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Membrane-bound dehydrogenases from *Gluconobacter* sp.: Interfacial electrochemistry and direct bioelectrocatalysis

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ABSTRACT

Although membrane-bound dehydrogenases isolated from *Gluconobacter* sp. (mainly PQQ-dependent alcohol and fructose dehydrogenase) have been used for preparing diverse forms of bioelectronic interfaces for almost 2 decades, it is not an easy task to interpret an electrochemical behaviour correctly. Recent discoveries regarding redox properties of membrane-bound dehydrogenases along with extensive investigations of direct electron transfer (DET) or direct bioelectrocatalysis with these enzymes are summarized in this review. The main aim of this review is to draw general conclusions about possible electronic coupling paths of these enzymes on various interfaces via direct electron transfer or direct bioelectrocatalysis. A short overview of the metabolism and respiration chain in *Gluconobacter* relevant to interfacial electrochemistry is given. Biosensor devices based on DET or direct bioelectrocatalysis using membrane-bound dehydrogenases from *Gluconobacter* sp. are described briefly with the emphasis given on practical applications of preparing enzymatic biofuel cells. Moreover, interfacial electrochemistry of *Gluconobacter oxydans* related to the construction of microbial biofuel cells is also discussed.

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1. Introduction

Bacteria of *Gluconobacter* sp. as well as *Acetobacter* sp. are long known in biotechnology for their unique oxidative metabolism. Their typical applications are the fermentative production of some industrially important metabolites, such as acetic acid, carboxylic acids, sorbose and other precursors of vitamin C. Lately, *Gluconobacter* has been widely used to develop biosensors and, most recently, biofuel cells. All of these applications fundamentally rely on *Gluconobacter*'s unique metabolism. As new applications are being developed, there is also a shift from the empirical use of *Gluconobacter* as a biocatalyst to the targeted use of dehydrogenases, respiratory chain components, electron transfer and other processes occurring at the subcellular level.

Although membrane-bound dehydrogenases isolated from the *Gluconobacter* sp. have been used to prepare diverse forms of bioelectronic interfaces for almost 2 decades, it has not been an easy task to interpret its electrochemical behaviour correctly. In the past, this topic has only been covered partly by the groups of Ikeda and Laurinavicius showing examples of the interfacial electrochemistry (direct or mediated bioelectrochemistry) of PQQ-containing enzymes by citing their own work [1,2]. This review summarizes all the published results on, and investigation of, direct electron transfer or direct bioelectrocatalysis of membrane-bound dehydrogenases isolated from *Gluconobacter* in an attempt to draw a general conclusion about possible electronic coupling paths of these enzymes on various interfaces. A short overview of the metabolism and respiration chain of *Gluconobacter* relevant to its interfacial electrochemistry is provided. Moreover, a number of practical applications of direct bioelectrocatalysis for the construction of biosensor devices and biofuel cells with these enzymes are discussed.

2. Metabolism in *Gluconobacter*

Gluconobacter sp. is one of the genera of acetic acid bacteria and taxonomically belongs to the *Acetobacteraceae* family with a pronounced ability to incompletely oxidize a wide range of alcohols, sugars and polyols, e.g., converting primary alcohols to acids, or secondary alcohols to ketones. Notoriously known is the process of oxidation of ethanol to acetic acid, characteristic of all genera of the acetic acid bacteria [3]. Another typical feature of *Gluconobacter* sp. is the ability to oxidize sugars to their corresponding carboxylic acids, e.g., 2-keto-L-gulonic acid and 2-keto- and 5-keto-gluconic acids, which can be easily converted to vitamin C [4–6]. Membrane-bound dehydrogenases responsible for the oxidation of substrates are localized in the cytoplasmic membrane with the active site facing the periplasm (Fig. 1). These oxidation reactions are referred to as “oxidative fermentation”, due to incomplete oxidation of

Abbreviations: ADH, alcohol dehydrogenase; AFM, atomic force microscopy; Au, gold; BFC, biofuel cell; CP, carbon paste; DET, direct electron transfer; DPV, differential pulse voltammetry; FAD, flavin adenine dinucleotide; FDH, fructose dehydrogenase; GaDH, gluconate dehydrogenase; GCE, glassy carbon electrode; KB, Ketjan black; LDH, lactate dehydrogenase; NHE, normal hydrogen electrode; PP, polypyrrole; Pt, platinum; PQQ, pyrroloquinoline quinone; Q_{10}, ubiquinone; SAM, self-assembled monolayer.

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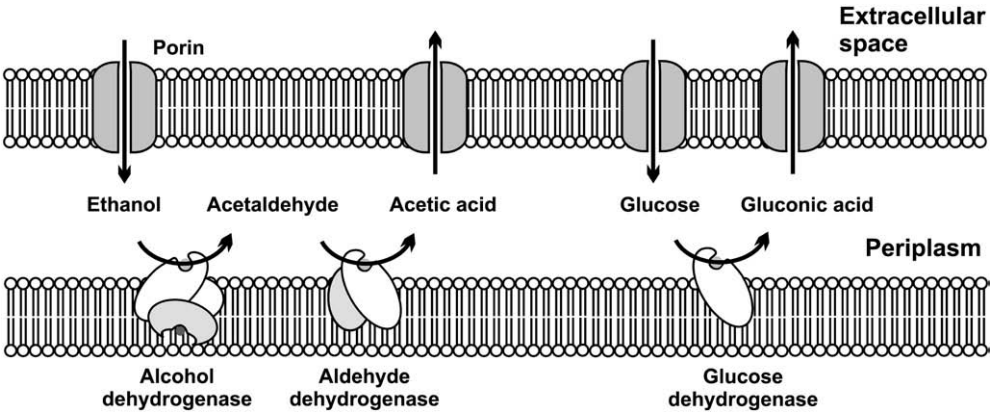


Fig. 1. Schematic model of localization of membrane-bound dehydrogenases in *Gluconobacter* sp.

substrates accompanied by accumulation of the corresponding oxidation products in large amounts in the growth medium. There is a possible evolutionary explanation for this type of metabolism. In the early growth phase, rapid production of acids that are difficult to assimilate by other microorganisms gives *Gluconobacter* sp. a big competitive advantage [7]. In the second phase, the bacteria can take up the incompletely oxidized substrates and channel them into the oxidative pentose phosphate pathway [8]. The ability of *Gluconobacter* sp. to oxidize a wide range of organic compounds with a low growth yield has resulted in many applications in biotechnology [9,10], such as asymmetric oxidation of various substrates [11] and preparation of several biosensor devices to name a few [12–14]. The exciting interfacial electrochemical properties (mainly DET and direct bioelectrocatalysis) of the *Gluconobacter* enzymes are recently being investigated for use in biofuel cells.

3. Respiratory chain of *Gluconobacter*

The broad substrate specificity of many dehydrogenases isolated from *Gluconobacter* is most likely the reason why the biosynthesis of these enzymes is not regulated by the availability of a carbon source, but rather their constitutive expression [8]. The respiratory chain of the *Gluconobacter* sp. consists of a large amount of redox-active biomolecules including PQQ-dependent dehydrogenases (the structure of the cofactor is shown in Scheme 1), cytochrome *c*, ubiquinone (Q_{10}, see Scheme 1) and terminal cytochrome *o* ubiquinol oxidase. The respiratory chain is branched at the level of ubiquinone, which results in the existence of a cyanide-sensitive or a cyanide-insensitive oxidase pathway [15].

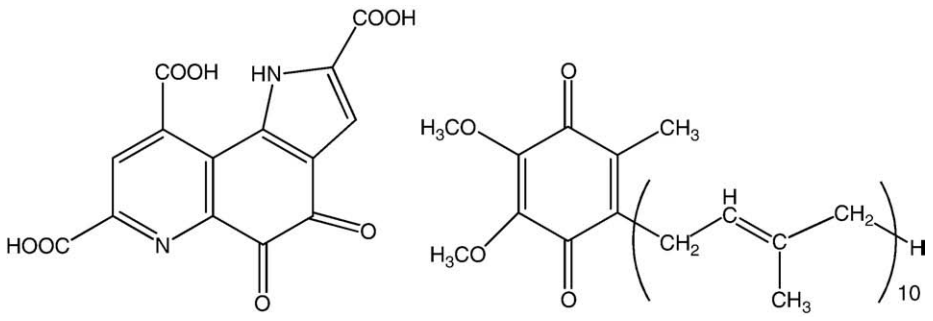
3.1. Cyanide-sensitive oxidase pathway

The PQQ-dependent alcohol dehydrogenase (ADH) plays an important role in both of these pathways. The cyanide-sensitive oxidase pathway can be defined by the catalytic action of ADH present in the cytoplasmic membrane of the *Gluconobacter* sp. For better clarity and the reader's convenience, all of the potential values cited in this review will be referred to the normal hydrogen electrode (NHE), unless specified otherwise. ADH oxidizes a substrate (alcohol) by the PQQ-containing subunit I (−23 mV) producing acetaldehyde. Electrons are then transferred through the cytochrome *c* site of subunit I (heme I, +265 mV) to the subunit II through heme II_1 (+428 mV), then to heme II_2 (+431 mV) and finally to quinone (a membrane soluble mediator), all potentials determined at pH 4.5 [16–18] (Table 1 and Fig. 2). The generated membrane-bound quinol is oxidized by quinol oxidase with oxygen being the final electron acceptor [15]. This cyanide-sensitive oxidase route is responsible for the generation of a proton gradient due to the accumulation of partially oxidized products outside the cell, which is later used for the production of energy in the form of ATP. Excessive generation of the electrochemical proton pool on the other hand disrupts the electron transfer pathway within the respiratory chain. An alternative way has evolved that keeps oxidizing the substrate at a high speed using the non-energy generating bypass oxidase system.

3.2. Cyanide-insensitive oxidase pathway

It has been shown that the subunit II of PQQ-dependent alcohol dehydrogenase plays a dominant role in the alternative and cyanide-insensitive oxidase pathways. It mediates electron transfer from the electron pool of ubiquinol and bypasses the oxidase system at the heme II_3 site (+496 mV, see Fig. 2). Under acidic conditions, the active form of ADH is turned into an inactive form with a preferential ubiquinol oxidizing activity (the ability to oxidize alcohol decreases 10-fold) [15]. The inactive form of ADH exhibits a loose conformation in a partially oxidized state, while a tight conformation and a fully reduced state are the dominant features of the active form of ADH.

ADH is able to donate electrons to ferricyanide *in vivo and in vitro* via all 4 heme c moieties. The ferricyanide activity is modulated by pH.



Scheme 1. Structure of PQQ (on the left) and Q_{10} (on the right).

Table 1
Redox potentials of fructose dehydrogenase (FDH) and alcohol dehydrogenase (ADH).

Redox group	Redox potential *vs.* NHE [mV]				
	ADH pH 7.0[a] [16]	ADH pH 4.5[a] [16]	ADH pH 7.0[b] [18]	FDH pH 7.0 [32]	FDH pH 4.5 [32,62]
PQQ	–	–	−167	−183	−23
Heme c I	+111	+265	+101	–	+277
II_1	+290	+428	+216	–	–
II_2	+429	+431	+370	–	–
II_3	+429	+496	+401	–	–

[a] ADH from *Acetobacter methanolicus*.
[b] ADH from *Gluconobacter suboxydans*.

Another physiological role of ADH in vivo is the mediation of the electron transfer from another primary membrane-bound dehydrogenase-glucose/aldose dehydrogenase through the subunit II of ADH to ubiquinone and finally to oxygen in the respiratory chain [19,20]. Interestingly, the redox potentials of all 4 heme groups are substantially altered by dissociation into separate subunits [7,16]. Subunit III of ADH exists freely in the periplasmic space with the exception of functioning as a molecular chaperone in the ADH complex [17,21]. The most recent study showed that the native form of ADH contains a tightly bound Q_{10} (Fig. 2) [17].

3.3. New physiological features

Recently, the complete genome sequence of *G. oxydans* 621H provided an in-depth view into its metabolic pathways, revealing the existence of 75 open reading frames that encode putative dehydrogenases or oxidoreductases of unknown functions [22]. Four new PQQ-dependent membrane bound dehydrogenases were confirmed in the genome with one PQQ-containing enzyme present in the periplasmic space. These results may be used to develop strategies for the employment of *G. oxydans* for a much greater variety of incomplete oxidations, possible new industrial applications, preparation of novel biosensor devices and biofuel cells.

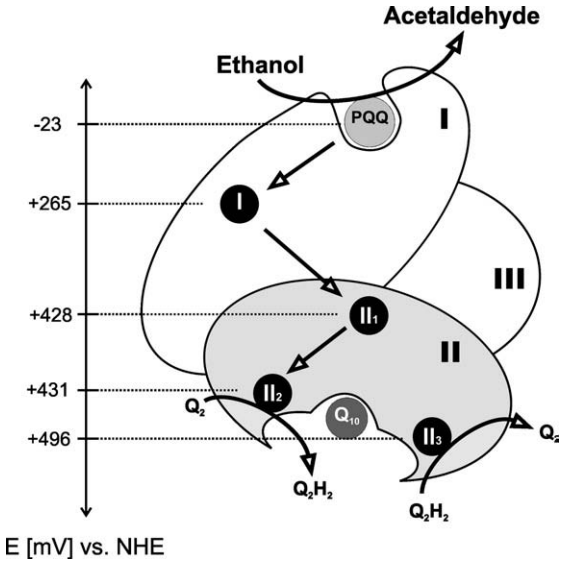


Fig. 2. Scheme of electron transfer from PQQ site of ADH through heme I, heme II_1 and heme II_2 site after substrate oxidation. The electron is transferred to membrane-bound quinone (Q_2) forming quinol (Q_2H_2) and finally to oxygen *via* quinol oxidase (not shown). This is the cyanide-sensitive oxidase pathway. Heme II_3 site is involved in cyanide-insensitive bypass oxidase pathway, when quinol (Q_2H_2) is oxidized to quinone (Q_2). In the scheme all 3 subunits of ADH are shown with a ubiquinone (Q_{10}) tightly bound in the subunit II of ADH. Redox potential of all redox sites of ADH was determined at pH 4.5.

4. Membrane-bound enzymes in *Gluconobacter*

Gluconobacter cells contain multiple membrane-bound dehydrogenases. Reports of the discovery, isolation and characterization of new dehydrogenases have been appearing in the literature for several decades. Especially the group of Prof. Adachi [23–28] has a long history and an impressive track record of isolation and characterization achievements, while the group of Prof. Ikeda pioneered the electrochemical investigation and characterization of these proteins [29–31]. There are two other groups in Japan; the first group led by Prof. Taniguchi is involved in the extensive characterization of the direct bioelectrocatalysis of PQQ-dependent dehydrogenases [32,33] and the second group led by Prof. Kano is active in the construction of biofuel cells [34–37]. Mediated and direct electrochemistry of PQQ dependent dehydrogenases was studied in detail by Prof. Laurinavicius' group [1,38–42]. A number of membrane-bound dehydrogenases have been studied extensively; in particular fructose dehydrogenase partly due to the fact that it is the only commercially available dehydrogenase isolated from *Gluconobacter*.

Membrane-bound enzymes have their active site oriented towards the periplasm (Fig. 1). Such a periplasm-localized oxidase system is characteristic of a truncated and thus less efficient energy-producing respiratory chain. Moreover, these enzymes oxidize the substrate directly with no energy consumed for the uptake of the substrate and the excretion of oxidized products through the cytoplasmic membrane [4]. Additionally, these enzymes are able to oxidize molecules harmful to cells [43], a feature important from the viewpoint of bioconversion and bioremediation or the construction of more robust biofuel cells.

Membrane-bound dehydrogenases of *Gluconobacter* can be divided into three categories. The first group contains quinoproteins such as glucose/aldose dehydrogenase having a PQQ redox site and only one subunit (see Table 2). The second group consists of quinohemoproteins having both a PQQ redox site and a few heme c sites with at least 2 subunits like ADH, aldehyde dehydrogenase and FDH. The third group of enzymes contains proteins with FAD and a few heme c sites (e.g. 2-D-gluconate dehydrogenase). Interestingly, a direct electron transfer or direct bioelectrocatalysis on various electrode materials was observed only with the proteins having at least two subunits and heme c sites (Table 2). However, DET of glucose/aldose dehydrogenase from *Gluconobacter* sp. can not be ruled out as suggested by a recent study showing direct bioelectrocatalysis of another membrane-bound glucose dehydrogenase (one subunit with only PQQ cofactor) isolated from *Erwinia* sp. [44].

PQQ-containing enzymes represent a new class of oxidoreductases containing an amino acid-derived *o*-quinone cofactor, recognized as a third redox site in the enzymes, in addition to pyridine nucleotide- and flavin-dependent dehydrogenases [4]. PQQ is coordinatively bound via Ca^{2+} (or Mg^{2+}) ions to the apoenzyme molecule and oxygen is not a natural electron acceptor of PQQ-dehydrogenases [38]. The importance of Ca^{2+} or Mg^{2+} for the thermal stability of the enzymes was experimentally verified with a suggestion to avoid the use of phosphate buffers [1].

Table 2
Characteristics of membrane-bound dehydrogenases present in *Gluconobacter* sp. relevant to bioelectrocatalysis.

Dehydrogenase	Subunits	M_w	Redox groups	DET	Reference
Alcohol	I, II, III	85, 49, 14	PQQ + cyt c	+	[23]
Aldehyde	I, II	86, 55	PQQ + cyt c	?	[24]
Gluconate	I, II, III	62, 45, 21	FAD + cyt c	+	[26]
Glucose/aldose	I	87	PQQ	−	[25]
Glycerol	I	80	PQQ	−	[27]
Fructose	I,II, III	67, 51, 20	PQQ + cyt c	+	[28]
Lactate	I,II ?	80, 60	PQQ + cyt c ?	+	[78]

DET — direct electron transfer or direct bioelectrocatalysis.

4.1. Direct electron transfer/bioelectrocatalysis

Achieving direct electronic coupling between redox enzymes and various conductive interfaces is not a trivial task, but it is gaining more attention, because it allows elucidation of the rates of catalysis and inhibition as a function of the potential applied. Thus, it introduces the "potential dimension" into the enzyme kinetics with the ability to control the driving force required to elicit bioelectrocatalysis [45,46]. Moreover, optimized interface modification protocols allowing direct electronic coupling are important for the development of practical applications (enzyme electrodes–biosensors, biofuel cells, bioelectroorganic synthesis and biomolecular electronic components) [46–48]. The absence of mediators gives such devices a superior selectivity, because they operate in a potential window close to the redox potential of the enzyme (they are less prone to interference) [49]. The first reports on DET using cytochrome *c* (small redox active protein) were published in 1977 by two independent groups [50,51], soon followed by the discovery that large proteins (e.g. laccase) can facilitate DET, as well [52,53]. There are two ways direct electronic coupling between the redox protein and the electrode can be investigated. The first one is a direct measurement of the electrochemical activity of the protein and the second possibility is elucidation of the catalytic response in the presence of the enzyme's substrate [54].

Membrane-bound enzymes isolated from *Gluconobacter* exhibiting DET are large proteins with the molecular weight ranging from 80 to 148 kDa. The proteins represent a uniform barrier for electron transfer (tunnelling) influenced by the distance between the donor and the acceptor (redox sites). A sharp (exponential) decay of the electron transfer rate (e.g. a 10^{12}-fold decrease over the distance of 2 nm within proteins with average decay constant β of 0.14 nm^{-1}) was observed [55]. More detailed analysis of the redox proteins have shown that electrons can be tunnelled over distances up to 1.4 nm within the protein shell at a rate faster than the rate of the substrate reaction [56]. Large redox proteins, thus, contain multiple redox sites with catalytic redox centres (e.g. flavins, quinones and pterins) interconnected by single electron redox site elements (e.g. hemes and FeS clusters) to facilitate an electron transfer across the protein [57]. Membrane-bound enzymes isolated from *Gluconobacter* confirm this general observation by having one catalytic redox site (PQQ or FAD) and a few electron redox site elements (heme c sites).

The interpretation of direct electron transfer of membrane-bound enzymes isolated from *Gluconobacter* is complicated by the fact that the redox potential of PQQ present in these enzymes is substantially lower (−167 or −183 mV, Table 1) than the redox potential of soluble PQQ (+117 mV) at pH 7.0 [58]. It is well known that the formal potential of the redox site can be modulated to a large extent by a ligand, coordination geometry and amino acid environment, and reactivity can be further adjusted by accessibility of the substrates to the active site [47]. A heme molecule with the formal potential varying from −270 mV (horseradish peroxidase) to+260 mV (cytochrome *c*) [49] is a nice example of this modularity. Moreover, heme exhibits various catalytic properties, which drastically change when incorporated into a protein [49]. A redox potential of PQQ bound is more positive compared to the formal potential of FAD being −210 mV or the redox potential of pyridine cofactor (NAD^+) of −320 mV [59,60]. This suggests quinoproteins are able to generate less energy from the substrate, a feature which may be important for the rapid oxidation rates observed [4]. Furthermore, it is obvious that the formal potential of PQQ has to be lower than the formal potential of the heme site present in the subunit I (+111 or+101 mV, Table 1 at pH 7.0) for efficient electron tunnelling from the PQQ redox site.

4.1.1. Fructose dehydrogenase (FDH)

FDH is a commercially available enzyme consisting of three subunits with an overall molecular weight of 138 kDa (Table 2). If the hard sphere model [61] is applied to this enzyme, the diameter of the protein can be calculated to be 7.0 nm, which is in agreement with an AFM (Atomic Force Microscopy) study of the protein [18]. The same model yields a theoretical coverage value of 3.9 pmol cm^{-2} for a fully packed monolayer of the protein. Although the redox potentials of all heme c sites present in FDH are not known, we can assume similar formal redox potential values of the heme c sites as determined for ADH by spectroelectrochemical or potentiometric titration based on the similarities between those two proteins. This hypothesis can be further verified by a direct comparison of the redox potential of the heme I site of ADH (+265 mV at pH 4.5) with the redox potential of DET of FDH obtained on various electrode surfaces at the same pH as follows:+277 mV on Pt,+277 mV on Au and+237 mV on GCE (glassy carbon electrode) [62]. Furthermore, the redox potential of a PQQ site in both proteins at pH 7.0 is very similar (Table 1).

The first report of the direct bioelectrocatalysis of FDH was published in 1991 by Ikeda's group with the protein immobilized on the surface of a carbon paste (CP) electrode contained behind a dialysis membrane [29]. The protein coverage on the electrode was estimated to be 2.4 pmol cm^{-2}, which is slightly lower than the theoretical coverage. The activity of immobilized FDH remained unchanged for 2 h at 25 °C or for 24 h at 5 °C. The biosensor device was successfully used for the detection of fructose in fruits with the response time of 50 s. When FDH was blended into the body of the CP, direct bioelectrocatalysis was obtained only in the presence of additives, with the highest current density obtained by using polyethyleneimine [63]. The biointerface showed very good operation stability (no decrease in 10 h) and a further decrease of the signal to 10% after 20 h was attributed to the negative influence of the electrodes being disconnected, although no such decrease was observed with the enzyme in a solution. The biosensor device was also used for the detection of fructose in honeys.

The first direct electron transfer (DET) of FDH without any substrate added was observed in 1991 on different electrodes [62]. The authors developed a new immobilization technique of FDH using voltage-induced (0.5 V *vs.* Ag/AgCl) enzyme adsorption. The redox process observed was incorrectly attributed to the electrochemistry of PQQ, a conclusion based on similarities between the observed redox potential and the redox potential of PQQ in a solution. The estimated surface coverage of 42.9–53.6 pmol cm^{-2} on various electrodes exceeded the theoretical coverage, suggesting the formation of multilayers of the protein on the surface. The largest anodic peak current for DET obtained by DPV (Differential Pulse Voltammetry) was observed on Au, followed by Pt and GC. The stability of the bioelectronic interface was rather poor and the signal dropped to 12% (Pt), 40% (Au) or 60% (GC) of the initial value after 24 h. In the following study, the authors proved that the presence of a polypyrrole (PP) film prepared by in-situ electropolymerization supports the protein layer in the correct orientation of the protein for more efficient electronic coupling (~1 order of magnitude) [64]. DPV revealed the presence of 2 well defined peaks, which is in good agreement with the previous study [62]. The response time of the FDH/PP interface of 3–5 s was very similar to that of the biointerface without the PP film (2–3 s) suggesting full access of the substrate to the catalytic site of the protein. The sensitivity of the biosensor device was much lower than that observed on a carbon paste electrode [29], suggesting a negative effect of metal surfaces on the direct electronic coupling of FDH.

Further studies confirmed the importance of the PP film in maintaining the stability and electrochemical activity of FDH adsorbed on Pt [65]. The sensitivity towards fructose increased from 48 nA mM^{-1} cm^{-2} on bare Pt to 200–400 nA mM^{-1} cm^{-2} with a thin PP film, and even to 1000 nA mM^{-1} cm^{-2}, when the PP film thickness increased 2-fold. The authors concluded that the poor stability of FDH on the bare Pt surface was the result of surface rearrangement of the enzyme rather than denaturation, with a negative effect of the open circuit potential during the adsorption of the enzyme on the bioelectrocatalytic signal observed.

Self-assembled monolayers (SAM) prepared by the chemisorption of thiols on gold surfaces is a very elegant strategy to deliver a wide range of functional groups with the ability to control the density of functional groups/charge on the surface in a reproducible way [66–69]. The effect of various terminal functional groups of SAMs on DET or direct bioelectrocatalysis of FDH has shown that a positively charged cystamine SAM on a gold surface is required for the electrostatic docking of the enzyme [70]. The DET of FDH using DPV with the enzyme in solution or in an adsorbed state appeared at the redox potential ranging from +39 mV to +108 mV with a submonolayer coverage (from 2.0 to 2.9 pmol cm^{-2}) on electrodes modified with various SAM layers. Interestingly, when the protein was kept in solution, the first DPV scan revealed the presence of a peak at +460 mV, most likely corresponding to the redox potential of the heme II_2 site in the second subunit of the enzyme. Upon continuous cycling, the peak disappeared completely. Another attempt to control the interaction of FDH with the surface on the nanoscale level was performed with colloidal gold (gold nanoparticles with $d=40$ nm) [71]. The bioelectrocatalytic current of FDH dropped sharply in 2 h to 50% of the initial response and the biosensor exhibited a response time of 15 s. A recent study showed that 2-mercaptoethanol (MET) is an optimal interface for docking FDH in order to achieve an efficient direct bioelectrocatalysis on an Au surface, gold nanoparticle modified Au surface and gold nanoparticle modified carbon paper [72].

Previous studies have shown that, when compared to metallic surfaces, carbon-based materials have a positive effect on efficient direct electron coupling of FDH while maintaining the stability of the protein. Thus, it is not surprising that the most recent studies performed by two Japanese groups led by Kano and Taniguchi were focused on the investigation of direct bioelectrocatalysis of FDH on carbon-based surfaces.

FDH bioelectrocatalysis on 4 different electrodes showed the largest current density on a plastic-formed carbon plate [32]. DPV on the basal plane of highly ordered pyrolytic graphite showed a large peak in the potential window from −200 to 0 mV depending on pH in a linear fashion with the slope of −59 mV/pH, a value typical for $2e^-/2H^+$redox reaction of PQQ [58]. A redox potential of −183 mV observed at pH 7.0 is in good agreement with the redox potential of PPQ determined in ADH at the same pH (−167 mV) [18]. Moreover, an AFM study has clearly shown that FDH is separated into subunits at a higher, non-physiological pH as judged by the protein height (3 nm *vs.* 7 nm). The same group extended the initial study and investigated the electrochemistry of FDH on a vertically grown multi-walled carbon nanotube forest on Pt with very good stability of the biointerface with no decrease of the response at 20 °C over 3 days [33].

The group led by Kano made an effort to maximize the current density of FDH-modified interfaces, a feature very important for the design of efficient biofuel cells. The first study showed that the current density dramatically increased from the initial 150 μA cm^{-2} to the final value of 4000 μA cm^{-2} upon prolonged incubation (12 h) with FDH on a Ketjan black electrode (KB, a carbon nanoparticle modified paste) [35]. The authors claim that the hydrophilic surface plays an important role in irreversible adsorption of FDH and the high current density observed. Further optimization of the amount of KB in the slurry used for the preparation of the electrode resulted in an impressive current density of 7000–10,000 μA cm^{-2} [34]. This current density is approximately one order of magnitude greater than the current density obtained with glucose oxidase or glucose dehydrogenase wired on a smooth, nonporous electrode surface using an Os hydrogel matrix (1–2 mA cm^{-2}) [73–75] and only 1–2 orders of magnitude smaller than the current density of methanol fuel cells (500 mA cm^{-2}) [76]. Although enzyme loadings ranging from hundreds to thousands of monolayers were predicted to be required to achieve the current density of 10 mA cm^{-2} [76,77], approximately the same current density was achieved with a single monolayer (4.6 pmol cm^{-2}) of FDH. In the calculation of the FDH surface density, the FDH activity of 1200 U mg^{-1} was taken into account, which translates to the activity of 5.5×10^3 electrons per second per enzyme molecule.

When no substrate is added to FDH immobilized onto the metallic surfaces (Au, Pt) (+0.5 V *vs.* Ag/AgCl), direct electron transfer can only be observed using a potential-assisted immobilization process [62,64]. Carbon-based materials are beneficial in achieving high current densities and a stable bioelectrocatalytic signal compared to the metallic surfaces. Based on the potential at which DET is observed, it seems that the preferred orientation of the enzyme facilitates an effective interaction between heme I and the electrode surface (Table 3). In some cases, it was possible to detect an interaction of other redox species of FDH such as the heme c II_2 site [70] or the PQQ cofactor [32].

4.1.2. Alcohol dehydrogenase (ADH)

PQQ-dependent ADH is an enzyme consisting of three subunits with an overall molecular weight of 148 kDa (Table 2). The enzyme has been extensively studied with the aim to elucidate the physiological functions of the enzyme, which are more complex than just the oxidation of ethanol into acetaldehyde, as discussed above. The redox properties of all redox sites of the enzyme were studied by either potentiometric titration or spectroelectrochemical titration and are summarized in Table 1.

The first direct bioelectrocatalysis of ADH on gold-plated platinum was observed in 1992 by Ikeda [30]. The initial study was later extended and direct bioelectrocatalysis of ADH on other electrode surfaces was evaluated [31]. The bioelectronic ADH-modified interface exhibited efficient electronic coupling as documented by the response time of 10 s obtained using this biosensor device. The authors discovered that ADH is strongly adsorbed onto metallic surfaces with the strength of adsorption decreasing in the following order: Ag>Au>GCE>basal pyrolytic graphite.

A novel immobilization approach using a PP film was used to entrap ADH inside the film by electropolymerization of pyrrole on Pt [39]. A low level of sensitivity towards ethanol was detected immediately after preparation, but after one day of storage at 20 °C, increased sensitivity and a broad dynamic range of the biointerface were observed. This was attributed to the rearrangement of the enzyme inside the layer, resulting in a more efficient electronic connection between the enzyme and PP. An increase of the anodic peak at potential of+390 mV suggests the involvement of the II_2 heme site in electronic coupling. The bioelectrocatalytic current started to rise from approximately +20 mV, also suggesting involvement of the heme I site. Interestingly, another peak

Table 3
Characteristics of FDH bioelectrocatalysis on various surfaces.

Immobilization	J_{max} (μA cm^{-2})	E_{start} (mV *vs.* NHE)	Sensitivity (μA mM^{-1} cm^{-2})	Year	Reference
On CPE	15	+150 (pH 4.5)	1.85	1991	[29]
Pt/PP	2–3	+150 (pH 4.5)	0.09	1992	[64]
Inside CPE	167	–	0.41 (FIA)	1996	[63]
Au nano/GCE	–	+150 (pH 4.5)	2.1	1997	[71]
Pt/PP	11	–	1.0	1997	[65]
PCP	60	+100 (+150) (pH 5.0)	–	2007	[32]
KB on CPa	4000	+150 (pH 5.0)	–	2007	[35]
KB on GCE	7000–10,000	+150 (pH 5.0)	–	2007	[34]
MWCNT on Pt	60	+150 (pH 5.0)	1.0	2008	[33]
MET on Au	170	+100 (pH 5.0)	–	2009	[72]
MET on AuNP on Au	3,250	+100 (pH 5.0)	–	2009	[72]
MET on AuNP on CPa	14,300	+100 (pH 5.0)	–	2009	[72]

CPE — carbon paste electrode, Pt/PP — polypyrrole on Pt, FIA — Flow Injection Analysis, PCP — plastic formed carbon plate, KB — Ketjan black, CPa — carbon paper, GCE — glassy carbon electrode, MWCNT on Pt — multiwalled carbon nanotube vertically aligned forest on Pt, MET — mercaptoethanol, AuNP-gold nanoparticles.

observed at approximately −200 mV was attributed to the redox switching of PP. This potential is very close to the redox potential of the PQQ redox center at the same pH of 6 (Table 1). The peak was not seen in the absence of a substrate and we can only speculate that the peak reflects the involvement of PQQ in the electronic coupling. An increase in the ionic strength of the buffer decreased the signal, suggesting that the electrostatic interaction between the protein and the PP film is crucial for direct bioelectrocatalysis.

Although no DET of dissolved ADH was observed on Au, a bioelectrocatalytic current increased in two steps starting at+100 mV and at+450 mV was observed [40]. This suggests that two different sites of ADH, e.g. the heme I and heme II_2 sites were involved in the coupling due to unrestricted interaction of the soluble enzyme with the surface. The authors claim that hydrophobic interactions were most likely involved, because the catalytic signal decreased in the presence of a surfactant. Interestingly, when ADH was adsorbed onto Au, a well resolved anodic peak at +400 mV was observed and increased its magnitude upon addition of EtOH. The redox chemistry of ADH adsorbed onto carboxyl and on succinimidyl ester terminated SAM was very similar to the bare gold electrochemistry with the start of the bioelectrocatalytic wave being positively shifted on a negatively charged SAM. A positively charged hexamethylcystamine SAM layer showed no electrocatalytic activity, suggesting the importance of a negative charge in the proper ADH adsorption. While the initial activity of the covalently immobilized ADH was much lower on the carboxylic SAM surface, the activity of the covalently bound ADH increased over an 8-day period most likely due to refolding of the protein into an active form.

Various forms of carbon-based electrode materials were used for ADH immobilization, with the most efficient direct bioelectrocatalysis observed on screen-printed electrodes using carbon black [41]. The bioelectrocatalytic current response was not influenced by an increased ionic strength suggesting a different mechanism of interfacial communication between the enzyme and the electrode compared to the PP-derived interface, which was sensitive to an increased ionic strength [40]. The biosensor exhibited a 24% signal loss after 12 h of continuous use (operational stability) with a quite stable response over the period of 6 days (long-term stability).

An extensive optimization study of the effect of the carbon black-modified electrode composition on direct bioelectrocatalysis was performed recently [42]. The study showed a start of the bioelectrocatalytic wave in the range of +220 to +400 mV at pH 6.0 depending on the electrode composition, suggesting the involvement of either heme I or heme II_2 in electronic coupling. The highest current density was obtained on the electrodes containing a set of surface functional groups and a set of nano-scale carbon structures.

It can be concluded that, in the absence of an added substrate, direct electron transfer can be seen on bare gold and on a gold surface modified by various SAM layers, most likely due to the involvement of the heme II_2 site in electronic coupling (anodic peak at+400 mV) [40]. A bioelectrocatalytic wave starts from +150 or +200 mV, suggesting the involvement of heme I site in the direct interaction with the electrode, as well (see Table 1). Moreover, when a PP film was co-deposited on the Pt electrode together with the enzyme, the strong entrapment in the conductive matrix allowed the enzyme to wire not only the heme I and heme II_2 sites, but likely also the PQQ site [40]. Carbon-based materials seem to be beneficial in achieving high current densities and a stable bioelectrocatalytic signal of ADH and FDH, when compared to metallic surfaces (Tables 3 and 4).

4.1.3. Gluconate dehydrogenase (GaDH)

GaDH is an enzyme consisting of three subunits containing a FAD cofactor and several heme c sites with an overall molecular weight of 128 kDa. The hard sphere model [61] reveals the diameter of the protein being 6.8 nm, with the theoretical coverage of 4.1 pmol cm^{-2} for a fully packed monolayer of the protein.

Table 4
Characteristics of ADH bioelectrocatalysis on various surfaces.

Immobilisation	J_{max} (μA cm^{-2})	E_{start} (mV)	Sensitivity (μA mM^{-1} cm^{-2})	Year	Reference
Pt	13 (10 mM E)	+150 (pH 6)	–	1992	[30]
Au/Pt	5 (10 mM E)	+150 (pH 4)	2.9	1993	[31]
Pt/PP	–	+17 (pH 6)	0.27	1999	[39]
Au	–	+100 (pH 6)	–	2000	[40]
Au/SAM	0.9 (2 mM E)	+200 (pH 6)	–	2000	[40]
SCE	214	+200 (pH 6)	179	2002	[41]
CBE	157	+220 to +400 (pH 6)	–	2005	[42]

E-ethanol, Au/Pt-gold plated platinum, Pt/PP — polypyrrole coated platinum, Au/SAM — self-assembled monolayer on Au, SCE — screen printed electrodes, CBE — carbon black modified electrode.

Kano's group has actively been working on the DET and direct bioelectrocatalysis of GaDH of *Gluconobacter frateurii* on various electrodes. Their first report describes the DET on an indium tin oxide electrode with the mid-potential of the anodic and cathodic potentials of+250 mV at pH 5.0, which most likely suggests the involvement of the heme c I site in the interaction. The amount of the electrochemically active GaDH was 4 pmol cm^{-2}, which is in good agreement with the theoretical coverage. A quartz crystal microbalance experiment revealed the coverage of 16 pmol cm^{-2}, suggesting the formation of multilayers (4 layers) of the enzyme on the electrode surface. The current density observed upon addition of 50 mM substrate was about 4 μA cm^{-2} with the enzyme kinetics being the rate-limiting step [36]. The second report showed a direct bioelectrocatalytic response of GaDH on bare and thiol-modified gold electrodes with the catalytic current rising from +150 mV at pH 4.0. Although the adsorption density of the enzyme was similar on all surfaces examined (from 2.1 to 3.1 pmol cm^{-2}), there were big differences in the electrochemical behavior of the enzyme. Favorable electrocatalysis was observed at a positively-charged surface with the current density of 13 μA cm^{-2} [37].

4.1.4. Lactate dehydrogenase (LDH)

Recently, the electrochemical behavior of a newly isolated PQQ-dependent lactate dehydrogenase was investigated on gold and on a screen-printed carbon electrode [78]. Gel electrophoresis revealed the presence of 4 different bands with two bands similar to other PQQ-containing dehydrogenases (80 and 60 kDa). The authors assumed that both subunits contain heme c moieties. The enzyme exhibited DET on a bare gold surface with the potential of an anodic peak of E_a +674 mV and the cathodic peak E_c at +612 mV, which results in the $E_{1/2}$ of +640 mV, a value much higher than any redox group of FDH or ADH. Interestingly, the current of both peaks increased upon addition of a substrate. It was also observed that DET occurred at lower values with the E_a of +361 mV and E_c of +77 mV ($E_{1/2}$ of +219 mV) on a screen-printed carbon electrode. This value is very similar to the redox potential of heme I of PQQ-dependent ADH or FDH (Tables 3 and 4). The current density of 120 μA cm^{-2} was observed with the enzyme immobilized on a Toray® carbon fibre paper in the presence of 50 mM lactate. The stability of the enzyme incorporated into a hydrophobized Nafion film was exceptional, and the performance of the bio-interface did not change during 45 days.

4.1.5. Interfacial bioelectrochemistry of Gluconobacter oxydans

Although the direct electron transfer between microbial cells and electrodes is now a well established mechanism facilitated in many different ways (outer surface cytochromes, microbial nanowires, conductive biofilm and soluble redox shuttles) [79,80], *Gluconobacter* has not been shown to facilitate DET to the electrodes. This may likely be due to the presence of the Gram-negative cell wall, which presents a 'long-distance' transport barrier for the electron transport. So far, the only reported way to achieve electron transport from intact

Gluconobacter cells is to wire active sites of dehydrogenases through the cell wall or porins.

The concept of wiring of intact cells of *Gluconobacter* with redox hydrogel (poly(1-vinylimidazole)$_{12}$-[osmium(4,4′-dimethyl-2,2′-dipyridyl)$_2$Cl$_2$]$^{+/2+}$) was pioneered by the group of Gorton [81]. Later, this concept was extended to wiring cells of *Pseudomonas putida* or *Pseudomonas fluorescens* [82]. Presently, it is not completely clear how this electronic wiring works (whether it is through the outer membrane or via porins). If a viable cell is wired, it is typically possible to access several dehydrogenases (alcohol, glucose/aldose and glycerol) in *Gluconobacter* by means of the redox hydrogel at once [81]. It was also shown that the wiring is not as efficient as when a soluble mediator (ferricyanide) is used, resulting in an approximate wiring efficiency of 30% for EtOH [81], 13% for glycerol [83] and 26% for 1,3-propanediol (1,3-PD) [83]. The maximum current density obtained varied from 2.7 μA cm^{-2} for glycerol to approximately 30 μA cm^{-2} for ethanol and 1,3-PD. The stability of the wiring was enzyme-dependent; 20% of the initial response was observed for glucose/aldose dehydrogenase and 60% for glycerol dehydrogenase and alcohol dehydrogenase after 24 h of continuous operation [81]. A more rapid decrease in the stability of redox polymer (0.66% h^{-1}) compared to ferricyanide (0.47% h^{-1}) suggests slow leaching of the Os redox polymer from the electrode surface or deterioration of the electronic coupling over time.

Gluconobacter cells can also be wired to the electrode surface by a series of hydrophobic mediators [84]. A closer examination has revealed the fact that acetylferrocene and ferrocenecarbaldehyde are poor mediators to wire glucose/aldose dehydrogenase of intact *Gluconobacter* cells. It has been shown that 2,5-dibromo-1,4-benzoquinone is catalytically the most efficient mediator, while the highest current density of 315 μA cm^{-2} was achieved by 2-methyl-1,4-benzoquinone. The next study showed that hydrophobic mediators (ferrocene and methylferrocene) perform better (redox potential generation) when used for the preparation of anodic half cells of biofuel cells compared to a soluble mediator (2,6-dichlorophenol indophenol) [85].

5. Biofuel cells

As the use of fossil fuels (oil and natural gas) is not a sustainable and green way of energy production, the current focus is on the development of alternative electricity production platforms using renewable resources without a net carbon dioxide emission. A technology based on fuel cells can convert chemical energy directly into an electrical current as long as the fuel is delivered into the system. Fuel cells operate at a lower temperature with a higher overall efficiency compared to combustion [77]. When enzymes are used as biocatalysts, the range of possible (bio)fuels that can be used to power biofuel cells (BFC) is significantly enhanced. BFC utilize redox enzymes (enzymatic BFC) or whole microbial cells (microbial BFC) for the oxidation of biofuel, and operate under milder conditions, which, in combination with the high selectivity of biocatalysts, gives them a key advantage over the conventional fuel cell technology [86].

The first enzymatic BFC was reported by Yahiro in 1964, but the power density output was fairly low [87]. A breakthrough in BFC design was reached in 1999, when it was proved that, unlike fuel cells, BFC can be constructed without a membrane due to the high selectivity of enzymes directly coupled to the electrodes (both anode and cathode) [88,89]. Even though the BFC technology has undergone a dramatic improvement in the performance characterized by the generated power density increase from less than 0.1 mW m^{-2} in 2000 to a power density of tens of W m^{-2} (e.g. 5–6 orders of magnitude in 8 years), the power density is still lower compared to the fuel cell technology [90]. It is still a few orders of magnitude lower than what is required by a cardiac pacemaker or to supply power to the electric grid [91]. Thus, there is a fair amount of scepticism about practical applications of BCF, which are believed to be limited to power small electronic devices such as sensor transmitters and implanted sensors with a minute power consumption [76,92]. A further increase in the power output can be achieved in two ways: engineered protein molecules (e.g. higher affinity to the substrate, higher catalytic activity and stability, smaller redox proteins with higher surface density etc.) or by an engineered immobilization environment (e.g. 3-D scaffold matrices or redox hydrogels, more protein-friendly matrices, etc.). Designing newly engineered proteins with better properties is a possible, albeit a very challenging job [93]. Thus, the current focus is on the design of a protein-friendly environment with increased stability [78,94,95], enhanced loading of proteins via 3-D matrix (chitosan scaffolds) [96–98] or using redox hydrogels [91,99,100]. For example, chitosan scaffold matrix can be easily made conductive by mixing with carbon nanotubes [101–103].

The upper limit of the power output of BFC ([current density]×[cell potential]) is not yet known due to several factors influencing both the maximum current density and cell potential of a BFC [104]. The theoretical maximal cell potential of BFC is 1.25 V based on the redox potential of −0.43 V for glucose oxidation and +0.82 V for oxygen reduction [86], but practically, the maximum cell potential of BFC achieved so far is 0.97 V (without any electric load) [94]. The highest power density of 43.9 W m^{-2} and the lifetime greater than one year for an enzymatic BFC [94] brings some hope and further development of BFC using nanotechnology (better control of electronic wiring and density of biocatalysts [60]) can drive this technology towards powering small electronic devices.

5.1. Gluconobacter oxydans biofuel cells

BFC typically consist of two compartments with the anode used for the oxidation of the biofuel by enzymes or microbes and the cathode for the reduction of oxygen, separated by a proton permeable membrane (Fig. 3). Microbial BFC have one big advantage over the enzymatic ones — their coulombic efficiency is up to 90% (e.g. 90% of the chemical energy of the biofuel is converted into electrical energy), [105] which comes at the expense of a low current density due to the slow electron flux through the cytosol and cytoplasmatic membrane. On the other hand, acetic acid bacteria are a promising candidate for the construction of efficient microbial BFC due to the periplasmic localization of the active sites of redox enzymes with a high turnover and current densities. Microbial BFC have the ability to adapt the

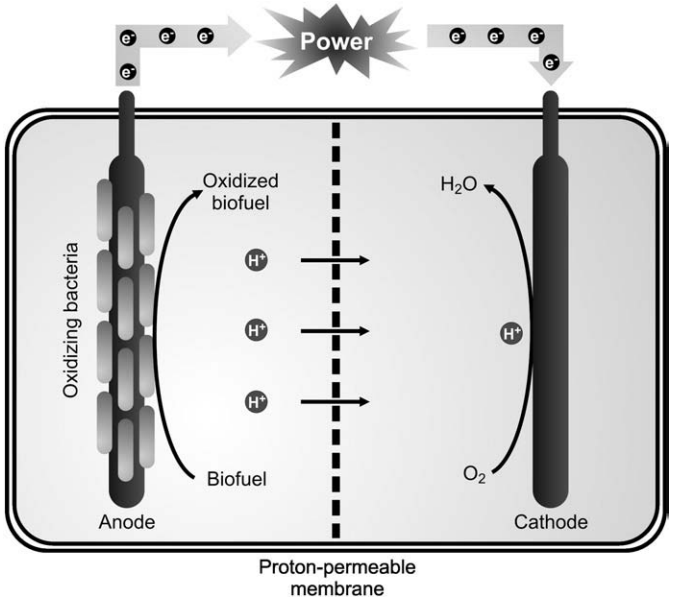


Fig. 3. General scheme of a microbial biofuel cell based on *Gluconobater oxydans* cells.

respiration machinery of the microbial cells to new conditions including new biofuels. The ability to oxidize glucose (coulombic efficiency and time needed to attain maximal current output) by *Gluconobacter oxydans* was influenced by the choice of the initial carbon source used for cultivation of the cells [106].

Microbial BFC are usually constructed with the aid of soluble redox shuttles (mediators) aimed to deliver electrons from the microbial cells towards the anode, as only a small number of microbial species are able to communicate with the electrode directly [105]. A soluble mediator 2,6-dichlorophenolindophenol was used to prepare the anode with a suspension of *Gluconobacter oxydans* cells, resulting in a potential of a half-cell of −55 mV *vs.* an unmodified working electrode [107]. It is believed that the low mediator concentration (34 μM) and a low cell density on the anode surface are the main causes of its low performance. The same group showed that immobilization of *G. oxydans* cells on the anode enhanced the performance of the half-cell to-75 mV using the same mediator [85]. The use of anode-bound hydrophobic mediators (ferrocenes) further increased the performance of the half-cell by increasing the current density and lowering the half-cell potential to −130 mV [85].

5.2. Biofuel cells derived from enzymes isolated from G. oxydans

As described in the previous chapters, membrane-bound dehydrogenases from *G. oxydans* exhibit a number of characteristics important for the preparation of efficient biofuel cells, such as direct bioelectrocatalysis and high stability on the surface of low-cost carbon electrodes with high current densities achieved at a low anodic redox potential. Direct bioelectrocatalysis allows working with no additional membrane; this not only increases the power output due to the decreased internal resistance, but also allows preparation of very small BFC devices.

ADH immobilized on the carbon anode was able to produce a potential of the half-cell of −115 mV, but the maximal potential (−190 mV) was achieved using 10 mM ferricyanide with ethanol as the biofuel, both values referred to the working electrode with an inactivated enzyme. The output potential of the half cell was pH dependent with the optimum performance at pH 6.0 [108]. DET of ADH was also used for the preparation of a full BFC with a combined cathode (glucose oxidase and microperoxidase) working in a DET mode. The potential of the anodic half-cell was −125 mV and the potential of the cathodic half-cell was +145 mV, but the authors did not mention what reference electrode was used. The overall potential of the full BFC of 270 mV was observed in the presence of both fuels (ethanol and glucose) with a limited half-life of the BFC (2.5 days) [109]. This study was recently extended using the same anode, but with alcohol oxidase and microperoxidase immobilized on the cathode, and the maximal open circuit potential of the full cell of 240 mV. Both the anode and cathode were powered by ethanol with a maximal power density of 0.02 W m^{-2} [110].

The limited stability of the PQQ-containing dehydrogenases isolated from *G. oxydans* was dramatically enhanced (the lifetime greater than 200 days) using their entrapment in a Nafion-modified membrane deposited on the anode surface [94]. Using this immobilization protocol, a BFC based on ADH and aldehyde dehydrogenase yielded the power density of 12.1 W m^{-2} and coulombic efficiency of 86% with glycerol as the fuel [111]. When a Nafion-modified membrane was used for the immobilization of the PQQ-dependent lactate dehydrogenase, the maximal power output of 0.22 W m^{-2} was achieved. The open circuit potential of the biofuel cell was 0.85 V and it was stable over a 45-day period [78].

Extensive optimization was performed to evaluate the influence of various factors on the performance of a FDH-based BFC. This resulted in the potential of the full BFC of 0.79 V (without the load) and maximum power density of 3.9 W m^{-2} achieved at 0.41 V. The performance of the BFC was further increased to 8.5 W m^{-2} (at 0.41 V) upon stirring [35]. The authors reported that the main limiting factor influencing the performance of BFC was the oxygen transfer towards the cathode. This problem can be circumvented using an air-breathing cathode with one side exposed to the ambient air [112]. In a recent study a gold nanoparticle modified carbon paper showed an efficient direct bioelectrocatalysis with FDH, not influenced by stirring, for preparing a bioanode with the current density of 14.3 mA cm^{-2} [72]. This value is claimed to be the highest ever reported current density for a bioanode based both on direct or mediated bioelectrochemistry.

6. Conclusions

Preferential electronic coupling of membrane-bound dehydrogenases from *Gluconobacter* sp. most likely occurs through the heme I site of subunit I, although another interaction with the electrode surface was observed in the case of enzymes weakly adsorbed on the electrode surface (e.g. solution based electrochemical investigation) probably via the heme II_2 site. It seems that the electric field influences the native structure of membrane-bound dehydrogenases to a large degree with the optimal orientation of an enzyme for direct bioelectrocatalysis achieved by an applied positive potential during immobilization. The enzymes are strongly and irreversibly adsorbed on metallic surfaces, resulting in a partial loss of the activity in the short term. The preferred electrode interfaces for immobilization of the enzymes are various forms of carbon due to the possibility of obtaining very high current densities and preserving enzymatic activities. Generally, the low stability of membrane-bound enzymes was successfully addressed by entrapment into a hydrophobized Nafion membrane. Moreover, membrane-bound dehydrogenase-modified interface can be used for the preparation of bioanodes of biofuel cells.

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