Enzymatic Bioelectrocatalysis
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ISBN 978-981-15-8959-1  ISBN 978-981-15-8960-7 (eBook)  
https://doi.org/10.1007/978-981-15-8960-7

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Electron transfer reactions in living organisms including photosynthesis and metabolisms play a central role in all living systems. Redox enzymes catalyze a variety of biological electron transfer reactions. Each enzyme has evolved over millions of years to catalyze one given reaction in high speed, high selectivity, and high specificity. Bioelectrochemistry is a field of study and application of biological electron transfer reactions in terms of thermodynamic and kinetics.

It has been established that redox enzymes can also work as efficient electrocatalysts. Over the last four decades, redox enzymes have received much attention for its use in the coupling of enzyme reactions having such intrinsic and powerful characteristics with non-specific electrochemical reactions. The coupled reaction is known as bioelectrocatalysis. It is worthy to note that the coupling gives a variety of biological redox functions to conventional electrode materials, and it is expected to open new ways for a wealth of fundamental researches and developing bioelectrochemical devices relating to amperometric biosensors, biological fuel cells (which is simply referred to as biofuel cells), and bioelectrochemical reactors (bioelectrosynthetic devices).

Recent progresses in this field have led to increasing sophistication in the approaches to the assembly of electrode surfaces, including immobilization and orientation of redox enzymes at the electrode surface, tuning of electrode surfaces, and protein mutations to facilitate electron transfer reactions. In addition, such improved bioelectrochemical techniques can be applied to study the electron transfer behavior of redox enzymes to address fundamental biological questions.

This book covers the fundamental aspects of the chemistry and electrochemistry of redox enzymes which underline the titled topic. Described are the basic concepts and theoretical aspects of bioelectrocatalysis, the different experimental techniques and materials used to study and characterize related problems, and also its various applications to bioelectrochemical devices. This work provides a unique source of information in this area, approaching the subject from a cross-disciplinary viewpoint.
I would like to thank all of my coauthors and collaborators for their supports in producing this book and for deep discussion to understand enzymatic bioelectrocatalysis. I hope that this book will inspire readers with interest to hold dream and interest in this exciting, important, interdisciplinary, and growing field.

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Acknowledgments

The present work has been performed mainly in Laboratory of Bio-Physical and -Analytical Chemistry, Division of Applied Life Science, Graduate School of Agriculture, Kyoto University with many collaborators in various universities and companies. The authors would like to express cordial thanks to the collaborators (colleagues, students, researchers). The authors would also express thanks to Mr. Shinichi Koizumi for strong and continual encouragement in writing and for editing.
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## Abbreviations and Symbols

### Enzyme

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| ADH          | Alcohol dehydrogenase                            |
| BOD          | Bilirubin oxidase                                |
| BSA          | Bovine serum albumin                             |
| cyt c        | Cytochrome c                                     |
| FDH          | d-fructose dehydrogenase                         |
| FoDH         | Formate dehydrogenase                            |
| Dp           | Diaphorase                                       |
| GADH         | d-gluconate dehydrogenase                        |
| GOD          | Glucose oxidase                                  |
| HRP          | Horseradish peroxidase                           |
| Lac          | Laccase                                          |
| MCO          | Multi-copper oxidase                             |
| PSI          | Photosystem I                                    |
| PSII         | Photosystem II                                   |
| SOD          | Superoxide dismutase                             |

### Redox Center

| Abbreviation | Description                                                                 |
|--------------|-----------------------------------------------------------------------------|
| CoQ          | Coenzyme Q (ubiquinone, 2,3-dimethoxy-5-methyl-6-poly prenyle-1,4-benzoquinone) |
| FAD          | Flavin adenine dinucleotide                                                |
| FeS          | Ion—sulfur cluster                                                          |
| FMN          | Flavin mononucleotide                                                       |
| Moco         | Molybdopterin cofactor                                                      |
| NAD(P)       | Nicotinamide adenine dinucleotide (phosphate)                              |
### Abbreviations and Symbols

| Abbreviation | Definition |
|--------------|------------|
| NHS          | N-Hydroxysuccinimide |
| PQQ          | Pyrroloquinoline quinone |
| T1Cu         | Type I copper site |
| T2/3Cu       | Type II/III copper cluster |

### Chemical

| Abbreviation | Chemical Name |
|--------------|---------------|
| ABTS         | 2,2’-Azinobis(3-ethylbenzothiazoline-6-sulfonate |
| ATP          | Adenosine triphosphate |
| BQ           | p-Benzoquinone |
| DMSO         | Dimethyl-sulfoxide |
| EDC          | 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide |
| GA           | Glutaraldehyde (1,5-pentanedial) |
| NHS          | N-Hydroxysuccinimide |
| PEI          | Poly(ethylenimine) |
| PEGDGE       | Poly(ethylene glycol) diglycidyl ether |
| PVI          | Poly(vinyl imidazole) |
| PVP          | Poly(vinyl pyridine) |

### Electrode

| Abbreviation | Description |
|--------------|-------------|
| AuE          | Gold electrode |
| CNT          | Carbon nanotube |
| CPE          | Carbon paste electrode |
| EPGE         | Edge-oriented pyrolytic graphite electrode |
| GCE          | Glassy carbon electrode |
| ITO          | Indium–tin–oxide electrode |
| KBE          | Ketjen Black-modified electrode |
| ME           | Microdisk electrode |
| RDE          | Rotating disk electrode |
| SHE          | Standard hydrogen electrode |

### Others

| Abbreviation | Description |
|--------------|-------------|
| DET          | Direct electron transfer |
| EPR          | Electron paramagnetic resonance |
| LFER         | Linear free energy relationship |
MET  Mediated electron transfer
RDS  Rate-determining step
SAM  Self-assembled monolayer

Symbols

Roman Symbols

\( a \) Activity
\( A \) Electrode surface area
\( c \) Concentration
\( D \) Diffusion coefficient
\( E^\circ \) Standard redox potential
\( E^{\oplus} \) Biological standard redox potential
\( E_{\text{cell}} \) Cell voltage
\( E_{1L} \) Lever's ligand parameter
\( F \) Faraday constant
\( \Delta_r G^\circ \) Standard Gibbs energy of reaction
\( \Delta_r G^{\oplus} \) Biological standard Gibbs energy of reaction
\( \Delta^\circ G_{X} \) Standard Gibbs energy of activation
\( i \) Current
\( i_s \) Steady-state current
\( j \) Current density
\( j_s \) Steady-state current density
\( k \) Reaction rate constant
\( k_2 \) Second-order reaction rate constant,
\( K \) Equilibrium constant
\( K_M \) Michaelis constant
\( K_S \) Semiquinone formation constant
\( m \) Number of protons in reaction
\( n \) Number of electrons in reaction
\( OCV \) Open-circuit potential
\( P \) Permeation coefficient
\( P_{\text{max}} \) Maximum power density
\( r \) Radius
\( R \) Gas constant or resistance
\( t \) Time
\( T \) Absolute temperature
\( x \) Distance from electrode surface
\( v \) Reaction rate
Greek Symbols

\( \alpha \)  Transfer coefficient  
\( \Gamma \)  Surface concentration  
\( \mu^\circ \)  Standard chemical potential  
\( \nu \)  Stoichiometric coefficient, kinematic viscosity  
\( \omega \)  Rotation rate in radian
Chapter 1
Redox Proteins and Bioelectrocatalysis

Abstract  This chapter starts by introducing the basic feature and thermodynamics of redox proteins including redox enzymes (oxidoreductases). Biochemical properties of organic and inorganic redox components in redox enzymes are detailed, and they are very important to understand bioelectrocatalysis. This chapter also introduces the basic concept of bioelectrocatalysis.

Keywords  Biological redox potential · Organic cofactors · Metallic ion cofactors · Hydride ion transfer · Electron transfer · Bioelectrocatalysis

1.1 Oxidoreductases [1, 2]

Oxidoreductases (redox enzymes) are concerned with widespread biological electron transfers, i.e. processes of respiration, fermentation, and photosynthesis to catalyze electron transfer reactions across the tree of life and to facilitate biologically driven fluxes of several essential elements such as hydrogen, carbon, nitrogen, oxygen, and sulfur on Earth [3]. The kinds of the redox enzymes are approximately one-quarter of all known enzymes based on the enzyme nomenclature [4]. The name of the majority of the redox enzymes is *dehydrogenase*, although sometimes *reductase* is used as an alternative. The first class of dehydrogenases that we shall meet are the enzymes that strictly utilize β-nicotine amide dinucleotide (phosphate) (NAD(P)) as a coenzyme and are called *NAD(P)-dependent dehydrogenases*. NAD(P)(H) shuttles back and forth between the NAD(P)-dependent enzyme and solution to transfer hydride ion (two-electron and single-proton, analogous to BH₄⁻ and AlH₄⁻). The specificity to NAD(P) is very rigid in NAD(P)-dependent enzymes. Other than these, NAD(P)-independent dehydrogenases including flavoproteins, quinoproteins, and metalloproteins utilize not NAD(P) but other redox compounds including redox proteins. NAD(P)-independent dehydrogenases catalyze the transfer of electron and/or hydride ion. NAD(P)-reducing dehydrogenases (e.g. NAD(P)-reducing hydrogenase (H₂ase) and NAD(P)-linked molybdenum (Mo)- (or tungsten (W)-)containing formate dehydrogenase (FoDH)) utilize NAD(P)⁺ as an electron acceptor, but NAD(P)⁺ can be replaced with other oxidizing compounds with electron-transfer property, because they have a flavin-containing diaphorase subunit that can catalyze both electron
Redox Proteins and Bioelectrocatalysis

and/or hydride ion; the flavin cofactor communicates with NAD(P)(H) in hydride ion transfer under natural conditions, but also with other redox compounds in electron transfer under artificial conditions. The names oxidase and peroxidase are only used where dioxygen (O₂) and hydrogen peroxide (H₂O₂) are the electron acceptors, respectively. Oxygenase and hydroxylase incorporate O₂ into the substrate being oxidized.

To understand and discuss the biological electron transfer, we frequently discuss phenomena in terms of the biological standard redox potential (E⊕) that measures the oxidizing strength of an oxidizing agent (Ox) at pH 7 in the following half reaction:

\[ \text{Ox} + mH^+ + ne^- \rightleftharpoons \text{Red}, \quad (1.1) \]

where \( m \) and \( n \) denote the numbers of protons and electrons in the half-redox reaction, respectively, and Red is the conjugated reduced form. \( E^\oplus \) can be converted from the standard redox potential (\( E^\circ \)) (at pH 0) by

\[ E^\oplus = E^\circ - \frac{2.303mRT}{nF} \times 7, \quad (1.2) \]

where \( R, T, \) and \( F \) denote the gas constant, absolute temperature, and Faraday constant, respectively (2.303RT/F = 0.05916 V at 298 K). \( E^\circ \) can be calculated from the standard Gibb energy of the half-reaction (\( \Delta_{r,\text{half}}G^\circ \)) by

\[ E^\circ = -\frac{-\Delta_{r,\text{half}}G^\circ}{nF} = -\frac{1}{nF} \sum \mp v_i \mu_i^\circ, \quad (1.3) \]

where \( v_i \) and \( \mu_i^\circ \) are the stoichiometric coefficient and the standard chemical potential of reactant, respectively, and the signs + and − denote the reactant(s) and product(s), respectively (i.e., \( v_{\text{Ox}} = -1, v_{\text{Red}} = 1 \) in Eq. (1.1). The \( E^\oplus \) values of some of biologically important redox couples are given in Fig. 1.1. Half-reaction 1 (reduction) couples with the reverse reaction of half-reaction 2 (oxidation) to construct an overall redox reaction:

\[ v_1\text{Ox} + v_2\text{Red} + (v_1m_1 - v_2m_2)H^+ + n_1n_2e^- \rightleftharpoons \text{Red}. \quad (1.4) \]

The biological standard Gibbs energy of the overall redox reaction (\( \Delta_rG^\oplus \)) is given by

\[ \Delta_rG^\oplus = -n_1n_2F(E_1^\oplus - E_2^\oplus). \quad (1.5) \]

Therefore, an electron transfer from a reductant with a negative side value of \( E_2^\oplus \) to an oxidant with a positive side value of \( E_1^\oplus \) is thermodynamically favorable (downhill) because of \( \Delta_rG^\oplus < 0 \). For example, the oxidation of ethanol with
1.1 Oxidoreductases

Fig. 1.1 Biological standard redox potentials of some biologically important redox couples and proteins

NAD\(^+\) (catalyzed by NAD-dependent alcohol dehydrogenase) is uphill and susceptible to product inhibition, while that of acetaldehyde with NAD\(^+\) (catalyzed by NAD-dependent acetaldehyde dehydrogenase) is strongly downhill.

Photosynthesis involves a series of electron transfer processes, splitting water to proton and O\(_2\) by sunlight energy that is the ultimate source of energy for all life on Earth, to generate high-energy reducing equivalents which are utilized in fixing carbon dioxide (CO\(_2\)) to glucose. Glucose is oxidized by O\(_2\) in metabolism to synthesize adenosine triphosphate (ATP) from inorganic phosphate and adenosine diphosphate (ADP). ATP is the essential energy carrier used in a wide range of biological processes. Energetics of life can be illustrated as an electron transport cycle between the two redox cycles: O\(_2\)/water and CO\(_2\)/glucose redox couples, as shown in Fig. 1.2 as a typical example. As judged from the data in Fig. 1.1, the electron transfer from glucose to O\(_2\) in metabolism is downhill (i.e., \(E_1^{\varnothing} - E_2^{\varnothing} = 0.815 \, \text{V} - (-0.425 \, \text{V}) = 1.24 \, \text{V}\)), while that from water to glucose in photosynthesis

Fig. 1.2 Schematic diagram of a typical electron transport cycle in energetics of life
is thermodynamically unfavorable (uphill) and requires sunlight energy. The electron transport cycle is regarded as a kind of battery; photosynthesis is a charging process and a coupled process of metabolism and respiration is a discharging process.

1.2 Redox Components

Redox enzymes use a palette of redox components called coenzymes, prosthetic groups, or cofactors: β-nicotine amide dinucleotides (phosphate) (NAD(P)⁺), flavins, quinones, hemes, ion-sulfur clusters, copper, molybdenum, nickel, etc. β-NAD- and β-NADP-dependent enzymes catalyze hydride ion transfers in the main streams of catabolism and anabolism, respectively. Core redox enzymes are ancient and highly diverse in amino acid sequence, and usually require specific transition metal(s) in their active site to catalyze (multi-step) single-electron transfers. On the other hand, flavoproteins and quinoproteins can catalyze both (two-step) single-electron transfer and hydride ion transfer and link the electron transfer between several organic redox substances (including NAD(P)(H)) and inorganic redox-centers in metal-containing redox enzymes. In the following, we describe the structures and redox reactions of these redox components.

1.2.1 NAD(P)(H) [1, 5]

Pyridine nucleotide coenzymes NAD(P)(H) undergo a hydride ion (H⁻) transfer (i.e. a single step two-electron one-proton redox reaction without involving its intermediate radical) in the biological system (Fig. 1.3A). NAD(P)-dependent enzymes catalyze the reversible transfer of hydride ion from NAD(P)H to carbon atoms on the oxidized substrate and vice versa (Fig. 1.3B). Thus, NAD(P)(H) are usually regarded as hydride ion-transferring coenzymes. Many NAD(P)-dependent dehydrogenases utilize ordered mechanisms; NAD(P)(H) is non-covalently and weakly bound to NAD(P)-dependent dehydrogenases during the enzyme reactions and is released into solution after the catalytic reactions. Therefore, the enzymatic reactions are frequently monitored spectrophotometrically at 340 nm due to the absorption of NAD(P)H in solution (Fig. 1.3C). NAD-dependent dehydrogenases are involved in catabolic processes, while NADP-dependent ones are involved in anabolic ones. NAD(P)-dependent enzymes show high specificity toward both the substrate and NAD(P)(H).

The biological standard redox potential \( E^{\oplus} \) for the NAD⁺/NADH couple is \(-0.315 \text{ V} \) versus the standard hydrogen electrode (SHE)* and the formal potential \( E^{\circ} \) shifts by \(-29.5 \text{ mV per pH at 298 K} \), according to Eq. (1.6):

\[
E^{\circ'} = E^{\oplus} - \frac{2.303mRT}{nF} \Delta \text{pH},
\]  

\[ (1.6) \]
1.2 Redox Components

**A**

![Diagram of NAD(P)⁺ and NAD(P)H](image)

During the two-electron reduction, the oxidation number of the N1 atom changes from $-2$ to $-3$, while that of the C4 atom changes from $-1$ to $-2$. H$_{α}$ and H$_{β}$ at the C4 position in NAD(P)H are not equivalent. NAD(P)H has a quinoid structure in N1–[(C2–C3)+(C6–C5)], which gives an absorption band at $\lambda_{\text{max}} = 340$ nm.

**B**

Hydride transfer from NAD(P)H to carbon of a ketone substrate.

**C**

The adsorption spectra of NAD$^+$ and NADH. The molar absorption coefficient of NADH is $6.23 \times 10^3$ cm$^2$ mol$^{-1}$ at 340 nm where $\Delta$pH is the shift in pH from the biological standard value (pH 7.0).

* The potential is referred to SHE in this book, otherwise stated.

1.2.2 Flavins [1, 6, 7]

Flavin redox cofactors are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fig. 1.4A); they have an isoalloxazine heterocyclic ring system, which is responsible for their redox activity. The $E^\circ$ value of the two-electron transfer of free FAD is $-0.219$ V. The two-electron transfer is coupled with a two-proton transfer and $E^\circ$ shifts by $-59.2$ mV per pH at 298 K under acidic and neutral conditions. At increased pHs, the fully reduced form (Red) of flavins undergo proton dissociation, and pH dependence of $E^\circ$ (of the two-electron transfer) becomes $-29.5$ mV per pH.
The first one of striking properties of flavins is their strong yellow-green fluorescence. The property is conveyed onto flavin-dependent enzymes. The second is related to an important difference between flavins and NAD(P) in the electro transfer property; flavins undergo two-step single-electron transfer and then exists either as oxidized (Ox), Red, or an intermediate semiquinone radical species (Sem) (Fig. 1.4A). The absorption spectra of the redox species are given in Fig. 1.4B. Therefore, flavins are able to carry out both single- and two-electron transfer reactions. This property allows the electron transfer to a variety of redox compounds including metal ions and O₂.

The thermodynamics of the property is frequently given by the semiquinone formation constant ($K_S$) for a comproportionation reaction: \( \text{Ox} + \text{Red} \rightleftharpoons 2\text{Sem} \),

$$K_S = \frac{a_{\text{Sem}}^2}{a_{\text{Ox}}a_{\text{Red}}} = \frac{RT}{F} \exp \left[ \frac{F(E_{1}^{\circ'} - E_{2}^{\circ'})}{RT} \right], \quad (1.7)$$

where \( a_X \) is the activity of a redox species \( X \), and \( E_{1}^{\circ'} \) and \( E_{2}^{\circ'} \) are the formal potentials of single electron transfer of the Ox/Sem and Sem/Red couples, respectively, as given by the following Nernst equations:

$$E = E_{1}^{\circ'} + \frac{RT}{F} \ln \frac{a_{\text{Ox}}}{a_{\text{Sem}}}, \quad (1.8)$$
and

\[ E = E_2^{′′} + \frac{RT}{F} \ln \frac{a_{\text{Sem}}}{a_{\text{Red}}}. \]  

(1.9)

The Sem state is thermodynamically unstable \((K_S < 1)\) in aqueous solutions.

The third is that flavins can also undergo hydride transfers; flavins are able to accept hydride at N5 of the flavin nucleus from NAD(P)H (Fig. 1.4c) and several organic substances. The functions to catalyze both two-step single-electron transfer and hydride ion transfer allow to link the electron transfer between several organic substances including NAD(P)(H) and inorganic-redox centers in metal-containing redox proteins.

The fourth is that, unlike NAD(P), flavins are very tightly bound to FAD (FMN)-dependent enzymes (called flavoproteins), and sometimes covalently attached to His, Cys or Try residue of enzymes, [8, 9] as show in Fig. 1.5 (e.g. Cys(S)-6-flavin in histamine dehydrogenase [10]). Therefore, the flavins remain bound to the enzymes throughout the enzymatic reactions; many flavoproteins utilize ping-pong mechanisms. In addition, due to the tight binding to the enzymes, flavoproteins show a wide range of \(E^{\oplus}\) of the cofactor (Fig. 1.1), and the Sem intermediate sometimes becomes stable in proteins compared with in aqueous solution. The \(K_S\) value of free flavins increases with an increase of pH. The rate of the autoxidation (oxidation with \(O_2\)) also increases drastically with an increase of pH, since Sem plays very important role in the autoxidation [11].

Diaphorase (Dp) is a homodimer containing an FAD for each subunit, and can catalyze oxidation of NADH and NADPH with almost equal efficiency by a variety of electron acceptors. The \(E^{\oplus}\) value of the FAD in Dp from rat liver is \(-0.159\) V.

Cytochrome P450 monooxygenases contain flavin and heme, and catalyze hydroxylation of organic molecules, for which there is little precedent in organic chemistry. The enzymes give a characteristic UV absorption at 450 nm upon treatment of the heme with carbon monoxide (CO).

Fig. 1.5 The structures of covalently bound flavins
1.2.3 Quinones [7, 12]

Quinones undergo sequential two-step single-electron transfer reduction coupled with single- or two-proton transfer (that is, both electron and hydride ion transfers), as in the case of flavins. Ubiquinone (coenzyme Q (CoQ)), menaquinone, and plastoquinone are not cofactors of redox enzymes but are involved in the biological energy transducing electron transport chain (Fig. 1.6). The $E^{\oplus}$ value of CoQ is 0.045 V. They have a long isoprenoid chain to freely diffuse in the lipid membrane, the number of the isoprenoid chain units in CoQ being in the range from six to ten depending on the organism. These quinones can couple the electron transport and the proton transport in transmembrane protein complexes, such as photosystem II, cytochrome bf complex, NADH-CoQ reductase complex (Complex I), and CoQ-cytochrome $c$ (cyt $c$) reductase complex (Complex III).

At the end of the 1970s, pyrroloquinoline quinone (PQQ) has been discovered as a novel organic redox cofactor [13, 14]. PQQ is biosynthesized from Tyr and Glu, and the prosthetic group of bacterial hydrogenases for methanol, higher alcohol, aldose sugars, aldehydes, and poly(vinyl alcohol), and also for hydroxylation of lupanine. The $E^{\oplus}$ of free PQQ is 0.066 V [15]. The PQQ in the dehydrogenases is not covalently bound to the enzymes.

After the discovery of PQQ, several quinone cofactors have been discovered, as shown in Fig. 1.7. These are protein-derived cofactors generated by post-translational chemical modification of amino acid residues that are susceptible to oxidation (Tyr, Cys, Trp, Glu, Lys etc.) and work as build-in cofactors that are integrated within the enzyme polypeptides as a part of amino acid side-chains and retain their covalent linkage to the enzymes. Tropa quinone (TPQ, 6-hydroxydopa quinone) is the prosthetic group of copper-containing amine oxidases in bacteria, yeasts, plants, and mammals [16]. The TPQ-dependent enzymes catalyze $O_2$-independent oxidation of amines to aldehydes and $H_2O_2$. Tryptophan tryptophylquinone (TTQ) is the
prosthetic group of bacterial amine dehydrogenases which catalyze O₂-independent oxidation of primary amines to aldehydes and ammonia [17]. Lysine tyrosylquinone (LTQ) is the prosthetic group of lysyl oxidase, a second type of copper-containing amine oxidase [18]. The LTQ-dependent enzyme is specific for lysyl groups in collagen and elastin. In 2001, cysteine tryptophanequinone (CTQ) has been found as the prosthetic group of quinohemoprotein amine dehydrogenases from *Paracoccus denitrificans* and *Pseudomonas putida* [19]. All these quinone cofactor-dependent enzymes are called quinoproteins, by the analogy with flavins and flavoproteins.

These quinone cofactors, especially free PQQ, are very susceptible to reactions with a variety of nucleophiles including acetone, hydrazines, amino acids, alcohols. Some examples of the reactions are given in Fig. 1.8 in the case of free PQQ [20, 21]. Even the covalently bound CTQ in *P. denitrificans* reacts with carbonyl reagents, 4-nitrophenylhydrazine and hydroxylamine, to produces an inactive oxime form, which exists in the cultured cells and is activated by its substrate or strong reductants (Fig. 1.9) [22].

Electrochemistry and spectroscopy of free PQQ, [15, 23, 24] model compounds of TPQ, [25] TTQ [26], and CTQ, [27] PQQ in soluble glucose dehydrogenase [28], and CTQ in quinohemoprotein amine dehydrogenase [29] can be referred to the literature.

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**Fig. 1.7** The structures of the quinone cofactors. The four figures in the parentheses indicate the year of the discovery.
Iron is the fourth most abundant element in the earth’s crust, and is also the most abundant transition metal found in biological systems. Many iron-containing enzymes appear to be variants of common structural motifs and undergo single electron transfers in biological processes. The iron-containing protein family constitutes approximately 50% of all metal-containing redox enzymes, and it is proposed that the family potentially catalyzed redox reactions in the Archean oceans [31].

One of the common types of a single-electron carrier is heme redox center, which has a porphyrin ring, comprising four pyrrole rings by methylene bridges, with a
single Fe ion coordinated in the center. There are three different types, $a$, $b$, and $c$, with different substitution pattern around the porphyrin ring (Fig. 1.10). The most common heme is heme $b$ (sometimes called protoporphyrin IX), which exist in e.g. myoglobin, hemoglobin, and horseradish peroxidase (HRP). Cyt $c$ contains $c$-type heme. Cyt $c$ oxidase is the key to aerobic life, and is the terminal electron acceptor in the respiratory chain, transferring electrons and protons to $O_2$. The enzyme contains heme $a$ as the prosthetic group.

Fe ion has up to six coordination positions. Coordination positions 1–4 of Fe ion in a porphyrin ring are occupied by the tetradentate porphyrin ligand. Position 5 is located on the proximal axial side of the heme and is most commonly occupied by a His residue in a protein. Position 6 is located on the distal axial side (the side where the active site is located). The position is commonly occupied by His for hemes $a$ and $b$, but usually Met for heme $c$.

Because these substituents are directly attached to the ring and affect the electronic state of the Fe ion, the $E^{\oplus}$ value of hemes can vary very widely; $E^{\oplus}$ values locate in the range from $-0.3 \, V$ in His/His coordinated hemes $c$ to $+0.4 \, V$ His/Met coordinated hemes $c$ (Fig. 1.1). The wide variation of $E^{\oplus}$ allows hemes to play roles at different stage of several redox chains.

The $E^{\oplus}$ values of hemes are sufficiently negative than $E^\circ$ of aqua Fe$^{3+/2+}$ redox couple ($+0.771 \, V$), due to strong electrostatic stabilization of Fe$^{3+}$ by the porphyrin and axial ligands compared with Fe$^{2+}$. In protein engineering approach, three factors tune the $E^{\oplus}$ values of heme groups: donor properties of the axial ligand, substituents of the porphyrin, and polarity of the surrounding environment [32].

It has been proposed that heme-binding domains are derived via modular evolutionary processes that ultimately form the backbone of redox reactions in both anaerobic and aerobic respiration and photosynthesis [31].

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**Fig. 1.10**  
A The structures of hemes $a$, $b$, and $c$. B The absorption spectra of the oxidized and reduced cyt $c$ at pH 7.0. The molar absorption coefficient of the $\alpha$ peak of the reduced form is $27.7 \, cm^2 \, mol^{-1}$.
1.2.5 **Ion-Sulfur Clusters** [1, 7]

Non-heme iron proteins contain ion-sulfur (FeS) clusters, which are also the common type of single-electron carrier. Cys, His, Glu, Asp, and Tyr are the most commonly observed ligand to the ion center(s). Rubredoxins contain an ion atom in a tetrahedral coordination environment of sulfur atoms provided by four Cys residues. The $E^\circ$ value of rubredoxins is modulated by the protein environment from $+0.02$ V to $-0.03$ V. Most of the FeS cluster contain ion atoms bound to both inorganic sulfur atoms and sulfur atoms on Cys residues of its FeS protein (Fig. 1.11). [2Fe2S] and [4Fe4S] clusters are commonly found in biological electron carriers known as ferredoxins, and in a number of redox enzymes. The $E^\circ$ value of [4Fe4S] clusters change in the range from $-0.65$ V to $+0.45$ V by changes in the protein environment adjacent to the clusters (Fig. 1.1). In Rieske clusters in the cytochrome $b$ and CoQH$_2$-cyt $c$ reductase complexes, two of Cys ligands in [2Fe2S] ligands are replaced with His. FeS clusters have ability to accept a single electron from a two-step single-electron donor such as flavins, and transfer the electron to another electron carrier or to the active site of a redox enzyme.

![Rubredoxin](image1)

![2Fe2S](image2)

![Rieske complex](image3)

![3Fe4S](image4)

![4Fe4S](image5)

**Fig. 1.11** The structures of FeS clusters
1.2.6 Copper [33]

Copper proteins and enzymes contain redox active copper ion(s) in their active sites in a permanently coordinated state. The functions of copper proteins are restricted to electron transfer port in photosynthesis and nitrogen metabolism and in oxygen transport, suggesting that copper ion gained biological importance only after the oxygenation in the atmosphere. \( \text{Cu}^{+} \) is a soft acid in the Person’s HSAB (hard and soft acid base) concept and prefers Cys, and Met. The harder acid \( \text{Cu}^{2+} \) can be coordinated by Tyr, Ser, Thr, His, OH\(^{-}\) and H\(_{2}\)O. In addition, when no steric hindrance occurs, \( \text{Cu}^{+} \) prefers tetrahedral coordination, while the ligand of \( \text{Cu}^{2+} \) arrange in a square planar configuration. In the copper ion binding sites of copper proteins, both their ligands and conformations considerably deviate from those preferred by \( \text{Cu}^{2+} \), leading to a destabilization of the oxidized state and then a rise in the \( E^\circ \) value of copper proteins and to reduction of the reorganization energy associated with electron transfer. There are six different types of copper sites in copper proteins and enzymes (Fig. 1.12).

Type I copper sites (T1Cu) are characterized by a mononuclear copper ion coordinated by two His residues and one Cys in a trigonal planar structure, and a variable axial ligand (e.g. Met, His, Gln) giving intense blue color with a molar extinction coefficient (\( \sim 5000 \text{ M}^{-1} \)) at \( \sim 600 \text{ nm} \) due to \( \text{S(Cys)} \rightarrow \text{Cu(II)} \) charge transfer and electron paramagnetic resonance (EPR) signal with an usually narrow hyperfine coupling. T1Cu is present in small blue copper proteins functioning as electron transfer proteins in photosynthesis and respiration (e.g. amicyanin, plastocyanin, azurin) and also in blue multi-copper oxidases (MCOs) and copper-containing nitrite (\( \text{NO}_2^- \)) reductase. The \( E^\circ \) value of bilirubin oxidase (BOD) from \textit{Myrothecium verrucaria} with axial Met is +0.66 V [34] and is more positive than \( E^\circ \) of aqua \( \text{Cu}^{2+/+} \) redox couple.

![Fig. 1.12 The structures of some copper sites](image-url)
(+0.153 V). On the other hand, stellacyanin from *Rhus vernicifera* with axial Gln shows the most negative side value of $E^{\circ}$ (+0.184 V) among blue copper proteins [35]. This is predominantly ascribed to the electrostatic attractive-interaction between Gln and Cu$^{2+}$. As anticipated from a simple electrostatics argument, replacement of the axial Met in BOD by Gln causes a $-0.25$ V-shift of $E^{\circ}$ of T1Cu [36]. Similar effects were reported for a Met510Gln mutant in copper efflux oxidase (CueO) (a shift of $-0.13$ V) [37], a Met121Asp mutant in azurin (a shift of $-0.19$ V) [38]. When the axial site of the T1Cu site is vacant (with Phe, Leu, or Ala at the corresponding axis residue position), $E^{\circ}$ of the T1Cu site shifts to the direction of the positive potential [37].

“Non-blue” type II copper sites (T2Cu) exhibit a square planar coordination by N or N/O ligand (of His and Tyr or H$_2$O), and are present in oxidases and oxygenases, nitric (NO$_3^-$) reductase, and Cu, Zn-superoxide dismutase (SOD). T2Cu gives normal EPR spectra with axial symmetry. Type III copper sites (T3Cu) consist of oxygen-bridged dimer of copper ion centers, each coordinated by three His residues. They occur in an oxygen-transporting protein hemocyanine and a monoxygenase tyrosinase. Trinuclear copper centers (T2/3Cu) are trinuclear copper ion sites coordinated by eight His ligands as a coupled two T2Cu and a T3Cu ligands. T2/3Cu is present in blue copper oxidases (i.e. MCOs) together with T1Cu. *Binuclear Copper A centres* (Cu$_A$) are found in cyt c oxidase and nitrous oxide (N$_2$O) reductase. The two copper ions are coordinated by two His ligands, one Met, one protein backbone carbonyl oxygen, and two bridging Cys residues. *Cupper B centres* (Cu$_B$) are also found in cyt c oxidase. The copper ion is coordinated by three His ligands in a trigonal pyramidal geometry.

Cupper ion also exists in non-blue oxidases (TPQ-containing amine oxidases and galactose oxidases).

### 1.2.7 Nickel [39]

Hydrogenases (H$_2$ases) are ubiquitous and key components among anaerobic (occasionally aerobic) bacterial and archaea, and catalyze the reversible oxidation of dihydrogen (H$_2$) and reduction of proton (H$^+$). One major family of H$_2$ases contain binuclear Ni-Fe active sites, in which two thiolates of Cys residues are bridging the two metals, and are called [NiFe] H$_2$ases (Fig. 1.13 [40]). NAD(P)$^+$-reducing [NiFe] H$_2$ases have a Dp subunit containing an FMN, which catalyzes electrochemical communication between electron-transfer-type FeS in the subunit and hydride ion transfer-type NAD(P)(H) in solution. Some [NiFe] enzymes have a selenocysteinyl (Sec) residue instead of one of the Cys residues supporting the Ni-Fe center and are called [NiFeSe] H$_2$ases. Other families of H$_2$ases are [FeFe] H$_2$ases and [Fe] H$_2$ases.
1.2 Redox Components

Fig. 1.13 A The structure of the standard [NiFe] hydrogenase. The small and the large subunits are shown in blue and light brown, respectively. The pathways of the electron transfer, H⁺ transfer, and H₂ gas are indicated by the arrows. B The structure of the Ni–Fe active site. The position marked with X indicates the third bridging ligand, which changes during catalytic reaction. Hydrogen bonds to the active site ligands are shown by the dotted lines. The dotted red line indicates a possible proton transfer pathway. Reproduced from Ref. 40 copyright (2016) with permission from Oxford University Press

1.2.8 Molybdenum [41]

Molybdenum (Mo) is quite rare in terrestrial organisms, but all living species use Mo at the active sites of specific redox enzymes, molybdenum enzymes. The enzymes are involved in a wide range of biochemical redox reactions including oxidation of xanthine (by xanthine oxidase) and other purines in animals, the reduction of NO₃⁻ to NO₂⁻ (by nitrate reductase), and the reduction of molecular nitrogen (N₂) to ammonia (NH₃) (by nitrogenase). Molybdenum enzymes (molybdoenzymes) effect the conversions in both organic and inorganic sulfur chemistry (e.g. sulfite oxidase, dimethyl-sulfoxide (DMSO) reductase) and also are essential for the C1 metabolism (e.g. formate dehydrogenase (FoDH), CO₂ reductase, CO dehydrogenase) and for ethanol metabolism (e.g. aldehyde dehydrogenase).
Molybdoenzymes are classified into two groups. The first group includes the majority of molybdoenzymes, each of which has a similar Mo cofactor called molybdopterin cofactor (Moco), of which the structure involves an Mo ion coordinated by one or two characteristic 6-substituted pterin(s). Table 1.1 summarizes the distribution of molybdopterin and its molybdopterin derivatives, of which the structures are given in Fig. 1.14. An example of the structure of Moco is given in Fig. 1.15. The second group consists of nitrogenases, in which Mo is part of iron-molybdenum-sulfur cluster known as FeMoco. Tungsten (W)-containing enzymes, in which W is coordinated by pterins in place of Mo, have been found mainly in thermophilic anaerobes.

Table 1.1 Distribution of molybdopterin and molybdopterin dinucleotides in molybdoenzymes

| Pterin* | Enzyme                        | Source                                      |
|---------|-------------------------------|---------------------------------------------|
| MPT     | Sulfite oxidase               | Animals                                     |
|         | Xanthine dehydrogenase        | Animals, *Pseudomonas putida*               |
|         | Nitrate reductase             | Corn, *Chlorella vulgaris*                  |
|         | Aldehyde ferredoxine oxidoreductase | *Pyrococcus furiosus*                     |
|         | Formate ferredoxine oxidoreductase | *Thermococcus litoralis*                  |
| MGD     | DMSO reductase, nitrate reductase | *Rhodobacter sphaeroides*                  |
|         | Nitrate reductase, formate dehydrogenase | *Escherichia coli*                        |
|         | Formate dehydrogenase         | *Methanobacterium formicicum, Methanosarcina barkeri* |
| MCD     | CO dehydrogenase              | *Pseudomonas carboxydoflava*               |
|         | Quinolone oxidoreductase      | *Pseudomonas putida, Rhodococcus sp.*      |
| MAD, MHD| Formate dehydrogenase         | *Methanobacterium thermoautotrophicum*     |

* See Fig. 1.14 for the spelled-out name of the abbreviations of molybdopterin and molybdopterin dinucleotides
1.2 Redox Components

Fig. 1.14 The structures of bacterial pterin and molybdopterin dinucleotides

\[ \text{--OR} = \]

\[ \text{--O}^- \text{ molybdopterin (MPT)} \]

\[ \text{--O} - \text{P} - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{N} - \text{NH}_2 \]

\[ \text{ molybdopterin guanine dinucleotide (MGD)} \]

\[ \text{--O} - \text{P} - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{N} - \text{NH}_2 \]

\[ \text{ molybdopterin cytosine dinucleotide (MCD)} \]

\[ \text{--O} - \text{P} - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{N} - \text{NH}_2 \]

\[ \text{ molybdopterin adenine dinucleotide (MAD)} \]

\[ \text{--O} - \text{P} - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{N} - \text{NH}_2 \]

\[ \text{ molybdopterin hypoxanthine dinucleotide (MHD)} \]

Fig. 1.15 The structure of molybdenum cofactor (Moco) of DMSO reductase

1.2.9 Others

Extraction of electrons from water in photosystem II (PSII) takes place at a manganese active site known as the oxygen evolution complex. Manganese is distributed
throughout mammalian tissues. Much of the manganese present functions as cofactors of several redox enzymes: pyruvate carboxylase, SOD, arginase, glutamate synthetase. Vanadium is involved in nitrogenase and haloperoxidases.

### 1.3 Bioelectrocatalysis

It is well known that redox enzymes are extremely efficient catalysts, but it has also been established that redox enzymes can work as efficient electrocatalysts. Over the last four decades, redox enzymes have received much attention for use in the coupling of the enzyme reactions with non-specific electrochemical reactions. The coupled reaction is known as bioelectrocatalysis. Several review articles have been published [7, 42–56] and they can be helpful for readers with interest in this field.

We would like to propose an important concept in the coupling that the electrode reaction is not hydride ion transfer, but electron transfer, therefore molecules that can communicate with electrodes should have the electron transfer ability. In addition, the essential features of redox enzymes for realizing the coupling are that most of redox enzymes except NAD(P)-dependent dehydrogenases show low substrate specificity for either substrate. The natural substrates with low specificity have the electron transfer ability, and can be replaced with artificial redox compounds (and in some cases with electrodes). For example, the natural electron acceptor of FDH is CoQ$_{10}$ [57]; however, it can be replaced with several other quinones, inorganic oxidants, and several electrodes [58], because the electron donating site is a heme in the enzyme. Similar situations are observed even in oxidases and peroxidases. For instance, O$_2$ as the natural electron acceptor of FAD-dependent glucose oxidase (GOD) can be replaced with several organic and inorganic oxidants, because the electro donating site in the enzyme is FAD that can undergo both electron and hydride ion transfers. On the other hand, bilirubin as the natural electron donor of bilirubin oxidase (BOD) can be replaced with several organic and inorganic reductants, and several electrodes [59], because the electro accepting site is T1Cu in the enzyme. Several organic and inorganic reductants (as well as electrodes) can work as electron donors of HRP [60], of which the electrodonatig site is a protoheme.

In these manners, most redox enzyme reactions can be coupled with electrode reactions via (artificial) redox compounds (called mediators) that shuttle electrons between enzymes and electrodes. This reaction is known as mediated electron transfer (MET)-type bioelectrocatalysis (Fig. 1.16). Since NAD(P)(H) cannot communicate with electrode in a reversible way (or with low over potentials), NAD(P)-dependent enzymatic reactions have to be coupled with electrode reactions by using redox mediators that can transfer both hydride ion and electron, such as flavins, quinones (especially o-quinones), and phenothiazines (such as Meldola’s blue). NAD(P)-dependent enzymatic reactions can also be coupled with electrode reactions by flavoproteins such as diaphorase and ferredoxin NADP$^+$ reductase. Microbes and organelles (containing such redox enzymes) also work as electrocatalysts in MET-type bioelectrocatalysis.
On the other hand, it is known that some metal-containing enzymes and flavoenzymes can directly exchange electrons with electrodes in the absence of any mediators in the catalytic reaction. Such a reaction is referred to as direct electron transfer (DET)-type bioelectrocatalysis (Fig. 1.16). This type of coupling makes it possible to develop more simplified systems that can also minimize thermodynamic overpotentials in the coupling; the overpotential required in MET-type reactions between the redox center of an enzyme and a mediator in the solution is not necessary to drive the reaction. However, the number of DET-type enzymes displaying clear bioelectrocatalytic activity is small, and a DET-type reaction can only proceed on suitably arranged electrode surfaces. This is because redox enzymes are large in size and the redox sites in many redox enzymes are deeply buried in the enzyme matrix and electrically insulated throughout the entire enzyme volume.

But that as it may, it is worthy of note that the coupling provides a variety of biological catalytic functions to conventional non-specific electrode reactions. It is expected to open new ways for a wealth of researches and developing bioelectrochemical devices relating to amperometric biosensors, biological fuel cells (which is simply referred to as biofuel cells), and bioelectrochemical reactors (bioelectrosynthetic devices). In addition, the technique used for the coupling can be utilized as a novel tool for evaluating kinetic parameters of the catalytic reaction and the inhibition as well as thermodynamic parameters of the redox enzymes. On the contrary, the electrochemistry can be used as a tool for mechanistic studies of redox enzymes. Current-potential curves obtained from DET-type reactions have various kinds of information on the catalytic reactions and the electrode reactions of the enzymes. To interpret bioelectrocatalytic waves correctly, it is necessary to derive analytical equations on suitable models of DET-type reactions in relation to the thermodynamics and kinetics of the interfacial and intramolecular electron transfers and the structural-biological information of the electrochemically communicating site.
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Chapter 2
MET-Type Bioelectrocatalysis

Abstract This chapter deals with theoretical features of MET-type bioelectrocatalysis with emphasis of steady-state serial reactions. The fundamental concept in the selection of mediators is described based on linear free energy relationship and diffusion-controlled kinetics. Basic features of redox potential tuning and immobilization of mediators including redox polymers are also introduced.

Keywords Steady-state current · Serial resistance model · Linear free energy relationship · Diffusion-controlled kinetics · Ligand parameter · Bifunctional reagents · Redox polymers

2.1 Reaction-Layer Approximation in a Homogeneous System

The simplest situation of MET-type bioelectrocatalysis is that a redox enzyme ($E$) and a mediator ($M$) exist homogenously in a quiescent solution. In such situations, the system essentially provides a steady-state sigmoidal wave of the catalytic reaction. For the oxidation of the substrate ($S$), the reaction proceeds as follows:

$$S + \frac{n_S}{n_M} \text{Enzyme} \overset{\text{P} + \frac{n_S}{n_M} \text{M}_{\text{red}}}{\longrightarrow} \text{M}_{\text{red}} \overset{\text{Electrode}}{\rightarrow} \text{M}_{\text{ox}} + n_Me^-,$$

where $M_{\text{ox}}$ and $M_{\text{red}}$ denote the oxidized and reduced forms of $M$, respectively; $P$ denotes the product, whereas $n_S$ and $n_M$ denote the number of electrons in $S$ and $M$, respectively.

The enzymatic reaction (2.1) proceeds only in the diffusion layer of the oxidized mediator ($M_{\text{ox}}$) regenerated by the electrode reaction (2.2). The current ($i$) corresponds to the concentration gradient of $M_{\text{ox}}$ at the electrode surface ($x = 0$) as follows:
\[
\frac{i}{n_M F A} = -D_M \frac{\partial c_{M_{ox}(0,t)}}{\partial x},
\]

(2.3)

where \(D_M\) denotes the diffusion coefficient of \(M\), \(A\) denote the surface area of an electrode, and \(c_{M_{ox}(x,t)}\) denotes the concentration of \(M_{ox}\) and is the function of the distance from the electrode surface \((x)\) and the time \((t)\) in general. It is difficult to get general analytical solution of the current in MET-type bioelectrocatalysis, because of non-linear relation in the enzymatic reaction (2.1) to the concentration of substrate(s). Therefore, the steady-state assumption and the simplification of the enzymatic reaction mechanism are frequently applied to express the catalytic current of MET-type bioelectrocatalysis [1, 2].

Under the steady-state conditions, the reaction–diffusion equation in the 1D symmetric system is as follows:

\[
\frac{\partial c_{M_{ox}(x,t)}}{\partial t} = D_M \frac{\partial^2 c_{M_{ox}(x,t)}}{\partial x^2} - k_M c_{M_{ox}} = 0,
\]

(2.4)

where \(k_M\) denotes the enzymatic reaction rate constant of \(M_{ox}\), respectively. Under the situation, Eq. (2.4) provides the concentration profile of the mediator as follows:

\[
c_{M_{ox}(x,t)} = c_{M_{ox}(0)} \exp \left( -\frac{x}{\mu} \right),
\]

(2.5)

where \(\mu (= \sqrt{D_M/k_M})\) is called the thickness of the reaction layer and this model is known as reaction layer assumption [1]. Equations (2.3) and (2.5) are solved to get an analytical solution of the steady-state current \((i_s)\) as follows:

\[
\frac{i_S}{n_M F A} = k_M c_{M_{ox}(0)} \mu (= \sqrt{k_M c_{M_{ox}(0)}}).
\]

(2.6)

Fig. 2.1 A typical example of concentration profiles in steady-state MET-type bioelectrocatalysis in a homogeneous solution.
Figure 2.1 shows concentration profiles of $M_{\text{ox}}$ and the reduced mediator ($M_{\text{red}}$) in steady-state MET-type bioelectrocatalysis in a homogeneous solution with a $k_M$ value independent of $c_{M_{\text{ox}}}(x,t)$. The area of the exponentially decaying curve of $c_{M_{\text{red}}}(x,t)$ is identical with that of the rectangle-shape with hatched lines, when $k_M$ is independent of $c_{M_{\text{ox}}}(x,t)$. This means that $i_s$ corresponds to the steady-state rate of the regeneration of $M_{\text{red}}$ at an electrode.

When $M$ undergoes reversible response at an electrode, $c_{M_{\text{ox}}}(0)$ is given by

$$c_{M_{\text{ox}}}(0) = \frac{K_M}{1 + K_M} c_M^0 = \frac{c_{M_{\text{ox}}}^0}{2} \left\{ \tanh \left[ \frac{n_M F (E - E_{M}^{\circ})}{2RT} \right] + 1 \right\}, \quad (2.7)$$

with

$$K_M = \left( \frac{c_{M_{\text{ox}}}}{c_{M_{\text{red}}}} \right)_{x=0} = \left( \frac{c_{M_{\text{ox}}}^0}{c_M^0 - c_{M_{\text{ox}}}^0} \right)_{x=0} = \exp \left[ \frac{n_M F (E - E_{M}^{\circ})}{RT} \right], \quad (2.8)$$

where $c_M^0 (\equiv c_{M_{\text{red}}} + c_{M_{\text{ox}}})$ and $E_{M}^{\circ}$ denote the total concentration and the formal potential of $M$, respectively. Since $c_{M_{\text{ox}}}$ sigmoidally changes with the electrode potential ($E$), as given by Eq. (2.7), steady-state voltammograms under these situations provide a sigmoidal shape with its half-wave potential identical to $E_{M}^{\circ}$.

Figure 2.2a shows a typical example of steady-state voltammograms of MET-type bioelectrocatalysis.

Assuming ping-pong bi-bi mechanism, the enzymatic reaction rate of $M_{\text{ox}}$ ($v_M$) is given by

$$v_M = k_M c_{M_{\text{ox}}} = \frac{n_S}{n_M} \frac{k_{\text{cat}} c_E}{1 + \frac{K_{M(S)}}{c_S} + \frac{K_{M(M)}}{c_{M_{\text{ox}}}}}, \quad (2.9)$$

**Fig. 2.2** A Steady-state linear sweep voltammograms of MET-type bioelectrocatalysis, in which $E$ is FAD-dependent GDH, $S$ is glucose, and $M$ is 9,10-phenanthrene quinone, which is successively added into the solution. B The $i_{\text{lim}}$ values in panel a as a function of $c_M^0$. The linear line is given by Eq. (2.12), while the curve is given by Eq. (2.14)
where \( k_{\text{cat}} \) denotes the catalytic constant in solution, and \( K_{M(X)} \) is the Michaelis constant of \( X \). In the presence of an excess amount of \( S \) (i.e. \( c_S >> K_{M(S)} \)), \( k_M \) is given by

\[
k_M = \frac{n_S}{n_M} \frac{k_{\text{cat}} c_E}{c_{M_{\text{ox}}} + K_{M(M)}}. \tag{2.10}
\]

Furthermore, when \( c_M^0 \ll K_{M(M)} \), \( k_M \) becomes independent of \( c_{M_{\text{ox}}} \) and the enzyme reaction becomes linear to \( c_{M_{\text{ox}}} \):

\[
k_M = \frac{n_S}{n_M} \frac{k_{\text{cat}}}{K_{M(M)}} c_E \equiv k_2 c_E, \tag{2.11}
\]

where \( k_2 \equiv (n_S/n_M) \frac{k_{\text{cat}}}{K_{M(M)}} \) denotes the second-order reaction rate constant between \( E \) and \( M \), and it is independent of \( S \). Under the limiting condition: \( E \gg E_M^{\text{eq}} \), \( c_{M_{\text{ox}}(0)} \) becomes \( c_M^0 \), and the limiting value of the steady-state current \( (i_s^\text{lim}) \) is given by

\[
i_s^\text{lim} = FA c_M^0 \frac{n_S n_M D_M k_2 c_E}{\sqrt{2}} = \sqrt{2} \left[ c_M^0 \frac{K_{M(M)}}{K_{M(M)} - \ln \left( 1 + \frac{c_M^0}{K_{M(M)}} \right)} \right]. \tag{2.13}
\]

The parameter \( k_2 \) to be obtained in this system is very important for characterizing the enzymatic reaction between \( E \) and \( M \) and can be easily evaluated from the slope of the linear dependence of \( i_s^\text{lim} \) on \( c_M^0 \) (the linear line in Fig. 2.2B). Successive addition of a solution containing \( M \) is acceptable in the electrochemical measurements of \( i_s^\text{lim} \), as shown in Fig. 2.2A. This technique has a contrastive benefit for evaluating \( k_2 \) compared to conventional photometric measurements.

When we consider Eq. (2.10), \( i_s^\text{lim} \) is given by

\[
\frac{i_s^\text{lim}}{n_M FA \sqrt{(n_S/n_M) D_M k_{\text{cat}} K_{M(M)} c_E}} = \sqrt{2} \left[ \frac{c_M^0}{K_{M(M)}} - \ln \left( 1 + \frac{c_M^0}{K_{M(M)}} \right) \right]. \tag{2.13}
\]

The experimental data in Fig. 2.2B is well reproduced by Eq. (2.13). In the case: \( c_M^0 >> K_{M(M)} \), \( i_s^\text{lim} \) depends on the square root of \( c_M^0 \) as follows [3]:

\[
i_s^\text{lim} = FA \sqrt{2 n_S n_M D_M k_{\text{cat}} c_E c_M^0}. \tag{2.14}
\]

Under the conditions, the reaction thickness \( \mu \) is given by

\[
\mu = \sqrt{\frac{D_M c_M^0}{2(n_S/n_M) k_{\text{cat}} c_E}}. \tag{2.15}
\]
2.2 Selection of Mediators

Because the reaction between E and M is non-specific in essentials, and then a linear free energy relationship (LFER) given below may hold [4]:

\[
\ln \frac{k_j}{k_i} = \alpha \ln \frac{K_j}{K_i} = -\alpha \frac{(\Delta_r G_j^\circ - \Delta_r G_i^\circ)}{RT},
\]  

(2.16)

where \(\alpha\) denotes the transfer coefficient (0 < \(\alpha\) < 1), \(k_X\) and \(K_X\) denote the rate constant and the equilibrium constant of the rate-determining step (RDS) in the electron transfer between E and X, respectively, \(\Delta_r G_X^\circ\) and \(\Delta_r G_X^\circ\) denote the standard Gibbs energy of activation and reaction of the RDS, respectively. Equation (2.16) can be rewritten for the relation between \(k_2\) of the reaction between E and M and the formal potential of M (\(E_M^\circ\)) as follows:

\[
\log \left( \frac{k_{2,j}}{k_{2,i}} \right) = \frac{\alpha n_{M,RDS} F}{2.303 RT} \left( E_{M,j,RDS}^\circ - E_{M,i,RDS}^\circ \right) \quad \text{(for oxidation of } E_{\text{red}} \text{)},
\]

(2.17)

\[
\log \left( \frac{k_{2,j}}{k_{2,i}} \right) = \left( \alpha - 1 \right) \frac{n_{M,RDS} F}{2.303 RT} \left( E_{M,j,RDS}^\circ - E_{M,i,RDS}^\circ \right) \quad \text{(for reduction of } E_{\text{ox}} \text{)}.
\]

(2.18)

where \(n_{M,RDS}\) denotes the number of electrons in the RDS (usually \(n_{M,RDS} = 1\) even though the total number of electrons (n) is 2 for quinones). The slope \(\frac{\alpha n_{M,RDS} F}{2.303 RT}\) in the plot between \(\log[k_2/(M^{-1} s^{-1})]\) and \(E_{M,RDS}^\circ\) is about 8.5 V\(^{-1}\) at \(\alpha = 0.5\), \(T = 298\ K\), and \(n_{M,RDS} = 1\) (\(M = \text{mol dm}^{-3}\)). To be exact, \(E_{M,RDS}^\circ\) denotes the formal potential of the RDS with \(n_{M,RDS}\), but it may be assumed to be close to the formal potential of the overall electron transfer step with \(n\) (i.e. \(E_{M,RDS}^\circ \approx E_M^\circ\) for quinones with \(n = 2\)). It is worthy of note that \(E_M^\circ\) is measurable and Eqs. (2.19) and (2.20) can be used in practice without any detailed knowledge of the RDS:

\[
\log \left( \frac{k_{2,j}}{k_{2,i}} \right) = \frac{\alpha n_{M,RDS} F}{2.303 RT} \left( E_{M,j}^\circ - E_{M,i}^\circ \right) \quad \text{(for oxidation of } E_{\text{red}} \text{)},
\]

(2.19)

\[
\log \left( \frac{k_{2,j}}{k_{2,i}} \right) = \left( \alpha - 1 \right) \frac{n_{M,RDS} F}{2.303 RT} \left( E_{M,j}^\circ - E_{M,i}^\circ \right) \quad \text{(for reduction of } E_{\text{ox}} \text{)}.
\]

(2.20)

The value of \(k_2\) can reach the limiting value at increased value of \(E_M^\circ\) because of sufficiently increased driving force in the electron transfer from \(E_{\text{red}}\) to \(M_{\text{ox}}\) (and vice versa for reduction of \(E_{\text{ox}}\)). The limiting value of \(k_2\) is almost identical to the diffusion-controlled second-order reaction rate constant \(k_d\) defined as [4]

\[
k_d = 4\pi (r_E + r_M)(D_E + D_M)N_A,
\]

(2.21)
Fig. 2.3 A typical example of the relation between \( \log[k_2/(M^{-1}\text{ s}^{-1})] \) and \( E_M^{\circ'} \) in MET-type bioelectrocatalysis in the system containing FAD-dependent GDH and quinones

where \( r_X \) denotes the radius of \( X \) and \( N_A \) denotes the Avogadro number. When we assume that \( r_E + r_M \approx 2 \text{ nm} \) and \( D_E + D_M \approx 2 \times 10^{-7} \text{ cm}^2\text{ s}^{-1} \), \( \log[k_{d}/(M^{-1}\text{ s}^{-1})] \) is calculated to be about 8.5. In this model, the reaction is assumed to occur at the entire surface of an enzyme. When the reaction site is limited on the surface, the \( k_d \) value decreases. On the other hand, electrostatic attractive interaction increases \( k_d \).

The first evidence of the LFER was reported diaphorase-catalyzed MET-type bioelectrocatalysis of NADH and NAD\(^+\) [5, 6]. Similar relations were observed in MET-type bioelectrocatalysis of bilirubin oxidase [7], laccase [8, 9], PQQ-dependent glucose dehydrogenase (GDH) [10], fructose dehydrogenase (FDH) [11], and W-containing formate dehydrogenase [12].

Figure 2.3 shows an example of the relation between \( \log[k_2/(M^{-1}\text{ s}^{-1})] \) and \( E_M^{\circ'} \) for a dehydrogenase. Most of the data are located close to the two broken lines given by Eqs. (2.19) and (2.21). Some data located further upward and downward from the lines indicate the occurrence of some specific attractive and repulsive interactions, respectively. This is mainly due to electrostatic interaction. For \( E_{\text{red oxidation}} \), \( M \) with less positive \( E_M^{\circ'} \) is recommended in order to minimize the overpotential in the MET-type reaction, while \( M \) with more positive \( E_M^{\circ'} \) is recommended in order to increase the current density (vice versa for \( E_{\text{ox reduction}} \)). This scenario is very important for selecting \( M \) for an enzyme and for optimizing the MET-type bioelectrocatalytic system.

2.3 Mathematical Model in Immobilized Layers

In this section, let us consider an enzymatic oxidation of \( S \) with \( M_{\text{ox}} \) in \( E^- \) and \( M^- \) immobilized layer, as a MET-type reaction system (Fig. 2.4). Mathematical models describe steady-state electrochemical currents and concentration profile of \( S \) and \( M \) (and \( E \) species). When the thickness of the immobilized layer (\( l \)) is smaller than \( \mu \), the concentration polarization of \( M \) occurs only within a finite thickness of the immobilized layer. Since the solution is usually stirred, the concentration polarization is restricted within the immobilized layer and the membrane. As a result, the catalytic
mass transfer  permeation  enzyme reaction  electrode reaction

\( \dot{i}_{s,mt} \quad \dot{i}_{s,perm} \quad \dot{i}_{s,enz} \quad \dot{i}_{s,elec} \)

Fig. 2.4 A serial resistance model of MET-type bioelectrocatalysis

current reaches a steady state after a certain period of time in any case. In the immobilized layer, the amount of mediator may be usually excess (i.e. \( c_{0}^{M} \gg K_{M(M)} \)) and the enzymatic reaction rate constant (\( k_{M} \)) and the thickness of reaction layer are given by Eq. (2.10) and Eq. (2.15), respectively. The boundary conditions are given by

\[ c_{M_{red}}(x=0) = 0, \quad \text{(2.22)} \]

and

\[ \left( \frac{\partial c_{M_{red}}}{\partial x} \right)_{x=l} = 0. \quad \text{(2.23)} \]

Integration of Eq. (2.3) with the boundary conditions of Eqs. (2.21) and (2.24) gives [13, 14]

\[ \frac{i_{s}^{\text{lim}}}{n_{M}FA} = \frac{n_{S}}{n_{M}} D_{M} c_{0}^{M} \frac{\mu}{\mu} \tanh \frac{l}{\mu}. \quad \text{(2.24)} \]

Equation (2.24) represents the relationship between \( l \) and \( \mu \). When \( l \ll \mu \), the Eq. (2.25) is reduced to

\[ \frac{i_{s}^{\text{lim}}}{n_{M}FA} = 2 \frac{n_{S}}{n_{M}} k_{\text{cat}} c_{E} l. \quad \text{(2.25)} \]

Conversely, when \( l \gg \mu \), the Eq. (3.24) is reduce to Eq. (2.14). These \( l \) dependence of \( i_{s}^{\text{lim}} \) was reported for PQQ-dependent GDH- and osmium (Os) complex-immobilized hydrogel prepared by layer-by-layer method [15].

On the other hand, when \( c_{S} \ll K_{M(S)} \), \( i_{s}^{\text{lim}} \) is observed at \( E \gg E_{M}^{\text{eq}} \) (for oxidation of \( S \)) under stirring and steady-state conditions given by equations similar to Eq. (2.4) for \( S \) and \( M \). \( i_{s}^{\text{lim}} \) is related to the concentration gradients of \( M_{\text{red}} \) and \( S \).

\[ \frac{i_{s}^{\text{lim}}}{n_{M}FA} = D_{M} \left( \frac{dc_{M_{red}}}{dx} \right)_{x=0} = D_{S} \left( \frac{dc_{S}}{dx} \right)_{x=l} = P_{S} \left( c_{S}^{*} - \frac{c_{S,x=l}}{\beta_{S}} \right). \quad \text{(2.26)} \]
where $P_S$ and $\beta_S$ are the permeability of the semipermeable membrane of $S$ and the distribution coefficient of $S$ between the immobilized layer and the membrane, respectively. The maximum current ($i_S^\text{lim,max}$) is given by

$$i_S^\text{lim,max} = n_M F A k_{\text{cat}} c_E l. \quad (2.27)$$

By using Eqs. (2.26) and (2.27), $i_S^\text{lim}$ values can be numerically obtained for a given value of $c_{S,x=l}$. Numerical calculation supports the fact that $i_S^\text{lim}$ versus the bulk concentration of $S$ ($c_s^b$) profiles exhibit Michaelis–Menten-type curved characteristics. The empirical equation is given by [16]

$$i_S^\text{lim} = \frac{i_S^{\text{lim,max}} c_s}{K_{M(S)} + c_s}, \quad (2.28)$$

where $i_S^{\text{lim,max}}$ and $K_{M(S)}$ are the apparent value of $i_S^\text{lim,max}$ and $K_M(S)$, respectively.

A mathematical model that explains the kinetic features of MET-type reaction in a variety of immobilized layers would be helpful to optimize the operational parameters of electrodes in their practical use. Bartlett and Pratt solved the reaction–diffusion equations and built the case diagram based on complete analytical solutions derived for a set of limiting cases [17]. In their study, they only considered a Michaelis–Menten kinetics for $S$ conversion.

More recently, analytical solutions were derived using homotopy perturbation method combined with the inversion conjuncture in the Laplace plane [18, 19]. While limiting cases mostly describe steady-state electrochemical responses, this method allowed describing current densities in chronoamperometry at different potentials both in the transient and steady states. It was applied to an electrode based on MET-type reaction of laccase in an Os redox hydrogel. The time to reach steady state was determined and proved to depend on the potential of the redox mediator. Moreover, two graphical procedures were proposed for the estimation of $K_M$ values. In both cases, the model was validated by fitting with experimental data of O$_2$-reducing laccase cathodes [19].

In the first study of Bartlett mentioned above, numerical simulations of the 1D model were performed based on the relaxation method and were in good agreement with the analytical study. This 1D model was then applied to an enzymatic O$_2$ reducing porous cathode [20]. The composite electrode was modeled as cylindrical conducting fibers around which the reaction layer film was coated. The influence of morphology (in terms of film thickness, composite porosity, and fiber diameter) was evaluated. The author also studied how to improve mass transport of the O$_2$ substrate and predicted that the maximum current densities could be close to 100 mA cm$^{-2}$, for O$_2$-saturated conditions [20]. While this study mainly focused on thick films, another group proposed to consider high-surface area electrodes grafted with thin polymer layers [21]. In that case also it was proposed that current densities around 100 mA cm$^{-2}$ could be achieved with porous electrodes.
A model of an actual MET-type glucose/O₂ biofuel cell composed of a cascade of enzymes and redox mediators at the anode and cathode has also been reported [22]. This model was used to describe a biofuel cell formerly reported by Sakai et al. [23], in which both enzymatic systems were immobilized on porous carbon-fiber electrodes. The simulated data agreed with the experimental results.

### 2.4 Serial Resistance Model for Steady-State Response in MET-Type Bioelectrocatalysis

More specific description of the steady-state response in MET-type systems can be derived using a serial resistance model. Here one considers a set of \( p \) reactions in series. Under the steady-state conditions, we can define a reaction resistance \((R)\) as

\[
\frac{dG}{dt} = Rv = \sum_1^p R_m v = \text{const.} \quad \text{or} \quad R = \sum_1^p R_m, \tag{2.29}
\]

where \( G \) denotes the Gibbs energy and \( v \) denotes the reaction rate. When the \( m \)th reaction is the RDS, the reaction rate \((v_m)\) can be expressed as

\[
v_m = \frac{Rv}{R_m} = k_m c, \quad \tag{2.30}
\]

where \( k_m \) denotes the first-order reaction rate constant when the \( m \)th reaction is RDS. Combining Eqs. (2.29) and (2.30), we can obtain the following equation for expressing the reaction kinetics of a set of \( p \) reactions in series [4]:

\[
\frac{1}{v} = \sum_1^p \frac{1}{v_m} \quad \text{or} \quad \frac{1}{k} = \sum_1^p \frac{1}{k_m}. \tag{2.31}
\]

The serial resistance model is essential in describing the steady-state current. When we assume a set of series reactions as illustrated in Fig. 2.4 for an MET-type reaction at an enzyme/mediator-immobilized electrode, the steady-state catalytic current \((i_s)\) can be expressed as

\[
\frac{1}{i_s} \approx \frac{1}{i_{s,mt}} + \frac{1}{i_{s,perm}} + \frac{1}{i_{s,enz}} + \frac{1}{i_{s,elec}}, \tag{2.32}
\]

where \( i_{s,mt} \) denotes the mass-transfer-controlled steady-state current and is given by Levich equation at a rotating disk electrode (RDE) at an angular rotation rate in radian \((\omega)\) with a kinematic viscosity \((\nu)\) as

\[
i_{s,mt(\text{RDE})} = 0.620 n_F A D_S^{2/3} \nu^{-1/6} \omega^{1/2} c_S, \tag{2.33}
\]
or by the following equation at a microdisk electrode with a radius \( r \):

\[
i_{s,mt(\text{microdisk})} = 4n_S F D_S r c_S.
\]  

(2.34)

The permeation-controlled steady-state current \( i_{s,\text{perm}} \) at a permeation coefficient \( (P_S) \) can be expressed as

\[
i_{s,\text{perm}} = n_S F A P_S c_S.
\]  

(2.35)

The enzyme reaction-controlled steady-state current \( i_{s,\text{enz}} \) is empirically given by a Michaelis–Menten-type equation under the assumption of the presence of an excess amount of \( M \):

\[
i_{s,\text{enz}} = \pm n_S F A \frac{k_{\text{cat}} \Gamma_E c_S}{c_S + K_M(S)},
\]  

(2.36)

where \( \Gamma_E \) denotes the surface concentration of \( E \). The (one-way) electrode reaction-controlled steady-state current \( i_{s,\text{elec}} \) can be expressed using a Butler–Volmer-type equation based on LFER as

\[
i_{s,\text{elec}} = n_S F A k_M^o c_S K_M^{1-\alpha} \text{(for oxidation)},
\]  

(2.37)

and

\[
i_{s,\text{elec}} = -n_S F A k_M^o c_S K_M^{-\alpha} \text{(for reduction)},
\]  

(2.38)

with

\[
K_M' = \left( \frac{c_{M_{\text{ox}}}}{c_{M_{\text{red}}}} \right)_{\text{RDS,eq}} = \exp \left[ \frac{n_{M,\text{RDS}} F}{RT} \left( E - E_M^{o,\text{RDS}}' \right) \right],
\]  

(2.39)

where \( k_M^o \) denotes the standard rate constant of the interfacial electron transfer in the RDS of \( M \) at an electrode, and Eq. (2.39) is the Nernst equation of the RDS of the interfacial electron transfer of \( M \) with a number of the electron \( (n_{X,\text{RDS}}; \text{usually } n_{X,\text{RDS}} = 1) \) and a formal potential of \( E_M^{o,\text{RDS}} \), which may often be assumed to be close to the formal potential \( \left( E_M^{o,\text{RDS}} \right) \) of the overall step involving \( M \) with \( n_M \). \( \alpha \) is the transfer coefficient of the RDS of \( M \) at an electrode, and it is ideally 0.5.

### 2.5 Redox Mediators

One of the most important challenges in MET-type bioelectrocatalysis is the use of redox mediators that shuttle electrons between the redox centre of enzymes and electrodes thus enhanced the electron transfer [24]. From this point of view, an ideal
A redox mediator should be able to react rapidly with enzymes and electrodes (i.e., as fast heterogeneous and homogeneous electron transfer), be stable in both oxidized and reduced forms under the working conditions, and do not participate in side reactions during the electron transfer [25]. On the other hand, as described above, $E^{\circ\prime}$ of redox mediators is another important consideration in the choice of mediators. Figure 2.5 summarizes $E^\circ$ of several redox mediators used in MET-type bioelectrocatalysis. Formal potentials of inorganic (Table 2.1) and organic compounds (Table 2.2) are summarized and would be useful for the selection of mediators. To provide an appropriate potential gradient for electron transfer, $E^{\circ\prime}$ of a mediator should be more positive than that of the electrochemically active site of an enzyme for oxidative bioelectrocatalysis (and vice versa for reductive reaction) [25]. The $E^{\circ\prime}$ values of some redox mediators can be tuned by several approaches: for example by changing the ligands of metal complexes by assuming that all ligand contributions are additive. The ligand effect has been parameterized and $E^{\circ\prime}$ can be empirically calculated with the parameters ($E_L$) [26]:

$$E^{\circ\prime} = S_M \sum E_L + I_M,$$  \hspace{1cm} (2.40)

where $S_M$ and $I_M$ are constants for a given metal complex. The $E^{\circ\prime}$ of Os complexes and redox polymers in solution can be predicted by using $E_L$ [27].

A huge number of compounds, including natural and artificial redox couples, have been utilized in MET-type bioelectrocatalysis. As cofactors of redox enzymes, FAD [28] and PQQ [29] can be employed as natural redox mediators for MET-type bioelectrocatalysis. On the other hand, artificial compounds ranging from organic to...
### Table 2.1 Formal potentials metal complexes in aqueous solution

| Compound*1 | $E^o/N$ versus SHE | pH | Conditions*2 | References |
|------------|---------------------|----|--------------|------------|
| Water soluble Fe porphyrin $^{*3}$(Fe: $4^+/3^+$) | 1.341 | 5.0 | $I = 0.2$ | [82] |
| [Os(terpy)$_3$]$^{3+/2+}$ | 0.987 | | | $^*_{[83]}$ |
| [Rh(CN)$_6$]$^{3--/4--}$ | 0.9 | (standard potential) | | $^*_{[83]}$ |
| [Os(terpy)(bpy)(py)]$^{3+/2+}$ | 0.871 | | | $^*_{[83]}$ |
| [Os(py)$_2$(bpy)$_2$]$^{3+/2+}$ | 0.834 | | | $^*_{[83]}$ |
| [Os(bpy)$_3$]$^{3+/2+}$ | 0.885 | 7.0 | 0.1 M phosphate | [84] |
| [IrBr$_6^{2+/3-}$ | 0.805 | | | $^*_{[83]}$ |
| [Os(terpy)py$_3$]$^{3+/2+}$ | 0.800 | | | $^*_{[83]}$ |
| [Mo(CN)$_8$]$^{3--/4--}$ | 0.778 | | | $^*_{[86]}$ |
| [Fe(bpy)$_2$]$^{3+/2+}$ | 0.76 | 5.3 | 8 mM MES | $^*_{[86]}$ |
| [Os(4,4'-Me$_2$-bpy)$_3$]$^{3+/2+}$ | 0.638 | 7.0 | 0.1 M phosphate | [84] |
| [Co(ox)$_3$]$^{3--/4--}$ | 0.057 $\pm$ 0.02 | | 1 M KCl | $^*_{[83]}$ |
| [Os(terpy)(bpy)Cl]$^{2+/1+}$ | 0.563 | | | $^*_{[83]}$ |
| [W(CN)$_8$]$^{3--/4--}$ | 0.52 | 7 | 0.1 M phosphate, 0.1 M KCl | [87] |
| [Os(2-Me-im)$_2$(bpy)$_2$]$^{3+/2+}$ | 0.502 | 7.0 | 0.1 M phosphate | [84] |
| Ferrocene dicarboxylate | 0.501 | 7.0 | 0.1 M phosphate | [84] |
| | 0.513 | 7 | 0.05 M phosphate, $I = 0.1$ | [88] |
| [Os(im)$_2$(bpy)$_2$]$^{3+/2+}$ | 0.487 | 7.0 | 0.1 M phosphate | [84] |
| [Os(4-Me-im)$_2$(bpy)$_2$]$^{3+/2+}$ | 0.453 | 7.0 | 0.1 M phosphate | [84] |
| OsBr$_6^{2--/3--}$ | 0.45 | | | $^*_{[83]}$ |
| [Fe(CN)$_6$]$^{3--/4--}$ | 0.710 | | 1 M HCl | [89] |
| | 0.560 | | 0.1 M HCl | [89] |
| | 0.443 | | | $^*_{[86]}$ |

(continued)
### Table 2.1 (continued)

| Compound*¹ | $E^{°}/N$ versus SHE | pH | Conditions*² | References |
|------------|----------------------|----|--------------|------------|
| ferrocene ethanol | 0.419 | 7 | 0.05 M phosphate, $I = 0.1$ | [88] |
| [Os(im)$_2$(4,4′-Me$_2$-bpy)$_2$]$^{3+/2+}$ | 0.351 | 7.0 | 0.1 M phosphate | [84] |
| [Co(edta)]$^{1+/2−}$ | 0.376 | * | [83] |
| [Co(pdta)]$^{1+/2−}$ | 0.36 | * | [83] |
| [Co(cydtta)]$^{1+/2−}$ | 0.36 | * | [83] |
| [Co(phen)$_3$]$^{3+/2+}$ | 0.40 | 0 °C | ** | [83] |
| | 0.327 ± 0.02 | 8 mM phen, 1 M KCl | * | [83] |
| | 0.349 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [OsCl(1-Me-im)(bpy)$_2$]$^{3+/2+}$ | 0.312 | 7.0 | 0.1 M phosphate | [84] |
| [OsCl(im)(bpy)$_2$]$^{3+/2+}$ | 0.304 | 7.0 | 0.1 M phosphate | [84] |
| [Co(5-Me-phen)$_3$]$^{3+/2+}$ | 0.293 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Co(trdta)]$^{1+/2−}$ | 0.29 | * | [83] |
| [Ru(NH$_3$)$_5$(py)]$^{3+/2+}$ | 0.305 | 1 M NaCl | * | [83] |
| | 0.289 | 5.0 | 5 mM acetate, 0.2 M NaCl | [85] |
| [Co(bpy)$_3$]$^{2+/3+}$ | 0.34 | 0 °C | * | [83] |
| | 0.281 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Ru(NH$_3$)$_5$(4-thmpy)]$^{3+/2+}$ | 0.281 | 5.0 | 5 mM acetate, 0.2 M NaCl | [85] |
| Fe$^{3+/2+}$, malonate | 0.26 | 4 | * | [91] |
| Fe$^{3+/2+}$, salicylate | 0.26 | 4 | * | [91] |
| [Ru(NH$_3$)$_5$(4-Me-py)]$^{3+/2+}$ | 0.252 | 5.0 | 5 mM acetate, 0.2 M NaCl | [85] |
| [Co(terpy)$_2$]$^{3+/2+}$ | 0.31 | 0 °C | * | [83] |
| | 0.237 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Co(4-Me-phen)$_3$]$^{3+/2+}$ | 0.236 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |

(continued)
| Compound*1 | $E^\circ/N$ versus SHE | pH | Conditions*2 | References |
|------------|------------------------|----|--------------|------------|
| [Co(5-NH$_2$-phen)$_3$]$^{3+/2+}$ | 0.221 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Co(4,7-(bhm)$_2$phen)$^{3+/2+}$ | 0.212 | 5.0 | 5 mM acetate, 0.2 M NaCl | [85] |
| [Co(5,6-Me$_4$-phen)$_3$]$^{3+/2+}$ | 0.211 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| $trans$-[Co(gly)$_3$]$^{0/1-}$ | 0.20 | | 1 M KCl | * [83] |
| [OsCl(1-Me-Im)(4,4$'$-Me$_2$-bpy)$_2$]$^{3+/2+}$ | 0.199 | 7.0 | 0.1 M phosphate | [84] |
| [OsCl(Im)(4,4$'$-Me$_2$-bpy)$_2$]$^{3+/2+}$ | 0.191 | 7.0 | 0.1 M phosphate | [84] |
| [Fe(edta)]$^{1−/2−}$ | 0.158 | 7 | 0.05 M phosphate, $I = 0.1$ | [88] |
| [Co(4,7-Me$_2$-phen)$_3$]$^{3+/2+}$ | 0.147 | 5.0 | 5 mM acetate, 0.2 M NaCl | [85] |
| [Co(4,7-Me$_2$-phen)$_3$]$^{3+/2+}$ | 0.145 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Co(3,4,7,8-Me$_4$-phen)$_3$]$^{3+/2+}$ | 0.122 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Co(NH$_3$)$_6$]$^{3+/2+}$ | 0.058 | | 7 M NH$_3$, 1 M NH$_4$Cl | * [83] |
| [Ru(NH$_3$)$_6$]$^{3+/2+}$ | 0.10 | | 0.01 M HClO$_4$ | * [83] |
| | 0.030 | 7.5 | 0.02 M Tris, 0.1 M KCl | [90] |
| [Fe(ox)$_3$]$^{3−/4−}$ | 0.005 | | $>0.2$ M Na$_2$C$_2$O$_4$ | * [83] |
| Water soluble Fe porphyrin*3(Fe: 3$^+/2^+$) | $−0.029$ | 5.0 | $I = 0.2$ | [82] |
| $cis$-[Ru(NH$_3$)$_4$Cl$_2$]$^{1+/0}$ | $−0.100$ | | | * [83] |
| $trans$-[Ru(NH$_3$)$_4$Cl$_2$]$^{1+/0}$ | $−0.180$ | | | * [83] |
| [Co(dien)$_2$]$^{3+/2+}$ | $−0.233 \pm 0.002$ | | 0.1 M dien, 1 M KCl | * [83] |
| [Mn(CN)$_6$]$^{3−/4−}$ | $−0.24$ | | | * [83] |
| [Mo$_2$O$_3$(edta)$]^{2−/3−}$ | $−0.453$ | 4.70 | 0.4 M acetate | * [83] |
| [Mo$_2$O$_2$S$_2$(edta)$]^{2−/3−}$ | $−0.462$ | 4.70 | 0.4 M acetate | * [83] |
| [MoO$_4$(edta)$]^{2−/3−}$ | $−0.633$ | 4.70 | 0.4 M acetate | * [83] |
| [Cr(edta)(H$_2$O)$_2$]$^{1−/2−}$ | $−0.99$ | | 0.1 M KCl | * [83] |
| [Mn(CN)$_6$]$^{4−/5−}$ | $−1.06$ | | 1.5 M NaCN | * [83] |

(continued)
Table 2.1 (continued)

| Compound*1 | \(E^\circ/N\) versus SHE | pH | Conditions*2 | References |
|------------|------------------------|----|--------------|------------|
| \([\text{Cr(CN)}_6]^{3−/4−}\) | −1.143 | 1 M KCN | *83 | |

*1bpy: 2,2′-bipyridine
dpy: 2,2′-dipyridine
phen: 1,10-phenanthroline
terpy: 2,2′:6′,2″-terpyridine
im: imidazole
py: pyridine
thmppy: 4-(tris(hydroxymethyl)methyl)pyridine
bhm:
bis(bis(hydroxymethyl)methyl)
dien: diethylenetriamine
ox: oxalate diaminantetraacetate tetraanion
gly: glycinate anion
ppta: propylenediaminetetraacetate tetraanion
trdta: trimethylenediaminetetraacetate tetraanion
cyda: 1,2-cyclohexanediaminetetraacetate tetraanion

*2MES: 2-(N-morpholino)ethane-sulfonate buffer
Tris:
\(M = \text{mol dm}^{−3}\)
\(I: \text{ionic strength}\)
tris(hydroxymethyl)aminomethane
*3[5,10,15,20-tetrakis(2,6-dichloro-3-sulfonato)porphyrinato]Fe(H_2O)(OH^-)

inorganic molecules, including methylene blue, prussian blue, toluidine blue, thionine, neutral red, quinone derivatives, ferrocene and its derivate, and inorganic redox ions such as ferri/ferrocyanide were frequently utilized as redox mediators for rapid electron transfer between enzymes and electrodes [30]. Besides, some small electron-transfer proteins like cytochromes [31–34], and protein nanowires [35, 36] also could act as electron carriers for MET-type bioelectrocatalysis.

2.6 Immobilization of Enzymes and Mediators

In order to avoid the leakage of mediators used, semipermeable membranes are often utilized in MET-type bioelectrodes. However, the use of semipermeable membranes may causes unexpected complexity in the electrodes or resistance in the mass transfer of enzyme substrates to the electrode surface. As a result, various methods for co-immobilization of enzymes and mediators have been developed in the past decades.
Table 2.2  Formal potentials of organic compounds in aqueous solution

| Compound                                                                 | $E^{\circ}/V$ versus SHE | pH | Conditions                        | References |
|-------------------------------------------------------------------------|--------------------------|----|-----------------------------------|------------|
| [5,10,15,20-tetrakis(2,6-dichloro-3-sulfonato)porphyrinato]            | 1.581                    | 5  | $I = 0.5$                         | [82]       |
| Fe(H$_2$O)(OH$^-$)                                                      |                          |    |                                   |            |
| ABTS (2,2'-azinobis-(3-ethylbenzthiozoline-6-sulfonic acid)(0/1^-)     | 1.097                    | 7  | 0.05 M phosphate, $I = 0.1$       | [92]       |
| Promazine ($n = 1$)[ammonium form]                                      | 0.53                     |    | (standard potential)              | *[93]      |
| chloramine-T                                                            | 0.9                      | 7  |                                   | [94, 91]   |
| TMPDA (N,N,N',N'-tetramethylphenyldiamine)(2+/1+)                       | 0.749                    | 7  | 0.1 M phosphate, $I = 0.1$; Reversible on CV at 1 V s$^{-1}$ | [95]       |
| porphyrexide                                                            | 0.718                    | 7  | 0.1 M phosphate, 0.1 M NaCl       | [96]       |
| 0.725                                                                  |                          |    |                                   |            |
| ABTS(1^-/2^-)                                                           | 0.63                     | 5.3| 8 mM MES                          | [86]       |
| 0.702                                                                  |                          | 7  | 0.05 M phosphate, $I = 0.1$       | [92]       |
| syringaldazine                                                          | 0.7                      | 5.3| 8 mM MES                          | [86]       |
| o-tolidine                                                              | 0.55                     | 7  |                                   | [91, 97]   |
| bacteriochlorophyll $a$                                                 | 0.52                     |    | methanol                          | [91, 98]   |
| dopamine                                                                | 0.386                    | 7  | 0.1 M phosphate ($I = 0.1$)       | [95]       |
| 2,5-dihydroxy-1,4-benzoquinone                                          | 0.38                     | 7  |                                   | [91, 97]   |
| $p$-aminodimethylaniline                                                | 0.38                     | 7  |                                   | [91, 97]   |
| o-quinone/1,2-hydroxybenzene(catechol)                                  | 0.35                     | 7  |                                   | [91, 97]   |
| $p$-aminophenol                                                         | 0.314                    | 7  |                                   | [91, 97]   |
| tetrahydroxy-$p$-benzoquinone                                           | 0.312                    | 7  | 0.1 M phosphate ($I = 0.1$)       | [95]       |
| 2,5-dichloro-$p$-benzoquinone                                           | 0.302                    | 7  | 0.1 M phosphate ($I = 0.1$)       | [95]       |

(continued)
Table 2.2 (continued)

| Compound | $E^{\circ}/V$ versus SHE | pH | Conditions | References |
|----------|-------------------------|----|------------|------------|
| 1,4-benzoquinone | 0.293 | 7 | | [91, 97] |
| | 0.28 | | | |
| | 0.293 | 7 | 0.1 M phosphate ($I = 0.1$) | [95] |
| TMPDA (1+/0) | 0.276 | 7 | 0.1 M phosphate ($I = 0.1$) | [95] |
| diaminodurene | 0.263 | 7 | 0.1 M phosphate ($I = 0.1$) | [95] |
| 2,5-dihydroxyphenylacetic acid | 0.26 | 7 | | [91, 97] |
| 2,6,2'-trichloroindophenol | 0.254 | 7 | | [91, 97] |
| indophenol | 0.228 | 7 | | [91, 97] |
| $o$-toluidine blue | 0.224 | 7 | | [91, 97] |
| DCPIP (2,6-dichlorophenolindophenol) | 0.24 | 7 | 0.1 M phosphate, 0.1 M KCl | [99] |
| | 0.227 | 7.1 | 0.05 M MOPS, 0.2 M NaNO₃ | [100] |
| | 0.217 | 7 | | |
| 2,6-dibromomindophenol | 0.216 | 7 | | [91, 97] |
| phenol blue | 0.224 | 7 | | [91, 97] |
| 3-aminothiazine | 0.208 | 7 | | [91, 97] |
| 1,2-naphthoquinone-4-sulfonate | 0.174 | 7.5 | 0.1 M phosphate | [95] |
| 2,6-dimethyl-$p$-benzoinonic | 0.169 | 7 | 0.05 M phosphate ($I = 0.1$) | [95] |
| 2,6-dibromo-2' methoxy-indophenol | 0.161 | 7 | | [91, 97] |
| 2,3-dimethoxy-5-methyl-1,4-benzoquinone | 0.159 | 7.5 | 0.1 M phosphate | [95] |
| 2,5-dimethyl-$p$-benzoquinone | 0.15 | 7 | 0.1 M phosphate ($I = 0.3$) | [95] |
| 1,4-dihydoxynaphthoic acid | 0.153 | 7 | 0.1 M phosphate ($I = 0.5$) | [95] |
| Compound                                      | $E^\circ/V$ versus SHE | pH | Conditions                      | References       |
|-----------------------------------------------|------------------------|----|---------------------------------|------------------|
| 2,6-dimethylindophenol                        | 0.148                  | 7  |                                 | [91, 97]         |
| 5-isopropyl-2-methyl-p-benzoquinone           | 0.147                  | 7  | 0.1 M phosphate ($I = 0.3$)     | [95]             |
| 1,2-naphthoquinone                            | 0.143                  | 7  |                                 | [91, 101]        |
| 1-naphthol-2-sulfonate indophenol             | 0.123                  | 7  |                                 | [91, 97]         |
| toluylene blue                                | 0.115                  | 7  |                                 | [91, 97]         |
| TTQ (tryptophan tryptophylquinone) model      |                        |    |                                 |                  |
| (3-methyl-4-(3′-methylindol-2′-yl)indol-6,7-dione) | 0.107                  | 7  | phosphate, 20 v/v % DMSO        | [102]            |
| Ubiquinone (coenzyme Q)                       | 0.1                    | 7  | 95% ethanol                     | [91, 103]        |
| PMS (N-methylphenazinium methosulfate)        | 0.08                   | 7  |                                 | [91, 98, 104]    |
| TPQ (topa quinone or 6-hydroxydopa quinone)   | 0.079                  | 7  | phosphate ($I = 0.5$)           | [105]            |
| PQQ (pyrroloquinoline quinone)                | 0.066                  | 7  | phosphate ($I = 0.5$)           | [106]            |
| thionine                                      | 0.09                   | 7  |                                 | [107]            |
| thionine-tetrasulfonate                       | 0.063                  | 7  |                                 | [91, 97]         |
| ascorbic acid                                 | 0.058                  | 7  |                                 | [91, 97]         |
| PES (phenazine ethosulfate)                   | 0.055                  | 7  |                                 | [91, 97]         |
| cresyl blue                                   | 0.047                  | 7  |                                 | [91, 97]         |
| 1,4-naphthoquinone                            | 0.036                  | 7  |                                 | [91, 97]         |
| toluidine blue                                | 0.027                  | 7  | 0.1 M phosphate, 0.1 M NaCl     | [96]             |
| thiazine blue                                 | 0.027                  | 7  |                                 | [91, 97]         |
| galloycyanine                                 | 0.021                  | 7  |                                 | [91, 97]         |
| Compound                              | $E^{\circ}/V$ versus SHE | pH | Conditions                                    | References |
|--------------------------------------|--------------------------|----|----------------------------------------------|------------|
| thioindigo disulfonate               | 0.014                    | 7  |                                              | [91, 97]   |
| methylene blue                       | −0.013                   | 7.1| 0.1 M MOPS, 0.2 M KCl                       | [100]      |
|                                     | 0.011                    | 7  |                                              | [91, 97]   |
|                                     | 0.011                    | 7  | 15 °C                                        | [108]      |
| vitamin K₃(2-methyl-1,4-naphthoquinone) | 0.009                   | 7  | (calculated from $E^{\circ}$)                | [91, 97]   |
|                                     | −0.010                   | 7  | 0.1 M phosphate ($I = 0.1$)                  | [109]      |
| pycocyanine                          | −0.034                   | 7  |                                              | [91, 104]  |
| indigo tetrasulfonate                | −0.046                   | 7  |                                              | [91, 97]   |
|                                     | −0.040                   | 7  | 15 °C                                        | [108]      |
| vitamin K₁(2-methyl-3-phyty-1,4-naphtoquinone) | −0.05                   | 7  |                                              | [91, 97]   |
| luciferin                             | −0.05                    | 7  |                                              | [91, 101]  |
| gallocyanine                         | −0.054                   | 8.5| 15 °C                                        | [108]      |
| methyl capri blue                    | −0.061                   | 7  |                                              | [91, 97]   |
| resorufin                             | −0.07                    | 7.4| 0.1 M phosphate, 36 °C                       | [110]      |
| 2-amino-3-carboxy-1,4-naphtoquinone  | −0.071                   | 7  | McIlvaine ($I = 0.5$)                       | [111]      |
| 2-farnesyl-3-methyl-1,4-naphtoquinone| −0.074                   | 7  | 0.1 M phosphate ($I = 0.1$)                  | [109]      |
| indigo trisulfonate                  | −0.081                   | 7  |                                              | [91, 97]   |
|                                     | −0.080                   | 7  | 0.05 M phosphate ($I = 0.1$)                 | [95]       |
|                                     | −0.060                   | 7  | 15 °C                                        | [108]      |
| 4-amino-1,2-naphthoquinone           | −0.096                   | 7  | 0.1 M phosphate ($I = 0.1$)                  | [109]      |
| 6,8,9trimethylisoalloxazine          | −0.109                   | 7  |                                              | [91, 97]   |
| Compound                          | $E^{\circ}/V$ versus SHE | pH | Conditions          | References   |
|----------------------------------|-------------------------|----|---------------------|--------------|
| chloraphine                      | −0.115                  | 7  |                     | [91, 97]     |
| indigo disulfonate               | −0.116                  | 7  |                     | *[112]       |
|                                  | −0.125                  | 7  |                     | [91, 97]     |
|                                  | −0.188                  | 9.3|                     | *[113]       |
|                                  | −0.100                  | 7  | 15 °C               | [108]        |
| nile blue                        | −0.120                  | 8  | 0.5 M phosphate     | [114]        |
| indigocarmine                    | −0.125                  | 8  | 0.5 M phosphate     | [114]        |
| 9-phenylisoalloxazine            | −0.126                  | 7  |                     | [91, 101]    |
| 2-hydroxy-1,4-naphthoquinone    | −0.139                  | 7  |                     | [91, 97]     |
|                                  | −0.153                  | 7.5| 0.1 M phosphate     | [95]         |
| thioglycolic acid                | −0.14                   | 7  |                     | [91, 101]    |
| 2-amino-$N$-methylphenazine methosulfate | −0.145              | 7  |                     | [91, 97]     |
| azure A                          | −0.153                  | 8  | 0.5 M phosphate     | [114]        |
| indigo monosulfonate             | −0.157                  | 7  |                     | [91, 97]     |
| anthraquinone-1,5-disulfonate    | −0.170                  | 7  |                     | *[115]       |
| alloxazine                       | −0.170                  | 7  |                     | [91, 97]     |
| brilliant alizarin blue          | −0.173                  | 7  |                     | [91, 97]     |
| crystal violet                   | −0.176                  | 7.5| 15 °C               | [108]        |
| 2-methyl-3-hydroxy-1,4-naphthoquinone | −0.180              | 7  |                     | [91, 97]     |
| patent blue                      | −0.180                  | 8  | 0.5 M phosphate     | [114]        |
| 9-methylisoalloxazine            | −0.183                  | 7  |                     | [91, 97]     |

(continued)
| Compound                        | $E''/V$ versus SHE | pH | Conditions                        | References |
|--------------------------------|--------------------|----|----------------------------------|------------|
| cibacron blue                  | −0.184             | 8  | 0.5 M phosphate                  | [114]      |
| phenol red                     | −0.188             | 8  | 0.5 M phosphate                  | [114]      |
| anthraquinone-2,6-disulfonate  | −0.225             | 7  |                                  | *[112]     |
| neutral blue                   | −0.184             | 7  |                                  | [91, 97]   |
| bromophenol blue               | −0.196             | 8  | 0.5 M phosphate                  | [114]      |
| anthraquinone-2,7-disulfonate  | −0.203             | 7  | 0.1 M phosphate ($I_0 = 0.3$)   | [95]       |
| quinoline yellow               | −0.203             | 8  | 0.5 M phosphate                  | [114]      |
| riboflavin                     | −0.208             | 7  |                                  | [91, 101]  |
| FMN (flavin mononucleotide)    | −0.211             | 7  |                                  | [91, 116]  |
| FAD (flavin adenine dinucleotide) | −0.207           | 7  | 0.1 M phosphate, 25 °C          | [113]      |
| anthraquinone-1-sulfonate      | −0.218             | 7  |                                  | [91, 97]   |
| anthraquinone-2-sulfonate      | −0.249             | 7  | 0.1 M phosphate ($I_0 = 0.3$)   | [95]       |
| phenoxyarilamide               | −0.252             | 7  |                                  | [91, 97]   |
| lipoamide                      | −0.280             | 7  |                                  | *[118]     |
| safranine T                    | −0.276             | 7  |                                  | *[112]     |
| lipoic acid                    | −0.289             | 7  |                                  | [91, 97]   |
| indulin scarlet                | −0.299             | 7  |                                  | [91, 97]   |
| 4-aminoacridine                | −0.301             | 7  |                                  | [91, 119]  |
## Table 2.2 (continued)

| Compound | $E^*/V$ versus SHE | pH | Conditions | References |
|----------|-------------------|----|------------|------------|
| acridine | $-0.313$          | 7  |            | [91, 119]  |
| NAD (nicotinamide adenine dinucleotide) | $-0.315$          | 7  |            | *[118]     |
|         | $-0.320$          | 7  |            | [91, 118, 120] |
| NADP (nicotinamide adenine dinucleotide phosphate) | $-0.320$          | 7  |            | *[118]     |
|         | $-0.324$          | 7  |            | [91, 91, 120] |
| neutral red | $-0.325$         | 7  |            | [91, 97]   |
| cysteine | $-0.340$          | 7  |            | [91, 120]  |
| benzyl viologen (2+/1+) | $-0.359$          | 7  |            | *[112]     |
|         | $-0.36$           | 7  |            | [91, 97]   |
|         | $-0.358$          | 7  |            | *[115]     |
|         | $-0.370$          | 9.3|            | *[113]     |
| 3-aminoacridine | $-0.369$         | 7  |            | [91, 119]  |
| 1-aminoacridine | $-0.394$         | 7  |            | [91, 119]  |
| methyl viologen(2+/1+) | $-0.435$          | 7  |            | *[115]     |
|         | $-0.446$          | 7  |            | [91, 97]   |
|         | $-0.44$           | 7  |            |           |
| 2-aminoacridine | $-0.486$         | 7  |            | [119, 121] |
| [5,10,15,20-tetrakis(2,6-dichloro-3-sulfonato)porphyrinato] Fe(H_2O)(OH^-) | $-0.709$          | 5  | $I = 0.5$ | [82]       |
| 2,8-diaminoacridine | $-0.731$         | 7  |            | [119, 121] |
| methyl viologen(1+/0) | $-0.770$          | 7  |            | *[95]      |
| 5-aminoacridine | $-0.916$          | 7  |            | [119, 121] |

Water soluble Fe porphyrine: [5,10,15,20-tetrakis(2,6-dichloro-3-sulfonato)porphyrinato] Fe(H_2O)(OH^-)

MES: 2-(N-morpholino) ethanesulfonate buffer
ABTS: 2,2’-azinobis-(3-ethylbenzthiozoline-6-sulfonic acid)
MOPS: 3-(N-morpholino) propanesulfonic acid
DMSO: dimethyl sulfoxide
2.6 Immobilization of Enzymes and Mediators

2.6.1 Bifunctional Reagents

For immobilization of enzymes and mediators, bifunctional reagents are usually employed. Amino and carboxy groups on the surface of enzymes and some mediators are feasible positions for covalent linkages. In addition, the surface of electrodes can also be functionalized with various groups by several approaches. In this part, we introduce some of common bifunctional reagents utilized for enzymes and mediators immobilization.

Glutaraldehyde (1,5-pentanedial, GA) is one of the most widely used bifunctional reagents with the capacity to polymerize and immobilize enzymes and mediators. GA may react with different moieties of enzymes, mainly involving primary amino groups, but it may eventually react with other groups such as thiols, phenols, and imidazoles [37]. The exact structure of GA is not fully clarified. Some of proposed structures in solution and their reactions schemes are given in Panel A in Fig. 2.6 [37]. With the aid of GA, HRP and thionine were co-immobilized onto an L-Cys self-assembled monolayer (SAM) gold electrode (AuE) for fabrication of an H$_2$O$_2$ biosensor [38]. Here, amino groups (i.e. L-Lys residues) on HRP, two primary amino groups on thionine, and the amino tails of L-Cys SAM are crosslinked by GA and finally formed covalent leakages (Fig. 2.6B). In a similar manner, a stable matrix at glassy carbon electrode (GCE) constructed by cross-linking HRP, thionine, and bovine serum albumin (BSA) with GA [39].

On the other hand, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) mediates the formation of covalent linkages between carboxy and amino groups to form amide bonds through activated ester intermediates. (Fig. 2.6C) [40] One of ferrocene derivatives, common electrochemical mediators, with a carboxy tail thus can form an amide bond with amino groups on the surface of enzymes [41]. A glucose-oxidizing bioanode was fabricated by co-immobilization with 1,1'-ferrocenedicarboxylic acid between GOD onto an aminated glassy carbon electrode with EDC/NHS [42]. In this case, 1,1'-ferrocenedicarboxylic acid with two carboxy groups act as a bridge for covalent-bonding of GOD to an aminated electrode by forming amide bonds with them.

Poly(ethylene glycol) diglycidyl ether (PEGDGE), which has two epoxy groups that can react with amino, hydroxy, and carboxy groups, is another common bifunctional reagents for enzymes and mediators immobilization (Fig. 2.6D) [43]. GOD was covalently linked to a ferrocenecarboxaldehyde functionalized linear poly(ethyleneimine) (PEI) with PEGDGE for construction of a amperometric glucose biosensors [44].
Fig. 2.6 Reactions of some bifunctional reagents. A GA reacts with amino groups in different ways for cross-linking reactions, B co-immobilization of HRP and thionine with GA on L-Cys-SAM AuE, C formation of an amide bond using EDC/NHS, and D reaction scheme of cross linking with PEGDGE.

### 2.6.2 Redox Polymers

Redox polymers are most widely applied for immobilizing both enzymes and mediators on an electrode in MET-type bioelectrocatalysis. In this section, we will introduce typical redox polymers that have redox couples working as mediators which are bound to the cross-linked polymer network through covalent or coordinative bonds.
2.6 Immobilization of Enzymes and Mediators

Such polymers act immobilization matrices to keep enzymes on electrochemical transducers and serves as electron conducting relays based on self-exchange reactions among the oxidized and reduced polymers, for the electrical wiring of redox-enzymes (Fig. 2.7). Enzymes are usually co-immobilized on electrodes with cross-linking reagents such as PEGDGE or GA. Polymer-based mediator can immobilize enzymes and mediators with retaining some extent of the mobility required for MET-type reactions. In addition, they are water-soluble and substrates and salts can penetrate them easily. Heller group firstly started to use Os-complex modified poly(vinyl imidazole) (PVI) and poly(vinyl pyridine) (PVP) for GOD to construct glucose sensors (Fig. 2.8) [45, 46]. Os-complex based mediators allow for a fine potential adjustment by designing novel ligand and the polymer has been widely used for various bioelectrocatalytic systems such as HRPs [47], GDHs, [48, 49] MCOs, [50] pyranose dehydrogenase [51], lactate oxidase [52], and FDH [53]. In recent years, a variety of polymers have been constructed by changing not only redox functional group but also backbone polymers. For example, redox polymer composed of PVI, PVP, methacrylates, acrylates or acrylamides, linear or branched PEIs, poly(vinylalcohol)s, poly(3,4-ethylenedioxythiophene) (PEDOT) that are modified with ferrocene [54–56], penta-cyanoferrate [57], cobaltocene [58], viologen derivatives [58–62], phenothiazine [63] or quinone derivatives [64–66] are available (Fig. 2.8). Therefore, one can broadly tune the redox potential [67], hydrophilicity [68], and electrical charge [69]. The design of redox polymers with adjusted properties becomes more and more important to construct efficient bioelectrodes. Biosensors with high selectivity have been reported based on the electrostatic and steric interactions between some redox enzymes and well-designed redox polymers with different charged groups [57, 70]. In addition, the film thickness has to be controlled to avoid disturbing the substrate.
diffusion. In fact, by using such redox polymers, over 100 mA cm\(^{-2}\) catalytic glucose oxidation current (Os-polymer) [49] and high power density of formate/O\(_2\) biofuel cells (viologen polymer) [62] were reported.

On the other hand, the new aspects of the redox polymer were reported. A viologen polymer can work to protect the air sensitive H\(_2\)ases form O\(_2\) damage [60, 61]. Other viologen polymer can work to protect the bioanode in the formate/O\(_2\) biofuel cells from crossover reaction of O\(_2\) and the cathodic mediator [62]. These effects can be explained as follows: the thickness of the MET-reaction layer is thinner than that of the enzyme/mediator-immobilized layers, and the dissolved O\(_2\) diffusing to the outer surface of the immobilized layer is reduced by the reduced mediator generated in the enzyme reaction, in which the substrate works as a sacrificial reagent. Furthermore, redox polymers combined with pH- [71] or thermo-responsive [72] moieties could be utilized to develop a switchable bioelectrocatalysis for special purposes like logic gate.

The redox polymers have been well reviewed by Heller [73] and Ruff [74].
2.7 Electrode Materials

Various materials including carbon, metals, and semiconductors can be utilized as platforms for MET-type bioelectrocatalysis. Although rapid electron transfer have been found between redox mediators and several planar electrodes such as glassy carbon (GC), Au disk and indium–tin-oxide (ITO), materials with larger specific surface area (in a precise sense, with large values for surface-to-weight ratio) have been proposed to improve the performance of MET-type bioelectrocatalysis because of the increased amounts of enzymes and mediators. Carbon cloths, carbon papers, and carbon felts, which are made with carbon fibers of different diameters, are usually utilized for MET-type reaction. Barton et al. were the first to report the use of a carbon cloth modified with Os polymers and laccase from *Rhus vernicifera* and *Coriolus hirsutus* [75, 76]. They attained a current density of 5 mA cm$^{-2}$ at 0.57 V vs Ag|AgCl in a pH 5 chloride-free citrate buffer at 37.5 °C. In this pioneering work, the authors demonstrated that a 50-fold increase in surface area lead to a multiplication of the O$_2$ reduction current by a factor of 5.

On the other hand, nanostructured material-based electrodes with porous surface were also often employed to furthermore improve the performance of MET-type bioelectrocatalysis. Tsujimura et al. reported high performance of MET-type bioelectrodes for both glucose oxidation [49] and O$_2$ reduction [77] by using magnesium oxide-templated mesoporous carbon electrode (MgOCE). A high limiting current density of 145 mA cm$^{-2}$ was recorded for HCOO$^-$ oxidation at FoDH modified Ketjen Black-modified electrodes (KBEs), by using methyl viologen (MV) as a free mediator [78]. An O$_2$ reduction current density of 27 mA cm$^{-2}$ was realized at neutral pH by co-immobilizing BOD and 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS) at a hollow CNTs modified carbon clothes gas-diffusion electrode [79]. The improved performance of MET-type bioelectrocatalysis could be explained by increased enzyme immobilization, effective electron and mass transfer at such microstructured electrodes.

In addition, functionalized materials have also been developed for MET-type bioelectrocatalysis. For an example, glassy carbon electrochemically grafted a mediator illustrated a good performance for MET-type bioelectrocatalysis of PQQ-depant GDH [80]. Yamaguchi et al. also reported hydroquinone functionalized carbon black for bioelectrocatalysis of GOD [81].

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Chapter 3
Fundamentals of DET-Type Bioelectrocatalysis

Abstract This chapter starts by introducing the history of DET-type non-catalytic and bioelectrocatalytic reactions with much attention to the distinction between DET- and MET-type bioelectrocatalytic reactions. Theoretical features of DET-type bioelectrocatalysis are detailed with emphasis of orientation effect of redox enzymes and curvature effect of mesoporous electrodes. One of analytical methods for steady-state catalytic waves of DET-type bioelectrocatalysis is also introduced.

Keywords Metal-containing redox enzymes · Flavoproteins · Steady-state current · Long-range electron transfer · Random orientation · Mesoporous electrodes · Data analysis

3.1 History of DET-Type Bioelectrocatalysis

Interfacial electrochemistry, i.e. non-catalytic direct electron transfer (DET), of redox proteins has been energetically studied in 1970s–1980s especially by Hill group, as reviewed in the literature [1–4]. During the studies of Hill group, the significance of use of edge-oriented pyrolytic graphite electrode (EPGE) [5] as well as redox inactive promoters were pointed out to facilitate DET-type reactions.

In 1978, an important event has been reported as a DET-type bioelectrocatalysis: catalytic reduction of O₂ by a multi-copper oxidase (MCO) laccase (Lac) adsorbed on carbon electrodes [6], and the event was well followed by Lee et al. [7]. The reports inspired researchers in this field, and clear DET-type bioelectrocatalytic voltamograms of redox enzymes with multi-redox sites have been reported one after another especially by Hill and Ikeda groups in 1988–1992: $p$-cresol methylhydroxylase from Pseudomonas putida at EPGE [8], methylamine dehydrogenase from bacterium W3A1 at EPGE [9], d-gluconate dehydrogenase (GADH) from Pseudomonas fluorescence FM-1 at carbon paste electrode (CPE) [10], d-fructose dehydrogenase (FDH) from Gluconobacter sp. at CPE [11], and PQQ-dependent alcohol dehydrogenase (ADH) type III from Gluconobacter suboxydans at gold electrode (AuE) [12]. Note here that GADH, FDH, and ADH are generated in acetic bacteria. This time of period is the beginning of the research field of DET-type bioelectrocatalysis.
MCO contains blue T1Cu and T2/3Cu cluster and catalyzes the reaction: \( \text{DH}_2 + \text{O}_2 + 2\text{H}^+ \rightarrow \text{D} + 2\text{H}_2\text{O} \), \( \text{DH}_2 \) being an electron donor. Electrons form \( \text{DH}_2 \) are accepted at the T1Cu site and transferred to the T2/3Cu cluster, at which \( \text{O}_2 \) is reduced to \( \text{H}_2\text{O} \). \( \text{p-Cresolmethylhydroxylase} \) catalyzes the reaction: \( 4\text{-cresol} + \text{A} + \text{H}_2\text{O} \rightarrow 4\text{-hydroxybenzaldehyde} + \text{AH}_2 \), \( \text{A} \) being an electron acceptor. Other name in common is 4-cresol dehydrogenase (hydroxylating). This enzyme is a flavohemoprotein with an \( \alpha_2\beta_2 \) structure containing one heme \( \text{c} \) in small subunit (8.5 kDa) and one FAD in large subunit (79 kDa). Methylamine dehydrogenase is a quinohemoprotein with an \( \alpha_2\beta_2 \) structure containing one covalently bound TTQ [13] in \( \alpha \)-subunit (43 kDa) and one heme \( \text{c} \) in \( \beta \)-subunit (14 kDa). This enzyme catalyzes the reaction: \( \text{RCH}_2\text{NH}_2 + \text{A} + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{AH}_2 \), \( \text{A} \) being amicyanine or cyt \( c_{552} \) in nature. GADH is a membrane-bound flavohemoprotein and catalyzes the 2-electron oxidation of \( \text{d-gluc} \) to 2-keto \( \text{d-gluc} \). This enzyme is a heterotrimer containing one covalently bound FAD, two hemes \( \text{c} \), and one FeS. FDH is a membrane-bound flavohemoprotein and catalyzes the 2-electron oxidation of \( \text{d-fructose} \) to 5-keto-\( \text{d-fructose} \). The enzyme is a heterotrimer containing one covalently bound FAD and three hemes \( \text{c} \); in the past days, the catalytic center of FDH was believed to be PQQ. PQQ-dependent ADH (type III) is a membrane-bound quinonehemoprotein and catalyzes the oxidation of ethanol to acetaldehyde. This enzyme is a heterotrimer containing one PQQ and one heme \( \text{c} \) in subunit I and three hemes \( \text{c} \) in subunit II, though subunit III has no prosthetic group. In the DET-type reactions mentioned above, all the enzymes have multi-redox sites, and a/the metallic prosthetic group in the enzymes is considered as the site that electrochemically communicates with electrodes, as shown in Fig. 3.1, where the electrochemically communicating site is regarded as “built-in mediator” [14]. After then, DET-type bioelectrocatalysis has evolved into an established pathway of electron transfer for many multi-redox site enzymes.

The process of DET-type bioelectrocatalysis require the following criteria [15]: (i) the redox cofactor(s) of the enzyme remains bound or associated with the enzyme, (ii)
a non-diffusive and/or synthetic electroactive species on the electrode does not participate in the electron transfer, and (iii) catalytic oxidation or reduction current of the substrate is observed upon its addition to the system. Therefore, NAD(P)-dependent enzymes (utilizing ordered mechanisms) are not considered to undergo DET-type bioelectrocatalysis, while NAD(P)-linked enzymes such as NAD(P)-linked H₂ase, and NAD(P)-linked FoDH can undergo DET-type reactions.

Some examples of DET-type reactions reported to date are summarized in Table 3.1. Most of the enzymes undergoing DET-type bioelectrocatalysis have multi-redox sites. However, some of the redox enzymes with single-metallic prosthetic group are also capable of DET-type bioelectrocatalysis; cyt c peroxidase from yeast [16] and horseradish peroxidase (HRP) [17] containing one heme b as the catalytic center catalyze DET-type reduction of H₂O₂ at potentials close to the formal potential (~0.7 V vs. Ag|AgCl) of Compound I (i.e. 2-electron oxidized form with oxyferrl iron).

In addition, ferredoxin-NADP⁺ reductase from *Chlamydomonas reinhardtii* containing one FAD catalyzes bidirectional NADP⁺-reduction and NADPH-oxidation [47]. Cellobiose dehydrogenase type I from *Myriococcus thermophilum*, a heterodimeric flavohemoprotein containing an FAD in dehydrogenase subunit and a heme b in cytochrome subunit, also shows a DET-type reaction at the FAD in the presence of Ca²⁺, while the enzyme usually undergoes a DET-reaction at the heme b [50]. DET-type catalytic behaviors were also reported for PQQ-dependent soluble GDH (PQQ-sGDH) containing non-covalently (and weakly) bound PQQ in each molecule [49, 51]. These reports support that organic cofactors in redox enzymes have a possibility to directly communicate with electrodes.

Although there are many reports claiming DET-type bioelectrocatalysis of FAD-GOD containing a non-covalently bound FAD per each molecule, several authors have questioned the interpretation [52, 53]. Sakai et al. have shown clear experimental evidences of two mechanisms: DET- and MET-type bioelectrocatalysis by NAD-linked FoDH containing non-covalently bound FMN, W-pterin, and FeS clusters [54]; one of FeS is the electrochemically active site of the enzyme in DET-mechanism, while free FMN liberated from the holoenzyme plays as a mediator in MET-mechanism. In the case of PQQ-sGDH also, a possibility of MET-type reaction mechanism via free PQQ liberated from PQQ-sGDH could not be ruled out, because the half-wave potential of the catalytic wave was ~0.4 V more positive than the averaged peak potential of non-catalytic DET peak of PQQ-sGDH. The difference seems to be too large. In addition, the peak potential of non-catalytic DET peak of the enzyme was almost identical to that of free PQQ, though spectroelectrochemical measurements indicated that a $E^{\circ'}$ value of PQQ-sGDH was ~0.06 V more negative than $E^{\circ'}$ of free PQQ (at pH 7.0) [55]. Further research is required to get a rigid conclusion on DET-type bioelectrocatalysis of PQQ-sGDH. Utilization of irreversible adduct formation of free PPQ (Sect. 1.2.3) may be utilize to remove free PQQ throughout the bioelectrocatalytic reaction by PQQ-sGDH.

In construct to the above questioning on DET-reaction of FAD-GOD and PQQ-sGDH, new concept has been reported to realize DET-reaction of FAD-GOD by using platinum (Pt) nanoclusters generated near the FAD of the enzyme (Fig. 3.2)
### Table 3.1 Performance of DET-type bioelectrocatalysis of several redox enzymes

| Enzyme group                     | Enzyme name (source)                                | Catalytic and redox center(s)* (subunit structure)                                                                 | Reaction (half-wave potential of the catalytic wave) | Electrode          | Refs. | PDB   |
|----------------------------------|----------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|--------------------|-------|-------|
| **Copper-containing enzyme**     |                                                    |                                                                                                                   |                                                     |                    |       |       |
| Laccase (Pycnoporus cinnabarinus) | Type II/III 3Cu cluster, Type I Cu (blue copper) [monomer]                                                  | $\text{O}_2 + 4e^- (0.45 \text{ V vs. SCE, pH 3})$                                                              | EPGE                                                | [7] 2XYB          |       |       |
|                                  |                                                    | $\text{O}_2 + 4e^- (0.7 \text{ V vs. SHE, pH 4})$                                                                | Anthracene-modified EPGE                           |                    |       |       |
| Bilirubin oxidase (Myrothecium verrucaria) | Type II/III 3Cu cluster, Type I Cu (blue copper) [monomer]                                             | $\text{O}_2 + 4e^- (0.55 \text{ V vs. Ag|AgCl, pH 7})$  | MWCNT                                               | [19] 2XLL          |       |       |
|                                  |                                                    | $\text{O}_2 + 4e^- (0.4 \text{ V vs. Ag|AgCl, pH 7})$                                                             | AuNP                                                |                    | [20]  |       |
|                                  |                                                    | $\text{O}_2 + 4e^- (0.5 \text{ V vs. Ag|AgCl, pH 5})$                                                             | MgO-templated porous carbon                        |                    | [21]  |       |
| Laccase (Pycnoporus cinnabarinus) | Type II/III 3Cu cluster, Type I Cu (blue copper) [monomer]                                                  | $\text{O}_2 + 4e^- (0.45 \text{ V vs. SCE, pH 3})$                                                              | EPGE                                                | [22] 2XYB          |       |       |
| Copper efflux oxidase (Escherichia coli) | Type II/III 3Cu cluster, Type I Cu (blue copper), Cu²⁺ [monomer] | $\text{O}_2 + 4e^- (0.3 \text{ V vs. Ag|AgCl, pH 5})$                                                             | KBE                                                 | [7] 4NER           |       |       |
| Nitrite reductase (Alcaligenes faecalis) | (Type II Cu, Type I Cu) $\times 2$ [homodimer] | $\text{NO}_2^- + e^- (0.1 \text{ V (?) vs. SHE, pH 7})$                                                          | EPGE                                                | [23] 4YSC          |       |       |
| [NiFe] hydrogenase (Chromatium vinosum) | (NiFe, 4Fe4S (proximal), 3Fe4S (medial), 4Fe4S (distal)) $2$ [a$_2$b$_2$ heterotetramer] | $2\text{H}^+ + 2e^- \rightleftharpoons \text{H}_2$ ($\sim 0.35 \text{ V vs. SHE, pH 6})$ | PGE (edge)                                        | [24]              |       |       |

(continued)
| Enzyme group Enzyme name (source) | Catalytic and redox center(s)* (subunit structure) | Reaction (half-wave potential of the catalytic wave) | Electrode | Refs. | PDB |
|---------------------------------|------------------------------------------------|--------------------------------------------------|-----------|-------|-----|
| [NiFe] hydrogenase (Desulfovibrio vulgaris Miyazaki F) | (NiFe, 4Fe4S, 3Fe4S, 4Fe4S)×2 [a2b2 heterotetramer] | 2H⁺ + 2e⁻ ⇌ H₂ (−0.5 V vs. Ag|AgCl, pH 5) | p-PDA-modified KBE | [25] | 1h2r |
| [NiFe] hydrogenase (Hydrogenovibrio marinus) | (NiFe, 4Fe4S, 3Fe4S, 4Fe4S)×2 [a2b2 heterotetramer] | H₂ − 2e⁻ (−0.4 V vs. Ag|AgCl, pH 5.5) | KBE | [26] | 3ayx |
| [FeFe] hydrogenase (Desulfovibrio desulfuricans) | (FeFe, 4Fe4S, 4Fe4S, 4Fe4S)×2 [a2b2 heterotetramer] | 2H⁺ + 2e⁻ ⇌ H₂ (−0.3 V vs. SHE, pH 6) | EPGE | [27] | |
| NAD-reducing [NiFe] hydrogenase (Hydrogenophilus thermoluteolus) | NiFe, 4Fe4S, 4Fe4S, 2Fe2S, 4Fe4S, FMN (non-covalent) [heterodimer] | 2H⁺ + 2e⁻ ⇌ H₂, NAD⁺ + 2e⁻ ⇌ NADH (−0.6 V vs. Ag|AgCl, pH 7) [heterodimer] | KBE | [28] | 5xFc |
| NAD-reducing formate dehydrogenase (Methyllobacterium extorquens AM1) | W-pterin, 4Fe4S, 4Fe4S, 2Fe2S, 4Fe4S, FMN (non-covalent) [heterodimer] | CO₂ + 2e⁻ ⇌ COO⁻, NAD⁺ + 2e⁻ ⇌ NADH (−0.55 V vs. Ag|AgCl, pH 7) | AuNP/KBE | [29] | |
| NAD-reducing formate dehydrogenase 1 (Syntrophobacter fumaroxidans) | W-pterin, 2Fe2S⁴, 4Fe4S⁴ [heterotrimer] | CO₂ + 2e⁻ ⇌ COO⁻, (−0.35 V vs. SHE, pH 6.4) | EPGE | [30] | |
| Enzyme group                              | Catalytic and redox center(s)* (subunit structure)                  | Reaction (half-wave potential of the catalytic wave) | Electrode | Refs. | PDB  |
|------------------------------------------|---------------------------------------------------------------------|-------------------------------------------------------|-----------|-------|------|
| Nitrate reductase *(Escherichia coli)*   | Mo-pterin, 4Fe4S, 4Fe4S, 4Fe4S, 3Fe4S, heme b, heme b [heterotrimer] | NO$_3^-$ + 2e$^-$ (0.05 V vs. SHE, pH 7)               | EPGE      | [31]  |      |
| NADH:ubiquinone oxidoreductase [subcomplex of Complex I] (bovine heart mitochondria) | FMN, FeS$^-$8 [heterononamer]                                       | NAD$^+$ + 2e$^-$ ⇌ NADH (−0.35 V vs. SHE, pH 8)       | EPGE      | [32]  | 5LDW |
| Fumarate reductase [succinate dehydrogenase, soluble part of Complex II] *(Escherichia coli)* | FAD (covalent), 2Fe2S, 4Fe4S,3Fe4S [heterotetramer]                | fumarate + 2e$^-$ ⇌ succinate (−0.1 V vs. SHE, pH 7)  | EPGE      | [33]  | 6awf |
| Carbon monoxide dehydrogenase *(Carboxydothermus hydrogenoformans)* | (NiFe, 3Fe4S)$^2$, 4Fe4S [homodimer]                                 | CO$_2$ + 2e$^-$ ⇌ CO (−0.51 V vs. SHE, pH 7)           | EPGE      | [34]  | 1su6 |
| Dimethyl sulfoxide reductase *(Escherichia coli)* | Mo-pterin, 4Fe4S, 4Fe4S [heterodimer]                              | DMSO + 2 e$^-$ (−0.1 V vs. SHE, pH 7)                  | EPGE      | [35]  |      |
| Arsenate oxidase *(Alcaligenes faecalis)* | Mo-pterin, 4Fe4S, 2Fe2S [heterodimer]                              | As$^{3+}$ − 2e$^-$ (0.3 V vs. SHE, pH 7)               | EPGE      | [36]  | 1g8j |
| *Flavohemoprotein*                       |                                                                     |                                                       |           |       |      |
| Cellulose dehydrogenase *(Phanerochaete chrysosporium)* | FAD (non-covalent), heme b [heterodimer]                           | cellulose − 2e$^-$ (−0.05 V vs. Ag|AgCl, pH 3–5) | Graphite electrode | [37]  | 1kdg |
|                                         |                                                                     |                                                       |           | 1d7b |      |
| Enzyme group Enzyme name (source) | Catalytic and redox center(s)* (subunit structure) | Reaction (half-wave potential of the catalytic wave) | Electrode | Refs. | PDB |
|----------------------------------|---------------------------------------------------|------------------------------------------------|----------|-------|-----|
| Glucose dehydrogenase *(Burkholderia cepacia)* | FAD (covalent), 3Fe3S, heme c(proximal), heme c(medial), heme c(distal) [heterotrimer] | glucose – 2e− (?) | Carbon screen-printed electrodes | [38] | 2y0e |
| Fructose dehydrogenase *(Gluconobacter japonicus)* | FAD (covalent), heme c (proximal), heme c (medial), heme c (distal) [heterotrimer] | fructose – 2e− (0.08 V vs. Ag|AgCl, pH 5) | KBE | [39] |
| | | | fructose – 2e− (0.25 V vs. Ag|AgCl, pH 5) | Mercaptoetanol-modified AuNP | [40] |
| Nitrate reductase *(Neurospora crassa)* | (Mo-pterin, heme, FAD)×2 [homodimer] | NO3− + 2e− (−0.15 V vs. SHE, pH 7) | PEI-PM/EPGE | [41] |
| Gluconate 2-dehydrogenase *(Gluconobacter frateurii)* | FAD (covalent), (heme e)×2 [heterotrimer] | gluconate – 2e− (0.1 V vs. Ag|AgCl, pH 5) | ITO | [42] |
| p-cresolmethyhydroxylase *(Pseudomonas putida)* | (FAD, heme)×2 [a2b2 heterotetramer] | p-cresol – 2e− (0 V vs. SCE, pH 7) | Cu(NH3)63+ (promoter)/EPGE | [8] |
| Quinohemoprotein Glucose dehydrogenase *(Ewingella americana)* | PQQ (non-covalent), heme c [monomer] | glucose – 2e− (−0.1 V vs. SCE, pH 7) | 4-aminothiophenol-modified AuNP | [43] |
| Alcohol dehydrogenase *(Pseudomonas putida HK5)* | PQQ (non-covalent), heme c [monomer] | glycerol – 4e− (−0.1 V vs. SCE, pH 7) | 4-aminothiophenol-modified AuNP | [44] | 1KV9 |

(continued)
| Enzyme group Enzyme name (source) | Catalytic and redox center(s)* (subunit structure) | Reaction (half-wave potential of the catalytic wave) | Electrode | Refs. | PDB |
|----------------------------------|--------------------------------------------------|--------------------------------------------------|-----------|-------|-----|
| Alcohol dehydrogenase (Gluconobacter sp.) | PQQ (non-covalent), heme $c$, heme $c$, heme $c$, heme $c$ [heterotrimer] | ethanol – 2e$^{-}$ (0 V vs. Ag|AgCl, pH 6) | polypyrrol-entrapped on Pt | [45] | |
| Methylamine dehydrogenase (bacterium W3A1) | (TTQ (covalent), heme $c$)×2 [a$_2$b$_2$ heterotetramer] | RCH$_2$NH$_2$ – 2e$^{-}$ (−0.15 V vs. SCE, 7.2) | EPGE | [9] | |
| Hemoprotein | | | | |
| Peroxidase (horseradish) | heme $b$ [monomer] | H$_2$O$_2$ – 2e$^{-}$ (0.6 V vs. Ag|AgCl, pH 7) | KBE | [17] | 1h58 |
| Cytochrome $c$ peroxidase (Nitrosomonas europaea) | heme $c$, heme $c$ [monomer] | H$_2$O$_2$ – 2e$^{-}$ (0.5 V vs. SHE, pH 7) | EPGE | [46] | 1IQC |
| Cytochrome $c$ peroxidase (yeast) | heme $b$ [monomer] | H$_2$O$_2$ – 2e$^{-}$ (0.65 V vs. SHE, pH 7) | EPGE | [16] | |
| Flavoprotein | | | | |
| Ferredoxin-NADP$^+$ reductase (Chlamydomonas reinhardtii) | FAD (non-covalent?) [monomer] | NADP$^+$ + 2e$^{-}$ ⇌ NADPH (−0.35 V vs. SHE, pH 8) | ITO/EPGE | [47] | Reference structure 1FNB (spinachi) 1GAW (cone) |
| Glucose oxidase (Aspergillus niger) | FAD×2 (non-covalent) [homodimer] | glucose – 2e$^{-}$ (−0.35 V vs. Ag|AgCl, pH 7) (?) | MWCNT/Pt | [48] | 1CF3 |
| Quinoprotein | | | | (continued) |
| Enzyme group | Enzyme name (source) | Catalytic and redox center(s)* (subunit structure) | Reaction (half-wave potential of the catalytic wave) | Electrode | Refs. | PDB |
|--------------|----------------------|---------------------------------------------------|--------------------------------------------------|-----------|-------|-----|
| Soluble glucose dehydrogenase | PQQ×2 (non-covalent) [homodimer] | glucose − 2e⁻ (0.25 V vs. Ag|AgCl, pH 7.2) | CCGE | [49] [92] | |

*Double underline: most possible candidate of the electrochemical active site, single underline: any one of them as a possible candidate of the electrochemical active site, no underline: no clear comment on the electrochemical active site

**Chemical:** NAD⁺ (nicotinamide adenine dinucleotide, oxidized form), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), PQQ (pyrroloquinoline quinone), p-PDA (p-phenylenediamine), PEI (polyethyleneimine), PM (polymyxin B)

**Electrode:** AuNP (gold nanoparticles), EPGE (edge-oriented pyrolytic graphite electrode), KBE (Ketjen black-modified electrode), MWCNT (multi-walled carbon nanotubes), ITO (indium tin oxide), CCGE (carbon cryogel electrode)

**Reference electrode:** Ag|AgCl (silver|silver chloride|saturate potassium chloride electrode = 0.197 V vs. SHE), SCE (saturated calomel electrode = 0.241 V vs. SHE), SHE (standard hydrogen electrode)
Pt nanoclusters were grown enzymatic reaction of the enzyme with PtCl$_6^{2-}$ as an electron acceptor and were enable electrical contact between the enzyme and an electrode. Single-walled carbon nanotubes are suggested to accelerate DET-type bioelectrocatalysis of FAD-dependent GDH [58]. Enzymatically implanted platinum nanoclusters on porous gold electrodes also worked well as scaffolds for DET-type bioelectrocatalysis of FAD-dependent GDH [59].

Another confusing issue on DET-type bioelectrocatalysis concerns heme peroxidases (in the following simply referred to peroxidases). Most of peroxidases have a heme $b$ (ferriprotoporphyrin IX) as the active site. The native catalytic cycle of peroxidase is given in Fig. 3.3 with black color; oxidation of ferric peroxidase by H$_2$O$_2$ generates Compound I with oxyferryl ion and a porphyrin $\pi$ cation radical. Compound I is reduced to the ferric form via two-step single-electron transfer via...
3.1 History of DET-Type Bioelectrocatalysis

Compound II. The $E^\circ$ values of ferric/Compound I and ferric/Compound II are +0.50 V and +0.48 V, respectively [59–61]. Since $E^\circ$ value of H$_2$O$_2$/2H$_2$O couple is +1.36 V, the onset potential or half-wave potential of DET-type bioelectrocatalytic waves of peroxidases should be close to $E^\circ$ of peroxidases (at pH 7), as reported in several papers [16, 17, 62–65].

Contrary to the native function of peroxidases, there are numerous papers dealing with a DET-type electrode reaction of the ferric/ferrous redox couple of peroxidases (Fig. 3.3, gray color). Most of the authors in the papers on this issue also attempted to show H$_2$O$_2$ concentration dependence of the cathodic wave, though the voltammetric waves of the ferric/ferrous redox couple show a peaked shape even in the presence of H$_2$O$_2$. The extremely weak peroxidase-like activity is explained by the oxidation of the ferrous form by H$_2$O$_2$ to Compound II, which is reduced to the ferrous form via the ferric form (Fig. 3.3, gray color) [66]. This catalytic cycle is completely different from the native cycle of peroxidases.

3.2 Theory of Steady-State Catalytic Current

As a model of DET-type reaction, one may consider a set of series reaction involving the steady-state mass transfer and the steady-state DET-type bioelectrocatalysis on the electrode surface (electro-enzyme reaction), where the substrate S is transferred to the electrode surface, and is transformed into the product P by a DET-type bioelectrocatalysis. Therefore, the steady-state catalytic current is given by the following equation:

$$\frac{1}{i_s} = \frac{1}{i_{s,mt}} + \frac{1}{i_{s,elec-enz}}, \quad (3.1)$$

where $i_{s,mt}$ is given, for example, by Eqs. (2.32) and (2.33). The parameter $i_{s,elec-enz}$ denotes the electro-enzyme reaction-controlled steady-state current, and can be expressed as

$$i_{s,elec-enz} = \pm nSFk_{elec-enz}\Gamma_E, \quad (3.2)$$

where $k_{elec-enz}$ denotes the first-order steady-state rate constant of the enzyme E in the DET-type bioelectrocatalysis.

By considering a Butler-Volmer-type equation for the electrode kinetics of E and a Michaelis–Menten equation for the enzyme reaction, one can consider the following serial reactions (for oxidation of S):

$$k_{ox} \equiv k^{*}K_{E}^{1-\alpha} \quad k_{c} \quad k_{-1}$$

(E$_{red}$)
where $\text{ES}$ denotes the enzyme–substrate complex, $k_1$ and $k_{-1}$ denote the rate constant of the forward and backward reactions of the $\text{ES}$ formation, respectively, and $k_c$ denotes the catalytic constant of the DET-type bioelectrocatalysis. Therefore, based on the serial resistance mode, $k_{\text{elec−enz}}$ can be expressed as

$$
\frac{1}{k_{\text{s,elec−enz}}} = \frac{1}{k_{\text{Ox}}} + \frac{1}{K'_{\text{E,Enz}}k_c} + \frac{1}{K'_{\text{Enz}}k_c} + \frac{1}{k_c},
$$

(3.4)

where $K'_{\text{Enz}}$ denotes the steady-state reaction quotient defined as

$$
K'_{\text{Enz}} \equiv \frac{c_{\text{ES}}}{c_{\text{EOx}}} = \frac{k_1c_S}{k_{-1} + k_c} = \frac{c_S}{K_{M(S)}}.
$$

(3.5)

$K'_{\text{E}}$ can be given by Nernst equation of the RDS of the interfacial electron transfer of the electrochemically active site in $\text{E}$ with number of the electrons ($n_{\text{E,RDS}}$; usually $n_{\text{E,RDS}} = 1$) and a formal potential ($E_{\text{E,RDS}}'$):

$$
K'_{\text{E}} = \left( \frac{c_{\text{EOx}}}{c_{\text{Ered}}} \right)_{\text{RDS,eq}} = \frac{k_{\text{ox}}}{k_{\text{red}}},
$$

(3.6)

where $k_{\text{ox}}$ and $k_{\text{red}}$, respectively, denote the rate constants of the oxidation of $\text{E}_{\text{red}}$ and reduction of $\text{E}_{\text{ox}}$, which can be expressed as

$$
k_{\text{ox}} = k^0 K_{\text{E}}^{1−\alpha},
$$

(3.7)

and

$$
k_{\text{red}} = k^0 K_{\text{E}}^{−\alpha},
$$

(3.8)

where $\alpha$ is the transfer coefficient of the RDS of $\text{E}$ at an electrode, and it is ideally 0.5. Strictly speaking, we have to also consider the interfacial redox reaction of $\text{ES}$. However, we do not mention further details on this matter.

In the presence of an excess amount of $\text{S}$, the serial reactions are simplified (for oxidation of $\text{S}$) as

$$
k_{\text{ox}} \equiv k^0 K_{\text{E}}^{(1−\alpha)} \quad \text{E}_{\text{red}} \rightarrow \text{E}_{\text{ox}} \rightarrow \text{(E}_{\text{red})},
$$

(3.9)

and the steady-state reaction quotient can be expressed as

$$
K'_{\text{E−Enz}} \equiv \left( \frac{c_{\text{Eox}}}{c_{\text{Ered}}} \right)_{\text{st}} = \frac{k_{\text{ox}}}{k_{\text{red}} + k_c}.
$$

(3.10)
Therefore, $k_{\text{elec-enz}}$ is simplified and given on the basis of the series resistant model by

$$\frac{1}{k_{\text{elec-enz}}} = \frac{1}{K'_{\text{E-Enz}}k_c} + \frac{1}{k_c^*}. \quad (3.11)$$

Equation (3.11) is often rewritten as [54]

$$k_{\text{elec-enz}} = \frac{k_c}{1 + \frac{k_{\text{ox}}}{k_{\text{red}}} + \frac{k_c}{k_{\text{red}}}} \left( \frac{k_c}{1 + \frac{1}{K'_{\text{E}}} + \frac{k_c}{k_c^*K'_{\text{E}}^{1-\alpha}}} \right) \quad (\text{for oxidation of } S) \quad (3.12)$$

or

$$k_{\text{elec-enz}} = \frac{k_c}{1 + \frac{k_{\text{ox}}}{k_{\text{red}}} + \frac{k_c}{k_{\text{red}}}} \left( \frac{k_c}{1 + \frac{1}{K'_{\text{E}}} + \frac{k_c}{k_c^*K'_{\text{E}}^{1-\alpha}}} \right) \quad (\text{for reduction of } S). \quad (3.13)$$

### 3.3 Random Orientation Model of Enzymes

Marcus theory gives long-range electron transfer kinetics that is defined for a simple case by [68–72]

$$k^o = k^o_{\text{max}} \exp[-\beta(d - d_0)], \quad (3.14)$$

with

$$k^o_{\text{max}} = \sqrt{\frac{4\pi}{\lambda RT}} \frac{\pi H_{\text{DA} (d = d_0)}^2}{h} \exp\left(\frac{-\Delta^1 G^o}{RT}\right), \quad (3.15)$$

where $d$ denotes the distance between the electron donor and acceptor (or the electrochemically communicating redox center of an enzyme and an electrode surface), $d_0$ denotes the distance of the closest approach, $\beta$ denotes the decay coefficient, $k^o_{\text{max}}$ denotes the standard rate constant of the interfacial electron transfer at $r = r_0$, $\lambda$ denotes the reorganization energy, $H_{\text{DA} (r = r_0)}$ denotes Hamiltonian at $r = r_0$, and $h$ denotes the Plank constant. The rate constant of the electron transfer reaction decreases exponentially with $d$ ($k_2/k_1$ for $\beta = 1.4$ Å$^{-1}$ being 1/4 and 1/16 at $\Delta d = 1$ and 2 Å, respectively [72] and the enzyme orientation varies the values of $d$. Therefore, the orientation of adsorbed enzymes on an electrode is very important in determining the rate of the DET-type bioelectrocatalysis.

The simplest model has been presented based on the random orientation on a planar electrode for a spherical enzyme with a radius $r$ [71, 72]. In the enzyme,
the electrochemically active center is located at \( r_{as} \) from the center of the enzyme (Fig. 3.4, (A)). By assuming the random orientation of the enzyme at a planar electrode, the electrode reaction center in the enzyme rotates in the range between \( r - r_{as} \) and \( r + r_{as} \) with a probability density function \( f \) given by [73, 74]

\[
f = \frac{1}{2r_{as}}. \tag{3.16}
\]

Substitution of Eq. (3.14) into Eq. (3.12) gives the steady-state DET current by the enzymes located at \( z = d - (r - r_{as}) \):

\[
i_{s,z=d-(r-r_{as})} = \frac{i_{s,lim}}{1 + \frac{1}{K_{E}^o} + \frac{k_o}{k_{max} K_{E} t^{-\alpha} \exp(-\beta(z+r-r_{as}))}}, \tag{3.17}
\]

with

\[
i_{s,lim} = \pm nSFAk_c \Gamma_E. \tag{3.18}
\]

The integration of the current gives the total current by the whole enzymes on the electrode as follows for oxidation of \( S \):

---

**Fig. 3.4** Schematic model of an adsorbed enzyme on (A) planar and (B) spherical mesoporous-electrodes (Reproduced from Ref. [67], Copyright (2016) with permission from The American Chemical Society)
3.3 Random Orientation Model of Enzymes

\[ i_{s,\text{elec-enz}} = \frac{1}{2r_{as}} \int_{0}^{2r_{as}} i_{s,z} \, dz = \frac{i_{s_{\text{lim}}}}{2r_{as} \left( 1 + \frac{1}{K_E} \right)} \ln \left( \frac{k_c}{k_{\text{max}} K_E^{1-\alpha}} + \left( 1 + \frac{1}{K_E} \right) \exp[-\beta(r - r_{as})] \right) + \left( 1 + \frac{1}{K_E} \right) \exp[-\beta(r + r_{as})] \right). \] (3.19)

and for reduction of S as

\[ i_{s,\text{elec-enz}} = \frac{i_{s_{\text{lim}}}}{2r_{as} \left( 1 + K_E' \right)} \ln \left( \frac{k_c}{k_{\text{max}} K_E^{1-\alpha}} + \left( 1 + K_E' \right) \exp[-\beta(r - r_{as})] \right) + \left( 1 + K_E' \right) \exp[-\beta(r + r_{as})] \right). \] (3.20)

One of the typical steady-state catalytic voltammograms on the basis of the random orientation model on a planar electrode is represented as the solid line in Fig. 3.5. The calculated voltammogram consists of a sigmoidally increasing region, a linearly increasing region known as a residual slope [73, 74], and a limiting region to give \( i_{s_{\text{lim}}} \). In the sigmoidally increasing region, relatively fast interfacial electron transfer reaction occurs between the electrode and enzymes with orientations suitable for the DET-type reaction. The linearly increasing region is generated by enzymes with orientations which are unsuitable for fast DET-type reactions (dot-dashed line in Fig. 3.5). For the exponentially decayed \( k^0 \) due to an increase in \( d \) of the enzymes with unsuitable orientations, \( k_{\text{ox}} \) increases exponentially with an increase in \( E \) for the oxidation of S and the number of enzymes satisfying \( k_{\text{ox}} >> k_c \) increases linearly with \( E \) (dotted line in Fig. 3.5), and vice versa, \( k_{\text{red}} \) increases exponentially with a decrease in \( E \) for the reduction of S, and the number of enzymes with \( k_{\text{red}} >> k_c \) increases linearly with a decrease in \( E \). Finally, all enzymes on the electrode surface

![Fig. 3.5](image)

**Fig. 3.5** Calculated DET-type bioelectrocatalytic steady-state waves for the random absorption model on (solid line) a planar electrode and (dashed line) a spherical electrode. Parameters are: \( r = 2 \text{ nm}, r_{as} = 0.6 \text{ nm}, k_c/k_{\text{max}}^0 = 10^{-10}, \) and \( R_p = 2.4 \text{ nm} \). DET-type bioelectrocatalytic waves for the random absorption model at the planar electrode are divided into the two contributions: (dotted line) the current generated by enzymes with poor orientations at \( 48^\circ < \theta < 180^\circ \) and (dot-dashed line) the current generated by the other enzyme with suitable orientations.
can participate in fast DET-type bioelectrocatalysis at $k_{\text{ox}} >> k_{c}$ in the limiting region for oxidation and vice versa for $k_{\text{red}} >> k_{c}$ in the reduction reaction.

Mesoporous electrodes are very effective and sometime essential for proceeding the DET-type bioelectrocatalysis. It is also necessary to consider orientations of enzymes in mesopores. Herein, we assume a spherical enzyme with a radius $r$ located in a spherical pore with a radius $R_p$, (Fig. 3.4b) [67, 75]. In this model, the distances in the directions of $z[= r_{as}(1 - \cos\theta)]$, radial, and $x$ axes are, respectively, defined as follows:

$$d_1 = \begin{cases} -R_p + z + r - r_{as} + \sqrt{R_p^2 - 2r_{as}z + z^2(z + r - r_{as} \leq R_p)} \\ R_p - z - r + r_{as} + \sqrt{R_p^2 - 2r_{as}z + z^2(z + r - r_{as} \geq R_p)} \end{cases}, \quad (3.21)$$

$$d_2 = R_p - \sqrt{2r_{as}z - z^2 + (R_p - z - r + r_{as})^2}, \quad (3.22)$$

and

$$d_3 = \sqrt{R_p^2 - (R_p - z - r + r_{as})^2 - \sqrt{2r_{as}z - z^2}}, \quad (3.23)$$

The shortest path would be suitable for DET-type reaction. The steady-state current is given by the following numerical summation of $i_{s,z}$ with the shortest path:

$$i_{s,\text{elec-enz}} = \frac{1}{2r_{as}} \sum_{z=0}^{z=2r_{as}} i_{s,z} \Delta z, \quad (3.24)$$

The dashed line in Fig. 3.5 denotes one of the steady-state catalytic voltammograms based on the random adsorption model in a spherical mesopore. Although the parameters used in the model with the exception of $R_p$ are identical with those used for the random orientation at the planar electrode (solid line), the catalytic wave height in a spherical mesopore is larger than that on the planar electrode; the sigmoidally increasing part increases, while the residual slope part decreases in the spherical mesopore compared with the planar electrode. This is the curvature effect, and the effect becomes evident when $R_p$ is getting close to the value of $r$, while some macropores (or mesopores with much larger $R_p$) are essential for the mass transfer of $S$, $P$, and other counter ions.

### 3.4 Data Analysis of the Steady-State DET-Type Bioelectrocatalytic Waves

The curvature effect is very important to precede DET-type bioelectrocatalysis. However, quantitative analysis of steady-state DET-type catalytic voltammograms
based on Eq. (3.24) is somewhat complicated owing to lack of detailed (and complicated) information on the size and distribution of mesoporous structures on an electrode surface. Therefore, DET-type steady-state catalytic waves were frequently analyzed using Eq. (3.19) or Eq. (3.20), for example for H$_2$ases [73, 74, 76]. In the analysis, Eq. (3.19) or Eq. (3.20) is fitted to the experimental data by a non-linear least square method using suitable adjustable parameters.

On the other hand, suppose that only enzymes with limited orientations on an electrode surface can participate in the DET-type bioelectrocatalysis even in the limiting region, the following equation, for oxidation of S, can be obtained [77]:

\[
i_{\text{s,elec-enz}} = \frac{i_{\text{lim}}}{\beta \Delta d \left(1 + \frac{1}{K_E^\alpha}\right)} \ln \frac{k_0}{k_c} \left(1 + \frac{1}{K_E^\alpha}\right) + K_E^{(\alpha-1)} \exp\left(-\frac{\beta \Delta d}{\delta_1 d}\right) \left(1 + \frac{1}{K_E^\alpha}\right) + K_E^{(\alpha-1)}
\]

(3.25)

and for reduction of S, we have:

\[
i_{\text{s,elec-enz}} = \frac{i_{\text{lim}}}{\beta \Delta d \left(1 + K_E^\alpha\right)} \ln \frac{k_0}{k_c} \left(1 + K_E^\alpha\right) + K_E^{(\alpha-1)} \exp\left(-\frac{\beta \Delta d}{\delta_1 d}\right) \left(1 + K_E^\alpha\right) + K_E^{(\alpha-1)}
\]

(3.26)

Instances of data analysis using Eqs. (3.25) and (3.26) were reported for FDH [78, 79] and an MCO, BOD [19, 77], respectively.

When the substrate depression (the concentration polarization) of S occurs near the electrode surface, either of Eqs. (3.19), (3.20), (3.25), or (3.26) can be coupled to Eq. (3.2) [26]. In addition, when the surface concentration of S is lower than $K_M(S)$, then the contribution of ES on the wave shape has to be considered and also whether to use Eq. (3.4) (instead of Eq. (3.11)) on the model given in Eq. (3.4). However, Eq. (3.4) becomes extremely complicated when the random or limited orientation is considered. Therefore, we may assume a Michaelis–Menten-type relation on the limiting current, and Eq. (3.18) can be modified to a more general form as [29, 80]

\[
i_{\text{s,lim}} = \pm n_S FA k_c \Gamma_E \frac{1}{1 + K_M(S)/c_S}.
\]

(3.27)

In DET-type bioelectrocatalytic measurements, it is often difficult to observe (or experimentally define) $i_{\text{s,lim}}$ due to low values of $k_{\text{max}}^o/k_c$ or the limitation of the potential window in experiments. Under such situations, one may use $i_{\text{s,lim}}$ as one of the adjustable parameters, which leads to unstable convergence in non-linear regression analysis of DET-type catalytic waves. When some sigmoidal part is observed, however, the following analysis may be adopted even in the absence of information on $i_{\text{s,lim}}$. As shown in Fig. 3.5, DET-type bioelectrocatalytic waves for the random absorption model at the planar electrode can be considered as the sum of the two contributions: the current generated by enzymes with poor orientations (dotted line) and that the current generated by the other enzymes with suitable orientations (dot-dashed line). The former provides residual slope part, and the latter provides sigmoidal part.
The half-wave potential of the sigmoidal part without the residual slope is almost identical with $E^{1/2}_{o}$ when the response is Nernstian, or the sigmoidal part gives kinetic information on the heterogeneous electron transfer of the enzymes with suitable orientations. Practically, we can consider a virtual current–potential linear line with a slope that is identical to the residual slope and going through the onset position of the DET-type wave (that is, almost identical with the dotted line in Fig. 3.5). The current due to the virtual linear voltammogram is subtracted from the experimental data (that corresponds to the solid line in Fig. 3.5) to give a sigmoidal wave (that is almost identical to the dot-dashed line in Fig. 3.5). An example of successful analyses was reported for W-containing FoDH [7].

As described in this section, rather quantitative analysis has been realized for the steady-state waves of DET-type bioelectrocatalysis. The analysis is very important, not only for proper understanding of the reaction and improvement of the reaction system, but also for thermodynamic and kinetic analyses of the enzyme. The electrochemical information to be obtained is very useful in the field of biochemistry.

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Chapter 4
Characteristic Properties of Redox Enzymes as Electrocatalysts

Abstract This chapter introduces specific features of redox enzymes as electrode catalysts compared with metallic small catalysts. Large sizes of redox enzymes causes inconvenient effects of the enzyme orientation on DET-type bioelectrocatalysis. The significant of mesoporous structures to minimize the orientation effects is emphasized. In addition, this chapter describes peculiar and important effects that can facilitate the interfacial electron transfer kinetics at the top edge of microporous structures. Some strategies for enzyme orientations to enhance the interfacial electron transfer are also introduced together with examples for individual enzymes. Bidirectional redox catalysis as one of the important features of enzyme catalysts is exemplified and the performance will be discussed in back-to-basic style based of Marcus theory.

Keywords Porous electrode · Curvature effect · Enzyme orientation · Electrostatic interaction · Electron transfer pathway · Bidirectional bioelectrocatalysis · Re-orientation energy

4.1 Introduction

In bioelectrocatalysis, redox enzymes work as electrocatalysts as in the case of metal-based inorganic molecules in view of electrochemistry. Significant advantageous properties of redox enzymes compared with metal-based catalysts are: (1) extremely high catalytic activity, (2) low reorganization energy, (3) high specificity, (4) high identicalness and uniformity (thanks to biological expression), and (5) enormous chemical versatility. These factors are very convenient from the viewpoint of application. In addition, redox potential of the electrochemically communicating site of redox enzymes can be definitely defined, which leads to more rigorous discussion on current–potential curves of catalytic waves. However, redox enzymes have huge size and are fragile. The size matter causes characteristic features in DET-type bioelectrocatalysis; the orientation of an enzyme is a key factor determining the distance between the electrode surface and the redox site located near the surface of the enzyme of a large size, since the interfacial electron transfer rate constant decreases exponentially with the distance between an electrode surface and the redox site of an enzyme (Eq. (3.14)). First of all, we will discuss this matter in the following.
4.2 Surface-Area Effect and Curvature Effects (or Cage Effect) on Mesoporous Electrodes

There are huge numbers of papers that verify the importance of porous structures to detect or magnify DET-type catalytic current [1]. Ketjen Black with the primary particles of a diameter of ~40 nm was found to be an effective material for DET-type bioelectrocatalysis of histamine dehydrogenase from Nocardioides simplex [2], while aggregated gold nanoparticles or porous gold electrodes were effective for DET-type bioelectrocatalysis of FDH [3–5]. The control of the porous structure of carbon cryogel was found to be effective to increase the DET-type catalytic current density of FDH [6]. An MgO-templated hierarchical mesoporous carbon electrode was effective for DET-reaction of BOD [7], HRP showed DET-type bioelectrocatalysis at a gold-nanoparticle-modified electrode [8]. Several cases are presented in the reference [1].

Such mesoporous materials are frequently utilized not only for DET-type reactions but for MET-type reactions, and also for inorganic catalyst-based electrode reactions. The major reason to use mesoporous materials appears to be an increase in the effective surface area against the projective one. We completely agree with the opinion for the case of MET-type reactions and inorganic catalyst-based reactions, because electrochemically communicating substances in those systems have low-molecular mass and the orientation is of no importance.

Some suitable control of the porous structure of electrodes was shown to be effective to increase the catalytic current density [7]. However, situations in the case of DET-type reactions appear to be contrastive to those of MET-type and inorganic catalyst-based reactions. Although DET-type bioelectrocatalysis of HRP and H2ase (Desulfovibrio vulgaris Miyazaki F) was not shown on planar electrodes, it proceeded very clearly on suitably tuned porous electrodes [8–10]. For BOD, DET-type catalytic waves were shown to be quite small on a planar electrode [11], but can easily be detected at suitable porous electrodes [8]; the ratio of the catalytic current versus non-Faradaic current was larger at porous electrodes than at planar electrodes. In addition, it was found that aggregated gold nanoparticles played a vital role as scaffolds for DET-type reactions [8, 12–14].

Figure 4.1 shows a typical example of the relationship between the DET-type catalytic current density \( j \) by BOD-catalyzed \( \text{O}_2 \)-reduction as a function of the ratio of electrochemically effective surface area of porous electrodes versus a planar electrode \( (A/A_{\text{plane}}) \), in which porous electrodes were prepared from gold nanoparticles on a planar surface [14] or by anodization of the gold surface [13], and \( A/A_{\text{plane}} \) values were evaluated from non-Faradaic current ratio. The \( j \) value increases with \( A/A_{\text{plane}} \) with some saturation characteristics. The increasing property seems to be explained simply due to an increase in \( A/A_{\text{plane}} \) (as expected in MET-reactions), while the saturation effect seems to be ascribed to the mass transfer inhibition occurring in multilayer mesoporous electrodes or to the situation that enzyme solutions could not become widespread into the deep part of the porous structure.

An important feature was observed in the region with small values of \( A/A_{\text{plane}} \), as given in the inset of Fig. 4.1. In such a small \( A/A_{\text{plane}} \) region, the relationship between
4.2 Surface-Area Effect and Curvature Effects (or Cage Effect) on Mesoporous Electrodes

Fig. 4.1 The relationship between the catalytic current density ($j$) at 0.1 V and the relative surface area ($A/A_{\text{plane}}$) at BOD-adsorbed porous Au electrodes. The inset shows an enlargement of the region with small $A/A_{\text{plane}}$. Reproduced from Ref. [13], Copyright (2019) with permission from Elsevier.

$|j|$ and $A/A_{\text{plane}}$ appeared to increase linearly with a negative intercept (or to retard the increase in $|j|$). Similar retarded increases in DET-type bioelectrocatalysis have been reported for O$_2$ reduction by Lac [15] and H$_2$ oxidation by H$_2$ase [14] on the deposited Au nanoparticles on electrodes and BOD on the porous carbon materials [16]. The appearance of a negative intercept indicates that the DET-type current does not merely increase with an increase in the (electrochemically) effective surface area of the nanoparticle-modified electrodes. The mesoporous structures formed by the aggregation of nanoparticles form holes or scaffolds suitable for DET-type bioelectrocatalysis in the porous structure. This is called curvature effect (Sect. 3.3) or cage effect.

Here, we will introduce a model to explain the curvature effect of the aggregated nanoparticles on DET-type bioelectrocatalysis. Figure 4.2A shows the structures of the spherical nanoparticles in a close-packed lattice on a planar surface. The packed spheres with a radius $r_s$ lead to the formation of several holes between nanoparticles. The tetrahedral and octahedral holes in the close-packed structure are filled with spheres of radius of $0.2r_s$ and $0.4r_s$, respectively. In tetrahedral and octahedral holes, the invading small spheres will be in contact with packed nanoparticles at four and six points, respectively. Because the diameters of primary particles of gold constructing the porous electrode are in the range of 15–50 nm [8, 12–14], several enzymes will be embedded in these holes. Furthermore, in these holes, the enzyme makes contact with nanoparticles at various points. Therefore, the probability of achieving an appropriate orientation of the enzyme will increase with an increase in the aggregation of nanoparticles on electrodes. As shown in Fig. 4.2B, the contact points (for enzymes) increase with the surface coverage ($\theta$) of spherical nanoparticles with a negative intercept. The feature is very similar to that given in the inset of Fig. 4.1, and the $A/A_{\text{plane}}$ value of 5 corresponds to $\theta = 3.3$ by assuming the aggregation of spherical
nanoparticles with a radius of 20 nm. Therefore, we can conclude that DET-type reaction occurred by enzymes embedded in the holes provided by the aggregation of spherical nanoparticles.

### 4.3 Interfacial Electrode Kinetics on Microporous Electrodes

Interfacial charge transfer reactions occur between electrodes and redox species in the electrical double layer at the electrode surface. The electrical double layer at the planar electrode may be described by the Gouy–Chapman theory. However, the theory is unsuitable for the porous electrode surface. Since most of DET-type bioelectrocatalytic reactions are observed only at mesoporous electrodes, it is important to clarify the effect of the curved surface on the electrical double layer [17]. Here, we demonstrate the numerically simulated electrical double layer around the microstructure at the electrode surface based on the Poisson–Boltzmann equation as follows:

\[
\nabla^2 \phi = -\frac{F}{\varepsilon \varepsilon_0} \sum z_i C_i. \tag{4.1}
\]
4.3 Interfacial Electrode Kinetics on Microporous Electrodes

Figure 4.3 shows the calculated electrical double layer around a micropore with a diameter of 2 nm. The electrical double layer on the curved surface shows exciting features. The thickness of the electrical double layer widens with a decrease in the electrolyte concentration. The thickness of the electrical double layer at 1 mM is thicker than the size of the micropore. Under these conditions, the electrical double layer in the micropore overlaps with each other. The overlapping of the electrical double layer decreases the electric field in the micropore. Moreover, the stretching of the electrical double layer enhances the electric field in the vicinity of the corner at the entrance of the micropore. Both the overlapping and stretching of the electrical double layer lead to the formation of an inhomogeneous electric field around the microstructure on the electrode surface. Theoretically, the electric potential at the reaction plane ($\phi_H$) will affect the kinetics of the electrode reaction, and these effects are known as the second Frumkin effects as following [18]:

$$\ln(k_{ox}) \propto \exp \left\{ \frac{(1 - \alpha)F}{RT} (E - \phi_H - E^\circ) \right\}. \quad (4.2)$$

Therefore, the heterogeneous potential profile around the curved surface at the porous electrode will provide the different electrode kinetics from the planar electrode. Additionally, the electric charge density ($Q$) is localized at the edge of nano-structures based on Poisson equation [19] and can be expressed as [20]:

$$Q = \frac{\epsilon A}{\varepsilon \varepsilon_0} \left( \frac{\partial \phi}{\partial n} \right).$$
\[
\ln \left( \frac{Q(\theta_0)}{Q(\theta_0 = \pi)} \right) = (\pi/\theta_0 - 1) \ln(r/m),
\]

where \(\theta_0\) denotes the angle of the edge and \(r\) denotes the radius of the edge. For instance, at an edge with \(r = 1\) nm and \(\theta_0 = 3\pi/2\), \(Q\) is intensified a thousand-fold compared with that at a planar electrode.

Therefore, the kinetics of the electron transfer reaction between an enzyme and an electrode seems to be affected by the porous electrode surface. The interfacial electron transfer kinetics must be improved under such an intensified electric field at the drastically charged edge of the nanostructure. Of course, this situation is valid even when using inorganic catalysts.

4.4 The Control of the Orientation of Adsorbed Enzymes by Surface Modification of Electrodes

The first factor is the control of the orientation of enzymes on the electrode surface by utilizing various kinds of specific interactions [21]. The electrostatic interaction is frequently utilized in redox enzymes in which the redox site is surrounded by surface amino acid residues with a positive charge (such as BOD from \textit{Myrothecium verrucaria} [22, 23]) or with a negative charge (such as an [NiFe] H\(_2\)ase from \textit{Desulfovibrio vulgaris} Miyazaki F. [23, 24]). The electrostatic interaction of such enzymes with the oppositely charged electrode surface improved the performance of the DET-type reaction [22, 23]. In contrast to such situations, the redox site is sometimes surrounded by non-charged and hydrophobic peptide. In such cases, the hydrophobic \(\pi-\pi\) stacking interaction between the redox active site of the enzyme and modifiers induced favorable orientation by modifying the electrode surface with polycyclic aromatic compounds [25, 26]. In addition, the orientation of BOD was improved by modification with bilirubin as the natural substrate of the enzyme [27], and methoxy-functionalized electrode improved the DET-type reaction of FDH [28] that prefers to interact with methoxy-substituent containing quinones [29].

\textit{Bilirubin oxidase (BOD)}

Electrode modification using BOD has been utilized to improve the orientation of adsorbed enzymes [27]. Although the adsorbed amount of BOD is decreased by modification with bilirubin, i.e., the neutral electron donor of BOD, the modification was effective for improving DET-type bioelectrocatalysis of BOD [27, 30]. This result demonstrates that the control of the orientation of the adsorbed enzyme is very effective to improve the performance of the DET-type reaction.

Studies on the effects of electrode modification on the orientation of BOD showed that, for controlling the orientation of BOD, attractive electrostatic interaction between the modifier and BOD was very effective. Interestingly, effective modifiers for the orientation of BOD depend on the origin of BOD, e.g., a negatively
charged modifier was shown to be effective for orienting BOD from *Myrothecium verrucaria* (*MvBOD*) [22, 30], while a positively charged modifier was effective for BOD from *Bacillus pumilus* [30]. The entire charge of *MvBOD* was negative at pH 7; however, the surface of the BOD around the T1 copper site was positively charged at pH 7. The effectiveness of the negatively charged modifier for orienting *MvBOD* demonstrates that the local charge of the surface of the enzyme or the dipole in the enzyme are crucial for controlling the orientation of the adsorbed enzyme. Therefore, *MvBOD* was shown to be effectively orientated on the electrode surface by modifying negatively charged aromatic compounds.

Both small molecules and nanomaterials are effective modifiers of the electrode for DET-type bioelectrocatalysis of BOD. The modified gold nanoparticles [12, 14, 31, 32] and carbon nanotubes [30, 33–35] have been shown to be effective scaffolds for DET-type bioelectrocatalysis of BOD.

**Hydrogenase**

Hydrogenase is a unique enzyme that catalyzes bi-directional reactions of hydrogen oxidation and proton reduction. Membrane-bound [NiFe] hydrogenases (MBH2ase) provide high DET-type bioelectrocatalytic activity [36, 37]. Moreover, MBH2ase has several redox centers such as [NiFe] cluster and iron-sulfur clusters (FeS) called the proximal, medial, and distal. The electronic communication between MBH2ase and electrode generally occurred at distal FeS [38, 39]. To improve the performance of DET-type bioelectrocatalysis of MBH2ase, we studied the effects of modification of the electrode surface.

Carbon nanotubes have been shown to be effective scaffolds of DET-type bioelectrocatalysis of MBH2ases from *Desulfovibrio fructosovorans* and *Aquifex aeolicus* [40, 41]. Because the shortening process of nanotubes improved DET-type activity, the edge parts or defects on the surface of carbon nanotubes seem to be conducive for DET-type bioelectrocatalysis. Moreover, the chemical interaction between MBH2ase and modifiers has been investigated. The adsorption of thiol on the gold surface forms a functional self-assembled monolayer (SAM). The SAM formed by a short and positively charged thiol was most effective for DET-type bioelectrocatalysis of MBH2ase from *Aquifex aeolicus* [41]. Furthermore, the positively charged modifier was found to be effective in the DET-type bioelectrocatalysis of MBH2ase from *Desulfovibrio vulgaris* Miyazaki F. This experimental observation agrees with the fact that the distal FeS is located on the negatively charged surface of the MBH2ase. The increase in ionic strength in the measurement buffer decreases with the DET-type activity of the MBH2ase adsorbed at p-phenylenediamine-modified electrodes. Moreover, the effect of the ionic strength has been found to support the expectation that the electrostatic interaction between the MBH2ase and electrode controls the orientation of the adsorbed MBH2ase [23, 24].

**d-Fructose dehydrogenase (FDH)**

FDH is a heterotrimeric enzyme for which one subunit containing three heme *c* moieties is the most electrochemically active site [29] and exhibits an extremely high DET-type bioelectrocatalytic activity. The mutations on the axial ligands of each
heme c revealed that one heme c moiety (the first one from the N-terminal) does not participate in DET-type bioelectrocatalysis [42]. Therefore, the variant FDH, lacking the heme c moiety, shows a high DET-type bioelectrocatalytic activity [43].

DET-type bioelectrocatalysis of FDH has been studied using a SAM-modified electrode [3]. SAM formed by 2-mercaptoethanol was found to be more effective for DET-type bioelectrocatalysis of FDH compared to charged and hydrophobic SAMs. The terminal hydroxy group changes the hydrophobicity of the electrode surface. The coexisting surfactant that will be used for solubilizing FDH forms a bilayer on the hydrophilic electrode surface. Therefore, the FDH seems to be firmly embedded in the surfactant bilayer on the electrode surface to communicate with the electrode [44].

The natural electron acceptor of FDH is ubiquinone, although FDH was able to donate an electron to various mediators based on the linear free energy relationship (LFER). However, ubiquinone derivatives were found to react with FDH with a much higher activity than expected in accordance with LFER [29]. Certain specific interactions of FDH with the structure of ubiquinone (2,3-dimethoxy-1,4-benzoquinone) were expected; thus, we investigated the effects of the surface modifier on DET-type bioelectrocatalysis of FDH at the KB electrode and found that the methoxy group in the modifier improved DET-type bioelectrocatalysis of FDH. Analysis of the catalytic wave based on Eq. (3.19) revealed that modification with 2,4-dimethoxyaniline reduced the $2\beta r_{as}$ values from 2.1 to 1.2 for the orientation of FDH. This indicates that the methoxy-substituent-functionalized surface increases the effective orientated FDH at the electrode surface [28].

In conclusion, nanostructures of the electrode surface are very important and probably essential for DET-type bioelectrocatalysis by redox enzymes. Important functions of nanostructures are the curvature effect of the mesoporous structure, the enhanced electric double layer effect at the edge of the microporous structure, and specific interaction between the electrochemically active site of enzymes and electrode surface as well as the mass transfer effect of the macroporous structure. Several specific interactions are very critical to improve the orientation of enzymes. The electrode surface can be suitably tailored for each redox enzyme in the near future by a better understanding of the effects described in this chapter. Protein engineering is essential and effective to suitably interact enzymes with nanostructured electrode surfaces.

### 4.5 DET-Type Bi-Way Bioelectrocatalysis

Progress has been made in tailor-made tuning of electrode surfaces for several kinds of enzymes to improve the DET-type bioelectrocatalytic performance by considering the above factors, and the number of redox enzymes that provide DET-type bioelectrocatalytic waves is growing. Among such DET-type redox enzymes, it can be noticed that several enzymes display single catalytic wave of bi-directional interconversion of oxidized and reduced substrates, in which the catalytic wave sharply intersects with the potential axis at the zero-current–potential ($E_i = 0$) that is identical
to the equilibrium potential ($E_{eq}$) of the substrate redox couple. Such reversible bi-directional inter-conversions are realized for: the $2H^+/H_2$ redox couple using several kinds of membrane-bound $H_2$ases usually with a [NiFe] cluster [37], the carbon dioxide ($CO_2$)/carbon monoxide redox couple by using CO$_2$ dehydrogenase with a [Ni4Fe–4S] cluster, the $CO_2$/formate (HCOO$^-$) redox couple by using FoDH with a W-pterin active site, and the NAD$^+$/NADH redox couple by using mitochondrial complex I [45]. Introductive interpretation of steady-state sigmoidal bioelectrocatalytic wave has been reported elsewhere [46]. These experimental findings clearly indicate that redox enzymes can work as extremely efficient electrocatalysts of several electrochemical reactions that are otherwise extremely poor in kinetics.

Interestingly, NAD-linked soluble $H_2$ase electro-catalyzes with two sets of reversible bi-directional inter-conversion between the $2H^+/H_2$ redox couple and between the NAD$^+$/NADH redox couple in a DET-type bioelectrocatalysis [47]. Similarly, a four-way DET-type bioelectrocatalysis was observed at a tailored mesoporous electrode that adsorbs NAD-linked FoDH with a W-pterin catalytic site from *Methylobacterium extorquens* AM1 (Fig. 4.4A, B) [48, 49]. The inter-conversion between CO$_2$ and formate can be catalyzed at the W-pterin site in the W-pterin-containing subunit, while the interconversion between NAD$^+$ and NADH can be catalyzed by using flavin mononucleotide (FMN) in the diaphorase subunit of FoDH. One of the FeS clusters in FoDH directly communicates with suitably-tuned porous

![Fig. 4.4 DET-type bioelectrocatalytic waves by FoDH for A HCOO$^-$ oxidation and CO$_2$ reduction at pH 6.6, and B NADH oxidation and NAD$^+$ reduction at pH 7.0 at 25 °C, $v = 10$ mV s$^{-1}$ under quiescent conditions. Panel C is a schematic potential profile of the reactants involved in the four-directional catalysis](image-url)
electrodes. By considering the fact that the formal potential of the CO$_2$/HCOO$^-$ redox couple ($E^{\circ}_{\text{CO}_2$/HCOO$^-$}) is 0.11 V more negative than that of the NAD$^+/$/NADH redox couple ($E^{\circ}_{\text{NAD}^+$/NADH}) under neutral conditions [50], schematic energetics can be illustrated in Fig. 4.4C, which indicates that the standard Gibb energy of the reaction ($\Delta_r G^\circ$) is quite positive for one of the intramolecular electron transfer processes. Even under such conditions, FoDH shows clear bi-directional DET-type bioelectrocatalytic reduction and oxidation waves of the CO$_2$/HCOO$^-$ and NAD$^+/$/NADH couples. This means that the rate constants of the self-electron-exchange reactions of the electron-donating and -accepting sites ($k_{\text{DD}}$ and $k_{\text{AA}}$) are very large at the redox sites in the enzyme, as evidenced by the Marcus cross reaction theory [51]:

$$k \approx \sqrt{k_{\text{AA}}k_{\text{DD}}} \exp\left(-\frac{\Delta_r G^\circ}{RT}\right),$$  

(4.4)

where $k$ denotes the rate constant of the intramolecular electron transfer in FoDH. By considering a quadratic function of the energy map of reactant and product, $\Delta^\ddagger G^\circ$ can be expressed with the reorientation energy ($\lambda$) as [52, 53] (Fig. 4.5):

$$\Delta^\ddagger G^\circ = \frac{(\Delta_r G^\circ + \lambda)^2}{4\lambda}.$$  

(4.5)

Because $\Delta_r G^\circ = 0$ at self-electron-exchange conditions, $\Delta^\ddagger G^\circ = \lambda/4$. This implies that large values of $k_{\text{DD}}$ and $k_{\text{AA}}$ are only realized at small values of $\lambda$. Polypeptides surrounding the redox sites play important roles in minimizing the conformation change in the electron transfer and then to minimize $\lambda$. In other words, such up-hill electron transfers seem to be frequent in redox enzymes, but would be very difficult for small molecules in solution because of large $\lambda$.

Fig. 4.5 Schematic view for understanding Eq. (4.5)
In addition, considering the fact that \( E_i = 0 \) value of the bi-directional catalytic wave corresponds to the \( E_{eq} \) value of the substrates (CO\(_2\)/HCOO\(^-\) and NAD\(^+\)/NADH), the standard rate constant of the interfacial electron transfer between the electron-donating/accepting site (FeS cluster) in FoDH and the mesoporous electrode is quite large when the enzyme is adsorbed in productive orientations.

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Chapter 5
Protein-Engineering Approach for Improvement of DET-Type Bioelectrocatalytic Performance

Abstract Recent advances in the area of protein-engineering have facilitated the production of mutated proteins predesigned to enhance the electronic coupling with electrodes. In this chapter, we will start from the motivation of protein engineering and introduce typical protein-engineering strategies for improvement of DET-type bioelectrocatalysis.

Keywords Protein-engineering · Mutants · Direct electron transfer · Bioelectrocatalysis

5.1 Motivation of Protein-Engineering

As discussed in Chap. 1, redox enzymes that are capable of direct electron transfer with electrodes is very limited in number, because the redox active sites are in many cases embedded by insulating peptides of the enzymes. Increasing attention in the field of electrochemistry of redox enzymes is driven by designing efficient interfaces for electron transfer between electrode surfaces and redox enzymes. Because a DET-type bioelectrocatalysis proceeds between an enzyme and an electrode, strategies to improve the performance should be just considered in views of two directions: enzymes and electrodes. The past decades have witnessed the development of various electrode materials, such as carbon nanotubes, mesoporous carbon materials, and metal nanoparticles, for improving the performance of DET-type bioelectrocatalysis [1–6]. Such nanomaterials improved the interfacial electron transfer kinetics of redox enzymes thanks to their nanostructures that increased the effective enzyme loading [7, 8]. Besides, rationally designed electrode surfaces with special properties are also widely studied to achieve productive orientation or attachment of enzymes [9–11].

In this chapter, we will focus on the protein-engineering approach for improvement of DET-type bioelectrocatalysis. Enzymes can be modified by several techniques of the protein-engineering. Remarkable progress has been made in protein-engineering of redox enzymes in the past decades and several reviews related to the protein-engineering for bioelectrocatalysis and bioelectrochemistry are found in the literature [12–15]. Protein-engineering through rational design, direct evolutions, and combined approaches, has been successfully utilized to reform the enzymes with
special properties, such as thermal stability, pH activity/stability, tolerance towards salts etc [16, 17]. In the view of DET-type bioelectrocatalysis, several enzyme modification approaches have been proposed: (i) trimming of the N- or C-terminals and deglycosylation to reduce the size of enzymes and to open up the redox active site [18], (ii) site-directed mutation at the redox active site to change its redox and catalytic characteristics [19], and (iii) insertion of tag sequences and site-specific mutation of amino acid residue to control the orientation of enzymes on electrodes [20].

5.2 Enzyme Trimming

Enzymes are biomacromolecules that have usually large and sophisticated three-dimensional molecular structures. An effective strategy to increase the interfacial electron transfer kinetics for DET-type bioelectrocatalysis is downsizing the dimension of enzymes to shorten the distance between the active sites and the electrode surfaces and to increase the surface concentration of enzymes.

Generally, carbohydrate of enzymes acts as an insulating shell and increases the distance between the redox active site of enzymes and the electrode surface. Therefore, a useful method to shorten the distance is deglycosylation. For an example, in previous researches, it was shown that deglycosylation of HRP [21] and tobacco peroxidase [22] shortened the distance between the prosthetic group and the electrode surface, thereby enhanced the DET kinetics. HRP is a ferric enzyme that catalyses the oxidation of various kinds of electron donating substrates with H2O2 as the electron acceptor. The carbohydrate content of HRP reaches 18 % of the total mass, which was considered to hinder the electron transfer between the active site and electrodes. Gorton et al. constructed carbohydrate-free recombinant HRP enzymes [21]. The engineered enzyme-modified polycrystalline gold electrodes exhibited high current responses of DET-type bioelectrocatalysis to H2O2, while a native glycosylated HRP-modified electrode didn’t show such catalytic currents. The improvement in the DET kinetics of the enzymes was ascribed to the absences of the carbohydrate shell on the recombinant HRPs.

Except to HRP, improved DET-type bioelectrocatalysis was also reported in other deglycosylated enzymes: cellobiose dehydrogenases (CDH) [23], pyranose dehydrogenase (PDH) [24], and glucose oxidase (GOD) [25]. GOD is a flavoenzyme catalyzing glucose oxidation and is widely utilized in glucose biosensors and biofuel cells. GOD from Aspergillus niger is a heavily glycosylated homodimer that contains one FAD per monomer as the cofactor (Fig. 5.1A). The molecular mass is ca. 160 kDa depending on the level of the glycosylation. The calculated value of the minimum distance between the FAD to the outside of the protein in GOD is ca. 1.7 nm, which may lead to sluggish direct electron transfer of GOD adsorbed at a solid electrode [26, 27]. Mano and his co-workers reported a nearly fully deglycosylated and highly purified GOD that exhibited clear DET response at a glassy carbon electrode (Fig. 5.1B) [25]. The onset potential of the catalytic glucose oxidation wave obtained at the deglycosylated GOx-adsorbed electrode was −0.49 V versus Ag|AgCl. The
5.2 Enzyme Trimming

Fig. 5.1  A Representation of the structure of GOD [26]. The yellow and water blue spheres show the FAD active site and glycosylated sites, respectively. B Cyclic voltammogram at a deglycosylated GOD-adsorbed GC electrode in a phosphate buffer (pH 7.0, 20 mM, 37 °C) containing 45 mM glucose under argon atmospheres at a scan rate of 5 mV s\(^{-1}\). The inset in plane B shows non-catalytic cyclic voltammograms of (dashed line) native GOD- and (solid line) deglycosylated GOD-adsorbed glassy carbon electrodes in the absence of glucose [25]. Reproduced from Refs. [25, 26]. copyrights (2009) and (2018) from Wiley and Elsevier, respectively.

oxidative current density reached to 235 \(\mu\)A cm\(^{-2}\) at \(-0.20\) V and at a glucose concentration of 45 mM. The half-wave potential of the glucose oxidation was \(-0.38\) V, which was more positive than the expected redox potential of non-catalytic waves as observed at deglycosylated GOD-adsorbed electrodes in the absence of glucose (Inset of Fig. 5.1B). Therefore, it seems to be difficult to exclude an possibility that the catalytic wave is due in part to free FAD molecules leaving from the redox center of the enzymes and acting as a mediator in a mode of a MET-type bioelectrocatalysis [28]. As a similar case as an example, a clear bioelectrocatalytic wave was observed at a glassy carbon electrode and at a high concentration of FoDH in the presence of HCOO\(^-\) but without any added mediators [28]. The observed catalytic wave was reasonably assigned not to a DET-type bioelectrocatalysis but to free FMN-mediated catalytic oxidation of HCOO\(^-\); FMN in FoDH lost touch with the enzyme.

In addition to deglycosylation, elimination of the domains that are not related to the electron transfer to downsize the enzyme is also an effective way to improve the performance of DET-type bioelectrocatalysis. Our group has focused on the DET-type bioelectrocatalysis of FDH, a heterotrimeric membrane-bound flavohemoprotein catalyzes a 2-electron oxidation of \(d\)-fructose to 5-keto-\(d\)-fructose and consisting of three subunits: subunit I (67 kDa), subunit II (51 kDa), and subunit III (20 kDa) [29]. FDH has a covalently bound FAD in subunit I as a catalytic center and three heme \(c\) moieties (hemes 1c, 2c, and 3c from the N-terminus) in subunit II as prosthetic groups [29]. The subunit I/III complex (\(\Delta c\_FDH\)) lacking subunit II did not show any DET-type bioelectrocatalytic activity, although \(\Delta c\_FDH\) retained catalytic activity in the solution [30]. The data indicates that subunit II was essential in DET-type bioelectrocatalysis of FDH. Further studies have proposed that the electron transfer in FDH occurs in sequence from FAD, through heme 3c to heme 2c to the electrodes without going through heme 1c (Fig. 5.2A left) [19]. Therefore, an
Fig. 5.2  A Proposed schematic of the orientations suitable for DET-reaction of FDH, Δ1cFDH, and C Δ1c2cFDH. As templates, FAD-GDH from *Aspergillus flavus* (PDB 4YNT) and thiosulfate dehydrogenase from *Marichromatium purpuratum* (PDB 5LO9) were used for subunit I (green) and II (cyan), respectively, in the homology modeling. The arrow indicates the presumable pathway of the electron transfer in the DET-type reaction. The subunit III is not shown in the modeling because of the lack of the structural information of similar proteins. B Original and C normalized CVs of d-fructose oxidation at the (native) r_FDH-, Δ1cFDH-, and Δ1c2cFDH-adsorbed electrodes in McIlvain buffer (pH 4.5) in the presence of 0.1 M d-fructose under anaerobic conditions at \( v = 10 \text{ mV s}^{-1} \). The broken line in panel B indicates the background current at the bare Au electrode. In panel C, the background current was subtracted. Reproduced from Ref. [32] copyright 2019 (with minor modification) with permission from Elsevier

FDH variant (Δ1c_FDH) lacking 143 amino acid residues involved in the coordination of the heme 1c moiety was constructed to shorten the distance from heme 2c to the electrode surface (Fig. 5.2A middle) [31]. As a result, increase in the catalytic current density and a negative shifted in the half-wave potential of the catalytic wave was observed at Δ1c_FDH-modified electrodes as compared with those at (native) r_FDH-adsorbed one (Fig. 5.2B). The increase in the catalytic current density is presumably thanks to an increase in the surface concentration of the enzyme on electrodes with proper orientations for the DET-type bioelectrocatalysis owing to the downsizing of the enzyme.
It is worthy of note that an FDH variant lacking 199 amino acid residues, including the heme 1c and 2c moieties, \(\Delta 1c2c_{\text{FDH}}\) showed clear DET-type catalytic activity and its electron transfer occurred at a more negative potential than that observed for \(r_{\text{FDH}}\) and \(\Delta 1c_{\text{FDH}}\) (Fig. 5.2A right and C) [32]. In the case of this mutant (\(\Delta 1c2c_{\text{FDH}}\)), the electron during the DET-type bioelectrocatalysis is transferred from heme 3c to the electrode, and the interfacial electron transfer kinetics was drastically improved, most likely as a consequence of the shortening of the distance between the electrode surface and heme 3c resulting from downsizing of the enzyme (Fig. 5.2A). However, due to the mentioned downsizing of the enzyme, the catalytic activity and the limiting catalytic current density of \(\Delta 1c2c_{\text{FDH}}\) decreased compared to \(r_{\text{FDH}}\) (Fig. 5.2B) [32].

5.3 Site-Directed Mutation

Site-direct mutation of selected amino acids in the active site or in close proximity to redox-active centers is a successful concept and has been well explored to improve the electrical communication between an electrodes and biocatalysts.

An example of site-direct mutations affecting DET-type bioelectrocatalysis is shown in multi-copper oxidase (MCO), a family of redox protein for 4-electron reduction of \(O_2\). The active site of MCOs contains 4 Cu atoms which are divided into three types based on their spectroscopic and magnetic properties: type I (T1), type II (T2), and type III (T3) coppers. The T1 Cu oxidizes electron donators and transfers the electron to the trinuclear center composed of one T2 Cu and two T3 Cu atoms where \(O_2\) is reduced to \(H_2O\). In a DET-type bioelectrocatalytic reaction, the T1 site accepts electrons from electrodes. DET-type bioelectrocatalytic property of several mutations of CueO, namely M510L, M510Q, and D439A was examined [33]. M510L and M510Q were prepared by replacing the Met510, the axial ligand of the T1 site of CueO, with Leu and with Gln, respectively. D439A was prepared by replacing the Asp439 with Ala, Asp439 forming a hydrogen bond with His443 coordinating to the T1 Cu. It has been found that M510L and D439A exhibit a positive shift, while M510Q exhibited a negative shift, in the onset potential of the DET-type bioelectrocatalytic waves, compare to the wild-type CueO. The result is accordance with a report related to BOD, in which substitution of the Met coordinated to the T1 Cu at the axial position with Gln decreased the redox potential of T1 from 0.46 to 0.23 V [34].

In addition to single-site mutation, Zhang et al. recently constructed a series of CueO mutants by direct evolution method and found that a double substitution mutant, D439T/L502K, significantly increased the onset potential up to 0.54 V versus Ag|AgCl [35] (Fig. 5.3). The positions, D439 and L502, are located in the second coordination spheres of the T1 Cu and form hydrogen bonds with coordinated ligands, H443 and C500, respectively. The substitutions are assumed to have altered the donating ability of His443 and C500 to the T1 Cu. Combination the double substitution CueO mutant-based biocathode with a GDH-based bioanode realized a
Effects of site-directed mutant of FDH on DET-type bioelectrocatalysis were also examined. As mentioned in 5.2, subunit II in FDH containing three heme c moieties was essential in DET-type bioelectrocatalysis. Three mutants (M301Q, M450Q, or M578Q), in which the sixth axial Met ligand of heme 1c, heme 2c, and heme 3c, respectively, was replaced with Gln. The three mutants exhibited significant differences in voltammograms (Fig. 5.4). As expected, M450Q exhibited a significant negative-shift in the onset potential for fructose oxidation [19]. Furthermore, by combination of the effects of site-directed mutant and downsize, a novel variant M450Q Δ1cFDH was constructed, in which 143 amino acid residues involving heme 1c were removed and M450 as the sixth axial ligand of heme 2c was replaced with Gln [36, 37]. As a result, a negative direction shift of the formal potential as well as increased DET-type bioelectrocatalitic current density was achieved at M450Q Δ1cFDH-adsorbed electrodes, compare with the native r_FDH-adsorbed electrode (Fig. 5.4d).

5.4 Protein Surface Modification

The control of the enzyme orientation on the electrode surface appears to be a major challenge in order to avoid random orientation that often results in poor electrode performance [9]. For oriented immobilization of enzymes, strategies involving an electrode surface modification have been developed [9, 38]. For an example, MvBOD [39, 40], H₂ase [41], FDH [42], and FoDH [43] were successfully adsorbed on rationally functionalized electrode surfaces. Such strategies are greatly influenced by the surface properties of the natural enzymes, and have some limitation in practice. The site-directed biological modification of a functional enzyme is one of the smart approaches to achieve an oriented assembly of the enzyme. The basic strategy is to utilize the genetic engineering technology and to modify an enzyme with linkers that provide oriented attachment of the enzyme on a specific electrode base.
Fig. 5.4  A–C Cyclic voltammograms of d-fructose oxidation at (broken line) a native FDH-adsorbed Au electrode and (solid lines). A M450Q, B M578Q, and C M301Q mutant-adsorbed Au electrodes in a McIlvain buffer (pH 4.5) containing 0.1 M d-fructose under anaerobic conditions at a scan rate of 10 mV s\(^{-1}\). The dotted lines indicate background voltammograms at bare Au electrodes. D Linear sweep voltammogram of d-fructose oxidation at the (native) \(r_{\text{FDH}}\) (dashed gray line), M450QFDH- (dashed black line), \(\Delta 1c_{\text{FDH}}\) (gray line), and M450Q\(\Delta 1c_{\text{FDH}}\)- (black line) adsorbed electrodes in a McIlvain buffer (pH 4.5) in the presence of 0.2 M d-fructose under anaerobic conditions at \(v = 10\) mV s\(^{-1}\). The dotted line indicates the background at the bare Au electrode. Reproduced from Refs. [19, 36] copyrights (2016) and (2019) from Elsevier and The Electrochemical Society of Japan, respectively.

Thiol-gold bonds are frequently used to assemble films on Au electrodes. Therefore, the introduction of a Cys residue at the N- or C-terminus of the enzyme peptide chain facilitates the oriented immobilization of the enzyme [44]. Recombinant laccases (\(r_{\text{Lac}}\)) with a Cys-6 × His (Cys-6 × His) tag at the N- or C-terminus were purified, and the recombinant laccase was assembled with suitable orientation on a gold electrode via Au–S bonds (Fig. 5.5) [20]. The orientation-dependent non-catalytic DET reaction of laccase was investigated. DET-type bioelectrocatalysis toward \(O_2\) reduction were also investigated, but the waves were rather different from those expected from the non-catalytic waves. The similar method was also utilized
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Fig. 5.5  Schematic representation of the orientations of r_Lac immobilized orientationally on a bare gold surface with a Cys-6 × His tag at its N-terminus (A) or C-terminus (B). Reproduced from Ref. [20] copyrights (2014) (with minor modification) from Elsevier

for a MCO from Pyrobaculum aerophilum to improve DET-type bioelectrocatalysis by shortening the distance between the active site and the electrode surface [45].

Besides to forming Au–S bonds, thiol-maleimide click reaction is also a convenient method to covalently bind a Cys residue on the redox enzymes surface to electrode surfaces. Bartlett et al. constructed a sites specific variant of MoBOD S362C, in which Ser362 at the MoBOD surface closed to the TI Cu was replacing by Cys residue [46]. (Fig. 5.6A) The distance between the T1 site and the Ser362 is ca. 1.33 nm. A thiol-maleimide click reaction between S362CMoBOD variant and maleimide-functionalized MWCNT was employed to construct a stable bioelectrode. A clear DET-type bioelectrocatalytic O2 reduction wave with higher current and more sigmoidal shape was obtained at a S362CMoBOD-modified electrode than that at a native MoBOD-modified electrode (Fig. 5.6B, C) [46]. Such improvements in DET-type bioelectrocatalytic performance can be explained by the oriented immobilization of the enzyme with a short distance between the active site and electrodes. In a similar manner, two CDH mutants, T701C and E522C, have been prepared and shown great improvement in the performance for DET-type bioelectrocatalytic glucose oxidation [47].

His tagging is a well-known strategy in metal-ion affinity chromatography that has also been employed to enable specific attachments between an enzyme and an electrode. For example, a r_HRP with an addition of six-His-tag at the C-terminus of the enzyme polypeptide adsorbed gold electrodes showed an increase in DET-type bioelectrocatalytic current as compared to His-tag-free r_HRP-adsorbed electrodes [21]. Most probably, the His-tag favours a productive orientation and self-assembly of HRP on the electrode. For the electrode surface modified by nitrilotriacetic acid (NTA) moiety, after complex formation with Ni2+, recombinant proteins engineered
to contain a stretch of six consecutive His residues at the N- or C-terminus were attached via their affinities to the Ni–NTA moiety. Successful construction of an oriented monolayer of cyt c oxidase on an Au electrode surface was demonstrated, and DET-type bioelectrocatalysis of cyt c oxidase was confirmed [48, 49].

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Chapter 6
Applications to Biosensors

Abstract Amperometric biosensors is one of applications of bioelectrocatalysis, and glucose biosensor is the most well-known and successful example of all biosensor devices. This chapter describes several proposals for bioelectrocatalysis-based biosensors: MET-type mass transfer-controlled biosensors, MET-type potentiometric coulometry, and bienzyme biosensing by coupling DET-type peroxidase bioelectrocatalysis and oxidase reaction without any mediators.

Keywords Amperometry · Mass transfer-control · Surface-confined redox reaction · Potentiometry · Bienzyme coupling

6.1 Introduction

Electrochemical sensors are constructed with the selective and transducing parts [1]. In electrochemical biosensors, the substrate specificity of redox enzymes ensures the selectivity of the substrate of the sensors [2–4]. The electrode reaction corresponds to the transducing part in which the substrates flow is converted to the electron flow. The early biosensors were constructed from oxidases and oxygen electrode and detected the oxygen consumption due to the oxidase reactions [5]. In conjunction with the development of bioelectrocatalytic systems and detection devices, the freedom has increased in the design of electrochemical biosensors.

In the early type of electrochemical biosensors, the current detection, that is, amperometric detection, was used. The current detection is suitable to rapid measurements and in situ monitoring of the concentration change. The detection of charge, that is, coulometric detection, was also applied to biosensing. Since the electricity corresponds to the amount of the target substrate, coulometry seems to be suitable to accurate or trace analyses in principle. In addition, potentiometry with field-effect transistors was also applied to biosensing [6, 7]. The improvement in the selectivity and sensitivity and the miniaturization of electrochemical biosensors are hot topics even now. This chapter introduces the recent development in electrochemical biosensors based on amperometry and coulometry.
6.2 Selectivity of Electrochemical Biosensors

The substrate specificity of enzymes is usually high, while the selectivity of the electrode reaction is quite poor, as evidenced by LFER-type Butler–Volmer equation (LFER holds only for non-specific reactions). Therefore, once undesirable redox active species in a sample solution contact with an electrode, the electrode would show some response to these species. The body fluid contains various types of electroactive species at unknown and non-constant concentrations. Therefore, for example, oxygen reduction, dopamine oxidation, ascorbate oxidation, and urate oxidation will interfere the response of electrochemical biosensors. Therefore, the detection potential should be set to avoid the interference by these reactions. At typical carbon electrodes, detection potentials of around 0 V versus Ag|AgCl|KCl(sat.) are suitable to avoid the interferences due to these reactions [3, 8]. On the other hand, in order to avoid the instability in the current detection due to the changes of the electrode kinetics and the reference electrode potential, the detection potential must be set in the limiting current region for the target species. Therefore, the selection of the detection potential is one of very important factors in the actual analysis.

When the selectivity of the electrode reaction is not guaranteed by the working potential setting unfortunately, one of typical strategies is the utilization of permselective membranes on the electrode surface [9]. For example, electropolymerized 1,2-diaminobenzene plays as a barrier for the penetration of interfering substrates and for the leaking of immobilized enzyme [10]. As a simpler method for the formation of permselective membrane, casting of Nafion® dispersion on enzyme-modified electrodes is frequently employed and effective to suppress the permeation of ascorbate and urate from test solutions to the electrode [11, 12]. Since Nafion® is a cationic exchanger resin, the suppression effect of Nafion® toward such interfering substrates seems to be caused by the electrostatic repulsion between the interfering species and the membrane. It is also important that the membrane does not hinder the permeation of the target substrate.

6.3 Electrodes for Amperometric Biosensing

Amperometric biosensors are most widely studied in bioelectrocatalysis-based devices [2, 3, 13]. In the amperometric sensing, the steady-state response must be required for accuracy reasons. In the case of MET-type amperometric biosensors, the steady-state current response \( i_s \) is given as follows (see also Sect. 2.4);

\[
\frac{1}{i_s} = \frac{1}{i_{s,elec}} + \frac{1}{i_{s,enz}} + \frac{1}{i_{s,perm}} + \frac{1}{i_{s,mt}},
\]  
(6.1)
where $i_{s,\text{elec}}$, $i_{s,\text{enz}}$, $i_{s,\text{perm}}$, and $i_{s,\text{mt}}$ are the limiting current controlled by the electron transfer, the enzymatic reaction, the permeation of membrane, and the mass transfer of substrate, respectively. The term $1/i_{s,\text{elec}}$ can be ignored by setting the detection potential at the limiting current conditions (sufficiently positive or negative compared with $E_M^{\circ}$ for the oxidative or reductive detection of analyte, respectively). The value of $i_{s,\text{enz}}$ is directly affected by a change in the enzyme activity which is inherently unstable and more or less decreases with time. The $i_{s,\text{perm}}$ value is not constant for biosensors with an outer membrane. Therefore, the diffusion-controlled steady-state response is required to construct stable and reliable amperometric biosensors, i.e., $i_{s,\text{mt}} \ll i_{s,\text{perm}}, i_{s,\text{enz}}, i_{s,\text{elec}}$.

The requirement that $i_{s,\text{mt}} \ll i_{s,\text{perm}}, i_{s,\text{enz}}$ can be satisfied by using sufficiently high concentrations of a redox enzyme near an electrode without any outer membrane. In order to realize the steady-state mass transfer of substrate, hydrodynamic technique using a rotating disk electrode (RDE) or non-linear diffusion at a microdisk electrode (ME) may be used as typical electrochemical methods. The steady-state limiting current density for RDE ($j_{s,\text{RDE}}$) and ME ($j_{s,\text{ME}}$) are formulated as follows:

$$j_{s,\text{RDE}} = 0.62 n F D^{2/3} v^{-1/6} c \omega^{1/2},$$  \hspace{1cm} (6.2)

where $n$, $F$, $D$, $v$, $c$, and $\omega$ are the number of electrons, the Faraday constant, the diffusion coefficient of substrate, the concentration of substrate, and angular velocity of RDE [14] and:

$$j_{s,\text{ME}} = \frac{4nFcDr}{\pi r^2},$$  \hspace{1cm} (6.3)

where $r$ is the radius of ME [15].

MEs have several advantages in the electrochemical sensing; they are easy to miniaturize the apparatus and have fast response. However, the amperometric response of ME is quite small and, for example, of the order of pico ampere at nm-size MEs. Since the detection of such small signals requires special attentions for protection against noise, the enhancement of the response of the sensor is an important subject in practice. Microband electrodes may have possibly to solve this issue as judged from the following equation on the limiting response ($i(\tau)_{\text{lim}}$) of a microband electrode at $\tau (\equiv Dt/w^2)$ [16]:

$$i(\tau)_{\text{lim}} = \frac{1}{\sqrt{nF D c l}} + 1 \left( \tau < \frac{2}{5} \right) = \frac{\pi e^{-2\sqrt{\pi\tau}/5}}{4\sqrt{\pi\tau}}$$

\[ + \ln \left[ \frac{\sqrt{nF D c l}}{\sqrt{64\pi e^{-0.5772156} + e^{5/3}}} \right] \left( \tau > \frac{2}{5} \right), \]  \hspace{1cm} (6.4)
where \( l \) and \( w \) denote the length and the width of the microband electrode, respectively. Equation (6.4) shows that the decrease in the thickness of the electrode makes the response fast without change in the intensity. In addition, the response of the microband electrode increases with an increase in \( l \).

Since too-long microband electrode is not realistic in practice, we developed an ultrathin ring electrode as shown in Fig. 6.1. A gold film of the ultrathin ring electrode was fabricated by sputtering and an insulator is a photocurable resin [17]. The constructed ultrathin ring electrode worked as a band electrode with \( l = 6 \text{ mm} \) and \( w = 100 \text{ nm} \). The response of the ultrathin ring electrode agreed well with the value expected from Eq. 6.4 and the quasi-steady-state response was of the order of micro ampere. In addition, the non-Faradaic current of the ultrathin ring electrode was sufficiently small for practical use. Therefore, such ultrathin ring electrodes seem to be suitable to general amperometric sensing.

### 6.4 Mass-Transfer-Controlled Amperometric Biosensing

FAD-dependent glucose dehydrogenase (FAD-GDH) from \textit{Aspergillus terreus} catalyzes the oxidation of glucose with benzoquinone (BQ) as a mediator in MET-type bioelectrocatalysis. A glucose sensor constructed with an ME, FAD-GDH, and BQ provided a pseudo-steady-state current which was proportional to the glucose
concentration. In addition, the upper limit of the linear range exceeded the BQ concentration. This means that quite fast MET-type bioelectrocatalysis occurred near the electrode surface. The response of the sensor agreed with the calculated value for the hemispherical diffusion of glucose at the ME. Therefore, the sensitivity of the sensor became reproducible and independent of any change in the enzyme activity [18].

The analysis of the diffusion-limited MET-type bioelectrocatalysis at an ME based on the diffusion reaction equations of mediator and enzyme shows that the enzymatic reaction occurs in the very thin reaction plane (Fig. 6.2) [19]. The reaction plane locates near the electrode surface. The concentration gradient of the reduced mediator locates only inside in the reaction plane. This situation corresponds to that the diffusion flux of the glucose is completely transferred to the diffusion flux of mediator at the reaction plane. In addition, the reaction plane plays as a virtual electrode which selectively reacts with the substrate (glucose). The position of the reaction plane is determined by the kinetics of the bioelectrocatalytic reaction. However, the current agrees with the diffusion limiting current of the substrate at the ME.

The extremely fast enzymatic reaction by FAD-GDH realizes the diffusion-limited response at microband electrodes (Fig. 6.3) [20]. Numerical simulation shows that

**Fig. 6.2** Cross-sectional view of calculated concentration profiles of A substrate, B reduced mediator, and C reduced form of enzyme around an ME at a substrate concentration of 4 mmol dm$^{-3}$, a mediator concentration of 1 mmol dm$^{-3}$, an enzyme concentration of 0.2 mmol dm$^{-3}$, a diffusion coefficient of substrate of $6 \times 10^{-10}$ m$^2$ s$^{-1}$, and a diffusion coefficient of mediator of $1 \times 10^{-10}$ m$^2$ s$^{-1}$. The profiles were calculated for the situation of 20 s after the potential step at the limiting current conditions. Reproduced from Ref. 19, Copyright (2014) with permission from Royal Society of Chemistry
the increase in the thickness of the electrode improves the upper limit of the detection due to a decrease in the current density.

The steady-state characteristics due to the spherical diffusion can be theoretically obtained by a planar disk electrode. In the case of NAD-dependent lactate dehydrogenase, Meldola’s blue-adsorbed disk electrode with \( r = 1.5 \text{ mm} \) was used to realize steady-state diffusion-controlled electrolysis of lactate in the presence of free NAD\(^+\). After about 2 min of the electrolysis, the spherical diffusion at the edge part of the planar electrode became predominant contribution to the current. Such diffusion-controlled system enabled simultaneous detection of enantiomers such as d- or l-lactates [21]. Therefore, the slow steady-state characteristics of the planar electrode seem to be useful in practical applications.

### 6.5 Potentiometric Coulometry

The coulometry is the one of the most accurate analytical methods. Even the biosensing, coulometric methods coupled with bioelectrocatalytic charge accumulation are familiar techniques [22–27]. However, the fact that the Faradaic current is rather smaller than the non-Faradaic current in the bioelectrocatalysis involved in chronoamperometry makes the estimation of the total accumulated charge difficult.

One of the solutions for this problem is the determination based on potentiometry. The electric charge accumulated into an immobilized redox mediator on the electrode changes the electrode potential (Fig. 6.4). According to the Nernst equation as shown by Eq. 6.5, the change in the electrode potential corresponds to the change in the ratio of the oxidized state against the reduced state of the mediator (\( \Gamma_{M,\text{ox}} / \Gamma_{M,\text{red}} \)).

\[
E_i = E_M^{\text{eq}} + \frac{RT}{n_M F} \ln \left( \frac{\Gamma_{M,\text{ox}}}{\Gamma_{M,\text{red}}} \right)
\]  
(6.5)
where $\Gamma_{M,ox}$ and $\Gamma_{M,red}$ are the amounts of oxidized and reduced forms of the mediator in the immobilized layer, respectively. Therefore, the accumulated charge is evaluated directly from the total amount of the immobilized mediator ($\Gamma_1$) and the equilibrated electrode potential. In this method, the interference of the non-Faradaic processes in the coulometric method can be eliminated.

However, when osmium complex-combined poly polyvinylimidazole was used as a mediator, the potential response deviated from the Nernst equation because of the strong electrostatic interactions between the redox sites in the polymer [28]. In order to decrease the interaction between the redox species at the electrode surface, a redox active thin liquid film was fabricated on the electrode surface [29]. The liquid film was constructed with a hydrophobic ionic liquid (1-ethyl-3-methylimidazolium bis(nonafluorobutanesulfonyl)imide), organic medium (dibutyl phthalate), and redox mediator (ferrocene). Since the higher ionic strength in the liquid film effectively decreases the electrostatic interaction between the redox-active species, the liquid film modified electrode plays as a reversible surface-confined system. In addition, the potential difference between the liquid film and the sample solution is kept constant by the low solubility of the ionic liquid [30]. Since $\Gamma_1$ can be evaluated separately, the total amount of the accumulated charge by the bioelectrocatalysis can be estimated from the Nernst equation and the change of the electrode potential without any calibration as follows;

$$Q = \frac{n_M F A \Gamma_1}{1 + K_{M,i}} - \frac{n_M F A \Gamma_f}{1 + K_{M,f}},$$

(6.7)

where $K_{M,i}$ and $K_{M,f}$ are $\Gamma_{M,ox}/\Gamma_{M,red}$ values at the initial and final electrode potentials in the liquid film. When the initial potential is sufficiently negative (for the oxidative detection), the charge can be evaluated from the final potential alone;

Fig. 6.4  Schematic illustrations of A the accumulation process and B the potentiometric measurement of a liquid-film-modified electrode. Reproduced from Ref. 29, Copyright (2016) with permission from Elsevier.
The dynamic range of the potentiometric coulometry could be regulated on the basis of the amount of the mediator. The interference due to the non-Faradaic processes on the coulometry could be eliminated by potentiometric coulometry.

### 6.5.1 Bienzyme Biosensing

MET-type bioelectrocatalysis is widely used in electrochemical biosensors. However, several problems caused in relation to mediators, such as stability and cost, have limited the design of biosensor. Therefore, since no external addition of mediator is required in ideal biosensors, DET-type bioelectrocatalysis is more suitable for the actual biosensing than MET-type bioelectrocatalysis. The problem is that the number of the enzymes that can perform DET reactions is limited. This problem can be partially solved by combining enzymes.

The combination of an oxidase and horseradish peroxidase (HRP) is a classical design of biosensors, since $H_2O_2$ is a product of oxidase reactions [31]. However, in the proposed biosensors, MET-type bioelectrocatalysis of HRP was used to reductively detect $H_2O_2$. Recently, DET-type activity of HRP-adsorbed electrode has drastically improved to realize the steady-state diffusion-controlled reduction of $H_2O_2$ [8]. Therefore, the combination of HRP-adsorbed electrode and oxidases became very useful to detect the substrate of the oxidase without any mediator. The oxidase-HRP (OP)-type biosensing has been applied for the detection of glucose [8], putrescine [32], and pyruvate [33].

Assuming serial steps of the reactions of the OP-type biosensing, the response is given by the following equation:

$$
\frac{1}{i_S} \sim \frac{1}{i_{S,mt(S)}} + \frac{1}{i_{S,mt(O_2)}} + \frac{1}{i_{S,oxD}} + \frac{1}{i_{S,HRP}} + \frac{1}{i_{S,elec}}, \tag{6.9}
$$

where each $i_{S,k}$ denotes the following process; $mt(S)$ is the steady-state mass transfer of the substrate, $mt(O_2)$ is the steady-state mass transfer of $O_2$, $oxD$ is the catalytic oxidation of the substrate by the oxidase reaction on the electrode surface, and $elec$ is the interfacial electron transfer from an electrode to the oxidized HRP. The kinetic analysis of the enzymatic reactions at an OP-type biosensor was essential to realize the mass-transfer controlled response. According to the analysis, the DET activity of HRP enables the mass transfer-limiting conditions at an ME [33]. Therefore, the mass-transfer controlled biosensor can be constructed by the immobilization of the sufficient amount of oxidase on the electrode surface.
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Chapter 7
Applications to Biofuel Cells and Bioreactors

Abstract This chapter starts by introducing several types of biofuel cells as an application of bioelectrocatalysis with the advantages and disadvantages. Challenging issues and outlook are also described. Photo-driven bioanodes and bio-solar cell are also introduced. Bioelectrochemical reactors are proposed as reverse reactions of biofuel cells. One of brilliant points of bioelectrocatalytic systems is the property that the systems can catalyze redox reactions bidirectionally and reversibly. The significance of this matter is discussed in view of hydrogen society.

Keywords Biofuel cells · Biosupercapacitors · Bioreactors · Bidirectional catalysis · Hydrogen society · C1 cycle · NAD(P)-dependent enzymes

7.1 From Bioelectrocatalysis to Biofuel Cells and Bioreactors

As mentioned in Chap. 1, bioelectrocatalysis utilizes redox enzymes to provide non-specific electrode processes with specific catalytic activities, and the system can be applied not only to biosensors (signal transformation) but to varieties of electrochemical devices for the transformation of chemical energy of fuels to electrical energy or vice versa. The former is usually called as biological fuel cells (which is simply referred to as biofuel cells) and, the latter is named as bioelectrochemical reactors (which is simply referred to as bioreactors). Although some microbes (strictly speaking, a set of cascade redox enzymes in the microbes) are also often employed for energy conversions, this chapter deals only with biofuel cells and bioreactors that utilize purified redox enzyme(s).

7.2 Biofuel Cell

The concept of biofuel cells was first introduced by Yahiro and co-workers in 1964 [1]. Generally, biofuel cells consist of a two-electrode setup: with the aid of corresponding redox enzymes, fuels are oxidized at the bioanode, and the electrons flow
through the external electric circular to the biocathode, at which the oxidants are reduced (usually O\textsubscript{2} to water) (Fig. 7.1). A proton exchange membrane may be occasionally used to separate the two electrode systems and to avoid the electronic crossover between the two electrodes while accelerating proton transfers, although it is not essential in biofuel cells in theory.

Biofuel cells utilizing redox enzymes as bioelectrocatalysts are expected to be one of the next-generation energy conversion systems, because they have several advantages as follows. Firstly, redox enzymes with uniform characteristics can be obtained from a wide range of living organisms by isolation and purification, and then they can be classified as renewable catalysts. Secondly, fuels are diverse in kind. In principle, sugars, alcohols, organic acids, hydrogen, and their mixtures that can be digested by living organisms, can be used as fuels for biofuel cells. Thirdly, operational conditions are very mild and safe. The characteristics of the enzymatic reactions allow biofuel cells to operate at physiological pH, room temperature, and ambient pressure. Besides, redox enzymes show exceptional specificity towards their natural substances, thus enabling the assembly of both bioanode and biocathode of biofuel cells without any proton-exchange membranes (as separators). Thus, it is possible to miniaturize biofuel cells down to extremely small sizes. The high specificity of enzyme reactions is also convenient to avoid the requirement of high purity of fuels. Accompanying with the biocompatibility of redox enzymes, biofuel cells consuming fuels in physiological fluids is proposed to be utilized as harmless power sources for implanted or wearable electric devices.

According to the electron transfer mechanism between enzymes and electrodes, biofuel cells can also be simply classified into two types: MET- and DET-type biofuel cells. Table 7.1 summarized the performance of typical MET- and DET-type biofuel cells reported in the past years. Interested readers may find many review articles related to biofuel cells in the past 5 years [2–9].

Sugar/O\textsubscript{2} Biofuel Cells

The most common fuels utilized in biofuel cells are sugars, since they are inexpensive, abundant, renewable, and safe to use. Among various sugar-based biofuel cells, glucose/O\textsubscript{2} biofuel cells were often reported. Typically, a glucose/O\textsubscript{2} biofuel cell

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**Fig. 7.1** Schematic of a biofuel cell
| MET | Table 7.1 Performances of typical MET- and DET-type biofuel cells |
|-----|---------------------------------------------------------------|
| Anode | Cathode | Conditions | $OCV/V$ | $P_{\text{max}}$/mW cm$^{-2}$ (at $E_{\text{cell}}$/V) | Stability | Refs. |
| Reaction* | Enzyme (stabilizer)/mediator/electrode | Reaction* | Enzyme (stabilizer)/mediator/electrode | | | |
| HCOO$^-$ + $2e^-$ $\rightarrow$ CO$_2$ | $Me$-FoDH/VP/KB/WPCC | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD/ABTS/KB/WPCC (GD, 1 bar O$_2$) | 0.3 M HCOONa, 1.0 M phosphate buffer, pH 7.0, 40 °C, quiescent | 1.2 | 12 (0.78) | – | [10] |
| glucose + $4e^-$ $\rightarrow$ 5-dehydro-gluconate | $B$-GDH, Gn5DH/NAD/Bs-DI/VK$_3$/CFS | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD/ANQ/CFS (GD, 1 bar air) | 2.0 M imidazole buffer, pH 7.0, 25 °C, quiescent | – | 10 (0.5) | – | [11] |
| glucose + $2e^-$ $\rightarrow$ gluconate | $B$-GDH/NAD/Bs-DI/VK$_3$/CFS | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD/ANQ/CFS (GD, 1 bar air) | 2.0 M imidazole buffer, pH 7.0, 25 °C, quiescent | – | 5.0 (0.5) | Continuous operation for 2 h | [12] |
| HCOO$^-$ + $2e^-$ $\rightarrow$ CO$_2$ | $Cb$-FoDH/NAD/N-doped AuNPs | $O_2/2 + 2e^- \rightarrow H_2O$ | $Tv$-Lac/ABTS/N-doped AuNP | 0.05 M HCOONa, 0.1 M phosphate buffer (pH 6.0), quiescent | 0.95 | 1.96 (0.4) | Stable $E_{OCV}$ for 30 d | [13] |
| 6glucose + $2e^-$ $\rightarrow$ gluconate | $B$-GDH/NAD/Bs-DI/VK$_3$/CFS | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD/Fe(CN)$_6^{3-}$/CFS (GD, 1 bar air) | 0.4 M glucose, 1.0 M phosphate buffer, pH 7.0, 25 °C, quiescent | 0.8 | 1.45 (0.3) | Continuous operation for 27 h at 1 mA cm$^{-2}$ | [14] |
| glucose + $2e^-$ $\rightarrow$ gluconate | $B$-GDH/NAD/Bs-DI/AQS/HRC | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD (raffinose)/ABTS/KB/VGCF (GD, 1 bar air) | 1.2 M glucose, 2.0 M phosphate buffer, pH 7.5, 25 °C, quiescent | – | 0.5 (0.9) | – | [15] |

(continued)
| Reaction* | Enzyme (stabilizer)/mediator/electrode | Reaction* | Enzyme (stabilizer)/mediator/electrode | Conditions | OCV/V | $P_{\text{max}}$/mW cm$^{-2}$ (at $E_{\text{cell}}$/V) | Stability | Refs. |
|-----------|-------------------------------------|-----------|-------------------------------------|------------|-------|---------------------------------|----------|------|
| glucose $- 2e^- \rightarrow$ gluconate | $A_n$-GOx/Os-polymer / | $O_2/2 + 2e^- \rightarrow H_2O$ | $Tv$-Lac/MWCNT (compressed homogeneous mixture) (1 bar air) | 5 mM glucose, phosphate buffer, pH 7.0, 37 °C, quiescent | 0.75 | 0.16 (0.4) | – | [16] |
| glucose $- 2e^- \rightarrow$ gluconate | $A_n$-GOx (+ catalase)/NQ/MWCNT (compressed homogeneous mixture) | $O_2/2 + 2e^- \rightarrow H_2O$ | $Tv$-Lac/MWCNT (compressed homogeneous mixture) (1 bar air) | 5 mM glucose, phosphate buffer, pH 7.0, 37 °C, quiescent | 0.75 | 0.16 (0.4) | 22% after 1 year (1 h discharge every day) | [17] |
| glucose $- 2e^- \rightarrow$ gluconate | GOx/TTF/KB/conductive carbon ink/Japanese paper | $O_2/2 + 2e^- \rightarrow H_2O$ | $Mv$-BOD/KB/conductive carbon ink/Japanese paper (DET, 1 bar air) | 0.1 M glucose, phosphate buffer, pH 7.0, RT, quiescent | 0.55 | 0.12 (0.4) | – | [18] |
| HCOO$^- - 2e^- \rightarrow CO_2$ | $Ec$-FoDH/BPV-LPEI/CP | $O_2/2 + 2e^- \rightarrow H_2O$ | $Tv$-Lac/ABTS/MWCNT | 0.05 M HCOONa, 1.0 M phosphate buffer (pH 7.6, anode)/Nafion 212/0.1 M citrate/phosphate buffer (pH 4.0, cathode), stirring | 1.28 | 0.018 | – | [19] |
| Anode Reaction* | Enzyme/electrode | Cathode Reaction* | Enzyme/electrode | Conditions | OCV/V | $P_{\text{max}}$/mW cm^{-2} (at $E_{\text{cell}}$/V) | Stability | Refs. |
|----------------|-----------------|-----------------|-----------------|------------|-------|-----------------|----------|-------|
| H$_2$ $-2e^-\rightarrow 2H^+$ | $Dv$-MF-H$_2$ase/KB/WPCC (GD, 1 bar H$_2$) | O$_2$/2 + $2e^-\rightarrow H_2$O | $Mv$-BOD/KB/WPCC (GD, 1 bar O$_2$) | 1.5 M citrate buffer, pH 5.0, 40 °C, quiescent | 1.14 | 8.4 (0.70) | -- | [20] |
| H$_2$ $-2e^-\rightarrow 2H^+$ | $Dv$-MF-H$_2$ase/PDA-modified KBE/WPCC (GD, 1 bar H$_2$) | O$_2$/2 + $2e^-\rightarrow H_2$O | $Mv$-BOD/ABA-modified KB/WPCC (GD, 1 bar air) | 1.5 M citrate buffer, pH 5.0, 25 °C, quiescent | 1.12 | 6.1 (0.72) | -- | [21] |
| fructose $-2e^-\rightarrow 5$-keto-fructose | FDH/CCG/CP | O$_2$/2 + $2e^-\rightarrow H_2$O | $Mv$-BOD/BL-adsorbed KB/CP (GD, 1 bar air) | 0.5 M d-fructose, 1.0 M citrate buffer, pH 5.0, 25 °C, quiescent | 0.79 | 2.6 (0.46) | -- | [22] |
| fructose $-2e^-\rightarrow 5$-keto-fructose | FDH/CNTf | O$_2$/2 + $2e^-\rightarrow H_2$O | Ts-Lac/CNTf (1 bar O$_2$) | 0.2 M d-fructose, McIlvaine buffer, pH 5.0, 25 °C, stirring | 0.77 | 1.8 (0.45) | 84% after 24 h of continuous operation | [23] |
| H$_2$ $-2e^-\rightarrow 2H^+$ | $Aa$-MBH/CNF/PGE (1 bar H$_2$) | O$_2$/2 + $2e^-\rightarrow H_2$O | $Bp$-BOD/CNF/PGE (1 bar O$_2$) | 0.1 M phosphate buffer, pH 6.0, 60 °C, a separator Nafion$^\text{®}$ 117, bubbling | 1.05 | 1.5 (0.7) | 40% after 24 h of continuous operation | [24] |
| fructose $-2e^-\rightarrow 5$-keto-fructose | FDH/ME-modified AuNP/CP | O$_2$/2 + $2e^-\rightarrow H_2$O | $Mv$-BOD/AuNP/CP (1 bar O$_2$) | 0.2 M d-fructose, 0.1 M acetate buffer, pH 6.0, 25 °C, stirring | 0.73 | 0.87 (0.3) | 85% after 12 h of continuous operation | [25] |

(continued)
| Reaction* | Enzyme/electrode | Reaction* | Enzyme/electrode | Conditions | OCV/V | $P_{\text{max}}$/mW cm$^{-2}$ (at $E_{\text{cell}}$/V) | Stability | Refs. |
|-----------|-----------------|-----------|-----------------|------------|------|--------------------------|----------|-------|
| fructose $-2e^-$ $\rightarrow$ 5-keto-fructose | FDH/KB/CP | O$_2$/2 $+ 2e^-$ $\rightarrow$ H$_2$O | Ts-Lac/CG/CP (1 bar O$_2$) | 0.2 M D-fructose, 1.0 M citrate buffer, pH 5.0, 25 °C, stirring | 0.79 | 0.85 (0.41) | 63% after 12 h; continuous operation for 60 d | [26] |
| H$_2$ $-2e^-$ $\rightarrow$ 2H$^+$ | Aa-MBH/AN-MWCNT/CC (1 bar H$_2$) | O$_2$/2 $+ 2e^-$ $\rightarrow$ H$_2$O | Bp-BOD/AN-modified MWCNT/CC (GD, 1 bar air) | phosphate buffer, pH 7.2, 45 °C, H$_2$-bubbling | 0.95 | 0.72 (0.6) | – | [27] |
| H$_2$ $-2e^-$ $\rightarrow$ 2H$^+$ | Ec-H$_2$ase 1/CMC/PGE (78% H$_2$—22% air mixture) | O$_2$/2 $+ 2e^-$ $\rightarrow$ H$_2$O | Mv-BOD/CMC/PGE (GD, 78% H$_2$—22% air mixture) | 0.1 M phosphate buffer, pH 6.0, 25 °C, quiescent | 1.15 | 0.56 (0.65) | 90% after 24 h and 54% after 7 d | [28] |
| H$_2$ $-2e^-$ $\rightarrow$ 2H$^+$ | Ec-H$_2$ase 1/PGE (1 bar H$_2$) | O$_2$/2 $+ 2e^-$ $\rightarrow$ H$_2$O | Mv-BOD/PGE (1 bar O$_2$) | 0.1 M pH 5.0 phosphate buffer, 25 °C, rotating electrode | 0.99 | 0.063 (0.51) | – | [29] |

*Proton and water out of the relation of their own redox reaction are omitted

**Electrochemical:** OCV (open circuit voltage), $P_{\text{max}}$ (maximum power), GD (gas diffusion-type), CFS (carbon fiber sheet), CF (carbon felt), VGCF (vapor-grown carbon fiber), HRC (hierarchical porous carbon)

**Enzymes:** Me-FoDH (W-containing formate dehydrogenase from *Methylobacterium extorquens* AM1), Ch-FoDH (NAD$^+$-dependent formate dehydrogenase from *Candida boidini*), Ts-Lac (laccase from *Trametes versicolor*), Mv-BOD (bilirubin oxidase from *Myrothecium verrucaria*), Bp-BOD (bilirubin oxidase from *Bacillus pumilus*), Dv-MF-H$_2$ase ([NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F), Aa-MBH (membrane bound hydrogenase from *Aquifex aeolicus*), FDH (fructose dehydrogenase from *Gluconobacter japonicas* NBRC3260), Ts-Lac (laccase from *Trametes* sp.). B-GDH (NAD-dependent glucose dehydrogenase from *Bacillus* sp.), Bp-DI (diaphorase from *Bacillus stearethomophilus*), Gn5DH (glucono 5-dehydrogenase), Gn5DH (gluconate 5-dehydrogenase), Ec-H$_2$ase 1 (O$_2$-tolerant membrane bound [NiFe] hydrogenase 1 from *Escherichia coli*), An-GOx (glucose oxidase from *Aspergillus niger*),

**Electrode:** KB (Ketjen black), WPCC (waterproof carbon cloth), CCG (carbon cryogel), CP (carbon paper), CG (carbon aerogel), AuNP (gold nanoparticles), MWCNT (multi-walled carbon nanotubes), CNF (fishborn carbon nanofibers), PGE (pyrolytic graphite electrode), CC (carbon cloth), CMC (compacted mesoporous carbon)

**Mediator:** VP (viologen-functionalized polymer), ABTS ([2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate]), BPV-LPEI ([N-benzyl-N-propyl-4,4'-bipyridinium],

**Chemical:** PDA (p-phenylenediamine), ABA (2-aminobenzoic acid), BL (bilirubin), NAD (nicotinamide adenine dinucleotide), VK$_3$ (2-methyl-1,4-naphthoquinone, vitamin K$_3$), AQ5 (anthraquinone-5-sulfonic acid), ANQ (2-amino-1,4-naphthoquinone), AT (anthrascene), AN (amino naphtholic acid), ME (mercaptoethanol), NQ (p-naphthoquinone), TTF (trithiafulvalene)
combined a glucose bioanode, in which GOD or GDH is usually employed as redox enzymes, and an O₂ biocathode, in which MCOs, such as BOD, Lac, and CueO are often utilized. For an example, a glucose/O₂ biofuel cell was reported by combination of an NAD-dependent GDH/diaphorase-based bioanode and a BOD-based O₂ biocathode; the cell provided a maximum power density ($P_{max}$) of 5 mW cm⁻² at an operational cell voltage ($E_{cell}$) of 0.5 V [30]. At the bioanode in the system, glucose is 2-electron-oxidized with NAD⁺ by NAD-dependent GDH, and FMN in Dp accepts the hydride ion from NADH and provides two electrons to 2-amino-1,4-naphthoquinone as a mediator, which transfer the electrons to the bioanode in a MET-type bioelectrocatalytic mode. At the biocathode, O₂ acts as the final electron-acceptor in a mode of a MET-type bioelectrocatalysis of BOD using K₃[Fe(CN)₆] as a mediator [30].

Fructose also is a common sugar utilized for biofuel cells. Due to the DET capability of FDH, a DET-type fructose/O₂ biofuel cell was reported with a $P_{max}$ of 0.85 mW cm⁻² at an $E_{cell}$ of 0.41 V, in which fructose was 2-electron-oxidized by FDH in a DET-type bioelectrocatalytic mode at the bioanode, while O₂ was 4-electron-reduced in a DET-type bioelectrocatalysis of Lac at the biocathode [26]. Furthermore, a $P_{max}$ density of 2.6 mW cm⁻² at an $E_{cell}$ of 0.46 V was achieved under quiescent conditions by combining an FDH-based fructose bioanode with a BOD-based air-breathing O₂ biocathode [22]. In addition, many other sugars including xylose, sucrose, and polysaccharides such as maltodextrin have been also used as fuels in biofuel cells.

$H₂/O₂$ Biofuel Cells

Dihydrogen ($H₂$) is also a common fuel for biofuel cells because it has the highest energy density value per mass and has been widely used in conventional fuel cells. $H₂/O₂$ fuel cells are known as clean and highly efficient energy conversion devices and have attracted increasing attentions for establishing the “hydrogen economy”. In $H₂/O₂$ biofuel cells, $H₂$ is oxidized by $H₂$ase at the bioanode and O₂ is typically reduced by MCOs at the biocathode, the product being just $H₂O$. The 2-electron oxidation of $H₂$ is a complete oxidation, unlike that of sugars and alcohol. Although $H₂$ase shows high catalytic efficiency, two major issues have to be considered in the utilization of $H₂$ase as an electrocatalyst: their O₂ sensitivity and the oxidative inactivation that occurs at positive electrode potentials [31, 32]. A useful approach to protect $H₂$ase from O₂ damage and high-potential deactivation was to entrap the enzyme into a specifically designed viologen-based redox polymer [33]; almost whole parts of the polymer were reduced in a MET-type reaction of $H₂$ase, and the reduced polymer can scavenge (or reduce) O₂ diffusing from the solution phase and sometimes reactivate (or reduce) $H₂$ase that is oxidatively deactivated at electrodes at positive potentials. An $H₂/O₂$ biofuel cell combining the viologen polymer-functionalized $H₂$ase bioanode and BOD biocathode provided a $P_{max}$ of 0.178 mW cm⁻² and an open circuit voltage (OCV) of 0.947 V [34].

In contrast to the viologen-based redox hydrogel, gas-diffusion systems were also proposed to prevent the $H₂$ase inactivation for DET-type bioelectrocatalysis by providing high-speed $H₂$-supply around the bioelectrodes [35] (Fig. 7.2). Since the oxidation of the ready-form enzyme with O₂ or at electrodes in the deactivation
Fig. 7.2 Schematic of a gas-diffusion bioelectrode

competes with the reduction of the enzyme with H\textsubscript{2} in the catalytic reaction, an increase in the concentration of H\textsubscript{2} can prevent the oxidative inactivation \[32\]. In addition, the true substrate for H\textsubscript{2}ases would not be solvated H\textsubscript{2} but gaseous H\textsubscript{2} \[36\]. Therefore, gas-diffusion systems are very useful to prevent the oxidative activation of H\textsubscript{2}ases. Actually, a dual gas-diffusion DET-type H\textsubscript{2}/air-breathing biofuel cell with a membrane bound-H\textsubscript{2}ase from \textit{Desulfovibrio vulgaris} Miyazaki F as an anode catalyst and BOD as a cathode catalyst showed a \( P_{\text{max}} \) of 6.1 mW cm\textsuperscript{-2} at \( E_{\text{cell}} = 0.72 \text{ V} \) and an \( OCV \) of 1.12 V at room temperature (25 ± 2 °C) \[21\] under quiescent air conditions. A similar H\textsubscript{2}/O\textsubscript{2} biofuel cell provided a \( P_{\text{max}} \) of 8.4 mW cm\textsuperscript{-2} at \( E_{\text{cell}} = 0.84 \text{ V} \) and an \( OCV \) of 1.14 V at 40 °C \[20\].

Formic acid is a stable hydrogen carrier and has been used to power some biofuel cells \[37, 38\], because it has a rather negative value of the formal potential that is close to that of 2H\textsuperscript{+}/H\textsubscript{2} redox couple, and it is liquid at room temperature and highly soluble in water, and it can easily be handled, stored, and transported. A MET-type high-power formate/O\textsubscript{2} biofuel cell combining FoDH/VP/KB/WPCC and BOD/ABTS/KB/WPCC exhibited a \( P_{\text{max}} \) of 12 mW cm\textsuperscript{-2} at an \( E_{\text{cell}} \) of 0.78 V under quiescent conditions and an \( OCV \) of 1.2 V \[10\]. Besides, alcohol, such as methanol, ethanol, and glycerol are another common fuels for biofuel cells, because they are accessible and easy to transport and store. An ethanol/O\textsubscript{2} biofuel cell combining an MET-type ADH-bioanode and a DET-type Lac-biocathode was reported to exhibited a \( P_{\text{max}} \) of 1.78 mW cm\textsuperscript{-2} at an \( E_{\text{cell}} \) of 0.68 V when using wine as fuels \[39\].

Biofuel cells are expected to be one of the next-generation energy conversion systems, because they utilize renewable biocatalysts and fuels, operate under mild conditions, and provide high energy-conversion efficiency in theory. Therefore, the application of biofuel cells is attractive. However, biofuel cells are limited in some aspects, such as low energy density, low power density, short lifetime, and small cell voltage. Such limited performances seem to hinder their real applications.

Firstly, low energy density, which usually caused by incomplete fuel oxidation, is an important problem of biofuel cells. Although with high catalytic activity, redox enzymes with high specify that catalyzes one-step reaction leading to the low fuel utilization efficiency and low energy density of biofuel cells. A rationally designed bioanode consisting of enzyme cascades or multi-step pathways has been proposed to improve the overall energy density. The first biofuel cell based on enzyme cascade
that can complete oxidation of alcohol was reported in 1998, in which three NAD-dependent enzymes: alcohol dehydrogenase, aldehyde dehydrogenase, and formate dehydrogenase, were utilized to fully oxidize methanol to carbon dioxide (CO$_2$) and water [40]. A glucose/O$_2$ biofuel cell utilizing NAD-dependent gluconate 5-dehydrogenase as well as NAD-dependent GDH in the bioanode for a 4-electron oxidation of glucose was reported to exhibit a $P_{\text{max}}$ of 10 mW cm$^{-2}$ [41]. The value was two times larger than that of a glucose/O$_2$ biofuel cell utilizing only GDH for the bioanode [30]. An in vitro 15-enzyme pathway that can complete oxidize co-utilize glucose, sucrose and fructose in biofuel cell was designed, achieving a faraday efficiency of approximately 95% for these three sugars and yielding a $P_{\text{max}}$ of 1.08 mW cm$^{-2}$ [42]. Such enzymatic cascades in biofuel cells is well reviewed [43].

Secondly, low power output of biofuel cells is a major issue that constrains their potential use to applications. The power density of biofuel cells reported so far was at most in the level of several mW cm$^{-2}$ and is still much lower than that of the conventional fuel cells. The principal reason for this matter is pure electrochemical connection between enzymes and electrodes. In a DET-type system, the electron transfer efficiency relies on the distance between the redox-active sites of enzymes and electrode surfaces. From this viewpoint, deep understanding of the interactions between enzymes and electrodes is essential to realize a rapid interfacial electron transfer based on oriented-orientation of enzymes at functionalized electrode surfaces [44, 45]. In particular, recent researches have shown that several electrodes with mesoporous structures significantly improve the interfacial electron transfer kinetics between enzymes and the electrodes in DET-type bioelectrocatalysis [46, 47]. While in the MET-type system, suitable mediators with high affinity to enzymes and high turnover efficiency in electrode are demanded [10, 48].

Thirdly, in typical enzyme-catalyzed systems, biofuel cells often suffer from poor operational stability, resulting in short lifetimes, and higher costs. Although relatively stable proton exchange membrane fuel cells, or microbial fuel cells catalyzed by self-reproduced microorganisms can be reused for months, most biofuel cells can operate only for hours or days. Instability arises not just with the enzymes, but with the use of soluble cofactors such as NAD, ATP, and coenzyme A, which are essential to drive several redox enzyme reactions, and of other components including mediators. A glucose/O$_2$ biofuel cells combined with pH reactivation of laccase biocathode illustrated one-year stability [17]. Besides, the complexity of the biological systems may frequently lead to negative effects on the stability of biofuel cells, such as an issue of the biofouling of the electrode for implantable biofuel cells, or of the inhibition ascribed to O$_2$ and positive electrode potentials as in H$_2$/O$_2$ biofuel cells.

For almost all biofuel cells reported to date, the voltage at which usable power can be extracted is below the minimal requirement to power commercially available electronic devices. This drawback is inevitable as, from a thermodynamic point of view, the maximum gap in the formal potentials between two bioelectrodes is ~1.2 V (e.g. for H$_2$/O$_2$ biofuel cells), and it is much less than that of lithium ion batteries (e.g. ~4.2 V). In many cases, the involvement of mediators leads to an additional decrease in the voltage output of biofuel cell. But, sophisticated optimization of
redox mediators in a MET-type formate/O₂ biofuel cell realized an OCV of 1.2 V [10]. In addition, serial assembly of biofuel cells can be employed to amplify the output voltage. A multi-stacked sheet-type glucose biofuel cell composed of 15 multi-stacked biofuel cell units connected 5 in parallel and 3 in series provided an OCV over 2.5 V, which allowed the successful operation of a radio at working voltages of ca. 1.6 V for over 6 h [15]. The development of self-charging biosupercapacitors or charge-storing biofuel cells has also attracted growing attention to overcome several issues of biofuel cells [49–53].

### 7.3 Photobioelectrochemical Water Splitting

Water splitting is one of the important technological breakthroughs to construct a hydrogen economy. The photosynthesis of photosynthetic organisms occurs in the thylakoid membrane in the chloroplast and converts CO₂ in the atmosphere to organic substances with excited electrons generated by photobiochemical splitting of H₂O (Eq. (7.1)):

\[
2\text{H}_2\text{O} + 4h\nu \rightarrow 4\text{e}^- + 4\text{H}^+ + \text{O}_2 \tag{7.1}
\]

The excited electrons can be transferred to an electrode in MET-type reactions. The bioelectrode may be called photo-driven bioanodes. Biocatalysts used for photo-driven bioanodes include: chloroplasts [54, 55], photosystem I (PSI) [56], PSII [57], photosynthetic microorganisms [58–60], and thylakoid membranes [61–63]. 1,2-Naphthoquinone (NQ) [64] and hexaammineruthenium (III) ([Ru(NH₃)₆]³⁺) [65] may be utilized as mediators. Characteristics required as suitable mediators are: low barrier in electrode kinetics, high stability, suitable solubility, and low redox potential (to minimize the overpotential), while large O₂-tolerance and high permeability of bio-membranes are also required. A photocurrent density of 0.18 mA cm⁻² was reported in a photo-driven bioanode utilizing thylakoid membranes and [Ru(NH₃)₆]³⁺ [65] at a light flux density of 1.5 mmol m⁻² s⁻¹, in which water-spread multi-walled carbon nanotubes (MWCNTs) were mounted by π–π staking on a light-permeable ITO electrode, on which thin Au film with a thickness of 4 nm was sputtered to improve the electrode kinetics of the mediator.

Electrochemical coupling of the photo-driven bioanode with a DET-type MCO-based biocathode yielded a bioelectrochemical photocell (called bio-solar cell) [65]. The cell exhibited an OCV of 0.61 V and a \( P_{\text{max}} \) of 50 μW cm⁻². This is a typical and ideal example of an electrochemical device to support the hydrogen economy, though further trial to decrease the overpotential of the photobioelectrochemical H₂O splitting is required in future. Thinning of cells is also very important in bio-solar cells as well as other biofuel cells.

The reaction of Eq. (7.1) is a reverse reaction of a 4-electron reduction of O₂, which can be effectively catalyzed by MCO enzymes in bioelectrochemical system. Therefore, bioelectrochemical systems can realized by directional conversion between O₂
and H$_2$O with relatively small overpotential.

$$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightleftharpoons 2\text{H}_2\text{O} \quad (7.2)$$

The characteristics of biological redox functions as bioelectrocatalysts are excellent compared with metal-based electrocatalysts.

### 7.4 Bioelectrochemical/Biochemical Hydrogen/C1 Economy

H$_2$ases catalyzes both H$_2$ oxidation and H$^+$ reduction, and the H$_2$ase-based bioelectrocatalytic system allows bidirectional electrochemical interconversion between H$_2$ and H$^+$;

$$2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2. \quad (7.3)$$

Since H$_2$ is an attractive energy source in the sustainable society, electrochemical synthesis of H$_2$ is also meaningful, as in the case of electrochemical utilization of H$_2$. The important point here is that the bioelectrochemical interconversion of Eq. (7.3) proceeds almost reversible under neutral conditions, since most H$_2$ase-based bioelectrocatalytic systems do not have any overpotential in the interconversion [36, 66]. Such a reversible electrochemical interconversion of 2H$^+/\text{H}_2$ is very difficult for metal catalysts with under neutral conditions. Therefore, the bioelectrochemical interconversion of Eq. (7.3) can be underpin a hydrogen economy. H$_2$ was also utilized with N$_2$ to produce NH$_3$ in an MET-type bioelectrochemical system [67], where H$_2$ oxidation was catalyzed by H$_2$ase, while N$_2$ reduction was nitrogenase.

Similar bidirectional bioelectrocatalyses were observed for other redox enzymes: W-containing FDH from *Methylobacterium extorquens* AM1 for two redox couples of CO$_2$/HCOO$^-$ (Eq. (7.4)) and NAD$^+$/NADH (Eq. (7.5)) [68–70] and ferredoxin-NADP$^+$ reductase (FNR) from *Chlamidomonas reinhardtii* for a redox couple of NADP$^+$/NADPH (Eq. (7.5)) [71, 72];

$$\text{CO}_2 + \text{H}^+ + 2\text{e}^- \rightleftharpoons \text{HCOO}^-, \quad (7.4)$$

$$\text{NAD(P)}^+ + \text{H}^+ + 2\text{e}^- \rightleftharpoons \text{NAD(P)H}. \quad (7.5)$$

Since reactions (7.4) and (7.5) are hydride ion transfer in nature, direct conversion at electrodes is very difficult. The corresponding redox enzymes can convert the hydride ion transfer into the electron transfer by flavins comprised as cofactors in the redox enzymes, and the electrons are transferred from the flavins to electrodes via some metal cofactor(s). Since the reorganization energy of the intramolecular electron transfer is very low in redox enzymes, almost reversible interconversions
are realized even under such multi-step electron transfer. It is noteworthy that the reversible electrode reaction of the NAD(P)^+/NAD(P)H redox couple can be easily coupled with NAD(P)-dependent dehydrogenase reactions. The coupling can create variety of applications including biosensors, biofuel cells, and bioreactors, since the NAD(P)^+/NAD(P)H redox couple is the most important in biological redox system and there are a huge variety of NAD(P)-dependent enzymes in nature.

Although H\textsubscript{2} has many excellent properties as fuel, H\textsubscript{2} has critical problems in its storage and transportation issues because of its gaseous properties. One of possible alternatives is HCOO\textsuperscript{−} [37, 38], and from the thermodynamic point of view, the interconversion between the two redox couples is very useful to support the sustainable society:

\[
\text{CO}_2 + \text{H}_2 \rightleftharpoons \text{HCOO}^- + \text{H}^+.
\] (7.6)

The spontaneous and reversible interconversion was realized by using H\textsubscript{2}ase from DvMF and FoDH from MeAM1 [73]. Utilization of C1 redox cycle(s) is very important to support the hydrogen economy. Such biotechnology would facilitate the storage and the transportation of the primary energy sources.

Similar interconversion of two redox couples are catalyzed by a variety of redox enzymes. In views of carbon capture and utilization (CCU), the reversible interconversion between CO\textsubscript{2}/HCOO\textsuperscript{−} and NAD(P)^+/NAD(P)H couples is very useful and can be catalyzed by FoDH from MeAM1:

\[
\text{CO}_2 + \text{NAD(P)}H \rightleftharpoons \text{HCOO}^- + \text{NAD(P)}^+.
\] (7.7)

The direction of the reactions is simply determined by concentrations of the reactants and products in view of thermodynamics in biochemical systems [73]. However, the reaction can be controlled in favorite way by adding external potential in the bioelectrochemical systems.

On the other hand, NAD\textsuperscript{+}-reducing H\textsubscript{2}ase (from Hydrogenophilius thermoluteolus) [74] catalyzes the reversible interconversion between 2H\textsuperscript{+}/H\textsubscript{2} and NAD(P)^+/NAD(P)H couples:

\[
\text{H}^+ + \text{NAD(P)}H \rightleftharpoons \text{H}_2 + \text{NAD(P)}^+.
\] (7.8)

The NAD(P)(H) redox couples can be coupled with a variety of NAD(P)-dependent enzymes, as is evidenced in the production of L-glutamate from 2-oxoglutarate and NH\textsubscript{3} by L-glutamate dehydrogenase [72, 75]. In such redox enzymatic coupling, it is very important to minimize the diffuse distance of redox cofactors (or mediators) [76]. For this purpose, an ITO electrode with 5–100 nm scale pores was utilized to trap NADP-dependent enzymes and FNR for bioelectroorganic synthesis [77].

As described in this chapter, the bidirectional characteristics of bioelectrocatalytic systems are very important and useful to construct, support, and extend the hydrogen economy (as hydrogen/C1 economy, Fig. 7.3). These redox enzymes and
related bioelectrocatalysis will attract lots of attention in near future. The proposed hydrogen/C1 economy is indeed an environmentally clean, cheap, and sustainable energy system.

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Conclusions and Outlooks

Electrochemistry of redox enzymes is rapidly evolving, and can be employed to construct a broadly applicable technology. Increasing attention in this area is driven by remarkable progress in designing electrode surfaces efficient for the interfacial electron transfer between redox enzymes and electrodes, protein-engineering of redox enzymes, and also important applications to biosensors, biofuel cells, and bioreactors.

The concept of the coupling of specific redox enzyme reactions with non-specific electrode reactions seems to be simple at first glance. However, deep understanding of redox enzymes and interfacial electron transfer is absolutely necessary for improvement of the performance and application of the bioelectrocatalysis. Loose substrate specificity of one of substrates of redox enzymes (excepting NAD(P)-dependent dehydrogenases) is essential for the coupling. Charge transfer at electrodes is the electron transfer, while that of NAD(P)(H), sugars, organic acids, alcohols, aldehydes, and other organic compounds is the hydride ion transfer. Flavins, quinones, and phenothiazines intermediate between the electron and hydride ion transfers thanks to the two-step single-electron transfer characteristics, and then can communicate with electrodes as in the case of metal ion-based redox cofactors in redox enzymes and metal ion-containing redox compounds.

In MET-type bioelectrocatalysis, the LFER concept is very important in the selection of mediators, since the interfacial electrode reaction is non-specific in essence. Further development of redox polymers that can immobilize and electrochemically communicate with redox enzymes is desired for practical applications.

Recent progress in the understanding and improvement of DET-type bioelectrocatalysis is remarkable. Since redox enzymes are huge in size compared with metal-based catalysts, orientation effects of redox enzymes often appear on DET-type signals. The effects are not convenient for DET-type bioelectrocatalysis. Significance of mesoporous structures of electrodes has become known to minimize the effects, as called curvature effect. Several modification of electrode surfaces and mutations of redox enzymes have also been examined to control the orientation of enzymes.
on the electrode surfaces to minimize the distance between electrochemically-communicating sites and the electrode surfaces. In addition, as a general concept, it is important to emphasize the significance of microstructures in the interfacial electron transfer. At the top edge of microstructures, the electric field is drastically strengthened because of the expansion of the electric double layer. In addition, the electric charge on electrodes is accumulated on the top edge of microstructures. Further development in theoretical discussion on this issue is desired. Both of the effects appear to accelerate the interfacial electron transfer kinetics including DET-type bioelectrocatalysis. Deglycosylation and down-sizing protein engineering of redox enzymes have also shown successful results in the improvement of DET-type electron transfer kinetics of redox proteins. Thanks to these understandings and improvements, redox enzymes that are capable of DET-type communication will increase in number in near future. On the other hand, we have to continue deep discussion on false interpretation as DET-type bioelectrocatalysis.

This book also emphasizes the importance of a series resistance model in the analysis of catalytic current-potential curves of bioelectrocatalysis; bioelectrocatalysis is very complicated and then the analysis of transient responses (time-dependent responses) requires so many adjustable parameters and is very complicated. Analytical information of bioelectrocatalytic curves, especially of DET-type reactions, are very useful for interpretation of the intramolecular electron transfer in redox enzymes, and the knowledge will be helpful to design and improve the bioelectrocatalytic system. Some redox enzymes such as H$_2$ase, FoDH, and FNR have catalytic activity for bidirectional reactions of the individual redox couple as the substrates. Therefore, the coupling the reactions of such redox enzymes with electrode reactions allows bidirectional electrode reactions of the redox couples without overpotentials in practice. Low organization energy in redox proteins is responsible for such bidirectional catalysis that is very difficult with metal-based catalysts. Fast biological electron transfers with minimum driving force or sometime in uphill conditions proceed thanks to low organization energy. Cascade electron transfer is also essential in the biological system to avoid decreased kinetics in the inverted region of the Marcus theory at increased driving forces.

A huge variety of biosensor devices based on bioelectrocatalysis have been reported. However, the practical application is limited in number, though glucose biosensor is the most well-known and successful example of all biosensor devices. In this book, our ideas to improve biosensor performances are introduced: MET-type mass transfer-controlled biosensors with ultra thin-layered electrodes, MET-type potentiometric coulometry with surface-confined redox mediators, and bienzyme biosensing by coupling DET-type peroxidase bioelectrocatalysis and oxidase reactions without any mediators. The proposals showed some successful results, but are not applicable to all biosensor configurations. In order to get steady-state signals on amperometric biosensors, development of the surface coverage technique with permeable membranes in high reproducibility is desired. Furthermore, multi-analyte detection will be getting attention in future; relative concentrations would be more important than absolute concentrations for samples with possible
dilution/concentration such as saliva and urine. For this purpose, development of two-electrode systems providing reliable and reproducible signals is required.

In biofuel cell application, gas-diffusion bioelectrodes is one of the most important contributions in our group, although the technique is still developing for practical purpose. Although biofuel cell technology might be considered as a fundamental research field, recent technical breakthroughs may make it possible to use biofuel cells on a commercially basis. Some important proposals of the practical utilization of biofuel cells would drastically improve the performance in future.

As the reverse reaction of biofuel cells, bioelectrocatalytic synthesis will become increasingly important in future. Thanks to H$_2$ase, FoDH, and FNR with bidirectional catalytic activity, three redox couples: $2\text{H}^+/\text{H}_2$, CO$_2$/HCOO$^-$, and NAD(P)$^+$/NAD(P)H, are reversibly interconverted electrochemically in bioelectrocatalytic system. Electrochemical 4-electron reduction of O$_2$ and photoelectrochemical water splitting have been also realized by using MCO and PSII, respectively. Bioelectrochemical combination of these principal reactions in nature will open a new world of extended hydrogen economy and of varieties of biosensing technologies.

A serious problem in bioelectrocatalytic system is fragile properties of redox enzymes. Many researchers are tackling a great challenge in understanding of the enzyme stability and increasing of the lifetime of redox enzymes. One of alternatives may over expression and/or cell-surface expression of target enzyme(s) in microbes and utilization of the microbes as electrocatalysts (especially as bags of enzyme(s)).

We hope further development in understanding and application of bioelectrocatalytic systems. All these efforts are also valuable in establishing bioelectrochemistry as a novel research tool for redox enzymes.