

Review

Recent Progress in Applications of Enzymatic Bioelectrocatalysis

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Abstract: Bioelectrocatalysis has become one of important research fields in electrochemistry and provided a firm base for an important technology for application to various bioelectrochemical devices such as biosensors, biofuel cells, and biosupercapacitors. The understanding and technology in bioelectrocatalysis have been greatly improved by introducing nanostructured electrode materials and protein-engineering methods over the last few decades. Recently, the electro-enzymatic production of renewable energy resources and useful organic compounds (bioelectrosynthesis) also attracts worldwide attention. In this review, we summarize recent progress in applications of enzymatic bioelectrocatalysis.

Keywords: bioelectrocatalysis; nanostructured electrodes; protein engineering; bioelectrosynthesis; photo-bioelectrocatalysis

1. Introduction

Oxidoreductases catalyze redox reactions between two sets of redox substrate couples, and are expected as industrially-useful catalysts thanks to their high activities and substrate specificities under mild conditions (room temperature, normal pressure and neutral pH). However, most of oxidoreductases other than nicotinamide cofactor (NAD(P))-dependent enzymes show low substrate specificities for one of the substrates. Such redox enzymes can accept or donate electrons from or to electrodes directly or via some artificial redox mediators. The coupled reaction is called bioelectrocatalysis; the catalytic function of the redox enzymes provides a variety of specific and strong catalytic activity to non-specific electrode reactions. [Bartlett2008, Wilson2008, Heller1992, Kano2019, Scheller1987]. Bioelectrocatalysis is a firm base for characterizing the redox enzyme reactions but also for applying the concept and the related technologies to useful bioelectrochemical devices such as biosensors [Thévenot1999, Martinkova2017, Bollella2018a, Kucherenko2019, Nguyen2019, Pinyou2019, Bollella2020, Wilner1997, Scheller1989], biofuel cells [de Poulpiquet2014, Pinyou2019, Barton2014, Cracknell2008, Meredith2012, Mazurenko2017, Mano2018, Xiao2019a], biosupercapacitors [Shleev2017], and other bioreactors [Krieg2014].

Bioelectrocatalytic reactions are classified into two types according to the mode of the electron transfer described above: direct electron transfer (DET) and mediated electron transfer (MET), as shown in Figure 1. These reactions proceed in the following schemes in the simple case of the substrate oxidation.

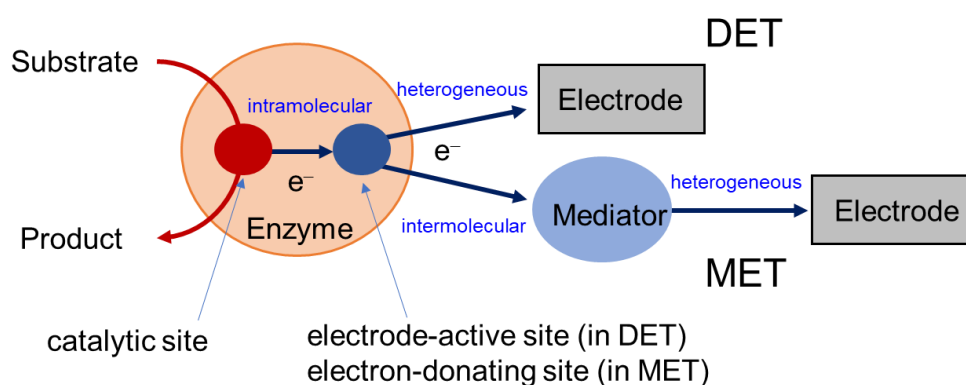
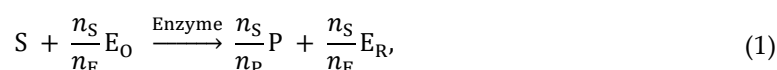


Figure 1. Schematic of electron transfer processes in DET- and MET-type bioelectrocatalysis for substrate oxidation. In this scheme, the enzyme is assumed to have both catalytic and electron-donating sites in the molecules. Some redox enzymes that have the catalytic site alone (without any other redox site (as electron-donating site)) are also able to show DET-type bioelectrocatalysis.

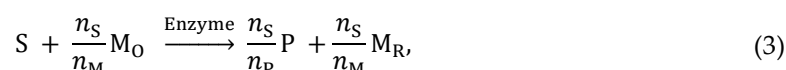
In DET:



and



In MET:



and



where S, P, E, and M indicate a substrate, a product, an enzyme, and a mediator, respectively. n_X is the number of electrons of chemical species X. X_O and X_R are the oxidized and reduced forms of X, respectively. In DET-type bioelectrocatalysis, it is easy to construct relatively simple systems with minimum overpotentials in the electron transfer between the electrode-active redox center of an enzyme and an electrode from the thermodynamic viewpoints. However, redox enzymes that are able to DET-type reactions are limited in number, because the electrochemical communication between an enzyme and an electrode occurs only when the electrode-active cofactor of the enzyme is close in distance to the electrode surface [Léger2008, Milton2017a, Adachi2020, Kitazumi2020]. In MET-type bioelectrocatalysis, on the other hand, a variety of enzymes can be used in principle, and suitable selection of mediators makes it possible to construct realistic systems. In addition, once both enzymes and mediators are stably immobilized on electrodes, the measurement systems work as pseudo-DET-type systems [Nygen2019, Pinyou2019, Gorton1992]. Particularly, redox polymers anchoring osmium complexes [Heller1992, Degani1989, Ohara1993, Timur2006, Nikitina2007, Nieh2013a, Alsaoub2019, Tsujimura2020, Xiao2019b], ferricyanide [Čnas1983, Nieh2013b, Nieh2013c], metallocenes [Merchant2010, Tran2011, Milton2016, Lee2020], and viologen units [Eng1994, Shiraiwa2018, Sakai2016a, Sakai2017a] are constructed as polymeric mediators

immobilized on electrodes. In summary, DET-type system is often more ideal than MET-type one, while it seems to be practical to utilize MET-type one in several objectives.

The main generation sources of the overpotential in bioelectrocatalysis are 1) the difference in the formal potential between the substrate and the electron-donating site and 2) slow kinetics in the heterogeneous electron transfer (Figure 1). There is no way to avoid the problem concerning the first issue, as long as one may utilize natural enzymes. Slow kinetics in heterogeneous electron transfer is compensated by so-called overpotential of electrode in DET-type reaction and by the large driving-force (that is, increased difference in the formal potential) between the enzyme and the mediator. In this review, we will describe several techniques for improving the performance of enzymatic bioelectrocatalytic systems and summarize recent studies on their applications.

2. Tuning of nanostructured electrodes and protein-engineering of redox enzymes

2.1. Electrode nanomaterials

In DET-type and enzyme/mediator-immobilized MET-type bioelectrocatalyses, immobilization of as many amounts of enzymes and mediators as possible on the electrode surface is, in principle, able to increase the current density of bioelectrocatalytic systems. Therefore, nanostructured electrodes with a large ratio of the effective surface area to the projective one are useful and frequently utilized for bioelectrocatalytic systems. In addition, it is suggested that the heterogeneous electron transfer kinetics at the top edge of microstructures of these electrodes is accelerated by the electric field strengthened by the expansion of the electric double layer [Kitazumi2013] and the charge accumulation as expected by Poisson equation [Kano2019]. This effect is very useful to decrease the overpotential due to the heterogeneous electron transfer in the DET-type reaction.

Electrode nanomaterials utilized in bioelectrocatalyses are roughly divided into two classes: carbon and metal. Carbon nanomaterials such as carbon nanotubes [Serafin2013, Holzinger2012, Tominaga2015], carbon blacks [Tsujimura2009], carbon cryogels [Flexer2011, Hamano2012], and MgO-templated carbons [Murata2014, Tsujimura2020, Mazurenko2018] have properties to physically adsorb a large amount of enzymes and mediators at hydrophobic sites, and are generally used as platforms favorable for bioelectrocatalyses. On the other hand, nanoporous gold constructed by anodization [Sakai2018, Takahashi2019a, Mie2019, Miyata2020] or dealloying [Siepenkoetter2016, Siepenkoetter2017, Xiao2018a] and metallic nanoparticles of gold [Serafin2013, Holland2011, Gutiérrez-Sánchez2012, Suzuki2012, Monsalve2015, Sakai2017b, Takahashi2019b, Hitaishi2020, Kizling2018a], silver [Murata2009, Zhang2018a, Zhang2018b], platinum [Yehezkeili2010, Trifonov2019, Adachi2020b], titanium oxide (TiO₂) [Zhang2018a, Zhang2018b], iron oxide (Fe₂O₃) [Nakamura2010, Kizling2018b], and indium tin oxide (ITO) [Rozniecka2011] are also widely used. Compared to carbon nanomaterials, the pore and particle sizes of metallic nanomaterials can be easily controlled according to several manufacturing methods.

Furthermore, nanostructured electrodes exert another positive effect on DET-type bioelectrocatalysis from the viewpoint of the enzyme orientation. The limited catalytic current density (j_{cat}) in DET-type reactions is expressed by Equation (5):

$$j_{\text{cat}} = \pm n_{\text{E}} F k_{\text{c,DET}} \Gamma_{\text{E,eff}} \quad (5)$$

where F is the Faraday constant, $k_{\text{c,DET}}$ is the catalytic constant in DET-type bioelectrocatalysis ($k_{\text{c,DET}} = (n_{\text{S}}/n_{\text{E}})k_{\text{c,DET(1)}}$, $k_{\text{c,DET(1)}}$ being the catalytic constant for single turnover of the enzyme), and $\Gamma_{\text{E,eff}}$ is the surface concentration of the effective enzyme. Here, the adjective “effective” means

being able electrochemically to communicate with electrodes. In other words, enzymes of which the electrode-active sites face away from the electrode are not included, because the long-range electron transfer kinetic constant (k°) exponentially decreases with an increase in the distance between the electrode surface and the electrode-active site in an enzyme (d) [Moser1992, Marcus1985, Marcus1993], as described by Equation (6):

$$k^\circ = k^\circ_{\max} \exp(-\beta d), \quad (6)$$

where k°_{\max} is the rate constant at the closest approach ($d = d_{\min}$) and β is the decay coefficient. Based on the random orientation model in which enzymes are assumed to be randomly oriented on electrodes [Léger2008, Sugimoto2016, Sugimoto2017], the enzymes can penetrate into nanostructured electrodes with mesopores, and the mesoporous electrodes can adsorb a large amount of enzymes suitable for DET-type reactions compared with planar electrodes, as illustrated in Figure 2. This is called curvature effect of mesoporous electrodes for DET-type bioelectrocatalysis and is also very effective to decrease the overpotential of DET-type bioelectrocatalysis. In practical applications, it is necessary to select and optimize electrode nanomaterials based on the estimated size, shape, and hydrophobicity of enzymes.

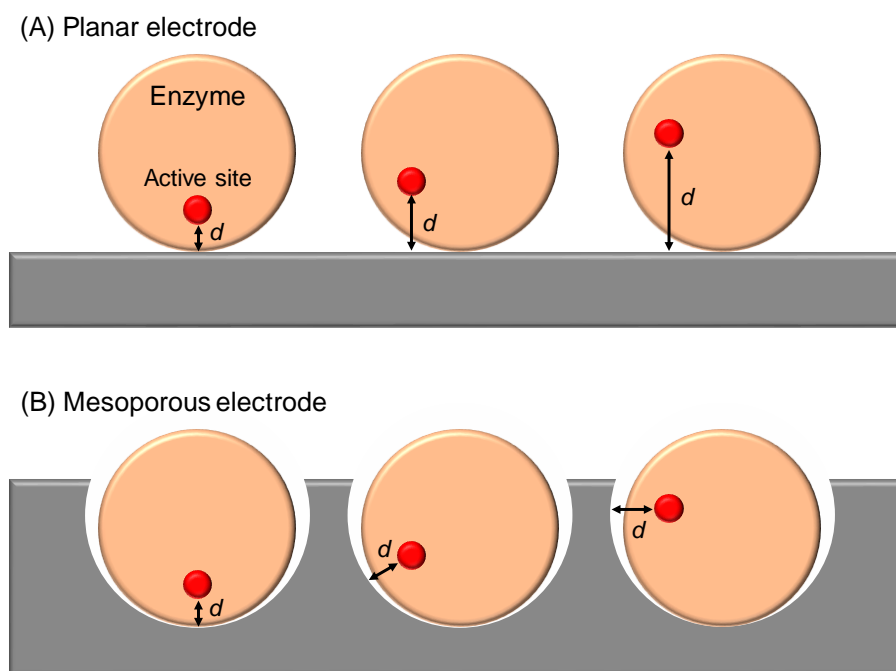


Figure 2. Schematic of enzyme orientations at (A) planar and (B) mesoporous electrodes.

On the other hand, chemical modifications of electrode surfaces are very effective to control the enzyme orientation by electrostatic or other specific interactions between the electrode-active site and the electrode surface, and therefore they are useful to increase the population of the enzyme orientations with minimum distances between the electrode-active site and the electrode surface [Hitaishi2018]. Such favorite orientations also contribute to decrease the overpotential in DET-type reaction. For example, [NiFe]-hydrogenase (H₂ase) from *Desulfovibrio vulgaris* Miyazaki F and copper efflux oxidase (CueO) expressed in *Escherichia coli* showed strong activity of DET-type bioelectrocatalysis at (positively charged) *p*-phenylenediamine-functionalized Ketjen Black-modified glassy carbon electrodes compared with Ketjen Black-modified electrode without any chemical functionalization and artificially introduced charged groups; the surfaces near the electrode-active sites of the enzymes are estimated to be negatively charged and the attractive

electrostatic interaction with positively charged electrode surfaces increases the probability of enzyme orientations favorable for the interfacial electron transfer [Xia2016a, Adachi2020c].

In contrast, bilirubin oxidase (BOD) from *Myrothecium verrucaria* showed strong DET-type bioelectrocatalytic activity at negatively charged electrodes; the surface near the electrode-active site of the enzyme is positively charged and the electrostatic interaction with negatively charged electrodes increases the probability of favorable orientations of the enzyme [Xia2016b]. On the other hand, DET-type bioelectrocatalysis of BOD was also improved by modifying an electrode with bilirubin (as the natural electron donor) which seemed to attractively interact with the electrode-active type I copper site as the electron-accepting site. The interaction increases the probability of favorable orientations of the enzyme [So2014]. In addition, membrane-bound D-fructose dehydrogenase (FDH) from *Gluconobacter japonicus* NBRC3260 showed strong DET-type bioelectrocatalytic activity at a 4-mercaptophenol-modified porous gold electrode, probably due to the stabilization of the enzyme layer by the hydroxy groups of 4-mercaptophenol [Bollella2018b]. FDH also showed the attractive and specific interaction with methoxy-substituents on the electrode surface, which resulted in the favorable orientation [Xia2016c]

In addition, gas-diffusion bioelectrodes are effective for enzymatic bioelectrocatalyses in which gaseous substrates such as dihydrogen, dioxygen, and carbon dioxide were used [So2017]. Since these gasses have low water solubility, the bioelectrocatalytic currents are often controlled and limited by diffusion processes of the substrates at usual electrodes. On the other hand, gas-diffusion bioelectrodes realize direct supplies of gaseous substrates from the gas phase to the reaction layer thanks to their suitable conductivity, hydrophobicity/hydrophilicity balance, and gas permeability, as illustrated in Figure 3.

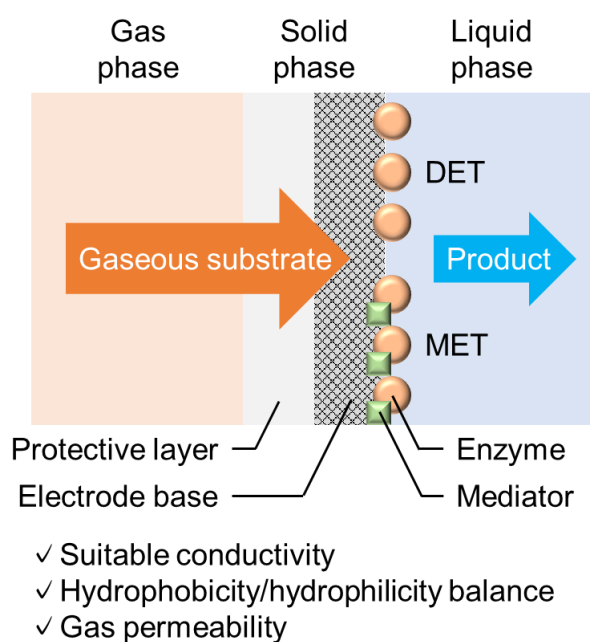


Figure 3. Schematic of a bio-three-phase interface of a gas-diffusion bioelectrode.

2.2. Protein-engineering approaches

Bioelectrocatalysis can be improved not only by functionalizing electrodes but also by engineering enzymes. Based on the crystal structure of enzymes, various mutations can be introduced into redox enzymes by protein-engineering methods [Xiao2019a, Wong2003].

2.2.1. Formal potential shift of electrode-active sites

The negative shift of the formal potential of the electron-donating site for the substrate oxidation (vice versa for the substrate reduction) by mutation is very effective to reduce the thermodynamic overpotential in the intramolecular electron transfer in redox enzymes having multi-redox sites (Figure 1). The formal potentials of metallic cofactors such as hemes and copper clusters are predominantly controlled by coordinated amino acid residues. Particularly, the point mutation of the axial ligands of hemes and blue copper clusters can easily tune the formal potentials of them. The direction of the potential shift depends in part on the electron-donating/accepting characteristics of the mutated amino acid side chains [Battistuzzi2002, Li2004]. Briefly in general, an axial ligand with a relatively strong electron-donating character shifts the formal potential of the cofactor in the negative potential direction due to the stabilization of the oxidized state of the cofactor, and vice versa. The formal potentials of redox enzymes are also greatly interfered by conformational changes caused by mutated amino acid residues.

FDH is a heterotrimeric enzyme composed of a flavin adenine dinucleotide (FAD)-containing large catalytic subunit, a three-heme *c* (called hemes 1*c*, 2*c* and 3*c* from N-terminus)-containing cytochrome subunit, and a small subunit, and proceeds a DET-type reaction by transferring electrons from FAD, heme 3*c*, heme 2*c*, to an electrode in this order [Kawai2014, Hibino2016, Adachi2019a]. Then, the sixth axial ligand (methionine 450) of heme 2*c* was replaced by glutamine having electro-donating characteristics to shift the formal potential of heme 2*c* in the direction of the negative potential. The FDH variant (M450Q_FDH) gave DET-type bioelectrocatalytic waves for the oxidation of fructose at a half-wave potential of approximately 50 mV more negative than that of the recombinant native enzyme, as shown in Figure 4A [Hibino2016, Adachi2019a]. Drastic change in the limiting catalytic current was not observed. This suggests that the rate constant of the intramolecular electron transfer from FAD to heme 2*c* in the enzyme is sufficiently large compared with the catalytic reaction rate constant at the catalytic center (FAD); the latter predominantly determines $k_{c,DET}$ in Equation (5).

On the other hand, such replacement is also effective for the axial ligand of the electron-accepting site (type I blue copper site) to negatively shift the formal potential. Replacement of the axial ligand methionine 467 in type I copper site of BOD with glutamine (M467Q_BOD) caused a large negative shift (approximately 0.23 V) in the half-wave potential of the DET-type bioelectrocatalytic waves. This means an increase in the overpotential in the intramolecular electron transfer in the enzyme. Fortunately, in this case, the catalytic limiting current density increased compared with that of the recombinant native BOD, as shown in Figure 4B (Note here that the catalytic wave in Figure 4B is illustrated for substrate oxidation) [Kamitaka2007]. Most probably, the catalytic rate constant of the dioxygen reduction at the type II/III catalytic site is sufficiently large compared with the rate constant of the intramolecular electron transfer from the electron-accepting type I site to the dioxygen-reducing type II/III site; the intramolecular electron transfer is the rate determining step to determine $k_{c,DET}$ in Equation (5). In addition, the electron transfer kinetics seems to obey the linear free energy relationship (LFER); the increase in the formal potential difference between type I and II/III sites (that is, the driving force of the reaction) results in an increase in the intramolecular electron transfer rate constant.

Even in MET-type bioelectrocatalysis, the redox potential shift of the electron-donating site (for substrate oxidation) can tune the overpotential in the intramolecular electron transfer process. In

addition, the intermolecular electron transfer kinetics between the electron-donating site in the enzyme and the mediator seems to be improved in theory by the potential shift mutation of the electron-donating site (for substrate oxidation) on the basis of the concept of LFER, though there is no report on this matter. Therefore, the mutational tuning of the redox potential of the electron-donating site (for substrate oxidation) may also expand the strategy of the mediator selection for MET-type bioelectrocatalysis.

2.2.2. Downsizing

As described by Equation (5), an increase in $\Gamma_{E,eff}$ is essential to getting a large bioelectrocatalytic current density. Then, $\Gamma_{E,eff}$ can be increased by downsizing enzymes from which the regions not deeply involved in bioelectrocatalysis are deleted, as shown in Figure 4C.

Downsizing effects were also investigated in FDH. Heme 1c of FDH is suggested to be uninvolved in DET-type reaction, and the downsized FDH without the heme 1c region ($\Delta 1c_FDH$) showed a larger DET-type bioelectrocatalytic current density than the recombinant native FDH (r_FDH) [Adachi2019a, Hibino2017]. In addition, further a downsized FDH without the heme 1c and 2c regions ($\Delta 1c2c_FDH$) also showed DET-type bioelectrocatalytic activity with reduced overpotential due to direct electrical communication between an electrode and heme 3c with the most negative formal potential [Adachi2019a, Kaida2019]. However, while $\Gamma_{E,eff}$ was suggested to be increased, j_{cat} of $\Delta 1c2c_FDH$ was as large as that of r_FDH (smaller than that of $\Delta 1c_FDH$), which seemed to be ascribed to a decrease in an enzymatic activity ($\approx k_{c,DET}$) of $\Delta 1c2c_FDH$ by excessive deletion [Adachi2019a, Kaida2019]. Thus, it is important to avoid conformational changes due to deletion and to retain an enzymatic activity as high as possible. Furthermore, a double mutant of downsizing and the potential shift (M450Q $\Delta 1c_FDH$) accomplished both an increase in j_{cat} and an overpotential reduction [Adachi2019a, Hibino2019, Kaida2020].

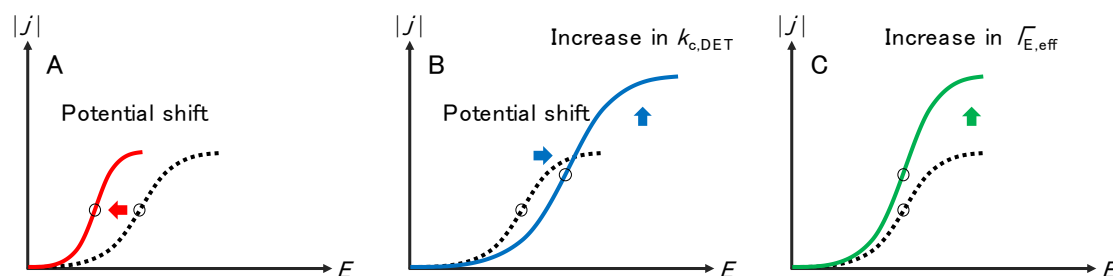


Figure 4. Illustrative drawing of the effect of mutations on steady-state bioelectrocatalytic wave in DET-type bioelectrocatalysis of “substrate oxidation”. (A) Negative potential shift in the formal potential of the electron-donating site of the DET-type redox enzyme reactions, in which the intramolecular electron transfer is not the rate determining process in the enzymatic catalytic reaction. (B) Positive potential shift in the formal potential of the electron-donating site of the DET-type redox enzyme reactions, in which the intramolecular electron transfer is the rate determining process in the enzymatic catalytic reaction. , and (C) downsizing without any change in the intrinsic enzymatic activity.

2.2.3. Surface amino acid mutation

It is desirable to tightly immobilize enzymes on electrodes in both DET- and MET-type bioelectrocatalytic reactions. Cross-linkers such as glutaraldehyde [Matsui2017], carbodiimide [Yehezkeili2010], and maleimide [Holland2011] are often used for covalent immobilization of enzymes [Mazurenko2020]. Mutation of amino acid residue(s) located on the enzyme surface also enhances the cross-coupling reactions and can control the orientation of the enzyme on suitable electrode surface. For example, cysteine introduction onto the enzyme surface close to the electrode-active site can increase the enzyme orientations suitable for DET-type reactions at thiol- and

maleimide-functionalized electrodes by forming (di)sulfide bonds. Holland et al. reported DET-type bioelectrocatalysis of cysteine-introduced glucose oxidase (GOD) conjugated with maleimide-modified gold nanoparticles on a gold electrode [Holland2011]. In addition, Ferapontova revealed that cysteine mutation of horseradish peroxidase (HRP) was effective for its orientation on gold electrodes to improve DET-type bioelectrocatalytic property [Ferapontova2002].

2.2.4. Fusion protein

DET-type bioelectrocatalysis is sometimes achieved by introducing an electrode-active domain into a native enzyme. Cellobiose dehydrogenase (CDH) is often used as a model of DET-type fusion enzymes. CDH has two domains: a larger catalytic dehydrogenase domain and a smaller electrode-active cytochrome domain. The domains are linked by a flexible polypeptide [Bollella2017]. The fused cytochrome domain mediates the electron transfer from the catalytic domain to electrodes, like a “built-in mediator.” Utilizing the fusion protein engineering methods, DET-type reactions by FAD-dependent glucose dehydrogenase (FAD-GDH) [Algov2017, Ito2019], pyrroloquinoline quinone (PQQ)-dependent GDH [Okuda2004], and flavodoxin [Gilardi2002] were reported.

3. Novel bioelectrochemical system

Major applications in enzymatic bioelectrocatalysis are biosensors and biofuel cells. Biosensors are utilized as analytical devices for food analyses and clinical diagnoses [Thévenot1999, Martinkova2017, Bollella2018a, Kucherenko2019, Ngyen2019, Pinyou2019, Bollella2020, Wilner1997, Scheller1989], and biofuel cells are ecofriendly energy conversion devices in which chemical energy of fuels are electrically extracted [de Poulpiquet2014, Pinyou2019, Barton2014, Cracknell2008, Meredith2012, Mazurenko2017, Mano2018, Xiao2019a]. Characteristics, properties, and progresses of these devices were previously summarized in the corresponding reviews cited above. Recently, in addition, novel methods for bioelectrocatalytic applications are reported as described below.

3.1. Biosupercapacitor

Biosupercapacitors are self-powered energy storage devices using bioelectrocatalysis to charge capacitors [Shleev2017]. In the biosupercapacitor, the electric power is generated by a biocathode and/or a bioanode. The fundamental difference between biosupercapacitors and biofuel cells is the separation of the output of the current and the bioelectrocatalytic reaction. Expressed as an equivalent circuit, biosupercapacitor is a biofuel cell and a capacitor connected in parallel. Regardless of the connection to the external circuit, the bioelectrocatalytic reaction injects the electrons into the supercapacitor. When the external circuit requires the current, a biosupercapacitor provides the current. An advantage of biosupercapacitors is that a large current can flow even if the kinetics of the bioelectrocatalysis is inadequate. Additionally, biosupercapacitors are compatible with the self-powered biosensors [Hanashi2009, Conzuelo2018].

The supercapacitor is classified into two types: the electrical double layer capacitor (EDLC) and the electrochemical pseudo capacitor (EPC). EDLC is the electrode having a large surface area, the electric charge is accumulated in the electrical double layer at the interface between the electrode and the electrolyte solution. EPC is constructed with the electrode and the reversible electrode-active redox species. The externally applied voltage shifts the electrode potential at an anode and a cathode in the negative and positive directions, respectively, as expressed in the Nernst equation.

Generally, redox active species are immobilized at the electrode surface. Since an electrochemical capacitor contains two electrodes and an electrolyte solution, electrochemical capacitors are also classified into three types: EDLC-EDLC, EPC-EPC, and EDLC-EPC [Falk2019].

Therefore, biosupercapacitors are basically classified into the three types. The EDLC is compatible with the DET-type bioelectrocatalysis and the MET-type bioelectrocatalysis is adapted to the EPC. In order to avoid the mixing of mediators, EPCs in the biosupercapacitor are frequently employed redox polymers as a mediator to immobilize at the electrode surface.

Electrochemically inactive porous electrodes in an electrolyte solution work as the EDLC. Additionally, DET-type bioelectrocatalytic activity is required for the EDLC-type biosupercapacitor. The reported materials suitable for the EDLC and the bioelectrode are carbon nanotubes [Agnes2014, Pankratov2014a, Pankratov2014b], porous gold [Xiao2018b], gold nanoparticles [Pankratov2018], and indium tin oxide nanoparticles [Bobrowski2017]. Porous electrodes often show high activity for the DET-type bioelectrocatalysis. Therefore, it is considered that the investigation in bioelectrodes for the DET-type biofuel cells will be directly useful for the biosupercapacitor.

Separation of oxidant and reductant is important in EPCs, since the mixing of the two species causes the cross-reaction discharge. Therefore, in the case of biosupercapacitors using the MET-type bioelectrocatalysis, a careful attention is required to head off the outflow of mediators. As mentioned above, redox polymers are employed as a mediator in biosupercapacitors [Pankratov2016, Alsaoub2017, Alsaoub2019, Zhao2019,]. Immobilization of redox active proteins at the electrode surface have been investigated for the improvement of the capacitance of EPC-type biosupercapacitors [Gonzalez-Arribas2018, Shen2019].

3.2. Bioelectrosynthesis

Bioelectrosynthesis is a coined term for generation of renewable energy resources and useful organic compounds [Wu2020]. Particularly, stereoselective enzymatic bioelectrocatalysis is desirable for a pharmaceutical use because pharmaceutical precursors are required to have high enantiomeric purity [Schmid2001, Zaks2001].

Some useful compounds are reduced forms of redox couples, and the reductive conversion is often the opposite reaction to that in metabolisms in vivo. In bioelectrocatalysis, on the other hand, there are several enzymes that can proceed bidirectional electro-enzymatic reactions (both the oxidation and reduction of substrate redox couples), in which the driving forces are reversed depending on pH. H₂ase [Vincent2007, Armstrong2011], formate dehydrogenase (FoDH) [Sakai2017b, Sakai2015, Armstrong2011, Reda2008, Bassegoda2014], carbon monoxide dehydrogenase (CODH) [Armstrong2011], diaphorase (DI) [Armstrong2011, Takagi1998], ferredoxine-NADP⁺ reductase (FNR) [Siritanaratkul2017], and some NAD(P)⁺-dependent dehydrogenases [Takagi1998, Siritanaratkul2017, Megarity2019, Morello2019, Alkotaini2018] show bidirectional bioelectrocatalytic activities. The characteristic of these enzymes is that the formal potentials of the substrate redox couples and the cofactors in the enzymes are relatively close to each other, and thus, the small up-hill intramolecular electron transfer proceeds in acceptable velocity [Adachi2020a]. Bidirectional bioelectrocatalyses are key reactions for constructing bioelectrochemical energy/compound conversion systems.

There are mainly two types of bioelectrosynthetic systems: a fuel cell-type [Morello2019, Wan2018, Wan2019, Lazarus2009, Adachi2018, Milton2017b, Chen2020] and an electrolysis-type [Shiraiwa2018, Siritanaratkul2017, Megarity2019, Alkotaini2018, Sakai2016b, Fukuda2008, Chen2019, Dong2020, Hickey2019]. The former realizes a spontaneous production of compounds without any external power supplies, while the latter proceeds relatively rapid reactions thanks to an optimally controlled electrode potential to realize diffusion-controlled conditions. We will show examples of bioelectrosyntheses in the following sections.

3.2.1. Dihydrogen production

Dihydrogen (H_2) is a typical clean energy which emits no harmful products in combustion. H_2 ase, a common anodic bioelectrocatalyst for H_2 oxidation, can also catalyze proton (H^+) reduction [Vincent2007, Armstrong2011]. The reversible reaction is biased to favor H_2 oxidation, and it is difficult to realize a large cathodic current density of H^+ reduction in DET-type system. Then, H^+ reduction with a large current density was achieved in MET-type system of H_2 ase using a viologen polymer as a redox mediator [Shiraiwa2018]. On the other hand, a dual DET-type bioelectrocatalytic water-gas shift reaction using CODH and H_2 ase ($H_2O + CO \rightarrow H_2 + CO_2$) was reported [Lazarus2009]. The system requires no external power supplies.

3.2.2. Formate production

Formate/formic acid ($HCOO^-/HCOOH$) is a liquid energy resource with a high energy density due to its property of being completely oxidized to CO_2 . FoDH interconverts $HCOO^-$ and CO_2 both in DET- and MET-type systems [Sakai2017b, Sakai2015, Armstrong2011, Reda2008, Bassegoda2014]. Sakai et al. reported efficient bioelectrocatalytic CO_2 reduction using FoDH from *Methylobacterium extorquens* AM1 and a synthesized redox mediator with the low formal potential on a gas-diffusion electrode [Sakai2016b]. In addition, spontaneous interconversion between $HCOO^-$ and H_2 without any external power supplies was achieved by coupling two bioelectrocatalyses by FoDH and H_2 ase ($H_2 + CO_2 \rightarrow HCOO^- + H^+$) [Adachi2018]. The direction of the reaction is controlled by pH- and concentration-dependent equilibrium potentials of the $2H^+/H_2$ and $CO_2/HCOO^-$ couples.

3.2.3. Ammonia production

Ammonia (NH_3) is an industrially beneficial compound used as a chemical raw material, a fuel, a hydrogen storage, and so on [Smith2002, Milton2017c]. Nitrogenase (N_2 ase) is an enzyme that catalyzes a nitrogen fixation from dinitrogen (N_2) to NH_3 using ATP hydrolysis energy in microorganisms under an anaerobic condition [Smith2002, Milton2017c]. Particularly, molybdenum-dependent N_2 ase, which comprises a catalytic MoFe protein and a homodimeric MgATP-binding Fe protein, has been mainly investigated in view of bioelectrocatalysis [Milton2017c, Cai2018]. N_2 ase reduces not only N_2 but also nitrite (NO_2^-) and azide (N_3^-) to NH_3 on electrodes, and the MoFe protein disassociated with the Fe protein shows the DET-type bioelectrocatalytic activity without ATP [Hickey2019]. Furthermore, ATP-independent NH_3 bioelectrosynthesis was improved in the MET-type system using N_2 ase and a cobaltocene-functionalized polymeric mediator [Lee2020]. On the other hand, Milton et al. reported a NH_3 -producing H_2/N_2 biofuel cell using MET-type reactions of N_2 ase and H_2 ase [Milton2017b]. If the fatal weakness of N_2 ase, a lack of oxygen tolerance, is improved, N_2 ase will be expected to have further industrial applications.

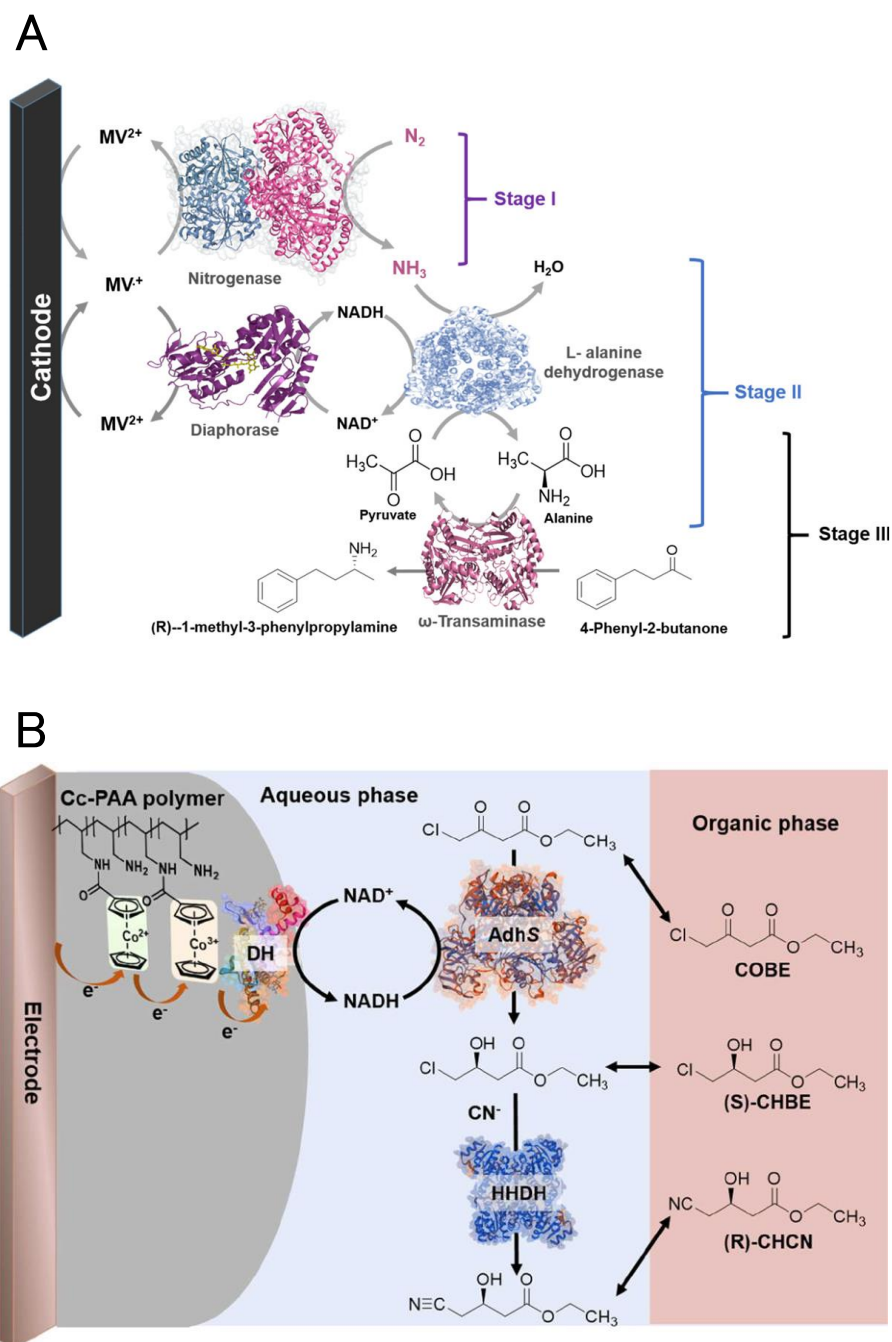


Figure 5. Schematic representation of multi-enzymatic bioelectrosyntheses of (A) chiral amine and (B) β -hydroxy nitrile. Abbreviations; DH: diaphorase, AdhS: alcohol dehydrogenase, HDDH: haloalcohol dehydrogenase, COBE: 4-chloroacetoacetate, CHBE: 4-chloro-3-hydroxybutanoate, CHCN: ethyl-4-cyano-3-hydroxybutyrate. Reprinted from Ref. [Chen2019, Dong2020], Copyright (2019,2020), with permission from ACS Publications.

3.2.4. NAD(P)⁺-dependent bioelectrosynthesis

Nicotinamide coenzymes (NAD(P)⁺/NAD(P)H) play essential roles in function of many NAD(P)⁺-dependent dehydrogenases, but the dehydrogenases need suitable redox mediators in single-step enzymatic bioelectrocatalysis, because the NAD(P)⁺/NAD(P)H interconversion requires high overpotentials at conventional electrodes due to its hydride ion transfer-type characteristics which are completely different from two-step single electron transfer-type electrode reaction. On

the other hand, introduction of DI and FNR, which are bioelectrocatalysts for the interconversion of the NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ redox couples, respectively, can realize various NAD(P)^+ -dependent bioelectrocatalyses with relatively low overpotentials [Takagi1998, Siritanaratkul2017].

The bi-enzymatic MET-type system of NAD^+ -dependent dehydrogenase and DI is kinetically and thermodynamically investigated and often applied to biosensors [Nikitina2007, Takagi1998, Antiochia2002, Lobo1997]. In addition, the coulometric electrooxidation of organic substances was achieved in the same multi-enzymatic system mimicking the tricarboxylic acid cycle [Fukuda2008]. On the other hand, reductive production of chiral compounds is useful in many cases. Minteer's group reported various NAD^+ -dependent multi-enzymatic cascade reactions for generating chiral compounds, as shown in Figure 5 [Chen2020, Chen2019, Dong2020]. They also demonstrated bioelectrosynthesis of polyhydroxybutarate with NADH regeneration by DI [Alkotaini2018].

In NADP^+ -dependent system, on the other hand, FNR shows the DET-type bioelectrocatalytic activity [Siritanaratkul2017]. Particularly, Armstrong's group has investigated NADP^+ -dependent bi-enzymatic bioelectrocatalysis using FNR. They reported reductive carboxylation of pyruvate to malate by malate dehydrogenase [Morello2019], reductive amination of 2-oxoglutamate to L-glutamate by glutamate dehydrogenase [Siritanaratkul2017, Wan2018], and enantioselective interconversion of the secondary alcohol/ketone couple by engineered alcohol dehydrogenase [Megarity2019, Wan2019].

3.3. Photo-bioelectrocatalysis

Photo-bioelectrocatalysis enables reducing a substrate with the lower formal potential by oxidizing a sacrificial reagent with a higher formal potential, which cannot spontaneously occur without solar energy. Electrons that photosensitizers accept from sacrificial reagents are excited by solar energy, and the electric potential is shifted in the negative direction, corresponding to the wavelength of the adsorbed light. Then, the electrons are donated to substrates via enzymes and mediators, as shown in Figure 6. Photosensitizers such as TiO_2 [Zhang2018a, Zhang2018b, Sokol2018a, Sokol2018b, Riedel2019], PbS quantum dots [Riedel2019], silver nanoclusters [Zhang2018a, Zhang2018b], and organic dyes [Sokol2018a, Sokol2018b] are incorporated in anodes of transparent electrode bases (ITO in general) with or without other catalysts. Particularly, in addition, the photosystem II (PSII) complex in the thylakoid membrane of cyanobacteria and higher plants is often used as a water-splitting anodic photo-bioelectrocatalyst [Sokol2018a, Sokol2018b, Riedel2019, Adachi2019b, Pankratov2017, Rasmussen2014a, Efrati2013, Kirchhofer2015, Pankratova2017, Yehezkeli2012, Calkins2013, Tsujimura2001, Mimcault1989, Carpentier1989, González-Arribas2017, Zhao2019, Rasmussen2014b, Saboe2016, Kanso2018, Hamidi2015, Hasan2017, Takeuchi2018, Hasan2014]. Bio-solar cells [Adachi2019b, Pankratov2017, Rasmussen2014a, Efrati2013, Kirchhofer2015, Pankratova2017, Yehezkeli2012, Calkins2013, Tsujimura2001, Mimcault1989, Carpentier1989] and solar biosupercapacitors [Pankratov2017, Pankratova2017, González-Arribas2017, Zhao2019], using PSII/I, thylakoid membranes, or cyanobacteria in anodes, and BOD or laccase in cathodes, realized the conversion from solar to electric energy without any sacrificial reagents in total. On the other hand, photo-bioelectrosyntheses, which is also called artificial photosyntheses, are reported. H_2 is generated by H_2 ase [Zhang2018a, Sokol2018a], and CO_2 is fixed by FoDH and CODH [Zhang2018b, Sokol2018b]. These cathodic reactions proceed at quite low potentials. Thus, in order to improve the Faradaic efficiency of these photo-bioelectrosyntheses, it is essential to reduce electron leakage to dissolved oxygen as much as possible, especially when oxygen-generating photosynthetic proteins or organisms are used as anodic photo-bioelectrocatalysts.

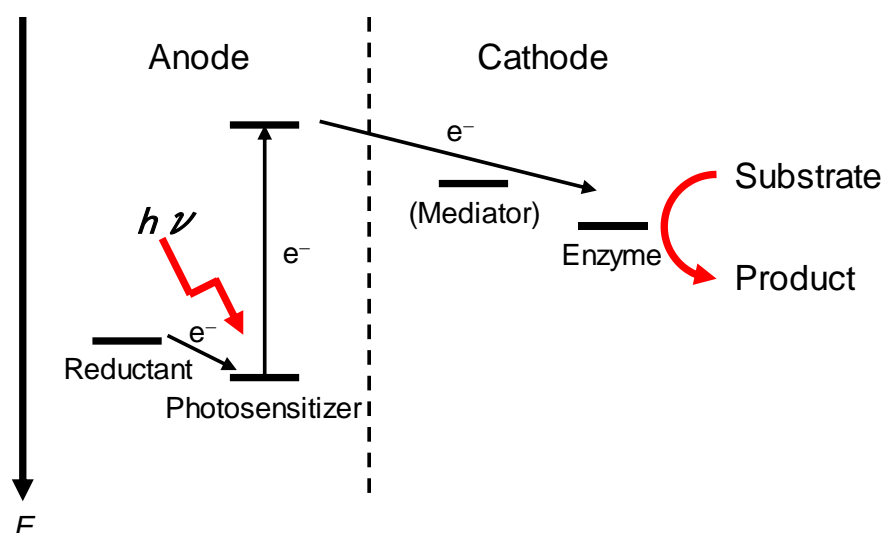


Figure 6. Potential profile in a simple photo-bioelectrocatalytic system.

4. Conclusions

Enzymatic bioelectrocatalysis is advancing day by day thanks to the finding of novel enzymes and the development of electrode nanomaterials. However, there are still issues to be solved such as thermostability, oxygen tolerance, and physical fragility, compared to inorganic electrocatalysts. Further understanding and consideration of the electrode interface and the interaction between enzymes and electrodes from kinetics and thermodynamics are demanded. Other perspectives will be also required for the improvement of present bioelectrochemical devices.

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