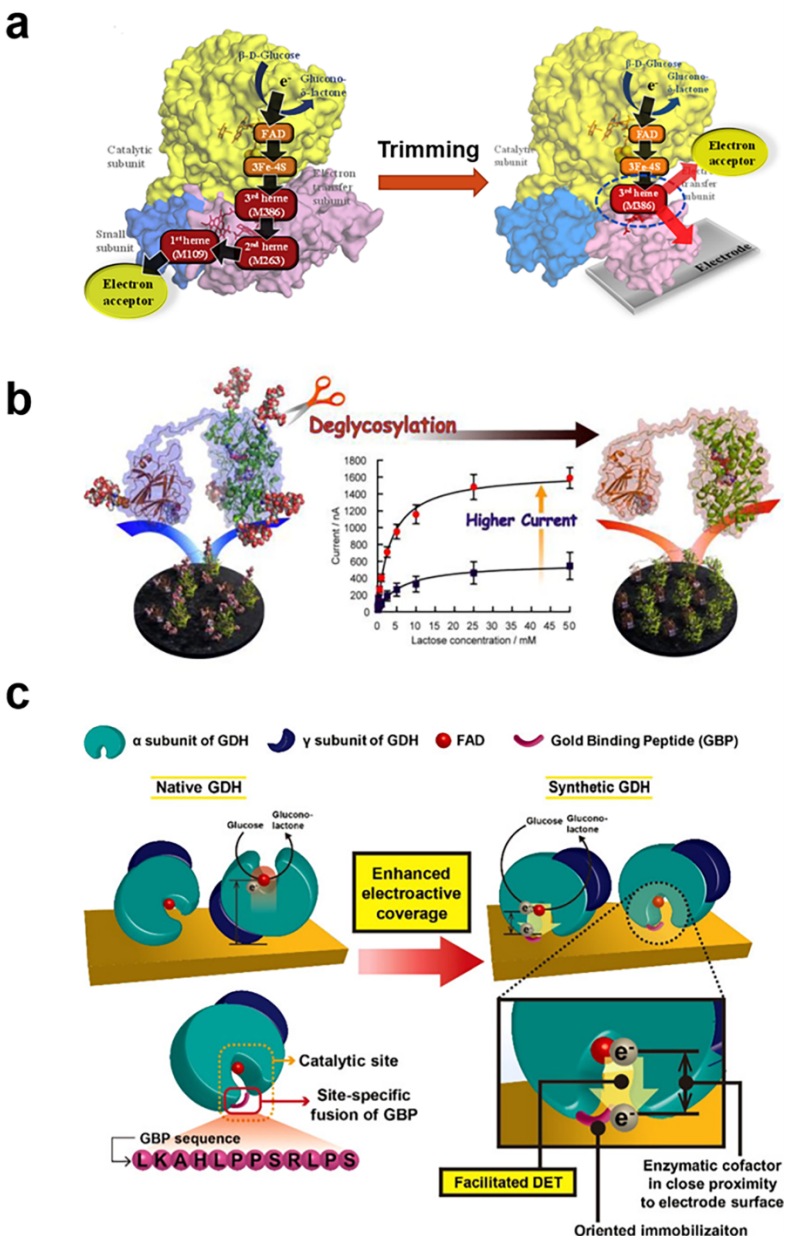

Figure 4. Four modification strategies based on rational protein design to enhance the electron transfer and electrochemical properties of oxidoreductase. (a) Trimming of oxidoreductase. The truncated β subunit could accept electrons from the FAD cofactor and exhibited directed electron transfer with the electrode. Reprinted with permission from ref. 187. Copyright 2018 Elsevier. (b) Surface modification. Deglycosylation leads to the downsizing of cellobiose dehydrogenase, decreases the distance between active site and electrode, and finally facilitates the directed electron transfer. Reprinted with permission from ref. 184. Copyright 2012 American Chemical Society. (c) Protein modification for oriented immobilization. The oriented immobilization facilitated by a site-specific gold binding peptide resulting in directed electron transfer. Reprinted with permission from ref. 195. Copyright 2018 American Chemical Society.

2.3.1.4. Protein Modifications for Oriented Immobilization. An oriented binding of an oxidoreductase without activity loss on the electrode surface is conducive to the efficient utilization of the electrode surface and the improvement of electrochemical communication between the oxidoreductase and the electrode. The fusion of a peptide sequence at the C- or N-terminus of protein is an effective method for achieving oriented immobilization. In Lee and co-workers' research (**Figure 4d**), glucose dehydrogenase was genetically fused with a gold binding peptide. Compared with natural glucose dehydrogenase, the modified counterpart enables apparent direct electron transfer across the enzyme-electrode interface, leading to a stable current generation.[195] Site-directed mutagenesis is also a useful strategy to realize oriented immobilization of the enzyme via the introduction of a new amino acid, which is able to form a new bond, ideally a covalent bond, with the electrode. The target protein can be immobilized at the electrode surface in a certain orientation, which would facilitate the directed electron transfer between the electrode and the prosthetic group of oxidoreductases. For instance, Holland and co-workers reported the introduction of cysteine to glucose oxidase via site-directed mutagenesis to display a free thiol group near its prosthetic group (FAD). The displayed free thiol group facilitated a site-specific and oriented attachment of maleimide-modified gold nanoparticles, thus enabling directed electron transfer between the conjugated glucose oxidase and the electrode.[196]

2.3.2. Directed Evolution. Different from the rational design of proteins, directed evolution does not require knowledge of the structure-activity relationship and allows for the tailoring of enzymes to defined target by mimicking Darwinian evolution.[197] In research involving directed evolution, a library of mutant enzymes is created via DNA mutations, and the generated enzymes with desired properties are identified and obtained by a

screening procedure. This iterative process is repeated until the desired trait is improved.[6] A vast array of enzymatic traits have been targeted, including catalytic activity, substrate specificity and promiscuity, stereoselectivity, stability, solvent variability, pH optima, and tolerance of harsh environmental and industrial conditions.[198] For the practical applications of bioelectrocatalytic systems, site-directed mutagenesis has been used to improve the kinetic parameters of glucose oxidase. Modified glucose oxidase has been further used to achieve high power outputs of glucose-powered enzymatic fuel cells.[199, 200] The O_2 activity of glucose oxidase has also been tuned via directed evolution to increase its activity for glucose oxidation and make it more applicable to use in enzymatic fuel cells and biosensors.[201, 202] For enzymatic fuel cells, the acidic operating conditions are beneficial to increase the proton concentration, thereby providing more available protons for the current generation. However, most oxidoreductases used in enzymatic fuel cells cannot tolerate acidic conditions. To solve this problem, Ma *et al.* successfully improved the stability of 6-phosphogluconate dehydrogenase under acidic conditions (**Figure 5a**). The modified 6-phosphogluconate dehydrogenase exhibited a 42-fold increase in catalytic efficiency at a pH of 5.4 compared with the wild-type dehydrogenase. The enzymatic fuel cell equipped with this modified 6-phosphogluconate dehydrogenase achieved a maximum power density of 0.13 mW cm^{-2} at pH 5.4, which was more than 10-fold higher than that with the same enzyme unit loading at pH 7.3.[203] Besides the catalytic properties, directed evolution can also be used to change the redox potential of a specific enzyme. *E. coli*'s copper efflux oxidase (CueO) has rarely been employed in the cathodic compartment of enzymatic biofuel cell due to its low redox potential (0.36 V *vs* Ag/AgCl) towards O_2 reduction. To address this, Zhang and co-workers used directed evolution to shift the onset potential of

CueO towards a more positive direction (**Figure 5b**). Firstly, random mutation and site-saturation mutation libraries were concurrently constructed. Meanwhile, a robust and efficient 8-channel electrochemical platform was used to evaluate CueO variants expressed in a 96-well microtiter plate. The residue positions at D439 and L502 that are adjacent to the coordinated ligands of T1 Cu site, have been identified as the main regions that contribute to improvement in the onset potential. A D439T/L502K mutant was constructed with a remarkable increase in onset potential of 0.54 V, and the enzymatic biofuel cell with a CueO D439T/L502K cathode generated a V_{oc} of 0.56 V, as well as a 1.72-fold enhancement in power output.[198]

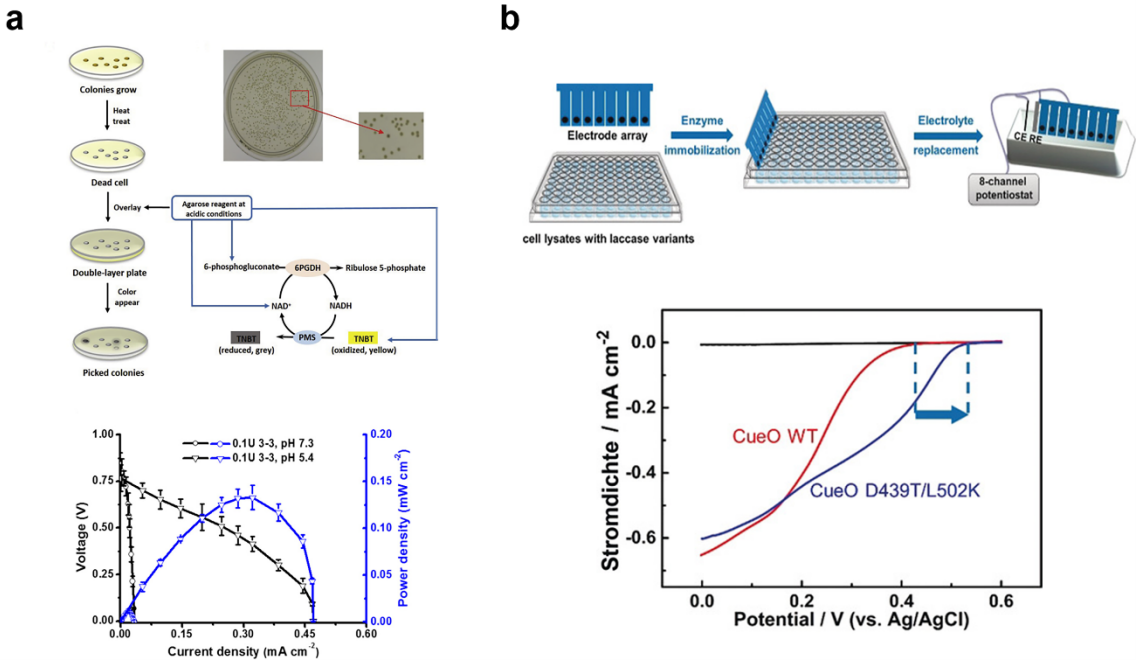

Figure 5. The modification oxidoreductase of based on directed evolution (a) Schematic of Petri-dish-based double-layer screening to identify the mutant 6-phosphogluconate dehydrogenase that can work at acidic condition. Reprinted with permission from ref. 203. Copyright 2019 Elsevier. (b) Schematic illustration of the electrochemical screening platform to get the mutant copper efflux oxidase with higher redox potential. Reprinted with permission from ref. 198. Copyright 2019 Wiley.

2.4. Modification of Microbial Cells for the Application in Bioelectrocatalytic Systems

The types of bioelectrocatalytic systems based on microbial cells are microbial biosensors, microbial fuel cells, and microbial electrosynthesis. An electrochemical microbial biosensor is an analytical platform that couples microorganisms with an electrode transducer to enable rapid, accurate, sensitive, and often quantitative detection of target analytes.[204] The microbial fuel cell is an energy conversion device that utilizes the biocatalytic abilities of viable microorganisms and a range of organic compounds as fuel sources to convert the chemical energy stored in chemical bonds into electrical current.[205] The typical microbial electrosynthesis (MES) process uses autotrophic microbes as the bioelectrocatalyst with a cathode as the electron donor and specific substrates as electron acceptors for target product synthesis.[4] However, two hurdles hinder further development and application in the real-world of microbial-based bioelectrocatalytic systems. One is the low electron transfer efficiency between microbial cells and electrodes. The other one is the low added value of the produced chemicals.[206] Three strategies can be used to tackle these two hurdles and create microbial cells with desired properties that are more applicable in bioelectrocatalytic systems. These strategies include (1) transplantation of a heterogeneous metabolic pathway for the production of products with high added-value into an electroactive microbial cell (including exoelectrogens and electrotrophs), (2) modification of native exoelectrogens to enhance the electron flux, and (3) incorporation of electron conduits in non-native exoelectrogens.[206-208] The field of synthetic biology combines the investigative nature of biology with the constructive nature of engineering. With the development of synthetic biology, the rational design and construction of novel

proteins, genetic circuits, and metabolic pathways have been feasible. Based on that, studies related to the rational rewiring and reprogramming of organisms, including electroactive microorganisms, have been widely performed.[209-211] Consequently, synthetic biology approaches can be employed to implement the three strategies discussed below.

2.4.1. Transplant a Heterogeneous Metabolic Pathway. Introducing a new production pathway into electroactive microbial cells is a practical approach to broaden the product scope of bioelectrocatalysis. However, the major hurdle is the limited toolset for genetic manipulation and metabolic modification, as the electroactive microorganisms represent non-model strains. Fortunately, the emergence of the new synthetic biology toolset, namely the development of RNA-guided genome editing by CRISPR-Cas9 to introduce precise genomic mutations,[212] provides new possibilities to address this issue.[206] For model electroactive microbial cells, namely *G. sulfurreducens*, *S. oneidensis* (Section 2.2.1), and *P. aeruginosa* (Section 2.2.2), genomic modification approaches have been used to improve the anodic current production in biofuel cells significantly.[213-215] So far, no study of the utilization of metabolic engineering approach for the production of chemicals with *Geobacter* species as the host has been reported due to low electron uptake rates, thin cathodic biofilms, lack of genetic manipulation methods, and knowledge gaps connecting electron uptake and intracellular metabolic activity.[206] For *S. oneidensis,* the Mtr pathway can be used to effectively transfer electrons from the electrode into the cell and drive metabolic reactions. Two studies, from Yang's group, used genomic modification approaches to endow the *S. oneidensis* cells with the ability to produce useful chemicals. Firstly, two genes of the Ehrlich pathway, *kiv*D and *adh* encoding ketoisovalerate decarboxylase and alcohol dehydrogenase, respectively, were introduced into *S.*

oneidensis. The two introduced genes endowed *S. oneidensis* the ability to produce iso-butanol. With a direct electron supply from the cathode, the engineered *S. oneidensis* strain provided 19.3 mg/L of *iso*-butanol within 100 hours of reaction.[216] Based on that, the researchers constructed a new engineered *S. oneidensis* strain that can convert butyrate to *n*-butanol. Three genes, *adhE2* encoding alcohol dehydrogenase, *ctfAB* encoding CoA transferase, and *acs* encoding acetyl-CoA synthetase, were introduced into *S. oneidensis* MR-1. The engineered strain exhibited the ability to produce *n*-butanol in the presence of 2% *N*-acetylglucosamine and 0.3% of butyrate. After approximately 100 hours of reaction, the highest *n*-butanol concentration achieved was 160 mg/L.[217] In research from Tefft and TerAvest, a hydrogenase-deficient *S. oneidensis* MR-1 cell was used as a chassis cell to integrate a heterologous proton pump (proteorhodopsin) and butanediol dehydrogenase. Based on the native extracellular electron transfer pathway in *S. oneidensis* MR-1, the exogenous electrons from the cathode were transported to the inner membrane quinone pool. Dependent on the supporting role of proteorhodopsin, NADH was regenerated with the consumption of reduced quinones. Finally, the generated NADH was used as reducing power to support the conversion from acetoin to 2,3-butanediol via butanediol dehydrogenase.[218]

2.4.2. Modification of Native Exoelectrogens to Enhance Electron Flux. The extracellular electron transfer (EET) pathway that bridges the electrons generated from the oxidation of substrates (carbon sources in the medium) and the electronic terminal receptor (electrode) can be divided into five successive steps (**Figure 6**): (i) the import and assimilation of an electron donor (carbon source) and the oxidation of an electron donor to release electrons; (ii) the transport of electrons to an intracellular electron carrier,

particularly NADH; (iii) the transport of electrons to a transmembrane electron transport pathway based on *c*-type cytochromes or (iv) soluble electron shuttle-mediated EET pathway; (v) cell attachment and biofilm formation on the electrode surface to enhance direct EET.[208] Synthetic biology approaches can be employed to modify every single step of the EET pathway and further to improve the electron transfer efficiency.

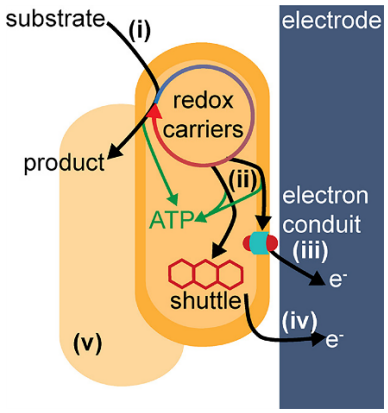

Figure 6. The engineering of specific targets to improve the current production by native exoelectrogens. The performance of exoelectrogens as industrial electrochemical catalysts is limited by several factors, which can be understood by dividing extracellular electron transfer into five distinct processes, namely (i) the oxidation of the initial electron donor; (ii) electron transfer from metabolic pathways to electron transfer components; (iii) electron transfer occurring through porin-cytochrome complexes; (iv) electron transfer through electron shuttles; and (v) cell attachment to surfaces of electrodes. Reprinted with permission from ref. 208. Copyright 2015 Wiley.

The first step of the EET pathway is the oxidation of the electron donor and the release of electrons. Some studies have demonstrated the enhancement of substrate oxidation in native exoelectrogens enables them to generate a higher current. A specific way to improve the oxidation of the electron donor is to employ synthetic biology approaches to improve the substrate uptake rate or introduce a new metabolic pathway into the native exoelectrogens to provide cells with the ability to utilize more diverse substrates. In *S. oneidensis*, the intracellular supply of electron donors, lactate, is limited under anaerobic conditions due to the lack of proton-motive force to drive substrate uptake.[219] To solve this

problem, Johnson and co-workers improved the substrate uptake rate of *S. oneidensis* via the recombinant expression of a light-driven proton pump (proteorhodopsin). The expressed proteorhodopsin increased the proton-motive force, achieving light-induced changes in the membrane potential and finally increasing the current production by approximately 2.5 times in the microbial electrochemical system.[220] In order to broaden the feedstock range, the metabolic pathways of new feedstock have also been introduced into exoelectrogens. *S. oneidensis* has been engineered to contain glucose, glycerol, even xylose utilization pathways from *Zymomonas mobilis*, *E. coli*, *Candida intermedia*, and *Clostridium acetobutylicum*, respectively, allowing it to use these compounds as a sole carbon and energy source in either aerobic or anaerobic conditions.[221-223] The reduced NADH is the primary intracellular reducing equivalent, which is the major releasable intracellular electron carrier. The regulation of the level of intracellular NADH could be employed to regulate the EET rate of exoelectrogens. In order to increase the intracellular releasable electrons, the heterologous overexpression of an *fdh* gene encoding the formate dehydrogenase from *Moraxella* spp. in the *S. oneidensis* MR-1 cells enabled the *S. oneidensis* MR-1 cells to produce increased current density in a microbial fuel cell.[224] Similarly, a *nadE* gene encoding the NAD synthetase was introduced into the *P. aeruginosa* cells. Through the overexpression of NAD synthetase, a three times higher electricity output was achieved.[225] A more in-depth study was reported by Song's group.[226] Based on the genomic and bioinformatic analysis, the researchers discovered and categorized three gene modules involved in the network architecture of NAD^+ biosynthesis in *S. oneidensis* MR-1. Among the three modules, five crucial genes, specifically *ycel*, *pncB*, *nad*M, *nad*D*, and *nad*E*, were identified. The overexpression of the five crucial

genes led to a 2.1-fold increase in the total intracellular NAD(H) level and a 4.4-fold increase of power density in the microbial fuel cell. To overcome the insulating characteristics of cell membranes and achieve EET, exoelectrogens employ diverse multiheme *c*-type cytochromes, consisting of transmembrane proteins and redox proteins for electrons transfer across the cell membrane to the extracellular electron acceptor.[227] As mentioned above in Section 2.4.1, *S. oneidensis* cells use the Mtr pathway, which consists of a series of *c*-type cytochrome proteins, including CymA, MtrA, MtrB, MtrC, and OmcA, to accomplish EET (Section 2.2.1). Correspondingly, the overexpression of the *c*-type cytochrome protein that makes up the Mtr pathway is a feasible strategy for improving the EET efficiency. Bretschger and co-workers demonstrated this strategy by overexpressing the MtrC protein in *S. oneidensis* cells. The engineered *S. oneidensis* strain generated 35% higher current in the microbial fuel cell than the wild-type strain.[146] The work from Min and co-workers went a step further. A metal-reducing conduit biosynthesis gene cluster *mtr*C-*mtr*A-*mtr*B encoding the component protein of the Mtr pathway was introduced and co-expressed in *S. oneidensis* MR-1. The engineered strain could produce 87% current density higher than that of the wild-type strain.[228] Exoelectrogens are able to utilize different inorganic (e.g., Fe^{3+}, H_2S, and H_2) or organic compounds (e.g., phenazines and flavins) as electron shuttles to realize EET.[229] For *S. oneidensis*, flavins are elucidated to be the electron shuttle. For *P. aeruginosa*, several phenazine derivatives play the function of an electron shuttle.[207] Some researchers have shown that promoting the synthesis and secretion of the electron shuttles via synthetic biology approach can be utilized to enhance the efficiency of EET. For *S. oneidensis*, the low concentration of endogenously secreted flavins limits the efficiency of EET. Correspondingly, a flavin biosynthetic pathway from

Bacillus subtilis was heterologously expressed in *S. oneidensis* MR-1, resulting in a 25.7-fold increase in secreted flavin concentrations and further caused the maximum power outward and inward power density was increased 13.2- and 15.5-times, respectively.[230] Likewise, increasing the electron shuttle, especially the production of phenazine metabolites, can also enhance the EET efficiency of *P. aeruginosa*. Some researchers indicated that the quorum sensing (QS) system could regulate the current generation and EET efficiency of the anode-respiring bacterium *P. aeruginosa* via regulating the production level of phenazines. Two studies showed that modifying QS systems could enhance phenazine production approximately 2-fold, which subsequently increased current density by 5-fold.[231, 232] Finally, the efficiency of EET can be improved by increasing the thickness of biofilms on the electrode, thus motivating researchers to modify exoelectrogens for enhanced biofilm formation.[233] In Liu and co-workers' study, a *ydeH* gene, a c-di-GMP biosynthesis gene, originated from *E. coli* was heterologously overexpressed in *S. oneidensis* MR-1 to enhance the production of cyclic-di-GMP, a key intracellular regulator for controlling biofilm formation. The MFCs inoculated with the engineered strains yielded an approximately 2.8-fold more power density than that of the wild-type strain.[214] In a study from Lovely's group, the gene GSU1240 encoding proteins with a PilZ domain were deleted. The engineered *G. sulfurreducens* strain CL-1 produced biofilms that were 6-fold more conductive than the wild-type biofilms. The power density was 70% higher than that of the wild-type *G. sulfurreducens* biofilms.[213]

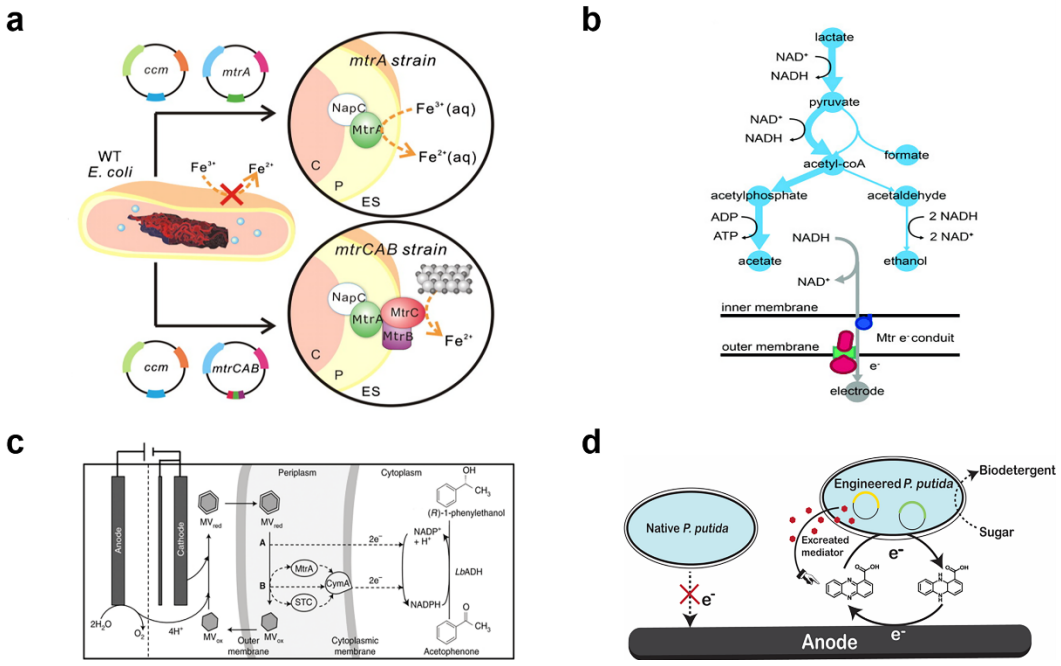

Figure 7. Incorporation of electron conduits in non-native exoelectrogens (a) Schematic depicting the engineered *E. coli* strain with *mtr*A and *mtr*CAB; this modified strain is capable of the soluble and extracellular metal reduction. Reprinted with permission from ref. 234. Copyright 2010 PNAS. (b) Electron transfer through the Mtr electron conduit alters substrate utilization in *E. coli*. Reprinted with permission from ref. 236. Copyright 2014 Wiley. (c) The cytoplasmatic NADPH-pool is linked to the cathode by using extracellular electron transfer through MV as a mediator and further periplasmatic cytochromes. In the cytoplasm, the enantioselective reduction takes place. MV: methyl viologen; *Lb*ADH: alcohol dehydrogenase from *Lactobacillus brevis*; MtrA, STC and CymA: proteins of the electron transfer pathway in *S. oneidensis* MR-1; OM: outer membrane; CM: cytoplasmic membrane. Reprinted with permission from ref. 237. Copyright 2019 Wiley. (d) The gene of phenazine synthesis genes from *P. aeruginosa* are transplanted into *P. putida*. The produced phenazine can mediate the electron transfer.

2.4.3. Incorporation of Electron Conduits in Non-native Exoelectrogens. In recent years, significant research efforts have been made to modify native exoelectrogens. Meanwhile, the modification of non-native exoelectrogens to create novel exoelectrogenic microorganisms is another research focus. Based on the cognition of the structure and EET mechanism, some non-native exoelectrogenic model strains that are widely used in industrial applications, such as *E. coli* and *Pseudomonas putida*, can be transformed into exoelectrogens via the heterologous construction of EET pathways of native

exoelectrogens. The advantages of this strategy include (1) the genetic background of model strain is clear, which facilitates complex genetic manipulations, and (2) the created exoelectrogens could be used as chassis strains to integrate new metabolic modification and ultimately obtain new cell factories.

As the Mtr pathway of *S. oneidensis* is well-understood (Section 2.2.1), an effective synthetic biology approach to creating novel exoelectrogens is to transplant the Mtr pathway into *E. coli* to construct an efficient EET, leading to varying extracellular electron transfer capabilities (Sections 2.4.1 and 2.4.2). Jensen and co-workers transplanted and expressed the Mtr pathway into *E. coli* cells (**Figure 7a**). After modification, the engineered *E. coli* strain was capable of reducing solid metal oxides (e.g., Fe^{3+} and solid Fe_2O_3) to ones that can by installing a synthetic electron conduit that bridges the cytosol to the extracellular space.[234] On this basis, a cytochrome *c* complex (CymA) that links the quinol pool and MtrA was further introduced into *E. coli*. This upgraded modification resulted in fast extracellular electron transfer rates. Furthermore, feeding of exogenous flavins allowed cells to couple Fe^{3+} reduction to growth under Fe_2O_3-reducing conditions.[235] Research from TerAvest showed that the heterologous expression of the Mtr pathway increased the power generation while also altering the metabolic fluxes to more oxidized products with improvement in the redox balance of *E. coli* (**Figure 7b**).[236] The transplanted EET *E. coli* cells can be further used as the chassis cells to integrate a new metabolic pathway for the production of useful chemicals. In the study of Mayr *et al*., the transplanted Mtr pathway effectively transported electrons into the *E. coli* cell to realize the intracellular regeneration of NADPH and support the asymmetric reduction of acetophenone (**Figure 7c**).[237] *P. putida* is another model strain, which is widely used in

industrial applications. Similar to *E. coli*, *P. putida* strains can also be modified to novel exoelectrogens via synthetic biology approaches. In Schmitz and co-workers' research, seven core phenazine (the electron shuttle) biosynthesis genes *phz*A-G and the two specific genes *phz*M and *phz*S to produce *P. aeruginosa* phenazines were introduced into *P. putida*. The engineered *P. putida* strain produced 33 μg/mL of phenazines and sustained strong oxygen-limited metabolism for up to 2 weeks at an anodic current density of up to 12 μA cm^{-2} (**Figure 7d**).[238]

3. THE BIOELECTROCATALYSIS SYSTEM

3.1. Electron Transfer Mechanisms

3.1.1. Electron Transfer Between Enzymes and Electrodes. In biological systems, fundamental metabolic processes rely on the complete catalytic cycle of constituent biomolecules, where the substrate binds to the active site of enzymes and is eventually transformed into a specific product. For oxidoreductases, these multi-step processes typically involve sequential electron transfers. By combining oxidoreductases with an electrode, electron acceptors and donors such as NAD(P)/NAD(P)H can be replaced. This simplified system provides a powerful tool in examining electron transfer mechanisms and constructing biosensors, biofuel cells, and bioelectrosynthetic devices. Theoretically, the interfacial electron transfer rate is highly dependent on the distance between the active site of the enzyme and the electrode surface. It has been proposed that the distance should be below 14 Å to support significant direct electron transfer (DET) by tunneling;[239] for systems where this is not possible, mediated electron transfer (MET) may be needed.

3.1.1.1. Direct Electron Transfer (DET). Direct (mediator-less) electron transfer occurs when electrons are transferred directly from the electrode to the substrate through the

enzyme active site. For several proteins, such as cytochrome *c*, ferredoxin, peroxidase, laccase, and azurin, the active site is well exposed. These proteins can undergo direct electron transfer in a process that has been investigated through electrochemical measurements for redox transformations.[240] However, these events are considered significantly slower on unmodified electrode surfaces (e.g., gold or platinum) compared to those which occur with its native electron transfer partners, and irreversible processes are often observed.[241] It is difficult to maintain the stability of enzymes in cases where they are close enough to the electrode surface for DET. A breakthrough was made by Eddowes *et al*., who discovered that the 4,4-bipyridyl modified gold electrode surface is able to interact with cytochrome *c* and enable reversible electron transfer to this enzyme.[242] Effective immobilization techniques have long been sought and have demonstrated to enhance rapid electron transfer rates. For example, direct adsorption of formate dehydrogenase, carbon monoxide dehydrogenase, or cytochrome P450 on a graphite electrode enables enzymatic turnover and also the study of enzyme kinetics.[243-245] A drawback of this strategy is that it requires the active site of the enzyme to be directly exposed to the electrode surface during immobilization. In addition, enzymes must be posed in the right orientation within a monolayer, which is not feasible in many cases and will largely decrease the quantity of active enzyme at the electrode surface.

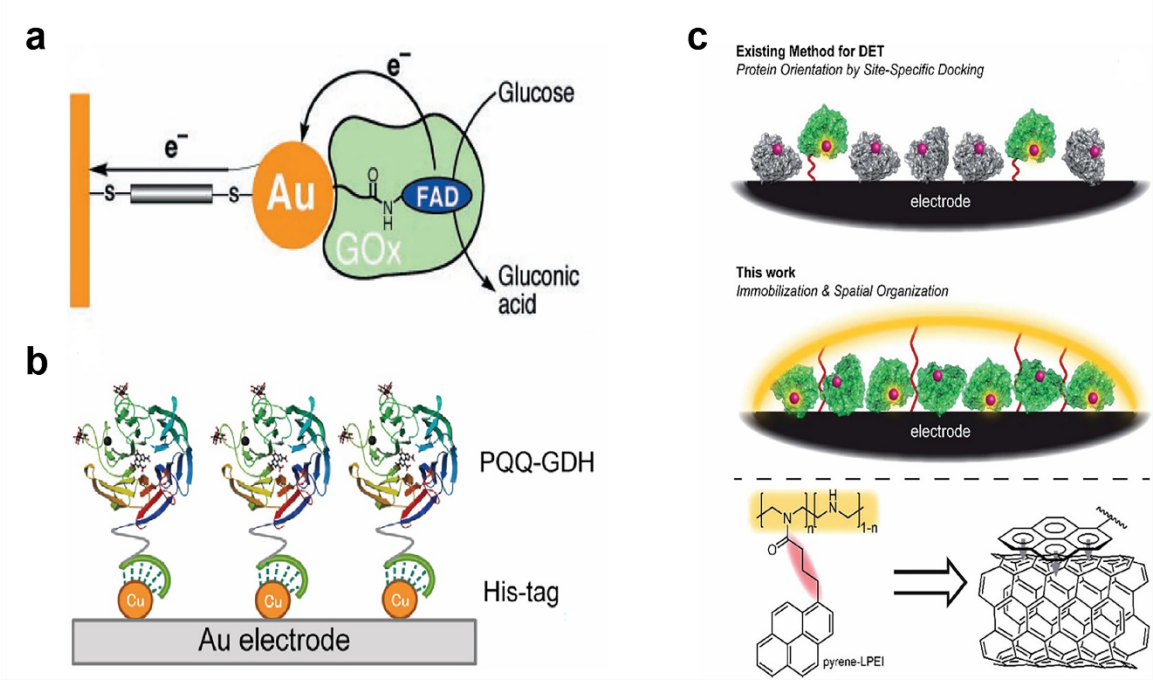

Figure 8. Approaches for direct electron transfer. (a) Glucose oxidase (GOx) incorporated with an "electrical nanoplug" (Au nanoparticles). Reprinted with permission from ref. 246. Copyright 2003 The American Association for the Advancement of Science. (b) Fixed enzyme orientation by interactions between His-tag and Cu nanoparticles. Reprinted with permission from ref. 247. Copyright 2014 Hilaris. (c) Electro-wiring enzymes using pyrene-LPEI as a conducting hydrogel. Reprinted with permission from ref. 251. Copyright 2018 Royal Society of Chemistry.

Approaches that minimize the distance between electrode and enzymes often involve docking motifs to fix enzymes at the right orientation. Xiao *et al.* developed a strategy to enhance DET by reconstitution apo-glucose oxidase with a gold nanocrystal functionalized with a FAD. The bioelectrocatalysis rate (~5000 s^{-1}) of the resulting "artificial" protein-enhanced significantly, where the gold nanoparticles served as the "electrical nanoplug" and efficiently wired the enzyme redox centers (**Figure 8a**).[246] Glucose dehydrogenases (GDH) with a His-tag decorated N-terminus were deposited at the Cu atoms modified electrode surface. A high-degree orientated GDH layer was achieved using the affinity binding of His-tag and Cu atoms (**Figure 8b**).[247] Meneghello *et al.* covalently immobilized cellobiose dehydrogenase (CDH) through the reaction of the maleimide modified electrode

surfaces and the thiol group of cysteine. The cysteine can be generated from anywhere on the surface of CDH by site-directed mutagenesis. This approach supports the study of the DET mechanism of multifactor in CDH while this enzyme is present at different orientations.[248] Other than cysteine, 4-azido-L-phenylalanine (an unnatural amino acid) has been incorporated into laccase to achieve enzyme orientation.[249] While His-tag only allows C- or N-terminus of enzyme immobilization, site-directed mutagenesis can mutate an amino acid into any position of proteins; thus, in principle, any enzyme orientation can be reached.[250] Other than enzyme orientation, an alternative DET approach is the development of conducting hydrogels. Hickey *et al.* fabricated a pyrene modified linear poly(ethylenimine) (pyrene-LPEI), which is able to preserve the residue activity of different enzymes despite their orientations at the electrode surface (**Figure 8c**). Using pyrene-LPEI as an electronic wire to connect remote enzymes with an electrode, a "plug and play" platform has been developed for a collection of electroactive proteins.[251]

3.1.1.2. Mediated Electron Transfer (MET). In mediated electron transfer processes, small, diffusive redox mediators or redox polymers create a bridge to shuttle electrons between the enzyme active site and the electrode surface. MET has been widely used as an alternative approach to realize the communication between enzymes and electrodes. Thermodynamically, the redox potential of the mediator should be within the range of the catalytic potential of the enzymes (at least ~50 mV difference is required to provide a sufficient driving force).[252] Mediators may also serve as a co-substrate that can be "recognized" by the enzyme, allowing facile access to the active site. Commonly used redox mediators, such as viologens, quinones, dyes, tetrathiafulvalene, and metal complexes, such as ferrocene, cobaltocene, osmium, ruthenium, and derivatives, are

coupled with a wide range of redox proteins in the applications of bioelectrosynthesis and biofuel cells.[41, 42, 253, 254] Implantable glucose fuel cells that use glucose oxidases and mediators layers to oxidize glucose, solely rely on the reaction of glucose and O_2 for energy supply in the human body (**Figure 9a**).[255] Milton *et al.* described an ammonia-producing H_2/N_2 fuel cell as an alternative to the highly energy-consuming Haber-Bosch process by using methyl viologen as electron donors and acceptors to support the turnover of nitrogenase and hydrogenase (**Figure 9b**).[41] Recently, small redox proteins (*e.g.*, cytochromes) have also been developed as redox partners in electrosynthesis and sensing applications. Cytochrome *c* not only serves as the physiological redox partner of many redox enzymes (e.g., lactate dehydrogenase and sulfite oxidase) but also can shuttle electrons to laccase, bilirubin oxidase, cytochrome P450 reductase, and ascorbate oxidase.[256] Dronov *et al.* co-immobilized bilirubin oxidase and cytochrome *c* in a polyelectrolyte multilayer. The designed protein architecture facilitated electron transfers within the non-natural protein partner matrix for O_2 reduction, which mainly increased the O_2 reduction rate.[257] Free cofactors such as NAD(P)H can be considered as mediators as they involved in electron transfers for the function of most oxidoreductases. Due to the high cost of these cofactors, regeneration strategies are particularly important in industrial enzymatic synthesis. However, because direct regeneration of cofactors on electrode surface requires large overpotential along with the formation of the biologically inactive dimers, the regeneration of cofactors is usually conducted by enzymes (e.g., diaphorase and lipoamide dehydrogenase) and redox mediators (e.g., viologen and cobaltocene) in the construction of a MET based cofactor regeneration system.[258]

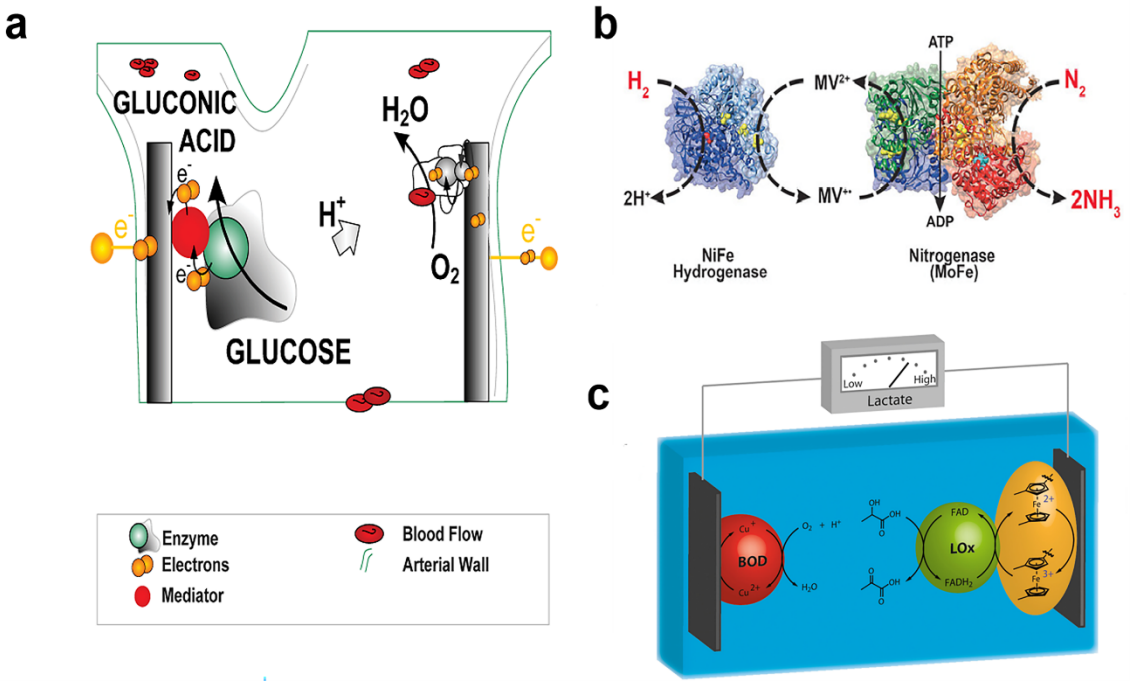

Figure 9. Approaches for mediated electron transfer. (a) Implantable glucose fuel cells. Reprinted with permission from ref. 255. Copyright 2010 MDPI. (b) Ammonia production by nitrogenase and hydrogenase using methyl viologen. Reprinted with permission from ref. 41. Copyright 2017 Wiley. (c) Schematic of a self-powered lactate sensor consisting of a bilirubin oxidase-based cathode and a ferrocene-mediated lactate oxidase-based anode. Reprinted with permission from ref. 263. Copyright 2016 Elsevier.

The drawback of diffusive redox mediators is that they can be toxic to the enzymes and may require separation for downstream applications. Redox polymers are materials that can "wire" enzymes on the electrode surface to create a 3D electroactive matrix in which electron transfer rates through the matrix are high due to self-exchange reactions between the redox pendants.[259, 260] The earliest work of this approach was carried out by Adam Heller and colleagues, where the 3D enzyme-polymer matrix was designed by crosslinking the osmium redox polymer chains with glucose oxidase for biosensor applications.[261] The number of active enzymes electronically linked to the electrode surface was largely increased with this approach, and the resulting enzyme-polymer film was stable, selective, and highly active toward glucose oxidation. Mao *et al.* designed a novel Os^{2+}/Os^{3+} complex

with a 13-atom carbon spacer linking to a polymer backbone. This redox hydrogel successfully wired glucose oxidase with FAD/$FADH_2$ in the active site, and the resulting polymer-enzyme film can electrooxidize glucose at a potential as low as –0.36 V *vs* Ag/AgCl.[262] In another example, a bioanode was constructed utilizing a ferrocene-modified redox polymer mediated lactate oxidase in the application of a self-powered lactate sensor (**Figure 9c**).[263] Polyphenazine and polytriphenylmethane redox polymers deposited with carbon nanotubes are widely used as biosensors in the detection of glucose, ethanol, sorbitol, and H_2O_2 using glucose oxidase, alcohol dehydrogenase, sorbitol dehydrogenase, and horseradish peroxidase, respectively.[259] Redox biofilms, inspired by the architecture of bacteria biofilms, have also been designed by Altamura and co-workers.[264] By self-assembly of a prion domain and a rubredoxin as the electron mediator, the highly organized redox biofilm was able to control the arrangement of redox protein, providing innovative technology for mediated electron transfer via bio-redox polymers.

3.1.2. The Electrical Interface Between Electrodes and Electroactive Microbial Cells. An essential concept in microbial-based bioelectrocatalysis is the electrochemical communication between bacterial microorganisms and electrode surfaces. This interconnection is driven by microbial metabolic pathways of electron transfer. The characteristics of electron transfer rates of microbe–electrode interfaces are critical in determining the reaction efficiencies of microbial bioelectrocatalytic systems. Realizing an effective electrochemical connection between a conductive electrode surface and a specific microbial cell type, qualitatively referred to as electron transfer rate, is reasonably complex as it depends on several factors. Notably, electrode surfaces need to be optimized chemically and morphologically to support fast electron transfer rates. The electrode

surface morphology and electrode chemistry can impact microbial-based bioelectrocatalytic reactions specifically regarding the formation and structure of microbial biofilms, as well as electron transfer mechanisms between microbes and electrodes. Progress in biocatalysis regarding electrode materials and surface adjustments has been made to improve (1) biocompatibility, (2) electrochemical surface area, (3) electron transfer rates, (4) conductivity, and (5) mass transfer between substrates and products.[265] The electrochemical communications between electroactive microbes and electrodes can be adjusted to elucidate these interactions on the nanometer and micrometer-scales.[266]

In facilitating the adhesion of electroactive bacteria on conductive surfaces, an essential property is the hydrophilicity of the electrode, which depends on electrostatic forces, van der Waals forces, and/or hydrogen bonding.[266, 267] Zhang and co-workers have demonstrated that positively charged electrodes assist in the formation of biofilms since the surfaces of electroactive microbes are typically negatively charged.[268] Additionally, Guo and co-workers analyzed the influence of surface hydrophobicity and charge on the biofilm buildup.[267] In this study, researchers investigated glassy carbon electrodes modifications with positive, neutral, and negative charges to construct either hydrophilic (–OH, $–SO_3^-$, $–N(CH_3)_3^+$) or hydrophobic ($–CH_3$) surfaces. Their results showed that positively charged hydrophilic electrode surfaces resulted in enhanced biofilm formation. Similarly, Picot and co-workers used electrode surfaces with positively charged phenylphosphonium cations and observed noteworthy improvements in the generation of anodic currents.[269]

In addition to the hydrophobicity of electrode surfaces, both the electroactive bacterial cell surface characteristics and the electrode morphology (e.g., roughness and porosity)

affect and determine bacterial cell adhesion and subsequent biofilm formation. The electrode surface to which microbes adhere not only acts as a support for the electroactive bacteria but is also involved in microbial metabolism via electron transfer processes.[266] Depending on the nature and identity of electroactive microbe, distinctions are observed in cell surface arrangements, surface charge densities, and polarizabilities, as well as number cellular attachments.[270, 271] The electrode morphology, in terms of its roughness, can provide attachment features for electroactive microorganisms.[272] Furthermore, the potential applied to the electrode can impact the surface charge, electric field, specific ion absorption, and migration and adhesion characteristics of the electrochemically-active microorganism.

Several methods for electrode surface modifications have been introduced, such as substituting 2D with 3D porous electrode material (e.g., felt, fiber brushes, foam), to significantly increase surface areas, thereby enhancing current density per electrode.[266] Utilizing interconnected carbon frameworks with large μm-size pores cannot prevent mass transfer limitations and also allow microbial organisms to penetrate through the porous structures and colonize biofilm.[273] Recent analyses of electrode surface properties (e.g., porosity, roughness) by Santoro and co-workers examined polytetrafluoroethylene-treated carbon paper electrodes. Their results demonstrated a positive relationship between electrode surface porosity (5–10 μm) and the number of bacterial cells anchored.[274] Extracellular polymeric substances, which are fundamental 3D structural components determining the characteristic of biofilms, also need to be considered.[275] Electrodes to which microbial cells attach are both electrochemically and metabolically unique environments for bacteria;[276] thus, the effectiveness of the bacteria-electrode

interconnection is impacted by various factors.[276] The electrode-microorganism interactions for selected electroactive microorganisms are discussed in previous subsections, specifically in terms of direct electron transfer. For the curious and engaged readers, we point to various extensive reviews on further details about electron transfer chains and mechanisms of well-studied electroactive bacterial cells.[85, 111, 134, 227] Since only very few microorganisms have to-date shown efficient direct electron transfer pathways, mediators have been used as electron shuttles to improve electrode-microbe communication.[277, 278] These types of electron transfer processes are discussed in detail in the next subsections in this review article.

3.2. Electrodes

Bioelectrocatalysis couples oxidation-reduction (redox) reactions (homogeneous) and electrode reactions (heterogeneous).[279] Electrodes represent not only the electrical current collectors but also a support base for the biological catalyst and a platform where the redox reaction occurs. Therefore, adequate electrode design, choice of electrode material, architecture, and physiochemical characteristics of electrode materials, all play a critical role in the biocatalyst-electrode interaction, which determines the overall performance of the bioelectrocatalytic system. Advanced electrode material designs with new structures, architectures, characteristics, and functionalities have been developed to enhance electrochemical communication between biocatalysts and electrodes.[280-288] An ideal electrode material should provide excellent electrical conductivity and long-term electrochemical stability. Additionally, it should have a biocompatible surface and a large accessible electroactive area for either redox enzymes or electroactive microorganisms. Excellent mechanical strength, low-cost, minimal environmental impact, corrosion

resistance, and scalability are other preferred electrode material properties.[266, 289-293] Herein, we discuss electrode materials that are commonly employed in bioelectrocatalytic systems. We also provide a detailed overview of electrode modification methods and biocatalyst immobilization strategies for both enzyme-based and microbial-based bioelectrocatalysis.

3.2.1. Electrode Materials. In order to meet the aforementioned electrode requirements for numerous bioelectrocatalysis applications, a wide range of electrodes have been developed, including gas diffusion electrodes,[294] graphite fiber brush anodes,[295] carbon-based fleece,[296] stainless steel,[297] carbon-based foams,[298] carbon nanotubes,[299] free-standing electrospun carbon nanofibers,[300, 301] graphite plates,[269] carbon paste and carbon paper,[302] and carbon nanochips.[303] Additionally, metallic electrode surfaces for bioelectrocatalytic systems are mainly based on gold, yet silver, copper, nickel, iron, titanium, and certain metal oxides have also been reported.[304-307] Due to their corrosive nature and high costs, certain metal electrodes are not commonly used.[308] Other metallic electrodes, which are made of gold, stainless steel, and titanium, are not corrosive. However, their smooth surfaces do not provide good adhesion for biological catalysts, which leads to subsequent low power and current densities.[309] In terms of enzyme-based bioelectrocatalytic systems, metallic gold electrodes are commonly employed,[310] and metal modification strategies are utilized to prevent potential enzyme conformation changes resulting from interactions of specific amino acids with bare metal electrodes.[304, 311, 312] For instance, studies performed with cellobiose dehydrogenase,[313] copper efflux oxidase,[314] and human sulfite oxidase,[315] have shown that self-assembled monolayers on metal surfaces are a great platform for bioelectrocatalysis. For microbial-based

bioelectrocatalytic systems, stainless steel is a promising material due to its inexpensiveness, resistance to corrosion, and high electrical conductivity. Yet the passive layer of this electrode material results in low biocompatibility, thus limiting the electron exchange between microorganisms and electrodes.[316] Carbon-based electrodes, including carbon paper, carbon cloth, carbon crush, carbon felt, carbon mesh, and carbon nanotubes, are among the most widely used electrodes for both enzymatic and microbial bioelectrocatalytic systems as they fulfill the majority of electrode material requirements.[317, 318] For interested readers on carbon-based electrodes and nanostructures, the following review articles are suggested.[319-321] For instance, porous carbon electrodes, with defined pore sizes and ability to facilitate electron transfer, have been shown to be adequate for the wiring of *E. coli*.[322] Additional research findings have reported usage of graphene-based electrodes for wiring both *E. coli* and *P. aeruginosa*.[323, 324] The use of these graphene electrodes resulted in enhanced electron transfer rates via (1) an expression of mediator molecules facilitating bacteria-electrode connection and (2) a favorable impact on bacterial growth rates. Although an abundance of new electrode materials has been developed, a single component cannot meet all electrode material requirements in most cases. While carbon is most commonly used as an electrode material, it gives the electrode surface hydrophobic features. This property often causes poor adhesions of bacterial colonies and/or redox proteins, resulting in a limited ability for electron transfer.[282] Consequently, various strategies to immobilize redox enzymes on electrodes, as well as electrode surface modifications with metal oxide nanocomposites and/or conductive conjugated polymers, have been developed to improve electron transfer kinetics, biocompatibility and/or bacterial cell adhesion.[282, 285]

3.2.2. Strategies for Electrode Modification and Bioelectrocatalyst Immobilization.

An effective strategy to improve the performance of bioelectrocatalytic platforms is to effectively modify the chemical and physical properties of electrodes to provide a means for enhanced bioelectrocatalysis attachments and fast electron transfer rates. The standard modification strategies to promote better electron transfer include gas treatments,[325] cyanuric chloride,[326] chitosan,[327, 328] melamine,[329] and 3-aminopropyltriethoxysilane.[330] Thermal treatment of electrodes allows for adjustments of surface porosity and roughness, which improves cell concentration and biofilm formation in the case of microbial bioelectrocatalysis.[331-333] These thermal surface modifications differ depending on the type of gas atmosphere used (e.g., oxygen, nitrogen, ammonia), and allow for the addition of hydrophilic functional groups on the electrode surface.[334] Additionally, chemical treatments are employed to introduce nitrogen- and oxygen-containing functional groups to promote enhanced biocatalyst attachment to electrode surfaces.[335-337] In chemically modifying carbon-based electrodes, numerous compounds, including nitric acid,[337] ammonium nitrate,[338] ammonium persulfate,[338] ethylenediamine,[337] 4 (N,N-dimethylamino) benzene diazonium,[336] and polyaniline,[335] have been utilized. Furthermore, thin metal layers, such as gold, palladium, and nickel coatings, have successfully been integrated to reduce the activation energy of electron transfer rates. Nanomaterial modifications (e.g., carbon nanotubes, nanoparticles) provide 3D conductive frameworks for enzyme attachments and growth of electroactive bacteria.[286, 339] Meanwhile, electrode modification strategies using redox polymers composed of the non-conductive backbones with redox-active side chains, have recently gained attention. Based upon the self-exchange-based electron conduction, these redox polymers can act as

electron shuttles.[41, 42] While most redox polymers are characteristic outer redox sphere species, such as ferrocene and transition metal complexes, many of these are organic redox molecules, including viologens, quinones, and 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO).[340] Bioelectrocatalytic systems utilizing carbon nanotube-polymer hybrids provide two significant advantages: (1) improved strength and electrical conductivity, and (2) a 3D nanostructure framework with a sizeable electroactive area, due to the exceptional properties and unique geometries of these hybrid structures.[259] In the following subsections, we discuss immobilization strategies for enzyme-based bioelectrocatalysis and electrode modification methods for microbial bioelectrocatalysis.

3.2.2.1. Enzymatic-based Electrode Modification and Immobilization. Efficient electrical communication between an enzyme and target electron acceptors is one of the key factors to optimize the performances of applicable enzymatic bioelectrocatalytic systems (EBS) such as bioelectrosynthesis,[41, 42] photosynthesis,[341] biofuel cells,[31, 342-350] biocapacitors,[351-353] and biosensors.[354-356] In general, oxidoreductases have cofactors embedded in a protein matrix, thereby shielding the electric conductance with the electrode and requiring a long-distance electron tunneling. The enzyme active site and electrode surface can be considered as donor-acceptor pair, and the electron transfer (ET) rate constant (k_{et}) between them relies on the ET tunneling distance [357, 358] (Eq. 1):

$$k_{et} = k_0 \exp\left(-\frac{\Delta G^{\neq}}{RT}\right), \text{where } k_0 = 10^{13}\exp\left(-\beta(r - r_0)\right) \quad (Eq.\,1)$$

where $\Delta G^{\neq}$ is the activation energy for ET, R is the gas constant, T is the absolute temperature, k_0 is the transmission coefficient for electron transfer at a fixed separation distance r of the donor-acceptor pair, β is the electron-coupling constant, r is the distance

between donor and acceptor, and r_0 is the van der Waals distance. Furthermore, the electromotive force between the enzyme cofactor and electron donor/acceptor[359] is represented by Eq. 2 and Eq. 3:

$$\Delta G = -nFE \quad (Eq. 2)$$

$$E = E^0 + \frac{RT}{nF} ln \frac{[O]}{[R]} \quad (Eq. 3)$$

where *G* is the Gibbs free energy, *n* is the number of electrons, *F* is the Faraday constant, *E* is the potential in terms of electromotive force (between the enzyme cofactor and electron donor/ acceptor), E^0 is the potential of the species at standard conditions, *O* is the oxidized species and *R* is the reduced species.

To achieve a facile and efficient ET of a given enzymatic system, three conditions must be established: (1) maintain the intrinsic properties of enzymes, (2) a high concentration of enzymes on the electrode surface, and (3) an efficient electric connection between the enzyme and the electrode are necessary. Immobilization, a strategy for placing a protein on an electrode surface, simplifies the diffusion process, minimizes the distance between the enzyme and the electrode surface, and increases the concentration of enzymes available for electron transfer on the electrode surface.[360]

There are two major categories of enzyme immobilization, namely (1) adsorption and (2) entrapment, both of which are shown in **Figure 10**. Adsorption of enzymes[361] on the electrode surface is an immobilization technique, which relies on intermolecular forces, such as dipole-dipole interactions, van der Waals forces, ionic interactions, and hydrogen bonding (**Figure 10a**). While this method retains the native enzyme structure and its enzymatic activities, it is usually relatively short-lived due to the weak intermolecular forces and selectivity towards enzymes that establish favorable affinity with the electrode

surface. Thus, modifications of the enzyme or the electrode surface (**Figure 10b**) or enzyme (**Figure 10c**) have been examined to establish a stronger enzyme-electrode affinity.[362] On the other hand, entrapment is an immobilization technique using polymeric structures (**Figure 10d-e**). The entrapment does not require a specific affinity between enzymes and polymeric structures, since it either covalently or noncovalently encapsulates enzymes in polymeric structures.[363, 364] Therefore, a variety of enzymes can be immobilized on the electrode surface. However, these entrapment strategies involve chemical cross-linking and rigid polymeric structures that significantly denature the enzyme structure and reduces its corresponding activity.

This variety of immobilization techniques enables scientists to choose the most suitable immobilization method for the selected enzymatic ET pathways (e.g., DET and MET). For a detailed discussion on specific ET pathway mechanisms, we refer readers to section 3.1 in this review article. Here, different types of enzyme immobilization are discussed, depending on the chosen ET pathway.

DET is heavily dependent on the achievement of close proximity between the enzyme and the electrode surface; specifically, the enzyme active site should be within 14 Å from the electrode for efficient DET.[71] Therefore, smaller enzymes are more likely to establish DET. To increase the rate of DET for more bioelectrocatalytic applications, adsorption via enzyme modification and electrode modification and entrapment via polymers have been studied and employed.

Adsorption of enzymes for DET can offer control over enzyme orientation to enhance the by creating a docking site.[365] For instance, Meredith and co-workers studied a substrate mimicking docking system with anthracene modified multi-walled carbon nanotubes (An-

MWCNTs)[366] and laccase. Since laccase has a substrate affinity towards aromatic groups, an aromatic moiety like anthracene acted as a docking site for laccase. Also, An-MWCNT established π-π stacking with the carbon electrode as an anchor for the electrode adsorption. However, this type of docking mechanism showed only 2% of electrochemical activity and required knowledge of the specific substrate-enzyme affinity. Thus, a different kind of docking system, such as modification directly on the electrode surface or on the enzyme was investigated to minimize the loss of enzymatic activities contributing to the overall EBS performance. Additionally, the Bilewicz research group has reported tailored, naphtylatene-modified single-walled carbon nanotubes (SWCNTs) to improve the direct communication of laccase with electrode surfaces.[367] Blanford and co-workers described a promising strategy to attach laccase to carbon electrode surfaces via use of the diazonium coupling reaction to provide aromatic functionalities that can bing to hydrophobic residues near the copper site.[365]

One example of adsorption-based immobilization that establishes a docking-anchor system to the electrode without a significant loss of enzymatic activity is a direct modification of multi-subunit enzymes.[368] Lee and co-workers engineered subunits of glucose dehydrogenase (GDH) on either N- or C- terminus with a site-specific gold binding peptide (GBP)[195] to immobilize enzymes while tuning the orientation on the gold surface (**Figure 4d**). Correspondingly, FAD-dependent GDH engineered with GBP to bind with highly tunable GNP arrays[369] implemented nanopatterning of enzymes for spatially controlled immobilization. Additionally, maleimide-modified gold nanoparticles (GNPs) were site-specifically attached to glucose oxidase (GOx).[196] Holland and co-workers engineered certain amino acids around the active site of GOx to cysteine in order to

recognize and bind the maleimide pre-modified on the GNPs via gold-thiol bonds. Thus, GOx-maleimide-GNP achieved DET by orienting the enzymes towards the gold electrode surface, as demonstrated in **Figure 11a**. Moreover, GNPs can also be used without enzyme modification. For instance, Ratautas and co-workers modified GNPs with 4-aminothiolphenol (4-ATP), since 4-ATP forms a quinone upon oxidation that can form a Schiff base with primary amine groups of enzymes for immobilization.[370] Small electron transferring enzymes can also substitute for GNPs. Algov and co-workers engineered cytochrome c to FAD-dependent GDH (FAD-GDH)[371] for DET as cytochrome c acted as an anchor connected to FAD-GDH. While the docking-anchor type of immobilization via modification of enzymes offer tuning of enzyme orientation to enhance DET rate without a significant loss of enzymatic activities, typically, these methods are best optimized using pre-established enzyme sequences, which are rarely known for novel enzymes. Thus, modification of the electrode surface was studied as s an alternative to sequential understanding of enzymes.

For electrode modification involving immobilization methods, the most commonly used electrode material is gold due to its ease in modification. Lee and co-workers modified gold electrode surfaces with thiol-containing chemicals, such as dithiobis(succinimigyle hexanoate) (DSH), dithiobis(succinimidyl octanoate) (DSO), and dithiobis(succinimidyl undecanoate) (DSU).[372] The thiol group of these chemicals formed chemical bonds on the gold surface due to the preferred affinity of gold-thiol bonds. The terminal succinimidyl group of these chemicals covalently bonded to the amino group of the FAD-dependent GDH, thus, creating an anchor on the gold surface and a docking site with enzymes for a complete adsorption immobilization. However, this method selectively works on surfaces

prone to modification, thereby limiting the choice of electrode materials. For more versatile immobilization that does not require fundamental knowledge of the chosen enzyme and/or specific electrode material, enzyme entrapment is employed.

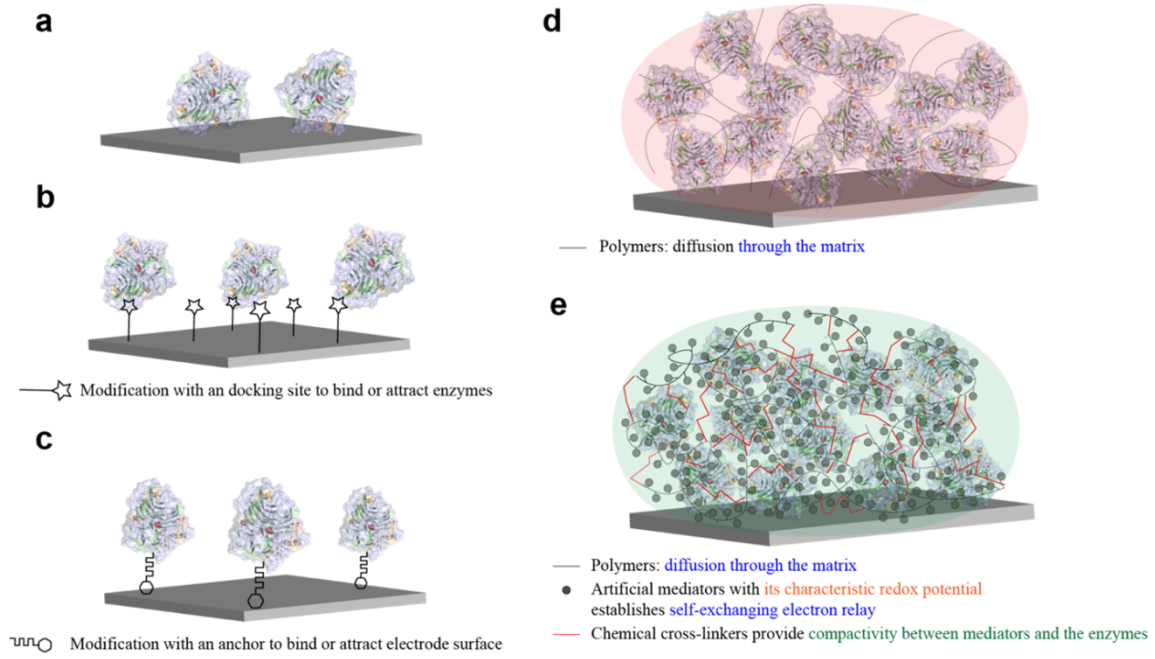

Figure 10. A scheme of enzyme immobilization techniques used in enzymatic bioelectrocatalysis systems (EBS). Enzyme adsorption immobilizes enzymes via intermolecular forces (a) that are short-lived. In order to enhance the adsorption rate, electrode modification (b) with a docking site for enzymes to bind or enzyme modification (c) with an anchor for electrodes to bind to can be engineered. A combination of both approaches can also be applied. Typically, these modifications allow for fine-tuning over the enzyme orientation, modifying either the electrode surface or the enzyme for a docking-anchor type of immobilization. Enzyme entrapment (d), on the other hand, immobilizes enzymes by ensnaring enzymes within polymers and small molecules that can still diffuse in and out of the polymer matrix. These polymers can be modified with artificial redox polymers (e) to establish mediated electron transfer. Blue, orange, and green texts represent characteristics affecting the current output, redox potential of EBS, and both, respectively.

Most of the enzyme entrapment methods focus on the random orientation of enzymes that relies on the probability of enzymes orienting correctly towards the electrode surface. Hickey and co-workers grafted pyrene moieties onto a polymer backbone (pyrene-LPEI).[251] This pyrene moiety approach uses the π-π stacking of pyrene to bring the

enzymes closer to the electrode surface. Thus, it is often coupled with carbon-based electrodes to maximize the π-π stacking. A simple polymer entrapment of enzymes with polymers like pyrene-LPEI is versatile and can be produced on the gram-scale. Other non-pyrene based polyelectrolyte electrode coating of cationic poly-guanidinyl-propyl-methacrylate (pGPMA, PG), as well as anionic inorganic polyphosphate sodium hexametaphosphate (P6), have been used to immobilize laccase on a carbon surface for DET, (**Figure 11b**).[373] The caveat of using polymers is the decrease in conductivity or an increase of resistance due to the non-conducting polymer backbone. Thus, hybrid composites of polymers and conductive materials can be used. For example, a hybrid nanocomposite of graphene and chitosan was used for enhanced conductivity for hemoglobin entrapment (**Figure 11c**).[374]

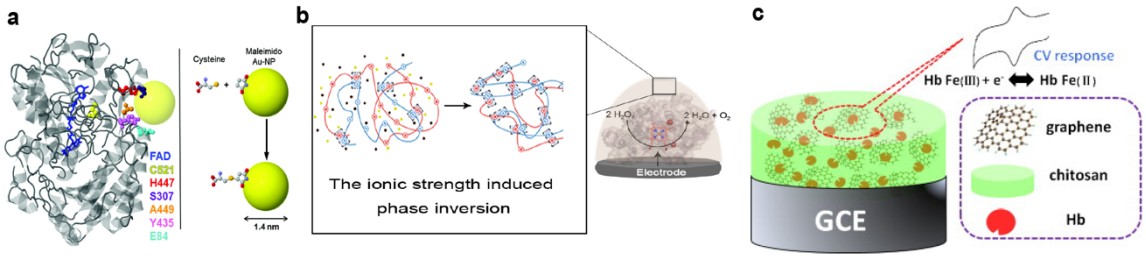

Figure 11. Adapted and modified schemes of gold modification of enzymes for adsorption and polymers for enzyme entrapment for direct electron transfer (DET). (a) Maleimide modified gold nanoparticles binding with cysteine modification. Reprinted with permission from ref. 196. Copyright 2011 American Chemical Society. (b) Enzyme entrapment with a nanocomposite of chitosan and graphene to increase conductivity. Reprinted with permission from ref. 373. Copyright 2020 Royal Society of Chemistry. (c) Polyelectrolyte coatings for a simple enzyme entrapment. Reprinted with permission from ref. 374. Copyright 2010 Elsevier. The cysteines (a) into glucose oxidase (GOx) were engineered as an anchor on gold surface and gold nanoparticles (GNPs), respectively. The enzyme modification was entirely avoided by entrapping enzymes within polymers. A polyelectrolyte coating (b) and a biopolymer (c), chitosan, were used to entrap enzymes on the electrode surface.

Conclusively, we have discussed three major parameters to consider when immobilizing enzymes for DET, namely (1) enzyme engineering with binding peptides to create an

anchor for the electrode surface, (2) electrode modification to create a docking site for enzymes and (3) enzyme friendly polymers for enzyme entrapment. However, regardless of many discoveries in immobilization for DET, establishing DET for bulkier enzymes is challenging as these enzyme types are inherently hindered from DET. Thus, immobilization for the mediated electron transfer (MET) system is discussed in the following subsection.

The benefit of establishing MET is based on its versatility, specifically one enzyme could use different mediators, and one mediator can facilitate MET of numerous enzymes. Therefore, the coupling of a correct mediator with the chosen enzyme can result in a low-overpotential and fast ET pathway. Furthermore, high concentration and effective wiring of the enzymes on the electrode surface via immobilization leads to a higher catalytic current density of the bioelectrochemical system (BES), thereby enabling higher power density biofuel cell applications. By definition, MET involves the use of artificial redox-active mediators as electron shuttles to enhance ET rates. Typically, reduction-oxidation stable dyes or organic compounds are used depending on the operational potential required for the chosen enzyme.[375] These mediators can be used as in-solution mediators or grafted onto an inert polymer backbone as a redox polymer.

The simplest and easiest method of establishing MET is to adsorb enzymes on the electrode surface via intermolecular forces and additionally incorporate an artificial mediator. For instance, 1,2-benzoquinone and 1,4- benzoquinones served as in-solution mediators to facilitate MET of pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) for a higher-performing MET by shortening the distance between the electrode surface and the PQQ active site with mediators (**Figure 12a**).[376]

Even when used as in-solution mediators, these mediators were adsorbed onto the electrode surface. While 1,4-benzoquinones operated as a mediator, 1,2-benzoquinone acted as an orienting agent, bringing the PQQ active site closer to the electrode surface. In this case, two mediators mediated one enzyme, but one mediator could be used for numerous enzymes. A four-enzyme cascade of nitrogenase, diaphorase, L-alanine dehydrogenase, and ω-transaminase with methyl viologen (MV) as an in-solution mediator facilitated MET of bioelectrosynthesis of a valued pharmaceutical precursor at –0.7 V *vs* SCE.[377] On a more positive potential range, Toluidine blue O (TBO) aided MET of aldehyde deformylating oxygenase at around –0.3 V *vs* SCE as an in-solution mediator.[378] These setups are relatively easy to assemble, but these in-solution mediators must to be reintroduced into the system every time a setup is changed, which further complicates any separation of enzymes and mediators in the solution for product analysis. Therefore, enzyme-mediator immobilization methods have gained a spotlight.

A docking system similar to DET can immobilize the enzyme on the electrode surface, but still utilize mediators to facilitate MET. This docking system makes product separation easier and increases the recyclability of both the enzymes and the mediators used. Patel and co-workers used pyrene modified nickel-complexes (Py-KDDD) to dock histidine-tagged nitrogenase and facilitate MET with MV as an in-solution mediator (**Figure 12b**).[37] In this study, a Nafion film entrapped nitrogenase on the electrode surface, and Py-KDDD acted as a docking site of nickel complex for nitrogenase while ensuring in the proximity to the electrode surface with the π-π stacking of pyrenes. Beyond MoFe nitrogenase, this method also offers the possibility to graft a wide range of His-tagged proteins. For a more specific docking system, a pyrrole-(2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)-

-pyrene (pyrr-(ABTS)-py) modification on carbon nanotubes immobilized both laccase and ABTS, the mediator to facilitate MET at 0.5 V *vs* SCE.[379] This pyrr-ABTS-py utilized a substrate docking system of pyrene for laccase and the electron mediation through the immobilized ABTS. Similar to the docking systems of DET, the MET docking system also requires previous knowledge of enzyme-substrate affinity or protein sequences that do not favor the utilization of newly found enzymes.

An alternative enzyme-mediator immobilization is the use of redox polymers where mediators are grafted onto inert polymeric backbones as a pendant. These pendants establish self-exchanging electron relays, conducting the electrons across the polymeric backbone. Milton and co-workers grafted naphthoquinone pendant onto the backbone of linear polyethyleneimine (NQ-LPEI) to facilitate MET for FAD-dependent GDH (**Figure 12c**).[380] Having multiple NQ pendants on LPEI created a self-exchange system of electron relays between NQ pendants, giving a higher power output. With a simplified diffusion system and highly concentrated surface area of mediators gave a facile MET. Other redox polymers with different pendants offer MET of different enzymes such as cobaltocene (**Figure 12d**),[258] and benzylpropylviologen[381] for MET of diaphorase and dimethylferrocene[382] for MET of FAD-dependent GDH. Unlike the docking mechanism, redox polymers give the versatility of immobilizing numerous enzymes for MET without requiring a fundamental knowledge of the ET of the chosen enzyme systems.

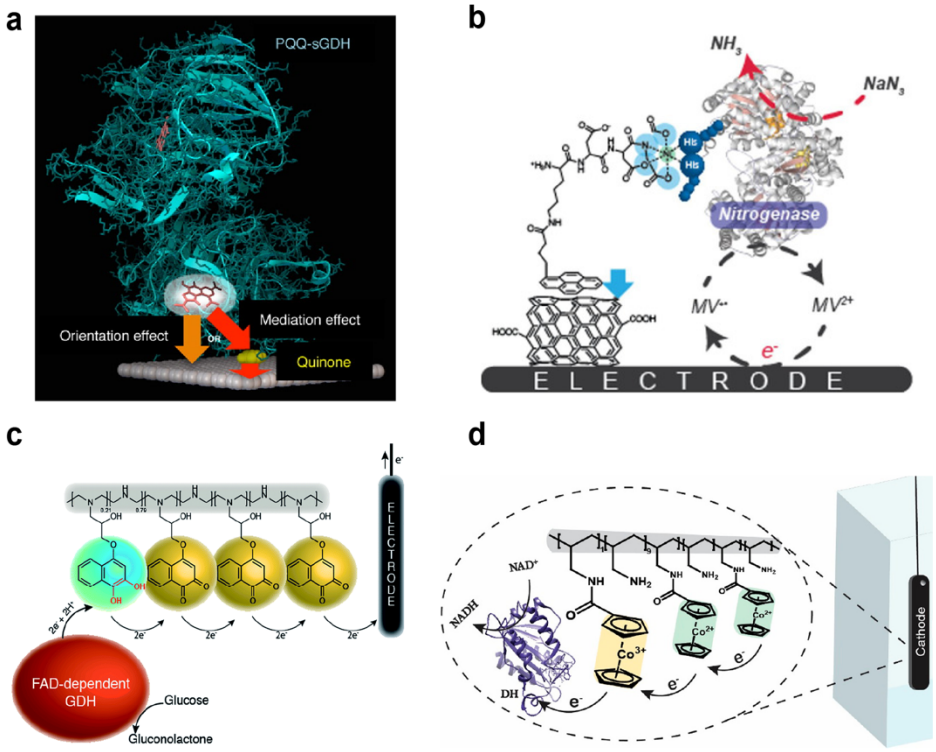

Figure 12. Adopted and modified diagrams of in-solution mediators with different enzyme immobilization and redox polymers, showing its self-exchanging electron relay. (a) In-solution 1,2-benzoquinone and 1,4- benzoquinones for PQQ dependent glucose dehydrogenase. Reprinted with permission from ref. 376. Copyright 2015 American Chemical Society. (b) Pyrene modified nickel complex to dock His-tagged nitrogenase. Reprinted with permission from ref. 37. Copyright 2019 Wiley. (c) Napthoquinone modified linear polyethyleneimine. Reprinted with permission from ref. 380. Copyright 2015 Royal Society of Chemistry. (d) Cobaltocene modified poly(allylamine). Reprinted with permission from ref. 258. Copyright 2019 American Chemical Society. Here, the in-solution mediators used were 1,2-benzoquinone and 1,4- benzoquinones (a), which acted as an orienting agent and an electron shuttle, respectively, after being absorbed onto the electrode surface. Methylviologen was used as a soluble mediator (b), nitrogenase was immobilized with Nafion and docked to the nickel complex linked to pyrene. The pyrene moiety acted as an anchor to the carbon surface and the nickel-complex docked the his-tags of nitrogenase, further enhancing the immobilization stability. In order to increase the recyclability of mediators, naphthoquinone (c) and cobaltocene (d) were grafted onto the inert polymer backbone as redox polymers. Both redox polymers facilitate MET by self-exchanging electron relay.

In addition, redox polymers can serve as a protection against oxygen for oxygen-sensitive enzymes,[383, 384] unwanted contributions from DET of contaminants, and high

potential deactivations,[385] that are limiting factors in further improving enzyme-based applications.[386] Specifically, Szczesny *et al*. built a bioanode using the viologen modified redox polymers to mediate hydrogenase.[387] Similarly, Ruff and co-workers used the same redox polymer to mediate a hydrogenase variant for oxygen protected bioanodes.[388] These polymer matrices work as mediation layers, and also protect against oxygen from the gas breathing setup and the detrimental overpotential that may deactivate the enzyme functionality.

Conclusively, MET offers an ET for bulkier enzymes that are inherently hindered from establishing DET. MET is a very versatile platform where one mediator can be used for multiple enzymes, or one enzyme can be coupled with several specific mediators depending on the operating potential. Thus, two major parameters should be considered for MET: (1) operating potential of enzymes and mediators, and (2) the need for enzyme-mediator immobilization such as redox polymers. For the use of redox polymers, please refer to previous subsections on the limitations and requirements of entrapment.

3.2.2.2. Microbial-based Electrode Modification. Electron transfer efficiency at the microbe-electrode interface plays a pivotal role in the performance of bioelectrochemical systems. As discussed previously, it is essential to optimize the topography and chemistry of electrode surfaces to provide fast electron transfer rates, which can be achieved via the selection of electrode material, and chemical and/or morphological alterations of electrode surfaces.[91, 94] These modifications facilitate attachment of electroactive microbial cells and biofilm development, therefore optimizing the electrochemical communication between bacterial cells and electrodes. Additionally, the chemical properties of the electrode

surface, along with biofilm formation, provide a means for improved electron transfer rates from bacterial microorganisms to electrodes.[389]

Carbonaceous electrodes, including carbon cloth, carbon felt, carbon paper, carbon mesh, and carbon nanotubes are amongst the most extensively used electrode materials in microbial-based bioelectrocatalysis.[317, 318] Besides their recognized antibacterial surface properties,[390-392] graphene materials have also been successfully used is bioelectrocatalytic schemes as they provide large electroactive area, conductivity, and sturdiness.[393-397] Although carbon is a widely used electrode material, its hydrophobic surface properties minimize cell adhesion, which results in limited electron transfer kinetics.[282] Therefore, carbon-based surfaces are often modified with metal oxide nanocomposites and conductive polymer conjugates to promote bacterial attachments and enhance electron transfer abilities.[282, 285] Zou and co-workers showed a successful combination of graphene oxide with titanium dioxide (TiO_2) nanocomposites, providing suitable conductive and hydrophilic characteristics, for an improved bioelectrocatalytic system with fast direct electron transfer kinetics and enhanced *Shewanella putrefaciens* growth.[398] Glassy carbon electrodes have been modified with multiwalled-carbon nanotubes doped with tin (IV) oxide (SnO_2) nanocomposites to significantly improve the performance of a microbial fuel cell using *E. coli*.[399] In addition, Zhu and co-workers reported modification of graphene nanosheets with indium tin oxide (ITO) and poly(allylamine hydrochloride) by self-assembly in a layer-by-layer fashion, which improved the conductive capability between bacterial cells and electrode, subsequently resulting in elevated electricity outputs.[400] Research findings have also reported the design of carbon cloth tailored with hematite (Fe_2O_3) nanocomposites as a high-performing electrode material for wiring *S.*

oneidensis.[401] This modification provides better contact of outer membrane *c*-type cytochromes and nanocomposite-modified electrode surface, resulting in enhanced conductivity and improved extracellular transfer efficiency. Luo and co-workers designed an electrode platform combining reduced graphene oxide and chitosan nanocomposites to provide a greater number of activation centers for *E. coli* adhesion, which resulted in better electrochemical activities and apparent direct electron transfer activity.[402]

Conductive polymers are also used as doping materials for electrodes, providing better bacterial adhesion. In addition, environmental durability and improved electrode performances have been observed when polymers were doped with nanomaterial composites.[282] In a study by Cui and co-workers, electropolymerization of polyaniline on microporous graphite felt resulted in a hydrophilic surface for the attachment of *S. putrefaciens* and a further controlled, electrophoretic deposition of carbon nanotubes increased both the electroactive area and conductivity.[403] In another study, Roh and Woo performed *in situ* chemical polymerization to dope polypyrrole on carbon nanotubes in order to create a carbon nanotube-polymer complex, which was subsequently deposited on carbon felt electrode.[404] This conducting polymer composite was employed as an anode in a microbial fuel cell, which resulted in decreased resistance and higher power density. The use of poly(3,4-ethylenedioxythiophene) has been reported to increase the electrochemical activity of modified carbon cloth electrodes with wired *Shewanella loihica*.[405]

Osmium redox systems (or similar redox polymers) have also been employed to wire bacterial microorganisms to electrodes in bioelectrochemical devices. The utilization of osmium polymer systems as electron shuttles offers possibilities to examine several microbial-based bioelectrocatalysts, including non-electroactive bacterial cells, for

bioanodes and biocathodes in bioelectrochemical platforms. The Gorton research group was the first to report a study on osmium systems for wiring of microbial cells.[406] In a pioneering study by Vostiar and co-workers, efficient electrochemical wiring was established between *Gluconobacter oxydans* and gold electrode surface with osmium redox system I, characterized by a short side chain and a high redox potential.[305] The efficient electrical connection was a result of electron transfer between the redox polymer and *G. oxydans*'s membrane-bound pyrroloquinoline quinone (PQQ)-containing dehydrogenases, able to oxide a variety of organic substrates. In another study from the Gorton group, Timur and co-workers reported the use of two osmium polymer systems I and II, which had lower redox potential and longer side chains providing motion flexibility, for the wiring of *P. putida* and *Pseudomonas fluorescens* on gold electrode surfaces.[407] In a follow-up study, the researchers demonstrated the use of carbon nanotube-modified carbon paste with osmium redox polymer system to design a microbial-based biosensor, using *P. putida*, for the detection of phenol.[408] Alferov *et al.* reported successful electrochemical communication between graphite electrodes containing osmium redox systems and cytochrome-enriched *E. coli* strains.[409] The use of these redox polymer systems I and II to establish contact with the electrode has also been demonstrated with Gram-positive *B. subtilis*. In this case, the polyanionic characteristics of the cell membrane, namely peptidoglycan and teichoic acids, likely contribute to interactions with the polycationic redox polymer systems, thereby allowing for electrochemical connection. Moreover, the Gorton group has reported the wiring of the purple bacterium *Rhodobacter capsulatus* with redox system III and examined the application of this system with biofuel cells and photobioelectrochemical platforms.[410] This study demonstrated that the bacterial

lipopolysaccharide enhanced the stability of the redox polymer matrix on the graphite and gold electrode surfaces. Patil and co-workers have also shown the modification of graphite electrode surfaces with osmium polymer system to improve the current generation in well-studied *S. oneidensis* MR-1.[411]

3.3. Reaction Medium

3.3.1. Single-phase Reaction Medium. Enzymatic biocatalysis is usually a type of homogeneous catalysis as most enzymes are dissolved in the aqueous phase, and hence the reaction medium is often water. There are two significant limitations of aqueous monophasic catalysis: (1) substrate solubility and (2) catalyst reuse. Many organic substrates or intermediates are poorly soluble in the aqueous reaction medium. They may also display inhibitory effects towards the enzymes, which inevitably leads to the loss of catalyst activity and productivity. The first problem can be addressed by adopting nonaqueous monophasic mediums (e.g., organic solvent, supercritical fluids, and gaseous solvents).[412] This alternative catalysis medium can increase the solubility of the nonionized formed substrates at the cost of partial inhibition of the biocatalyst. Although nonaqueous monophasic enzymatic catalysis is feasible with cautious system design, such as an adequate choice of solvent type and the development of a more stable, yet active, enzyme derivative like multipoint covalent attachment, the solvent inhibitory effect on the enzyme is not negligible.[413] Another possible solution is to add a co-solvent like dimethyl sulfoxide (DMSO) or ethanol to facilitate the dissolving process of the hydrophobic substrate in the aqueous phase.[414] However, the maximum loading capacity of the substrate is still minimal, and this homogeneous catalysis system cannot address the substrates or intermediates inhibitory effects towards the biocatalyst nor the recycling issue of the biocatalyst. As such,

it is not an ideal solution for biocatalysis involving cofactor regeneration, or product synthesis that require complex, multi-enzymatic metabolic pathways.

3.3.2. Biphasic Reaction Medium. A better way to tackle these challenges is to separate the biocatalyst and substrates into two immiscible phases.[415, 416] Usually, biphasic systems contain an aqueous phase where the enzymes dissolved in and an added nonaqueous phase as a reservoir for substrates, intermediates, and end-products.[417-420] The added phase can also be an aqueous phase consisting of a polymer solution with a buffer solution or two different sorts of polymer solutions.[416, 421] Other less common solvent combinations are fluorous solvents,[422] supercritical CO_2,[423] and ionic liquids.[424, 425] The use of aqueous/apolar medium biphasic systems not only addresses the issue of poor substrate solubility in conventional homogeneous catalysis but also maintains a low level of possible inhibitory compounds in the reaction phase. Additionally, the end-product can be extracted to the apolar phase *in situ* as the bioconversion occurs, shifting the thermodynamic equilibria to enhance the productivity as well as to simplify the downstream separations for product processing and biocatalyst recovery. However, the solvent selection with both suitable physicochemical properties and appropriate biocompatibility is yet a challenging area.

Two of the central considerations for solvent selection are high biocompatibility and product recovery capability. Other criteria such as high stability, fewer emulsions with the aqueous phase, non-biodegradability, non-hazardous to human and environment, and low market price are also worth consideration. Efforts have been made to correlate the biocompatibility of a variety of solvents to their physicochemical characters, including dielectric constant, dipole moment, polarizability, and the polarity of the solvent.

Pioneering work by Brink and Tramper described the first rules to classify biocompatibility of organic solvents for multiphasic biocatalysis.[426] In this study, the Hildebrandt solubility parameter (δ) was used to correct the polarity of the solvent, and they discovered that only relatively apolar (δ<8) solvents with a molecular weight above 150 are generally appropriate for organic biosynthesis. However, the Hildebrandt solubility parameter is not a good indicator of solvent polarity as its value relies significantly on the latent heat of vaporization of the solvent which depends on polar interactions and the current experimental information is not yet adequate to assess their validity to estimate solvent biocompatibility through polarity. Over the years, other parameters were adopted for correlation with solvent biocompatibility. Laane *et al.* used the Hansch factor (logarithm of the partition coefficient) of the solvents to correlate their bioactivity.[427] Strictly speaking, the Hansch parameter denotes more of hydrophobicity than polarity; nonetheless, it exhibited an improved correlation of the solvents with the catalytic rates of the biocatalyst in it. This method has been widely employed in the pharmaceutical industries as a part of drug activity studies.[428]In the screening of solvents, another issue that cannot be ignored is the extraction efficiency of the solvent for the target product. The product recovery capacity can be quantified by the partition number, which is defined as the ratio of compounds concentration in the organic phase with that of the water phase. The higher the partition coefficient is, the more efficient is the product recovery. For the physical solvents extractive process, the interaction between the product and the solvents is governed by the solvation process through various sorts of unspecific and weak donor bonds. While with the chemical solvents extractive process, a specific and robust relationship or even a new compound will be formed between the product and the solvents.

Numerous research studies have been conducted to create databanks for methodical analysis of solvent extracting efficiency in order to calculate the distribution of the reaction species in each phase.[429, 430] Some of these biphasic extractive equilibrium predictions programs have been employed in the pharmaceutical industries and the hydrophobic food additives biosynthesis, including cholesterol,[431] taxols,[432] or vanillic acid.[433]

To date, the majority of the biphasic biocatalysis studies have been focused on enzymes;[418, 434, 435] however, the use of living cells in biphasic biocatalysis is also an attractive area, particularly for cofactor tangled bioconversions, or multi-enzymatic biocatalysis based fermentations.[421, 424, 432] The consumption of high stoichiometric amounts of the expensive reduced cofactor is a great concern for cofactor tangled bioconversions. Bioelectrocatalysis offers a promise to regenerate reduced cofactor to ensure smooth reactions efficiently. It has many inherent benefits, including reaction tenability, low cost and recyclable electrodes, and excellent selectivity. However, combining a bioelectrocatalytic system with the biphasic system is a rather complicated case. The effect of the added organic phase on the enzyme-based bioelectrodes, the lifetime of bioelectrodes, especially the polymer-modified ones, Faradaic efficiency, and mass transportation across the phases are all challenging issues. Compared with bioelectrocatalysis, the application of biphasic systems in organic electrocatalysis is less complicated. Organic electrochemistry is a very straightforward methodology utilizing current to generate reactive intermediates to drive nonspontaneous organic reactions. The fundamental limitations for organic electrocatalysis are conductivity and the use of costly catalysts along with mediators. Mediators are often employed in organic electrocatalysis to form a stable intermediate at the electrode surface. Transition metal complexes and ionic

halides represent two common types of mediators and are usually not cheap.[436] The use of the biphasic system offers the advantages of higher electrical conductivity, easier handling and cycling of the catalyst and mediator, and improved current yield. For example, Mitsudo *et al.* employed a PEG/MeCN thermomorphic biphasic system in an electro-oxidative Wacker-type reaction, where the palladium catalyst can be recycled in the PEG phase upon the completion of the response.[437] However, heterogeneous electrochemical processes often suffer from the high kinetic barrier near the electrode surface, which leads to accumulation of radical cations and anions that might decompose and trigger electrode deactivation, hampering further reactions. This electrode passivation also results in higher current density and electrical energy consumption. To address this issue, redox mediators are often employed to transport electrons to the non-conductive phase, and support electrocatalysis occurs there. Although choosing suitable mediators that can function across different phases is still challenging in real-world applications of organic electrosynthesis. Creative works by the Chiba group have been focusing on developing soluble tag-assisted chemistry, applying the tagged substrates for biphasic electrochemical reactions.[438, 439] Direct anodic oxidation of the labeled substrate and an indirect mediated mechanism has been demonstrated as feasible when using oxidative disulfide bond formation as a model.[440] This tag-assisted phase-transfer methodology, along with many other creative applications, could considerably improve the utility of biphasic organic electrocatalysis. Due to the advantages, biphasic systems have been wildly applied in several organic electrocatalysis reactions, such as the typical Kolbe reaction,[441] Wacker oxidation,[437] oxime oxidation,[442] and selective oxidation of aromatic alcohols.[443] Compared with non-enzymatic electrocatalysis, very few attempts on biphasic

bioelectrocatalysis have been reported. The very first one was the asymmetric synthesis of chiral alcohols,[418, 444] which yielded a total turn number (TTN) of mediator that is two-times higher compared to the one-phase approach. Minteer group has recently developed a biphasic system coupled with a cofactor regenerating bioelectrode for asymmetric bioelectrosynthesis of chiral β-hydroxy nitriles (**Figure 13**).[445] The biphasic approach in bioelectrosynthesis decouples the cofactor regeneration from the substrate concentration, therefore making the retention of the cofactor in the aqueous phase possible. The added organic phase also serves as a reservoir for the substrates, and continuously extracts the product from the reaction phase, as well. This not only results in a higher product yields but also easier downstream processing, which is a promising strategy to push the bioelectrosynthesis on an industrial scale.

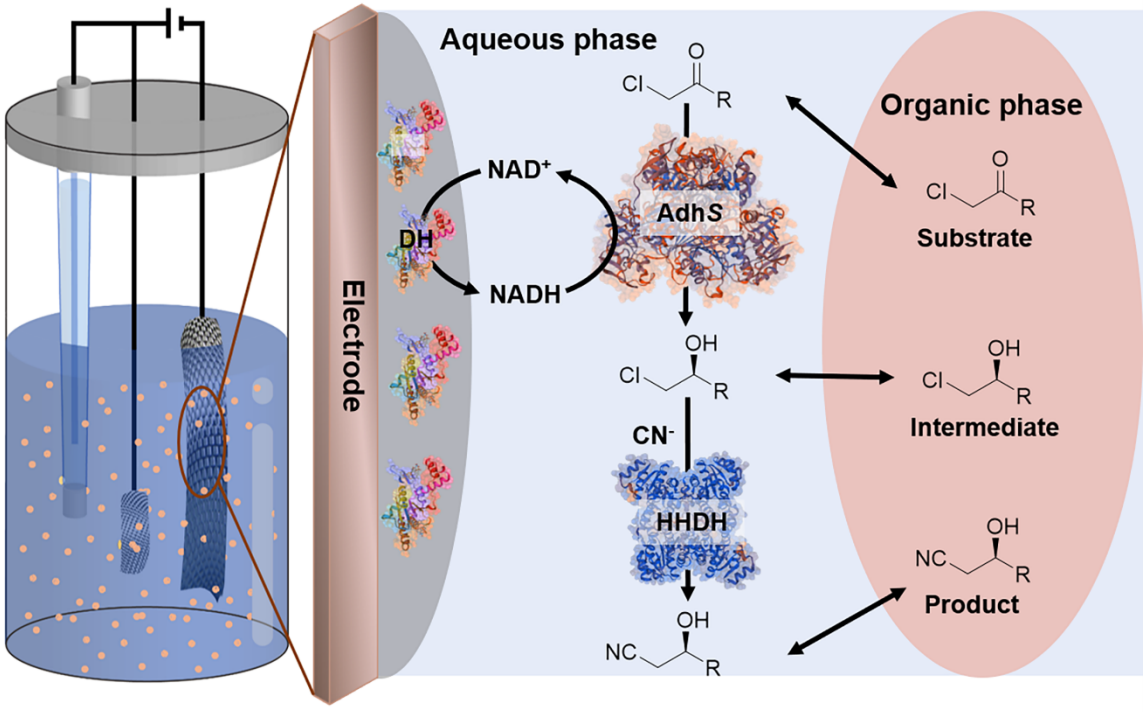

Figure 13. Schematic representation of the biphasic bioelectrocatalytic system for the preparation of chiral β-hydroxy nitrile. Reprinted with permission from ref. 445. Copyright 2020 American Chemical Society.

4. THE APPLICATIONS OF BIOELECTROCATALYSIS

4.1. Biosensors

The field of bioelectrocatalysis has extensive functional applications, such as energy conversion systems and electrochemical biosensors for analytical sensing. Electroanalytical biosensors are analytical platforms that integrate a biological recognition element with an electrochemical transducer to produce a measurable signal that is proportional to the analyte concentration.[204, 446-450] Electrochemical biosensors have demonstrated a vast potential as devices in medical diagnostics and several biotechnological industries, including food and beverage quality control, pharmaceutical, agricultural, and environmental.[449, 450] In 1962, Leland Clark developed the first enzyme-based biosensor for the detection of glucose.[451] Since then, significant research has focused on addressing electrode biofouling issues,[452-455] allowing for glucose detection *in vivo*. Biosensors have also been developed for noninvasive glucose measurements in tears,[455] sweat,[456] or saliva samples.[456, 457] Additionally, electrochemical-based biosensors have been designed as biomedical devices for the detection and monitoring of clinically relevant species, such as uric acid,[458, 459] lactate,[460] cholesterol,[461-464] pyocyanin,[172, 465, 466] creatine,[467, 468] dopamine,[469, 470] and nitric oxide.[471] As such, these biosensors are powerful tools in detecting developing infections at early stages allowing for adequate treatment strategies.[450, 472] Biosensor technologies are also useful for rapid and low-cost analyses of food and beverages to confirm quality. Electrochemical biosensor devices have been developed to evaluate the contents of glucose,[473-475] lactose,[476, 477] fructose,[478] sucrose,[479] vitamin C (ascorbic acid),[473] and pesticides,[480, 481] in different beverages, foods, and biological environments. Additionally, biosensors have been established for the

measurement of alcohols and polyphenols in drinks.[482-486] Electrochemical sensors have applications for real-time monitoring of environmental systems, such as water supplies, rivers, and wastewater treatment facilities. Specifically, environmental biosensor platforms have been constructed for the detection of various pesticides, such as organophosphates,[487-489] herbicides,[490] heavy metals, and pollutants.[491, 492]

Electrochemical biosensors offer a means for quantitative or semi-quantitative analytical information using enzymes, antibodies, protein receptors, organelles, or microorganisms as biological sensing elements.[204] The major components of the biosensor determine its performance, which is characterized by the analytical figures of merit, including detection limit, signal-to-noise ratio, selectivity, specificity, linear dynamic ranges, and response times.[472] To effectively convert the biological response resulting from the interaction between analyte and bioreceptor into an electrical signal,[204, 446, 493] the biocatalyst recognition elements must be in direct spatial contact with the transducer.[494] Consequently, suitable methods to immobilize the biocatalyst on the electrochemical transducer play a key role in the design of biosensors. Standard strategies, such as adsorption, covalent binding, and crosslinking, for improved contact between biocatalyst and electrode surfaces, are discussed in previous sections of this review. Biorecognition elements have been combined with several transducer types, including voltammetric, amperometric, conductimetric, and potentiometric.[204, 448, 495, 496] Based on these configurations and electroanalytical methods, electrochemical biosensors can function via measurement of signal in the form of open-circuit voltage, current, or power, which changes accordingly to variations in concentrations of the analyte of interest. The simplest and most commonly employed type is an amperometric biosensor, in which the working

electrode where the enzymatic or microbial process (e.g., reduction or oxidation of an electroactive metabolic product and/or intermediate) occurs at a fixed potential while the current is recorded.[204] Potentiometric biosensors measure a potential difference between a species-elective working electrode (e.g., ion-selective electrode) and a reference electrode, where the potential signal is concentration-dependent. Although these biosensor types exhibit excellent selectivity and sensitivity, they require the use of a highly accurate reference electrode with excellent stability, which sometimes limits their application in designing microbial-based biosensors.[494] In addition, voltammetric biosensors monitor the resulting current as the potential is varied, where peak currents correlate to distinct analytes of interest. Voltammetric techniques allow for the simultaneous detection of multiple analytes with different peak potentials.[494]

Depending on the electron transfer mechanism used for the measurement of the biochemical signal, biosensors are divided into three categories, or so-called 'generations' of biosensors (**Figure 14**).[497, 498] The first generation of biosensors (or mediator-less biosensors), where the reaction product diffuses to the transducer generating an electrical response, is based on the electroactivity of the bioreceptor substrate or product. In this biosensor class, the biocatalyst, which is most commonly either an oxidase or a dehydrogenase enzyme, is immobilized on the electrode surface. Since oxidases require molecular oxygen as a second substrate, oxidase-based biosensors are O_2-dependent. Thus, the first-generation biosensors that require O_2 as an electron acceptor are subject to errors in sensor response arising from low and/or changing concentrations of dissolved oxygen, which limits their applications.[499] The first-generation biosensors have characteristic high sensitivities and fast response times (~1 sec).[500] However, this biosensor generation

requires matrix effect corrections due to interferents, as well as electrode pretreatment steps to yield reproducible electrode surface and signal responses.[501] The second generation of biosensors involves the use of specific redox mediators) to act as electron carriers between the reaction and the transducer to yield improved responses. The most commonly used mediators include ferrocene, ferricyanide, methyl violet, Prussian blue, thionine, methylene blue, and phenazines,[502] which can be used either free in solution or immobilized with the biomolecule on the electrode surface. In the third generation of biosensors, the reaction itself causes a signal response due to direct electron transfer between an electroactive molecule and the electrode surface. The third generation of biosensors commonly uses redox polymers to wire the electroactive centers of the sensing biocatalysts to the surface of the electrode to improve sensor performance.[503]

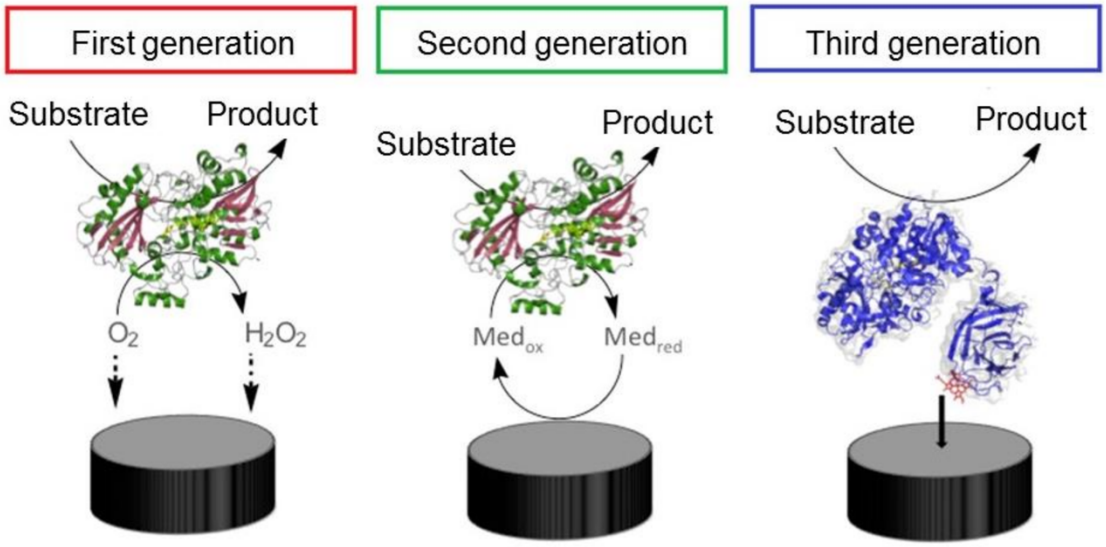

Figure 14. The three generations of electrochemical (here, enzymatic) biosensors divided based on electron transfer mechanisms. Reprinted with permission from ref. 497. Copyright 2017 MDPI.

Dependent on the biological recognition element type, biosensors characteristically belong in two major categories: (1) enzymatic, and (2) microbial electrochemical

biosensors, where the biorecognition elements used are particular enzymes and whole microbial cells, respectively. In enzyme-based biosensor, enzyme catalysts, such as glucose oxidase, horseradish peroxidase, and alkaline phosphatase, are commonly used for reactions accompanied by generation or consumption of detectable species (e.g., molecular oxygen, carbon dioxide, ammonia, hydrogen peroxide) or by enzyme activation or inhibition activity.[450] Most common biosensors for practical and clinical applications utilize enzymes,[29, 504] due to their high specificity and selectivity.[504, 505] The primary benefit of using enzymes as biorecognition components is the ability to genetically engineer the enzyme active site to suitably modify substrate specificity for the detection of a variety of analytes.[450] Enzyme-based biosensors, however, use purified enzymes, which require time-consuming, difficult, and expensive enzyme purification steps, and/or use of multiple enzymes or cofactor/coenzyme to generate detectable products.[204] Additionally, enzymatic biosensors are associated with limited enzyme stabilities due to their dependence on various factors, such as ionic strength, temperature, and pH, which can affect biosensor performance.[450] On the other hand, microbial biosensors using microorganisms as biological recognition entity, providing several advantages, including reduced costs, a wide range of substrates, and mass production.[504] Unlike enzyme-based biosensors, bacteria-based biosensors metabolize complex molecules under aerobic or anaerobic conditions, releasing detectable ammonia, carbon dioxide, hydrogen ions. In comparison to enzymes, microorganisms do not require expensive and time-costly purifications. However, microbial biosensors have specific issues, including relatively poor selectivity and sensitivity, as well as slow responses due to reduced diffusion of substrates through cell walls.[494] Additionally, microbial-based biosensors have other disadvantages, such as

unreliable operation in complex biological environments, which results in inconsistent responses of microbe cells. Yet, genetic engineering modifications can be employed to tailor bacterial microorganisms to enhance certain enzyme activities or express external proteins/enzymes.[204, 506, 507] Herein, we focus mainly on reviewing enzymatic and microbial biosensors that have been developed for various chemical and analytical bioelectrocatalytic sensing applications. Biosensors based on other biological recognition elements, such as nucleic acids (e.g., DNA/RNA) or antibodies, are only briefly mentioned as they are not the main focus in this review article. For engaged readers in this area, the following review articles are recommended.[508-512] For readers with a specific interest in nanomaterials for biosensing applications, we suggest the following excellent review article by the Cosnier group.[513]

4.1.1. Electrochemical Enzymatic Biosensors. Electrochemical enzymatic biosensors (EEBs) correlate the electrons used for the enzymatic redox reaction and the concentration of the substrate, referred to as analytes, in the solution.[514-516] The enzyme either oxidizes or reduces the specific substrate, and the electrons from that enzymatic redox reaction are observed and quantified through the established electron transfer (ET) on the electrode surface. Furthermore, these detected electrical signals can be processed into a user-friendly output, correlating back to the analyte concentration in the solution (**Figure 15**). Moreover, these EEBs can be easily fabricated as portable devices with an incase of wireless technologies. The glucose strip,[517] commonly used for people with diabetes, is the most famous example of portable EEBs. Glucose oxidase immobilized on screen-printed electrode (SPE) strips, catalyzes the oxidation of glucose in blood samples, and the electrons used in the oxidation are processed into a signal readout that reflects the

concentration of glucose in the blood sample being analyzed. While many other non-biological sensors can achieve lower detection limits relative to EEBs, the most significant advantage of EEBs is their selectivity and specificity for target analytes, which depend on the capture enzyme and the utilization of highly selective and sensitive enzyme-analyte affinity. For details on ET mechanisms and also enzyme immobilization strategies required for EEBs, please refer to previous subsections. Additional benefits of EEBs include low cost and their implementation as point-of-care platforms.

Primarily, two types of current outputs could be monitored for EEBs: (1) catalytic currents[518] resulting from an increase of the total electrons from the baseline of the given enzymatic redox reaction and (2) inhibitory currents[519-522] resulting from a decrease of the total electrons used in the redox reaction from its maximum enzymatic activity. For catalytic currents, the target analyte undergoes oxidation or reduction by the paired enzyme, resulting in a net increase in the electrons monitored at the electrode surface. On the other hand, for the inhibitory currents, the target analyte hinders the oxidation or reduction of the paired enzyme, resulting in the net decrease in the electrons monitored at the electrode surface. In both cases, the analyte is selectively recognized by the enzyme. Here, analytes of interest are briefly discussed based on its corresponding enzyme-analyte system and the current output type, amperometric output. For readers with specific interests in impedance-based EEBs outputs, we recommend these articles.[523-529]

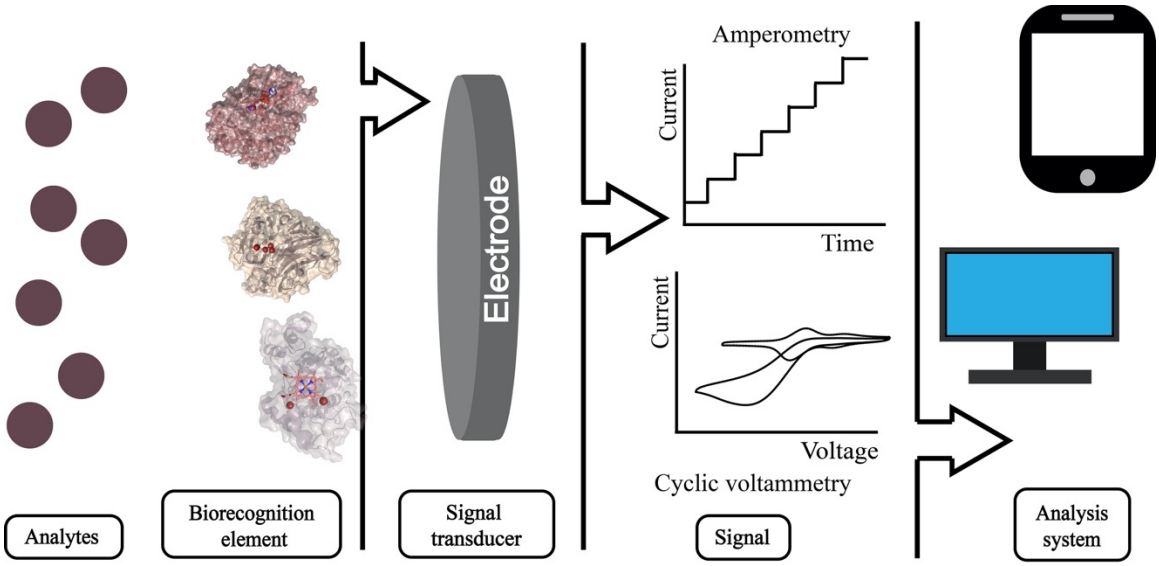

Figure 15. A scheme of electrochemical enzymatic biosensors. A particular enzyme with its specific selectivity affinity towards analytes of interest is used as the biorecognition element, immobilized on the surface of a signal transducer (e.g., an electrode). Refer to Section 3.2.2.1. for a detailed discussion on enzyme immobilization. Once the enzyme establishes an electron transfer pathway with the signal transducer, a detectable signal corresponding to the redox reaction with the analyte is observed. Refer to Section 3.1. for a detailed explanation of electron transfer pathways. These signals are further processed to quantify the concentration of analytes in the system.

4.1.1.1. Electrochemical Enzymatic Biosensors for Chemical Sensing in Water. Unintentional addition of chemicals such as pesticides, detergents, pharmaceutical residues, toxins, slip into everyday products or food, increasing the health risks for citizens. To analyze water quality, several EEB technologies have been developed. Thus, this section provides an overview of EEB platforms developed for the detection of unwanted chemicals in water samples. Water is a critical component of human life, but it also carries many contaminants that need to be tested and filtered out properly before human consumption.

One of the most common water contaminants are pesticides, which are crucial in farming industries to support the food demand for the global population. However, many

of the pesticides are harmful to humans upon consumption; thus, accurate detection of trace pesticides or maximum pesticide concentration for workers is needed. Carbofuran, a banned pesticide in the US, is still a problem encountered in the ecosystem. The inhibitory current of esterases from *Eupenicillium shearii* FREI-39 immobilized on halloysite nanotubes[530] reflected the carbofuran concentration. For this sensor platform, the linear range of carbofuran detection was 5.0–1000.0 μg L^{-1} with a limit of detection (LOD) of 5.13 μg L^{-1}. Arduini and co-workers detected a phenoxy-acid herbicide-2,4-dichlorophenoxyacetic acid (2,4-D), and triazine herbicide-atrazine by its inhibitory effect on alkaline phosphatase and tyrosinase, respectively on a 3D paper-based origami electrode device (**Figure 16a**).[531] This origami electrode device had a linear range of 10–100 ppb for atrazine and 15–200 ppb with 50 ppb as the LOD for 2,4-D in a complex surface water samples.

Another toxic contaminant of interest, commonly present at ppb, is arsenic.[532-534] For instance, Wang and co-workers immobilized laccase on the electrode surface with anthracene modified multi-wall carbon nanotube (An-MWCNT) (**Figure 16b**).[535] Based on its inhibitory current, the linear range was 0.5–5 mM for arsenite and 0.5–8 mM for arsenate. The LOD for arsenite was 13 μM, and for arsenate was 132 μM. While these detections specifically recognize arsenic, the limit of detection is too high, not yet suitable for reliable real-life detection of arsenic in wastewater.

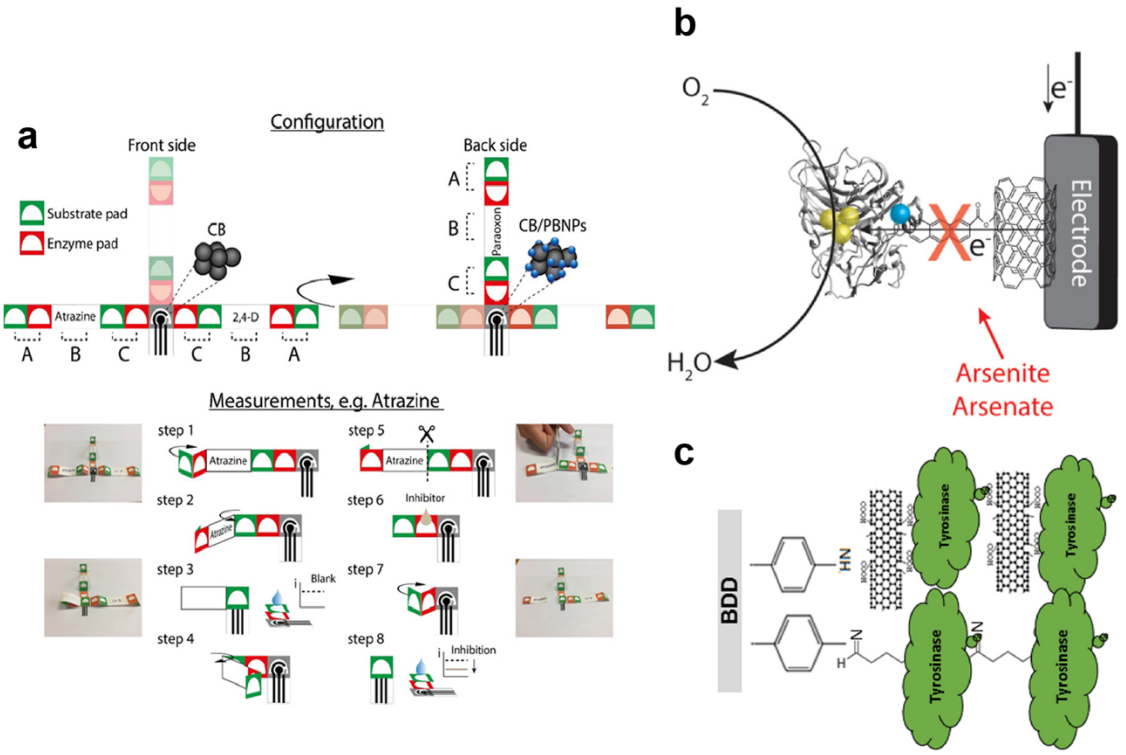

Figure 16. Adopted and modified schemes of electrochemical enzymatic biosensors for the chemical sensing of water samples. The 3D-origami setup (a) was used to detect numerous pesticides by immobilizing different enzymes on the electrode surface. This setup was tested with wastewater samples, showing a promising selective detection. On the other hand, lab-based electrochemical enzymatic biosensors for the detection of arsenic (b) and bisphenol A (c) have been developed and reported. In these examples, arsenic was detected in the form of arsenite and arsenate inhibition towards laccase, while bisphenol A was detected by its redox reaction with tyrosinase. Reprinted with permission from (a) ref. 531. Copyright 2019 Elsevier. (b) ref. 535. Copyright 2016 American Chemical Society. and (c) ref. 538. Copyright 2015 Elsevier.

Lastly, phenolic compounds are widely used among a variety of manufacturers and due to their toxicity, it is essential to develop a platform for their detection.[536] Wee and co-workers immobilized tyrosinase on screen-printed electrodes (SPEs) with carbon nanotubes for catechol and phenol detection based on its catalytic current.[537] The linear ranges were 1.5–8 μM and 0.5–5.5 μM, and the LODs were 14 nM and 35 nM for catechol and phenol, respectively. Similarly, Zehani and co-workers immobilized tyrosinase on a diazonium-functionalized boron-doped diamond (BDD) electrode modified with

MWCNTs for a highly sensitive bisphenol A (BPA) detection (**Figure 16c**).[538] The determined linear range was from 0.01–100 nM with a LOD of 10 pM. Since the same enzyme can be used for different analyte detection, a precise calibration curve of a mixture is needed to develop a practical EEB based on tyrosinase. Rahemi *et al*. investigated the use of TiO_2 to generate reactive oxygen species (ROS) in the presence of hydrogen peroxide (H_2O_2).[539] The researchers reported an original method with a short pre-activation step of TiO_2-horseradish peroxidase (HRP) to design an HRP-based biosensor that works in solutions without H_2O_2. As such, this EEB works as a reagent-less biosensor that can detect phenols, as well as aromatic amines, without the need to add co-substrates in the measuring solutions. On the other hand, Nazari and co-workers immobilized laccase on a polyaniline electrodeposited glassy carbon electrode via glutaraldehyde coupling.[540] Based on its catalytic current, a linear range for catechol was 3.2–19.6 μM with a LOD of 2.07 μM. However, laccase is known to have detections for a variety of phenolic analytes.[541-548] Thus, an explicit calibration of a specific analyte-laccase affinity in a solution of all possible interference is necessary for future practical applications. Undeniably, EEBs show capability in selectively recognizing chemicals in the water as a point-of-care system. Still, a comprehensive study on a selective detection among all possible interferences in a real-life sample is lacking. Thus, a well-established background and controls will be needed to launch a feasible EEB for real-life detection using wastewater.

4.1.1.2. Electrochemical Enzymatic Biosensors for Biomedical Diagnostics. EEBs have received a significant amount of attention as biomedical devices providing a means for the detection of clinically relevant chemicals,[549, 550] disease biomarkers,[253, 462, 551-554] and continuous, real-time monitoring of health status[253, 555-557] of individuals in a clinical

setting. As such, they provide real-time information, at the molecular level, for patient's health, performance, or stress. The ability for continuous monitoring addresses disadvantages with current time-consuming clinical methods (e.g., cell culturing, molecular-based detection methods, blood tests) and offers ways for optimizing therapeutic strategies. Herein, we discuss EEBs for the detection of numerous chemicals linked to health status.

One of the most common ways to introduce foreign, undesired chemicals into the human body is through food and/or beverage consumption, which can subsequently cause health issues. Zhou and co-workers reported an EEB sensor in which protein phosphatase was immobilized on a poly-o-aminophenol-carbon nanotubes-modified SPE (PoAP-SPE) for the detection of okadaic acid, a common diarrhea inducing toxin, in the supernatant of shellfish cells.[558] The linear range of this okadaic acid biosensor was 1–300 μg L^{-1} with a limit of detection (LOD) of 0.55 μg L^{-1}. In another study, del Torno-de Román and co-workers used tyrosinase and gold nanoparticles (GNPs) immobilized on the screen-printed carbon electrodes (SPCEs) for the detection of sulfamethoxazole, an antibody used to treat bacterial infections in veterinary clinics (**Figure 17a**).[559] The LOD was 22.6 μM with the linear range of detection was roughly 20–200 μM.

In addition to food toxins, drugs designed to enhance human health can also be detected using EEB platforms. Kurbanoglu *et al*. detected methimazole, an antithyroid agent, with a low sample volume of 6 μL within 20 seconds by immobilizing a nanocomposite of iridium oxide nanoparticles functionalized magnetic nanoparticles and tyrosinase on an SPE.[560] The LOD of this biosensor was estimated to be 0.006 μM for a batch mode and 0.004 μM for flow mode. Alvau and co-workers detected CPT-11 (irinotecan), an

antineoplastic drug for the treatment of colorectal cancer, by its inhibitory effect on an enzymatic relay of acetylcholine esterase (AChE) and choline oxidase (ChOx) (**Figure 17b**).[561] In this enzymatic relay, AChE oxidizes acetylcholine to choline that was further oxidized by ChOx to hydrogen peroxide (H_2O_2) and betaine aldehyde. Finally, H_2O_2 was also electrochemically detected on this device. With the addition of CPT-11, AChE is inhibited, thus no more choline for ChOx to produce H_2O_2. The biosensor LODs were determined to be 1.6 ng mL^{-1} and 1.5 ng mL^{-1} in phosphate-buffered saline (PBS) and fetal bovine serum (FBS), respectively. De Wael and co-workers designed a catalase-based electrochemical biosensor for the detection of H_2O_2.[562] In this innovative work, the researchers immobilized gelatin films on glassy carbon electrode surfaces using a spin coating strategy, which resulted in reproducible current responses from the uniform micrometer-size, biocompatible layers. Lastly, acetaminophen, a common painkiller, was detected using polyphenol oxidase in carbon paste[563] and tyrosinase on SPE covered with graphene.[564] For the polyphenol oxidase system, the LOD was determined to be 5 μM with a linear range of 20–200 μM, while the LOD for tyrosinase was 1.1 μM. Overall, EEBs offer a unique detection of foreign chemicals or the drugs itself to prevent overconsumption of toxins and understand the effect of drugs in determining better treatment strategies.

The most significant benefit of EEBs likely dwells in the selective and sensitive detection of biomolecules found in the body, especially if those biomolecules indicate the health status marker. As the most widely known example, glucose was detected as a biomarker of diabetes countless times with glucose oxidase,[552, 565] or glucose dehydrogenase.[566, 567] Moving past glucose, Kuretake *et al*. used HRP and alcohol oxidase (AOD) to detect ethanol as a universal biomarker for the level of intoxication.[568] Ethanol

was oxidized to acetaldehyde by AOD, and hydrogen peroxide, the byproduct, was reduced by HRP. The linear range was from 50–500 ppm. Moreover, Verma and co-workers detected uric acid, a biomarker of wound healing and gout, using uricase and GNPs decorated graphene oxide nanocomposites.[569] The LOD was approximated to be 7.32 μM with a linear range of 50–800 μM. Regarding more serious disease-related biomarkers, Si *et al*. detected hypoxanthine, a novel biomarker for cardiac ischemia,[570] using layer-by-layer assembly of xanthine oxidase, carbon nanotubes, and graphene complexes on SPEs.[571] The LOD was 4.04 μM with a linear range of 5–50 μM. Moreover, cancer biomarkers are of high interest at lower detection limits for early diagnosis. Mandli and co-workers detected microRNA (miRNA), as a possible biomarkers[572] for cancer diagnosis, therapy, and prognosis, based on a unique DNA sandwich form (**Figure 17c**).[573] The pencil graphite electrode (PGE) was modified with GNPs for the thiol terminal side of the probe 1 (SH-P1) to bind via gold-sulfur bonds. The SH-P1 bonded to the half of miRNA-21 while the other half bonded to probe 2 (B-P2), where streptavidin-conjugated alkaline phosphatase was immobilized on. Finally, a substrate, 1-naphthyl phosphate, was added for the enzymatic reaction. In this setup, only the alkaline phosphate that is bound to the B-P2, miRNA-21, and SH-P1 can establish an electron path, providing an electrical signal indicative of the presence of miRNA-21. The LOD was 100 pM with the linear range of 200 pm–388 nM. Additionally, the Millner research group reported an amperometry-based biosensor for the detection and quantification of lactate using pre-impregnated Prussian Blue screen-printed carbon electrodes and polyethylenimine (PEI) polymer for lactate oxidase immobilization.[574] The lactate biosensor demonstrated promising performance for detection of lactate in post-operative patient drain fluid samples. In another work, Pita *et*

al. demonstrated the use of gold nanoparticle-modified gold disk electrodes for covalent immobilization of bilirubin oxidase to design an oxygen biosensor.[575] This amperometric biosensor had a detection limit of 6 ± 1 μM with a linear range of 6–300 μM, thus exceeding the physiologically relevant oxygen levels in human fluids. Irrefutably, EEBs show an excellent substrate-affinity towards the biomolecules naturally occurring in human bodies as an indicator of health status. Moving forward, the improvement of EEB sensitivity via material variance,[462, 576, 577] data collection methods,[578, 579] and stability via bioengineering[580-582] would pave the path towards a stable shelf life for easier industrial manufacturing, early diagnosis, and prognosis of severe diseases. A summary of EEBs overviewed herein is provided in **Table 1**.

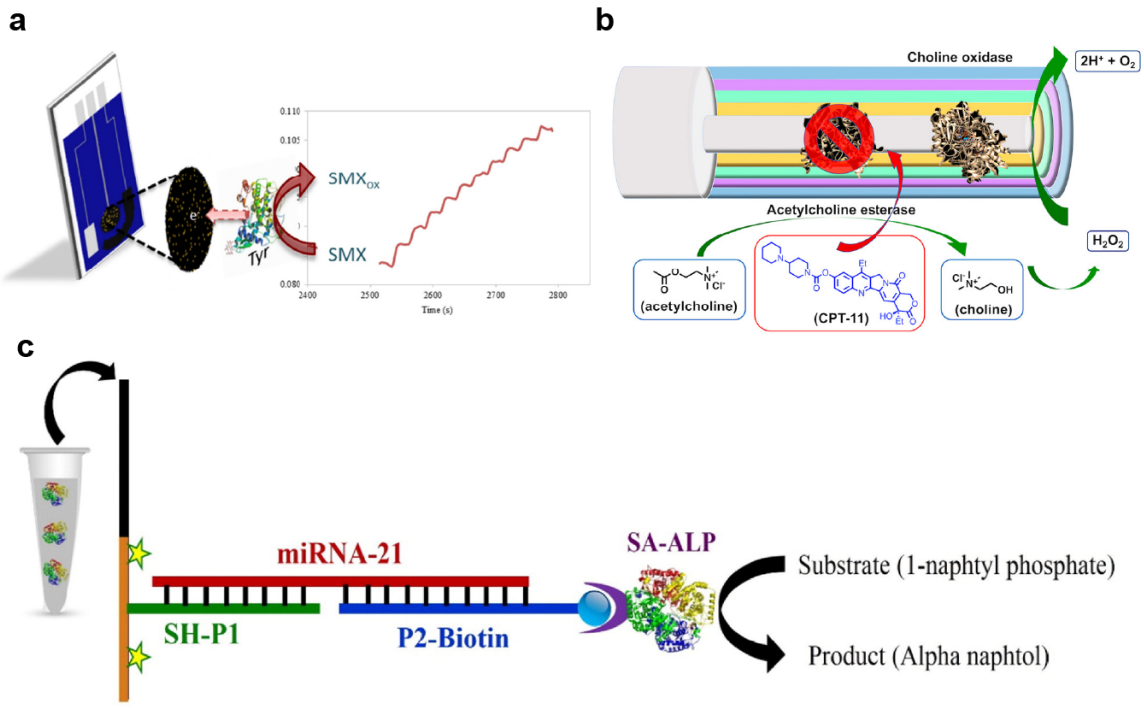

Figure 17. Adopted figures summarizing three unique electrochemical enzymatic biosensor setups for chemical sensing in biomedical fields. (a) Tyrosinase (Tyr)-based electrochemical enzymatic biosensor for the detection of sulfamethoxazole (SMX), an antibiotic, was realized by a simple immobilization of the enzyme on the screen-printed carbon electrode (SPCE) surface. Reprinted with permission from ref. 559. Copyright 2016 Elsevier. (b) A bi-enzyme relay of acetylcholine esterase and choline oxidase was used to detect an antineoplastic drug, CPT-11, used for the treatment of colorectal cancer. Reprinted with permission from ref. 561. Copyright 2018 American Chemical Society. (c) A DNA-sandwich setup was built to detect microRNA-21 (miRNA-21). One probe (SH-PI) was designed to immobilize on the electrode surface while binding to the half of miRNA-21. The other probe (P2-biotin, B-P2) was designed to immobilize streptavidin-conjugated alkaline phosphatase (SA-ALP) while binding to the rest of the miRNA-21. Only the SA-ALP bound to the DNA-sandwich of P2-biotin, miRNA-21, and SH-P1 gave electrochemical signals of the 1-naphthyl phosphate redox reaction. Reprinted with permission from ref. 573. Copyright 2017 Elsevier.

Table 1. Summary of electrochemical enzymatic sensors (EEB) reviewed.

Target Analyte(s)	Sensing Strategy	Enzyme(s) Used	LOD	LR	Ref.
Carbofuran pesticide	Voltammetry; Halloysite nanotubes	Esterase	5.13 μg L^{-1}	5.0–1000.0 μg L^{-1}	530
Phenoxy-acid herbicide-2,4-dichlorophenoxyacetic acid (2,4-D); Triazine herbicide-atrazine	Amperometry; 3D paper-based origami electrodes	Alkaline phosphatase; Tyrosinase	50 ppb (2,4-D); not specified for Triazine herbicide-atrazine	15–200 ppb (2,4-D); 10–100 ppb (Triazine herbicide-atrazine)	531
Arsenite; Arsenate	Self-powered biosensor; Anthracene modified multi-wall carbon nanotubes	Lacasse	13 μM (Arsenite); 132 μM (Arsenate)	0.5–5 mM (Arsenite); 0.5–8 mM (Arsenate)	535
Catechol; Phenol	Voltammetry; Screen-printed electrodes with carbon nanotubes	Tyrosinase	14 nM (Catechol); 35 nM (Phenol)	1.5–8 μM (Catechol); 0.5–5.5 μM (Phenol)	537
Bisphenol A	Amperometry; Diazonium-functionalized boron-doped diamond electrode modified with multi-wall carbon nanotubes	Tyrosinase	10 pM	0.01–100 nM	538
Catechol	Voltammetry; Polyaniline electrodeposited onto a glassy carbon electrode	Lacasse	2.07 μM	3.2–19.6 μM	540
Okadaic acid	Voltammetry; poly-o-aminophenol-carbon nanotubes modified screen-printed electrode	Phosphatase	0.55 μg L^{-1}	1–300 μg L^{-1}	558
Sulfamethoxazole	Amperometry; screen-printed carbon electrodes with gold nanoparticles	Tyrosinase	22.6 μM	20–200 μM	559
Methimazole	Amperometry; Screen-printed electrodes modified with iridium oxide nanoparticles	Tyrosinase	0.006 μM (batch mode); 0.004 μM (flow mode)	Not specified	560

Table 1. (*Contd*.)

CPT-11 (irinotecan)	Amperometry; Platinum electrode modified with glutaraldehyde, polyethyleneimine, and poly-o-phenylenediamine	Acetylcholine esterase; Choline oxidase	1.6 ng mL^{-1} (in phosphate-buffered saline); 1.5 ng mL^{-1} (in fetal bovine serum)	Not specified	561
Hydrogen Peroxide (H_2O_2)	Voltammetry; Glassy carbon electrodes modified with gelatin films	Catalase	Not specified	Not specified	562
Acetaminophen	Voltammetry; Carbon paste	Polyphenol oxidase	5 µM	20–200 µM	563
Acetaminophen	Amperometry; Screen-printed electrodes	Tyrosinase	1.1 µM	Not specified	564
Ethanol	Amperometry; Screen-printed carbon electrodes	Horseradish oxidase; Alcohol oxidase	Not specified	50–500 ppm	568
Uric acid	Voltammetry; Graphene oxide nanocomposites modified with gold nanoparticles	Uricase	7.32 µM	50–800 µM	569
Hypoxanthine	Voltammetry; Screen-printed electrodes with carbon nanotubes and graphene complexes	Xanthine oxidase	4.04 µM	5–50 µM	571
microRNA	Voltammetry; Pencil graphite electrodes modified with gold nanoparticles	Streptavidin-conjugated alkaline phosphatase	100 pM	200 pm–388 nM	573
Lactate	Amperometry; pre-impregnated Prussian Blue screen-printed carbon electrodes with polyethylenimine (PEI) polymer	Lactate oxidase	Not specified	Not specified	574
Oxygen	Amperometry; Gold nanoparticle-modified gold disk electrodes	Bilirubin oxidase	6 µM	6–300 µM	575

4.1.2. Electrochemical Microbial Biosensors. Electrochemical microbial biosensors are analytical instruments that incorporate a microorganism to detect a target analyte by converting the measured signal into a quantifiable electrochemical response (**Figure 18**).[504, 583] Bacterial microorganisms have developed advanced sensing mechanisms in regulating their cell growth and behaviors. Microbes can sense not only environmental factors and changes, such as pH, temperature, and/or nutrients, but can also detect variations in their metabolism.[584] Their mechanisms can detect cellular-based signals, which are then transduced in an electrochemical manner. Using bacterial microorganisms for the development of sensors offers several advantages over using enzymes. Specifically, microbial biosensors utilizing bacteria provide a means for cost-effective analyses as microbes can be grown in vast quantities via simple cell culturing methods. Additionally, microbial biosensors can detect multiple target analytes; also, microorganisms can be genetically engineered using synthetic biology strategies to adapt microbes for specific substrates.[584] Microbial-based sensors typically have high stabilities as microorganisms have excellent abilities to adapt to several environmental conditions. However, microbial-based sensors have certain disadvantages due to a few inherent limitations of bacteria, including relatively low sensitivity and poor selectivity for detection in multiplexed environments and/or samples.[585, 586]

An appropriate selection of the type of microorganism for the detection of an analyte substance of interest is a significant question when designing electrochemical microbial biosensors.[587] To date, substrate specificity characteristics of several bacterial strains have been investigated with a number of substrates, such as organic acids, carbohydrates, and alcohols, and compiled in a database.[588] These data enable the selection of appropriate

microorganisms in selecting microbial biosensors components. For instance, a few research studies have used substrate specificity properties from this database for *Gluconobacter* species in the development of potentiometric and amperometric microbial biosensors for the detection of xylose.[589, 590] Additionally, genetic engineering strategies (e.g., gene modifications, genetically manipulated cells) have been employed in the microbial biosensor platforms to create specific bacterial strains for improved biosensor selectivity and sensitivity.[591, 592] In addition to the selection of bacterial strain, microbial biosensor performance relies on proximity between the biological catalyst and the electrode surface. Thus, effective immobilization approaches (section 3.1.2) are required for the successful function of microbial biosensors. Here, we provide an overview of several electrochemical microbial-based biosensors, however, for interested readers in this particular topic, several review articles published on microbial biosensors, focusing on technologies, electroanalytical methods, and/or specific applications,[204, 446, 504, 583, 587, 593] are recommended.

The first microbial biosensor, described by Divies in 1975, was combined the use of *Acetobacter xylinum* and an oxygen electrode,[594] which became the foundation for the development of microbial biosensors for many biotechnological purposes, including environmental monitoring, clinical diagnostics, and food examination industries. Electrochemical microbial biosensors have been developed as inexpensive and quick analytical instruments for monitoring environmental pollutants, specifically organic and inorganic toxicities, such as heavy metals that can cause diseases as they accumulate in living organisms.[366] Singh and co-workers designed a *Chlorella* sp. whole-cell biosensor over a glassy carbon electrode for the detection of mercury as it can inhibit the activity of

phosphate enzymes located in *Chlorella* sp. cell wall.[595] This amperometric biosensor demonstrated a lifetime of 14 days with selectivity over silver, alkaline earth metals, and transition metals. In another research study, Alpat and co-workers developed a microbial biosensor with *Circinella* sp.-modified carbon paste electrode for the voltammetric detection of copper (Cu^{2+}) in real samples.[596] Carbon paste electrodes have also been modified with *Porphyridium cruentum* biomass to design a voltammetric microbial sensor for the detection of arsenic (Ar^{3+}) in contaminated water.[597] Additionally, voltammetric-based microbial biosensors have been developed for sensitive determination of lead (Pb^{2+}) using carbon paste electrodes prepared with *Rhizopus arrhizus*[598] and *P. aeruginosa* biomass[599] from aqueous solutions. Microbial biosensors have also been developed for the detection of various organic contaminants. For instance, a microbial biosensor based on *Pseudomonas* sp. strain ASA86 immobilized on a porous cellulose nitrate membrane on a chloride ion electrode for the detection of a typical soil and groundwater pollutant trichloroethylene.[600] For detection of trichloroethylene, Hnaien and co-workers developed an impedimetric-based bacterial biosensor by immobilizing *P. putida* F1 strain on gold microelectrodes, which were functionalized with single-walled carbon nanotubes connected to anti-*Pseudomonas* antibodies via covalent linkage.[601]

In addition to electrochemical microbial biosensors for environmental monitoring, bacteria-based biosensors have been developed as rapid and affordable tools for food and drink analyses, as well as fermentation. As ethanol is essential in fermentation procedures, electrochemical bacterial sensors have been designed for sensitive detection and monitoring of ethanol during fermentation. For instance, an amperometric microbial biosensor was constructed by Valach and co-workers for the detection of ethanol in flow

injection analysis, with a linear biosensor range of 10 μM–1.5 mM and a 3 min response time; in this sensor design, *G. oxydans* microorganisms were immobilized on the surface of a glassy carbon electrode combined a silver/silver chloride (Ag/AgCl) electrode.[602] Similarly, Akylimaz *et al.* developed an amperometric biosensor utilizing *Candida tropicalis* bacteria for sensitive determination of ethanol. The *C. tropicalis* cells, containing alcohol oxidase, were immobilized in gelatin via the use of glutaraldehyde.[603] Wen and co-workers also reported the design of ethanol microbial biosensor using *Methylobacterium organophilium* attached to an eggshell membrane and oxygen electrode.[604] Electrochemical microbial biosensors have also been developed as devices for assuring the quality of coffee via the rapid and sensitive detection of caffeine. Babu and co-workers designed an amperometry-based bacterial biosensor for caffeine detection by attachment of *Pseudomonas alcaligens* MTCC 5264 strain, which is capable of degrading caffeine, on a cellulose acetate membrane with a Clark oxygen electrode.[605] With a readout time of 3 min, this biosensor platform showed the ability for rapid detection of caffeine and also a high specificity for this target analyte in the presence of interfering compounds, such as paraxanthine, theobromine, and sugars, as *P. alcaligens* MTCC 5264 has specific ability to degrade caffeine.[605] Furthermore, Li and co-workers reported the development of a voltammetric microbial biosensor for the detection of two common food sweeteners, D-xylose and D-glucose.[606] In their sensing device, the researchers co-immobilized xylose dehydrogenase and glucose oxidase and loaded XDH-bacteria on electrodes modified with nanocomposite films of multi-walled carbon nanotubes. Research studies have also reported the construction of electrochemical cell-based biosensors for the detection of target analyte compounds of pharmaceutical value. For example, Akyilmaz and co-workers

recently reported the creation of a *C. tropicalis*-based biosensor for the selective detection of L-Ascorbic acid.[607] In this sensor fabrication, *C. tropicalis* yeast cells were attached with o-aminophenol to create a film layer on a platinum electrode via an electropolymerization method. Using both amperometry and differential pulse voltammetry, the researchers quantified levels of L-ascorbic acid in real samples.

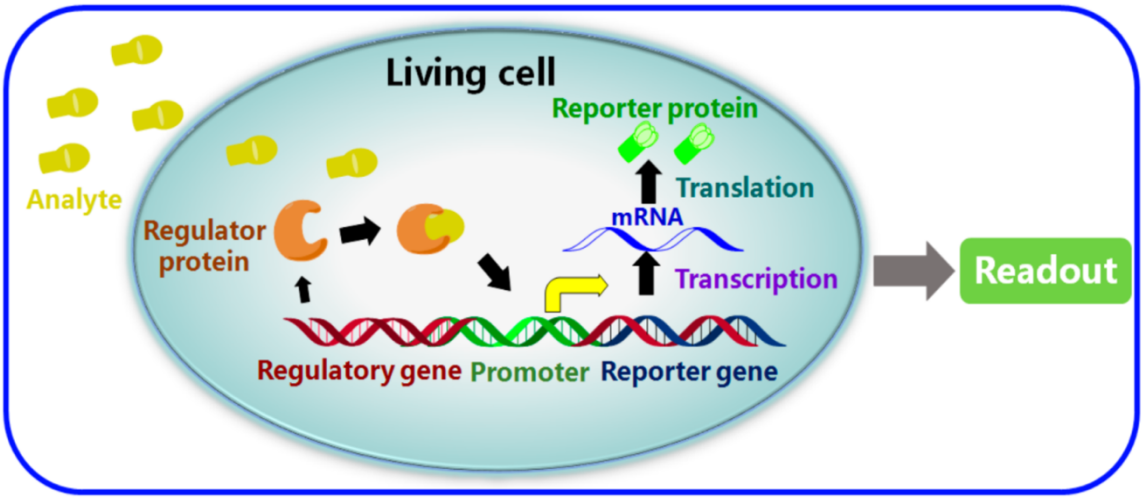

Figure 18. Electrochemical microbial biosensors. A diagram portraying the general working mechanism of a typical whole-cell biosensor, which detects a specific analyte and is subsequently amplified into an electrical signal. The resulting readout is detectable via the immobilization and/or use of living bacterial cells as the biological unit providing molecular recognition elements for biosensor. Reprinted with permission from ref. 583. Copyright 2017 MDPI.

Furthermore, electrochemical bacterial biosensors have shown promise as clinical diagnostic devices for rapid detection and monitoring of developing pathogenic infections.[472] Namely, bacterial sensing platforms offer a means for fast and accurate detection of DNA, pathogens, and/or hormones. Tuncagil *et al.* reported the development of a microbial biosensor for the detection of glucose utilizing *G. oxydans* cells, which were immobilized on 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine conducting polymer coating the surface of a graphite electrode.[608] Similarly, Cevik and co-workers reported the fabrication of an amperometric biosensor based on whole *G. oxydans* cells,

also, for the detection of glucose.[609] In this sensor assembly, a glassy carbon electrode was coated with a polymer film of 10-(4*H*-dithiyeno [3,2-b:2',3'-d]pyroll-4-il)decan-1-amine by electropolymerization to form the interface between the electrode surface and bacteria. Additionally, Akyilmaz and co-workers demonstrated construction of a voltammetry-based microbial biosensor for the detection of epinephrine by attachment of *Phanerochaete chrysosporium* ME446 strain in gelatin on a platinum electrode via glutaraldehyde crosslinking agent.[610] In this biosensor, the increasing current responses were a result of epinephrine converting into epinephrine quinone through a reduction-oxidation activity catalyzed by lactase in the fungal *P. chrysosporium* ME 446 cells. Its application was examined and demonstrated for sensitive epinephrine in pharmaceutical ampules. Additionally, Smutok *et al*. reported the design of a selective microbial-based biosensor for the detection of L-lactate using permeabilized cells of genetically engineered *Hansenula polymoprpha*.[611] In this biosensor design, the researchers immobilized the genetically engineered bacteria on graphite electrodes using phenazine methosulfate as the diffusing redox mediator. In particular clinical diagnostics applications, electrochemical biosensors have also been designed to detect the presence of electrochemically active pathogens in clinical samples from infected patients.[472] In these cases, there is no biological catalyst on the electrode surface; however, the bacteria present in clinical samples will act as a catalyst via the bacterial secretion of redox-active mediators, which give a signal only when pathogen of interest is present. For instance, the design and application of carbon ultramicroelectrode arrays were recently demonstrated for the real-time electrochemical detection of the human pathogenic microorganism, *P. aeruginosa,* via the generation of electroactive phenazine metabolites.[172, 466] Therefore, electrochemical microbial

biosensors have been extensively used for environmental monitoring, evaluation of food and drink quality, as well as for medical diagnostics due to their versatility, stability, fast response, and low-cost. Based on these attractive characteristics, future directions in the development of biosensors, including miniaturization and portability, as well as wearable and self-powered biosensor devices, are discussed in the following sections. Slow responses associated with microbial biosensors have been attributed to cell membrane diffusion issues. To overcome these challenges, future work needs to focus on genetic engineering strategies to modify microbes to expresses specific enzymes of interest, thereby improving the response times and biosensor sensitivity. The genetic engineering methods have the potential also to increase the biosensor specificity via the expression and/or activation of preferred metabolic pathways and suppressing undesirable ones.

4.1.3. Single-molecule Sensors Based on Nanopores. Minimizing the sensor's detection limit to the single-molecule level has always been a pursued goal in analytical chemistry. In comparison with ensemble measurements (comprising of thousands or millions of entities), single-molecule sensing provides much richer information as it can detect and quantify rare, aberrant species, which would be lost in the noise of an analytical device during ensemble measurements.[612, 613] In recent decades, with the long-term development of single entity sensing, the sensing of a single cell and single nanoparticles has become a reality.[614] Electrochemical methods have a vital role in single entity sensing, because they enable precise monitoring of electron/charge transfer processes by a designable and controllable sensing interface on the nano-scale, which is comparable to the size of single entities. Therefore, electrochemical sensing in a confined space is becoming a promising measurement in single entity sensing. Electrochemically confined spaces (e.g.,

nano/microelectrodes, nanopipettes, nanopores) provide a means for useful analytical analysis of single entities and nanointerfaces with high selectivity and sensitivity. They provide a tiny geometric space for extracting one entity from an ensemble system and also focus on various energies (e.g., photo, electrical, and chemical energies) to command single entities.[615] The group of Yi-Tao Long carried out of fruitful work around the concept and applications of confined space, and the following corresponding publications are recommended as important references on this topic.[614-617]

A nanopore is a commonly used confined space to investigate dynamic processes at a single-molecule level. In general, the nanopore locates at the interface of two electrolyte solutions and act as the only mass transfer channel.[618] The application of an electric potential difference between two electrolytes via two electrodes generates an ionic current that is able to drive an individual molecule into the pore. Correspondingly, each molecule's transient stay in the confined nanopores will block the ionic current flow through the pore. Such a dynamic action will cause the blockage current via the volume-exclusion effect. In this process, the specific nanopore-analyte interactions can be converted into detectable ionic signals, which can specifically correlate to analytes' critical structural information at the single-molecule level, such as size, shape, and conformation.[619, 620] In initial attempts, researchers used a biological nanopore, which was based on a single-membrane protein molecule. The biological nanopore possesses a single-biomolecule interface for achieving high sensitivity and selectivity.[621] More recently, various synthetic materials were developed, such as glass nanopipettes, silicon nitride membranes, graphene and DNA scaffolds, to construct single solid-state nanopores and even hybrid nanopores.[617, 622]

The nanopipette is a subclass of solid-state nanopores, which generally refers to quartz and glass pipets with a hollow needle-like geometry and a sharp tip with a diameter of a few nanometers. The nanopipette has a unique advantage in single-cell analysis. Based on its needle-like geometry and nano-sized sharp tip, nanopipette-based biosensors penetrate a single cell with minimal invasion to monitor cellular processes and metabolic activities via bioelectrochemical reactions and electron transfer processes under normal physiological conditions.[623] Nascimento and co-workers employed a nanopipette as a nano-sized glucose biosensor to detect intracellular glucose levels of a single cancer cell with high spatiotemporal resolution.[624] In their work, the researchers modified the nanopipette's inner surface with glucose oxidase, which was able to catalyze the oxidation of glucose and convert glucose to gluconic acid. The generation of gluconic acid caused a noticeable drop in pH, leading to a change in the impedance. Thus, a direct correlation between intracellular glucose concentrations and impedance changes in the nanopipette orifice was established. In another study, Song *et al.* functionalized a nanopipette with G-quadruplex DNAzyme was used to detect and quantify levels of the intracellular reactive oxygen species (ROS). The immobilized G-quadruplex DNAzyme catalyzed the oxidation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) with intracellular ROS as an oxidant and resulted in a change in surface charges of the nanopipette.[625] The nanopipette-based nanoelectrode can be employed to detect the intracellular redox-active species, as well. In research work by the Long group, an asymmetric wire nanopore electrode was designed, where the unbiased gold layer on the inner surface was polarized under bias potential, triggering bipolar electrochemical reactions at the two extremities with the formation of an H_2 bubble on the orifice side. Consequently, the ion current was

successfully amplified by at least three orders of magnitude due to the H_2 bubble generation. Finally, this system achieved NADH sensing and an electron transfer process in single living cells by dynamic ion current.[626]

The stochasticity of ion mobility inside a nanopore causes the production of nonlinear and nonstationary current responses, which have complex and transient frequency features. However, revealing the interactions and kinetics of single molecules require a large number of statistical data sets composed of single-molecule information. Consequently, the development and applications of improved analytical and statistical methods and algorithms are critical to improve the accuracy of detection and reveal hidden kinetics during dynamic motion and the electron transfer of single molecules.[617] In recently reported research studies, a Hilbert-Huang transform algorithm was employed to analyze nonlinear and nonstationary nanopore data and achieve valuable results.[627, 628] Specifically, the recorded current is disintegrated into a series of individual mono-components, called intrinsic mode functions, by performing ensemble empirical mode decomposition. Then, the Hilbert transform is applied to extract the instantaneous frequencies in each intrinsic mode function. Therefore, the Hilbert-Huang transform frequency-based analysis converts two-dimensional signals from the time domain into three-dimensional spectra. These spectra show energy-frequency-time distributions, thereby revealing dynamic information hidden behind big datasets.

4.2. Biofuel Cells

The uneven geographical distribution of fossil fuels and the environmental pollution problem caused by the combustion of fossil fuel combustion forces humankind to pursuit novel green and sustainable energy sources. In this respect, fuel cells as effective

electrochemical devices to convert chemical energy into electrical energy without intermediate steps are considered as a promising technical means.[629] The production of energy from renewable resources, waste, and the environment (energy harvesting) has been a recent focus of many research groups due to the need for cheap, environmentally friendly, renewable fuel and catalysis.[630] Biofuel cells are an approach for such clean energy production as they employ the electrode surfaces to harness the flow of electrons produced and consumed by redox enzymes. These electrons can then be fed into an electric circuit, where they can be used to do work.[631] Biofuel cells can be categorized by the type of bioelectrocatalyst: enzymatic fuel cell and microbial fuel cell. In the enzymatic fuel cell, single isolated oxidoreductases, enzymatic cascades, or multi-enzyme complex are usually used as bioelectrocatalysts to perform the conversion from chemical energy to electrical energy. Additionally, some organelles (especially mitochondria and chloroplast), the subcellular microcompartmentalization structure of living cells, can also be used as bioelectrocatalysts in the enzymatic fuel cell. These organelles contain a series of oxidoreductases to form electron transfer chains, which have electrochemically active species, such as ubiquinone or cytochrome *c*, that establish electrical communication with the electrode.[7-11] Essentially, organelles contain multi-enzyme complexes formed in a microcompartmentalized membrane structure. Consequently, organelle-based fuel cells are still part of the enzymatic fuel cell family. Microbial fuel cells are devices that use bacteria as the biological catalysts to oxidize organic and inorganic matter and generate current.[94] Microbes generally carry out their metabolic activities (anabolism and catabolism) either in the presence of O_2 or in the absence of O_2.[94]

4.2.1. Enzymatic Fuel Cells. In an enzymatic fuel cell, either one or both electrodes, e.g., the bioanode and/or the biocathode, utilize enzymes, specially purified enzymes, to bioelectrocatalytically oxidize the fuel and to reduce the oxidant. The enzymatic fuel cell can use a broad range of chemical compounds as fuels, including methanol, ethanol, glycerol, pyruvate, and glucose, in increasing order of carbon number in the compounds.[632] In the enzymatic fuel cell, the enzymes are used for fuel oxidation at the anode and oxidant reduction at the cathode. The power output of the enzymatic fuel cell is the product of the cell voltage and the current. Cell voltages depend on the selection of fuel and oxidant, pH of the fuel compartment and the cathode compartment, the rate of electron transfer, the flowing current, resistances within the cell (e.g., Ohmic losses), and mass transport processes. The maximum cell voltages for enzymatic fuel cells are usually determined by the difference between the formal redox potential of the redox enzyme cofactors, in the active site, utilized for the anode and cathode.[633]

The overall performance of enzymatic fuel cells depends on the efficiency of electron transfer between the selected enzyme and the electrode. In direct electron transfer processes, electrons are directly transferred from the enzyme to the electrode, which, as previously discussed, requires a specific distance between the enzyme and the electrode surface for electron tunneling to occur. In other cases, when the distance requirement is not satisfied, mediators are used as electron shuttles (mediated electron transfer), including methylene green,[634] methyl viologen,[635] ferrocene,[636] neutral red,[637] and ferricyanide,[638] to allow for fast electron transfer rates. However, the utilization of these redox mediators introduces challenges to enzymatic fuel cell systems, such as poor biocompatibility, stability, and increased system cost.[635, 639] In addition to the type of electrode material used

in enzymatic fuel cells, the electron transfer mechanism also relies on the structure and/or type of enzymes as biocatalysts. In enzymatic fuel cells, the most commonly used enzymes at the bioanode include glucose oxidase,[640] glucose dehydrogenase,[567, 641] lactate oxidase,[263] lactate dehydrogenase,[634] cellobiose dehydrogenase,[642] alcohol dehydrogenase,[643] fructrose dehydrogenase,[644] pyranose dehydrogenase,[645] and hydrogenase.[646, 647] Enzymes, such as glucose oxidase, have deeply buried redox centers, thereby requiring the use of mediators to establish an electrochemical connection between the enzyme redox center and the electrode surface.[648] A challenge with the use of glucose oxidase is that it can use molecular O_2 as an electron acceptor; the high potential necessary to oxidize oxygen can potentially cause interferences with other species.[567] Research studies have also examined the use of alternative enzyme catalysts that have capabilities for direct electron transfer mechanisms. For instance, cellobiose dehydrogenase has demonstrated promising direct electron transfer in glucose/oxygen enzymatic fuel cells.[649] However, this heme-based enzyme requires engineering strategies to not only improve its selectivity for glucose but also to reduce interferences with lactose, maltose, or other sugars.[650] Additionally, some research studies have utilized glucose dehydrogenase as an alternative to glucose oxidase; however, glucose dehydrogenase, unable to use oxygen as an electron acceptor, transfers electrons to redox cofactors (e.g., nicotine adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), pyrroloquinoline quinone (PQQ)).[382, 567] Among the redox cofactors, FAD is bound more tightly to the enzyme, which prevents its dissociation over time, thus improving the enzyme lifetime.[651] Additionally, enzymatic fuel cells employing FAD-dependent glucose dehydrogenase could achieve higher power outputs as FAD-dependent glucose dehydrogenase exhibits

lower redox potential. In contrast to bioanodes, biocathodes in enzymatic fuel cells are typically modified with laccase[652, 653] or bilirubin oxidase,[638] as enzymes that are capable of reducing oxygen to water at high redox potentials,[654] although there are examples of peroxidases.[655, 656]

Optimization of both the energy density and the power density is critical when designing enzymatic fuel cell systems. While many enzymatic fuel cells employ a single enzyme for partial fuel oxidation, the complete oxidation of most fuels requires a combination of multiple enzyme systems to utilize the available fuel energy.[657] A significant challenge with the development of enzymatic fuel cells with high energy density is the successful enzymatic cascade for complete fuel oxidation. For example, to achieve complete glucose oxidation to CO_2, our research group has designed a bioanode consisting of a six-enzyme cascade.[658] The bioanode contained (1) PQQ-dependent enzymes extracted from *Gluconobacter sp*., (2) aldolase from *Sulfolobus solfataricus*, and (3) oxalate oxidase from barley. In addition to oxidizing glucose to carbon dioxide, this bioanode also eliminated the use of mediators as it showed the capability to perform direct electron transfer. The use of enzyme cascade systems, however, increases the complexity of fuel cells, and enzymes with limited stability can minimize the overall stability of the enzymatic fuel cell.

For highly engaged readers interested in this topic of enzymatic fuel cells, we highly recommend a recent and impactful review article from Xiao and co-workers, which systematically and comprehensively summarizes the latest progress of enzymatic fuel cells, especially the strategies for achieving high energy density, increasing power density, improving stability, and improving cell voltages.[635] This review article mainly focuses on

the application of isolated enzymes, enzymatic cascades, and multi-enzyme complexes in enzymatic fuel cells.

4.2.1.1. Organelle-based Biofuel Cells. Herein, we specifically focus on the implementation of organelles, namely mitochondria, in biofuel cells. The broad availability of mitochondria (animals, plants, and fungi sources), their easy isolation, and the presence of all the necessary enzymes and redox carries to accomplish complete fuel oxidation in their matrix motivated the interest in utilizing them as a biocatalyst for the development of biofuel cells. In 2008, a pioneering study by Arechederra and Minteer showed that mitochondria-based bioanodes coupled to a Pt-based cathode allowed obtaining biofuel cells with outstanding open circuit potential (about 1.0 V) and the capability to operate for up to 60 days.[8] Importantly, complete oxidation of pyruvate to CO_2 was demonstrated, without the requirement of exogenous redox mediators, as direct electron transfer between the immobilized mitochondria and the electrode surface was accomplished. Accordingly, the immobilization of mitochondria on carbon paper electrodes utilizing a hydrophobically modified Nafion membrane enabled combining the advantages of enzymatic based biofuel cells (high voltage) and microbial fuel cells (long term stability and complete fuel oxidation). A drawback of the developed biofuel cell was the limited power density (~ 200 $\mu W\ cm^{-2}$). However, an interesting aspect of utilizing mitochondria for the development of biofuel cells is that various compounds can inhibit their activity, and other chemicals are capable of decoupling the inhibition. This feature was utilized by Germain *et al*. to develop a mitochondria-based pyruvate/O_2 biofuel cell enabling the self-powered detection of an explosive nitroaromatic compound (nitrobenzene) down to a concentration of 1 pM in a self-powered on/off the sensor.[659] Specifically, nitrobenzene allowed decoupling the

inhibition effects of an antibiotic (1μM oligomycin, which inhibits pyruvate metabolism), resulting in a power output variation of more than one order of magnitude compared to inhibited mitochondria (~25 and 0.6 μW cm^{-2}, respectively). Later studies focused on unveiling the effects of various parameters on the electrochemical performance of mitochondria-based fuel cells, including substrate type and concentration, temperature, pH, and use of different inhibitors and decouplers.[660-663] Interestingly, it was shown that the presence of oxygen in the electrolyte could strongly affect the performance of pyruvate/O_2 biofuel cells.[661] The oxygen sensitivity of mitochondria based fuel cells is due to cytochrome c oxidase using electrons to reduce O_2. One possibility to decrease the inhibiting effects of O_2 on the current density obtained from the mitochondria-based fuel cells is to inhibit cytochrome c oxidase. The use of cyanide or carbon monoxide as inhibiting agents increased current density output of 3.66-fold and 4.83-fold, respectively.[661]

The application of pyruvate/O_2 mitochondria-based fuel cells for self-powered biosensing was expanded to eleven different explosive nitroaromatic compounds, including 2,4,6-trinitrotoluene (TNT).[660] This was accomplished by employing inhibitors targeting various components of mitochondria metabolism and eleven explosives acting as uncoupling agents. The mechanism of uncoupling is illustrated in **Figure 19**. Furthermore, mitochondria-based fuel cells enabled the fast and cost-effective study of drug-induced toxicity, providing direct evidence of the effects of drugs on mitochondrial metabolism,[662] as well as for the sensing of different pesticides, achieving an impressive limit of detection for atrazine[663] and malathion.[664]

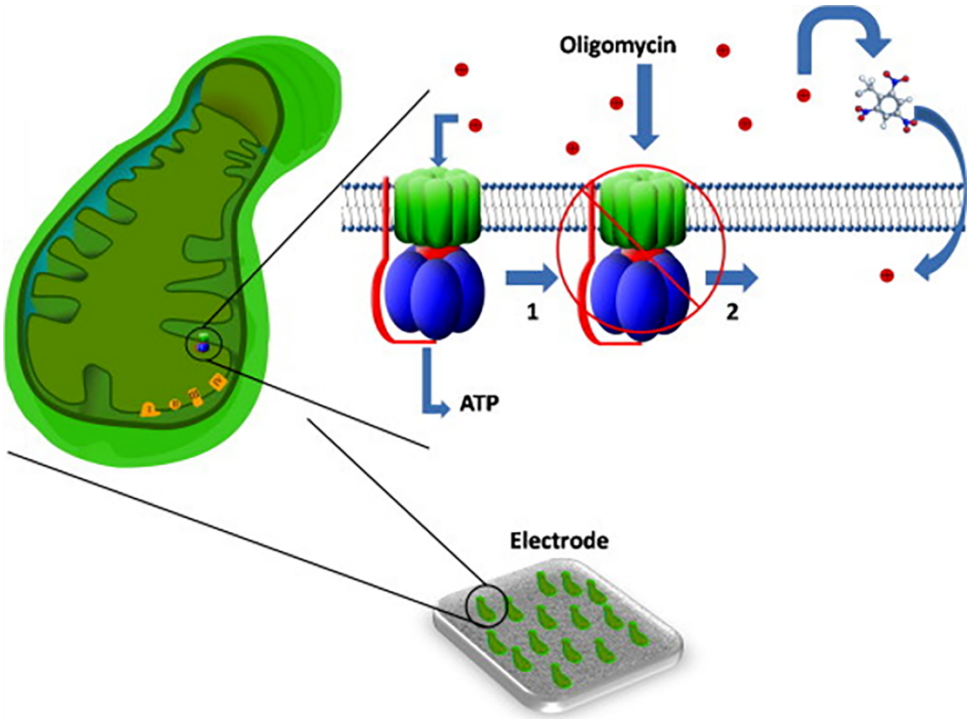

Figure 19. Scheme of mitochondria immobilized at the anode electrode of the biofuel cell. 1) ATP synthases transporting protons across the membrane during pyruvate metabolism; 2) the presence of the antibiotic oligomycin inhibits ATP synthases, blocking proton transport; 3) the presence of 2,4,6-trinitrotoluene uncouple the mitochondrial membrane by transporting protons into the matrix. Reprinted with permission from ref. 660. Copyright 2010 Elsevier.

4.2.2. Microbial Fuel Cells. Microbial fuel cells (MFCs) have gained significant attention in microbial bioelectrocatalysis due to the need for environmentally friendly, inexpensive, renewable fuels, and catalysts. These bioelectrochemical systems, offering a promise for renewable energy generation, use electroactive bacteria as bioelectrocatalysts to directly convert chemical energy into electrical energy via complex reduction-oxidation transformations during bacterial respiration.[665-669] Electrochemically-active microorganisms catalyze the oxidation of organic substrates (fuel),[215, 670] such as glucose, lactose, sucrose, xylose, and malic acid. The basic working principle of MFCs relies on redox half-reactions, which typically occur in two compartments separated by a membrane and linked by an external wire. Electroactive bacteria catalyze the oxidation half-reaction

of the organic fuel in the anode compartment. Electrons, which are released from cellular respiratory metabolism, flow through an external electrical circuit from the anode to the cathode electrode, generating electrical current (**Figure 20**).

MFC systems are developed similarly to enzymatic fuel cells; however, instead of specific redox enzymes, the anode is modified with intact bacterial cells as biological catalysts. The bioanode of the microbial fuel cell oxidizes a crude waste product, passing electrons through an electrical circuit to a cathodic reaction, often the oxygen reduction reaction (ORR). In comparison to enzymatic fuel cells, MFCs are less substrate specific as different metabolisms of electroactive bacteria can achieve complete oxidation of a wide range of fuels, which is often more energy efficient. Additionally, bacterial cells are living and self-replicating, which gives MFC systems long-term stability. In constructing enzymatic fuel cells, immobilization strategies are required to attach redox enzymes to the anode surface, whereas in MFCs, bacteria self-adhere to anodes, typically forming thick electroactive biofilms. Moreover, metabolic pathways in microbes often contain a series of oxidation steps, similar to a multi-step enzyme cascade biofuel cell. For instance, Speers and co-workers explored the metabolic pathways in *G. sulfurreducens*, in which electrons were harvested from sequential oxidation reactions in the tricarboxylic acid cycle. In their study, lactate (the fuel) was first oxidized to pyruvate, which then reacted to form acetyl coenzyme A (acetyl-Co-A) that enters the tricarboxylic acid cycle.[671] In addition to increasing the number of electrons transferred per mole of a substrate, these multi-step metabolic pathways can give rise to a variety of highly modified products, expanding the possibilities for applications of MFCs.

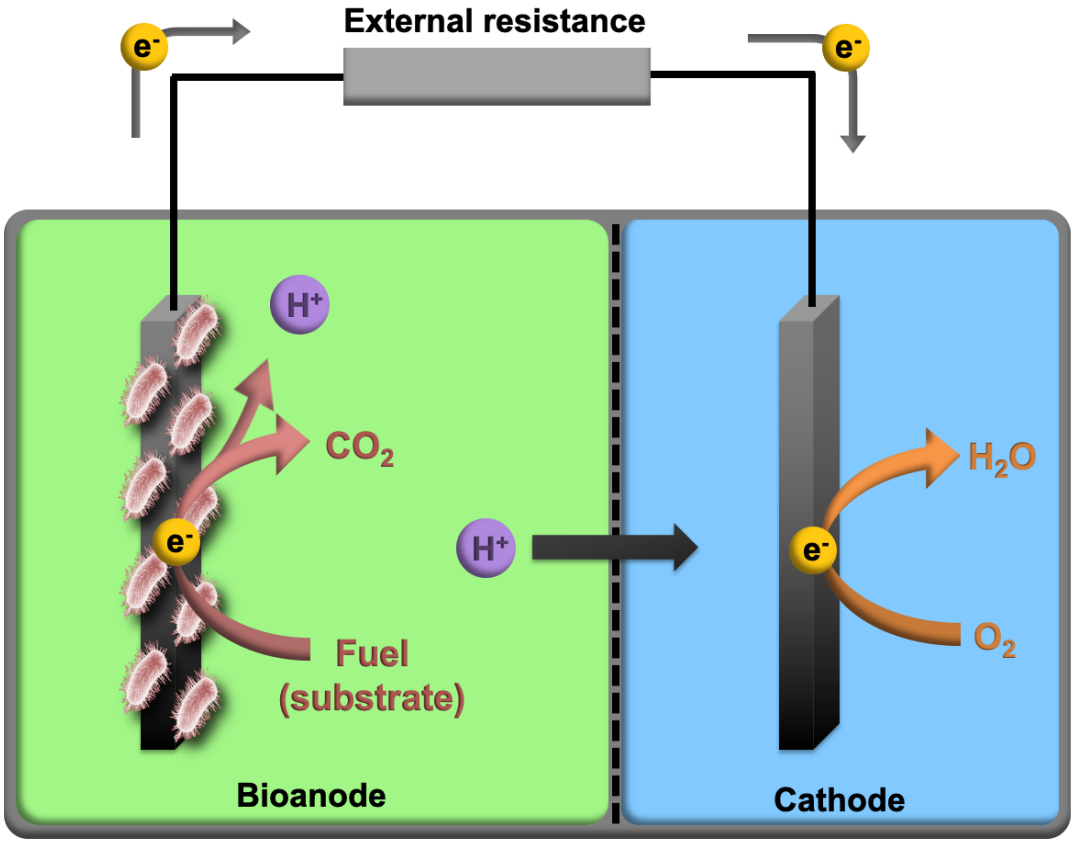

Figure 20. Microbial fuel cell (MFC) and its main components: bacteria, electrodes (anode and cathode), and a membrane. The anode, under limited-oxygen conditions, contains a convertible organic substrate (herein, fuel is malic acid, electron donor) while the cathode contains molecular oxygen (electron acceptor). The working principle of MFCs relies on redox half-reactions, which occur in the two compartments separated by a membrane and linked by an external wire. Electroactive microorganisms catalyze the oxidation half-reaction of an organic fuel in the anode compartment. Electrons, released from cellular respiration activity, flow from anode through an external electric circuit to cathode, generating electricity. At the cathode, oxygen (electron acceptor) is reduced. To establish charge neutrality, proton ions (H^+) are transported across the membrane to the cathode as electrons are released at the anode, both of which react with O_2 on the cathode to generate water as a byproduct. Current flowing through the external circuit and voltage difference of half-reactions generate power output.

MCFs offer an enormous promise as bioelectrochemical systems for various applications, including bioremediation, wastewater treatment, and biofuel production.[273, 668, 672-675] Namely, the target industries for MFC applications include municipal,[676, 677] industrial,[678] and agricultural wastewater treatment strategies,[679] which contain notable

amounts of organic compounds that can be oxidized and degraded by microbes. The primary purpose of MFC applications relies on the conversion of waste products to biofuels, hydrogen gas, methane, and other valuable organic and/or inorganic chemicals and also the production of small amounts of bioelectricity. The generated electricity, in turn, can be put directly back into the system (e.g., self-powered biosensors),[680] or resold to the grid for profit in as little as five years.[681] Despite their numerous applications, the practical function of MFCs remains limited due to the relatively low MFC power densities (mW m^{-2}). To achieve a fast conversion of chemical energy to electrical energy, the components of a microbial fuel cell (anode and cathode electrodes, bacteria, and membranes) have to be carefully engineered.[673, 682] Research studies have focused on the optimization of electrode materials and designs of membranes.[665] However, the enhancement of the slow extracellular electron transfer rate (EET) from bacteria to anode electrodes is necessary since EET plays a fundamental role in MFC performance.[683]

The two EET mechanisms, discussed in previous sections, are (1) direct electron transfer from inside the cells, or (2) indirect, mediated electron transfer via exogenous or endogenous electron redox mediators. In direct electron transfer, anodes are in physical contact with redox-active proteins on cellular surfaces, facilitating electron transfer.[684, 685] However, most electroactive bacteria do not have these redox surface proteins, and as such, only a few bacterial strains can achieve direct electron transfer mechanisms to the electrode. Namely, *S. oneidensis* has gained popularity in microbial electrochemistry, as studies have shown it undergo direct electron transfer via specific protein complexes embedded in the cell membrane, the *Mtr* pathway (Section 2).[108] Bioengineering efforts have exploited this natural machinery by genetically modifying other bacterial species

(e.g., *E. coli*) traditionally incapable of direct electron transfer, to express *S. oneidensis Mtr* genes, and achieve extracellular electron transfer.[281] In a more recent study, *E. coli* cells were modified with type IV pilus genes from *G. sulfurreducens* (described in Section 2.2.1), resulting in a mutant that could perform extracellular electron transfer via electrically conductive protein nanowires.[686]

Since only a limited number of microorganisms have redox-active surface protein for direct electron transfer mechanisms, MFCs require the use of artificial, redox-active mediators. Research studies have developed MFCs employing exogenous mediators (e.g., neutral red, thionine) to increase electron transfer kinetics and enhance power output.[687, 688] For example, Park and co-workers demonstrated that the amount of electrical energy produced by MFCs can be increased approximately 1000-fold by incorporating exogenous electron mediators into graphite electrodes.[688] Additionally, Grattieri and co-workers recently employed different quinones as exogenous mediators directly into electrolyte solutions of *R. capsulatus* mediated electron transfer systems to investigate the generation of biophotocurrent in photosynthetic purple bacteria.[689] Their results showed the largest photocurrent density with the use of *para*-benzoquinone.[689] However, this method based on exogenous mediators is expensive as it requires high concentrations of electron shuttle mediators, which can be toxic to the microbial cells.[690] Thus, another strategy is to design MFCs using endogenous electron mediators, such as flavins, phenazines, and quinones, synthesized by microbes.[172, 225, 466, 691] For example, certain electroactive bacteria, such as *P. aeruginosa*, are capable of self-generating various redox-active phenazine metabolites as electron shuttles that directly transfer electrons to anodes.[172, 692] This approach enables for a simplified MFC design and device operation at high-sustained activity levels,

reducing operational expenses, and also eliminating other downsides (e.g., toxicity to cells). Ali and co-workers have characterized current generation potential in *P. aeruginosa*-based MFC using glucose, fructose, and sucrose as organic substrates.[693] The researchers demonstrated that *P. aeruginosa* can effectively use pentose and hexose sugars via anode respiration, with the highest power density of 136 ± 87 mW m^{-2} generated from glucose.[693] Additionally, Islam and co-workers explored the synergistic effects of a defined co-culture system consisting of *P. aeruginosa* and *Klebsiella variicola*, showing three times higher MFC current density compared to MFCs with either of the two bacteria species alone.[694] Specific metabolite analysis showed that the production of a *K. variicola* fermentative metabolite (1,3-propanediol) stimulated the production of higher amounts of *P. aeruginosa* phenazine metabolites via synergistic interactions, resulting in enhanced MFC performance.[694] Investigating methods to establish direct and/or mediated electron transfer mechanisms from bacteria to anodes remains an active area of research in designing MFC systems with improved overall performances. In advancing MFC technologies, future work needs to focus on bridging new design platforms to genetically engineer electron transfer pathways in non-electroactive microorganisms, to improve EET rates and MFC power yields.

When employing MFCs for practical applications *in situ*, several environment-related factors can arise, which can subsequently inhibit MFC efficiency. Namely, saline wastewater, which comprises about 5% of the world's total wastewater,[695] can dehydrate bacterial cells, resulting in cell death. Implementing MFC systems that are tolerant to salinity has been very challenging, as bacteria have evolved mechanisms to saline resistance involving adaptations in membrane structure and charge balance, both of which

can impact electron transfer. Recently, Gaffney and co-workers combined electrochemistry with bioinformatics in a pioneering study to elucidate the relationship between gene expression and electron transfer processes in the halotolerant bacterium *R. capsulatus*.[696] The findings from this study demonstrated that saline adaptation plays a significant role in the electrocatalytic response of *R. capsulatus* and variations in gene expressions after salt adaptation, thus providing a better understanding of bioelectrochemical systems under saline conditions.

4.2.3. Biosolar Cells. Current overwhelming reliance on finite, highly carbon dioxide (CO_2)-emissive fossil fuels to cater to the growing global energy demand necessitates the utilization of alternative energy sources such as solar energy. Ubiquitous solar irradiation provides 310^{24} J of energy per year to the earth, which makes energizing energy-exhaustive processes like CO_2 reduction to value-added carbon compounds (C compounds) plausible.[697] Solar energy is renewable, green, and sustainable compared to high carbon-footprint energy sources. However, solar-to-electric energy conversion by photovoltaic devices presently contributes a meager ~1% to the global energy consumption compared to fossil fuels (85%).[698] Contemporary solar fuel cells are mainly inorganic catalyst-based, such as solid-state junction photovoltaic devices made of doped forms of silicon and dye-sensitized solar cells (DSSCs) constituting ruthenium- or platinum-based photosensitizers.[699, 700] However, strenuous fabrication processes, use of scarce, expensive, and toxic components raise limitations associated with inorganic catalysts.[701] Moreover, inorganic metal-, semiconductor- or conducting polymer-based fuel cells that photo-reduce CO_2 are mostly limited to producing C_1 compounds, such as carbon monoxide and formate. Using these fuel cells to electrosynthesize complex C compounds that require multiple

proton-coupled electron transfers remain inaccessible, poorly selective, or impeded by the susceptibility of particular electrocatalysts to photocorrosion.[702, 703] Therefore, low cost, eco-friendly, selective, and durable solar fuel cells that circumvent existing limitations to efficient solar energy harvesting are compulsory. Biosolar cells are a promising comprehensive solution in that respect.

Photoautotrophic microorganisms such as cyanobacteria, purple bacteria, and algae are evolutionarily optimized biocatalysts, which energize bioelectrocatalytic processes utilizing solar energy (**Figure 21**).[704] They possess elevated solar absorption, high extinction coefficients, and photoelectric properties.[705] Respective genetic, enzymatic, and cellular compositions in phototrophic metabolism facilitate highly product-specific bioelectrocatalytic reactions under mild ambient conditions (*vide supra*).[704, 706, 707] These photosynthetic microorganisms also have the metabolic sophistication to form valuable precursors from CO_2 reduction, which leads to more complex C compounds.[708-710] Moreover, whole cell-based photocatalysts retain their self-sustainability and repairing abilities, making them more pliable in practical applications.[704, 711]

Co-cultures of compatible but functionally differentiated heterotrophic and photosynthetic bacteria streamline the performance of photo-microbial fuel cells (PMFCs). Synergistic syntrophic interactions between the two biotic components overcome the additional metabolic expenses attributive to simultaneous photo- and catalytic-based functions in a singular microorganism.[711-713] A PMFC of cyanobacteria, *Leptolyngbya,* and green algae, *Acutodesmus,* has been shown to degrade 90% of organic waste from wastewater, 100% ammonium nitrogen (bioremediation) coupled to direct electricity production of 55 Wh m^{-3}.[714] Wei and co-workers designed a self-sustaining micro-sized

photo-microbial cell composed of *Synechocystis* sp. PCC 6803 strain and *S. oneidensis*.[712] The cell generated a photocurrent of 8 mA m^{-2}, which is a seventy-fold current increment compared to singular photosynthetic bacteria.[712] This improvement is collectively attributed to the microstructure that assists in mass transport, reduces the internal resistance, and co-culture synergy. PMFC miniaturization is also desirable for energizing small scale applications under limited resources and remote settings.[712, 715]

Biophotovoltaic (BPV) devices are biological solar cells, which generate electricity from photosynthetic activities of living organisms (e.g., algae). The biophotovoltaic cell lifetime and cost-effectiveness are being improved by modulating proton exchange membrane (PEM), electrolyte, and charge mediators, etc.[716] Cyanobacteria have been inexpensively 'printed' onto sheets and incorporated into BPV cells to potentially energize low power LED lights and alarm clocks.[717, 718] While these optimizations increase BPV power generation, solar-to-electricity conversion efficiencies remain meager (~1%) compared to the typical photosynthetic energy conversion efficiencies of green algae (8%).[711, 712] Kim and co-workers designed a biohybrid system containing cyanobacteria, *Synechococcus* spp., and an inorganic photosensitizer, γ-Fe_2O_3-neodymium iron boride magnet nanoparticles.[719] The resultant BPV demonstrated amplified electronic contact and solar harvesting, in the absence of charge mediators.[719] Peak power densities during the light and dark reactions of the resultant BPV cell are 0.806 and 0.235 W m^{-2}, respectively. These power densities are well-above typical BPV values, which range between 0.015–220 mW m^{-2} and the highest power density reported for a *Synechococcus* spp-based system (0.610 W m^{-2}).[720]

A recent study by Joshi and co-workers reported the creation of a bionic mushroom-type architecture by closely linking cyanobacteria with graphene nanoribbons onto an umbrella-like mushroom pilus for the generation of photosynthetic bioelectricity.[721] The graphene nanoribbons mediate extracellular electron transfer from cyanobacteria, thereby generating photocurrent. In this creative work, the researchers also employed 3D-printing technology to assemble cyanobacteria in densely packed bacterial structures and geometries to create density-dependent cell populations, resulting in an approximately 8-fold increase in the generated photocurrent compared to non-3D-printed cyanobacteria colonies. While this study did not demonstrate the incorporation of 3D-printed cyanobacteria in biosolar cells, the 3D-printing technology used in this study provides interesting questions for future research investigations in this area and bioelectrochemical systems based on cyanobacteria.

In addition, photo-microbial desalination cells (PMDCs) couple desalination and electricity generation. Only 2.5% of the global water reserves are freshwater while 96.5% are ocean waters, which makes the prospects of desalinating seawater to address the freshwater and energy crises enticing potentially.[722] Al-Mamun and co-workers reported a PMDC containing graphite electrodes, synthetic wastewater with aerobic sludge as the anolyte and a mineral solution with microalgae as the biocatalyst.[723] The PMDC yielded high external resistance, 40% desalination efficiency and a power density of 84 mW m^{-3}.[723] Zhang and co-workers reported the first hybrid PMDC containing zinc-doped hematite and TiO_2 photoanode, along with a bilirubin oxidase biocathode to recycle the water/oxygen redox couple.[724] This biocathode gave a maximum power density of 21.4 μW cm^{-2} as opposed to a platinum mesh electrode (0.32 μW cm^{-2}). Liang and co-workers designed a

newer generation of PMDCs consisting of a *Geobacter*-α-hematite bio-photocatalyst anode and graphite felt cathodes.[725] The resultant current density of 8.8 A m^{-2} and 96% salt removal are well-above the typical desalination efficiencies of microbial fuel cells (MFCs) (<80% salt removal).

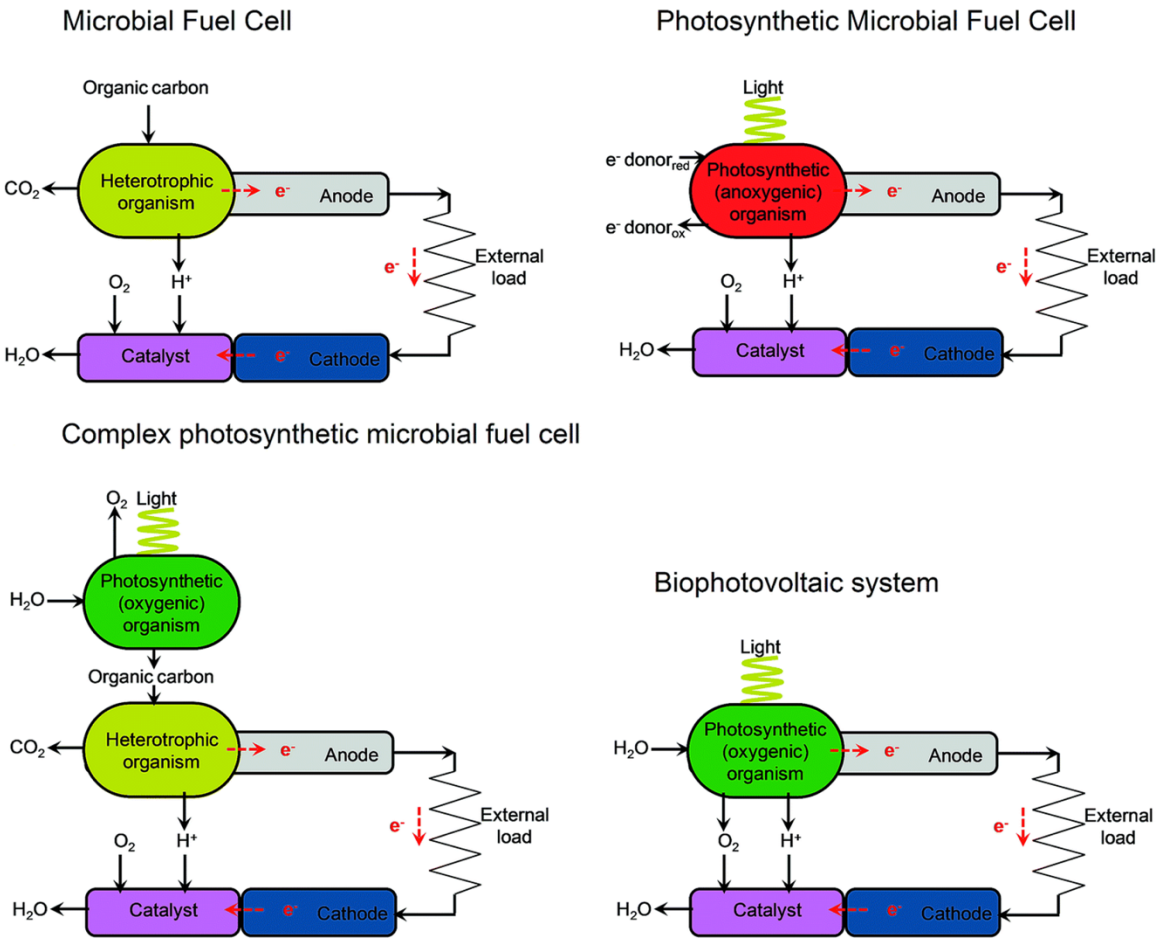

Figure 21. An adopted and modified scheme of bioelectrochemical cell depicting the distinct solar biofuel cell configurations. A hybrid solar biofuel cell is constructed with a bioanode based on a photosynthetic microorganism (e.g., cyanobacteria, purple bacteria, algae) and an inorganic cathode for oxygen reduction. Bioanodes of complex photosynthetic fuel cells couple heterotrophs with photosynthetic microorganisms to function synergistically. The cathode can be substituted with biological entities to make an all-biological solar biofuel cell. Moreover, the nature of the microbial cell, organelle, and enzyme determines how each biological entity is incorporated into the solar biofuel cell and the respective scope of applications and efficiencies. Reprinted with permission from ref. 726. Copyright 2015 Royal Society of Chemistry.

Relative to contemporary photovoltaic materials, phototrophic biocatalysts are easily cultured, versatile, and adaptable.[718, 726] In DSSCs, microbial pigments, including chlorophylls[727] and carotenoids,[728] are substitutable to some of the toxic, scarce, and expensive photosensitizers.[699, 700] Donoso and co-workers utilized pigments extracted from Antarctic bacteria *Hymenobacter* sp. A9A5 and *Chryseobacterium* spp. for DSSCs. These pigments possess UV-resistance in addition to the intrinsic photostability of non-photosynthetic microbes,[729, 730] which improves photostability under continuous irradiation.[731-733] Bacteriorhodopsin protein photosensitizer in DSSCs has shown consistently high external efficiency (0.94%).[734-736] However, labor-intensive isolation, purification of microbial pigments and proteins, retention of long-term biological functionality, and stability have restricted the growth of microbial photosensitizers. Therefore, a new generation of genetically modified whole cell photosensitizers has been introduced. Yadav and co-workers engineered *E. coli* to heterologously overexpress a gene cluster of lycopene photosensitizer dye synthesis, and in turn, interfaced the *E. coli* onto TiO_2 semiconductors using a supramolecular porous organic mesh.[737] The resultant photosensitizer yielded a total external efficiency of 0.057% that is comparable to pigment-based DSSCs like chlorophyll (0.055%).[727] Within the context of efficiency of contemporary DSSCs (13%), the significance of whole-cell photosensitizers lies in their easy, green fabrication (that sidesteps enzyme/pigment isolation) and low fabrication costs.

Chloroplasts can also be employed to construct biosolar cells. Chloroplasts containe thylakoid membranes where all the protein complexes responsible for photosynthesis are comprised, allowing the conversion of sunlight energy (photons) into chemical energy (sugars). Furthermore, they have various mechanisms of self-repair to protect them against

photodamage,[738] and can be easily extracted for broadly available plants, making them a promising candidate for developing biosolar cells. In a photo-biofuel cell, water acts as the electron donor, being oxidized to oxygen thanks to photons absorption in the chloroplast, and the generated photoexcited electrons are diverted to the anode and utilized at a cathode were oxygen reduction is performed. Accordingly, chloroplast fuel cells constitute a promising approach to expand sunlight energy harvesting. However, only a few reports of chloroplast-based fuel cells are available in the literature. In a 1981 pioneering study by Bhardwaj *et al.*, a chloroplast fuel cell was reported, where an exogenous redox mediator (2,6-dichlorophenolindophenol) was utilized to harvest the photoexcited electrons at a Pt electrode.[739] Despite the design of the cells being relatively simple and the use of redox mediators, both for the anodic and cathodic reactions, the device allowed a maximum power output in the range of 380 μW and a power conversion efficiency of approximately 2-3%. In a later study, Okano *et al.* reported the possibility to immobilize chloroplasts on transparent SnO_2 electrodes using a 2% agar gel, with methyl viologen utilized as a diffusible redox mediator.[740] The chloroplast photoanode, coupled to a Pt cathode operating in 0.5 M H_2SO_4, allowed a chloroplast fuel cell with a solar energy conversion efficiency of approximately 1 to 2%.

These initial reports of chloroplast fuel cells presented some limitations in terms of limited current density and power output, as well as the presence of diffusible redox mediators. In order to tackle these limitations, Ryu *et al.* explored the possibility of utilizing an ultrasharp nanoelectrode inserted in the chloroplast of the single-celled alga *Chlamydomonas reinhardtii* to harvest the photoexcited electrons without the need of diffusible redox mediators.[741] The approach allowed the direct harvesting of photoexcited

electrons, by reaching proximity with the quinone pool or ferredoxin in the chloroplast. However, the accurate insertion and positioning of the nanoelectrode introduce limitations for the scale-up of the system. With the aim to simplify the chloroplast fuel cell setup, Amao *et al.* immobilized chloroplasts on a nanocrystalline TiO_2 film on indium tin oxide electrodes modified with 12-aminolauric acid, allowing direct transfer of the photoexcited electrons.[742] The modified chloroplast photoanode coupled to a Pt-based cathode enabled a remarkable short-circuit photocurrent of approximately 10 μA cm^{-2}. In a recent study, Hasan *et al.* investigated the possibility to utilize a bio-inspired redox polymer, where the redox moieties are bound to the polymer backbone, to harvest the photoexcited electrons from the chloroplast.[743] Specifically, the polymer comprises naphthoquinone redox moieties, resembling the quinone redox intermediates in thylakoid membranes. By employing the redox polymer, a 5-fold enhancement in photocurrent generation was obtained compared to the chloroplast in direct electron transfer conditions, achieving a current density of 5.7 ± 0.3 μA cm^{-2}.

4.3. Investigation of Oxidoreductase Catalytic Mechanisms via Bioelectrocatalytic Methods

Bioelectrocatalysis depends on the biocatalysis of redox reactions, which occur at the electrode-electrolyte interface where the electrode plays the role of an electron donor/acceptor to the biocatalyst. The substrate conversion, the binding of redox partner, and the intramolecular electron transfer can produce different electrical signal changes via electrical communication between oxidoreductase enzymes and electrode surfaces. The corresponding changes of electrical signals can provide useful information for the investigation of the thermodynamic, kinetic, and catalytic mechanisms.

Protein film voltammetry, developed by Fraser Armstrong's group, refers to a concept that an oxidoreductase is configured as a film on an electrode surface and probed by various electrochemical methods. As the oxidoreductase molecules are immobilized on electrode surfaces, the modulations of the electrode potential or catalytic turnover cause the electron transport to, from, and within the oxidoreductase molecule, which can be detected as a current response in characteristic ways with time and potential. Therefore, protein film voltammetry is a useful methodology to study the catalytic mechanisms of oxidoreductases and significantly promotes the progress of bioelectrocatalysis. Applying protein film voltammetry, Christophe Léger and Fraser Armstrong carried out in-depth research works studying catalytic mechanisms, inhibition kinetics, and intramolecular electron transport of hydrogenase.[744-749] The Hirst and Reisner research groups investigated the CO_2 reduction mechanism and formate oxidation using Mo-dependent formate dehydrogenase by employing protein film voltammetry.[243] Protein film voltammetry provided a new perspective on redox-coupled reactions by distinguishing the potential and time domains compared to standard solution kinetics experiments. In addition, the binding properties of inhibitors to the reduced and oxidized active sites were characterized (**Figure 22**).[750] Besides hydrogenase and formate dehydrogenase, the catalytic mechanisms of nitrate reductases,[751] acetyl-CoA synthase,[752] and cytochrome c peroxidase,[753] have also been investigated by using protein film voltammetry. As the protein film voltammetry is an extensive research area, many review articles have provided a detailed and comprehensive summary of the principle, characteristics, and applications of this technology. For engaged readers in this particular area, these review articles are highly recommended.[33, 754-758]

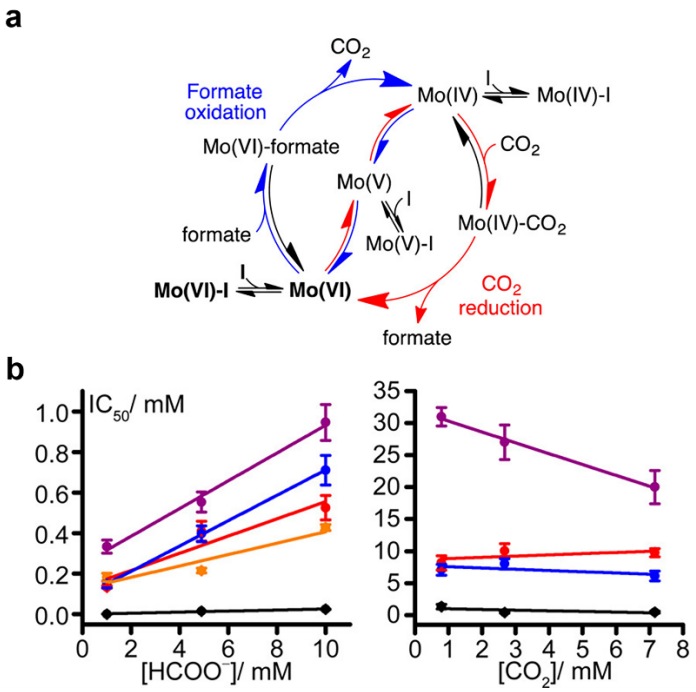

Figure 22. (a) Model for the inhibition of Molybdenum-containing formate dehydrogenase H from *E. coli* electrocatalysis. (b) Dependence of inhibitor IC50 values on substrate concentration for formate oxidation and CO_2 reduction. NO_2^- was reduced by the electrode and thus was omitted from the CO_2 reduction graph. Black, N_3^-; red, OCN^-; blue, SCN^-; purple, NO_3^-; orange, NO_2^-. Conditions: 23.5 °C, pH 7, −0.1 V *vs* SHE (formate), −0.6 V *vs* SHE (CO_2). Reprinted with permission from ref. 750. Copyright 2017 American Chemical Society.

Nitrogenase is the only enzyme known to enable NH_3 production from biological N_2 reduction. It is a multi-protein complex, which consists of an electron-transferring ATP-hydrolyzing iron protein (Fe protein) and a catalytic molybdenum-containing protein (MoFe protein) where N_2 is reduced. There are two alternative nitrogenase systems, which employ vanadium or iron-only (VFe and FeFe) proteins.[5, 67] The MoFe protein is a dimer of dimers containing a [Fe_8S_7] cluster (P-cluster) and a [Fe_7MoS_9C] cluster (FeMoco).[759] The activity of nitrogenase *in vivo* depends on a [Fe_4S_4] cluster-containing Fe protein as a unique electron donor.[760] During catalysis, electrons are initially transferred from the P-cluster to FeMoco upon binding of Fe protein to MoFe protein via a deficit spending mechanism, in which electrons are subsequently back-filled into the P-cluster from Fe

protein.[761] While the kinetics of isolated nitrogenase have been extensively studied, little is known about the thermodynamics of its cofactors under catalytically relevant conditions. Recently, a collaborative study between the Minteer, Seefeldt, and Einsle research groups reported the direct measurement of reduction potentials associated with each metallocofactor of the nitrogenase complex (**Figure 23**).[34] In this study, the researchers functionalized a polymer (linear polyethylenimine, LPEI) with pyrene moieties capable of establishing a coherent bioelectrochemical interface to drive catalysis of several metalloenzymes without the need for exogenous electron mediators to directly measure redox potentials for each of the cofactors in nitrogenase under biologically relevant conditions. The nitrogenase metallocofactors were observed by square wave voltammetry at approximately −0.23 V *vs* NHE for the P cluster and −0.59 V *vs* NHE for the FeMoco, respectively. The redox potential of FeMoco was observed to be more reducing than that of the P-cluster, suggesting a requisite for endergonic electron transfer during the catalytic turnover of nitrogenase. Incorporation of the Fe protein into pyrene-LPEI films resulted in a distinct shift in the FeMoco redox potential to −0.43 ± 0.02 V. In addition, a nearly identical shift in redox potential for FeMoco was observed in the complete absence of H_2 gas. The voltammetric analysis of MoFe protein in the absence of Fe protein revealed that electrochemically driven catalysis could only be observed when atmospheric H_2 content was below ~1.8%. These results potentially indicate that the shift in the potential of FeMoco is the result of an interaction between MoFe protein and H_2 where binding of Fe protein prevents this interaction.

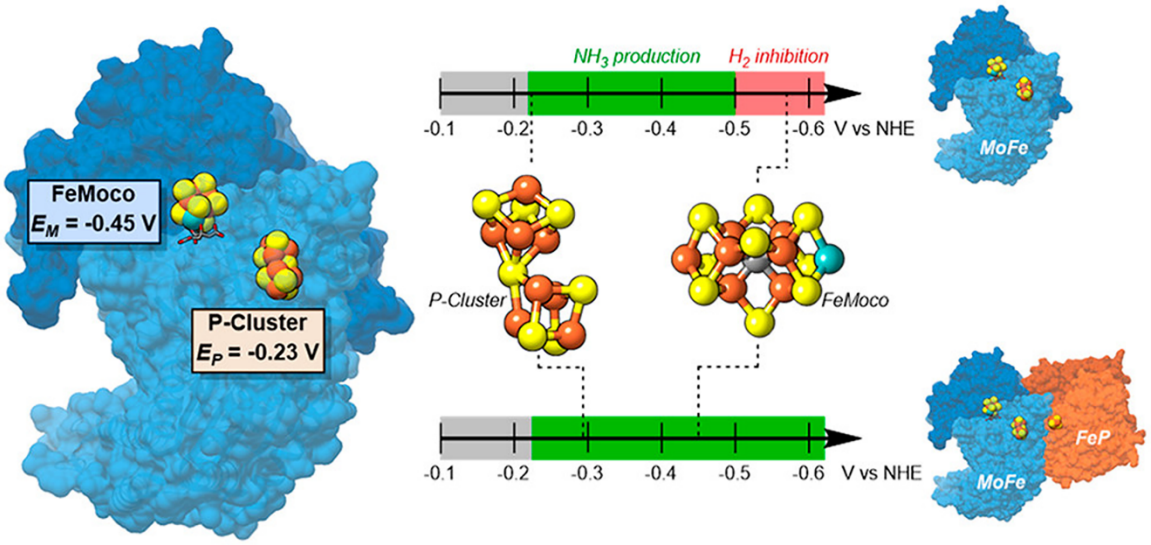

Figure 23. The thermodynamic landscape for the active site of Mo-dependent nitrogenase: direct reduction potential measurement of either the [Fe8S7] (P-cluster) or [Fe7MoS9C] (FeMoco) cofactors. Reprinted with permission from ref. 34. Copyright 2019 American Chemical Society.

The Abad group achieved an electrode surface modification through the reaction with a biphenyl dithiol self-assembled monolayer and gold clusters capped with thioctic acid to facilitate the electrochemical communication between a gold electrode and galactose oxidase (GOase).[762] As a result, the hybrid system showed an effective electrocatalytic response for oxygen reduction with the formal potential of the Goase redox reactions and rate constants for electron transfer to Goase protein, preserving their natural enzymatic activities and enhancing electron transfer (ET) rates. Since glucose is ubiquitous and abundant in most living organisms, a significant amount of enzymatic bioelectronics research has focused on investigating proteins that utilize glucose as a substrate. The Alfonta group established a detection of direct electrochemical glucose oxidation signal by the addition of minimal cytochrome domain to c-terminus of GDH from *Burkholderia cepacia*. Cyclic voltammetric and square wave voltammetric current-potential responses resulted in enhanced electrocatalytic current by fusion GDH catalyzed glucose oxidation.[371] In addition, electrochemical K_M^{app} and i_{max} from the steady-state

amperometric analysis provide the affinity of fusion glucose dehydrogenase (GDH) toward glucose. The Mano group developed a highly porous carbon cryogel-based electrode assembled with pyrroloquinoline quinone-soluble glucose dehydrogenase (PQQ-sGDH), facilitating the detection of the direct catalytic signal. Bioelectrocatalytic activity for glucose and maltose oxidation was shown including the intrinsic enzyme kinetics, the maximum rate of heterogeneous electron transfer, and the substrate accessibility to the enzyme's active center.[694]

4.4. Bioelectrosynthesis

Bioelectrosynthesis refers to the process of utilizing bioelectrocatalysts to produce desired products in bioelectrocatalysis systems. In contrast to biofuel cells, which generate electrons by oxidizing fuels at the anode, bioelectrosynthesis focuses on the cathode, where the substrate is converted to the desired product with the consumption of external electrons.[695] The electrochemical communication between the bioelectrocatalyst and the electrode can be performed via either DET or MET.[46] For traditional biocatalysis, the reduced equivalent is generated by the addition of a second enzymatic reaction, which involves a second enzyme and the second substrate *in vitro* for constructing an intracellular reduced equivalent regeneration pathway. Compared with traditional biosynthesis, bioelectrosynthesis has the merit of requiring no additional approaches for the regeneration of reduced equivalents as the electrode can be employed as the electron donor to support the synthesis of the target product(s).

4.4.1. Enzymatic Electrosynthesis. Enzymatic electrosynthesis is a bioelectrocatalytic process in which isolated enzymes or multi-enzyme cascades are driven by electrons from the electrode for the synthesis of target products.[763] Enzymatic electrosynthesis uses

renewable enzymes as bioelectrocatalysts. Depending on the high activity and high selectivity, enzymatic electrosynthesis can be used to synthesize a variety of useful chemicals.[18] The single enzyme bioelectrosynthetic system is mainly used for the synthesis of the simple compounds or the introduction of functional groups and chiral centers. Bioelectrosynthetic systems with enzymatic cascades can be used to perform multi-step conversion processes and the synthesis of products with complicated structures.

4.4.1.1. Hydrogenase and Enzymatic Electrosynthesis of Dihydrogen (H_2). Molecular hydrogen (H_2) is a carbon-neutral energy carrier, which has excellent advantages for replacing fossil fuel-based liquids as it is both clean and renewable.[64] Beside being used as a fuel, H_2 is also useful for hydrogenation of a variety of products and applications, including ammonia for fertilizers, and food and heavy oils in gasoline production.[764] In nature, many microorganisms are able to produce hydrogenase, a metalloenzyme, which catalyzes the reversible oxidation of H_2. Hydrogenases are classified into [FeFe]- and [NiFe]-hydrogenases based on the metal clusters at their catalytic sites.[765] The [FeFe]-hydrogenase active site cluster is composed of a regular [4Fe-4S] sub-cluster (H-cluster) bridged to a 2Fe sub-cluster via a bridging cysteine thiolate. [NiFe]-hydrogenase has a similar structure where one of the Fe ions of the 2Fe sub-cluster is bridged to a Ni atom. The Ni atom is, in turn, terminally coordinated by two additional cysteine thiolates. Some [NiFe]-hydrogenases in which one of the two terminal cysteines are replaced by selenocysteine are called [NiFeSe]-hydrogenases.

Although hydrogenases are very promising perspective biocatalysts for H_2 production and H_2 oxidation, a major obstacle to the application of hydrogenase is their sensitivity to O_2.[766] Many research studies have been performed to investigate the oxidative inactivation

mechanism of hydrogenase.[767-771] To use hydrogenase under aerobic condition, researchers have adopted three strategies, namely (1) the discovery of novel hydrogenases that naturally resist O_2,[772, 773] (2) the modification of hydrogenases to enhance O_2 tolerance via protein engineering methods,[774-776] and (3) the integration of the hydrogenases into redox polymer films that provide a self-activated shield.[777-779] In bioelectrocatalysis, the application of redox polymers is a simple and effective method to protect hydrogenase under aerobic conditions. In this field, Wolfgang Schuhmann's research group, in collaboration with Nicolas Plumeré, Wolfgang Lubitz, and Adrian Ruff, have performed fruitful research works. Under a hydrogen fuel cell architecture, the researchers designed a viologen-functionalized redox polymer and immobilized an O_2-sensitive [NiFe]-hydrogenase on the surface of the electrode with this redox polymer. The electrons generated from the H_2 oxidation catalyzed by hydrogenase induced the viologen-catalyzed O_2 reduction at the surface of the redox polymer to prevent the oxidative inactivation of [NiFe]-hydrogenase. Meanwhile, the electrons could also be transferred to the anode surface via the viologen moieties to generate current and power output in the presence of O_2.[777] On this basis, they further improved the structure of the viologen-based redox polymer and successively developed two new redox polymer, poly(3-azido-propylmethacrylate-co-butyl acrylate-co-glycidyl methacrylate)-viologen and poly(glycidyl methacrylate-co-butyl acrylate-co-poly(ethylene glycol)methacrylate)-viologen. The two redox polymers were used to immobilize hydrogenase, prepare a two-layer bioanode, and protect the hydrogenase from high potentials and O_2 damage. In the integration with an oxygen-reducing bilirubin oxidase gas-breathing biocathode, the formed H_2/air biofuel cell showed a current density of up to 8 mA cm^{-2}. A maximum power

density of 3.6 mW cm-^{2} at 0.7 V and an open circuit voltage of up to 1.13 V were achieved.[387]

An increasing number of recent studies have suggested that hydrogenase-based H_2 evolution can also be achieved via electrochemical methods. The first and critical step for the hydrogenase-based electrochemical H_2 production is the preparation of hydrogenase modified bioelectrodes.[780] To date, [FeFe]- and [NiFe]-hydrogenases have been applied on many different solid electrodes, including single-walled carbon nanotubes,[781] carbon felts,[782] TiO_2 electrodes,[783] and CdTe nanocrystals.[784] Qian and co-workers used a mixture of montmorillonite clay and poly(butylviologen) as a sandwich layer to immobilize hydrogenase on glass carbon electrodes. This modified electrode can be used for efficient bioelectrochemical H_2 evolution.[785] In another study, Morra and co-workers employed an anatase TiO_2 electrode to absorb [FeFe]-hydrogenases. The immobilized hydrogenase demonstrated the ability to perform DET to and from the electrode surface and catalyzed the evolution of H_2 with a current density of approximately 2 mA cm^{-1}. The H_2 evolution occurred with a Faradaic efficiency of ~98%.[780] In very recent research, [FeFe]-hydrogenase from *Clostridium pasteurianum*, as well as [NiFe]- and [NiFeSe]-hydrogenase from *Methanococcus maripaludis*, were respectively immobilized on the surface of cathode with cobaltocene-functionalized polyallylamine redox polymer to perform H_2 evolution based on mediated electron transfer. Faradaic efficiencies of H_2 evolution of over 80% were achieved for all the three hydrogenases.[786] Additionally, photoelectrochemical methods based on the application of semiconductors can be used for H_2 production. The capture and storage of optical energy in the form of H_2 via water-splitting is a promising H_2 production method, which has been demonstrated by great

research work from the Reisner group. The researchers also developed novel photoelectrodes, TiO_2-coated p-Si and lead halide perovskite photocathodes, for the generation of photocurrent for the reduction of protons to H_2. Specifically, [NiFeSe]-hydrogenase was first immobilized on a TiO_2-coated p-Si photocathode. The p-Si|TiO_2|hydrogenase photocathode exhibited visible-light-driven H_2 production. After 1 hour of reaction in this experimental setup, a charge of 5.1 mC had passed, and 25 nmol of H_2 were detected, corresponding to a 95% Faradaic efficiency.[787] After that, the [NiFeSe]-hydrogenase was immobilized on a triple cation mixed halide perovskite to form a perovskite-hydrogenase photocathode. At 0.8 V *vs.* RHE onset potential, the perovskite-hydrogenase cathode was combined with a $BiVO_4$ water oxidation photoanode to form a self-sustaining, bias-free photoelectrochemical tandem system for water-splitting and H_2 production. The photoelectrochemical tandem system produced 21.1 μmol cm^{-2} H_2 after 8 h of controlled potential photoelectrolysis with Faradaic efficiency of 82%.[788] On the other hand, the Reisner research group designed photoelectrochemical systems that couple semi-artificial photosynthesis and H_2 production. In the architecture of these photoelectrochemical systems, photosystem II was immobilized on anodes to perform water oxidation upon illumination. The generated electrons were then transferred from photosystem II bioanode to hydrogenase biocathode to realize the reduction of proton and H_2 production.[789, 790] In more recent work from the Reisner group, the photosystem II was co-immobilized with osmium-based redox polymers on a diketopyrrolopyrrole dye TiO_2 photoanode to enable complementary panchromatic solar light absorption. Coupled with the [NiFeSe]-hydrogenase modified indium-tin-oxide electrode, the photoelectrochemical system was able to catalyze bias-free H_2 production from water.[791] Although the viability

of these photoelectrochemical systems is limited due to the low efficiency of photodegradation and photosystem instability, these examples still provide an interesting proof-of-concept model for future studies in bioelectrocatalysis based on photoelectric conversion.

4.4.1.2. Enzymatic Electrosynthesis of Nitrogen Fixation-based Chemicals. Dinitrogen (N_2) is the most abundant natural gas and the ultimate source of nitrogen for nitrogenated industrial and natural compounds.[792] However, the reductive conversion of N_2 to active and useful nitrogenous compounds, especially ammonia (NH_3), is challenging due to the inertness of N_2.[793] Currently, the majority of NH_3 is produced via the Haber-Bosch process, which consumes 1-2% of the global energy output and produces about 3% of the global CO_2 emission.[794, 795] The electrochemical NH_3 production based on nitrogenase at ambient conditions is an alternative technology to the Haber-Bosch process. Molybdenum nitrogenase is a multi-protein complex, which consists of a Fe protein and a MoFe protein where N_2 is reduced. There are two alternative nitrogenase systems employing vanadium- or iron-only (VFe and FeFe) proteins.[5, 42] The most widely studied and well-understood nitrogenase is MoFe nitrogenase, which contains MoFe cofactor. The conversion from N_2 to NH_3 by nitrogenase follows the reactions below (Eq. 4) under optimal conditions (where P_i is the inorganic phosphate).

$$N_2 + 8H^+ + 16\,MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16\,Pi \quad (Eq.\ 4)$$

The Minteer group established a bioelectrocatalytic N_2 fixation and NH_3 production system based on the utilization of isolated MoFe nitrogenase and Fe protein in the architecture of hydrogen (H_2) fuel cell (**Figure 24a**).[41] In the cathodic NH_3 producing chamber, methyl viologen (MV) was used as an electron mediator to transfer the electrons

from the electrode to the Fe protein, which subsequently delivered electrons to the MoFe protein alongside the requisite for the hydrolysis of ATP. In the anodic chamber, the H_2 was used as an electron donor. The electrons from the oxidation of H_2 catalyzed by hydrogenase flowed through the external electric circuit to the cathodic chamber to support the reduction of N_2. In this bioelectrosynthetic system, NH_3 was produced from H_2 and N_2 with the simultaneous production of electrical current and power output. The achieved Faradaic efficiency of the NH_3 production was 26.4%. This system demonstrates the possibility of employing renewable energy to support bioelectrochemical N_2 fixation and NH_3 synthesis. In order to eliminate the need for Fe protein and expensive ATP, the Minteer research group developed an alternative bioelectrosynthetic route for ammonia production based on the immobilization of MoFe protein to bypass the reducing and ATP hydrolyzing of the Fe protein. First, MoFe protein was immobilized by poly(vinylamine) and ethylene glycol diglycidyl ether on the electrode surface whereby the unnatural electron mediator, cobaltocene (bis(cyclopentadienyl)cobalt (III)), is able to shuttle electrons from electrode to MoFe protein. This system realized the conversion from N_3^- to NH_3 and NO_2^- to NH_3 without the addition of Fe protein and the consumption of ATP.[34] Then, a DET-based bioelectrocatalytic N_2 fixation system was investigated.[34, 251] In order to achieve the Fe protein- and ATP-free N_2 fixation, a novel polymer consisting of a linear (poly)ethylenimine (LPEI) backbone functionalized with pyrene moieties (pyrene-LPEI) was synthesized and employed. The MoFe protein was immobilized in a pyrene-LPEI hydrogel on a carbon electrode. Via this immobilization strategy, MoFe protein was observed to perform ATP-free and Fe protein independent, direct electroenzymatic reduction of N_2 to NH_3.

Herein, a noteworthy issue is that NH_3, the end-product of N_2 fixation based on nitrogenase or the Haber-Bosch process, is a bulk chemical with low added-value. The conversion of the generated NH_3 to nitrogenous chemicals with high added-value still requires subsequent tedious chemical synthesis steps catalyzed by precious metal catalysts.[796] Therefore, the Minteer group further developed an upgraded bioelectrocatalytic N_2 fixation system in which the generated NH_3 could be converted *in situ* by an enzymatic cascade to intermediates with high added-value, which could be used as building blocks for the synthesis of pharmaceuticals or other biotechnological chemicals (**Figure 24b**). Specifically, NH_3 generated from N_2 reduction catalyzed by nitrogenase was further upgraded in a multi-enzyme cascade composed by diaphorase, *L*-alanine dehydrogenase, and ω-transaminase. In this way, the generated NH_3 could be transferred to ketone substrates to produce chiral amines (e.g. (*R*)-1-methyl-3-phenylpropylamine). The MV acted as the electron mediator to transport electrons to nitrogenase for N_2 reduction and diaphorase for NADH regeneration that required by *L*-alanine dehydrogenase.[377] This concept was recently improved to a self-powered H_2/α-keto acid enzymatic fuel cell in which the chemically inert N_2 could be converted to chiral amino acids powered by the oxidation of H_2 (**Figure 24c**). The electrons generated from the oxidation of H_2 at the anode was coupled to an N_2 reduction to NH_3 at the cathode; the produced NH_3 was subsequently coupled with diaphorase and leucine dehydrogenase to achieve the asymmetric aminations of α-keto acid to produce chiral amino acids with high Faradaic efficiency and enantiomeric excess.[797] The establishment of the upgraded bioelectrocatalytic N_2 fixation system demonstrates another new exciting outlook for the application of nitrogenase in future bioelectrosynthesis.

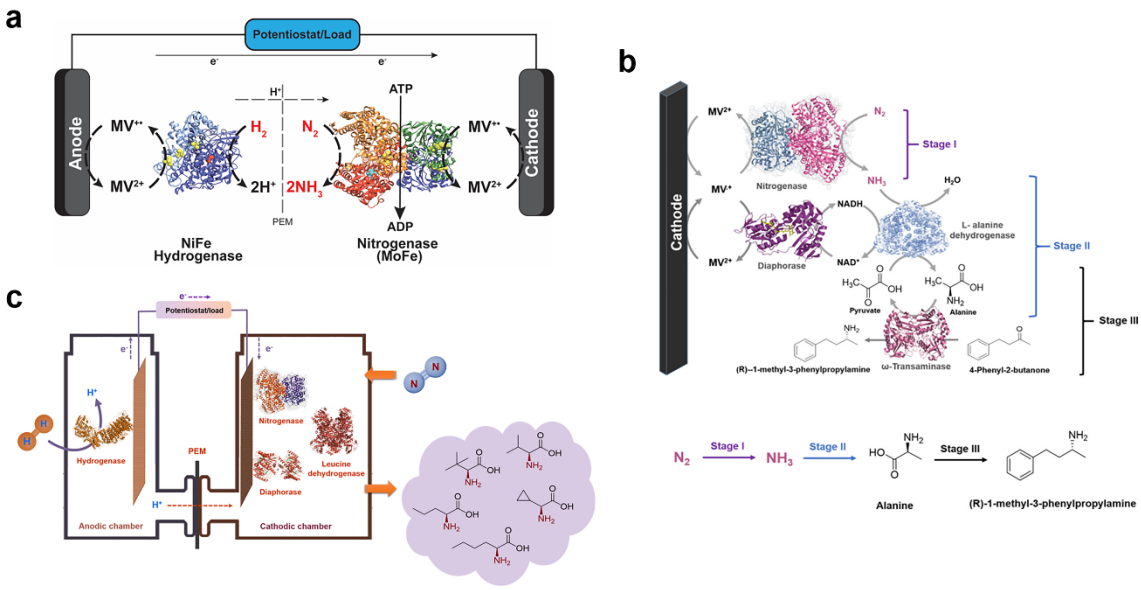

Figure 24. Bioelectrocatalytic N_2 fixation and upgraded nitrogen fixation based on the utilization of nitrogenase. (a) Compartmentalization of hydrogenase and nitrogenase Fe/MoFe proteins by the use of a proton exchange membrane (PEM) leads to an enzymatic fuel cell (EFC) configuration that is able to utilize MV as the electron mediator in both chambers and simultaneously produces NH_3 and electrical energy from H_2 and N_2 at room temperature and ambient pressure. Reprinted with permission from ref. 41. Copyright 2017 Wiley. (b) Schematic representation of the upgraded bioelectrocatalytic N_2 fixation system and the conversion route from N_2 to the chiral amine intermediate. Reprinted with permission from ref. 377. Copyright 2019 American Chemical Society. (c) Schematic representation of bioelectrocatalytic conversion from N_2 to chiral amino acids in a H_2/α-keto acid enzymatic fuel cell. Reprinted with permission from ref. 797. Copyright 2020 American Chemical Society.

4.4.1.3. Enzymatic Electrosynthesis of CO_2 Fixation-based Chemicals. The excessive emission and accumulation of CO_2 from fossil fuel combustion have become a global crisis as the atmospheric accumulation of CO_2 plays a crucial role in global warming and climate change.[798] On the other hand, atmospheric CO_2 has been identified as a cheap and abundant carbon feedstock, the consumption of which is considered advantageous due to this radical environmental change.[799] Thus, technological development to enhance the effective fixation and utilization of CO_2 has become a significant research focus. Among the most recent bio-inspired strategies, the reductive enzymatic electrochemical capture and fixation

of CO_2 are considered to be effective approaches. In enzymatic electrochemical systems, CO_2 can be reduced to a variety of useful chemicals, such as formate, methanol, ethanol, and hydrocarbons, with the utilization of different bieoelectrocatalysts.[800, 801]

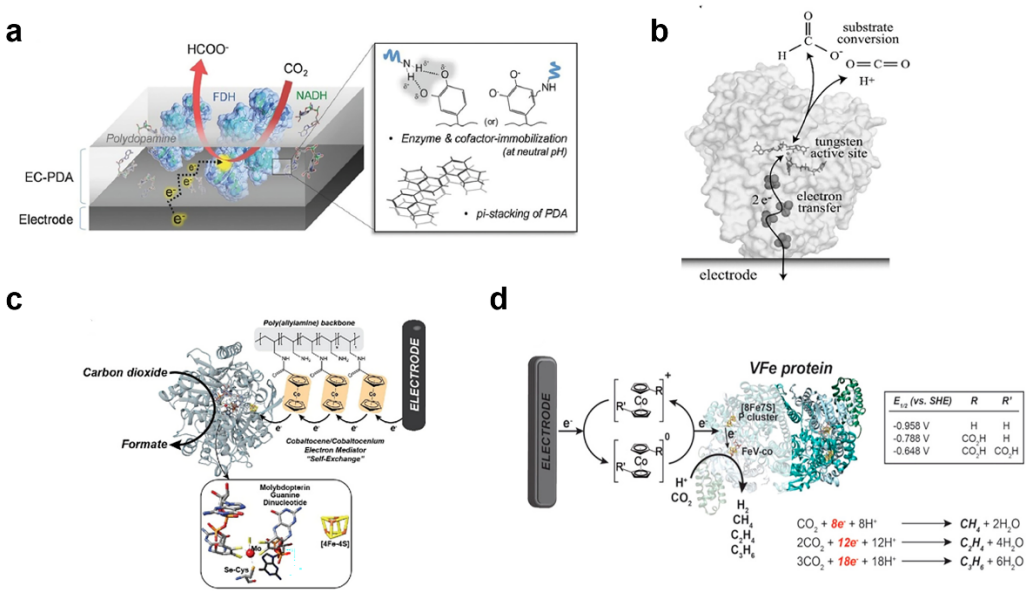

Figure 25. (a) NADH-dependent formate dehydrogenase (FDH) and cofactor (NADH) co-immobilization with poly dopamine (PDA) to enable efficient CO_2 reduction. Reprinted with permission from ref. 809. Copyright 2016 Wiley. (b) W-FDH adsorbed to a pyrolytic graphite edge electrode; DET is enabled by the iron-sulfur cluster present in the enzyme. Reprinted with permission from ref. 811. Copyright 2008 PNAS. (c) Efficient mediated electron transfer (MET) type of CO_2 reduction by Mo-FDH immobilized with cobaltocene modified poly(allylamine) backbone (Cc-PAA) polymer. Reprinted with permission from ref. 39. Copyright 2018 Wiley. (d) MET of VFe by cobaltocene derivatives for the synthesis of CH_4, C_2H_4, and C_3H_6. Reprinted with permission from ref. 40. Copyright 2018 American Chemical Society.

Formate is the most common product of CO_2 fixation catalyzed by formate dehydrogenase (FDH) at the low redox potential of –0.42 V *vs* SHE.[802] Formic acid is of commercial value as a chemical feedstock, an efficient carrier of hydrogen, and suitable for direct use in fuel cells.[803] To date, two types of FDHs, metal-dependent and NADH-dependent, have been identified. The metal-dependent FDHs utilize Mo or W atom as the active sites to catalyze CO_2 reduction. Additionally, the metal-dependent FDHs contain several Fe-S clusters to facilitate the electron transport to the active site, which contains a

central Mo or W atom. Different from metal-dependent FDHs, NADH-dependent FDHs do not have metal ions or other permanent redox centers, and instead use NADH directly.[804] For the NADH-dependent FDHs, NADH or the artificial electron mediators are the source of electrons for the CO_2 reduction. Therefore, the efficient supplement and regeneration of reduced electron mediator are essential for formate production. In some previous research, specific free artificial electron mediators, such as neutral red, $[Cp^*Rh(bpy)Cl]^+$ complex, and 1,1′-Trimethylene-2,2′-bipyridinium dibromide, can act as electron shuttles to transfer electrons from the electrode to NAD^+ and support the reduction of CO_2 catalyzed by FDHs.[805-807] In a study by Choi *et al*., the artificial electron mediator methyl viologen (MV) was able to directly transport electrons from the cathode to the FDH and support the production of formate.[808] Compared with the use of free electron mediators, the co-immobilization of FDHs and electron mediators is another effective strategy. The well-characterized NADH-dependent FDH from *Candida boidinii* and its coenzyme, NADH, were embedded in a polydopamine (PDA) film by copolymerization. The PDA matrix with nanoscale thickness facilitates electron transfer for the production of formate with 99.18% Faradaic efficiency and unprecedentedly prolonged catalytic enzyme stability for about two weeks (**Figure 25a**).[809] In a recent study, Yuan and co-workers utilized a low-potential (E^0 of –0.576 V *vs* SHE) redox polymer, which was synthesized by a facile method, containing cobaltocene grafted to poly(allylamine) backbone (Cc-PAA), for CO_2 reduction with a 99% Faradaic efficiency (**Figure 25c**). The FDH was immobilized by the Cc-PAA polymer on the surface of the cathode. The pendant of Cc-PAA, cobaltocene, effectively mediated the electrons transfer from the electrode to the immobilized FDH. The resulting bioelectrode reduces CO_2 to formic acid with high Faradaic efficiency of 99%.[39] Besides

mediated electron transfer (MET), direct electron transfer (DET) can also be employed to support CO_2 reduction and format production. For the metal-dependent FDHs, the Fe-S clusters in the vicinity of this metal-binding domain facilitate DET between the active site and the electrode. If the oriented immobilization of FDH is achieved, DET can be observed. In a DET design, a gold nanoparticle-embedded Ketjen black-modified glassy carbon electrode was treated with 4-mercaptopyridine to facilitate the oriented immobilization of W-FDH and the improvement of interfacial electron transfer kinetics. [810] Another important study for CO_2 reduction based on DET was reported by Reda and co-workers(**Figure 25b**).[811] In this study, the W-containing FDH was adsorbed to a freshly polished pyrolytic graphite edge electrode. Using this enzyme-modified electrode, the researchers observed CO_2 reduction to formate at below –0.8 V *vs* Ag/AgCl with 97% Faradaic efficiencies. Furthermore, they suggested an electron transfer mechanism among the electrode, the enzyme, and CO_2 for the subsequent reduction reaction. Two electrons are transferred from the electrode to the active site (the active site is buried inside the insulating protein interior) via the Fe-S cluster, to reduce CO_2 to formate and form a C-H bond. Conversely, when formate is oxidized, the two electrons are transferred from the active site to the electrode.

Nitrogenases, including MoFe, FeFe, and VFe nitrogenase, are also capable of CO_2 reduction. Seefeldt and colleagues immobilized MoFe and FeFe nitrogenase, respectively, with polyvinylamine hydrochloride and pyrene on a glassy carbon electrode. With cobaltocene as a mediator, the immobilized nitrogenase can convert CO_2 to formate with 9% Faradaic efficiency for MoFe protein and 32 % for FeFe-protein.[812] An interesting and important finding was reported by the Minteer group (**Figure 25d**),[40] in which the

electroenzymatic C-C bond formation from CO_2 catalyzed by VFe nitrogenase originated from *Azotobacter vinelandii*. In this research, two cobaltocene electron mediators, 1,1'-dicarboxy-cobaltocenium and 1-carboxy-cobaltocenium, were employed. The bioelectrocatalytic VFe system can reduce CO_2 to ethylene (C_2H_4) and propene (C_3H_6), without the requirement of CO as the substrate and forming C-C bonds. The products were detected and quantified after the passage of 4 coulombs of charge at –0.86 V *vs* SHE in a 2 mL reaction system. The generation of 25 nmol C_2H_4 and 42 nmol C_3H_6 per μmol VFe was observed.

The conversion from CO_2 to methanol is a challenging process that requires six electrons. The single-enzyme catalyst is not able to carry out this complicated conversion process. To mimic microbial multistep reactions, multi-enzyme *in vitro* systems have been explored for various catalytic reactions where single enzyme catalysis is not effective.[813] Consequently, the multi-enzyme cascade in which three NADH-dependent enzymes including FDH, formaldehyde dehydrogenase (FLDH) and alcohol dehydrogenase (ADH) was constructed to perform the production of methanol from CO_2.[814] In Yoneyama and colleagues' study, the electrolysis of a CO_2-saturated solution containing MV, FDH, and ADH simultaneously produced formaldehyde and methanol. Through the replacement of MV with PQQ, methanol was exclusively produced.[815] Ji and colleagues developed a unique nano-architecture strategy involving poly(allylamine hydrochloride)-doped hollow nanofibers co-integrated with an electron mediator, photosensitizers, and the encapsulated three enzyme cascade. The photoregeneration of NADH under visible-light irradiation effectively supports the conversion from CO_2 to methanol catalyzed by FDH, FLDH, and ADH. The yields of methanol improved from 35.6 % to 90.6 % under specified

conditions.[816] Park and co-workers also used photoelectrochemical method to realize the conversion from CO_2 to methanol. Specifically, they employed a photoelectrochemical cell (PEC) using a photoanode (Co-Pi/α-Fe_2O_3) that oxidizes H_2O and transfers electrons to the photocathode ($BiFeO_3$), where the NADH is generated by a rhodium mediator. This PEC was integrated with the three-enzyme cascade composed by FDH, FLDH, and ADH to achieve effective methanol production powered by solar energy.[817]

4.4.1.4. Enzymatic Electrosynthesis of Fine Chemicals. Enzymes have excellent chemo-, regio-, and stereo-selectivities and catalyze organic synthetic reactions mostly without side reactions. In particular, the oxidoreductase-catalyzed redox reactions offer yields and selectivity that are often not achievable with chemical syntheses.[818] Electron supply or removal is always required when using oxidoreductases. This function is fulfilled by a variety of cofactors that have been mentioned above. To ensure a smooth reaction, the sufficient supply and effective regeneration of cofactors are essential. Regeneration of the desired cofactors depends on the type of enzyme and its cofactor and can be realized in different ways. The most commonly used method is the addition of extra enzymes, such as alcohol dehydrogenase, glucose dehydrogenase, formate dehydrogenase, and their corresponding sacrificial co-substrates. Through the oxidation of the sacrificial co-substrates, the cofactors can be regenerated.[819, 820] Compared to the enzyme-coupled coenzyme regeneration, bioelectrocatalytic regeneration method does not require the addition of extra enzyme and sacrificial co-substrate as electricity can be the electron source for coenzyme regeneration without the production of byproduct.[821, 822] Based on the above, the combination of the excellent catalytic properties of oxidoreductases and the capability of cofactor regeneration of the electrosynthesis system makes enzymatic

electrosynthesis a promising approach for the production of a variety of useful chemicals, especially fine chemicals with high added-value. In addition to the effective regeneration of cofactors, the electrosynthetic system can also be used *in situ* to generate the substrate, such as H_2O_2, for the oxidoreductases.[823, 824] The reaction type of oxidoreductases that can be used in enzymatic synthesis systems to produce fine chemicals, especially chiral chemicals, include the hydroxylation of carbon-hydrogen bonds, the reduction of carbonyls, the reductive amination of carbonyls, the epoxidation of olefins, and the reduction of olefins.

The Hydroxylation and Halogenation of C-H Bonds

Direct C-H activation is of vital importance, in particular, due to the prevalence of the production of chiral alcohols or halide in pharmaceuticals, natural products, and fine chemicals.[825] However, the selective activation of C-H bonds is still a big challenge in organic synthesis.[826, 827] Specifically, balancing the reactivity of the oxygen-transfer reagent with selectivity is a largely unresolved issue of organic catalysts, while it is an inherent property of many oxidative enzymes such as monooxygenases, peroxygenases, and chloroperoxidase.[828, 829] The electrosynthesis system can be used to generate enough reduced cofactors to support the hydroxylation of the C-H bond catalyzed by monooxygenase. Furthermore, the substrate of peroxygenase and chloroperoxidase, hydrogen peroxide (H_2O_2), can also be in situ generated in the electrosynthesis system to support the hydroxylation and halogenation of the C-H bond.

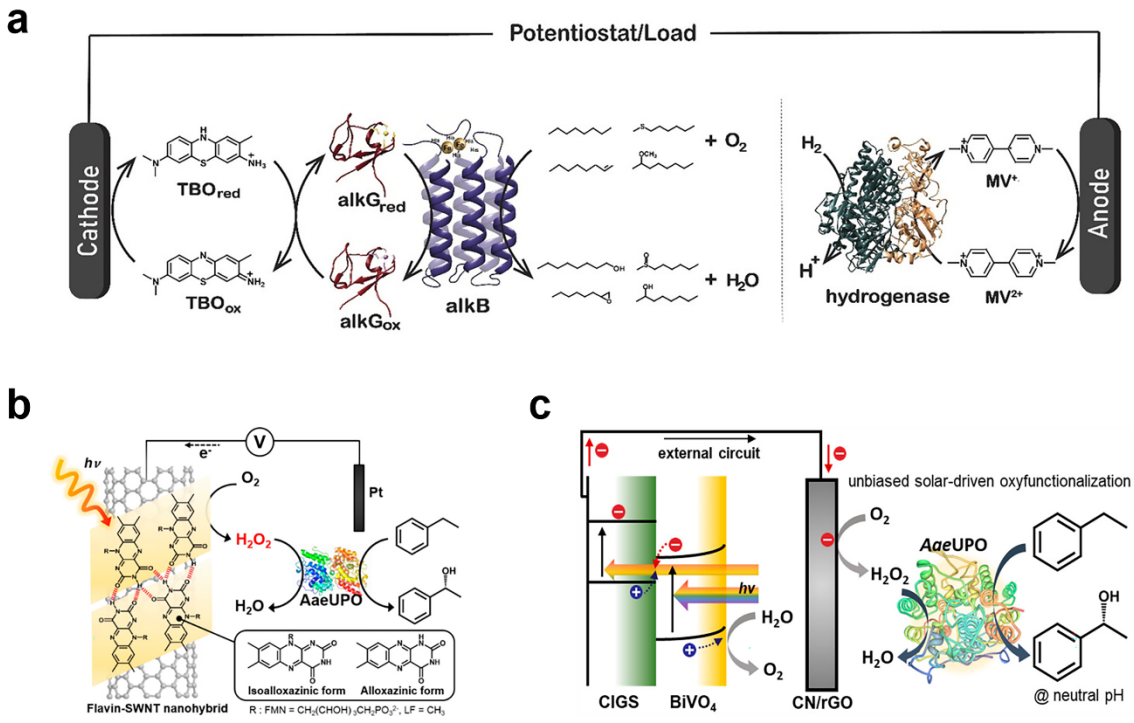

Figure 26. (a) The enzymatic fuel cell (EFC) with an alkB/alkG biocathode and Nafion-separated hydrogenase bioanode for hydroxylation, epoxidation, sulfoxidation, and demethylation. Reprinted with permission from ref. 835. Copyright 2020 Wiley. (b) Flavin-single wall carbon nanotube-based photoelectrochemical platform enabled peroxygenases-catalyzed, selective hydroxylation reactions. Reprinted with permission from ref. 824. Copyright 2017 American Chemical Society. (c) Bias-free *in situ* H_2O_2 generation in a photovoltaic-photoelectrochemical tandem cell for biocatalytic oxyfunctionalization catalyzed by peroxygenase. Reprinted with permission from ref. 839. Copyright 2019 American Chemical Society.

Cytochrome P450 monooxygenases are able to catalyze the hydroxylate the hydroxylation of the C-H bond. In the enzymatic electrosynthesis system, both the natural cofactor (NADH) and the artificial cofactor (e.g., cobalt(II) sepulchrate trichloride, cobalt(III) sepulchrate, and cobaltocene) can be employed as electron mediators and regenerated to shuttle electrons from electrode to P450 monooxygenase. The corresponding hydroxylation reaction includes the specific *ortho*-hydroxylation of *α*-substituted phenols, the ω-hydroxylation of fatty acids, and the hydroxylation of steroids.[830-833] Besides mediated electron transfer (MET), direct electron transfer (DET)

can also be used to support the hydroxylation reaction of P450 monooxygenase. The classic examples are the immobilization of P450cam on the antimony-doped tin oxide-coated glass slides electrode or the indium tin oxide electrode to produced hydroxylated camphor without the requirement of any electron mediators.[832, 834] In the research by Fantuzzi, a monolayer coverage was obtained on gold modified with cystamine/maleimide that covalently linked surface accessible cysteines of P450 2E1. This bioelectrode can be used to catalyze the hydroxylation of p-nitrophenol via DET.[745] Recently, the Minteer group reported a selective electroenzymatic oxyfunctionalization catalyzed by monooxygenase in a hydrogen fuel cell (**Figure 26a**).[835] Specifically, the alkane monooxygenase from *P. putida* is employed to catalyze the difficult terminal oxyfunctionalization of alkanes under mild conditions. Toluidine blue O (TBO) acts as the electron mediator that continuously transfers electrons from the cathode to the alkane monooxygenase, thereby replacing both NADH and the redox partner of alkane monooxygenase. Finally, by coupling monooxygenase biocathode with a hydrogenase bioanode and consuming H_2 as an electron donor, they successfully developed an enzymatic fuel cell capable of oxyfunctionalization while simultaneously producing electricity. In recent years, it has been found that the peroxygenase, which excels in terms of substrate scope and specific activity compared with traditional monooxygenase, is a promising biocatalyst to realize the hydroxylation of the C-H bond in preparative organic synthesis.[829, 836] Different from the P450 monooxygenase, peroxygenases do not rely on complicated and susceptible electron transport chains delivering reducing equivalents to the heme active site needed for reductive activation of molecular oxygen and therefore are not subject to the 'oxygen dilemma.[837] The H_2O_2 is employed to directly regenerate the catalytically active oxyferryl heme species of

peroxygenase. Therefore, the reduced equivalent is not required in the catalytic process of peroxygenase. However, peroxygenases suffer from a pronounced instability against H_2O_2. The electrosynthesis system can use O_2 as a substrate to realize the *in situ* generation of H_2O_2, which is conducive to release the inhibition of H_2O_2 on the activity of peroxygenase at excess concentrations. In research by Horst *et al*., the hydroxylation of C-H of ethylbenzene catalyzed by unspecific peroxygenase (UPO) was performed in an enzymatic electrosynthesis system. The carbon-based gas diffusion electrode was employed as the working electrode to perform the conversion from O_2 to H_2O_2 (**Figure 26b**). The *in situ* supply of H_2O_2 ensured high reaction efficiency. Total turnover numbers (TONs) of up to 400,000 $mol_{product}$ mol_{UPO}^{-1} and space-time-yields of up to 25 g L^{-1} d^{-1} were achieved in the enzymatic electrosynthesis system.[838] Park's group studied the utilization of photoelectrochemical method to realize the in-situ generation of H_2O_2 to support the hydroxylation of ethylbenzene catalyzed by peroxygenase. On one hand, they developed a flavin-hybridized, single-walled carbon nanotube (SWNT) photoelectrodes to reduce the overpotential needed for the reduction of O_2 to H_2O_2 by 170 mV. Under illumination, flavins allowed for a marked anodic shift of the oxygen reduction potential. Finally, the TON of photoelectroenzymatic hydroxylation of ethylbenzene achieved was 123,900.[824] On the other hand, they also constructed a photovoltaic-photoelectrochemical tandem cell for the oxyfunctionalization catalyzed by peroxygenase (**Figure 26c**).[839] The photovoltaic-photoelectrochemical tandem cell consisted of a FeOOH/$BiVO_4$ photoanode, a Cu(In,Ga)Se_2 solar absorber, and a graphitic carbon nitride/reduced graphene oxide hybrid cathode for light-driven H_2O_2 generation. Powered by sufficient photovoltage generated by the solar absorber, the photovoltaic-photoelectrochemical tandem cell generates H_2O_2

in situ via the reductive activation of O_2 using H_2O as an electron donor. The TON of ethylbenzene to (*R*)-1-phenylethanol achieved 43,300 with high optical purity ee_p > 99%.

The *in situ* system for H_2O_2 electrogeneration can also be employed to achieve the halogenation of target substrates. Dirk Holtmann's group carried out some related studies in this direction. In Holtmann's studies, the H_2O_2 was generated *in situ* at a gas diffusion electrode. Chloroperoxidases then acted as the biocatalyst to facilitate the production of hypohalides from H_2O_2 and a halide. These reactive hypohalides are then able to participate in a variety of halogenation reactions.[840] First, the researchers used thymol-equilibrated gas diffusion electrode to provide chloroperoxidase with appropriate amounts of H_2O_2 to sustain high hypochloride generation rates while minimizing H_2O_2-related biocatalyst inactivation. Then, the generated hypochloride can spontaneously react with different substrates to produce chlorothymol, chlorocarvacrol, and bromothymol, as well as dichlorothymol with high efficiency.[828] In their more recent study, an oxidized carbon nanotube-modified gas diffusion electrode was employed to perform *in situ* H_2O_2 generation at low overpotentials.[823] The modification of oxidized carbon nanotube reduced the working potential of H_2O_2 production from –350 mV to –250 mV *vs* Ag/AgCl. Hypobromite was generated by chloroperoxidase with H_2O_2 consumption and reacted with 4-pentenoic to form bromolactone.

The Reduction of Carbonyls

The reduction of carbonyl (ketone substrate) is a beneficial reaction in organic synthesis for the production of alcohol, especially chiral alcohol. Among many kinds of biocatalysts, carbonyl reductases (also referred to as alcohol dehydrogenases or ketone reductases) have been used to catalyze the asymmetric reduction of carbonyl to prepare chiral alcohols.[841-

[843] Similar to other oxidoreductases, carbonyl reductases also consume NAD(P)H to perform the reduction of the carbonyl. This electrosynthetic system can use electricity as the electron donor to realize the effective regeneration of reduced cofactor for the reduction of carbonyl catalyzed by carbonyl reductases.

In previous studies, mediated electron transfer (MET) based on the utilization of free electron mediator is the dominant method to perform the regeneration of NAD(P)H. $Cp^*Rh(bpy)L$ has been used as an electron mediator to facilitate the reduction of acetophenone, cyclohexanone, 4-phenyl-2-butanone to produce (*R*)-phenylethanol, cyclohexanol and (*S*)-4-phenyl-2-butanol.[418, 844-846] The methyl viologen (MV) coupling with diaphorase can be used to regenerate NADH, which has been applied in the reduction of cyclohexanone, 2-methyl-cyclohexanone, pyruvate, and benzoylformate to produce cyclohexanol, (1*S*,2*S*)-(+)-2-methylcyclohexanol, *D*-lactate and (*R*)-mandelate.[847-849] Recently, the Minteer research group developed a biphasic bioelectrocatalytic synthesis method to prepare chiral β -hydroxy nitriles.[445] In their research, diaphorase was immobilized by a cobaltocene-modified poly(allylamine) (Cc-PAA) redox polymer on the surface of cathode (DH/Cc-PAA biocathode) to achieve an effective bioelectrocatalytic NADH regeneration. The generated NADH effectively facilitated the reduction of ethyl 4-chloroacetoacetate to ethyl (*S*)-4-chloro-3-hydroxybutanoate catalyzed by alcohol dehydrogenase. The conversion ratio of 30 mM ethyl 4-chloroacetoacetate after 10 hours of the reaction was close to 100%. The generated (*S*)-4-chloro-3-hydroxybutanoate was further involved in the dehalogenation and cyanation substitute catalyzed by halohydrin dehalogenase and finally was converted to the (*R*)-ethyl-4-cyano-3-hydroxybutyrate, a useful active ingredient of Lipitor.[850] Besides MET, some ketone substrates can also

directly be reduced on the surface of the electrode. A representative example is the enzymatic electrosynthesis of *L*-3,4-dihydroxyphenylalanine (*L*-DOPA), which is a precursor of the neurotransmitter dopamine and a widely used drug in the treatment of Parkinson's disease.[851-853] *L*-DOPA is the hydroxylation product of tyrosine catalyzed by tyrosinase. But, the monophenols by cresolase activity of tyrosinase is able to further catalyze the generation of peroxidation products of *L*-DOPA, *L*-DOPAquinone. In the enzymatic electrosynthesis system, the generated *L*-DOPAquinone can be directly reduced back to *L*-DOPA at the reductive potential of *L*-DOPA by the electrons supplied from the electrode without the requirement of electron mediators.

Reductive Amination of Carbonyls

Enantioselective reductive amination of carbonyls is an important and widely used approach for the synthesis of chiral amine and biologically relevant molecules.[854] In biocatalysis, this type of reaction is mainly used to prepare chiral amino acid via the asymmetric amination of α-keto acid. Amino acid dehydrogenases are the enzymes that catalyze the reductive amination of α-keto acids to *L*-amino acids in the presence of the cofactor NADH, which acts as an electron donor. The amino acid dehydrogenase is capable of utilizing the free ammonium, rather than amino compounds, as a substrate to catalyze the formation of chiral amino acid, which is conducive to reduce the production cost.

The enzymatic electrosynthesis of *L*-glutamate catalyzed by glutamate dehydrogenase via the reductive amination of oxoglutarate is a representative example. In the electrosynthesis system, NADH was regenerated by using the artificial mediator accepting oxidoreductase (AMAPORS) and methyl viologen (MV) as an electron shuttle.[855-857] In recent years, the photoelectrochemical method has been developed for the synthesis of

glutamate. Lee and co-workers developed an unbiased photoelectrochemical tandem assembly of a photoanode ($FeOOH/BiVO_4$) and a perovskite photovoltaic to provide sufficient potential for the NADH-dependent reductive amination of α-ketoglutarate and glutamate production catalyzed by glutamate dehydrogenase. Specifically, the tandem photoelectrochemical system consisting of a nanostructured $FeOOH/BiVO_4$ photoanode, an organometallic perovskite-based photovoltaic cell, and a carbon nanotube (CNT) film cathode. FeOOH worked as a water oxidation catalyst on the $BiVO_4$ photoanode to enhance the extraction of photogenerated holes and the efficiency of water oxidation, as well as to improve the photoanode's stability. The perovskite solar cell with a light absorber containing triple cation perovskite compositions made of Cs, formamidinium, and methylammonium, absorbs the transmitted light through the $FeOOH/BiVO_4$ photoanode, providing an additional photovoltage to satisfy the thermodynamic requirement for both water oxidation and supply of electrons for NADH regeneration. On this basis, the conductive CNT modified cathode performed the reduction of a Rh-based electron mediator, $[Cp^*Rh(bpy)H_2O]^{2+}$, which finally realized the regeneration of NADH. This enzymatic photoelectrosynthesis system significantly facilitated the synthesis of glutamate. The total turnover number and a turnover frequency of the enzyme achieved were 108,800 and 6200 h^{-1}, respectively.[858] Beside glutamate dehydrogenase, Leucine dehydrogenase can also be used in enzymatic electrosynthesis system. Minteer's group applied leucine dehydrogenase in an enzymatic electrosynthesis system (H_2/α-keto acid enzymatic fuel cell) to produce *L*-norleucine and derivatives.[377] The electrons generated from H_2 oxidation at the hydrogenase anode transported to the cathode and supplied enough reducing equivalents for the NH_3 production and NADH recycling catalyzed by nitrogenase and

diaphorase, respectively. The generated NH_3 and NADH were consumed *in situ* by the leucine dehydrogenase to generate *L*-norleucine with 2-ketohexanoic acid as the NH_3 acceptor. This H_2/α-Keto acid enzymatic fuel cell can also be used to produce *L*-norvaline, *L*-valine, *L*-ter-leucine, and *L*-cyclopropylglycine with high enantiomer excess value.

The Epoxidation of Olefins

Asymmetric epoxidation is a useful method for the synthesis of biologically active pharmaceuticals and fine chemicals. The introduction of two C-O bonds in one reaction results in the formation of two chiral centers and also provides access to a diverse array of key intermediates due to the possibility of facile opening of the epoxide ring.[419] An effective way for the preparation of chiral epoxy compounds is the utilization of flavin-dependent monooxygenases to catalyze the epoxidation reactions. Monooxygenases frequently exhibit high conversion rates at excellent enantioselectivities, are active at ambient reaction conditions, and use molecular oxygen as oxidant.[75] For enzymatic electrosynthesis, the $FADH_2$-dependent styrene monooxygenase from *Pseudomonas* sp. VLB120 is the most commonly used bioelectrocatalyst to catalyze the specific *S*-epoxidation of styrene derivatives.[859] This monooxygenase is composed of an $FADH_2$-dependent oxygenase component (StyA) that performs the epoxidation reaction and an NADH-dependent reductase component (StyB) that transfers reducing equivalents from NADH to StyA.

In initial studies of the application of $FADH_2$-dependent styrene monooxygenase for enzymatic electrochemical epoxidation, Schmid and co-workers established an electrochemical method to regenerate $FADH_2$ to substitute for the complicated native

regeneration cycle composed by StyB and NADH. The cylindrical carbon felt electrode served as the cathode to supply electrons for the reduction of FAD at –550 mV *vs* Ag/AgCl. The epoxidation rate of enzymatic electrosynthesis system was much lower than that of reaction with the natural $FADH_2$ regeneration cycle. The slow electrochemical reduction rate of the flavin and fast aerobic reoxidation accounted for the low reaction rate.[860] For settling this problem, Schmid's group further developed a highly porous reticulated vitreous carbon electrodes to maximize the volumetric surface area. This improved electrode was used in a flow-through mode to increase the regeneration rate of $FADH_2$. Finally, the space-time production rate of (*S*)-styrene oxide increased from 0.143 mM h^{-1} to 2.2 mM h^{-1}.[419] Ultimately, the Schmid group developed a novel flow-through reactor equipped with a porous, three-dimensional reticulated vitreous carbon electrode with exceptionally large surface areas. This system improved mass transfer rates. The reduction rate of FAD was up to 93 mM h^{-1}. The space-time production rate of (*S*)-styrene oxide can be kept at 1.3 mM h^{-1}.[861] In addition to work by the Schmid group, Yoo's group also performed related studies.[420] The researchers employed a zinc oxide/carbon black composite electrode. The attractive interaction between zinc oxide and styrene monooxygenase lead to the high local concentration of styrene monooxygenase around the electrode surface, and also increased the accessibility of $FADH_2$ from the electrode surface to the enzyme. By adjusting the reaction conditions, such as oxygen solubility, high Faradaic efficiency of 65% was obtained.

Reduction of Olefins

The enzymes catalyzing the asymmetric hydrogenation of olefins generating up to two stereogenic centers are known as ene-reductases. They are subdivided into four enzyme

classes, namely (1) old yellow enzymes (OYEs), (2) enoate reductases, (3) medium-chain dehydrogenases/reductases (MDRs), and (4) flavin-independent short-chain dehydrogenase/reductases (SDRs). Ene-reductases require the use of NAD(P)H as a cofactor for hydride donation.[862] For the enoate reductases and OYE enzyme family, electroenzymatic methods for coenzyme regeneration have been developed to support the asymmetric reduction of olefins. In Simon and co-workers' research, the asymmetric synthesis of (2*R*)-2-methyl-3-phenylpropionate by an enoate reductase from *Clostridium tyrobutyricum* was combined with electrochemical regeneration of reduced MV. The reduced MV was used as an electron mediator to transfer electrons from the cathode to the enoate reductase. After 80 h reaction, the conversion ratio of 80 mM substrate achieved was approximately 95%.[863] The same enoate reductase was further immobilized, coupled with electrochemical regeneration of MV to perform the asymmetric reduction of (*E*)-2-methyl-3-phenyl-2-propenoate and (*E*)-2-methyl-2-butenoate to their (*R*)-enantiomeric products. The enzymes were either immobilized on a cellulose filter or immobilized directly on the carbon felt electrode. The ee_p of both of the generated (*R*)-enantiomeric products were >98%.[864] The photoelectrochemical system has also been employed to perform the enzymatic reduction of olefins. Son *et al*. constructed a photoelectrochemical cell equipped with a protonated graphitic carbon nitride (p-g-C_3N_4) and carbon nanotube hybrid (CNT/p-g-C_3N_4) film cathode, and a FeOOH-deposited bismuth vanadate (FeOOH/$BiVO_4$) photoanode for the reduction of ketoisophorone to (*R*)-levodione catalyzed by OYE. In the biocatalytic photoelectrochemical cell platform, photoexcited electrons provided by the FeOOH/$BiVO_4$ photoanode are transferred to the robust and self-standing CNT/p-g-C_3N_4 hybrid film that reduced FMN. The p-g-C_3N_4 promotes a two-

electron reduction of FMN coupled with an accelerated electron transfer by the conductive CNT network. The reduced FMN subsequently utilized by OYE for the asymmetric reduction of ketoisophorone to (*R*)-levodione. Finally, the (*R*)-levodione was synthesized with the enantiomeric excess value of above 83%.[865]

4.4.2. Microbial Electrosynthesis. Microbial electrosynthesis refers to a novel bioenergy approach in which electricity is used as the energy source for the reduction of CO_2 catalyzed by microbial cells to single-carbon or multi-carbon organic compounds that can serve as transportation fuels or other useful organic chemical commodities.[268, 866] The conversion of electrical energy to extracellular, multi-carbon chemicals is an attractive option and has great significance for energy storage and distribution.[867] Since microbial electrosynthesis offers a great potential for the generation of renewable biofuels and commodity chemicals, the understanding and cognition of the type of microbes and the metabolic pathway mechanisms are critical to improving the performance of microbial electrosynthesis systems. Furthermore, this work has profound environmental implications, including the understanding of ecological aspects of one-carbon metabolism and extracellular electron transfer relevant to global biogeochemical cycling.[868] A wide variety of value-added products can be produced in microbial electrosynthesis systems, such as hydrogen, ethanol, methane, acetate, butanol, and hydrogen peroxide.[869] Currently, microbial electrosynthesis of acetate from CO_2 has achieved high production (>10 g/L).[870] Besides acetate, more valuable products including butyrate,[871] caproate,[872] and polyhydroxybutyrate (PHB),[873] could also be generated via CO_2 fixation performed by microbial electrosynthetic systems. The wide diversity of products generated from CO_2 in microbial electrosynthesis represents a new direction for the synthesis of materials and

chemicals.[874] Apart from wild-type microbial cells, some engineered strains have also been applied in the microbial electrosynthetic systems with the development of metabolic engineering and synthetic biology. Through the introduction of a new synthetic pathway or the modification of the existing metabolic pathway, the engineered strains are able to synthesize products with higher added value, such as chiral compounds, succinate, PHB, and natural products, as well.

4.4.2.1. Microbial Electrocatalytic H_2 Production. The microbial electrolysis cell is an emerging technology that combines the metabolism of microbial cells with electrochemistry to realize H_2 production.[764] Two modes, specifically a biocathodic and a bioanodic mode, can be used in the microbial electrolysis cell for H_2 production. In the biocathodic mode, microorganisms with the ability to express hydrogenase are immobilized on cathodes as electrocatalytic agents to catalyze proton reduction and H_2 production. Tatsumi *et al.* prepared a biocathode by using polycarbonate membranes to immobilize *Desulfovibrio bulgaris* cells on a glassy carbon electrode. In this study, the researchers utilized methyl viologen as the electron mediator to shuttle electrons between the cathode and the hydrogenase inside the cell, achieving H_2 evolution.[875] In a similar research work from Lojou and co-workers, *D. bulgaris* were immobilized onto an electrode with a dialysis membrane and could perform the H_2 production with methyl viologen as electron mediator.[876] Villano *et al.* reported the use of hydrogenophilic dechlorinating bacteria, *Desulfitobacterium* and *Dehalococcoides*, which were applied in a cathodic chamber to catalyze H_2 production via proton reduction.[877] *Desulfitobacterium*- and *Dehalococcoides*-enriched cultures produced H_2 at rates of 12.4 μeq/mgVSS/d (where VSS is volatile suspended solids). Moreover, the *Desulfitobacterium*-enriched culture was

able to catalyze H_2 production via DET at –0.75 V *vs* SHE with H_2 production rate at 13.5 μeq/mgVSS/d. In the bioanodic mode, anode-respiring microbial cells, including *Geobacter*, *Shewanella*, *Pseudomonas*, *Clostridium*, *Escherichia*, *Desulfuromonas*, and *Klebsiella*, are immobilized on anodes. These immobilized microbial cells can oxidize a variety of organic compounds, such as glucose, cellulose, ethanol, acetate lactate, butyrate, and propionate, and transfer electrons to the anode.[878] The electrons travel to the cathode via an external electrical circuit, where the electrons reduce H_2O to realize H_2 evolution.[764] In this process, a power supply is required to boost the voltage of electrons reaching the cathode. Wastewater is a commonly used substrate in microbial electrolysis cells for H_2 production. Through the oxidation of organic compounds in wastewater, H_2 production occurs while simultaneously achieving wastewater treatment. In reported studies to date, domestic wastewater,[879] swine wastewater,[880] fermentation effluent,[881] industrial and food processing wastewater,[882] and winery wastewater[883] have been used for in microbial electrolysis cell for H_2 production. In a research work by the Bernet group, a biofilm-based two-chamber microbial electrolysis cell with a volume of 4 L was continuously fed with acetate under saline conditions for more than 100 days. The current density achieved was 10.6 A m^{-2}_{Anode}. The H_2 production rate was up to 0.9 $m^3_{H2}\, m^{-3}\, d^{-1}$.[884] In a study by Montpart *et al*., synthetic wastewater, containing glycerol, milk, and starch, was evaluated in a single chamber microbial electrolysis cell. With the simultaneous degradation of the three substrates, the current intensity achieved was 150 A m^{-3}. The H_2 production rate was determined to be as high as 0.94 $m^3\, m^{-3}\, d^{-1}$.[885]

4.4.2.2. Microbial Electrocatalytic N_2 Fixation and Ammonia Production. In addition to isolated nitrogenase, some microbial cells can also be used as bioelectrocatalysts to

catalyze N_2 fixation and ammonia production. Algal and cyanobacteria are the conventional catalysts for ammonia production. Leddy and Paschkewitz used an SA-1 mutant of *Anabaena variabilis* immobilized on a glassy carbon electrode with a hydrophobically modified Nafion film to electrochemically produce ammonia from N_2.[886] It was shown that ferredoxin mediates nitrogenase bioelectrocatalysis in the cell when SA-1 mutant of *A. variabilis* is immobilized on indium tin oxide coated polyethylene. The intracellular nitrogenase is being constantly reproduced by the cell and is protected from oxygen through the formation of heterocyst (**Figure 27a**).[60] In another study, Chong Liu and co-workers constructed a hybrid inorganic–biological system to synthesize NH_3 from N_2 and H_2 generated from electrocatalytic water splitting at ambient conditions (**Figure 27b**).[887] Specifically, a constant voltage was applied between a cobalt–phosphorus alloy hydrogen evolution cathode and a cobalt phosphate oxygen evolution anode for water splitting and H_2 generation. The hydrogenase of an H_2-oxidizing bacterium, *Xanthobacter autotrophicus*, oxidized the generated H_2, driving the CO_2 fixation in the Calvin cycle and N_2 fixation by nitrogenase. As the generated NH_3 can diffuse extracellularly, *X. autotrophicus* cells can be used as electrogenerated biofertilizer and added to soils to improve the growth of cherry belle radish by up to approximately 1,440% in terms of the storage root mass. This research demonstrated that the H_2 acts as the electron carrier in N_2 fixation can be generated *in situ* from electrochemical water splitting.

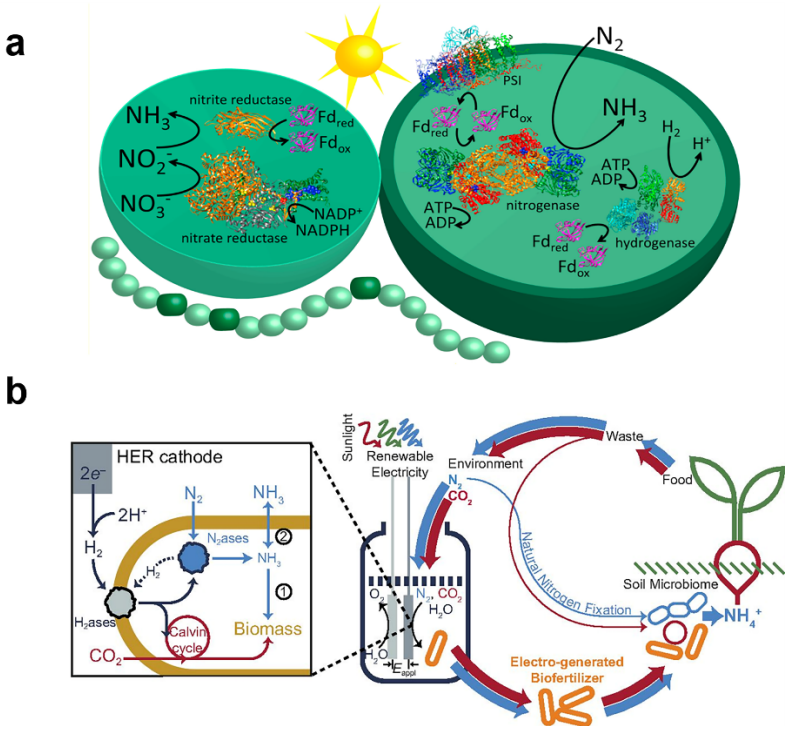

Figure 27. (a) Representation of the main enzymes and reactions involved in biological nitrogen fixation in the vegetative and heterocyst *A. variabilis* cells. Vegetative cells are represented by the pale green circles, and heterocysts are represented by the larger, dark green circles. Vegetative and heterocyst cells are linked together to form filaments. Reprinted with permission from ref. 60. Copyright 2017 Elsevier. (b) Schematic of the electroaugmented nitrogen cycle. A constant voltage (E_{appl}) is applied between CoPi OER and Co-P HER electrode for water splitting. Hydrogenase of *X. autotrophicus* oxidizes the H_2, fueling CO_2 reduction in the Calvin cycle and N_2 fixation by nitrogenase. The generated NH_3 is typically incorporated into biomass (pathway 1) but can also diffuse extracellularly by inhibiting biomass formation (pathway 2). *X. autotrophicus* forms an electrogenerated biofertilizer that can be added to soil to improve plant growth. The red pathway indicates carbon cycling; blue pathways indicate nitrogen cycling. CoPi, oxidic cobalt phosphate; OER, oxygen evolution reaction; HER, hydrogen evolution reaction. Reprinted with permission from ref. 887. Copyright 2017 PNAS.

4.4.2.3. Microbial Electrosynthesis of Methane. Methane is a noble means of storing energy, and it is also easy to transport.[38] For the microbial electrosynthesis of methane-based on the electro-reduction of CO_2, the role of the anode is to provide a complete circuit in physics. There are many oxidation reactions on the anode, which include the oxygen evolution reaction or the oxidative decomposition of organic compounds. The electroactive microorganisms accomplish CO_2 electromethanogenesis on the biocathode. HCO_3^-/CO_2

can be used as a substrate for the CH_4 production catalyzed by the microorganisms. Usually, the electron transfer mode for the CO_2 electromethanogenesis and CH_4 production include direct (Eq. 5) and indirect electron transfer (Eq. 6 and Eq. 7).[888]

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \quad E = -0.244\ V\ vs\ SHE \quad (Eq.5)$$

$$2H^+ + 8e^- \rightarrow H_2 \quad E = -0.421\ V\ vs\ SHE \quad (Eq.6)$$

$$CO_2 + 3H_2 \rightarrow CH_2 + 2H_2O \quad (Eq.7)$$

Electromethanogenesis is a research field that has rapidly developed in recent years. The first study of electromethanogenesis was carried out using a two-chamber reactor with an abiotic anode and biotic cathode. *Methanobacterium palustre* was used as the bioelectrocatalyst for the reduction of CO_2 and the production of CH_4 at a set potential of less than −0.5 V *vs* SHE. Although at this operation potential hydrogen could be generated, the electron transfer mechanism was still believed to be DET without solid evidence.[889] The electromethanogenesis based on DET had remained to be a hypothetical reaction model.[101, 890] In some studies, the hydrogenase-disrupted mutant of a methanogen can still produce CH_4 in a BES reactor in an applied-voltage-dependent manner.[891] In subsequent research, a *Methanothermobacter*-related methanogen and synergistetes- and thermotogae-related bacteria were selected during the acclimation in the two-chamber electrochemical reactor at high temperature to improve the production rate of CH_4. An important finding is that methane can be produced at –0.35 V *vs* SHE at a rate of 206 mM L^{-1} day^{-1}. The midpoint potential of the catalytic wave of the biocathode, presenting supporting evidence for the direct electron transfer (DET)-mediated mechanism.[892] Meanwhile, research by Zhen *et al*. also proved the directed electron transfer between *Methanobacterium* and electrode, which facilitated CH_4 production.[893] The DET-mediated electromethanogenesis

can be coupled with the water treatment process. Some useful attempts have been carried out to synchronously realize the CH_4 production and the treatment of domestic wastewater,[894] dairy farm wastewater,[895] and seawater-based subsurface aquifer in a natural gas field.[896] For the mediated electron transfer (MET)-mediated electromethanogenesis depending on H_2 production, Marshall and co-workers first reported the reduction of CO_2 to a mixture of methane, acetate, hydrogen, and formate, using enriched mixed co-cultures consisting of >93% *Methanobacterium* and ~5% *Methanobrevibacte* originated from brewery wastewater under the potential of −0.59 V *vs* SHE.[897] In subsequent research, a novel electrode design consisted of porous nickel hollow fibers, which acted as an inorganic electrocatalyst for hydrogen generation from proton reduction and as a gas-transfer membrane for direct CO_2 delivery to CO_2-fixing hydrogenotrophic methanogens on the cathode through the pores of the hollow fibers. These unique electrode structures create a good environment for the enrichment of methanogens and the H_2-mediated CH_4 production.[898] In recent research, Beak and co-workers investigated the development of a biocathode from non-acclimated anaerobic sludge in an electromethanogenesis cell at a cathode potential of −0.7 V *vs* SHE over four cycles of repeated batch operations. The conversion rate of CO_2 to CH_4 increased to 97.7% as the number of cycles increased, suggesting that a functioning biocathode developed during the repeated sub-culturing cycles. The CO_2-resupply test results suggested that the biocathode catalyzed the formation of CH_4 via both direct and indirect (H_2-mediated) electron transfer mechanisms.[899]

4.4.2.4. Microbial Electrosynthesis of Acetate. Acetate is another major product of CO_2 reduction in microbial electrosynthesis, which can be produced by pure or mixed

acetogenic cultures.[900] Acetate can be electrochemically produced at −0.28 V *vs* SHE (Eq. 8).

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O \quad E = -0.28\,V\ vs\ SHE\ (Eq.8)$$

In the first report of electroacetogenesis, Nevin and co-workers found that when the graphite cathode was acclimated by *Sporomusa ovata, Sporomusa sphaeriodes, Sporomusa silvacetica, Clostridium ljungdahlii, Clostridium aceticum, and Moorella thermoacetica* at potential −0.4 V *vs* SHE, the electrons derived from the cathode could reduce CO_2 and produce acetate, 2-oxobutyrate, or formate.[901] Although most studies of electroacetogenesis reported acetate production lower than 1 g L^{-1} d^{-1},[902] some studies made breakthroughs. It was found that a graphite granule packed bed cathode could obtain a high area to volume ratio and achieved 3.1 g L^{-1} d^{-1} in the production rate.[903] Moreover, a study used galvanostatic control to overcome the reducing power limitation. The final production rate reached 18.72 g L^{-1} d^{-1}.[904] Some studies indicated that the higher production rate of acetate and current density could be obtained in mixed culture than pure culture within each cathode potential, which was likely due to the syntrophic interactions among different communities.[905, 906] Hydrogen can also act as the electron donor to support the production of acetate (Eq. 9).

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O\ (Eq.9)$$

Recently, some novel materials have been developed and applied to fabricate new electrodes (**Figure 28a**). These novel electrodes have been used to facilitate water splitting and *in situ* hydrogen generations for the reduction of CO_2. For example, some non-precious metal cathodes, including cobalt-phosphide (CoPi), molybdenum-disulfide (MoS_2), and nickel–molybdenum alloy (NiMo) cathodes, have been used to perform

durable hydrogen evolution. The integration of the non-precious metal cathodes and *S. ovata*, which metabolizes CO_2 and H_2 to acetate, achieves coulombic efficiencies close to 100% without accumulating hydrogen. Moreover, the one-reactor hybrid platform is successfully used for efficient acetate production from electricity and CO_2.[907] Almost simultaneously, Chong Liu's group reported a biocompatible biological–inorganic hybrid system with high efficiency for electricity-driven CO_2 reduction and acetate production.[908] Specifically, H_2O is split to O_2 by a cobalt phosphate anode, and H_2 is produced by a cobalt–phosphorous alloy cathode. The generated H_2 is utilized by the hydrogenase of *S. ovata* as an electron donor to drive the reduction of CO_2 and acetate generation. In order to solve the problem of the low solubility of H_2, a biocompatible perfluorocarbon nanoemulsion was used as an H_2 carrier. The production of acetate was increased by 190%. The average acetate titer of 6.4 g L^{-1} was achieved in four days with close to 100% Faradaic efficiency. Another innovative research is from Peidong Yang's group (**Figure 28b**), which reports the construction of a photoelectric conversion system based on the silicon (Si) and titanium dioxide (TiO_2) nanowire arrays with large surface area as the light-capturing units that enabled a direct interface with *S. ovata* as the cellular catalyst. Reducing equivalents was generated from the light-harvesting electrodes to power the *S. ovata* cell, which allowed the conversion of CO_2 to extracellular acetate. The photoelectrochemical production of acetate can be achieved under aerobic conditions with low overpotential (η <200 mV), high Faradaic efficiency (up to 90%), and long-term stability (up to 200 h). The highest concentration of produced acetate was achieved to be ~6 g L^{-1}.[710, 909]

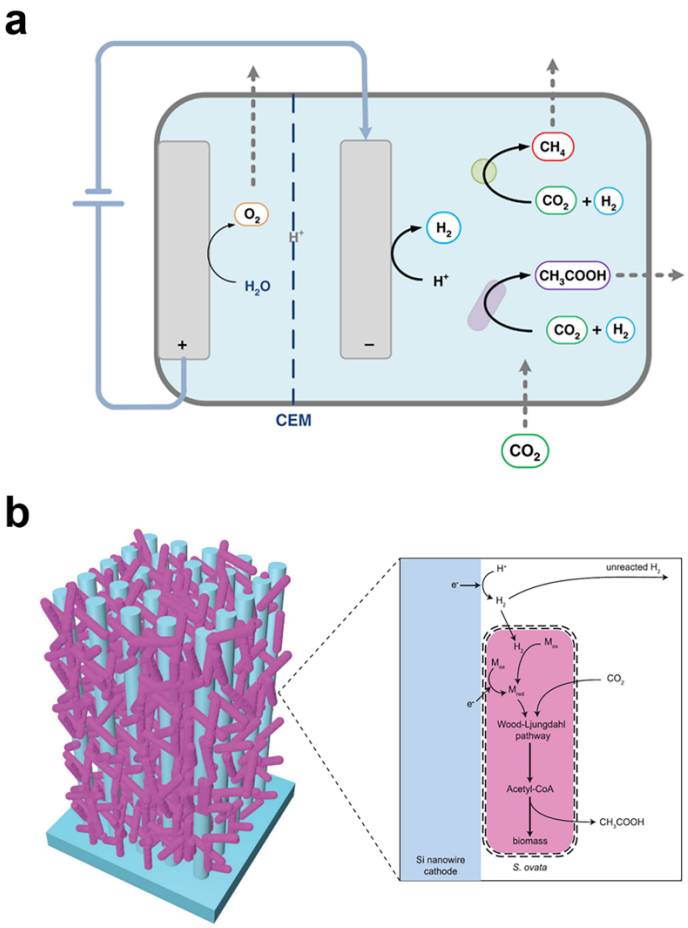

Figure 28. (a) Integrated bioelectrochemical reactor; anode (+) and cathode (−) compartments are separated by a proton-exchange membrane (CEM). The target reaction on the cathode is the evolution of hydrogen as an electron donor for the microbial reduction of CO_2. The displayed microbial catalysts are homoacetogenic bacteria (purple rod) and methanogenic archaea (green cocci). Reprinted with permission from ref. 907. Copyright 2019 Springer Nature. (b) Schematics of the close-packed nanowire-bacteria hybrid system (Left) and the reaction pathway (Right). The electrons are transferred (via either direct pathway or H_2-mediated pathway from the Si nanowire cathode to *S. ovata* to generate the intracellular reducing equivalents (M_{red}). The reducing equivalents are finally passed on to the Wood-Ljungdahl pathway to produce acetate and biomass. Reprinted with permission from ref. 909. Copyright 2020 Elsevier.

4.4.2.5. Microbial Electrosynthesis for the Production of Alcohols and Volatile Fatty Acids. Microbial electrosynthesis is a type of microbial electrocatalysis in which an electrochemical cathode supplies electron to living bacteria via applied electric current; the microorganisms use the supplied electrons to reduce carbon dioxide (CO_2) yielding

products of industrial relevance. Thus, the fascinating aspect of microbial electrosynthesis[866, 910, 911] is its ability to utilize unwanted waste, CO_2,[45, 912-915] and produce value-added biofuels, such as ethanol by the bacterial metabolic pathways.[916] In the microbial electrosynthesis for alcohol production, ethanol is of particular interest due to its potential replacement for gasoline.[917] Birjandi and co-workers electrochemically facilitated ethanol fermentation from CO_2 by *Saccharomyces cerevisiae*, producing 11.52 g L^{-1} in 40 hours of operation.[918] On the other hand, Liu and co-workers used *Clostridium scatologenes* ATCC 25775T, an anaerobic bacterium, to produce ethanol with a maximum yield of 0.015 g L^{-1} during 7 days of operation along with other products, such as butyric and acetic acids.[919] Moreover, Ammam and co-workers improved the microbial electrosynthesis of ethanol by *S. ovata* from 1.5 ± 1.0 mM without any tungstate to 13.2 ± 1.2 mM with the addition of tungstate.[920] Various tungsten amounts were examined where the concentrations were multiplied by 1× (0.01 μM), 5×, and 10× (0.1 μM) tungstate, resulting in increased ethanol production when 10× tungstate was used. The tungstate addition to this system also improved the production of acetate, 1-propanol, and 1-butanol. Similarly, Harrington *et al*. used neutral red (NR) to mediate the microbial electrosynthesis of *E. coli, Klebsiella pneumoniae, Z. mobilis.*[921] Except for *Z. mobile,* the addition of NR enhanced the production of *E. coli* and *K. pneumoniae*; namely, *K. pneumoniae* produced 93% more ethanol compared to the control group. Additionally, microbial communities consisting of various bacterial species have also shown capabilities for alcohol production.[922] Srikanth and co-workers obtained mixed culture from a corroded metal surface to utilize CO_2.[923] This mixed microbial community produced methanol, ethanol, and butanol triggered by the precedent production of acids, including formic acid and acetic

acid. At the end of a 90-day operation, about 61% of the total production (~34.28 g L^{-1}) was alcohol. Vassilev and co-workers used the mixed reactor microbiome for CO_2 reduction to produce a mixture of carboxylic acids and the corresponding alcohols.[924] During the first 60 days of operation, ethanol was the major product, accumulating 28.1 mM. However, starting on the 90th day, butanol production increased; ultimately, by the end of the experiment (462 days), butanol was the major alcohol produced along with isobutanol, ethanol, and hexanol.

Moving forward in further increasing alcohol production of microbial electrosynthesis, a direct increase of cofactors[925] such as nicotinamide adenine dinucleotide (NAD) involved in ethanol metabolic pathways, genetic engineering[206, 926, 927] to either inhibit competing reactions[928, 929] or to introduce foreign genes[930-933] to facilitate particular reactions are experimentally tested. He and co-workers increased the production of butanol in *Clostridium beijerinckii* IB4 with NR, an electron carrier by increasing the concentration of NADH available for butanol metabolic pathway.[934] The production of butanol from this system increased from 9.36 g L^{-1} to 10.05 g L^{-1} when consuming 50 g L^{-1} of glucose. On the other hand, Bajracharya and co-workers inhibited methanogensis of *C. ljungdahlii* in mixed culture for more efficient CO_2 reduction leading to productions of acetate, acetic acid, ethanol, and butyrate.[935] To avoid methanogenesis, the mixed culture was heated at 90 °C for an hour to eliminate any heat-tolerant methanogens and was regrown in a media with sodium 2-bromoethanesulfonate (NaBES) as a methanogenesis inhibitor. While this inhibition of competing reactions and direct increase of cofactors used in the metabolic pathway can direct microbial electrosynthesis towards CO_2 reduction to ethanol (while

avoiding methane production), the complex microbial system hinders the groundbreaking selective guidance in the cell.

Microbial electrosynthesis can also be employed for the synthesis of volatile fatty acids are organic acids composed of six or fewer carbon atoms. These acids (1) are used as synthetic building blocks for numerous commercially valued chemical products, and (2) have several applications in the production of bioenergy, pharmaceutical, and food industry.[936, 937] Among these volatile fatty acids are acetic acid, isovaleric acid, propionic acid, butyric acid, and isobutyric acid.[938] Although the standard, commercial, chemical synthesis of volatile fatty acids is based on the use of non-renewable petroleum,[939] this synthesis method is a rising concern with the reduction of fossil fuels.[940, 941] As a promising, environmentally-friendly alternative to the petroleum-based production of volatile fatty acids is microbial electrosynthesis due to degradability, sustainability, and renewability as it can use renewable carbon-based sources as raw materials. Namely, volatile fatty acids can be synthesized via microbial fermentation processes as they are the end products of fermentation and biosynthetic pathways.[942] Several microorganisms, including *Acetobacter*, *Clostridium*, *Moorella*, and *Kluyveromyces*, are known to use a range of carbon sources for the synthesis of volatile fatty acids, under anaerobic conditions.[943-946] Research studies have investigated a variety of carbon sources for the production of volatile fatty acids via microbial fermentation; most studies have employed pure sugars,[947-949] such as glucose and xylose, which result in high productivity with fewer side products, thus lowering purification costs.[950, 951]

Acetic acid, or ethanoic acid, can be synthesized by the following microbe families: *Acetobacter*, *Acetomicrobium*, *Thermoanaerobacter*, *Clostridium*, and *Acetothermus*.[945,

[952, 953] Microorganisms in the *Acetobacter* family are frequently employed in industrial syntheses of acetic acid, where bacteria use several sugars, including ribose, glucose, arabinose, galactose, and xylose.[954] In a research study, Ehsanipour and co-workers reported the bioproduction of 17 g L^{-1} of acetic acid using *M. thermoacetica* and lignocellulose sugars as an abundantly available carbon source.[943] Ravinder *et al*. showed the production of 30.98 g L^{-1} acetic acid from cellulose using *Clostridium lentocellum* SG6.[955] To reduce production costs, acetic acid has also been simultaneously synthesized with other products, such as gluconic acid using thermotolerant *Acetobacter* species.[956] Nayak *et al*. have also shown production of 96.9 g L^{-1} of acetic acid using *Acetobacter aceti* fermentation proves and cheese whey as a carbon source.[945] An electrosynthesis procedure for the synthesis of 11 g L^{-1} acetic acid from CO_2 using a well-acclimatized and enriched microbial consortium and a new 3D porous electrode material prepared via electrophoretic deposition method.[948] Additionally, a few genetic engineering strategies have been utilized to enhance the microbial-based production of acetic acid, namely overexpression of alcohol dehydrogenase and acetic acid exporter.[957, 958] Modestra and co-workers demonstrated the use of a double-chambered bioelectrochemical system, in which the cathode chamber contained enriched homoacetogenic microorganisms, for the synthesis of carboxylic acid/volatile acids with a major production of acetic acid (12.57 mM).[959]

Another volatile fatty acid of interest is propionic acid, which is used as an intermediate to produce several chemicals of industrial relevance. Its biosynthesis is environmentally friendly and performed by *Propionibacterium* spp. (e.g., *P. acidipropionici*, *P. freudenreichii*, *P. thoenii*)[852,859] using glucose, xylose, and lactose as the carbon sources.[855] For instance, Liang and co-workers have demonstrated the production of 68.5 g L^{-1}

propionic acid using immobilized *P. acidipropionici* stable bacteria via eight repeated fermentation cycles.[944] In another study, Quesada-Chanto and co-workers have demonstrated the use of *P. acidipropionici* with sugarcane molasses as the carbon source for the synthesis of 30 g L^{-1} propionic acid.[960] In a more recent study, Wang *et al*. proposed *P. freudenreichii*-based co-fermentation using glycerol and glucose as carbon sources to synthesize propionic acid and B_{12}.[961]

Butyric acid is a significant building block in the production of industrially valued chemicals. For the microbial synthesis of butyric acid, different microorganisms from various biological habitats have been isolated. These include microbes such as *Sarcina*, *Megasphaera*, *Clostridium*, *Butyrivibrio*, *Fusobacterium*, among which *Clostridium* is most commonly employed due to its ability to use a wide range of carbon sources.[962, 963] For example, Baroi and co-workers used *C. tyrobutyricum* strain with glucose and xylose to concurrently synthesize butyrate.[824] Immobilized *C. tyrobutyricum* in a fibrous fed bioreactor yielded 26.2 g L^{-1} butyric acid with cane molasses and 20.9 g L^{-1} butyric acid with sugar bagasse as the carbon sources.[946, 964] Dwidar and co-workers used a microbial co-culture of *Bacillus* strain using sucrose and *C. tyrobutyricum* ATCC 25755 to give levansucrase enzyme, hydrolyzing sucrose into fructose and glucose, which were then fermented into 34.2 g L^{-1} butyric acid by *C. tyrobutyricum*.[965]

To select the most effective method and microorganism for microbial electrosynthesis of volatile fatty acids, several factors that impact productivity, cost of raw materials, product yield, and side products, need to be carefully considered. Genetic engineering strategies to modify a bacterial metabolism could be employed to (1) reduce side products, (2) use different carbon sources, and (3) increase the productivity of volatile fatty acids.

However, the lack of genetic engineering methods for anaerobic microbes to produce volatile fatty acids remains a significant challenge in altering metabolic pathways. In addition to metabolic engineering, research on isolation, identification, and characterization of new microbial species that provide higher productivity is necessary. A summary of research on the microbial electrosynthesis of ethanol and volatile fatty acids is given in **Table 2** and **Table 3**, respectively.

Table 2. Summary of works reviewed on the microbial electrosynthesis for the production of alcohols.

Alcohol	Microorganism Used	Substrate	Yield	Ref.
Ethanol	*Saccharomyces cerevisiae*	CO_2	11.52 g L^{-1} in 40 h	918
Ethanol	*Clostridium scatologenes*	CO_2	0.015 g L^{-1} in 7 days	919
Ethanol	*Sporomusa ovata*	CO_2	Not specified	920
Methanol Ethanol Butanol	Mixed microbial community	CO_2	34.28 g L^{-1} total alcohol in 90 days	923
Ethanol Butanol Isobutanol	Mixed microbial community	CO_2	28.1 mM ethanol as major product	924
Butanol	*Clostridium beijerinckii*	Glucose	10.05 g L^{-1}	934
Ethanol	*Clostridium ljungdahlii*	CO_2	Not specified	935

Table 3. Summary of works reviewed on the microbial electrosynthesis for the production of volatile fatty acids.

Volatile Fatty Acid	Microorganism Used	Substrate	Yield	Ref.
Acetic acid	*Moorella thermoacetica*	Sugarcane straw hydrolysate	17.2 g L^{-1}	943
Acetic acid	*Clostridium lentocellum*	Paddy straw	30.98 g L^{-1}	955
Acetic acid	*Acetobacter aceti*	Cheese wey	96.9 g L^{-1}	945
Propionic acid	*Propionibacterium acidipropionici*	Jerusalem artichoke hydrolysate	68.5 g L^{-1}	936
Propionic acid	*Propionibacterium acidipropionici*	Sugarcane molasses	30 g L^{-1}	944
Propionic acid	*Propionibacterium freudenreichii*	Glycerol and glucose	0.71 g g^{-1}	961
Butyric acid	*C. tyrobutyricum*	Cane molasses and sugar bagasse	26.2 g L^{-1} (with cane molasses) 20.9 g L^{-1} (with sugar bagasse)	946 964
Butyric acid	*Bacillus* strain *C. tyrobutyricum*	Sucrose	34.2 g L^{-1}	965

4.4.2.6. Application of Engineered Strains in Microbial Electrosynthesis. All the cases of microbial electrosynthesis mentioned above are based on the use of wild-type electroactive microbial cells. In recent years, with the development of metabolic engineering and synthetic biology technologies and the continuous enrichment of gene manipulation methods, the combination of electrochemical technology and metabolic engineering or synthetic biology is becoming a new research hotspot. This combination endues the genetic engineering of model microorganisms, such as *E. coli*, *S. cerevisiae,* and *B. subtilis*, to utilize exogenous electrons to alleviate redox imbalances during the synthesis of biochemicals and biofuels.[966] Meanwhile, depending on the diversity of the engineered synthetic pathway, the product scope of microbial electrosynthesis can be significantly

expanded. The products of microbial electrosynthesis system will no longer be limited to the conventional fuel chemicals but can be further extended to a variety of fine chemicals with higher added value.

The first strategy to construct an engineered strain is the introduction of an electron transfer pathway to establish the electrochemical communication between the bacterial cells and electrode. In a study by Wu *et al*., the electron transport proteins MtrABC, FccA, and CymA from *S. oneidensis* MR-1 were expressed in *E. coli* T110 to construct an electroactive cell factory, which can utilize electricity to reduce fumarate and produce succinate. The electroactive *E. coli* T110 strain was further improved by incorporating a carbon concentration mechanism (CCM). This strain was fermented in a microbial electrosynthesis system with neutral red as the electron carrier and supplemented with HCO_3^-, which produced a succinate yield of 1.10 mol/mol glucose.[967] Sturm-Richter and co-workers used a similar strategy in which heterologously expressed *c*-type cytochromes CymA, MtrA, and STC from *S. oneidensis* in *E. coli* cells to construct the electron transport pathway.[968] This electroactive *E. coli* can be used as a chassis cell to integrate a new synthetic pathway. Consequently, Mayr and co-workers integrated an NADPH-dependent alcohol dehydrogenase from *Lactobacillus brevis* into this electroactive *E. coli* chassis cell to perform the asymmetric reduction of acetophenone and the synthesis of (*R*)-1-phenylethanol. The import of exogenous electrons effectively ensured the regeneration of NADPH and the production of (*R*)-1-phenylethanol.[237] Another strategy to improve the electron transfer efficiency is to employ the use of electron mediators. The Hao Song group established a microbial electrosynthesis system based on the utilization of neutral red as an electron shuttle. In their research, they employed neutral red mediated extracellular

electron transfer pathway between the electrode and recombinant *S. cerevisiae* harboring 7α-hydroxylase to facilitate the intracellular NADPH regeneration. The shortcut enhanced the biotransformation from dehydroepiandrosterone (DHEA) to 7α-OH-DHEA catalyzed by P450 monooxygenase.[969] In another study, the researchers introduced ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) into the wild-type *Ralstonia eutropha* via metabolic engineering approach to endow the *R. eutropha* cells the ability of CO_2 fixation. Neutral red is used as an electron mediator to deliver electrons from the cathode into *R. eutropha*, facilitating the efficiency of CO_2 reduction and PHB production. Upon application of the cathode potential at –0.6 V *vs* Ag/AgCl and the engineered *R. eutropha*, the final concentration of PHB achieved was determined to be 485 ± 13 mg L^{-1}.[45]

5. OUTLOOK AND FUTURE DIRECTIONS

5.1. Future Directions for Biosensor Design

Electrochemical enzymatic and microbial biosensors are a central application of bioelectrocatalysis due to their extensive use as analytical devices for (1) monitoring environmental samples, (2) evaluating food and beverages, and (3) medical diagnostics. In the following sections, we briefly summarize biosensing platforms, which have developed as future biotechnological trends in bioelectrocatalysis. Namely, recent progress has been directed toward miniaturization, multiplexed detection analysis, as well as applicable expansion to wearable sensing technologies (e.g., paper-based biosensors and tattoo-based biosensors), which have minimal sample pre-treatment steps and low power requirements. Additionally, self-powered biosensors have been designed as simple and low-cost devices to meet the increased demand for personal analyses and health monitoring. Although noteworthy advances have been made in the development of innovative biosensors, future

work requires improvement and integration of biosensor assemblies to address barriers with stability, sensitivity, reliability, and simplicity, for practical applications and commercialization of electrochemical biosensors. Strategies for the attachment of bioelectrocatalysts on electrode surfaces require further examination to (1) enhance electron transfer rates and (2) provide extended sensor stability. Additionally, the elucidation of electron transfer mechanisms in bioelectrocatalysts is required to effectively optimize the sensitivity of biosensing platforms. Appropriate biocatalyst selection should also be examined, especially since microorganisms and enzymes respond to changes in environmental factors to generate measurable signals. Future developments, particularly with electrochemical microbial biosensors, should focus on designing devices that can achieve detection under extreme conditions, such as highly acidic, saline, and/or extreme temperature settings. Therefore, the selection and adaptation of microbes that can survive in such harsh conditions is an important future direction.[970] A major challenge with biosensors is their application for *in vivo* sensing as most of the developed biosensors demonstrate only proof-of-concept studies. In complex biological environments, interfering chemicals found in the sample matrix can cause electrode biofouling, which decreases the signal responses and selectivity of the biosensor. To address these disadvantages, genetic engineering methods, and also optimization of biomaterials, have been employed to minimize interferences.

In addition to electrochemical-based biosensors, optical-based biosensing methods can provide qualitative analyte information via non-destructive analyses of solutions of interest.[971] Electrochemical methods (e.g., cyclic voltammetry, differential pulse voltammetry, square wave voltammetry) allow for real-time, *in situ*, qualitative monitoring

of redox reactions, reaction reversibility, and electrical current responses.[472] As such, these methods typically provide high sensitivity close to the transducing electrode's surface. Optical and imaging techniques, such as surface plasmon resonance, Raman spectroscopy, fluorescence-based microscopy, and scanning probe microscopy can be combined with electrochemical biosensing strategies to provide an enhanced understanding of bio-interfacial events.[972-974] Towards these goals, new configurations of transducer elements configurations have been designed, such as indium tin-coated glass fiber optics or transparent carbon electrodes.[172, 465, 471, 975, 976] These provide a means for the same probe to detect and measure analytes using both electrochemical and optical strategies in parallel. In addition to potential improvements in the response and sensitivity of biosensors, future studies on designing devices based on the combination of electrochemical and optical techniques could provide prosperous sets of data and additional means to control and monitor specific analyte in sensing environments of interest.

5.1.1. Miniaturization of Biosensors. In creating biosensors for practical monitoring applications, portable microfabricated devices have become attractive, promoting the miniaturization of electrochemical biosensors. These miniaturized biosensor systems use electrodes at the micro- and nanometer dimensions, which are typically designed using micro/nanofabrication methods.[465, 975, 977] Reducing the electrode size to micro- and nano-size dimensions offers substantial benefits for biosensors, specifically high analytical selectivity, rapid response times, high signal-to-noise ratios, and increased mass transport at the electrode surface as the diffusion profiles become mainly radial.[465, 975, 977-979] Consequently, recent studies have aimed to promote the fabrication of miniaturized electrochemical biosensors.

Popovtzer and co-workers employed photolithography to create miniature electrodes for microbial biosensors to monitor water toxicity by detecting ethanol and phenol.[980] In this sensor design, eight miniaturized chamber cells, each consisting of a gold working electrode, a reference electrode, and a counter electrode, were incorporated on a single disposable chip. In a later study, Popovtzer *et al.* described a mathematical model for kinetic properties of microbial enzymatic reactions in response to toxins and the diffusion of redox species to the miniaturized electrochemical platform.[981] The simulation data were in agreement with the measured results, thereby showing a promise for the development of biosensors on the miniature scale. Electrochemical biosensors using microelectrodes have also been integrated with microfluidic devices. Ben-Yoav *et al.* reported the design of a whole-cell biosensor incorporating four microchamber biochips for the detection of water genotoxicity.[982] Miniaturizing the chamber size resulted in decreased diffusion distance between redox molecules to the electrode, subsequently enabling rapid and sensitive analyses of nanoliter sample quantities.

When combined with biosensors, screen-printing technologies allow for the design of miniaturized biosensors that are appropriate for analytical applications. In addition to distinct printing materials, screen-printing provides simple fabrication steps. Screen-printed electrodes have also been used to enhance the attachment of microorganisms on the surface of working electrodes.[983] For instance, Hua and co-workers recently reported a disposable electrochemical enzymatic biosensor utilizing screen-printed carbon electrodes for the amperometric detection of organophosphorus pesticides.[984] Acetylcholine esterase was immobilized onto a screen-printed carbon electrode modified with multi-walled

carbon nanotubes, chitosan, and gold nanoparticles, achieving high sensitivity due to synergistic effects between carbon nanotubes and gold nanoparticles.

Additionally, micro- and nano-electrode arrays have been developed consisting of tens to thousands of interconnected electrodes.[985-988] The fabrication of these electroanalytical platforms is relatively simple; these platforms offer advantages to biosensing technologies, including low ohmic potential drops, high spatial resolution, and capability for multi-analyte detection.[977] The first amperometric biosensor using microelectrode arrays was introduced by Ross *et al*., where different enzymes (e.g., glucose oxidase, choline oxidase, and lactate oxidase) were immobilized in a conducting polymer (e.g., polypyrrole) for environmental monitoring.[989] The Stevenson group has reported the development and facile fabrication of a low-cost, versatile electrochemical biosensing platform based on carbon ultramicroelectrode arrays,[975, 977] which were recently applied for the real-time electrochemical detection of multiple redox-active phenazine metabolites from *P. aeruginosa* strains.[172, 466] Additionally, Buk and co-workers introduced an enzymatic biosensor using gold microdisk array electrodes (20 μm diameter) decorated with carbon quantum dots and gold nanoparticles for the sensitive detection of glucose.[990] Although significant progress has been made to design miniaturized electrochemical biosensors, future research work needs to focus on their optimization for practical applications in real samples.

5.1.2. 3D-printed Biosensor Devices. Three-dimensional (3D)-printing, an additive manufacturing method, has recently received attention in the area of bioanalytical sensors.[991, 992] Specifically, this emerging technology has been employed to fabricate and design smaller and more efficient electrodes as biosensing platforms with various

advantages, including low-cost, manufacturing speed, multiplex sensing capabilities, as well as controllability and flexibility of the fabrication process, enabling tailored sensor geometries, shapes and architectures.[993, 994] 3D-printing technology has been employed to design electrochemical biosensors via (1) incorporation of commercially available electrode devices into 3D-printed structures or (2) 3D-printing biosensing platforms. Additionally, 3D-printing has been used, in combination with conductive materials, to fabricated electrodes of different geometries[995] with a high potential for electroanalytical sensing applications. However, 3D-printing technology is a relatively uninvestigated area for electrochemical biosensors because only a few studies have demonstrated the successful functionalization of 3D-printed conductive electrodes with biological recognition elements.[991]

Dong and co-workers recently demonstrated the development of a fully 3D-printed amperometric biosensor for lactate detection.[996] In this sensor design, the researchers printed thin silver electrodes on flexible polyethylene terephthalate (PET) substrate, which was biofunctionalized by deposition of a lactate oxidase layer. The electrodes were 3D-printed via direct ink writing method based on the use of highly viscous silver nanoparticle ink. This 3D-printed electrochemical biosensor demonstrated a linear dependence of 1–20 mM lactate for *in vitro* studies. The Gozen and Lin research groups reported the first 3D-printed flexible electrochemical biosensor for glucose detection using direct-ink-writing technology.[997] The researchers used a novel two-step process approach for printing the enzyme with the electrodes. First, the bare 3D-printed electrode was fabricated using a commercial Prussian blue conductive carbon ink. Second, the researchers developed an enzyme ink by introducing glucose oxidase into a tetraethoxysilane:H_2O:ethanol:HCl

(60:13:13:1 *v/v*) solution, which was subsequently dissolved in a hydroxypropyl cellulose solution. This enzyme ink was then printed on the 3D-printed carbon electrodes to construct the glucose biosensor, which has a limit of detection of 6.9 μM and a linear range of 0.1–1.0 mM. In another inventive study, Katseli *et al.* reported a functional and fully integrated electrochemical sensor for glucose detection, fabricated using a single-step 3D-printing approach.[998] This sensing device was manufactured through a fused deposition modeling as a 3D-printing method to print three conductive polymer electrodes (working, counter, and pseudo-reference) using two filaments: (1) a carbon-loaded polylactic acid conductive filament, and (2) an insulator polylactic acid non-conductive filament. To obtain the glucose biosensor, the researchers immersed the 3D-printed working electrode in a 1:1:2:2 (*v/v)* solution of glucose oxidase, Nafion, ethanol, and hydrogen peroxide. This sensor was coupled with chronoamperometry for the indirect quantification of glucose via the detection of hydrogen peroxide derived from enzymatic oxidation of glucose.

Certain studies have demonstrated a comparison of 3D-printed electrochemical biosensors with screen-printed biosensor devices, where the 3D-printed electrodes were characterized with a broader linear range and higher sensitivity.[997] This result is likely associated with the smooth, distinct, conductive edges with minimal defects achieved with 3D-printing methods. As such, 3D-printing approaches show a promise as a feasible technology for further advances in the design of electrochemical biosensors with enhanced performance characteristics. Future research studies need to carefully examine the lifetime and fragility of 3D-printed electrochemical biosensors, both of which would depend not only on the nature of the biological recognition element but also on the functionalization strategy used to incorporate or immobilize these bio-components on electrode surfaces.

5.1.3. Paper-based Biosensor Devices. Biosensing technologies have given significant consideration to paper-based analytical devices as they offer a promise for point-of-care analytical testing and onsite analysis. The first paper-based device was introduced for the quasi-quantitative detection of glucose in urine samples.[999] Paper-based devices can be easily combined with instrumental electrochemical detection methods.[1000] While there are a plethora of studies that have reported paper-based analytical tools for various applications, these sensors show a particular promise for biomedical diagnostics.[1000, 1001] Several methods for fabricating sensing systems based on electrochemical paper-based biosensors have been employed, such as photolithography, wax printing, wax screen-printing, and wax dipping.[1002-1007] Despite the different printing strategies available, the most common method is wax screen-printing due to its cost-effectiveness and operation simplicity.[1008] Dungchai and co-workers demonstrated the use of printing methods for the fabrication of paper-based microfluidic electrode devices for the detection of glucose, uric acid, and lactate in biological samples using glucose oxidase, uricase, and lactate oxidase, respectively.[1003] A similar paper-based device was fabricating using wax screen-printing to measure glucose levels in blood samples.[1008] Electrochemical paper-based sensors and the ink's physicochemical characteristics enable simple modification using various nanomaterials (e.g., metallic nanoparticles, carbon-based nanomaterials, conducting polymers), which increase the biosensor conductivity.[1009-1012]

Ruecha and co-workers reported a screen-printed paper-based biosensor, which was modified with nanocomposite composed of graphene, polyaniline, and polyvinylpyrrolidone, to enhance the conductivity and increase the biosensor surface area, resulting in increased biosensor sensitivity. The researchers attached cholesterol oxidase to

the nanocomposite-modified paper biosensors for the amperometric detection of cholesterol.[1013] In another work, Sun and co-workers reported an electrochemical enzymatic biosensor based on microfluidic paper-based device decorated with gold nanorods for the sensitive detection of microRNA utilizing cerium dioxide-gold-with-glucose oxidase as the electrochemical probe to amplify the signal.[1014] As such, this biosensor device represents a platform for microRNA detection and point-of-care diagnostics in a clinical setting. In a recent study, Cao *et al.* reported the fabrication of a 3D paper-based microfluidic screen-printed electrode sensor for the quantitative detection of glucose in human sweat and blood,[1015] using immobilized glucose oxidase. Mohammadifar and co-workers described an enzymatic electrochemical biosensor for the semi-quantitative screening of glucose levels in urine samples.[1016] This biosensor consisted of a paper-based sensing strip and an amplifier circuit with visual readouts, thereby providing a simple yet powerful glucose biosensor for use in point-of-care diagnostics. Future work with paper-based electrochemical biosensors needs to focus on developing platforms that provide a means for equipment-free analytical sensing and analyses.

5.1.4. Wearable Biosensor Devices. An exponentially growing area that has generated tremendous interest is the development of wearable electrochemical sensing technologies.[22, 303, 482, 1017-1020] Wearable biosensors have recently expanded the scope from monitoring mobility and vital signs (e.g., heart rate, steps) to noninvasive detection of critical biomarkers indicative of human health.[1018] These biosensors allow for real-time noninvasive identification of biomarkers in biological fluids (e.g., saliva, sweat, tears).[1017, 1021, 1022] As such, these devices can replace the standard tests required for obtaining health

information, opening opportunities to change hospital-based systems to home-based personalized instruments, and thus significantly reducing healthcare-associated costs.

Kim and co-workers reported a wearable electrochemical biosensor for continuous monitoring of salivary metabolites.[1023] In this work, the researchers integrated a printable enzymatic electrode onto an easily removable mouthguard for noninvasive amperometric lactate monitoring. The biosensor was established on an immobilized lactate oxide and low potential detection of peroxide from human saliva samples. Three separate layers were screen-printed on a flexible polyethylene terephthalate (PET) substrate where the conductive silver/silver chloride ink was printed as the reference electrode, whereas Prussian blue-graphite ink was introduced as the working electrode and the counter electrode. Lactate oxidase was then immobilized on the working electrode surface via electropolymerization-based entrapment in a poly(ophenylene-diamine) film. This mouthguard biosensor can provide information about health status and stress level, thus offering promise for biomedical applications. The Wang group reported the first example of an electrochemical temporary tattoo-based biosensor for the real-time noninvasive lactate monitoring in human perspiration using lactate oxidase.[1024] The researchers successfully used this biosensor for real-time continuous and dynamic monitoring of lactate from human sweat during prolonged cycling exercises, giving temporal lactate profiles from sweat. Moreover, tear-based electrochemical enzymatic biosensors have also been reported.[485, 1025] A tear-based biosensor for lactate detection was designed by placing carbon paste electrodes into a contact lens (**Figure 29a**). Dimethyl-ferrocene redox polymer and lactate oxidase were immobilized onto the carbon electrode surface.[634, 1026]

This biosensor was constructed as a biofuel cell with bilirubin oxidase immobilized onto anthracene-modified multi-wall carbon nanotubes.

Furthermore, Mishra and co-workers developed a flexible epidermal tattoo and textile-based biosensor for the voltammetric detection of vapor-phase organophosphorus nerve agents.[1027] This wearable biosensor was fabricated on elastic conducting inks printed on tattoo papers and transferred to the skin. Using square-wave voltammetry, the researchers detected organophosphorus molecules, specifically *p*-nitrophenol generated from the organophosphorus hydrolase enzymatic reaction. The acquired voltammetric responses were transferred wirelessly to a mobile device (e.g., phone) via Bluetooth, thereby allowing for timely and effective detection of skin exposure to organophosphorus species. Moreover, a wearable electrochemical enzymatic biosensor was developed on a glove platform for a fingertip detection of organophosphorus nerve compounds.[1028]

Given the need for glucose monitoring in diabetes patients, tattoo-based electrochemical platforms have been developed for measuring glucose by immobilization of glucose oxidase on Prussian blue-carbon electrodes.[1029] A saliva-based glucose sensor was fabricated as a mouthguard[1030] using glucose oxidase (**Figure 29b**). Similarly, a sweat-based glucose sensor[1031] used immobilized glucose oxidase, where the reduction of the byproduct hydrogen peroxide was qualitatively correlated to glucose concentrations in sweat. This configuration was integrated with smartphones, giving high accessibility and portability of this sensor (**Figure 29c**). Biosensors have been developed to serve as smartphone platforms and also as simple band-aid forms not only for glucose[1032] but also for uric acid.[1033]

Additionally, electrochemical tattoo biosensors have been designed for alcohol monitoring in stimulated sweat.[1034] In a research work by Kim and co-workers, alcohol oxidase was attached to anodic iontophoretic electrodes to quantify alcohol in sweat induced via iontophoretic delivery of pilocarpine drugs. Tattoo-based biosensors for measuring both alcohol and glucose have been integrated into a single platform for continual multi-analyte detection.[1035] Additionally, sensors using breath condensate or gas-capture techniques have been investigated for breath biomarkers.[1036] A study reported the use of gas-phase breath samples for ethanol detection by depositing horseradish peroxidase and alcohol oxidase onto a gas chromatography paper.[1037] In this biosensor, ethanol in the gas phase breath was blown onto the chromatography paper on a screen-printed electrode. The immobilized alcohol oxidase oxidized ethanol to produce acetaldehyde and hydrogen peroxide as the byproduct, which was further reduced by horseradish peroxidase.

While an extensive number of noninvasive wearable biosensors have been developed for the detection of health-related biomarkers and environmental monitoring, these biosensors have specific challenges, such as achieving low detection limits and sensor compatibility to monitor chemical and biological processes indicative of patient's health. Another primary concern with wearable biosensors is to minimize electrode biofouling that often occurs with prolonged biosensor operation in oral cavities and/or human skin. In general, *in vivo* analyses are challenging due to reduced signal responses and selectivity, which result from biofouling interferants in the biological samples.[1038] At present, most electrochemical wearable devices are in the proof-of-concept prototyping stage, thus requiring future efforts to optimize these biosensors for real applications in biofluids and biological environments. Detailed studies on how these biosensors perform under varying

temperature and pH conditions are necessary to provide accurate data. Large-population validation studies are also required to promote a broader acceptance of wearable biosensors and allow for their transition to clinical applications.

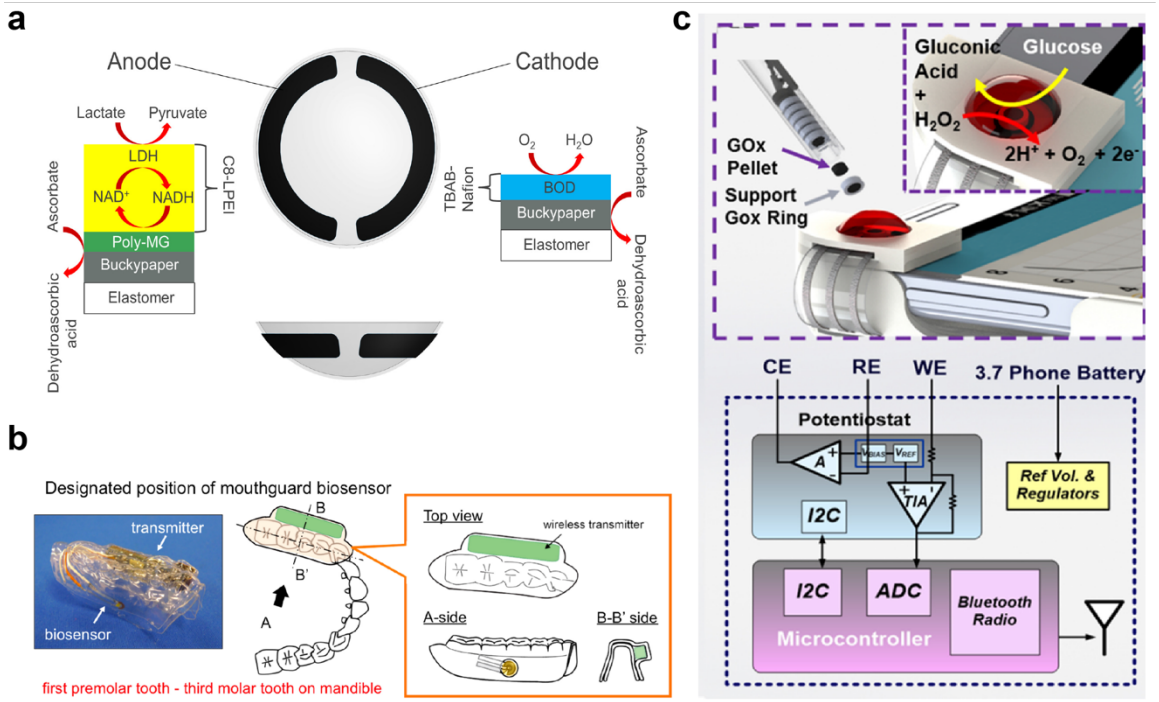

Figure 29. Adapted and modified schematics depicting wearable platforms based on electrochemical enzymatic biosensors. (a) Tear-based lactate monitoring from a contact lens platform. Reprinted with permission from ref. 634. Copyright 2015 Elsevier. (b) A saliva-based glucose monitoring from a mouthguard platform. Reprinted with permission from ref. 1030. Copyright 2016 Elsevier. (c) Sweat-based glucose monitoring from a smartphone platform. Adapted and reprinted with permission from ref. 1031. Copyright 2018 Elsevier.

5.1.5. Self-powered Biosensors. A general limitation of the aforementioned electrochemical wearable biosensors is the requirement for an electrical circuit and/or a power source for data recording. On the other hand, biofuel cells that are based on either enzymes or microorganisms to produce and store energy from the human body can provide an appropriate power supply for the development of wearable biosensors. Self-powered biosensor prototypes utilizing biofuel cells, therefore, do not need an external electrical

source of energy as they can generate power output that scales accordingly with analyte concentration.

Self-powered biosensors received consideration after Katz and co-workers reported a layer-by-layer assembly of bioelectrocatalytic electrodes to design an innovative glucose/O_2 biofuel cell element.[1039] The biofuel cell power output increased with increasing fuel concentrations (either lactate or glucose). Self-powered enzymatic biosensors have also been developed for the detection of ethylenediaminetetraacetic acid (EDTA)[1040] and nitroaromatic explosives.[659] Krikstolaityte *et al.* described a self-powered biosensor, which was a single compartment biofuel cell based on anode and cathode powered by the same fuel glucose.[1041] Glucose oxidase from *Aspergillus niger* was used as glucose-consuming biocatalyst for both the anode and cathode in the fuel cell.[1041] In this self-powered biosensor, a graphite rod electrode, cross-linked with glucose oxidase and modified with 5-amine-1,10-phenanthroline, was the bioanode while a graphite rod electrode co-immobilized with glucose oxidase and horseradish peroxide was used as the biocathode. The addition of glucose to the fuel cell, caused the oxidation at the bioanode, followed by a subsequent hydrogen peroxide reduction at the biocathode. The maximum power density generated was proportional to the glucose concentration. In another research work, a self-powered biosensor for quantifying cholesterol levels was designed using a single enzyme where an identical substrate powered both the anodic and the cathodic bioelectrocatalytic processes.[1042] This cholesterol self-powered biosensor is particularly attractive as it uses cholesterol oxidase as a single enzyme to oxidize cholesterol for mediated bioelectrocatalysis at the bioanode while producing peroxide for Prussian blue electrocatalysis as the bioanode.

Research studies have also reported the development of self-powered biosensors established on detection by enzyme inhibition effects, which can have an impact on the biofuel cell outputs. In this biosensor type, the detected signal decreases with increasing analyte concentration as a result of biocatalyst inhibition. The Minteer group first reported experimental findings of laccase enzymatic inhibition by both arsenate (As^{5+}) and arsenite (As^{3+}).[535] Laccase bioelectrodes were employed in a glucose/O_2 enzymatic fuel cell to yield a self-powered biosensor for arsenite and arsenate. The biofuel cell was composed of flavin adenine dinucleotide glucose dehydrogenase (FAD-GDH)-based bioanode and a laccase based biocathode. In the presence of arsenite, FAD-GDH facilitates the oxidation of glucose on the bioanode, while a decrease in O_2 reduction on biocathode caused a decline in power output. This conceptual self-powered biosensor showed decreasing power densities with increasing arsenite and arsenate concentrations in the 1–20 mM and 1–8 mM ranges, respectively. The detection limits were 13 μM for arsenite and 132 μM for arsenate. This biosensor operated at a 10% current draw from the maximum current density of the enzymatic fuel cell. Majdecka and co-workers reported an integrated self-powered biosensor based on a hybrid biofuel cell and a sensing device with a three-electrode cell setup.[1043] This self-powered biosensor, containing zinc-plated bioanode and a carbon nanotube-modified carbon paper discs as the biocathode with either laccase or bilirubin oxidase, was applied for the chronoamperometric detection of catechol analytes and oxygen sensing. The micro-biosensors, designed to be portable and small, were powered by the hybrid biofuel cell, generating sufficient data for wireless transmission systems.

Self-powered biosensors offer advantages compared to traditional electrochemical biosensors (e.g., no need for a potentiostat, a simplified 2-electrode instead of a 3-electrode

setup). However, there are some limitations, including higher noise relative to the low potential mediated system. Future research needs to tackle challenges with the stability of enzymatic self-powered biosensors, as well as response times and selectivity of microbial self-powered biosensors. For engaged readers with a particular interest in self-powered biosensors, a recently published review by Grattieri and co-workers is highly suggested.[20]

5.2. Future Directions of Biofuel Cells

5.2.1. Future Directions of Enzymatic Fuel Cells. As one of the few alternative energy conversion strategies, enzymatic fuel cells exhibit unique merits. Without the involvement of noble metal and toxic solvent, enzymatic fuel cells are environment-friendly devices that can operate at room temperature and yet still possess a high fuel conversion efficiency. Enzymes, with their irreplaceable substrate specificity, remove the need for membranes in fuel cells and are therefore used to circumvent the issues of fuel crossover and membrane degradation.[1044] Enzymatic fuel cells can also be scaled down to self-powered implantable devices because of the flexible fuel compacity of enzymes; fuels become inexhaustible as glucose, lactate, O_2, etc. are easily accessible in physiological fluids (e.g., blood).

Although enzymatic fuel cells have many benefits, they are not able to challenge or replace the existing power system, by far, considering the performance and stability of enzymatic fuel cells. There are several challenges to overcome that require multidisciplinary research efforts from electrochemists, biologists, material scientists, and engineers. The biggest challenge is the longevity of enzymes. Once isolated from living organisms, enzyme activity typically diminishes in hours (depending on the environment). Currently, only thermostable enzymes derived from thermophilic microorganisms have found large-scale applications in industry. These enzymes are thermostable due to the

existence of more intermolecular interactions (e.g., van der Waals forces, hydrogen bonds). Meanwhile, protein engineering has achieved moderate success in adding more interactions to improve enzyme stability, for example, by creating surface disulfide bridge or introducing aromatic residues.[1045] Protein engineering can also improve other properties of enzymes, such as substrate specificity, reaction efficiency, and catalytic potential. All these factors play an essential role in the evaluation of the performance of enzymatic fuel cells, including fuel selection, power generation, and open circuit potential. Recently, the bio-mimic catalyst, which takes advantage of elegantly designed catalysts from nature but gets rid of the protein shell, opens up a new prospect in the development of enzymatic fuel cells to solve the enzyme stability issue.[1046-1049]

The design of novel materials for efficient enzyme immobilization, which is another bottleneck in this field, aids in the generation of adequate and stable power of enzymatic fuel cells. Approaches to solving the problem are put forward from different angles. Higher and more stable power generation can be reached by the immobilization of enzymes, which decreases aggregation, unfolding, and autolysis. Immobilization of enzymes on porous support can decrease the inactivation of enzymes by gas bubbles and prevent interactions with proteases.[1049] Oxygen damage can also be solved; Plumeré *et al.* have designed viologen-based redox polymers to protect hydrogenase from high-potential deactivation and oxygen damage.[777] Nanostructured materials such as carbon nanotubes, graphene, polymers, and metal nanoparticles are able to significantly increase the electrode surface area and conductivity;[287] these merits are utilized to achieve better enzymatic fuel cells performance. Through the combination of enzymatic fuel cells and supercapacitors, the electrical power can be stored in supercapacitors, and a high-power output can be reached

in a short burst.[654] Other than the limitations mentioned above, the cost of electrode materials and biocatalyst, biocompatible issues for operation in body, and the low concentration of substrate (e.g., limited O_2 in aqueous solution) also need further investigations. Future developments in protein engineering and material design are critical in the commercialization of enzymatic fuel cells that hold promise in changing the way of power generation.

5.2.2. Future Directions of Organelle-based Biofuel Cells.

Compared to the use of intact organisms (e.g., bacterial cells), organelle provides various advantages, such as the easier establishment of direct electron transfer with an electrode surface, better transport of substrates through their membrane, and increased stability compared to the isolated enzymes responsible for their metabolisms. All of these aspects make the application of organelle in biofuel cells extremely interesting. Despite the relatively low power generation obtained from these systems, they have proven to be particularly interesting for various promising applications. Mitochondria-based biofuel cells allowed the development of self-powered biosensors having remarkably low limits of detection and providing easy-to-use devices for the *in situ* sensing of explosives and other toxic compounds. Another promising application of mitochondria biofuel cell is to develop systems for cost-effective preliminary screening of drugs, with the possibility to gain insights into the mechanisms of inhibition thanks to changes in bioelectrocatalysis. An important aspect that should be taken into consideration for future studies is that maximizing the electrochemical performance of organelle-based biofuel cells would benefit all the various applications of these devices, not only for their use as micro-low power generation tools. In fact, higher current generation allows higher sensitivity in self-

powered biosensors and could expand their applications towards performing more quantitative analysis.

5.2.3. Future Directions of Microbial Fuel Cells. Over the last 15-20 years, the field of microbial fuel cells has seen impressive advancements, leading to unprecedented power productions, stable operation, and cost-effective electrodes.[273] As a result, reports of microbial fuel cells applied in the field are now available, where these bioelectrochemical systems are utilized for wastewater decontamination,[1050] field-bathroom with self-powered illumination,[1051] or for powering remote sensors.[1052, 1053] Furthermore, proof-of-concept for emerging applications of microbial fuel cells have been recently reported, with their employment for water desalination,[1054, 1055] treatment of high-salinity wastewater (which inhibit traditional biological decontamination plants),[1056-1059] self-powered biosensing of various pollutants in both industrial effluents or freshwater,[20, 1060-1062] operation as supercapacitors,[1063, 1064] and for powering microbial electrochemical cells.[164, 1065]

These exciting new applications of microbial fuel cells pave the way for several future research directions. However, a critical aspect to underline is that, besides the tremendous improvements in the field of microbial fuel cells, the mechanistic understanding of the extracellular electron transfer process at the basis of the technology remains limited.[22, 162, 1066] Accordingly, it is critical that future studies will be focused on unveiling and clarifying the extracellular electron transfer process. In fact, gaining a detailed understanding of the process will enable the rational design of optimized systems. With this issue in mind, the recent advancements in the field of computational methods applied to the study of microbial electrochemical systems, such as modeling, bioinformatics, and quantum mechanical calculations,[1067] will provide critical tools to deepen our understanding of this

fascinating field. Finally, another critical aspect, especially for the application and commercialization of microbial fuel cells, will be gaining a better understanding of the complex network of microorganisms and their interactions, commonly referred to as "microbial ecology."[1068] Also, in this case, various computational methods, such as bioinformatics analysis, will play an important role combined with electrochemical and/or other analytical tools (e.g., fluorescence).

5.2.4. Future Directions of Biosolar Cells. Metabolic versatility of phototrophic microbes has been adapted into various energy-related applications through bio-photocells. These biosolar cells present a green, sustainable, cost-and energy-efficient approach to readdress our dynamic energy demands. Matching the performance of current solar technologies and further realizing the full potential of biosolar cells relies on optimizing the corresponding solar-to-electric and solar-to-biomass conversion efficiencies.[718] While phototrophs are specialized in absorbing solar irradiation at specific wavelengths at quantum efficiencies approximating unity, their insulating biofilms hinder the conduction of resultant photoelectrons to the abiotic electrodes.[704, 706] 'Biotic-abiotic interfacing' remains one of the main challenges. Therefore, improving 'electronic contact' to facilitate efficient photoelectron conduction between biotic and abiotic components,[134, 708] and enhancing compatibility between functional components in biohybrids[723] (e.g., prevent fouling, leaching) are broad solutions (*vide supra*). As of now, the limitations pertaining to biosolar cells are being addressed by their hybridization with supplemental semiconductors, metal, and conducting polymers.[726] However, applying synthetic biology tools to phototrophs to bioengineer highly specific and prolific microbial function that circumvents metabolic expenses during microcellular housekeeping is an alternative outlook.[709, 1069, 1070] Apart

from the biocatalytic components, the comprehensive biosolar cell architectures, namely electrodes, electrolytes, membranes, cell designs, and fabrication techniques, need to be optimized to enhance the performance efficiency and cost-effectiveness in large scale utility.[722] Commercialized organic solar cells and microbial fuel cells are a source of inspiration in this pursuit.[913]

On the other hand, the bioengineering of more useful and adaptable microbes is limited by the insufficient mechanistic understanding of the multicomponent, case-sensitive biosolar cell operations.[704] Fundamental studies of microbes and miniaturized biomimetic systems to model biosolar cells could potentially bridge that knowledge gap.[1071, 1072] Overall, biosolar cells utilize the metabolic versatility of biocatalysts in order to harvest sustainable and green solar energy to facilitate multiple useful reactions. Although many of the corresponding energy applications are currently a subject of research and on a small scale, potentially scaling up by elucidation of the essential function required of biosolar cells and optimization of the biosolar cell architecture is commercially and environmentally lucrative.

5.3. Future Directions of Bioelectrosynthesis

5.3.1. Making the Best Use of Protein Engineering in Enzymatic Electrosynthesis. Oxidoreductases are the functional core component of enzymatic electrosynthesis systems. Improvements in the catalytic properties of oxidoreductases can directly lead to the enhanced performance of enzymatic electrosynthesis systems. In future research, the performance of oxidoreductases that needs to be improved to meet the requirement of electrosynthesis mainly includes the following aspects. (1) Enhancing the electron transfer efficiency for direct electron transfer (DET). For the specific strategies that can be taken,

refer to section 2.3.2. (2) Improving the affinity of oxidoreductases towards artificial electron mediators or changing the coenzyme preference from natural coenzymes to biomimetic coenzymes. Most artificial electron mediators are not the natural substrate of oxidoreductases. Improving the affinity towards artificial electron mediators (e.g., reducing the K_m value and increasing k_{cat} value) via protein engineering is conducive to enhance the electron transport efficiency and reduce the amount of artificial electron mediators used. The development of synthetic biomimetic nicotinamide coenzyme has led to a breakthrough not only in the field of biocatalysis but also in organic chemistry and for medicinal applications. Due to the high cost of natural nicotinamide cofactors[1073] and in the interest of bioorthogonality, the renewal of interest in biomimetic coenzymes in oxidoreductase-catalyzed reactions is showing a great promise.[1074] The application of cheaper and more stable biomimetic coenzymes would effectively reduce costs, simplify the product separation process, and extend the system operating time. Meanwhile, constructing enzyme-cofactor/mediator conjugates is also an effective approach to improve the electron transport efficiency.[1075] (3) Improving the stability of oxidoreductases used in enzymatic electrosynthesis systems. The poor stability of oxidoreductases due to deactivation results in short lifetimes and higher costs. Protein engineering is an effective and widely used method to improve the stability of different oxidoreductases.[1076] The oxidoreductases with high structural stability are able to remain active for a long time and finally can be used to improve the operational stability of enzymatic electrosynthesis systems.

5.3.2. The Combination of Microbial Electrosynthesis and Synthetic Biology. Synthetic biology is an interdisciplinary branch of biology, chemistry, and engineering that

combines the investigative nature of biology with the engineering design principles, ultimately giving the life forms new functions and traits.[1077] Currently, the research of synthetic biology has been focused on the design and construction of artificial biological pathways or the redesign and modification of natural biological systems for the effective production of new drugs, complicated natural products, biochemicals, and bioenergy.[1078] For *in vivo* synthetic biology systems, a critical issue is a balance between consumption and supply of reduced equivalents. Bioelectrochemical techniques offer a novel, efficient, and promising method to alleviate, and also to eliminate, the redox imbalances during the synthesis of target biochemicals and biofuels. Specifically, the reduced equivalents can be generated by the bioelectrochemical system and imported into the cell, therefore manipulating the redox balance of the cell. The additional reducing power available inside the cell is conducive to produce desired products.

To combine bioelectrochemical techniques and synthetic biology tools, the first strategy is to establish effective electrochemical communication between the electrode and the cells, especially for the non-electroactive microbes. In this area, some progress has been achieved. With the introduction of the Mtr pathway (Sections 2.4.1 and 2.4.2), engineered *E. coli* can be used as the electroactive chassis cells that are able to utilize the exogenous electrons from the electrode to catalyze target reactions.[237, 968] However, in these systems, the intracellular electron mediators are still the natural coenzymes ($NAD(P)^+$). The diversion of the coenzyme to the exogenous synthetic pathway can still disturb the redox balance inside the cell, which would slow down, or even stop, the entire system.[1079, 1080] One possible solution is to develop and integrate bio-orthogonal redox systems based on biomimetic coenzyme into the electroactive chassis cell with the expression of nucleotide

transporters.[1081] In detail, the coenzyme preference of the oxidoreductase that makes up the synthetic pathway can be reversed from a natural coenzyme to the biomimetic cofactor (e.g., nicotinamide mononucleotide, nicotinamide riboside, or nicotinamide cytosine dinucleotide) via protein engineering approaches.[1082] The biomimetic coenzyme can be transported into the cell by the nucleotide transporter, reduced by the exogenous electrons, and ultimately consumed by the synthetic pathway. The exogenous synthetic pathway is completely driven by exogenous electrons, which avoid the interference with the intracellular redox balance, thoroughly rid the exogenous synthetic pathway from dependence on natural coenzyme. It is more conducive to long-term cell survival and efficient production of target products. Another strategy is to integrate the synthetic pathway into native electrochemical cells. The study by Peidong Yang's group is an enlightening work (**Figure 30**).[710, 909] Namely, the acetate from CO_2 photoelectric CO_2 conversion system was utilized *in situ* by genetically engineered *E. coli* to achieve the conversion from CO_2 to n-butanol, polyhydroxybutyrate (PHB), and natural products. Herein, we can conceive that the conversion pathway of the produced acetate could be introduced into the electroactive microbial cell, rather than to employ supernumerary engineered *E. coli*. The generation of acetate could be converted to acetyl-CoA by acetyl-CoA synthetase inside the cell. With the role of nodes of acetyl-CoA in the metabolic network, the generated CO_2 could be converted to a variety of useful chemicals, for instance, glucose via gluconeogenesis, fatty acid via the reversed pathway of β-oxidation, or the isopentenyl pyrophosphate (the precursor of isoprenoids natural products) via mevalonate acid-dependent pathway. Based on this, it is expected to construct a real sense of the "artificial photosynthesis" system built on photoelectric conversion. All of these still

depend on the further developments of genetic manipulation toolkit for non-model microorganisms.

In the studies of metabolic engineering and synthetic biology, the detection of metabolic intermediates at the single-cell level, especially the intracellular concentration of unstable metabolic intermediates at the metabolic node, is critical to understand cellular function, monitor gene expression, identify the metabolic rate-limiting step, and the formulation of metabolic regulation strategy.[1083] At present, the *in vivo* real-time monitoring of metabolic intermediates at the single-cell level remains a challenge. The single-cell sensing based on the utilization of nanopipettes provides new possibilities for solving this problem. The nanopipette-based biosensors have been utilized for real-time sensing of the cellular processes and metabolic activities with minimal invasion via bioelectrochemical reactions and electron transfer processes under normal physiological conditions.[624-626] Moreover, the nanopipettes can also be explored as pressure-driven fluid manipulation tools for a reproducible sampling of nanoliter liquid volumes from living single cells. By integrating it with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS), intracellular metabolites have been characterized by high sensitivity.[1084] All these research advances are based on wild-type cells. It can be expected that the novel single-cell sensing technologies based on nanopipettes, described earlier, can be applied in the real-time monitoring of metabolically engineered cells modified using synthetic biology toolset. This combination will play a significant role in (1) assessing expression levels of heterologous proteins, (2) understanding cellular behaviors of engineered cells, and (3) formulating effective regulatory strategies.

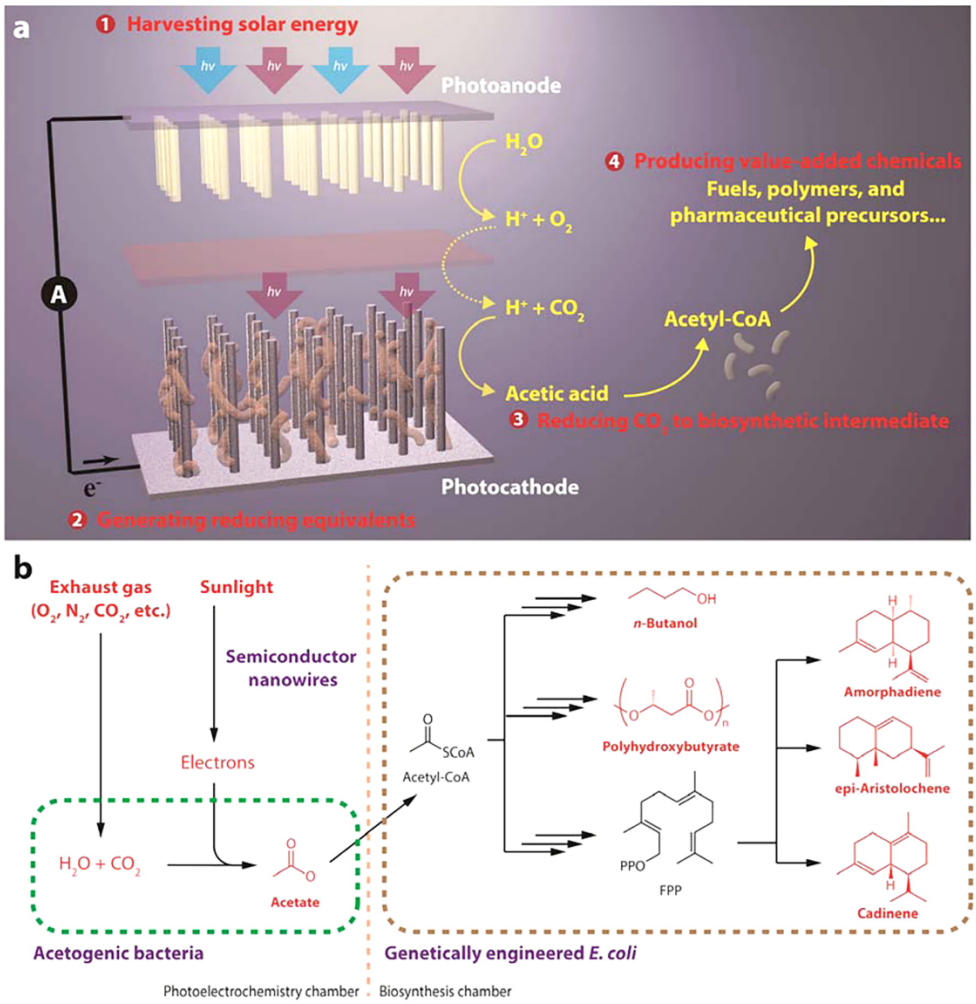

Figure 30. Schematics of a general artificial photosynthetic approach. (a) The proposed approach for solar-powered CO_2 fixation includes four general components: (1) harvesting solar energy, (2) generating reducing equivalents, (3) reducing CO_2 to biosynthetic intermediates, and (4) producing value-added chemicals. The integration of materials science and biology is an approach that combines the advantages of solid-state devices with living organisms. (b) As a proof of concept, it is demonstrated that, under mild conditions, sunlight can provide the energy to directly treat exhaust gas and generate acetate as the biosynthetic intermediate, which is upgraded into liquid fuels, biopolymers, and pharmaceutical precursors. For improved process yield, *S. ovata* and *E. coli* are placed in two separate containers. FPP: farnesyl pyrophosphate. Reprinted with permission from ref. 710. Copyright 2015 American Chemical Society.

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