

## Direct Electron Transfer-type Bioelectrocatalysis of Redox Enzymes at Nanostructured Electrodes

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### Abstract

Direct electron transfer (DET)-type bioelectrocatalysis, which couples electrode reactions and catalytic functions of redox enzymes without any redox mediator, is one of the most intriguing subjects studied over the past decades in the field of bioelectrochemistry. In order to realize the DET-type bioelectrocatalysis and to improve the performance, nanostructures of the electrode surface have to be carefully tuned for each enzyme. In addition, enzymes can also be tuned by protein engineering approach for the DET-type reaction. This review summarizes the recent progresses in this field of the research, while taking into consideration of the importance of nanostructure of electrodes as well as redox enzymes. Described are basic concepts and theoretical aspects of DET-type bioelectrocatalysis, significance of nanostructures as scaffolds for DET-type reactions, protein engineering approached for DET-type

reactions, and concepts and facts of bidirectional DET-type reactions, from a cross-disciplinary viewpoint.

**Keywords:** direct-electron transfer-type bioelectrocatalysis; nanostructures; mesoporous electrodes, curvature effect; protein engineering; bi-directional bioelectrocatalysis; hydrogenase; fructose dehydrogenase; bilirubin oxidase; formate dehydrogenase; ferredoxin-NADP<sup>+</sup> reductase

## 1. Introduction

Electron transfer reactions in living organisms including photosynthesis and metabolisms play a central role in all living systems. Such biological electron transfer reactions are catalyzed by a variety of redox enzymes in high speed, high selectivity, and high specificity. Over the last four decades, redox enzymes have received much attention for use in the form of the coupling of enzymatic reactions with electrochemical reactions. The coupled reaction is called bioelectrocatalysis [1–11]. The coupling provides a variety of biological redox functions to conventional electrode materials and will open new ways for a wealth of fundamental researches and developing bioelectrochemical devices relating to biosensors [12,13], biological fuel cells [2,4,6,8,9], bioelectrochemical reactors [10,14], and biosupercapacitors [15].

Most redox enzymatic reactions can be coupled with electrode reactions via (artificial) redox compounds (called redox mediators) that shuttle electrons between enzymes and electrodes. This reaction is known as mediated electron transfer (MET)-



**Figure 1.** Schematic view of DET-type bioelectrocatalysis

type bioelectrocatalysis. Some redox enzymes can directly communicate with electrodes in the catalytic reaction. Such reaction is referred to as direct electron transfer (DET)-type bioelectrocatalysis (Figure 1). This type of the coupling makes it possible to develop more simple systems that can minimize thermodynamic overpotentials in well-organized couplings. However, DET-type enzymes providing clear bioelectrocatalytic waves are still small in number, and DET-type reactions proceed only 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.

Recent progresses in this field increased sophistication in approaches to the assembly of electrode surfaces, tuning of electrode surfaces, and protein mutations to facilitate electron transfer reactions. On the other hand, in order to interpret bioelectrocatalytic waves, rigid analysis based on analytical equations on suitable models of DET-type reactions is required in relation to the thermodynamics and kinetics

of bioelectrocatalyses (that is electro-enzymatic reactions) in view of the interfacial and intramolecular electron transfers and the structure of redox enzymes.

In this review, from a cross-disciplinary viewpoint, we will describe basic concepts and theoretical aspects of DET-type bioelectrocatalysis, significance of nanostructures as scaffolds for DET-type reactions, protein engineering approaches for DET-type reactions, and concepts and facts of bidirectional DET-type reactions.

## 2. Theoretical aspects for the DET-type bioelectrocatalysis

The simplest reaction scheme for a substrate (S) oxidation by DET-type bioelectrocatalysis is expressed by the following enzymatic reaction:



and the electrode reaction:



where  $E_R$  and  $E_O$  are reduced and oxidized forms of an enzyme, respectively; S and P are its substrate and product, respectively; and  $n_E$  is the number of electrons of the enzyme. Parameters  $k_f$ ,  $k_b$ , and  $k_c$  correspond to the rate constants of the forward electrode reaction, the reverse electrode reaction, and the enzymatic reaction, respectively. Under steady-state conditions (defined by  $\partial c_i / \partial t = 0$ ) without concentration polarization of the substrate, the steady-state catalytic current ( $i_s$ ) is given as follows [16]:

$$i_s = \frac{i_s^{\lim}}{1 + \frac{k_c}{k_f} + \frac{k_b}{k_f}}, \quad (3)$$

where  $i_s^{\text{lim}}$  is the limiting steady-state current of the system at  $k_c \ll k_f$  and  $k_b \ll k_f$  and is given by a Michaelis–Menten-type equation:

$$\frac{i_s^{\text{lim}}}{n_E F A} = \frac{v_E k_c \Gamma_E}{1 + \frac{K_S}{c_S^*}}. \quad (4)$$

The electrode kinetics is given by Butler–Volmer equation for substrate oxidation:

$$k_f = k^o \exp \left\{ \frac{(1 - \alpha) n'_E}{RT} (E - E_E^{o'}) \right\}, \quad (5)$$

and

$$k_b = k^o \exp \left\{ -\frac{\alpha n'_E F}{RT} (E - E_E^{o'}) \right\}. \quad (6)$$

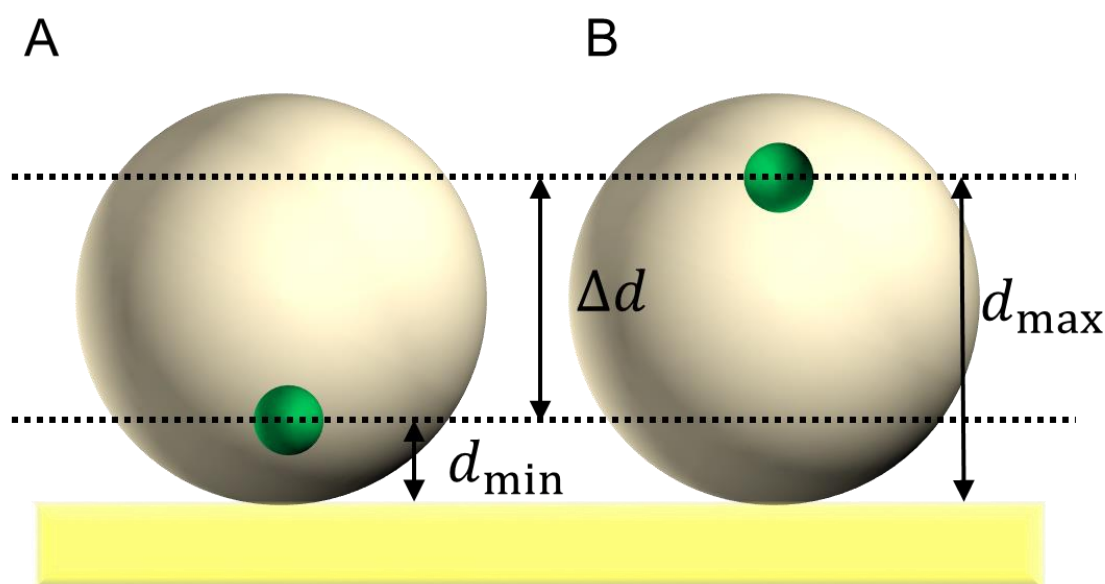
In these equations,  $v_E (= n_S/n_E)$  is the ratio of the stoichiometric number of the substrate against the that of the enzyme ( $n_S$  being the number of electrons of the substrate),  $K_S$  is the Michaelis constant of the substrate,  $\Gamma_E$  is the surface concentration of the adsorbed enzyme,  $c_S^*$  is the substrate concentration in the bulk phase,  $n'_E$  is the number of electrons in the rate-determining step of the heterogeneous electron transfer, (usually  $n'_E = 1$ ) and  $E_E^{o'}$  is the formal potential of the electrode-active redox center in the enzyme.

The situation that  $i_s = i_s^{\text{lim}}$  can be realized at  $E \gg E_E^{o'}$  for substrate oxidation in theory. However, it is often difficult to observe such potential-independent regions to give  $i_s^{\text{lim}}$  within the potential windows of practical measurements. The large size of enzymes and eccentric location of the electrode-active redox center, and the random orientation of the enzymes are major causes of such experimental observations. In DET-type reactions, the distance between an electrode and an electrode-active redox center in an enzyme ( $d$ ) is a critical value, because the electron transfer reaction rate constant should be dominated by the distance from the electrode. The rate constant of a long-range electron transfer reaction exponentially depends on  $d$  as follows [17]:

$$k^{\circ} = k_{\max}^{\circ} \exp(-\beta d) , \quad (7)$$

where  $k_{\max}^{\circ}$  is the rate constant at the closest approach ( $d = d_{\min}$ ) and  $\beta$  is the decay coefficient. Since the orientation of an adsorbed enzyme obviously affects  $d$ , the orientation of the adsorbed enzyme at the electrode is crucial for DET-type bioelectrocatalyses.

Herein, we demonstrate a simple orientation model, which is based on the random orientation of enzymes at a planar electrode. Figure 2 shows a schematic of the model, which assumes a spherical enzyme. In the enzyme, the electrode-active redox center locates at  $d$  from the electrode. When the orientation of the enzyme is random on the planar electrode, the electrode-active redox center in the enzyme locates at distances between  $d_{\min}$  and the farthest approach ( $d = d_{\max}$ ) with a constant probability [18–20].



**Figure 2.** Schematic model of an adsorbed spherical enzyme on a planar electrode in (A) the closest approach of the electrode active redox center in an enzyme (small spheres) and (B) the farthest approach of the electrode active redox center.

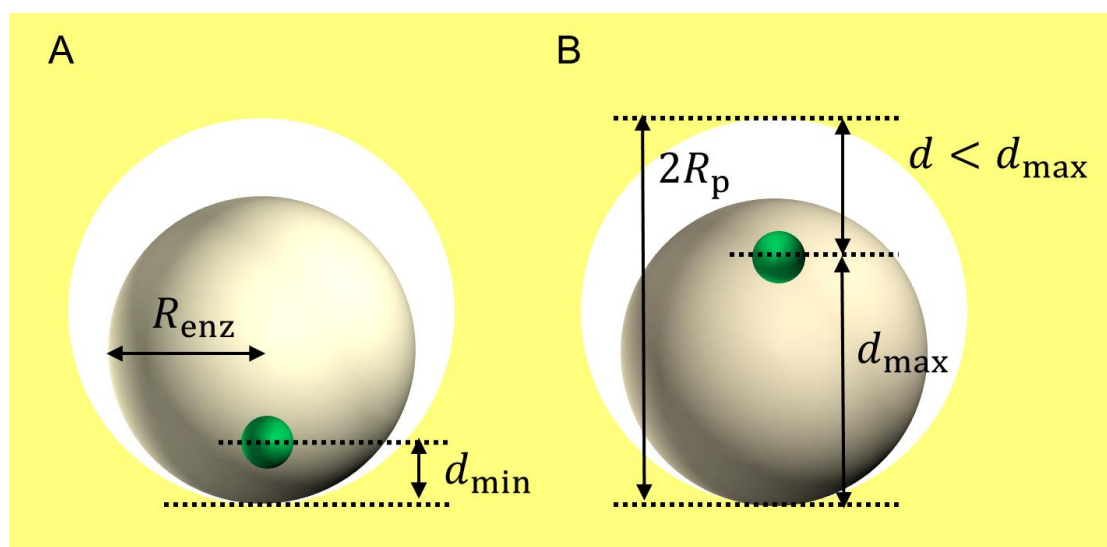
According to this model, the steady-state DET-type catalytic current by the randomly adsorbed enzyme is expressed by the following equation:

$$i_s = \frac{i_s^{\text{lim}}}{2\beta\Delta d(1 + \eta^{-1})} \ln \left| \frac{\frac{k_c}{k^o\eta^{1-\alpha}} + (1 + \eta^{-1}) \exp(-\beta d_{\min})}{\frac{k_c}{k^o\eta^{1-\alpha}} + (1 + \eta^{-1}) \exp(-\beta d_{\max})} \right|, \quad (8)$$

where  $\eta$  is the Nernst equation for the rate-determining step of the heterogeneous electron transfer:

$$\eta = \exp\left(\frac{n'_E F(E - E_E^{o'})}{RT}\right). \quad (9)$$

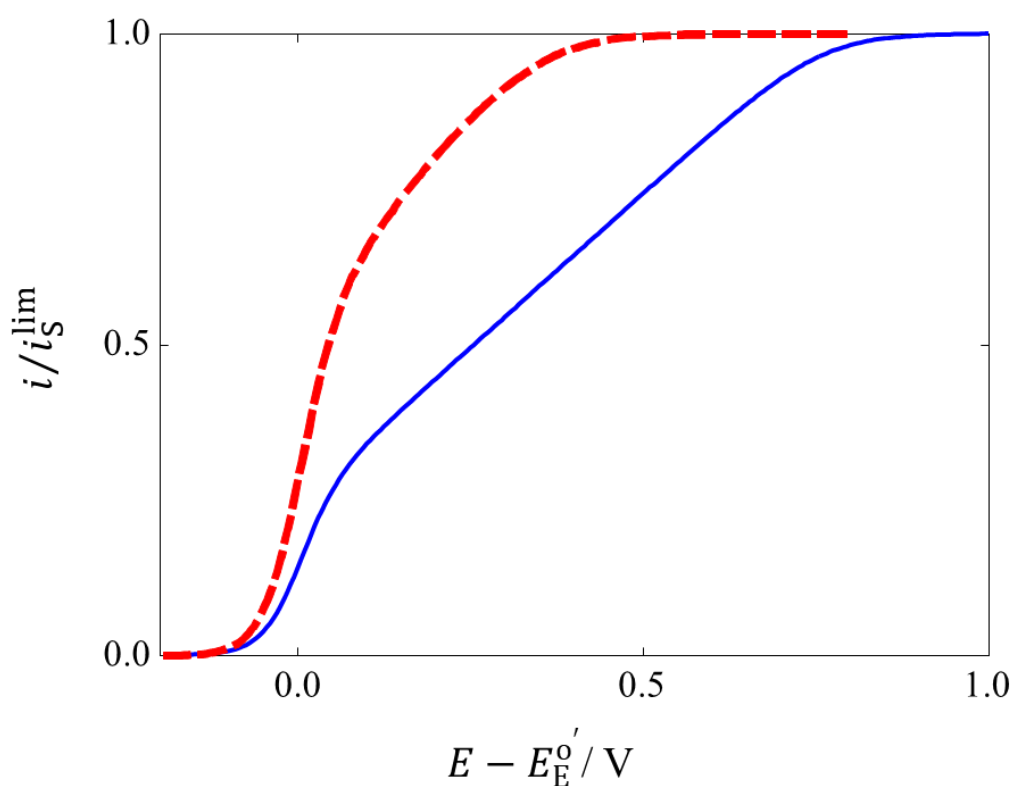
When the electrode surface has porous structures, the concave surface inside the pore seems to be effective to the electron transfer kinetics. Figure 3 shows a schematic image of a decrease in the distance between the redox center in the enzyme and the electrode surface provided by a porous structure on the electrode [20]. According to the porous model, when the radius of pore ( $R_p$ ) is equal to the radius of the



**Figure 3.** Schematic model of an adsorbed spherical enzyme with a radius of  $R_{enz}$  in a micropore with a radius of  $R_p$  on electrodes. (A) and (B) show the closest and farthest approaches of the redox center (small spheres), respectively.

enzyme ( $R_{enz}$ ), the efficiency of the DET-type bioelectrocatalysis becomes maximum and the orientation effect disappears in theory.





**Figure 4.** Steady-state bioelectrocatalytic voltammograms calculated for planar (solid line) and porous electrodes (dashed line). The parameters used are:  $d_{\min} = 1.6$  nm,  $d_{\max} = 2.6$  nm,  $R_{\text{enz}} = 2$  nm,  $k_c/k_{\text{max}}^0 = 10^{-10}$ , and  $R_p = 2.7$  nm.

The theoretical model enables to calculate voltammograms for DET-type bioelectrocatalyses. Calculated voltammograms are given in Figure 4. The solid line corresponds to a steady-state voltammogram calculated for a DET-type bioelectrocatalysis of spherical enzymes adsorbed at a planar electrode. The voltammogram for the planar electrode shows an exponentially increasing region, a linearly increasing region, and a limiting current region. In the exponentially increasing region, fast electron transfer reactions predominantly occur between the electrode and

the enzymes with orientations suitable for the DET-type bioelectrocatalysis. In the linearly increasing region, poorly and randomly oriented enzymes contribute the catalytic current; the exponentially decayed  $k^\circ$  with an increase in  $d$  (Eq. (7)) of the poorly oriented enzymes is canceled with an exponential acceleration of  $k_f$  with an increase in  $E$  for substrate oxidation (Eq. (5)):

$$k_f = k_{\max}^o \exp \left\{ \frac{(1 - \alpha)n_E'}{RT} (E - E_E^{o'}) - \beta d \right\}. \quad (10)$$

The linearly increasing region is called residual slope [18], which is frequently observed in voltammograms of DET-type bioelectrocatalyses. The current in the limiting current region is controlled by the enzymatic reaction by all of the effective enzymes adsorbed on the electrode; the current in the limiting current region is given by Eq. 4. Therefore, the observation of the limiting current is very helpful and important for the analysis of steady-state catalytic waves of DET-type bioelectrocatalyses.

However, it would be frequently difficult to observe such limiting regions in DET-type bioelectrocatalytic waves. According to the random orientation model, the appearance of the residual slope region in steady-state catalytic waves indicates the existence of randomly oriented enzymes on the electrode surface. Based on this concept, elimination of the contribution by the randomly and unsuitably oriented enzymes from the voltammogram by subtracting the residual slope seems to be a reasonable approach to analyze the contribution from productively (or suitably) oriented enzymes [21,22].

The voltammogram of a DET-type bioelectrocatalysis by enzymes in the spherical pore electrode is shown as a dashed line in Figure 4. The value of the limiting steady-state current on the spherical model is identical with that calculated for the random orientation model on the planar electrode because the calculations were

performed for the identical amount of adsorbed enzymes (per electrochemically effective area) in the two models. However, the steady-state catalytic current on the spherical model is higher than that on the planar model except the limiting region. Especially, the exponentially increasing region is enlarged and the width of the residual slope region becomes narrow on the spherical model. Such changes in the steady-state wave indicate a drastic increase in  $k^o$  of the enzymes located in the mesopore. This may be called a curvature effect of mesoporous electrodes for the DET-type bioelectrocatalysis. However, the precise relationship between the curvature of mesoporous electrodes and the efficiency of the DET-type bioelectrocatalysis seems to be complicated for actual cases because the pore size distribution on porous electrodes will be unclear. Examples of DET-type bioelectrocatalyses at electrodes with controlled structures are introduced in the following section.

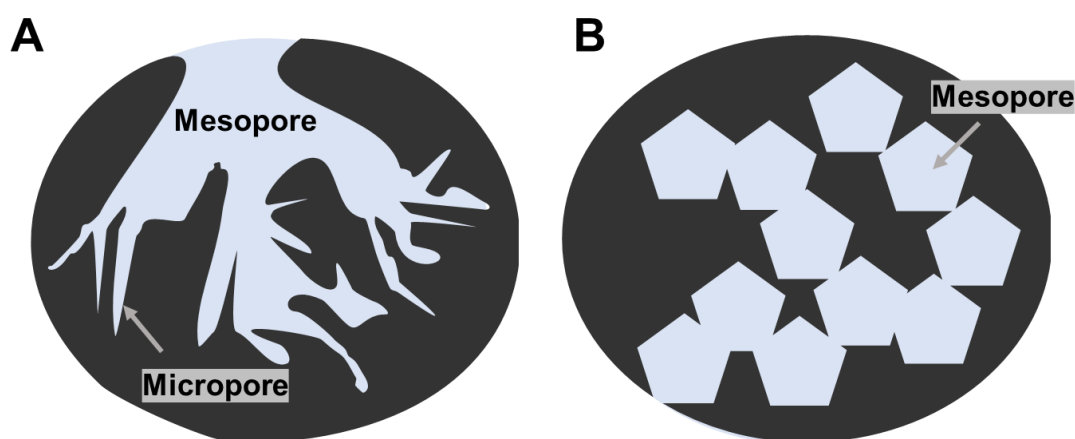
### **3. Significance of nanostructures of electrodes for DET-type bioelectrocatalysis**

There are very few systematic investigations on effects of nanostructures of electrodes on the DET-type bioelectrocatalysis since the control of nanostructures on the electrode surface is difficult and will be one of future challenges. However, techniques to construct nanostructures at carbon and gold materials have relatively well examined. The chemical state on the surface of these materials can be controlled. In this section, some outlines for DET-type bioelectrocatalyses at structure-controlled porous carbon and gold materials are introduced.

#### **3.1 Porous carbon electrodes**

Activated carbons have complex and irregular structures in the materials

(Figure 5A). Therefore, such porous carbon materials are frequently characterized in terms of the pore-size distribution and the effective surface area. Systematic syntheses of porous carbon materials are reviewed by many researchers [23–25]. Although effects of nanostructures on DET-type bioelectrocatalyses seem to be difficult to clearly understand, few works were published in systematic investigation on effects of the pore size on the DET-type bioelectrocatalysis. Recently, a technique to control the size of macro- and meso-pores in carbon materials by magnesium oxide (MgO) particles as templates was developed (Figure 5B) [26,27]. MgO-templated carbons showed interesting properties as scaffolds for redox enzymes. DET-type bioelectrocatalyses at some porous carbon materials are exemplified in this section.



**Figure 5.** Schematic images of (A) typical activated-carbon and (B) MgO-templated carbon.

### *Fructose dehydrogenase*

Membrane-bound D-fructose dehydrogenase (FDH) is one of DET-type redox

enzymes [28]. FDH is a heterotrimer with a molecular mass of 138 kDa, composed of three subunits; subunit I (67 kDa) contains a covalently bound flavin adenine dinucleotide (FAD) and oxidizes D-fructose to 5-keto-D-fructose, subunit II (51 kDa) includes three hemes *c* (heme 1*c*, 2*c* and 3*c* from the N-terminus), and subunit III (20 kDa) exhibits chaperonic function [29]. Carbon cryogels (CCGs) with various pore sizes were systematically synthesized as electrode materials. [30] The adsorption and desorption isotherms of nitrogen gas on the CCGs indicated that the materials had the pores with radiuses of 5, 11, 16, 16, 26, and 40 nm. Although the Brunauer–Emmett–Taller (BET)–surface areas of the CCG-modified electrodes were almost same with each other, the oxidative catalytic current by the adsorbed FDH strongly depended on the pore size of the CCGs. The increase in the pore size resulted in an increase in the loaded amount of FDH on the electrode surface. The large pore seems to be convenient for FDH to penetrate the porous structure. Unfortunately, the steady-state catalytic waves of the DET-type bioelectrocatalysis of FDH depended on the electrode potential in the whole potential region examined; each wave had a residual slope without a limiting current region. The characteristics indicate that FDH molecules adsorb in a widely dispersed pattern of the orientation (random orientation) on the CCG electrodes. The surface modification of the porous carbon electrode with a methoxy substituent-containing compounds was effective to improve the orientation of FDH [31]. Not the electrostatic interaction but the chemical interaction seems to affect the orientation of FDH; the enzyme has a specific interaction with methoxy substituent of ubiquinones [32].

MgO-templated carbons with various pore sizes were investigated as scaffolds of a DET-type bioelectrocatalysis of FDH [33]. The sizes of the mesopores studied were

in the range from 10 to 100 nm. This study showed that porous materials with large radius were significant for the penetration of the enzyme into the porous electrode material. On the other hand, porous materials with small radius comparable with that of the enzyme enhanced the thermal stability of the adsorbed enzyme. The limitation of the enzyme mobility seems to improve the stability. The fine tuning of the pore-size distribution and the size of the interconnection between the pores in porous electrode materials is important to develop high performance bioanodes.

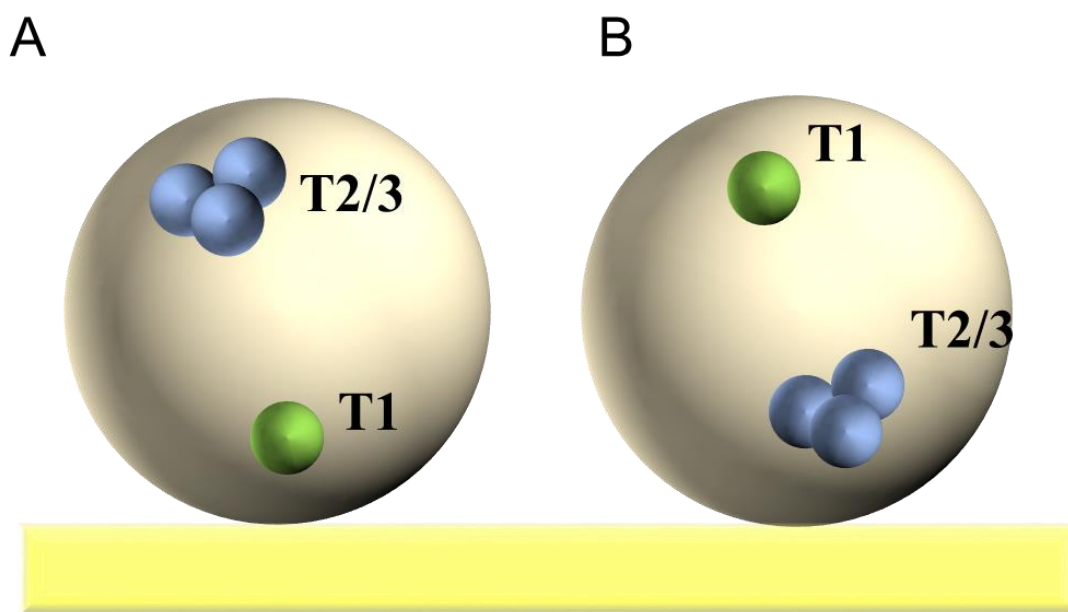
### *Bilirubin oxidase*

Bilirubin oxidase (BOD) from *Myrothecium verrucaria*, one of the multi copper oxidase family, reduces O<sub>2</sub> to water in a DET-type bioelectrocatalysis [16]. Because of its relatively high activity and relatively positive reduction potential for a four-electron dioxygen (O<sub>2</sub>) reduction, BOD is an attractive catalyst for biocathodes in biofuel cells [9]. BOD contains four copper (Cu) atoms classified into a Type 1 Cu (T1) site (T1) and a T2-3 Cu (T2/3) cluster. O<sub>2</sub> is reduced at the T2-3 Cu cluster. According to the redox titration and site-specific mutation of BOD [16,34], an electrode-active redox center was assigned to the T1 Cu site. Therefore, an orientation in which the T1 Cu site faces the electrode (Figure 6A) is suitable for the DET-type bioelectrocatalysis compared with an opposite orientation in which the T2/3 Cu cluster faces the electrode (Figure 6B). It is well known that the orientation of adsorbed BOD (from *Myrothecium verrucaria*) was controlled by the surface modification of electrodes with negatively charged aromatic compounds [19,35,36]. Although the DET-type bioelectrocatalytic activity of BOD at planar glassy carbon electrodes is low, the activity was drastically enhanced at porous electrodes [9,37]. Because of a limited solubility of O<sub>2</sub> in aqueous

solutions and a very high DET-type activity of BOD, the catalytic reduction current density of O<sub>2</sub> easily reaches the mass-transfer limited value. In such cases, analysis of steady-state catalytic waves obtained at rotating disk electrodes is very useful to get the information of the orientation of BOD at porous electrodes. MgO-templated carbon electrodes with systematically controlled size pores (10 nm to 200 nm) were investigated as scaffolds for BOD by rotating disk voltammetry [36]. The steady-state O<sub>2</sub> reduction currents of the DET-type bioelectrocatalysis of BOD depended on the pore size and the rotating speed. The adsorbed amount of the enzyme per unit weight of the carbon material increased with an increase in the pore size. Moreover, the adsorption amounts depended on the incubation time for the adsorption of BOD. When the pore size was small, the adsorption quickly reached the saturation. The result shows that the penetration of the enzyme into the porous structures on the electrode surface is an important process in the adsorption of the enzyme on mesoporous electrode surfaces. The recorded voltammograms showed residual slope regions. The residual slopes at the voltammograms recorded at the MgO-templated carbon materials depended on the size of the porous structures; the contribution of the residual slope region on the voltammogram became large at electrodes with large sizes of pores (> 100 nm). The situation is similar to that observed on planar electrodes. On the other hand, when the pore sizes were small (10 and 20 nm), the shape of the steady-state catalytic wave became a typical sigmoidal shape. This is a typical curvature effect. The small pore seems to be effective to increase the probability of the orientations suitable for the DET-type bioelectrocatalysis. The stability of the adsorbed BOD was also affected by the pore size. Comparison of catalytic waves at electrodes with 40 nm- and with 150 nm-pores revealed that the 40 nm-MgO templated carbon was a more stable scaffold for the

BOD adsorption [38].

### *Hydrogenase*

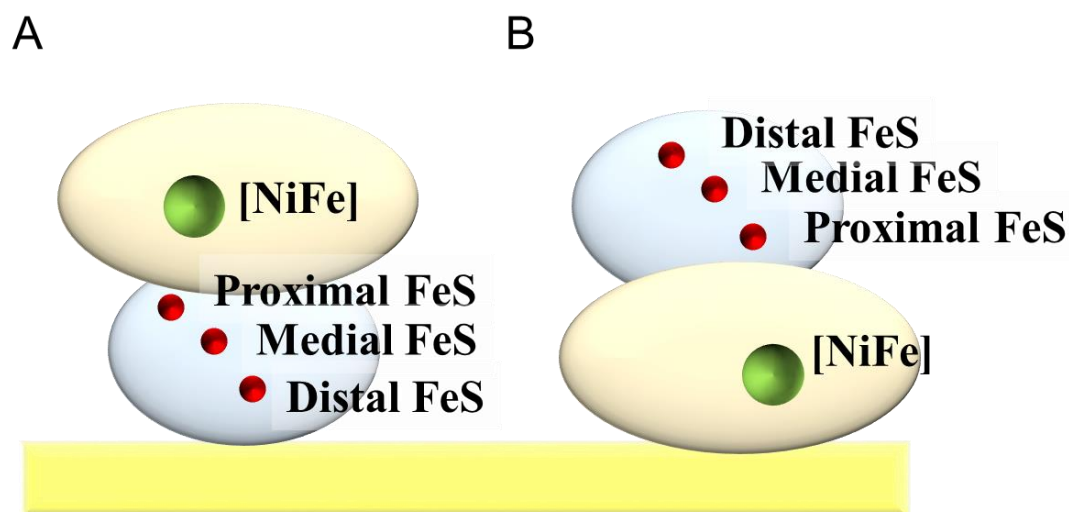


**Figure 6.** Schematic illustrations for the adsorbed BOD at flat electrode in (A) effective and (B) invalid orientations for the DET-type bioelectrocatalysis.

Hydrogenase ( $H_2ase$ ) catalyzes a reversible reaction between hydrogen ( $H_2$ ) and proton ( $H^+$ ).  $H_2ase$  is classified into three types ( $[Fe]$ ,  $[FeFe]$ , and  $[NiFe]$ ) on the basis of its redox center.  $[NiFe]$   $H_2ase$  is an attractive catalyst for biocathodes of  $H_2/O_2$  biofuel cell thanks to its  $O_2$  tolerance [5,39]. For example, a standard  $[NiFe]$   $H_2ase$  from *Desulfovibrio vulgaris* Miyazaki F is constructed with large and small subunits. The large subunit contains  $[NiFe]$  cluster, while the small subunit contains three FeS clusters which are called proximal, medial, and distal (Figure 7). The distal FeS cluster is considered to be the electrode-active redox center [40].



DET-type bioelectrocatalysis of  $H_2ase$  from *Aquifex aeolicus* was also investigated at MgO-templated carbon electrodes [41]. In this work, the bioelectrocatalytic behaviors at the electrodes of two pore-sizes (35 nm and 150 nm) were compared with each other; here the size of the  $H_2ase$  in solution was determined to be 18 nm by dynamic light scattering. The results showed that the pore-size affected the orientation and the amounts of the adsorbed enzymes on the electrode surface. The initial rising-part in the steady-state catalytic wave at the electrode with the small pores was sharper than that with the large pores, indicating that the curvature effect was more evident on the electrode with the small pores. On the other hand, the amount of the adsorbed enzyme at the electrode with the large pores was demonstrably larger than that with the small pores. Analysis of the adsorption kinetics indicated that the large pores were convenient for the enzyme to penetrate deeply into the porous structures. For the



**Figure 7.** Schematic illustrations for the adsorbed  $H_2ase$  at flat electrode in (A) effective and (B) invalid orientations for the DET-type bioelectrocatalysis.

enzyme, it seemed to be difficult to penetrate the small pores, of which the size is approximately doubled of the enzyme size. It seems be important to give it time to adsorb increased amounts of enzymes on porous electrodes with rather small radius.

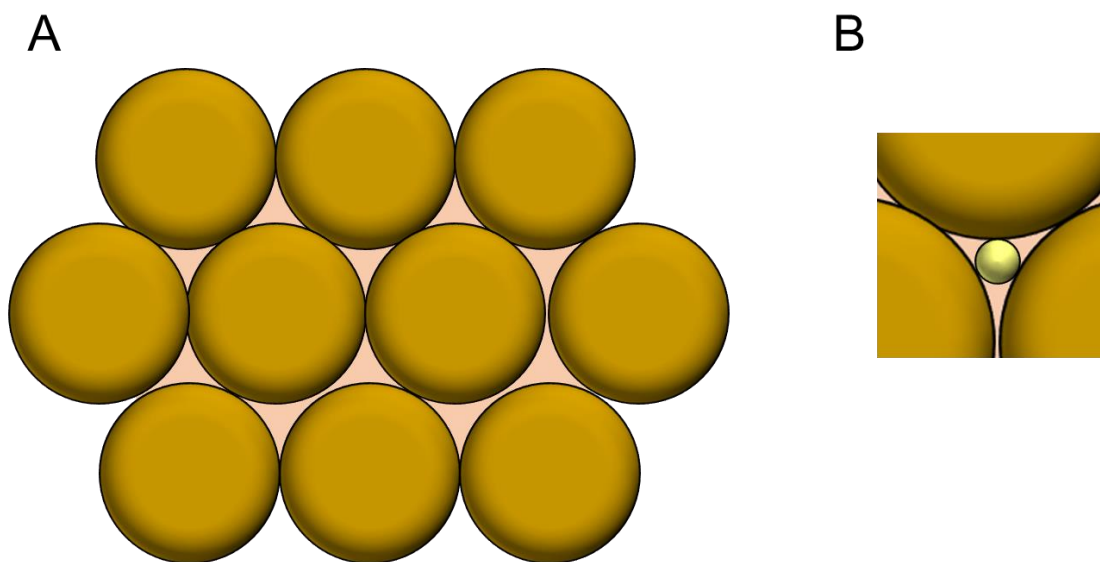
In the case of the H<sub>2</sub>ase also, the adsorption on the porous carbon electrodes stabilized the enzyme. In addition, the stabilization effect was more evident at the electrode with the small pores than that with the large pores. Restricted movement of the enzyme in mesoporous structures with the size close to the enzyme size seems to stabilize the enzyme.

#### *Histamine dehydrogenase*

Histamine dehydrogenase from *Nocardioides simplex* (HmDH) is one of soluble iron-sulfur flavoproteins with a molecular mass of 152 kDa, and contains flavin mononucleotide (FMN), [4Fe-4S] cluster, and adenine diphosphate (ADP) as cofactors [42,43]. HmDH is capable of catalytically transferring electrons from histamine to electrodes. The electrode active site in HmDH seems to be the [4Fe-4S] cluster. Porous electrodes fabricated from three carbon materials were investigated as scaffolds for the DET-type bioelectrocatalysis of HmDH [44]: Ketjen Black with a diameter of 40 nm, carbon aerogel with a pore size of 10 nm, and carbon microsphere with a diameter of 100 nm. Among the carbon materials, DET-type bioelectrocatalysis of HmDH was detected only at the Ketjen Black-based porous electrode. The size of the carbon aerogel mesopore seems to be too small for HmDH to penetrate into the pore, while the size of the carbon microsphere macropore seems to be too large for HmDH to enjoy the curvature effect.

### 3.2 Nanoporous metals and aggregated metal nanoparticles

The synthesis of monodispersed nanoparticles has developed for long time [45,46]. The aggregation of monodispersed spherical nanoparticles will provide ordered mesopores (gaps) among the surrounding nanoparticles, as shown in Figure 8A. The maximum diameter of inscribe spheres that can be fitted in the mesopore is about approximately 0.3 size of the diameter of the nanoparticles (Figure 8B). Such a modification of the electrode surface with monodispersed nanoparticles is regarded as an easy method to give controlled mesoporous structures. By considering the curvature effect, the most effective mesopores among the surrounding nanoparticles for adsorption of enzymes may be provided by nanoparticles with radii of three to four times larger than the size of a target enzyme; multipoint contact between an enzyme in the



**Figure 8.** (A) A simple model of the aggregated nanoparticles. (B) Schematic view of an inscribed sphere in a pore between the closed packed spheres.

mesopores and the surrounding nanoparticles enjoy the curvatures effect for DET-type bioelectrocatalysis.

On the other hand, the mesopores among the surrounding nanoparticles is not sphere. This structure seems to be very important for the mass transfer of substrates and products.

As a result, such porous structures constructed by large nanoparticles are generally effective for DET-type bioelectrocatalysis than the planar structure [47]. Some examples of such productive effects of nanoparticles on DET-type bioelectrocatalysis are described in the following.

### *Hydrogenase*

Monsalve et al. demonstrated that layered gold nanoparticles with a diameter of 25 nm were effective scaffolds for DET-type bioelectrocatalytic oxidation of H<sub>2</sub> by H<sub>2</sub>ase from *Aquifex aeolicus* [48]. When the adsorbed amount of the nanoparticles was small, that is, when the electrochemically active surface area was less than 10 times of the projected area (the surface area of the planar electrode), very small current density (per projected surface area) of the DET-type bioelectrocatalysis of the H<sub>2</sub>ase remained unchanged in spite of an increase in the electrochemically active surface area. However, clear catalytic waves were observed when the electrochemically active surface area became over 10 times of the projected one, and the current density (per projected surface area) increased with the electrochemically active surface area at least up to 25 times of the projected one. The results show that coarse aggregations constructed with insufficiently packed nanoparticles is not suitable scaffolds for DET-type reaction of the adsorbed enzyme. In contrast, the mesoporous spaces constructed among densely

packed nanoparticles are suitable scaffolds for the DET-type bioelectrocatalysis. In addition, the aggregates constructed with the gold nanoparticles with a diameter of 25 nm provided scaffolds for DET-type reaction of the H<sub>2</sub>ase than those with a diameter of 35 nm; the diameters of the mesoporous spaces seem to be too large for the H<sub>2</sub>ase to enjoy the curvature effect. All these results demonstrate that the size of mesoporous space constructed in gaps in nanoparticle aggregates is a very important factor for the DET-type bioelectrocatalytic activity of H<sub>2</sub>ases.

#### *Bilirubin oxidase*

DET-type bioelectrocatalysis of BOD at gold nanoparticle submonolayer-modified electrodes was systematically investigated by Pankratov et al. [49]. The sizes of the nanoparticles investigated were 20, 40, 60, and 80 nm. The kinetic analysis based on the real surface area showed that the electro-enzymatic kinetics was almost independent of the size of the nanoparticles. This result suggests that only the densely aggregated nanoparticles provide scaffolds suitable for DET-type bioelectrocatalysis of BOD.

On the other hand, some methodologies to fabricate microporous gold electrodes have been developed. Anodization of gold in a buffer solution containing glucose as a reductant is one of the fabrication methods of microporous gold materials [21]. The microscopic observation of the fabricated microporous gold electrode fabricated in a glucose solution showed that microporous structures were constructed from the gold nanoparticles [21,47]. The constructed microporous gold electrodes work as effective scaffolds for the DET-type bioelectrocatalysis of BOD. On the other hand,

electrodes with low densities of microporous structures were not effective for DET-reaction of BOD. All these results show the importance of three-dimensional mesoporous structure constructed with nanoparticles, but not nanoparticles themselves.

Nano-porous gold (NPG) electrodes constructed by dealloying silver/gold alloy in nitric acid were investigated as scaffolds for DET-type bioelectrocatalysis of BOD [50]. The pore sizes of the NPG materials were controlled by dealloying conditions to be 9, 15, 24, 41, 45, and 61 nm as average. The residual slope on the voltammogram showed that the porous gold materials with an average pore size of 9 nm were the most suitable scaffolds for the DET-type bioelectrocatalysis of BOD. Based on the crystal structure of BOD [51,52], the shape of BOD can be considered as a sphere with a diameter of 5 nm. Comparison of the size between the best most effective pore and the enzyme supports the curvature effect of the mesoporous structure on the DET-type bioelectrocatalysis.

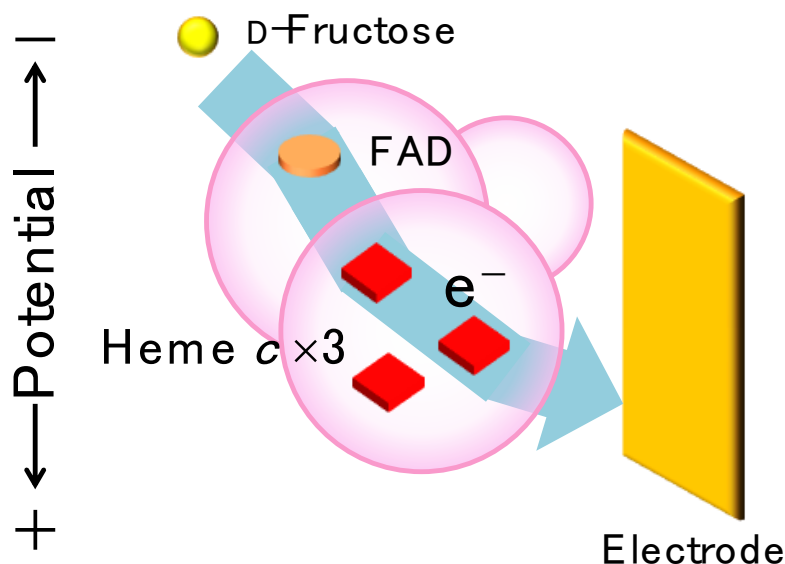
### *Fructose dehydrogenase*

NPG electrodes constructed by dealloying a silver/gold alloy were investigated as scaffolds for DET-type bioelectrocatalysis of FDH [53]. The average pore sizes of the NPG materials examined were 9, 18, 42, and 62 nm. The catalytic current density (per projected area) increased with an increase in the pore size from 9 to 42 nm, while the current density decreased on the electrode with an average pore size of 62 nm. The highest current densities were obtained with NPG electrodes with an average pore size of 42 nm. In the experiments, the shape of the steady-state catalytic waves was almost independent of the average pore size. Therefore, the difference in the current density seems to be ascribed predominantly to the difference in the adsorbed amount of FDH.

#### **4. Protein engineering methods for improvement of DET-type bioelectrocatalytic performance**

For practical application of bioelectrocatalysis to bioelectrochemical devices such as biosensors, bioreactors, biofuel cells, and biosupercapacitors, enzyme-functionalized electrodes are required to satisfy the following two improvements; one is an increase in a catalytic current density and the other is a decrease in overpotential. The former may be solved by getting the catalytic constant of enzymes higher or increasing the amount of enzymes adsorbed on the electrode surface. The latter may be realized by shifting the formal potential of the electrode-active redox site of enzymes negatively for oxidative reactions of substrates (and positively for reductive reactions). Some enzymes are tuned by protein engineering methods to improve DET-type bioelectrocatalytic properties.

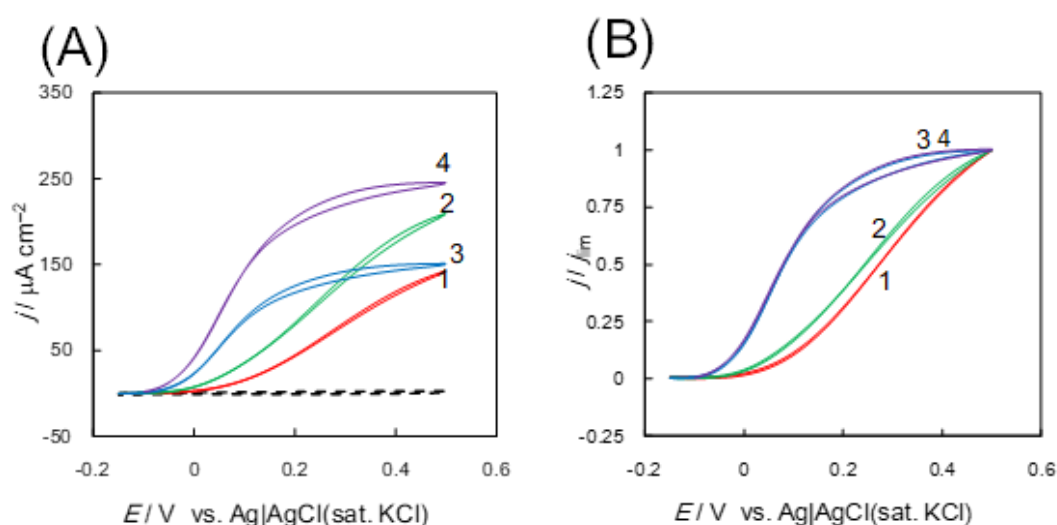
It has been reported that some FDH variants show better DET-type bioelectrocatalytic performance than the wild-type FDH [28]. Considering the comparison of the formal potentials of hemes *c* and the onset potential of the steady-state catalytic wave of the DET-type bioelectrocatalysis, it was suggested that electrons were transferred from D-fructose to an electrode via FAD, heme 3*c*, and 2*c* in this order, and heme 1*c* seemed to be uninvolved in the intramolecular electron transfer in the DET-type reaction, as shown in Figure 9 [32].



**Figure 9.** Schematic view of proposed electron transfer pathway from D-fructose to an electrode in aDET-type reaction of FDH. The electrons are transferred from substrate-reduced FAD to heme 3c, heme 2c, and an electrode without going to heme 1c. Reprinted from [28], Copyright (2019), with permission from Elsevier.

These suggestions were proved by investigating FDH variants (M450Q and  $\Delta 1c$ \_FDH). The M450Q variant replaces M450, which is the sixth axial ligand of heme 2c, with glutamine in order to shift the formal potential of the heme in the negative direction, and the half-wave potential of the steady-state catalytic wave of the M450Q variant shifted negatively as expected [54]. The  $\Delta 1c$ \_FDH variant lacking the heme 1c





**Figure 10.** Original (A) and normalized (B) cyclic voltammograms (CVs) of D-fructose oxidation at Au electrodes on the surface of which had been adsorbed the D-fructose dehydrogenase variants; the recombinant FDH (1, red),  $\Delta 1c$ \_FDH (2, green),  $\Delta 1c2c$ \_FDH (3, blue), and M450Q $\Delta 1c$ \_FDH (4, purple), in a McIlvaine buffer (pH 4.5) containing 0.1 M D-fructose under anaerobic conditions at a scan rate ( $v$ ) of 10  $\text{mV s}^{-1}$ . The dashed line in panel (A) represents a background voltammogram obtained using a bare Au electrode. Reprinted from [28], Copyright (2019), with permission from Elsevier.

moiety showed an increase in the limiting catalytic current density probably thanks to the downsizing of the enzyme [55]. In addition, a double variant (M450Q $\Delta 1c$ \_FDH) showed improved characteristics observed for both M450Q and  $\Delta 1c$ \_FDH [56].

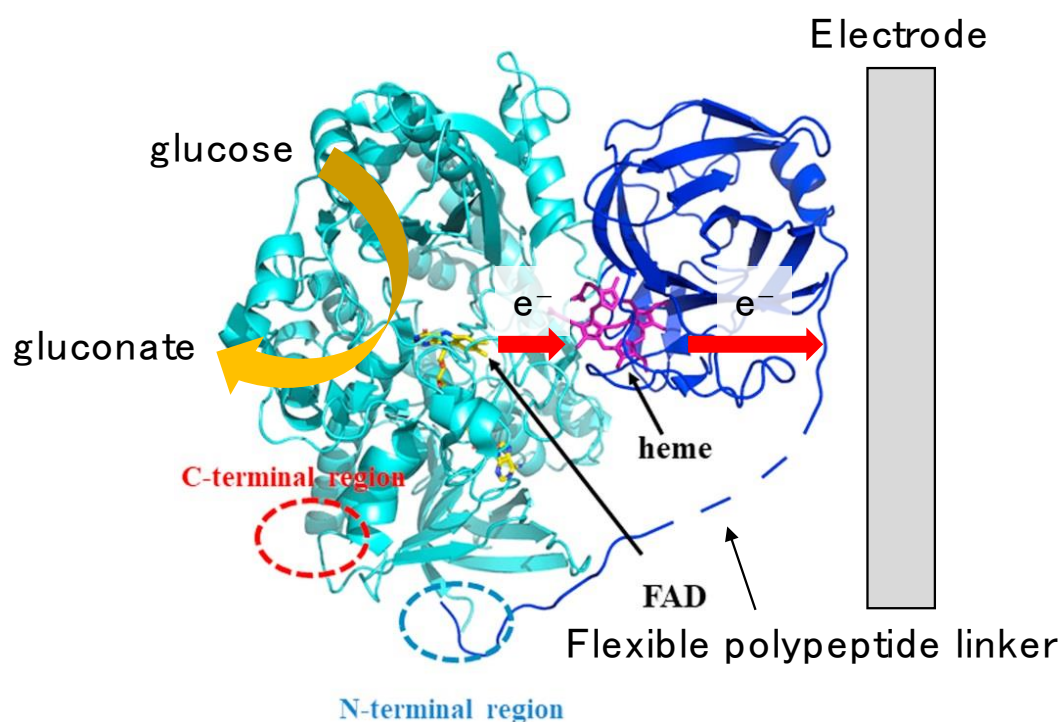
On the other hand, a  $\Delta 1c2c$ \_FDH variant, which lacks both the heme 1c and 2c moieties, showed DET-type bioelectrocatalytic activity similar to that of M450Q $\Delta 1c$ \_FDH were realized [57]: an increase in the limiting catalytic current density caused by the downsizing of the enzyme and a decrease in the overpotential

thanks to the heterogeneous electron transfer from heme 3c with the most negative formal potential to an electrode. DET-type bioelectrocatalytic waves of these FDH variants are shown in Figure 10.

As well as FDH, FAD-dependent glucose dehydrogenase (FAD-GDH) from *Burkholderia cepacia* belongs to the bacterial FAD-dependent dehydrogenase family [58,59], and DET-type bioelectrocatalysis of FAD-GDH was investigated [60–63]. FAD-GDH is a heterotrimer consisting of three subunits:  $\alpha$  subunit (60 kDa) harboring a FAD catalytic center and an iron-sulfur (FeS) cluster (3Fe-4S),  $\beta$  subunit (43 kDa) containing three hemes *c* (heme 1c, 2c, and 3c from the N-terminus), and  $\gamma$  subunit (18 kDa) working as a chaperon [64,65]. Based on the mutagenesis studies of FAD-GDH, the electrons produced by glucose oxidation were proposed to pass to an electrode through FAD, 3Fe-4S, heme 3c, and 2c in this order [66]. Based on the proposal, an FAD-GDH variant (tr $\beta$ ), of which heme 1c and 3c moieties are truncated, was designed and tr $\beta$  showed improved water solubility and DET-type bioelectrocatalytic activity with lower overpotential than that of the wild-type FAD-GDH [59]. These results can be interpreted as in the case of FDH. In addition, FAD-GDH variants (Ala472Phe and Asn475Asp) improved substrate specificity toward glucose [67].

On the other hands, fusion protein engineering is also a general method for achieving DET-type bioelectrocatalysis. A model enzyme was cellobiose dehydrogenase (CDH), which is a DET-type bioelectrocatalyst and consists of two domains: a larger catalytic dehydrogenase domain (DH<sub>CDH</sub>) which includes a non-covalently bound FAD and oxidizes substrates, and a smaller cytochrome domain (CYT<sub>CDH</sub>) which contains heme *b* and communicates with electrodes [68]. The DH<sub>CDH</sub> and CYT<sub>CDH</sub> are connected through a flexible polypeptide linker and the intramolecular

electron transfer between two domains occurs easily [68]. As a mimic of CDH, several redox enzymes were fused with cytochrome domains of other enzymes through a linker and DET-type reactions of them were realized or improved: e.g. pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (PQQ-GDH) from *Acinetobacter calcoaceticus* [69], FAD-GDH from *Burkholderia cepacia* [70] and from *Aspergillus flavus* [71], and flavodoxin from *Desulfovibrio vulgaris* [72]. The cytochrome domain worked as a “built-in mediator” and improved the heterogeneous electron transfer kinetics between the enzyme and electrodes, as shown in Figure 11.



**Figure 11.** Model structure and the electron transfer pathway from glucose to an electrode in a direct-electron-transfer-type reaction of a fusion protein of FAD-GDH from *Aspergillus flavus* and CYT<sub>CDH</sub>. The electrons are transferred from FAD to an electrode via heme *b*. Reproduced from Ref. [71], Copyright (2019), with permission from Elsevier, and modified in part.

In addition, site-directed mutagenesis of enzymes are incorporated to improve DET-type bioelectrocatalytic performance. In order to increase the stability of enzymes and bioelectrochemical devices, disulfide bonds are often introduced. A PQQ-GDH variant in which one of amino acids was replaced with cysteine showed increased stability compared with the wild-type thanks to its dimeric structure constructed by enhanced intermolecular disulfide bonds [73]. The importance of dimeric or oligomeric structure for the enzyme stability was supported by the following works; 1) Other PQQ-GDH variants in which hydrophobic amino acids were introduced, which seemed to be a dimer by enhanced hydrophobic interaction, also showed high stability [74], 2) chemically oligomerized FAD-dependent glucose oxidase (FAD-GOD) exhibited higher thermal stability than the native one [75]. The limitation of the enzyme mobility seems to improve the stability, as observed in the case that enzymes were embedded (adsorbed) in mesoporous structures with size close to that of the enzymes.

Cysteine-introduced variants are also effective for forming covalently bonds or promoting the interaction between enzymes and electrodes or conductive materials. It was reported that FAD-GOD variants in which the surface cysteine mutation was introduced formed thioether bonds with maleimido-modified gold nanoparticles and perform DET-type reaction [76]. In addition, Gorton et al. investigated effects of cysteine mutations on DET-type bioelectrocatalysis of recombinant horseradish peroxidase (rHRP) on gold electrodes, and claimed that the surface cysteine controlled the orientation of enzymes adsorbed on the electrode and several variants improved the DET-type performance [77]. On the other hand, enzymes expressed in *E. coli* lack glycosylation shells and the downsizing of the enzymes may shorten the distance

between the electrochemical active site and electrodes, which may lead to improvement of DET-type bioelectrocatalysis [78].

On the other hand, directed evolution of enzymes is also useful for DET-type bioelectrocatalysis [78–80]. For example, laccase variants engineered by directed evolution showed a shift of the optimum pH from acidic to neutral conditions and also the chloride tolerance, which seemed to be favorable for practical use under physiological conditions [81].

## 5. Interconversion of redox couples by bidirectional DET-type bioelectrocatalysis

Some redox enzymes catalyze two-way reactions (both oxidation and reduction) of substrate redox couples. One of the requirements for the two-way reaction is that the formal potentials of the substrate redox couple and the cofactor in enzymes are close with each other, in order to minimize the kinetic barrier of uphill intramolecular electron transfer of one of the two-way reactions in the enzymes. Here, the rate constant of the electron transfer reaction ( $k$ ) are expressed by the following Marcus cross relation [82]:

$$k \cong \sqrt{k_{DD}k_{AA} \exp\left(-\frac{\Delta_r G^\circ}{RT}\right)}, \quad (11)$$

where  $k_{DD}$  and  $k_{AA}$  are the rate constants of the self-electron-exchange reactions at the redox site in the enzyme as electron-donating and -accepting sites, respectively.  $\Delta_r G^\circ$  is the standard Gibbs energy of a reaction and is given by:

$$\Delta_r G^\circ = -nF(E'^{\circ}_{AA} - E'^{\circ}_{DD}), \quad (12)$$

where  $E'^{\circ}_{AA}$  and  $E'^{\circ}_{DD}$  are the formal potential of electron-accepting and -donating sites, respectively. In order to decrease  $\Delta_r G^\circ$  in uphill processes ( $\Delta_r G^\circ > 0, E'^{\circ}_{AA} < E'^{\circ}_{DD}$ ),  $E'^{\circ}_{AA}$  should be close to  $E'^{\circ}_{DD}$ . This is the reason of the requirement mentioned above. The other requirement for the fast electron transfer of uphill reactions is that  $k_{DD}$  and  $k_{AA}$  should be very large, as expected from Eq. (11). Since the rate constant of a self-exchange reaction ( $k_{self}$ ) is related to the reorganization energy ( $\lambda$ );

$$RT \ln \frac{k_{self}}{Z} = -\Delta^\ddagger G^\circ_{self} = -\frac{\lambda}{4}, \quad (13)$$

Low value of  $\lambda$  is essential for uphill electron transfer. Here,  $Z$  is a coefficient of the absolute kinetics and  $\Delta^\ddagger G^\circ_{self}$  is the standard Gibbs energy of activation. Low  $\lambda$  value is realized in a minimum change in geometry during the redox reaction. Peptide matrix

frequently plays an important role to minimize the geometry change. Therefore, an uphill electron transfer may be observed in intramolecular electron transfer in redox proteins in some instances.

Bidirectional bioelectrocatalysis is useful to construct effective energy conversion systems. Examples of the interconversion system reported for H<sub>2</sub>ase, formate dehydrogenase (FoDH), and ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) reductase (FNR) are shown in the following section.

### *Hydrogenase*

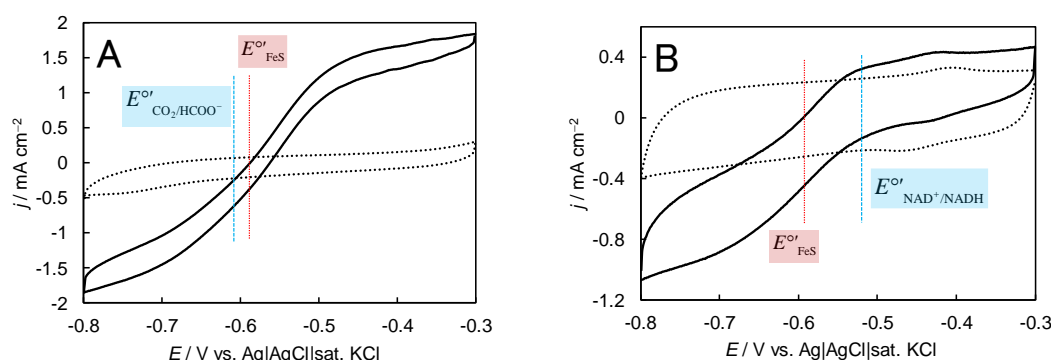
It is well known that H<sub>2</sub>ase catalyzes H<sub>2</sub> oxidation and H<sup>+</sup> reduction, and communicates with an electrode directly at the distal FeS cluster [5,39]. The formal potential of the 2H<sup>+</sup>/H<sub>2</sub> couple is −0.614 V vs. Ag|AgCl|sat. KCl at pH 7, which is close to that of the distal FeS cluster (−0.54 V for H<sub>2</sub>ase from *Desulfovibrio fructosovorans* [83]). The closeness of the formal potentials is responsible for the reversibility of the H<sub>2</sub>ase reaction. Moreover, nicotine adenine dinucleotide (NAD<sup>+</sup>)-reducing [NiFe]-hydrogenase, which has a diaphorase unit, shows bidirectional DET-type bioelectrocatalysis interconverting not only 2H<sup>+</sup>/H<sub>2</sub> but also NAD<sup>+</sup>/NADH [84,85].

### *Formate dehydrogenase*

Tungsten or molybdenum-containing FoDHs from *Methylobacterium extorquens* AM1, *Syntrophobacter fumaroxidans*, and *Escherichia coli* were reported to show DET-type bioelectrocatalytic interconversion between carbon dioxide (CO<sub>2</sub>) and formate (HCOO<sup>−</sup>) [86–88]. FoDHs have a tungstopterin or molybdopterin as a catalytic center and FeS clusters as the electrode-active redox site [86–88] with a formal



potential of  $-0.59$  V for *Methylobacterium extorquens* AM1 [86], which is close to that of the  $\text{CO}_2/\text{HCOO}^-$  couple ( $-0.63$  V at pH 7.0 [89]). In addition, FoDH from *Methylobacterium extorquens* AM1 (FoDH1), which has a non-covalently bound flavin



**Figure 12.** (A) Cyclic voltammograms (CVs) for  $\text{HCOO}^-$  oxidation and  $\text{CO}_2$  reduction at FoDH-modified mesoporous carbon electrodes constructed with 4-mercaptopyridine-modified gold nanoparticles in 1.0 M phosphate buffer (pH 6.6) containing 10 mM  $\text{HCOONa}$  under  $\text{CO}_2$  gas-saturated and quiescent conditions at 25 °C and  $\nu = 10 \text{ mV s}^{-1}$ . The dotted lines represent CVs in the absence of the substrate.  $E^{\circ'}_{\text{CO}_2/\text{HCOO}^-}$  (the blue line) and  $E^{\circ'}_{\text{FeS}}$  (the red line) are the formal potentials of the  $\text{CO}_2/\text{HCOO}^-$  couple and the electrode-active FeS cluster in FoDH, respectively. (B) CVs for  $\text{NADH}$  oxidation and  $\text{NAD}^+$  reduction at FoDH1-modified mesoporous carbon electrodes constructed with 4-mercaptopyridine-modified gold nanoparticles in 1.0 M phosphate buffer (pH 7.0) containing 50 mM  $\text{NAD}^+$  and  $\text{NADH}$  under argon-saturated and quiescent conditions at 25 °C and  $\nu = 10 \text{ mV s}^{-1}$ . The dotted line represents the CV in the absence of substrate.  $E^{\circ'}_{\text{NAD}^+/\text{NADH}}$  (the blue line) and  $E^{\circ'}_{\text{FeS}}$  (the red line) are the formal potentials of the  $\text{NAD}^+/\text{NADH}$  couple and the electrode-active FeS cluster in FoDH, respectively. Reprinted from [86], Copyright (2017), with permission from Elsevier, and modified in part.

adenine dinucleotide (FMN), catalyzes a bidirectional interconversion between  $\text{NAD}^+$  and  $\text{NADH}$  (the formal potential of the  $\text{NAD}^+/\text{NADH}$  couple is  $-0.52 \text{ V}$  at pH 7.0 [86]),

and four-way DET-type bioelectrocatalysis ( $\text{CO}_2/\text{HCOO}^-$  and  $\text{NAD}^+/\text{NADH}$ ) was shown at mesoporous carbon electrodes constructed with 4-mercaptopyridine-modified gold nanoparticles; the modified gold particles controlled the orientation of FoDH and enhanced the heterogeneous electron transfer kinetics between the enzyme and the electrode [86]. The DET-type bioelectrocatalytic waves in four-way reaction of FoDH1 are shown in Figure 12. Besides that, external power-less bioelectrochemical interconversion between  $\text{HCOO}^-$  and  $\text{H}_2$  was accomplished by coupling two bioelectrocatalytic reactions of  $\text{H}_2$ ase and FoDH (note that FoDH plays as a MET-type bioelectrocatalyst) [90].

#### *Ferredoxin-NADP<sup>+</sup> reductase*

FNR from *Chlamidomonas reinhardtii* is a monomeric flavoenzyme with a molecular mass of 45 kDa and catalyzes the two-electron reduction of  $\text{NADP}^+$  to NADPH at the end of the photosynthetic electron transport chain [91]. In spite of that the enzyme has only a single redox cofactor (a non-covalently bound FAD), unlike the enzymes described above, FNR performed a reversible DET-type reaction of the  $\text{NADP}^+/\text{NADPH}$  redox couple and the reaction was coupled with  $\text{NADP}^+$ -dependent enzymes to construct bioreactors with minimum overpotentials [91,92].

## Conclusions

Bioelectrocatalysis is rapidly evolving and will be employed to establish a broadly applicable technology. Increasing attention in this area is driven by remarkable progress in designing electrode surfaces for fast electron transfer kinetics between redox enzymes and electrodes, protein-engineering of enzymes. We may make use of a variety

of redox enzymes as electrocatalysts in practice, although we have to tackle a great challenge in increasing the lifetime of redox enzymes in future. Further investigations for more deep understanding of electro-enzymatic reactions will be also required. All these efforts are valuable in establishing bioelectrochemistry on bioelectrocatalysis as a practical technique for practical applications and also as a novel tool for redox enzyme researches.

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